Interplay between inflammation and calcification in cardiovascular diseases

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INTERPLAY BETWEEN INFLAMMATION AND CALCIFICATION IN CARDIOVASCULAR DISEASES

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2022

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Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2022

ISBN 978-91-8016-601-0
Cover illustration: Molecular networks in cardiovascular diseases.
Created with BioRender.com.

INTERPLAY BETWEEN INFLAMMATION AND CALCIFICATION IN CARDIOVASCULAR DISEASES THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Nikolaos – Taxiarchis Skenteris

The thesis will be defended in public at Maastricht University, Maastricht, The Netherlands, online on May 31st, 2022 at 13:00 hours

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The research presented in this dissertation was funded with a grant from European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant (agreement No 722609), INTRICARE

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The thesis will be defended in public at Karolinska Institutet, Andreas Vesalius lecture hall, Campus Solna, Berzelius väg 3 on June 3rd, 2022 at 09:00 hours

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The research presented in this dissertation was funded with a grant from European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant (agreement No 722609), INTRICARE

Στην αγαπημένη μου οικογένεια

To my beloved family

« Πάντα στὸ νοῦ σου νἄχῃς τὴν Ἰθάκη.
Τὸ φθάσιμον ἐκεῖ εἶν' ὁ προορισμός σου.
Ἀλλὰ μὴ βιάζῃς τὸ ταξείδι διόλου.
Καλλίτερα χρόνια πολλὰ νὰ διαρκέσει.
Καὶ γέρος πιὰ ν' ἀράξῃς στὸ νησί,
πλούσιος μὲ ὅσα κέρδισες στὸν δρόμο,
μὴ προσδοκώντας πλούτη νὰ σὲ δώσῃ ἡ Ἰθάκη.

Ἡ Ἰθάκη σ' ἔδωσε τ' ὡραῖο ταξίδι.
Χωρὶς αὐτὴν δὲν θἄβγαινες στὸν δρόμο.
Ἄλλα δὲν ἔχει νὰ σὲ δώσει πιά.

Κι ἂν πτωχικὴ τὴν βρῇς, ἡ Ἰθάκη δὲν σὲ γέλασε.
Ἔτσι σοφὸς ποὺ ἔγινες, μὲ τόση πείρα,
ἤδη θὰ τὸ κατάλαβες ἡ Ἰθάκες τί σημαίνουν.»

Ἰθάκη - Κωνσταντῖνος Π. Καβάφης

" Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaca gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaca won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithacas mean."

Ithaca - Constantine P. Cavafy[1]

[1] C. P. Cavafy, "Ithaca" from *C.P. Cavafy: Collected Poems* (Princeton University Press, 1975). Translated by Edmund Keeley and Philip Sherrard. Translation Copyright © 1975, 1992 by Edmund Keeley and Philip Sherrard.

ABSTRACT

Cardiovascular calcification has been linked to all-cause mortality and is a broadly adopted predictor of cardiovascular (CV) events. Rather than a mere by-product of the changing disease environment, calcification impacts actively the disease progression and pathogenesis as it predominates both in early- and late-stages, through mediating tissue biomechanical destabilisation and directly impacting tissue inflammation. However, its clinical contribution to the fate of the disease remains to be elucidated. Emerging body of evidence from both basic and clinical research has demonstrated the significance of the innate immune system in cardiovascular diseases (CVDs). Here, inflammation and calcification are engaged in a vicious cycle particularly at early-stages, whereas in advanced-lesions, large calcifications linked with suppressed inflammation and plaque stability. However, this interaction during disease progression remains largely elusive. The aim of this thesis is to investigate the interplay between inflammation and calcification in advanced atherosclerosis and calcific aortic valve disease (CAVD).

Study I explores gene and protein expression signatures and biological pathways of advanced CAVD lesions in order to characterise the underlining mechanisms associated with the disease pathology. Multi-omics integration of overlapping transcriptome/proteome molecules with miRNAs, identified a unique CAVD-related protein-protein 3D layered interaction network. After addition of a metabolite layer, Alzheimer's disease (AD) was identified in the core of the gene-disease network. This study suggests a novel molecular CAVD network potentially linked to amyloid-like structures formation.

Study II characterises osteomodulin (OMD) in the context of atherosclerosis, chronic kidney disease (CKD) and CAVD. Plasma OMD levels were correlated with markers of inflammation and bone turnover, with the protein being present in the calcified arterial media of patients with CKD stage 5. Circulating OMD levels were also associated with cardiac valve calcification in the same patients and its positive signal was detected in calcified valve leaflets by immunohistochemistry. In patients with carotid atherosclerosis, plasma OMD levels were increased in association with plaque calcification as assessed by computed tomography. Transcriptomic and proteomic data analysis showed that OMD expression was upregulated in atherosclerotic compared to non-atherosclerotic control arteries, and particularly in highly calcified plaques, where its expression correlated positively with markers of vascular smooth muscle cells (VSMCs) and osteoblasts. *In vivo*, OMD was

enriched in VSMCs around calcified nodules in aortic media of nephrectomised rats and in plaques from *ApoE*$^{-/-}$ mice on warfarin. *In vitro* experiments revealed that exogenous administration of recombinant human OMD protein repressed the calcification process of VSMCs treated with phosphate by maintaining the VSMC contractile phenotype along with enriched extracellular matrix (ECM) organisation, thereby attenuating VSMC osteoblastic transformation.

Study III analyses OMD expression in human carotid plaques and particularly its link with future CV events. Transcriptomic analysis revealed that OMD levels were increased in plaques from asymptomatic patients compared to symptomatic ones, with high levels being associated with fewer CV events in a follow-up analysis.

Study IV investigates the link between mast cell (MC) activation and key features of human plaque vulnerability, and the role of MC in VSMC-mediated calcification. Integrative analyses from a large biobank of human plaques showed that MC activation is inversely associated with macrocalcification and positively with morphological parameters of plaque vulnerability. Bioinformatic analyses revealed associations of MCs with NK cells and other immune cells in plaques. Mechanistic *in vitro* experiments showed that calcification attenuated MC activation, while both active and resting MCs induced VSMC calcification and triggered their dedifferentiation towards a pro-inflammatory- and osteochondrocyte-like phenotype.

Overall, this thesis demonstrates that the underlying mechanisms of CVD related to inflammation and calcification can be comprehensively characterised by integration of large-scale multi-omics datasets along with cellular and molecular assays on one side, and disease specific biomarkers and advanced diagnostic imaging tools on the other. In summary, these studies not only indicate that advanced-calcification is a stabilising factor for plaque and disease progression but also, unveil novel insights into the cardiovascular calcification pathobiology, and offer promising biomarkers and new therapeutic avenues for further exploration.

LIST OF SCIENTIFIC PAPERS

I. Heuschkel MA*, Skenteris NT*, Hutcheson JD, van der Valk DD, Bremer J, Goody P, Hjortnaes J, Jansen F, Bouten CVC, van den Bogaerdt A, Matic L, Marx N, Goettsch C.
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II. Skenteris NT, Seime T, Witasp A, Karlöf E, Wasilewski GB, Heuschkel MA, Jaminon AMG, Oduor L, Dzhanaev R, Kronqvist M, Lengquist M, Peeters FECM, Söderberg M, Hultgren R, Roy J, Maegdefessel L, Arnardottir H, Bengtsson E, Goncalves I, Quertermous T, Goettsch C, Stenvinkel P, Schurgers LJ, Matic L.
Osteomodulin attenuates smooth muscle cell osteogenic transition in vascular calcification.
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III. Goncalves I*, Oduor L*, Matthes F, Rakem N, Meryn J, Skenteris NT, Aspberg A, Orho-Melander M, Nilsson J, Matic L, Edsfeldt A, Sun J*, Bengtsson E*
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IV. Skenteris NT, Hemme E, Delfos L, Karlöf E, Lengquist M, Kronqvist M, Zhang X, Maegdefessel L, Schurgers LJ, Arnardottir H, Biessen EAL, Bot I, Matic L.
Mast cells participate in atherosclerotic plaque calcification and smooth muscle cell reprogramming.
Manuscript

*authors contributed equally

OTHER RELATED PUBLICATIONS

I. Waring OJ*, Skenteris NT*, Biessen EAL, Donners MMPC.
Two-faced Janus: The dual role of macrophages in atherosclerotic calcification.
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II. Petri MH, Thul S, Andonova T, Lindquist-Liljeqvist M, Jin H, Skenteris NT, Arnardottir H, Maegdefessel L, Caidahl K, Perretti M, Roy J, Bäck M.
Resolution of Inflammation Through the Lipoxin and ALX/FPR2 Receptor Pathway Protects Against Abdominal Aortic Aneurysms.
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*authors contributed equally

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LIST OF ABBREVIATIONS

αSMA	α-Smooth muscle actin
ACE	Angiotensin-converting enzyme
AD	Alzheimer's disease
AFX	Amaurosis fugax
AGEs	Advanced glycation end-products
ALP	Alkaline phosphatase
AS	Asymptomatic
AVR	Aortic valve replacement
BGLAP	Osteocalcin
BiKE	Biobank of Karolinska Endarterectomies
BMP	Bone morphogenetic protein
CAC	Coronary artery calcium
CAE	Carotid artery endarterectomy
CALC	Calcification
CAVD	Calcific aortic valve disease
cEV	Calcifying extracellular vesicle
CKD	Chronic kidney disease
CNN1	Calponin
COMP	Cartilage oligomeric matrix protein
CTA	Computed tomographic angiography
CVD	Cardiovascular disease
DEGs	Differentially expressed genes
EC	Endothelial cell
ECM	Extracellular matrix
EndoMT	Endothelial to mesenchymal transition
GAGs	Glycosaminoglycans
GF	Growth factor
HAoSMC	Human aortic smooth muscle cell
HCoSMCs	Human coronary smooth muscle cell
ICAM-1	Intercellular Adhesion Molecule 1
IPH	Intraplaque hemorrhage
LDL	low-density lipoprotein
LRNC	Lipid rich necrotic core
MC	Mast cell
MGP	Matrix Gla protein

MSX2	Msh Homeobox 2
MYH11	Smooth muscle myosin heavy chain 11
MYOCD	Myocardin
NK	Natural killer
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
OGN	Osteoglycin
OMD	Osteomodulin
OPN	Osteopontin
OPG	Osteoprotegerin
OSX	Osterix
PDGF	Platelet-derived growth factor
PG	Proteoglycan
PPi	Pyrophosphate
RAGE	Receptor for advanced glycation end-products
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
S	Symptomatic
ScRNAseq	Single-cell RNA sequencing
SLRP	Small leucine-rich proteoglycan
SMTN	Smoothelin
SOX9	SRY-box transcription factor 9
SRGN	Serglycin
TCF21	Transcription factor 21
TGFβ	Transforming growth factor beta
TIA	Transient ischemic attack
TNAP	Tissue non-specific alkaline phosphatase
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion protein 1
VEC	Valvular endothelial cell
VIC	Valvular interstitial cell
VSMC	Vascular smooth muscle cell
WHO	World Health Organisation
WNT	Wingless/integrated

1 INTRODUCTION

The presence of cardiovascular calcification predicts patient morbidity and mortality. Calcium deposits within the soft cardiovascular tissues disrupt the biomechanical function of the diseased vessel and lead to complications such as heart failure, myocardial infarction, and stroke. Emerging notion in the field supports that pathophysiology of cardiovascular calcification is an active process triggered mainly by pro-inflammatory cues. Both inflammation and calcification are engaged into a vicious cycle that is hypothesised to drive further disease progression, causing atherosclerotic arterial and valvular calcification. The current doctoral thesis attempts to summarise established knowledge in the field of cardiovascular calcification with special interest for inflammation and calcification and provide new concepts for the underlying disease mechanisms.

1.1 CARDIOVASCULAR DISEASE, EPIDEMIOLOGY, RISK FACTORS AND CLINICAL PERSPECTIVES

Cardiovascular disease (CVD) is the number one cause of death worldwide, with an estimation of 32% reported deaths worldwide [1]. Myocardial infarction and stroke account for approximately 85% of these deaths [2,3]. The prevalence of CVD is increasing in developing countries due to longevity and growth of population [1]. According to WHO, CVD is an umbrella term which includes a cluster of disorders of the blood vessels and heart such as: coronary heart disease causing heart failure or myocardial infarction, vascular disease-causing stroke and aneurysm rupture, peripheral arterial disease-causing ischemia in the arms and legs [2]. The majority of those CV events are the atherosclerotic manifestations. The most well-established risk factors for developing heart disease and stroke are unhealthy diet, male sex, tobacco use, alcohol consumption and physical inactivity, with hypertension, obesity, hyperlipidemias, diabetes and age to be referred as “intermediate risk factors” [4]. Presence of cardiovascular calcification is considered as a prognostic marker of all-cause risk and CVD mortality [5]. The risk for myocardial infarction or death is significantly higher in individuals with coronary artery calcium (CAC) score higher than 300 compared to those with calcium score between 1-100 [6].

1.1.1 Atherosclerosis

Atherosclerosis remains the major killer from vascular disease globally [7]. Atherosclerosis, meaning the hardening and narrowing of large arteries caused by plaques, can occur in different vascular beds, including coronary, carotid and iliac arteries (Figure 1) [8].

Classification of the atherosclerotic plaques is based on histological descriptions used as a standard protocol from American Heart Association (AHA types I-VIII) [9]. Its major clinical manifestations include acute coronary syndromes such as myocardial infraction and ischemic strokes or chronic conditions including angina pectoris, and transient cerebral ischemic attacks [10]. Increased CAC score is related to atherosclerotic plaque burden and is linked with all-cause mortality, rendering this a broadly adopted predictor of CV events [5,11]. Due to the slow progression of the disease, the majority of people with atherosclerotic plaques in carotid arteries do not develop symptoms for decades. When symptoms arise, they are associated with decrease in blood flow caused by stenosis of arterial lumen or development of thrombotic obstruction. If carotid artery is severely occluded, the atherosclerotic plaque is prone to rupture, where small plaque elements may break off and go to the brain, causing sudden transient loss of vision in one or both eyes, a condition called amaurosis fugax (AFX), a major or minor stroke and transient ischemic attack (TIA). While accurate disease-related predictors as well as optimal treatment for the disease are still lacking, the primary goal for disease management is to prevent stroke. Thus, the patient is advised about optimal behavioral treatment and controlled blood pressure, glucose and cholesterol levels. In addition, combination of medication is prescribed for plaque stabilisation to lower the risk for development of clinical symptoms, such as antithrombotic therapy (acetylsalicylic acid or clopidogrel), cholesterol-lowering therapy (HMG-CoA reductase-inhibitors i.e statins or ezetimibe) and anti-hypertensive therapy (angiotensin-converting enzyme [ACE] inhibitors and angiotensin II receptor blockers) [12]. When the carotid artery is severely occluded, surgical intervention is highly recommended, where the atherosclerotic plaque is surgically excised in a procedure named carotid endarterectomy (CEA), in order to restore the arterial blood flow. CEA is secured as primary (asymptomatic) and secondary (symptomatic patient with

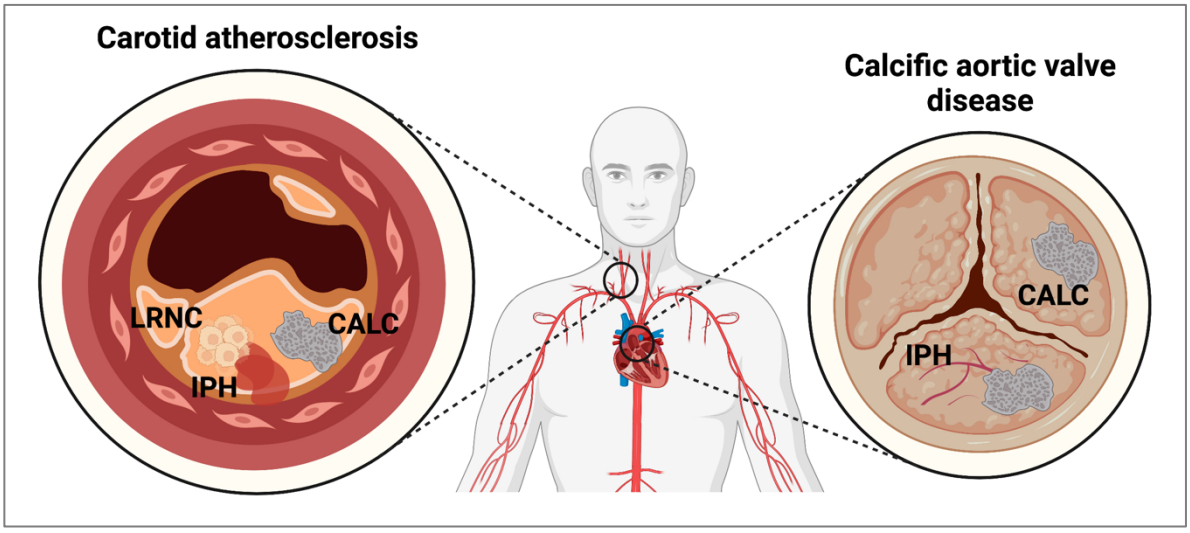

Figure 1.Carotid atherosclerosis and calcific aortic valve disease; among the major clinical manifestations of CVDs.

recent stroke or TIA within the prior 6 months) prevention of stroke in individuals with severe carotid artery stenosis [13].

1.1.2 Calcific aortic valve disease

Calcific aortic valve disease (CAVD) is a progressive pathology that spans from mild aortic valve leaflet thickening without concomitant blood flow interference, referred as "aortic sclerosis", to severe valve mineralisation accompanied with leaflet dysfunction, or "aortic stenosis". CAVD is the most prevalent heart valve pathology in Western countries and the third most frequent CVD after systemic arterial hypertension and coronary artery disease (Figure 1) [14]. The global burden of CAVD is expected to increase in the coming decades [15], as a result of the population longevity and the lack of appropriate therapeutic tools to reduce disease burden. Genetic and clinical risk factors have been linked with CAVD onset development and progression. Congenital leaflet abnormality (bicuspid) along with aging remain the two strongest risk factors for CAVD. Moreover, metabolic syndrome's comorbidities and elevated plasma levels of lipoprotein(a) have also been linked with elevated risk of CAVD [16]. Male sex, smoking, high blood pressure, kidney disease, mineral metabolic disturbances and secondary hyperparathyroidism further increase the risk of disease incidence [14]. Valvular calcification is recognised as a significant contributor to morbidity and mortality, particularly in patients with CKD [17,18]. Similar to atherosclerosis, patients with aortic valve stenosis may not develop symptoms for many years. Currently, pharmacological intervention does not exist to effectively halt CAVD progression or reverse the calcium deposition within the valve leaflets. Traditional drugs explored in several clinical trials failed to modulate the disease progression [19,20], with aortic valve replacement (AVR) being the only sufficient treatment for severe CAVD.

1.2 CARDIOVASCULAR CALCIFICATION – PATHOPHYSIOLOGY

Cardiovascular mineralisation is an active pathophysiological procedure characterised by the deposition of Ca^{2+} and PO_4^{3-} ions into amorphous calcium phosphate (Ca/P) particles, which further transform into hydroxyapatite crystals over time [21], both in blood vessels and heart valves. Vascular calcification is associated with atherosclerosis [22], diabetes mellitus, CKD [23], aging and CAVD [24]. Traditionally, ectopic calcification is classified into two forms: micro- and macrocalcification, which usually occur side-by-side during disease progression. Microcalcification is largely observed in earlier-stage lesions [25], while macrocalcification predominates in late- or advanced-stages of the disease [26,27]. Microcalcified particles, defined as smaller than 50 μm in size [28], are developed in a four-stage process, involving calcifying extracellular vesicle (cEV) accumulation, aggregation, membrane fusion, and finally,

mineralisation in endogenous collagen matrix [26]. During mineralisation, amorphous Ca/P transforms into mature crystal-like form hydroxyapatite "microcalcification" present in spherical and needle-like morphology types, 0.5–15 μm in size [29]. When these hydroxyapatite crystal-like forms coalesce into larger sheet-like or nodular structures, up to several millimeters in diameter, they are defined as "macrocalcification" and classified into a) punctate/fragmented calcification (< 3 mm), which corresponds to "speckled calcification" (≥15 μm to ≤2 mm in diameter) or fragmented calcification (< 3 mm); and b) sheet/nodular calcification (>3 mm), which corresponds to diffuse mineralisation on radiographs [29,30]. In addition, cardiovascular calcification is classified according to the location where the minerals are deposited, as in the intima or media of arteries or in aortic valve leaflets, resulting to vascular and valvular mineralisation-related pathologies, respectively.

1.2.1 Arterial calcification

The vessel wall consists of three layers termed *tunica intima*, *tunica media* and *tunica adventitia* that are homed by several different cell types. Endothelial cells (ECs), present in the innermost layer towards the lumen, form the barrier between the vessel wall and blood. Vascular smooth muscle cells (VSMCs), which are embedded in extracellular matrix (ECM) components such as elastin and collagen in the *tunica media*, facilitate the vessel dilatation and constriction processes. Fibroblasts, pericytes and mesenchymal stem cells, located in the outer ECM-rich adventitial layer, maintain the structural integrity of the vessel wall under mechanical load [31,32]. Arterial calcification being present in both intimal and medial layers is classified as either atherosclerotic intimal calcification or medial Mönckeberg arterial calcification (also referred to as Mönckeberg's sclerosis). Such calcifications represent distinct pathological conditions which occur independently but often coincide and overlap.

1.2.1.1 Atherosclerotic intimal calcification

Calcification in the *tunica intima* layer of the arterial wall can be found in a varying distribution throughout the vasculature, co-localising more frequently with atherosclerosis, but it is also seen in patients with diabetes mellitus and CKD. Atherosclerosis is a slow progressing, chronic inflammatory disease that affects several arterial beds (i.e medium- and large-sized arteries) and is characterised by the accumulation of fatty and fibrous elements together with structural VSMCs and immune cells in the intimal layer of the arterial wall upon endothelial injury [10]. The term atherosclerosis origins from the Greek words ἀθήρα (athḗra), meaning "gruel" and σκλήρωσις (sklerosis), meaning "hardening". Atherosclerosis is considered a multifaceted process which involves processes such as lipid deposition, chronic inflammation, dynamic cellular transdifferentiation and mineralisation (Figure 2).

Initially, the endothelial monolayer is activated in response to both pro-inflammatory stimuli and local hemodynamic environment at preferential sites in the vasculature, such as turbulent or oscillatory stress [33]. Circulating blood monocytes can bind to the adhesion molecules i.e VCAM-1 and ICAM-1, of activated ECs, roll and transmigrate into the arterial wall. The infiltrating monocytes then mature into macrophages attaining pro-inflammatory phenotype characteristics [34]. In addition to immune cells and pro-inflammatory mediators, cumulative infiltration of low-density lipoprotein (LDL) is also considered an important contributor for disease initiation and progression [10]. In the intima, the LDL particles are trapped on ECM proteoglycans, undergo enzyme and biochemical modifications which further exacerbate the inflammatory responses with procalcifying properties [35]. Modified lipids can be effectively taken up by VSMCs and directly induce their migratory activation, foam cell formation and osteoblastic-like transdifferentiation [36-38]. Moreover, injured endothelium leads to impaired

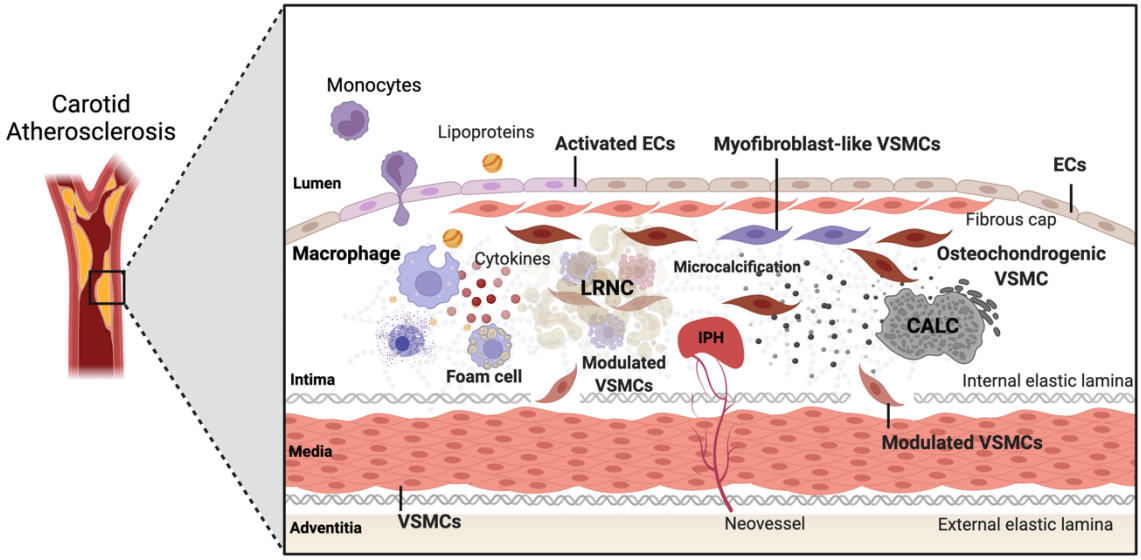

Figure 2.Pathophysiology of atherosclerotic intimal calcification.

release of endogenous vasodilator nitric oxide (NO), early during atherogenesis [39]. Of note, NO inhibits calcification of VSMCs and differentiation of VSMCs into osteoblastic cells by blocking transforming growth factor beta (TGFβ) signalling pathway [40], while its loss in combination with the aggravating inflammatory responses can further induce endothelial to mesenchymal transition (EndoMT) [41], contributing thus to calcification [42]. Chemokines released from activated ECs and pro-inflammatory macrophages further enhance the recruitment and migration of monocytes and other circulating immune cells into the arterial wall. In turn, macrophages express scavenger receptors allowing them to bind and engulf modified LDL particles transformed them to "foam cells". At this initial step of atherosclerosis, the accumulating foam cells with the ECM proteins can be observed in histopathological analysis and these structures are called "fatty streaks" or "xanthomas" [9]. T and B lymphocytes, as well as MCs and other immune cells which enter the lesion, can further

modulate VSMCs, which start to proliferate and migrate into intima layer forming the fibrous cap [43]. Established atherosclerotic plaques continue to accumulate lipids, which are internalised by macrophages and VSMCs, leading to a release of apoptotic bodies (50–5000 nm in diameter). In addition, failure of macrophages to properly clear apoptotic bodies leads to secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), which has been described as a potent inducer of osteogenic gene expression in VSMCs [44,45]. Modulated VSMCs along with macrophages can release cEVs (30–300 nm in diameter), which facilitate the formation of Ca/P crystals [45,46]. The releasing apoptotic bodies and cEVs predispose the sites for hydroxyapatite nucleation and microcalcification formation in the dysregulated ECM [26,47-49]. Highly inflamed plaques contain microcalcification and inflammatory cells such as macrophages, MCs, platelets and neutrophils, that release inflammatory cytokines and ECM degrading enzymes, contributing thus to further enlargement of lipid rich necrotic core (LRNC) and thinning of the fibrous cap, rendering the plaque prone to rupture [50]. Microcalcified particles being present in the fibrous cap and the lesion shoulders depict a dynamic inflammation-triggering procedure associated with disease progression, greater atheroma burden and increased risk of plaque rupture as the result of strong mechanical forces [51,52]. Vulnerable plaques are characterised by a large LRNC, ongoing inflammation accompanied with microcalcification, neovessels formation with intraplaque hemorrhage (IPH) and a thin fibrous cap (Figure 3) [53]. Accumulation of calcified nodules into larger macrocalcified structures localised deeper in the necrotic core and the surrounding collagenous matrix has been linked with more reparative healing responses and plaque stability processes [27,54]. In advanced atherosclerotic plaques, neo-angiogenesis in the adventitial *vasa vasorum* gives rise to neovessels formation inside plaques. IPH as a result of immature leaky neovessels contributes to the ongoing inflammation, necrotic core growth and plaque instability [55,56]. Emerging body of studies revealed a direct association of MCs with IPH [57], which in turn has been linked with calcification [58-60]. Lastly, calcification

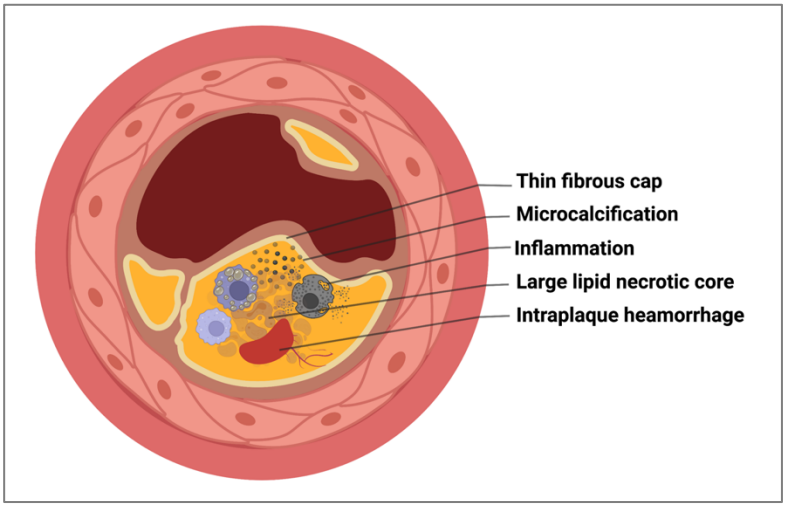

Figure 3. Schematic image showing the morphological features of vulnerable atherosclerotic plaque.

quantity and location are considered as independent indicators for IPH in carotid atherosclerotic plaques [59].

1.2.1.2 Medial calcification

Medial calcification consists of a group of different etiologies and pathologies which lead to mineralisation of the medial layer of the arteries [61]. Medial calcification is distributed more diffusely throughout the vascular tree, at sites with or without atherosclerosis and is a prominent pathological characteristic found in high prevalence in patients with diabetes [62], chronic kidney disease [17], peripheral artery disease [63], and it is associated with aging [64]. It leads to changes in the blood flow dynamics and vessel wall mechanics, such as systemic manifestations of increased arterial stiffening and impaired blood perfusion of high blood-demanding organs, increasing the risk for stroke, heart attack, renal insufficiency, limb ischemia, as well as dementia [61,65-67]. The phenotypic landscape of VSMCs is adequately understood as they are quite dynamic and participate in phenotype switching. Deposition of hydroxyapatite crystals in medial layer occurs usually in the absence of infiltration of both lipids and inflammatory cells, with the latter to be recruited mainly in the initial stages [68]. Mineral disturbances, such as hyperphosphatemia in patients with CKD, lead to abnormal increase of calcium and phosphate in the blood, which in turn they are deposited in the form of hydroxyapatite crystals in the remodelled ECM and primarily in the fragmented/degraded elastic lamina [69]. In the presence of high phosphate, modulated VSMCs release cEVs and collagen-enriched ECM [70-72]. Additionally, the production of pro-inflammatory, -apoptotic and -fibrotic cytokines as well as reactive oxygen species (ROS) activate medial VSMCs dedifferentiation for initiation and propagation of medial calcification [61,73]. Apart from oxidative stress, mitochondrial impairment, cell apoptosis and loss of endogenous calcification inhibitors are also considered the main drivers for medial calcification [74,75]. Genetic aberrations also promote medial mineralisation as loss of the secreted enzyme CD73, which converts adenosine monophosphate (AMP) to adenosine, has been linked with a rare autosomal recessive genetic vascular calcification disease (ACDC - arterial calcification due to deficiency of CD73) [76,77]. Uremic toxins and advanced glycation end-products (AGEs) are considered the main contributors to renal- [78] and diabetic-induced medial calcification [79]. Progressive calcium deposition in media may distort its architecture, as calcification is extending deeper into the inner layers of the arterial wall. This results to circumferential rings of calcification in media and secondary changes in the intimal layer such as subendothelial hyperplasia characterised by increased cellularity (myofibroblasts presence). To this end, large calcification deposits in the media often contain chondrogenic-like cells, osteocytes and

multinucleated giant cells [80]. Despite the important knowledge that has been acquired, arterial calcification pathophysiology remains largely unknown. Experimental approaches in combination with omics analyses can add new insights into the molecular pathogenesis and prognosis of this clinically slowly progressing disease feature.

1.2.2 Valvular calcification

CAVD was previously believed to be a passive degenerative process caused by time-dependent movements of valve leaflets and passive nucleation of calcium and phosphate [81]. Recent experimental and clinical studies have changed this notion suggesting that CAVD is actually an active multifactorial process which involves lipoprotein infiltration and retention, chronic inflammation, myofibro- and osteoblastic trans-differentiation of valvular interstitial cells (VICs) and active deposition of calcium on the surface of aortic valve leaflets [14]. Aortic valve calcification involves a complex interplay of the leaflet structure, cellular

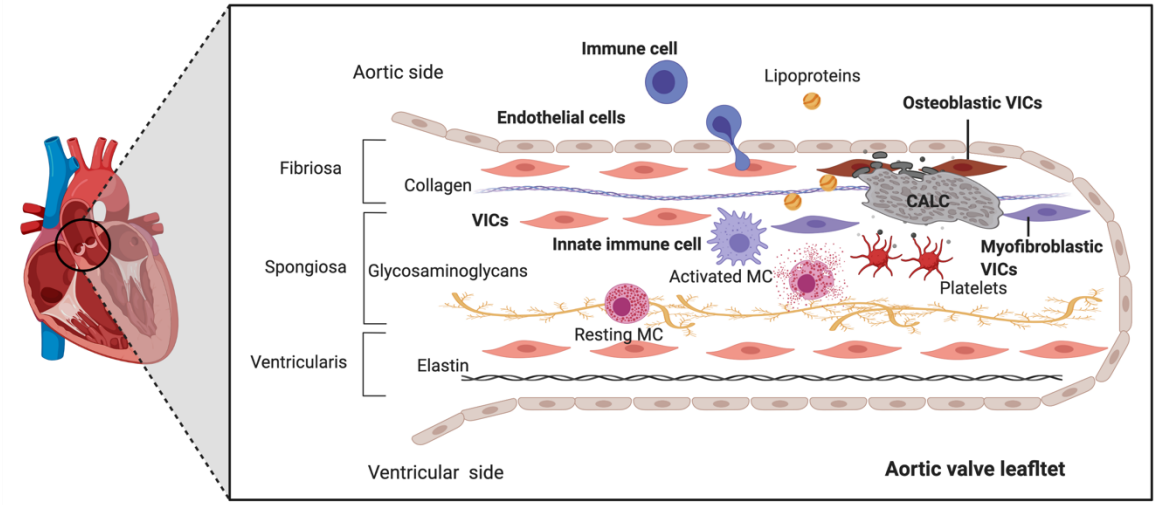

Figure 4. Pathophysiology of CAVD.

differentiation, ECM composition and secretory profile of several cell types. The aortic valve is composed of three leaflets, each one has a three-layer composition that defines the biomechanical functions of the aortic valve (Figure 4) [82]. The layer that faces the aorta is named *fibrosa* and is rich in circular collagen type I and III fibers, the middle layer is named *spongiosa* and is enriched with proteoglycans and glycosaminoglycans, whereas the one towards the ventricle is named *ventricularis* and is enriched with collagen and radially oriented elastic fibers [83]. VICs are the main cell type in the valve leaflets, with valvular endothelial cells (VECs) to cover the outer aortic and ventricular surface, serving as a membrane between the blood and the aortic valve [84]. Pathophysiology process of CAVD can be distinguished into separated phases: from inflammation to fibrosis and finally to calcification and severe stenosis. The initiation phase shares similarities with atherosclerosis [85], since both are triggered by EC damage/activation and inflammatory responses [86,87], while

fibrosis and calcification are being key features of the propagation phase [88]. A large spatiotemporal study revealed cellular, molecular and pathway heterogeneity within the valvular tissue during the calcification process [89]. The initiation phase is primarily ignited by biomechanical stress and injury in the aortic side of the valve leaflets, which cause VEC damage and activation leading to lipid infiltration and modification. Similarly to atherosclerosis, the oxidised lipoproteins, mainly LDL and Lp(a), initiate an inflammatory response within the valvular endothelium, resulting to infiltration of macrophages, MCs and lymphocytes which induce ultimately VICs activation and dedifferentiation towards an osteoblast-like phenotype [36,90,91]. Platelet-derived TGFβ1 and other releasing factors, in response to elevated aortic shear stress, have shown to modulate VIC phenotypic switching towards an osteogenic-like state promoting valvular calcification [92,93]. Similarly to atherosclerosis, NO released by VECs blocks the pathogenic differentiation of VICs into myofibroblast [40] *via* activation of NOTCH [94,95] and inhibition of TGFβ signalling pathways [40]. Moreover, in response to injury, VECs can undergo EndoMT and progressively transform into osteoblast-like cells [96]. Mineralisation of the aortic valve leaflet is caused by a series of events including oxidative stress, cEVs and apoptotic bodies. It starts in the *fibrosa* layer and is restricted close to the lipid deposition and retention sites [87,97]. Calcification triggers an even more prominent immune response, creating a positive feedback loop, which consequently leads to fibrosis and extensive ECM mineralisation, key characteristics of the propagation phase. This rapid expansion of calcified nuclei ignites CAVD progression by recruitment of additional pro-inflammatory immune cells and VICs differentiation towards ECM synthetic and osteoblastic phenotype. The fibrotic stage is the main difference between the CAVD and atherosclerosis, since collagen accumulation is beneficial in atherosclerosis to stabilise the plaque and prevent rupture and thrombosis [98]. In CAVD, activated VICs and other immune cells, including MCs, produce ECM degrading enzymes, whereas myofibroblastic-like VICs actively participate in collagen production leading to leaflet thickening and stiffening, and ultimately resulting in blood flow obstruction [99,100]. In response to persistent pathological stimuli, myofibroblastic-like VICs transdifferentiate further towards osteoblastic-like VICs, leading to formation of bone-like structures in the valve leaflets; a highly regulated process in a similar fashion as skeletal bone formation [88,101,102]. Wingless/integrated (WNT), bone morphogenetic protein (BMP) and TGF are the most well-described governing signalling pathways for VICs osteogenic transition and therefore, matrix mineralisation [103,104]. Lastly, neovascularisation and intraleaflet hemorrhage may further facilitate the recruitment of inflammatory and osteoprogenitor cells into the aortic valves [105]. Research has shown that iron accumulation in the leaflet hematoma induces global inflammation and VIC osteoblastic

modulation, facilitating the calcification process and accelerating the disease progression [106,107]. Overall, aortic valve inflammation, fibrosis and neovascularisation are associated with tissue remodeling and mineralisation processes.

1.2.3 VSMCs in arterial calcification

VSMCs represent a diverse cell population in regards to their developmental lineages as they originate from multipotent precursors such *neural crest* for VSMCs in ascending aorta, aortic arch, head and neck vessels, *somites* for those in thoracic aorta, *splanchnic mesoderm* for those in abdominal aorta and lastly, *pro-epicardium* for coronary artery SMCs [108]. Despite their different origin, contractile VSMCs share functional characteristics including morphological features and expression of "VSMC-specific" markers such as α-smooth muscle actin (αSMA), transgelin (TAGLN or sm22 alpha), smooth muscle myosin heavy chain (SMMHC; also known as myosin 11 (MYH11)), calponin (CNN1) and smoothelin (SMTN) as well as elastins, collagens and proteoglycans in the ECM [108]. Myocardin (MYOCD), for instance, is a master transcriptional regulator of the VSMC lineage [109]. A previous study from our group identified several new sensitive markers of VSMCs related to their actomyosin cytoskeleton, including PDLIM7 and LMOD1 [110]. A number of similar studies are based on the expression profiles of the above markers at gene or protein level for identification of contractile VSMCs [111]. Early studies indicated that VSMCs exhibited another distinct phenotype; the historically termed "synthetic" phenotype, recognised as a prerequisite for progression of vascular disease. However, emerging evidence suggests that VSMCs exhibit a considerable phenotypic plasticity by representing a spectrum of several phenotypes that may coexist in both normal and diseased vessel wall [112]. These different types are often accompanied by markedly different patterns in cell morphology and expression of "VSMC-specific" markers [113,114]. Recently, VSMC research demonstrated that VSMC clusters populate the plaque by a selective clonal expansion process and they exhibit important functional characteristics which contribute in different ways to disease fate [115,116]. As a result, VSMCs can go through multiple differentiation

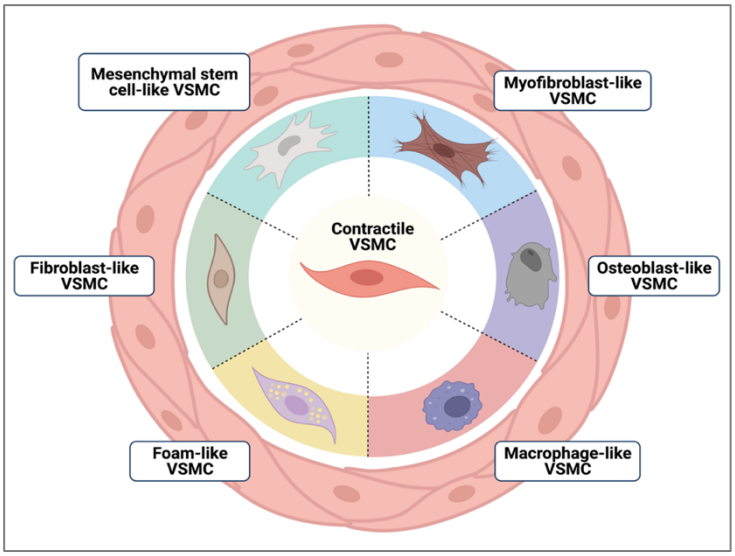

Figure 5. VSMC phenotypic plasticity.

procedures, often in parallel, at different stages and regions in the lesion. Therefore, in response to milieu stimuli, VSMCs can undergo phenotypic modulation into various lineages (Figure 5), including senescent-, foam- and macrophage-like cells, mesenchymal stem cell-like cells, fibroblasts and myofibroblasts, adipocyte-like cells and particularly osteochondrocyte-like cells [111,114,117-119]. Fibroblasts play a key role in tissue mineralisation as they can transdifferentiate into myofibroblasts with enhanced abilities of matrix proteins production, proliferation and migration under the stimulus of numerous cytokines and growth factors (GFs) [120-122]. In response to pro-inflammatory and osteogenic enviroment, myofibroblasts co-express "VSMC-specific" markers with osteogenes, suggesting that transdifferentiation of myofibroblasts into the osteogenic lineage may contribute to vascular calcification [123-125]. This myofibroblast phenotype can be acquired after fibroblasts treatment with elastin degradation products and TGFβ1 [126]. Such phenotypic switching of VSMCs is mainly characterised by lower expression of contractile proteins and myofilament density form one side, but increased proliferation and expression of pro-inflammatory cytokines as well as ECM-remodeling proteins from the other side [111], and it appears to be dependent on transcription factor 21 (TCF21) [127,128], kruppel-like factor 4 (KLF4) [115,118] and TGFβ signalling [116,129]. Methodological development of high-throughput omics technologies such as single-cell RNA sequencing (scRNAseq) along with cell lineage tracing techniques have set the space for in-depth VSMC phenotypic characterisation [118,129,130]. Tissue spatial analysis provides clear evidence that VSMC phenotypic modulation and clonal expansion are distinct and independent processes within the media layer [131]. The most intensively explored modulated VSMCs are the osteochondrogenic-like cells, which are the main cells that orchestrate vascular mineralisation in both intima and media [132]. The rise of such phenotype is accompanied by loss of "VSMC-specific" cytoskeletal markers and gain of osteochondrogenic markers including Runt-related transcription factor 2 (RUNX2), SRY-box transcription factor 9 (SOX9), Msh Homeobox 2 (MSX2), osterix (OSX), osteopontin (OPN), osteocalcin (BGLAP), alkaline phosphatase (ALP), and Type II, and X collagen [111]. Importantly, VSMC osteochondrogenic transdifferentiation precedes and is required for arterial calcification with several factors to drive the mineralisation process including oxidative stress and mitochondrial dysfunction, development and release of cEVs and apoptotic bodies, loss of calcification inhibitors, cellular senescence, uremic toxins, mineral disturbances and inflammation [74].

1.2.4 Mechanisms of VSMC-mediated arterial calcification

Although there are no osteoblast-like cells in the normal artery wall, ECs in the aortic intima, pericytes in the microvessels, myofibroblasts in the adventitia, several resident cells like VSMCs in the media or mesenchymal stem and progenitor cells have the potential to differentiate or transdifferentiate into osteoblast-like cells in response to stimuli [123,125]. Such osteochondrocyte-like cells have been found to co-localise with Ca/P deposits within atherosclerotic lesions [133]. The mechanisms that promote the initiation and progression of cardiovascular matrix mineralisation share similarities with the ones occurring in bone formation by interrelated processes (Figure 6) [134-136].

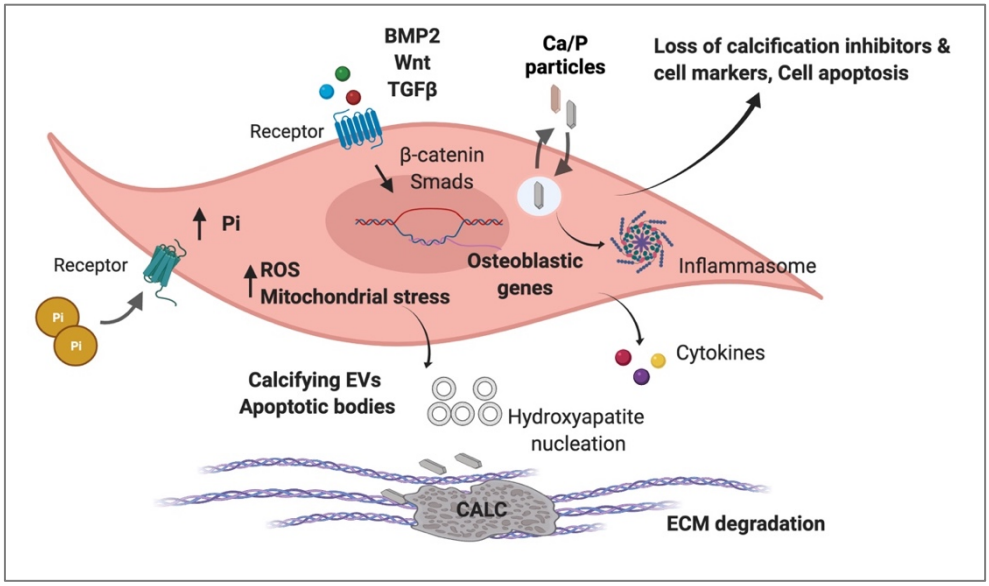

Figure 6.Mechanisms of VSMC-mediated calcification.

1.2.4.1 Phosphate-induced calcification

The physiological blood phosphate concentration in adults varies from 2.5 to 4.5 mg/dL (0.81 to 1.45 mmol/L). Elevated phosphate levels in the plasma (hyperphosphatemia), both in the form of minerals (high phosphate model as inorganic phosphate) and in biologically active form incorporated in biomolecules (β-glycerophosphate model as organic phosphate), are contributing to ectopic calcification in cardiovascular structures [137,138]. Hypersphosphatemic milieu induces phenotypic changes in VSMCs towards osteochondrogenic-like cells [137,139], including upregulation of the aforementioned osteogenic transcription factors (i.e RUNX2, MSX2) [140,141], as well as the chondrogenic transcription factor SOX9 [142]. RUNX2-specific deletion in VSMCs attenuates vascular calcification [143], whereas its accumulation in response to DNA damage adds another layer of disease complexity by bridging the DNA damage signalling to osteogenic gene upregulation [144]. In high phosphate conditions, RUNX2 upregulates the transcriptional expression of downstream target genes that regulate bone development including BMP2, ALP and Type 1 collagen. In turn, BMP2 can further induce

RUNX2 expression via a feedback loop [143]. In addition, MSX2, a well-documented BMP2 gene target in osteoblasts [145], induces the expression of both RUNX2 and OSX [146]. Under the same sources, activated WNT signalling pathway allows β-catenin translocation to the nucleus, facilitating the transcription of the osteoblast-like-related genes [104], a necessary step for RUNX2 and MSX2 downstream effects [147]. Interestingly, exposure of VSMCs to high phosphate conditions represses alkaline phosphatase (ALPL) expression and tissue non-specific alkaline phosphatase (TNAP) activity [148], whereas organic phosphate sources induce its expression [71]. In addition, high phosphate induced oxidative stress [149] and mitochondrial ROS production in VSMCs [150] trigger their osteochondrogenic dedifferentiation. In turn, elevated Ca^{2+} in mitochondria leads to secretion of cEVs into the extracellular milieu [151], interaction of which with glycosaminoglycans initiates ECM mineral deposition [46]. Lastly, both hyperphosphatemia and uremic toxins can trigger VSMC osteochondrogenic transition [148,152] and induce apoptosis and necrosis by releasing apoptotic bodies which serve as nidus for Ca/P deposition [153,154] particularly in the vessels of patients undergoing dialysis [155].

1.2.4.2 Ca/P particle-induced calcification

Ca/P nanocrystals induce VSMC osteoblastic-like differentiation and the expression of key osteogenes (for example BMP2, RUNX2, BGLAP) that modulate the ECM mineralisation [156-158], *via* upregulation of BMP2 and OPN *in vitro* [156,159]. Of interest, Ca/P particles are engulfed in the lysosomes of VSMCs leading to either increase in intracellular Ca^{2+} levels and subsequent apoptosis [47,159,160] or NLR family pyrin domain containing 3 (NLRP3) inflammasome activation [161]. Engagement of NLRP3 inflammasome is required for the phosphate-induced VSMC calcification [162]. Lastly, VSMCs cultured on hydroxyapatite crystals and calcified elastin increase their expression of RUNX2 and ALPL, demonstrating that matrix alone is able to influence VSMC phenotype [158,159,163].

1.2.4.3 Pro-osteogenic biomolecules

Members of the TGFβ and BMP families participate in osteoblast-like differentiation of VSMCs [164], engaging WNT/β-catenin signalling pathway in order to exert their pro-calcifying effects [146,165]. TGFβ1 is described as a pronounced inducer of osteochondrogenesis and calcification of VSMCs [164] *via* SMAD2/3 protein phosphorylation [166], and SOX9-mediated up-regulation of RUNX2 [167]. Similarly, BMPs binding to their receptors (BMPR1 and BMPR2) activate SMAD2/3 proteins, which then translocate from the cytoplasm into the nucleus to control the transcription of their target genes [164,168].

1.2.4.4 Loss of VSMC endogenous calcification inhibitors

VSMCs can dynamically express a variety of proteins that both promote and inhibit calcification, modulating their transcriptional program and phenotype [38,169]. The osteogenic inducers are balanced by inhibitors of calcification including matrix Gla protein (MGP), fetuin A, klotho, OPN, osteoprotegerin (OPG) and pyrophosphate (PPi) [170]. Klotho plays a significant role in Ca/P balance as in synergy with its co-receptor (fibroblast growth factor-23; FGF23) decreases phosphate reabsorption and synthesis of 1,25(OH)2 vitamin D [171]. In CKD patients [172] as well as in animal models that resemble this disease [173], klotho protein is reduced, whereas its transgenic overexpression in animal models inhibits CKD-induced medial mineralisation [174]. OPN is considered one of the earliest regulators of mineralisation process found in the vessel wall, although its mechanism of action remains elusive. Knockout studies revealed that it acts as endogenous inhibitor of calcification, since *Opn*-deficient mice developed calcifications [175,176]. In addition, it has been shown that OPN binds to positively charged calcium ions in hydroxyapatite and eliminates its growth by osteoclast-mediated hydroxyapatite dissolution [175]. Lastly, OPN deficiency in VSMCs triggers enhanced susceptibility to calcification under increased phosphate conditions both *in vitro* [177] and *in vivo* [178].

1.3 PROTEOGLYCANS IN CALCIFICATION

The ECM is the 3D architectural scaffold of the vascular wall, which allows the latter to resist to a variety of mechanical stresses, while preserves its shape and integrity [179,180]. In addition, ECM proteins can provide biochemical cues and initiate signalling cascades modulating a variety of cell processes such as survival, migration, proliferation and differentiation [181]. Dysregulation of these processes can lead to several pathologic conditions [182] including cardiovascular diseases [183,184]. The core ECM proteins comprise of glycoproteins, collagens and proteoglycans (PGs) as well as other ECM-associated proteins [181]. PGs possess essential role in lipid retention and both immune system and VSMC activation [185]. Particularly, the interaction between VSMCs and PGs is bidirectional, meaning that PGs are primarily synthesised by VSMCs, mainly upon TGF signalling, and they regulate VSMC phenotype switching. In addition, PGs actively participate in tissue inflammation, fibrosis and remodeling procedures [186,187]. PGs, based on localisation and homology, are categorised into four families as *i) intracellular ii) cell surface iii) pericellular* and *iv) extracellular* [188], which consist of a core protein with covalently-attached negatively-charged glycosaminoglycans (GAGs). The sulfated and carboxylated GAGs give rise to four types of GAGs, namely heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate

(KS), thus forming distinct PG families: HSPGs, DSPGs, CSPGs and KSPGs [188]. All these PGs are found in different locations and amounts within lesions and contribute diversely to the disease progression. Glycosylation, a post-translational process of the enzymatic addition of a saccharide compound to another saccharide, protein, or lipid, has been shown to play a key role in vascular diseases [184] and calcification [189]. Despite that PGs are synthesised by almost all cells, they only constitute a small percentage of the total extracellular matrix proteins in normal healthy arteries. On the contrary, their expression is dramatically increased during the onset of the disease [184]. Recent studies from our group identified the abundance and implication of proteoglycan 4 in VSMC and VIC osteogenic differentiation during intimal [190] and valvular [191] calcification, respectively. Moreover, biglycan, a KSPG, induces BMP2 and TGFβ1 and pro-osteogenic reprogramming of VICs [192], while decorin co-localises in calcified regions and promotes VSMCs calcification [193] *via* TGFβ signalling [194]. Emerging research supports that ECM remodeling proteins may provide prognostic and diagnostic value of plaque vulnerability and outcome [195,196]. For example, tissue osteoglycin, (OGN; also called mimecan) is linked with carotid plaque vulnerability and risk for future CV events [197], while its serum levels correlate with coronary heart disease [198] and arterial stiffness [199]. Recently, circulating levels of both cartilage oligomeric matrix protein (COMP) and its COMPneo- fragment were assessed in patients with carotid atherosclerosis, with the latter being proposed as a new biomarker to identify symptomatic carotid stenosis [200].

1.3.1 Osteomodulin

Osteomodulin (OMD, also known as osteoadherin) is a 47 kDa small leucine-rich proteoglycan (SLRP; KS-SLRP) which was firstly isolated from bovine bone extracts and characterised as a cell-binding KSPG [201]. The N- and C-terminal regions of the protein contain six and two closely-spaced tyrosine sulfate residues, respectively [202], which bind heparin-binding proteins and GFs [203]. In addition, OMD presents a unique pattern of alternative glycosylation profile among KS-SLRPs, which varies among the biomineralisation processes (Figure 7). In non-mineralised ECM, OMD is non-glycanated and N-glycosylated, whereas in mineralised ECM of developing bones, the KS substitution of OMD becomes more apparent [204]. Several studies have shown that OMD is restrictively expressed in calcified

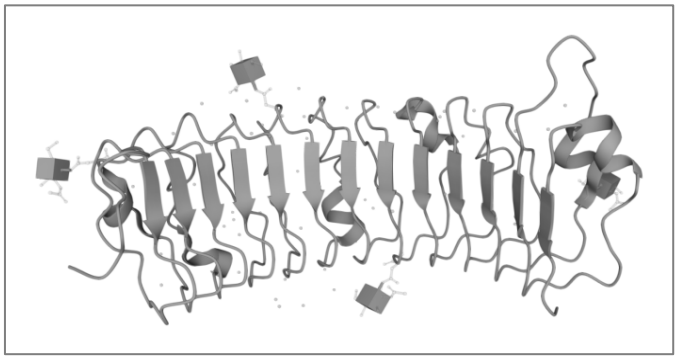

Figure 7. 3D structure of OMD core protein modelled after X-ray crystallography.

tissues, such as bones [201,205,206] and in the predentin towards the mineralisation front in the developing tooth [207-213], displaying a high binding affinity to hydroxyapatite *via* its negatively charged C-terminal domain [201,205]. In addition, extracellular matrix OMD orchestrates the diameter and shape of collagen type I fibrils [214,215]. Gene regulatory element exploration identified binding sites for osteogenic RUNX2 [216] and OSX, which both regulate OMD transcription [217]. To this end, it has been shown that OMD expression was highly upregulated during osteoblast differentiation of bone marrow- and adipose tissue-derived mesenchymal stem cells compared to adipocyte or chondrocyte differentiation [218]. OMD enhances mature osteoblast differentiation and mineralisation [206,219,220], while its expression is elevated upon stimulation with either TGFβ1 [221] or BMP2 [213,219,222] and increased osteoclastic activity [206]. Moreover, overexpression of OMD increases osteoblast viability and decreases caspase activity [222]. Studies have described OMD as an osteoblastic mechanosensitive gene [223], where its expression is increased in regions exposed to high mechanical forces such as valve leaflets [224]. ScRNAseq analysis of publicly available data of patients with hypertrophic cardiomyopathy (HCM) found OMD as a core gene in an mRNA-miRNA network [225]. Furthermore, gene ontology analysis showed that OMD expression is enriched with processes related to ECM organisation, cell-matrix adhesion and ossification, but it is suppressed with immune responses, suggesting that OMD may participate in the pathogenesis of HCM and may serve as a potential biomarker. In agreement, plasma OMD levels were upregulated in pediatric dilated cardiomyopathy compared to adult one [226]. Reports of large-scale plasma proteomic profiling of cardiovascular disease cohorts have revealed that OMD may serve as a potential novel circulating biomarker associated with cardiovascular risk traits [227] and type 2 diabetes [228]. Despite OMD implication in osteoblast maturation and several cardiovascular-relared pathologies, its function in cardiovascular calcification has yet to be fully elucidated.

1.4 INFLAMMATION IN CARDIOVASCULAR CALCIFICATION

Cardiovascular calcification lies in the intersection of chronic inflammation with bone mineralisation [22,229]. Inflammation is considered an important trigger of cardiovascular mineralisation, as several studies have shown that it precedes the development of both arterial and valvular calcification [86,230]. Both inflammation and microcalcification are engaged in a vicious cycle during the early-stage of atherosclerosis [52,231], both of which can trigger VSMCs differentiation to produce larger and more stable macrocalcifications (Figure 8). Both the innate and the adaptive immune system actively participate in the mineralisation process [232-234]. Despite the vast repertoire of immune cells, only macrophages are so extensively studied

in the context of atherosclerotic calcification [45,233]. Similarly to VSMCs, macrophages also exhibit a remarkable plasticity and functional heterogeneity which render them very adaptive to microenvironment stimuli [235,236]. The M1/M2 macrophage model has been shown to present opposing effects on extracellular endogenous mechanisms of calcification and may respond differently to a calcifying environment [45,237]. However, it should be noted, that this model is now considered a simplification of the macrophage's full phenotypic spectrum [238]. Inflammatory macrophage activity accelerates plaque calcification through many mechanisms including lipid handling procedures, cell apoptosis/necrosis, release of cEVs, which are coupled to plaque growth and risk of rupture [45]. In addition, macrophage-derived

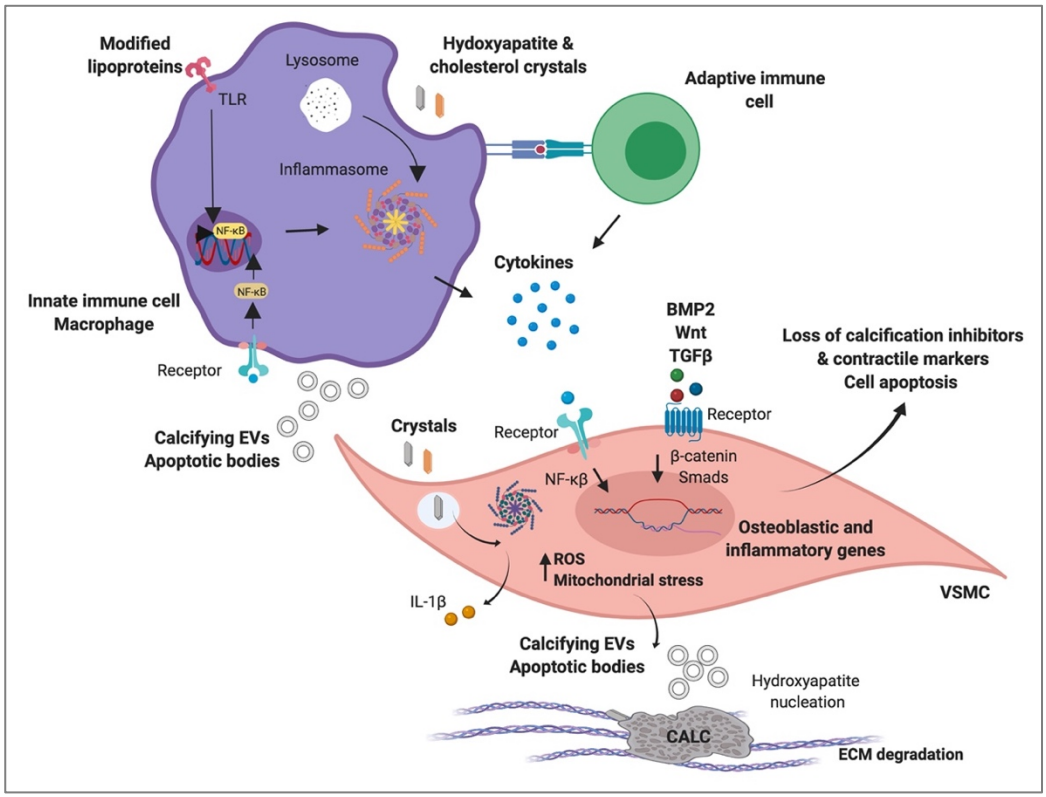

Figure 8. Crosstalk between cells from innate and adaptive immune system and their engagement with VSMC-mediated atherosclerotic plaque calcifiication.

inflammatory mediators impact VSMC endogenous calcification inhibitors and promote VSMCs transdifferentiation into osteochondrocyte-like cells [38,45,231,233]. In reverse, microcalcification exacerbates inflammation as Ca/P crystals activate macrophages to release inflammatory cytokines [45,231]. Apart from pro-inflammatory molecules [239], macrophages release several pro-osteogenic cytokines that also modulate VSMC phenotypic switching [240]. Genetic lineage reprogramming of osteochondrogenic VSMC phenotype engages secretion of cEVs, upregulation of osteogenic markers, while downregulation of "VSMC-specific" markers [45,233]. Moreover, interenalisation of cEVs can further induce calcification of recipient VSMCs [151]. Reduction of microcalcification formation and enhancement of pro-fibrotic activities are associated with well-established plaque stabilisation processes. In heavily

calcified atherosclerotic plaques, calcification-related macrophage subtypes are appeared [45] particularly in the surrounding of macrocalcification areas, where they acquire less pro-inflammatory, but more reparative osteoclast-like features, contributing thus to regression of calcification (Figure 9) [241-243]. Transcriptomic pathway analysis of these advanced-calcified atherosclerotic plaques has shown that inflammation processes are heavily suppressed while the VSMC-related processes are upregulated, contributing to plaque stability [27,244]. Despite that many macrophage mechanisms contributing to either progression or regression of vascular calcification have been studied, their heterogeneity may lead to rather unexplored effects. In addition to macrophages, other not well studied immune cells including dendritic,

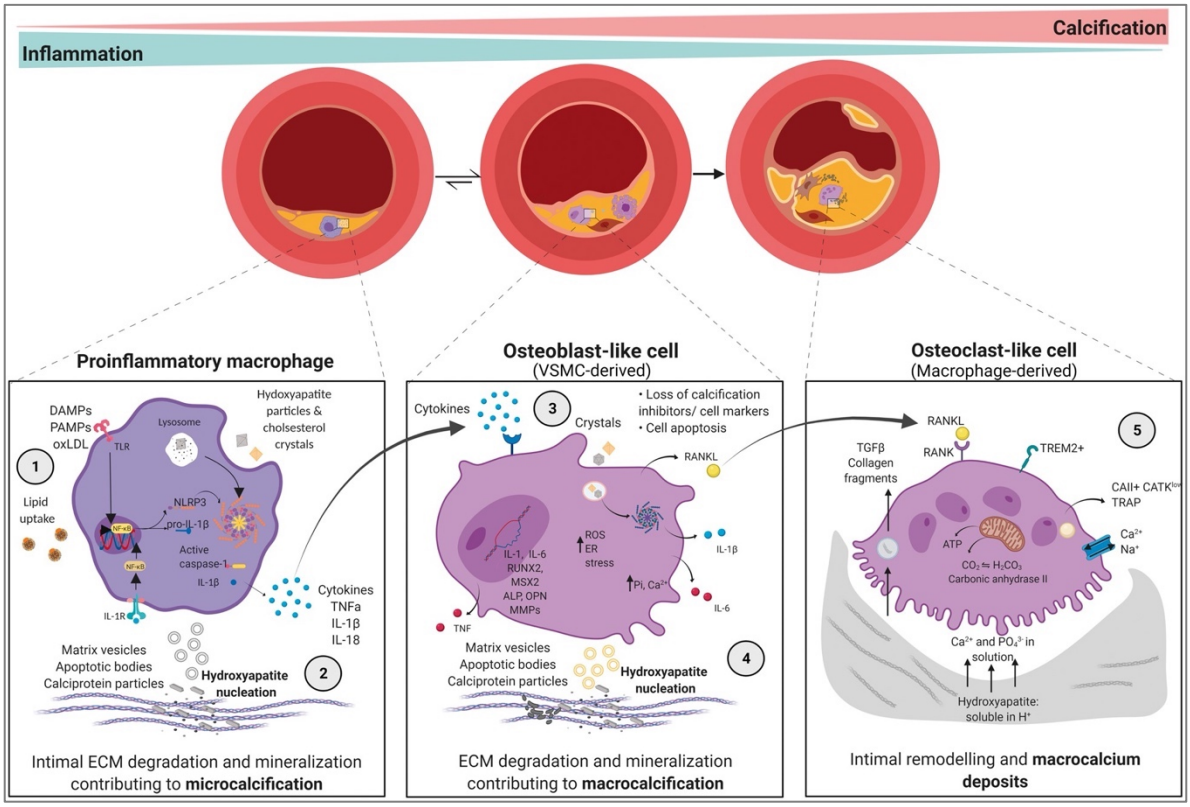

Figure 9. Interplay between macrophages and calcification in atherosclerotic plaque from early to late disease stages [45].

NK cells, T cells and MCs [233], participate in calcification. Each of them exhibits a great phenotypic plasticity and functional diversity, leading to pleiotropic effects in cardiovascular calcification [236,245].

1.4.1 Mast cells in calcification

Mast cells (MCs) are hematopoietic cells derived from progenitor cells that circulate in the blood. After their recruitment into tissues, MC progenitors mature in response to specific stimuli within the tissues [246]. MCs are diverse inflammatory cells that act in the first line of defense primarily against pathogens and they have been found to be located within the cardiovascular system, including the myocardium, the aortic valve and the atherosclerotic

plaque. Activation of MC, predominantly by cross-linking of FcεRI with IgE, can contribute to the pathogenesis of CVDs [247-249], with high cell numbers present in human calcified leaflets to be associated with disease severity [250]. MC activation occurs when ligands such as antibodies (including IgE and IgG), lipopolysaccharide, complement peptides, substance P, or neuropeptide Y bind and interact with their respective receptors (mainly Fcε receptor-1, Fcγ receptors and Toll-like receptors) on the cell surface [247]. Additionally, interaction of MCs with adjacent cell types in the plaque can also modulate their phenotype [251]. MC activation initiates a signalling cascade which leads to either rapid secretion of stored in granules mediators in a process termed "degranulation", or *de novo* synthesis and release of cytokines, chemokines and eicosanoids [252]. The secretory granules contain a whole array of mediators, including histamine, proteoglycans (for example heparin and serglycin - SRGN), neutral proteases (including chymase and tryptase), cathepsins and a variety of pro- and anti-inflammatory cytokines and GFs (like TGFβ1) [253]. Tryptase and chymase are the most precise markers to decipher MC phenotypic heterogeneity in tissues. All human MCs contain the cell-specific protease tryptase, specifically expressed by MCs in atherosclerotic tissues, while a fraction of them (around 40%) contains chymase and other granule proteases (including carboxypeptidase A3 and cathepsin G) [252,254]. In turn, the secreted molecules act on the adjacent cells and influence the surrounding microenvironment by shaping their functions and responses. In atherosclerotic plaque, activated MCs are predominantly found in the "shoulder" regions and particularly at the place of erosion or rupture in patients who died from myocardial infarction [255-257]. Human studies have revealed an association of MCs with plaque neovascularisation, microvessel density, IPH and thrombus formation, features that increase the risk of adverse events [258-262]. Particularly, higher plasma levels of MC tryptase were found in carotid atherosclerotic patients with CV events [262]. Animal experimental studies have illuminated the crucial role of MCs in atheroprogression and plaque vulnerability [263,264]. In advanced lesion of atherosclerotic mice, MC activation resulted to plaque vulnerability by inducing cell apoptosis including endothelial cells, macrophages and VSMCs [247,265]. In line with this, MC-secreted chymase induced matrix degradation which led to VSMC apoptosis [266], while SRGN, an intracellular PG found as a secreted complex in the ECM, is utilised for the assembly and packaging of the several mediators in the granules [267]. Moreover, it has been shown that degranulated proteases trigger neutrophil recruitment at the site of inflammation [268], while MCs can interact with dendritic cells [269], T cells [269,270], and $CD4^+$ cells in a direct manner as antigen presenting cells [271]. Worth noting that MCs are localised proximal to calcified regions within human plaques [272], and a connection between MC activation and carotid plaque macrocalcification was recently identified in human studies

based on data analysis of the large Biobank of Karolinska Endarterectomies (BiKE) [27], however its role and the molecular mechanisms involved in VSMC-mediated calcification have yet to be elucidated.

1.5 UNMET NEEDS IN CARDIOVASCULAR CALCIFICATION

"We are drowning in information, but starved for knowledge"

John Naisbitt

Up to date, prompt diagnosis and specific treatment strategies are not available to reduce disease burden and mortality, result of our lack of understanding to the fullest those pathophysiological processes by which minerals are deposited in cardiovascular tissues. The precise molecular mechanisms and biomarkers that allow for a better characterisation of plaques vulnerable features and predict patient disease progression are still an unmet need and an urgent problem to be solved. These gaps in knowledge illustrate clearly the need for a multidisciplinary, integrative and translational experimental strategy that can better model the complex molecular processes and improve the characterisation and classification of the risk patients [273]. Technological advances in characterising the molecular and cellular heterogeneity of the diseased tissue have unleashed a great potential for deeper understanding of cardiovascular calcification complexity.

1.5.1 Multi-omics data analysis

Although the prevalence of CVDs continues to increase significantly worldwide, a detailed understanding of the underlying mechanisms resulted to disease development is still lacking. The discovery of insightful mechanistic players and biomarkers in single-omics studies have benefited all fields of science, including cardiology [274,275]. However, this classical analysis of individual layer of disease is restricted to superficial explanations, whereas the complete understanding of such complex human disorders requires an assemblage of several omics layered networks [276]. Recent technological advances have logarithmically amplified the spectrum of available omics data with the latter to include genome, epigenome, transcriptome, proteome, metabolome, lipidome to even microbiome. A holistic view of the disease requires an integration of multiple layer analysis across several omics in order to achieve an individualised network-based precision medicine (Figure 10) [277,278]. Therefore, the collection of multi-omics data strongly contributes to elucidate the interactions across disease layers and helps to reconstruct detailed biochemical networks, which can be further tested in high-throughput molecular and functional studies. Then, accurate interpretation of the relationship between multidimensional data promises the development of tailored

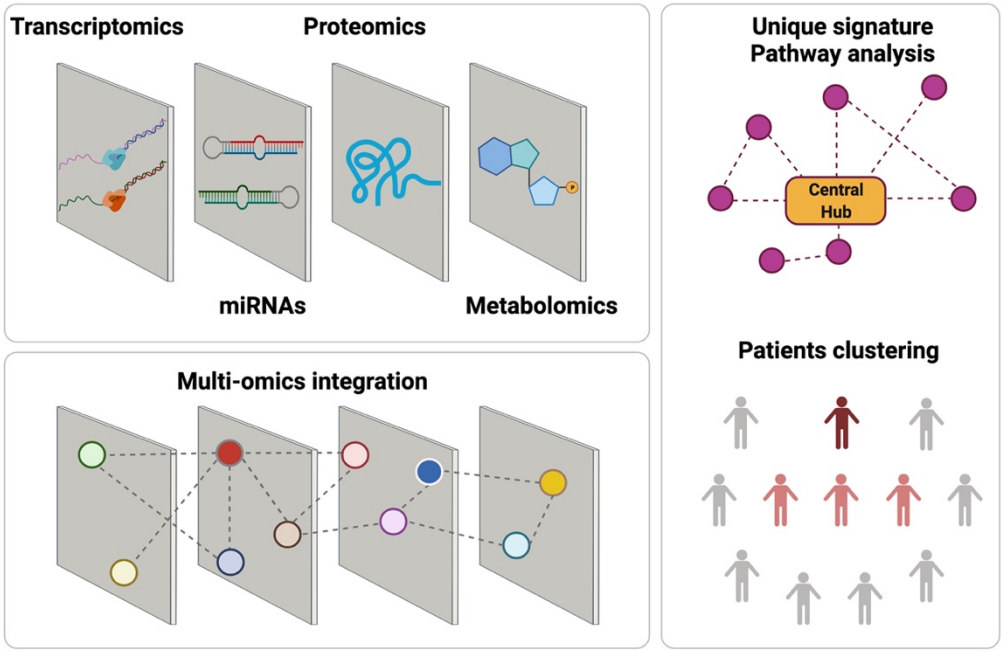

Figure 10. Integration of multi-omics data reveals unique regulatory networks and molecules significant for disease pathogenesis.

therapeutics [278,279]. However, the current available methodological tools are still immature and initial steps are being taken [280]. Utilisation of multiple computational methods including artificial intelligence, deep learning and high-resolution imaging will dramatically improve the quality and reliability of predicted underlying biological processing [281]. Recently, our lab reported the first virtual transcriptomics methodology to predict carotid plaque gene signature based on plaque morphology data generated from pre-operative computed tomographic angiography (CTA) imaging analysis [282]. In addition, approaches to decipher the complexity of vascular calcification by multi-omics integration, network-based medicine and use of artificial intelligence have already been reported [283-285]. Lastly, far beyond the classical analyses, multi-omics approaches allow the characterisation of ECM composition [286] and identification of a large number of circulating biomarkers in order to predict CV events, progression of subclinical atherosclerosis [287,288] and CAVD [89,289] and molecular pathways as targets for therapeutic intervention.

1.5.2 Diagnostic imaging tools

Carotid artery disease and CAVD are commonly diagnosed with conventional non-invasive diagnostic methods including ultrasonographic (US) examination that determine tissue anatomy and blood flow parameters and magnetic resonance imaging (MRI) or CTA, which both can evaluate the degree of stenosis, excluding plaque characteristics. CTA is particularly good for estimating calcification, where the density of the tissue is measured in Hounsfield Units (HU) of the pixels in the radiological image. Due to enrichment of current understanding of cardiovascular calcification and inflammation as well as their significant

role in plaque morphology and disease fate, recent technological modalities in imaging and computational fields have been developed. These in turn provide valuable information for tissue morphological structures in great resolution and high reproducibility [290]. Such methodologies have revolutionised the classical imaging tools which in combination with advanced histological microscopy and artificial intelligence allow a 3D reconstruction of vessel architecture, uncovering important information including vessel volume, geometry, cellular and acellular morphological components [244]. In addition, MRI holds great potential in plaque imaging due to its capability to detect features of soft-tissue like LRNC, neovessels, IPH as well as fibrous tissue [291]. Positron emission tomography (PET)-CT imaging in combination with [^{18}F]-fluorodeoxyglucose (^{18}F-FDG) and [^{18}F]-sodium fluoride (^{18}F-NaF) allow the tracing of inflammation and (micro-) calcification, respectively, and therefore, both tracers can be used as imaging biomarkers to detect vulnerable plaques [292,293]. Optical coherence tomography (OCT), an intravascular imaging approach, offers the possibility due to its micron scale resolution to identify key features of plaque vulnerability such as thin fibrous cap, LRNC, macrophage density, the presence of thrombus, microvessels, and calcific nodules [294]. Overall, advances in both basic research and imaging tools help to functionally annotate morphological features and molecular and cellular components of diseased tissue, relevant to cardiovascular calcification and inflammation, which can add thus valuable knowledge for disease outcome.

1.5.3 Disease specific biomarkers

Calcification is considered an independent predictor of atherosclerosis-related CV events with CAC and extra coronary calcium scores being the only tools for quantitative evaluation of cardiovascular calcification by CTA images [295]. In addition, CAC score is used as a tool to stratify individuals into high- or low-risk categories [296]. Despite that our knowledge regarding the role and the drivers of cardiovascular calcification has momentously increased, accurate biomarkers are still lacking. Far from calcification, blood biomarkers related to systemic inflammation, such as high-sensitive C-reactive protein, TNF-α, interleukin 6 (IL-6) [297], or N-terminal pro-brain natriuretic peptide (NT-proBNP) are used routinely in clinical practice [298]. Recent advances in proteomic and metabolomic fields have empowered significant progress towards the investigation of the role of ECM in atherosclerosis [195,196]. Several matrix components can differentiate asymptomatic *vs*. symptomatic carotid plaques, and thus be used as biomarkers for cardiovascular risk prediction [299]. Released cEVs enriched with pro-calcific proteins could be used as potential biomarker in serum [300], while levels of several soluble circulating calcification markers such as klotho, MGP, OGN, OPG, fetuin A,

sclerostin, and sortilin could also be used to prognose vascular calcification and provide valuable information for high-risk patient identification and stratification [301-303]. Recently, large-scale proteomic data proposed OMD as a potential novel circulating biomarker, associated with cardiovascular risk traits [227] and type 2 diabetes [228]. However, none of these aforementioned biomarkers have been introduced into routine laboratory practice and such remains challening. The clinical relevance of each newly proposed marker must be evaluated in regards a) to which phage of the disease a specific biomarker may be more informative, and b) to which cohort it must be applied in order to predict plaque vulnerability prior to symptoms' manifestation.

Overall, we are currently facing an overwhelming amount of research data generated by multi-omics and imaging analysis, biomarker exploration and patient clinical data, without this emerging knowledge being translated into patient care. Despite this progress, our understanding is still restricted to surface interpretation of the key regulatory mechanisms that drive disease pathogenesis. Combination of molecular networks and morphological features implies that a unified model of vulnerable atherosclerotic plaque is not representative and forces for re-consideration of the diversity of vulnerable phenotypes [304]. The current thesis aims to provide new insights into the vulnerable lesion components by means of molecular, cellular, morphological and clinical data integration.

2 RESEARCH AIMS

This PhD project is part of the EU Marie Curie International Training Network program INTRICARE with primarily focus to understand the earliest processes associated with vulnerable plaque formation, and ultimately to develop novel preventive, diagnostic, and therapeutic tools that will improve current clinical practice.

The general aims of the thesis were to:

- Unveil novel molecular pathways and networks in calcification
- Understand the underlying mechanism between cellular phenotypic switching and vunlerable plaque morhological features
- Identify and functionally characterise markers of calcification and link those with future CV events

More specifically, the objectives of this thesis were to:

I. Identify a novel molecular calcification signature of CAVD (***Study I***)
II. Investigate the role of OMD in cardiovascular calcification and assess its role as a potential biomarker (***Study II***)
III. Relate carotid OMD gene expression with future CV events (***Study III***)
IV. Explore the link between MC phenotype and key vulnerable plaque morphological and cellular features and clinical symptoms (***Study IV***)

3 MATERIALS AND METHODS

3.1 HUMAN BIOBANKS

Biobanks of human specimens constitute a valuable and indispensable resource for successful biomedical research. Well-documented and extensive sample collection and repository give the possibility to study numerous urgent medical questions and initiate international research collaborations. All studies used in the current thesis were approved by the local ethical review boards and were carried out in accordance with the principles of the Declaration of Helsinki. The individual human data are protected by General Data Protection Regulation enforced by EU and ethics laws that regulate the privacy of individuals who participated in the studies.

The Biobank of Karolinska Carotid Endarterectomies (BiKE) was used in *Studies II* and *IV*. BiKE prospectively enrolls patients undergoing surgery for carotid stenosis (>70% NASCET) [305], and it is consisting of more than 1500 patients' materials and medical records. After CEA, the excised carotid plaques are collected and stored in combination with patients' plasma. Particularly, the proximal half of the carotid plaque is sent for microarray profiling and the distal part is used for histological evaluation. In addition, BiKE contains genetic, transcriptomic, proteomic and metabolomic signature of carotid plaques as well as an ImageBank with quantified diagnostic images by CTA/US. Moreover, medication-related data, such as cholesterol-lowering (ezetimibe, statins), anti-diabetics (metformin, insulin) and anti-hypertensives (ACE inhibitors, calcium antagonists, beta-blockers, diuretics, angiotensin II blockers), are also included. In BiKE, about 70% of the patients experienced symptoms (S) defined as minor stroke (MS), TIA and AFX (retinal TIA). Patients without qualifying symptoms within 6 months prior to surgery were categorised as asymptomatic (AS) based on results from the Asymptomatic Carotid Surgery Trial (ACST) [306]. Calcification and other plaque morphological features were estimated by the VascuCAP software, Elucid, USA after processing of the CTA images.

In *Study III*, carotid plaques were collected from patients with or without ipsilateral symptoms during CEA at the Vascular Department of Skåne University Hospital (Malmö, Sweden) between October 2005 to June 2015 (The Carotid Plaque Imaging Project – CPIP). Patient's carotid arteries were examined with a pre-operative ultrasound to evaluate the degree of stenosis. Plaques associated with ipsilateral symptoms (TIA, AFX or ischemic stroke) and stenosis >70%, or plaques not associated with symptoms (AS) but with >80% stenosis were considered for surgery. Patients were followed up for CV events and death retrieved from Swedish national registers. All causes of death were confirmed through the

National Population Register. Follow-up time was 57 months for CV event and 70 months for CV death.

In *Study II*, findings related to CKD were explored based on a special cohort comprised of inferior epigastric artery biopsies and blood samples from CKD stage 5 patients undergoing kidney transplant surgery at the Karolinska Hospital, Sweden. The biobank's material can be used for a wide range of clinical analyses, including biomarker discovery. Circulating OMD levels were explored in a subset of 98 fasting blood samples which were collected prior to the surgical procedure. Patient selection was based on histological von Kossa staining evaluation of vascular media calcification score (CS): grade 0 exhibits no media calcification (n=25), grade 1 represents minor media calcification (n=25), grade 2 exhibits moderate media calcification (n=24) and finally grade 3 represents severe media calcification (n=24). The two predominant comorbid conditions that the patients presented were CVDs (25%; cerebrovascular, cardiovascular, and/or peripheral vascular disease) and diabetes mellitus (19%). A great majority of the patients were treated with phosphate binders (90%) and erythropoiesis-stimulating agents (82%), while 42% were on cholesterol-lowering drugs. Antihypertensive medications included ACE inhibitors and/or angiotensin II receptor antagonists (55%), betablockers (58%) and calcium-channel blockers (55%).

In *Study II,* a cross-sectional observational study of was used to investigate further the relation of OMD with aortic valve calcification. Human aortic valves were obtained from patients with CAVD scheduled for aortic valve replacement at Maastricht University Medical Center+ (MUMC+), The Netherlands.

3.2 TISSUE GENE EXPRESSION PROFILING

The most commonly used methodologies for transcript profiling are quantitative Polymerase Chain Reaction (qPCR), microarrays, RNA sequencing (RNAseq) and single cell RNA sequencing (scRNAseq). qPCR is broadly used as a validation method of the mRNA expression because it offers high specificity and sensitivity.

3.2.1 Microarrays

Microarrays, (*Study II* and *IV*), have become an indispensable tool for global transcriptome analysis of differentially expressed genes (DEGs). They offer the advantage of creating a low-cost gene expression profile signature, providing comparable data of the molecular processes and pathways associated with disease or healthy state. Still, gene microarray technique is used to some extent in new projects to facilitate a broad exploration of the underlying mechanisms. In *Study II* and *IV*, RNA, extracted from isolated carotid plaques, was first examined for quality with respect to its purity and integrity. Then, it was processed

by the Karolinska Institutet Bioinformatics and Expression Analysis core facility. The microarray global gene expression signature was conducted by using the Affymetrix, Human Transcriptome Array (HTA) 2.0 chip.

3.2.2 RNA sequencing

As next-generation high-throughput sequencing technologies have emerged, RNAseq is used more often than microarrays to profile the complete set of transcripts of the under investigation sample. In *Study III*, RNAseq libraries were prepared from human carotid atherosclerotic plaques and samples were sequenced using the Illumina HiSeq2000 and the NextSeq platforms. RNAseq analysis provides a high sensitivity measurement of the absolute expression levels and it is capable of identifying new genes and transcript isoforms compared to microarrays. Additionally, RNAseq reduces the background noise because the cDNA sequences can be precisely mapped to the genome [307].

3.2.3 Single cell RNA sequencing

Beyond bulk tissue RNAseq, which fails to resolve specific cell types transcript signature, scRNAseq data analysis of human carotid and coronary plaques were used in *Study II*. ScRNAseq methodology quantifies the transcriptome of individual cell, offering thus an enormous potential for *de novo* discovery by deciphering the cellular heterogeneity and plasticity in healthy and atherosclerotic arteries [308]. Development of this technology has distinct advantages and applicability especially in combination with spatial tissue omics characterisation. However, the high running costs and the generation of large and complex datasets that need further bioinformatic processing are the main obstacles to get scRNAseq widely used.

3.2.4 Microarray deconvolution data

To enumerate the relative abundance of cellular populations in the plaque, mRNA deconvolution strategy was applied in *Study IV* to tissue microarrays by using previously described procedure *via* the CIBERSORT software (https://cibersort.stanford.edu) [309]. In brief, a "cell-type signature matrix" of 22 well characterised immune cell populations was defined by RNAseq or scRNAseq analysis. Then, BiKE tissue bulk microarray data were used to estimate the relative frequencies of these hematopoietic immune cell populations in the plaque (Figure 11) [309,310]. CIBERSORT is a useful methodology for accurate high throughput characterisation of diverse cell subsets deriving from bulk mixtures with unknown cell populations.

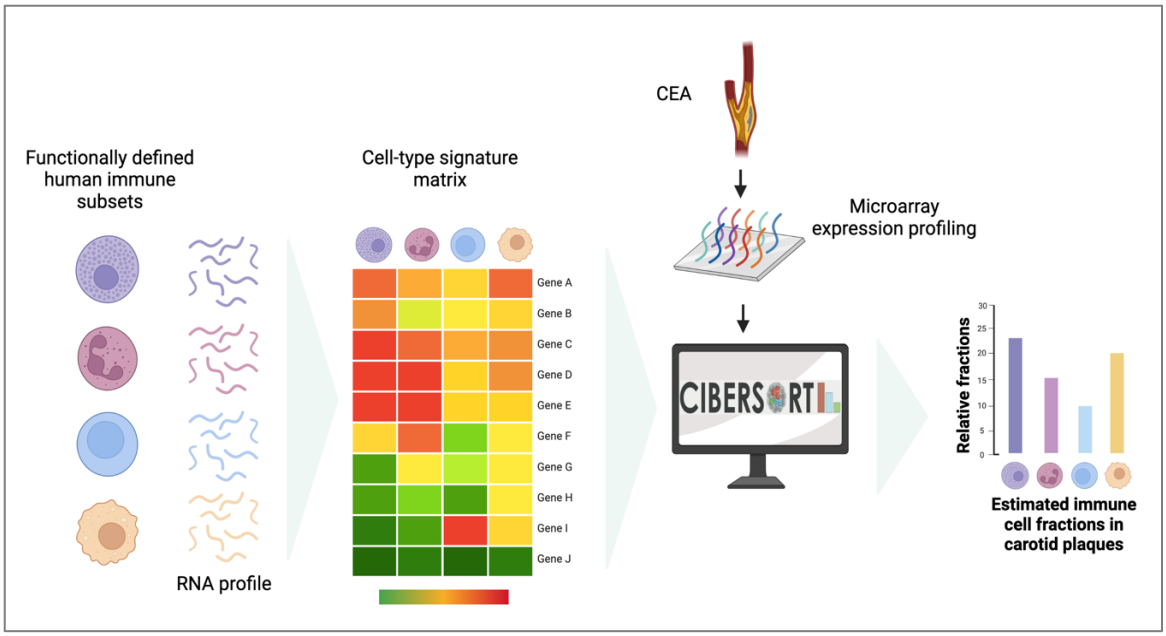

Figure 11.CIBERSORT workflow. CIBERSORT requires as input a "cell-type signature matrix" that is enriched for each cell type of interest. This matrix in turn can be applied to characterise cell type proportions in bulk tissue expression profiles.

3.3 BIOINFORMATIC ANALYSES

Gene expression profiling provides unique opportunities to study molecular and biological patterns of gene expression regulation. Over the past years, numerous methodologies have arised for gene expression data analysis. Large-scale transcriptomic experiments generate huge raw data that must be processed in order to obtain biological function. Thus, the primary aim is to identify a set of DEGs between two or more conditions. Such DEGs are identified through a multiple testing adjustment which is the optimal way to handle the thousands of genes represented on an array [311]. In high-throughput gene expression studies, statistical methods that control the multiple test error rate have emerged as powerful tools including the false discovery rate (FDR). FDR represents the expected proportion of wrong rejections among the rejected hypotheses. Bonferroni adjustment is another way to control experiment-wide error probabilities when multiple comparisons are being made, and it identifies the pairwise comparisons where a DEG is significant. Then, the set of filtered genes are ordered in a ranked list (up- or down-regulated), according to their differential expression (fold change) between the two or multiple conditions [312]. The gene list can then be imported to the public web-based programs, which give a powerful analytical method for interpreting gene expression data by clustering them according to common biological function, cellular compartmentalisation, chromosomal location, or regulation [313]. Of note, the web-based portal SubCell BarCode served for querying single gene subcellular localisation (www.subcellbarcode.org). The significantly up- and down-regulated genes associated with specific biological processes are defined by the comparison between the two phenotypes.

Both several omics bioinformatic and pathway enrichment/overrepresentation analyses have multiple advantages since they reduce the dimensionality from thousands of genes to a smaller number of DEGs and pathways, eliminating at the same time the multiple hypothesis testing error. In addition, they provide informative results for further experimental exploration and interpretation of the causal disease mechanisms.

3.3.1 Pathway analysis

For the pathway analysis in *Study I*, ConsensusPathDB database (http://consensuspathdb.org/) was used employing the canonical pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and Biocarta. Fisher's exact test was applied for computing significance of the annotation sets with respect to input molecules. Pathways with adjusted q-value<0.05 were considered as significantly enriched. To identify the KEGG pathways of the differentially expressed miRNAs, an *in-silico* analysis was conducted using the web database: DIANA Tools mirPath v.3 (http://www.microrna.gr/miRPathv3). Combination of predicted (DIANA-microT-CDS) and experimentally (DIANA-TarBase v7.0) supported interaction algorithms were used for prediction of microRNA functional annotation. In *Study II*, the up- and down-regulated gene lists were imported to the Enrichr program (https://maayanlab.cloud/Enrichr/); a comprehensive gene set enrichment analysis web tool based on Fisher's exact test. Enrichment analysis of DEGs is mainly limited to gene ontology (GO) terms. Alternatively, gene lists are projected onto known protein–protein interaction networks and signalling molecular pathways. The latter include genes participating in the pathway databases such as KEGG or Reactome [312]. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org/) 11.0 database was used to both identify interacting proteins based on experimental data and built the protein-protein interaction network. Lastly, based on gene ontology origin, pathways can be fused so to reduce their redundancy.

3.3.2 Multi-omics approaches

The large data sets produced by omics approaches can also be integrated for further discovery of novel molecules and pathways. In *Study I*, multi-omics data integration was performed using the web-based tool OmicsNet (https://www.omicsnet.ca). Genes and proteins were annotated according to their official gene symbol and microRNAs according to miRBase ID. The layered network was built as previously described [314]. Combination of several databases was used to build an accurate 3D layered network primarily based on experimentally validated data according to a) IntAct database (manually curated experimentally validated PPI), b) miRNet (experimentally validated miRNA targets information based on TarBase and

miRTarBase) and c) ENCONDE (transcription factor (tF)-gene interactions (TGI) derived from ENCODE CHIP-seq data). In case of a network bigger than 3000 nodes, the minimum network setting was taken into account for further evaluation. Only interactors that interact directly with seeded nodes were considered for further pathways characterisation through the network pathway analysis using KEGG and Reactome databases. Finally, MetaboAnalyst v4.0 (https://www.metaboanalyst.ca/) was used for integration of seeded molecules and metabolites into a metabolite–gene–disease interaction network based on their biological function.

3.4 *EX VIVO* CULTURE OF HUMAN CAROTID ATHEROSCLEROTIC PLAQUES

Ex vivo culture of patient tissues facilitates the characterisation of supernatant profile or the screening of the tissue response to candidate agents and compounds, contributing thus to more individualised therapeutic approaches. In *Study II* and *IV*, freshly isolated carotid atherosclerotic plaques were used obtained from patients undergoing CEA at Karolinska University Hospital, Solna. On the day of operation, the plaque was collected at room temperature, washed and cut into small pieces (~2 mm^3) with forceps and scissors. The pieces were distributed into a petri-dish and immediately incubated in RPMI 1640 medium supplemented with 10% FBS at 37 ^oC in 5% CO_2. After 24 h, the pieces and the supernatant were harvested and stored at -80^oC for further analysis. The supernatants were particularly used for cytokine profile assessment by ELISA.

3.5 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a specific and robust methodology suited for quantitative evaluation of secreted proteins in supernatants and blood or its constituents. ELISA is widely used both in research and in clinical routines as it provides valuable information for diagnosis of various diseases. In Study *II*, a sandwich OMD ELISA assay was used to quantify the protein OMD levels in the plasma of carotid atherosclerotic and CKD patients. In addition, a sandwich ELISA assay was used to quantify the IL-1β levels in calcified VSMCs in *Study II* and *IV*.

3.6 *IN VITRO* MODELS OF CALCIFICATION

Experimental *in vitro* studies for calcification can illuminate its complex pathobiology and serve as a promising tool for drug screening [315,316]. Both inorganic and organic phosphate models are widely used for *in vitro* calcification assays. Alternatively, calcium, alone or in combination with phosphate, can also be used. Above a certain concentration of phosphate

(~2mM) in cell medium, CaP crystals begin to precipitate [317]. Worth noting that organic phosphate in the form of β-glycerophosphate acts as a substrate for TNAP enzyme which causes β-glycerophosphate degradation into inorganic phosphate in the culture media. Ascorbic acid, an essential co-factor for a variety of enzymes, induces ECM synthesis and remodeling in culture, while dexamethasone triggers osteogenic differentiation of VSMCs by inhibiting endogenous calcification inhibitors. In the current thesis, to induce calcification cell medium was supplemented with either 2.6 mM phosphate for up to 12 days or 10mM β-glycerophosphate in combination with 0.1 mM l-ascorbate phosphate and 10 nM dexamethasone for 14 days [318]. In both cases the medium was refreshed every 3 days. Commercial primary human aortic smooth muscle cells (HAoSMCs) and human coronary artery smooth muscle cells (HCoSMCs) as well as HAoSMCs isolated from a non-diseased region of aortic tissue obtained from patients undergoing surgical thoracic aortic aneurysm repair were used for *in vitro* calcification assays.

3.7 *IN VIVO* MODELS OF CALCIFICATION

In addition to *in vitro* studies, *in vivo* experiments provide information encompassing the whole systemic pathophysiological context of calcification. Thus, preclinical models using mice and rats have been generated and established, offering a broader overview of the whole organism. Since rodents are resistant to vascular calcification, several intervention procedures including genetic manipulation, surgery, specific diets and drugs are needed for induction of a certain degree of disease.

3.7.1 Mouse model of atherosclerotic intimal calcification

Common animal models for atherosclerosis are the hyperlipidemic apolipoprotein E (ApoE)- or low-density lipoprotein receptor (LDLr)-deficient mice, especially under high-fat diet [319]. Global *ApoE* gene deletion results in atherogenic aggregation of cholesterol-rich remnants [320], clearance of which requires functional apolipoprotein E, and development of calcification in highly advanced lesions of innominate arteries of aged chow-fed mice [321]. Hyperlipidemic *ApoE*$^{-/-}$ mice under specific diets (for example Western type diet) accelerate calcification process. Additionally, the pharmacological use of warfarin is linked with an increase in systemic vasculature calcification in humans [322,323] and rodents [324,325]. The observed increase is due to inhibition of MGP [326], a vitamin K-dependent protein that prevents systemic calcification by cleaning CaP in the tissues. In *Study II*, calcification of atherosclerotic plaques was induced as previously described [327], C57BL/6 *ApoE*$^{-/-}$ mice fed with a vitamin K-deficient Western type diet enriched with warfarin (Figure 12A). In brief, the control group

received vitamin K1 (100 µg/g) while the warfarin group received combination of warfarin (3.0 mg/g) and vitamin K1 (1.5 mg/g). Low amounts of vitamin K1 introduced to avoid warfarin deleterious manifestations on the liver and thus prevent extensive bleeding, but establishing vitamin K-deficiency in the periphery [328]. Mice aortic arch and the innominate artery were collected at different times (7, 9 and 13 weeks) for further immunohistochemistry analysis.

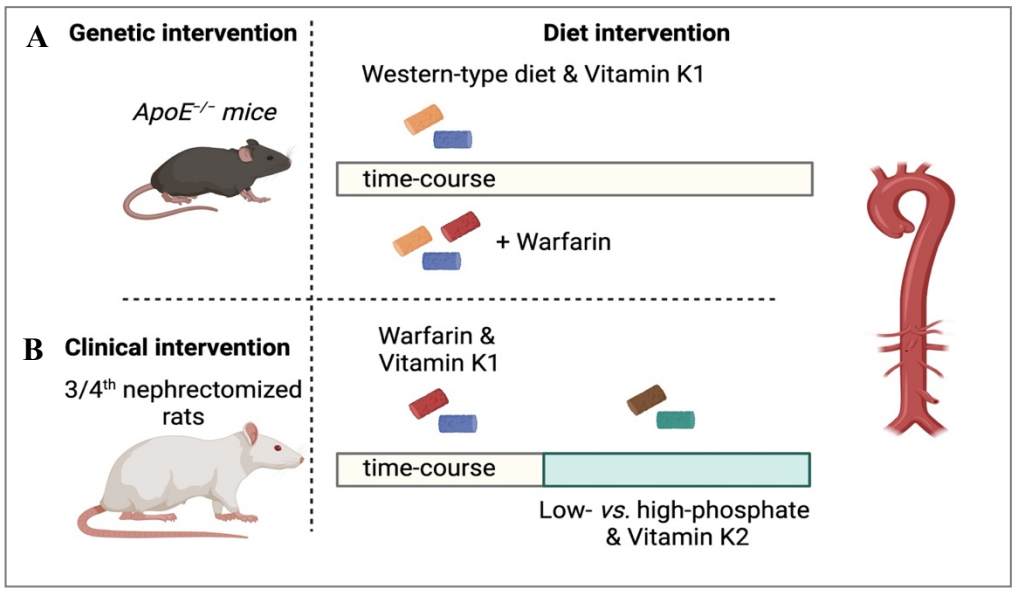

*Figure 12. In vivo models of intimal **(A)** and medial **(B)** arterial calcification.*

3.7.2 Rat model of vascular medial calcification

In line with the calcification development in uremic milleu in humans, rodents develop analogous medial mineralisation under such conditions. Rats with normal lipid levels are subjected to uremia *via* a surgical unilateral 5/6th nephrectomy [329] in order to mimic renal failure *in vivo*. Due to high mortality rate after a 5/6th nephrectomy, in *Study II*, male Sprague-Dawley rats were subjected to a 3/4th nephrectomy that resulted in an increase of urea and creatinine values, underscoring the validity of this model for studying advanced CKD [330]. In addition, special dietary supplements are required to further enhance the disease associated medial calcification in the vasculature. To this end, increase in dietary calcium and phosphorus content [331] as well as warfarin [325] are most commonly used ways. The extent of arterial calcification depends on the diet used and the duration of the treatment. In *Study II*, one week after the nephrectomy, rats were switched on diet containing 0.76% calcium, 0.45% phosphate, 3 mg/g warfarin and 1.5 mg/g vitamin K1 in order to deplete the levels of vitamin K and thus prime calcification (Figure 12B). After three weeks, the diet was changed to a purified diet consisting of 1.34% calcium, 1.2% phosphate and equal number of rats were allocated into groups with either high (100 µg/gram) or low (5 µg/gram) vitamin K2 for another 8 weeks. Vitamin K2 diet supplementation was used to improve the function of renal

artery and prevent further outgrowth of artery mineralisation [332-334]. Thoracic aortas were collected and fixed for further histological evaluation. The highly demanding surgery procedures of this model results to a large variation of the disease severity and post-operative complications.

3.8 ETHICAL CONSIDERATIONS

This thesis follows a translational strategy relying on human biobank data and tissue collections, such as detailed medical records, human atherosclerotic carotid plaques, epigastric arteries, aortic thoracic explants and plasma for further experimental assessments. The exploration of established biobanks consisting of human tissues and detailed records demands the participation of well-educated medical and paramedical staff. All included subjects were asked permission to participate in the research and got informed about all the research protocols, in order to get a more integrated overview of the way that their specimens will be used. The decision for participation in the research studies is taken autonomously where the patients or organ donor guardians sign an informed consent. The subjects have the right to withdraw their participation from the study at any time, even after signing the informed consent. The personal data are pseudonymised in order to ensure that they are not attributed to an identified or identifiable person. A code was given for each one of the samples and picture was taken for the database before continuing the processing of specimens. Pseudonymisation of samples is a good way to encrypt subject's valuable information from potential leak which could harm its life in the future. Only the data controller gets access to the fully personal data, whereas the data processor processes merely the data according to instructions and under the authority of data controller. All human data used in the current thesis are protected by GDPR and ethics laws that govern the privacy of the participants in the studies. The ethical permits were approved by the regional Ethical Committee accompanied with an ethical permit number. All studies were conducted in accordance with the principles of the Declaration of Helsinki.

The use of human material in research is very important and invaluable because the results could directly be applicable to clinical practice. The use of animal models, especially in complex and multi-factorial diseases, is not always optimal due to the difficulties of transferring the finding to humans. However, when the use of animal models is the only way for addressing demanding research question, the welfare of the experimental model and the 3R concept - Replacement, Reduction and Refinement – should be followed. Replacement of animal models with another method, such as *in vitro* experiments or use of *in-silico* models (such as bioinformatic analyses in *Study I*). Additionally, artificial organoids or *ex vivo*

culture of human specimens (*Studies II* and *IV*) would reduce the number of animals and refine the way that the animals are treated in order to minimise the impact of experimental procedure on them. In order to reduce the number of required for research animals, utilisation of pre-existing material can be used by studies designed under another research purpose, but fit to the research questions (*Study II*). Establishment of global biobanks and databases containing data and material from experimental models in which researchers could share their findings would further promote the aim of the 3R concept. All animals used in the thesis lived under well-monitored conditions in an institutional animal facility. Moreover, well-trained professionals and caretakers eliminated the impact of treatments and surgeries and improved their welfare. Animals anesthetised prior to any surgery procedure and received pain relievers afterwards. Animals were sacrificed using a quick and painless method according to acceptable guidelines in case of surgery complications or for tissue harvesting purposes. All animal walefare and experimental procedures were conducted according to the official guidelines for use of laboratory animals in research and the methodologies were certified by the local animal ethics committees.

4 MAIN RESULTS AND DISCUSSION

In the present PhD thesis, an integrative approach was applied to delineate cardiovascular calcification complexity by utilising basic and clinical research methodologies. Use of molecular and cellular assays along with *in-silico* analysis of large-scale omics data from one side, and diagnostic imaging tools with patient medical records from the other, aimed to form a translational perspective in order to comprehensively elucidate the pathophysiological mechanisms underlying cardiovascular calcification.

4.1 MULTI-OMICS 3D LAYERED NETWORK REVEALED THE PRESENCE OF AMYLOID-LIKE STRUCTURES IN CAVD

In *Study I*, publicly available multiple omics datasets were explored to investigate pathway–phenotype associations in CAVD. Application of the inclusion and exclusion criteria resulted to 32 miRNAs, 596 mRNAs and 80 proteins, eligible for the subsequent bioinformatic analyses (Figure 13).

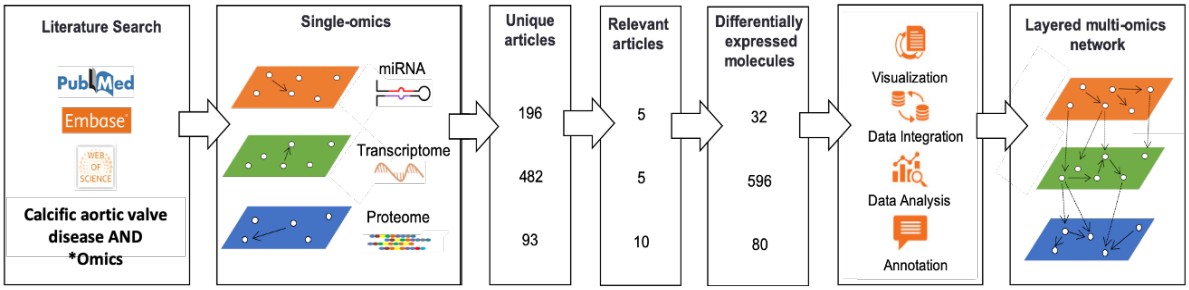

Figure 13. Schematic representation of the strategy followed in the Study I. Literature search was performed for CAVD and omics. Single-omics studies were included and the results were processed for further bioinformatic analyses.

Functional analysis of each layer revealed platelet degranulation and complement/coagulation cascade as top impaired pathways. Intersection of mRNAs and proteins revealed nine common genes that form a PPI network. Pathway analysis of the formed PPI network revealed similar dysregulated pathways with immune system, platelet degranulation/activation/aggregation, complement/coagulation cascade and extracellular matrix being over-represented. The complement/coagulation-platelet crosstalk occurs in innate immunity [335] and early atherogenesis [336], may also promote CAVD progression. Patients with aortic stenosis exhibit impaired platelets [337], which in turn initiates the complement/coagulation cascade [338], activating further the platelets in a positive feedback function [339]. In agreement with platelet activation in CAVD, results of a recent study indicate that degranulated platelets exacerbate the progression of aortic stenosis in mice by triggering VIC osteogenic differentiation and ECM mineralisation [93]. Construction of a multi-omics 3D

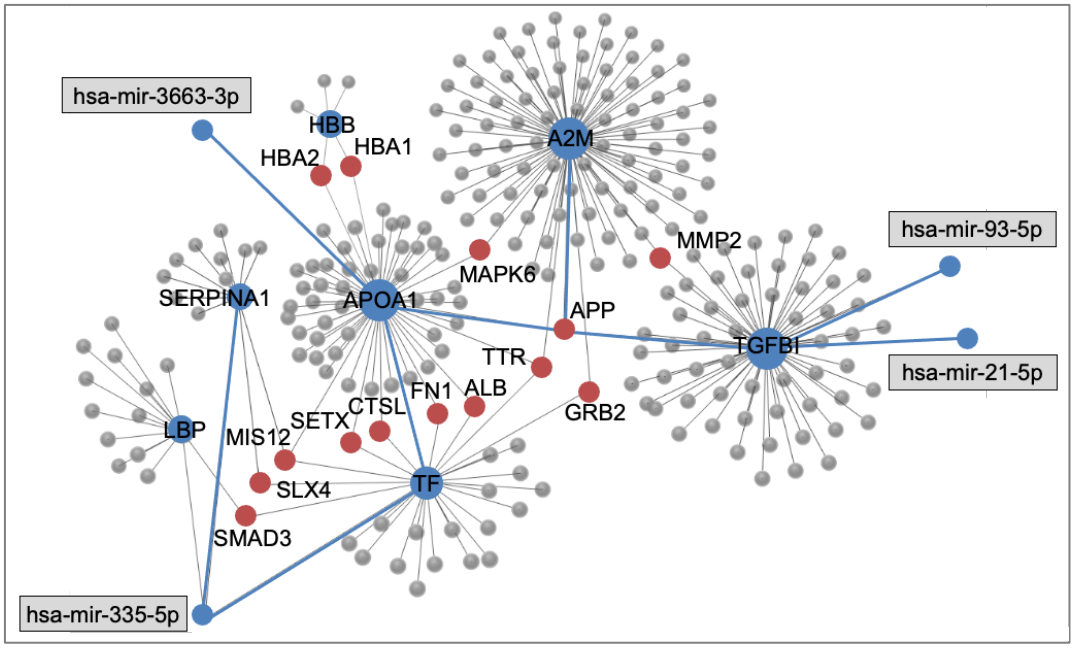

Figure 14. Multi-omics 3D layered PPI network in CAVD containing the differentially expressed miRNAs and the common molecules between transcriptome and proteome.

layered network between the nine identified molecules and miRNAs revealed that 7 seed molecules interacted to 4 different seed miRNAs into a PPI network (Figure 14). In addition, this multi-omics 3D layered network is supported by several genes including SMAD3 and MMP2 [87,97], which are involved into the mineralisation process. The seed molecules were further mapped in the layers of the recently published aortic valve molecular atlas [89]. Some of the seed molecules were observed in fibrosa; the calcification-prone layer, whereas, others were present within the spongiosa, a rather unexplored aortic valve layer in CAVD. Recent, machine learning and bioinformatic analysis confirmed the presence of FN1 in calcified valves associated with innate immune cells such as macrophages, MCs and NK cells [340]. Moreover, two interesting molecules, the amyloid beta (Aβ) precursor protein (APP) and transthyretin (TTR), were located in the center of the layered PPI network. Many of the observed genes are known to engage in amyloid plaque formation. This new molecular network points towards an occasionally reported but largely unexplored link between CVDs and AD [341]. APOA1 is a significant contributor of amyloid presence within calcified valves [342], while APP is the main molecule of amyloid plaques observed in AD patients [343]. TTR - another amyloidogenic molecule - has neuroprotective functions, since it binds to Aβ in order to block its fibrillation [344]. Functional annotation of the 3D layered biological network demonstrated that lipoprotein metabolism, platelet activation/degranulation and TGFβ signalling were the most significantly over-represented pathways (Figure 15); finding that comes in line with the current research in CAVD [345]. In addition, recent multi-omics' reports identified hub genes and pathways, pinpointing the significance of platelet activation and complement and coagulation cascades for the CAVD development [346,347]. Integration to the

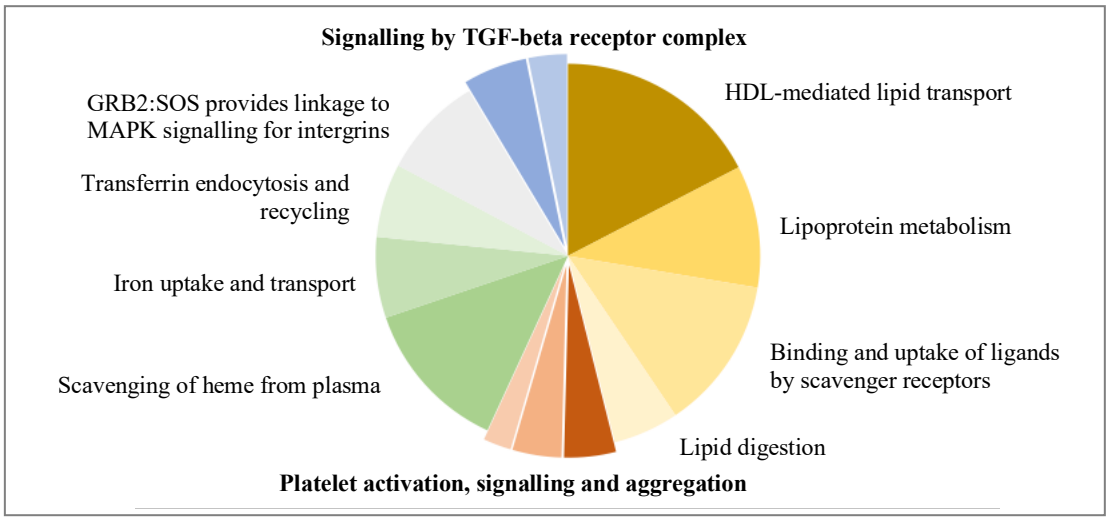

Figure 15. Reactome pathway enrichment analysis for multi-omics layered PPI network.

existing PPI network by an additional layer of dysregulated metabolites revealed that AD was located in the center of the formed network. Staining of human valves confirmed the presence of the TTR and APP molecules as well as the Aβ-like structures in the calcified regions. It should be emphasised that both AD and CAVD are aging-related complex conditions characterised by low-grade inflammation. Therefore, the APP molecule, found in advanced human carotid plaques [348], is mainly secreted from alpha granules during platelet degranulation and stimulates pro-inflammatory signalling exacerbating the atherosclerotic burden [348]. In turn, APP-deficiency in mice reduces atherosclerotic plaque size [349], suggesting that APP can be secreted from degranulated platelets and may get involved in the initiation of calcification process by acting as a hydroxyapatite nesting place. Interestingly, Aβ was found in close proximity to calcified regions in aortic stenotic valves [350]. Moreover, mineralisations found in coronary artery are associated with increased prevelance of dementia in elderly people [348], suggesting a potential common mechanism between Aβ and CaP deposition. Recent study confirmed that Aβ40 induced VIC osteoblastic differentiation *in vitro*, *via* activation of the receptor for AGEs (RAGE) [351]. In addition, TTR precipitations were detected in calcified leaflets. It is worth mentioning that a link between amyloidosis and calcification has previously been reported in

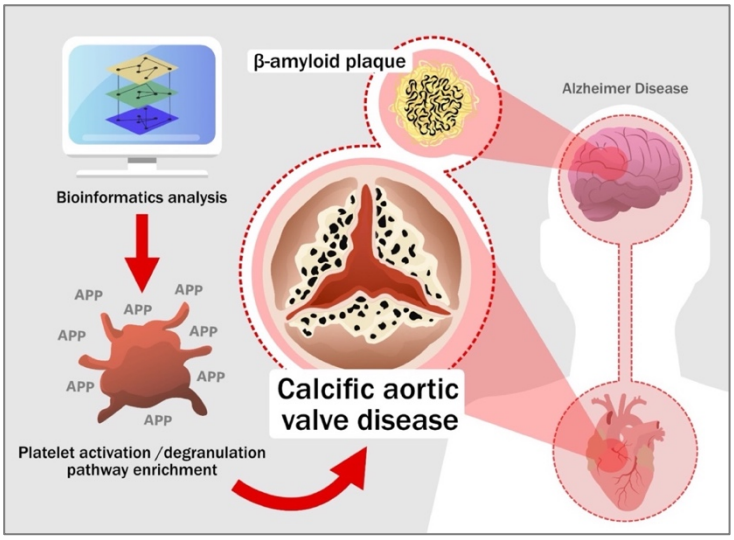

Figure 16. Identification of a novel molecular CAVD network that is linked to the formation of Aβ -like deposits, known from AD, potentially though the platelet degranulation pathway.

autopsy studies [352]. An independent association between dementia onset and the occurrence of CVDs was reported [341], leading towards the notion that a common molecular pathogenesis may exist between CVDs and AD. To this end, AD patients had impaired heart function as a result of Aβ deposits in their hearts [353]. In addition, these AD exhibited elevated aortic peak velocity which is used as an indicator for aortic stenosis [353].

Overall, *Study I* implies a potential link between calcification process in CAVD and Aβ plaques in AD in a way that these two diseases might share common pathophysiological mechanisms through platelet activation/degranulation processes (Figure 16).

4.2 OMD EXPRESSION IS ASSOCIATED WITH CALCIFICATION, PLAQUE STABILITY AND FEWER CV EVENTS

In *Studies II* and *III*, an integrative approach between human biobanks was followed to investigate the role of OMD in cardiovascular calcification. OMD emerged as a significantly upregulated gene in human carotid atherosclerotic plaques but its expression was downregulated in plaques from S *vs*. AS patients (Figures 17A, B). Interestingly, stratification of global gene expression data into low- *vs.* high-calcified plaques, as they were evaluated by CTA image analysis [27], revealed that OMD gene expression was significantly increased in high-calcified plaques (Figure 17C). In addition, high OMD gene expression levels were associated with a reduced risk for future CV events and cardiovascular death (Figure 17D). To confirm the association between OMD and calcification, circulating OMD levels were quantified in plasma of patients with carotid stenosis and CKD by ELISA. Results showed that circulating OMD protein levels were reduced in peripheral plasma from atherosclerotic patients compared to control individuals. In addition, multiple linear regression analysis between plasma OMD protein levels and other plaque morphological features, as they quantified by diagnostic carotid CTA scans, showed an independent statistically significant positive association between tissue calcification and plasma OMD levels (Figure 17E). To further strengthen this finding, circulating OMD protein levels were quantified in CKD patients. The results showed an increase of OMD levels in the plasma of patients with advanced (grade 3) medial calcification of epigastric arteries compared to those with intermediate medial calcification (grades 1 and 2). Interestingly, circulating OMD levels were positively correlated with the aortic valve calcification score from the same patients. The above findings were further confirmed by the presence of OMD positive signal in calcified regions of specimens obtained from patients with carotid atherosclerosis, CKD and CAVD. Moreover, global microarray gene expression analysis of human carotid endarterectomies revealed that OMD transcript is correlated positively with markers for

VSMC contractility (MYOCD, MYH11, ACTA2), collagen and BMP2, involved in matrix mineralisation and remodeling.

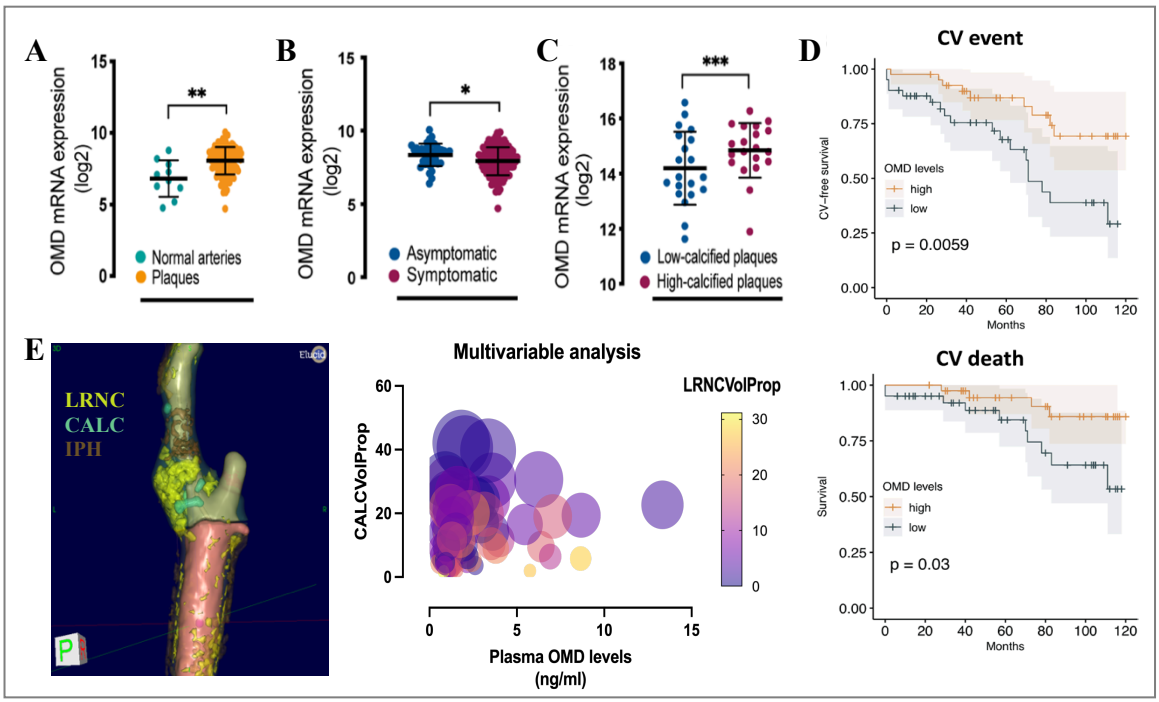

Figure 17. ***A)*** *OMD gene expression in microarrays from carotid atherosclerotic plaques compared to normal arteries, and* ***(B)*** *in plaques from S patients vs. AS ones.* ***C)*** *OMD gene expression in microarrays from high- vs. low-calcified human carotid atherosclerotic plaques, where calcification was assessed by TeraRecon analysis software.* ***D)*** *Associations of OMD below and above median with CV events and death during follow-up analysed by Kaplan-Meier curves.* ***E)*** *Multiple linear regression analysis was used to estimate the association between plasma OMD levels and CALC (circle size), LRNC (color grade) and other morphological features as estimated by vascuCAP analysis software.*

To further consolidate the role of OMD in cardiovascular calcification, murine models were utilised in order to resemble both atherosclerotic- and CKD-induced intimal and medial calcification, respectively. Hyperlipidemic *ApoE*$^{-/-}$ mice received a Western diet enriched with warfarin and vitamin K1 [327] and Sprague–Dawley rats subjected to 3/4th nephrectomy fed with a diet rich in warfarin and phosphate [354,355]. Immunohistochemistry of aortas of the above animals revealed that OMD protein was strongly expressed in developing intimal and medial calcification, and found in the matrix regions enriched in α-SMA^{+} and RUNX2^{+} cells. These results are consistent with previous reports showing that OMD is restrictively expressed in highly mineralised tissues, particularly bones [201,205,206] and at the mineralised front of the developing tooth [207-213].

4.3 OMD IS EXPRESSED BY FIBROMYOBLASTS AND INHIBITS ECM CALCIFICATION BY ATTENUATING OSTEOBLASTIC VSMC TRANSITION

To identify the cell populations that govern OMD expression, bioinformatic analysis of scRNAseq data from both human coronary and carotid atherosclerotic plaques was employed. The analysis revealed that OMD was expressed by fibromyocytes, fibrochondrocytes and fibroblasts, which are defined by a gradually reduced expression of ACTA2 compared to classical VSMCs. Therefore, OMD was co-expressed with other markers known for VSMC phenotypic modulation, the macrophage marker galectin 3 (LGALS3) as well as the fibromyocyte- and fibrochondrocyte-related markers, osteoprotegerin (TNFRSF11B) and fibronectin 1 (FN1), respectively [127,356-358]. These results indicate that the VSMCs that express OMD slowly loose their specific markers and transdifferentiate into an intermediate state with multipotent features. Therefore, the function of OMD in human VSMC cultures was further explored, considering that VSMCs are the main cells that undergo phenotypic modulation during mineralisation process. *In vitro* investigations with primary HAoSMCs showed that OMD gene expression is increased after exposure to both pro-osteogenic (BMP2 and TGFβ1) and pro-inflammatory (IL-4, IL-6 and IFNγ) stimuli as well as under high phosphate conditions (Figure 18). Given that OMD gene is increased throughout the course in β-glycerophosphate osteogenic medium (Figure 19A), the molecular role of OMD in VSMC modulation was further explored. To achieve this, VSMCs were treated with either siRNA to inhibit OMD expression or recombinant human OMD (rhOMD) protein. Silencing of OMD gene expression in HCoSMCs cultured in β-glycerophosphate osteogenic medium resulted to a significant upregulation of osteogenic-related genes, and ultimately increased of matrix calcification (Figure 19B). This finding is also depicted by the upregulation of osteogenic-related pathways such as TGFβ, WNT and innate immunity (Figure 19E). Additionally, since it was observed that OMD mRNA levels were downregulated in HAoSMCs under high phosphate culture media already after 3 days (Figure

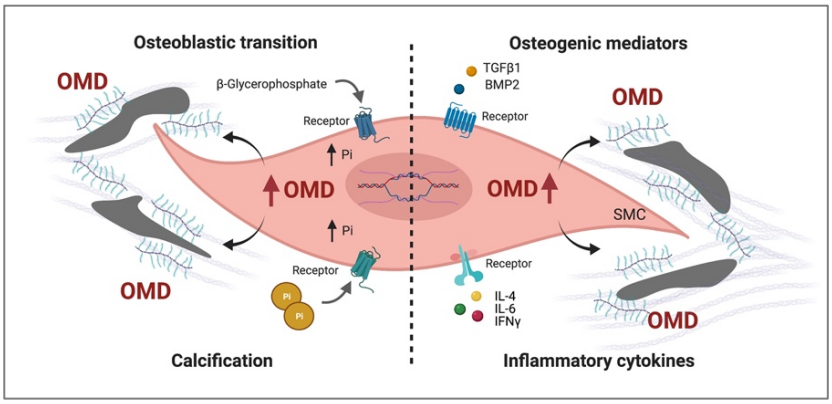

Figure 18. Schematic representation of the mechanisms by which OMD gene expression is induced in VSMCs.

19C), HAoSMCs were exogenously stimulated with full length rhOMD for 12 days. This resulted to attenuation of ECM calcification (Figure 19D) and reprogramming of HAoSMCs towards an intermediate cell state. At this state, the HAoSMCs seem to maintain their contractile phenotype and simultaneously upregulate their ECM secretory phenotype [359]. Both the contractile markers of typical VSMCs [110], and the dedifferentiated VSMC markers of myogenic- or fibroblast-like cells were upregulated, while both the pro-inflammatory and -osteogenic markers, which participate in matrix mineralisation, were significantly downregulated in rhOMD-treated VSMCs. In confirmation of these findings, the biological pathways were also changed in a similar way. Therefore, ECM organisation, proteoglycans biosynthesis, collagen and elastic fiber formation and smooth muscle contraction were among the top identified biological pathways of upregulated genes, whereas, TGFβ, BMP and WNT signalling pathways–related to osteoblastic differentiation and calcification were repressed (Figure 19F). This integrative analysis suggests that administration of exogenous OMD in osteogenic medium holds VSMCs in a contractile mode by retarding their transition towards an osteoblastic phenotype. At the same time exogenous OMD induces a reprogramming of VSMCs into a protective matrix secretory myofibroblast-like state (Figure 20A) [111,127,356]. Worth noting that, myofibroblasts have typically been defined by key phenotypic features including the expression of both "VSMC-specific" and ECM-related markers [360]. Activated myofibroblasts mediate the deposition of matrix proteins leading to tissue fibrosis and thus, they participate in arterial matrix remodeling processes [361]. Moreover, they are characterised by increased proliferation and decreased migration properties, effect that may be elicited by their increased PDGF-BB expression [43,114]. In addition to PDGF, TGFβ signalling pathway is also responsible for myofibroblast differentiation [362]. Taking together the implication of BMP2 signalling to mineralisation and results of a recent article showing a direct interaction between BMP2 and OMD in bone ossification [213], further experiments were designed to investigate the above interaction in VSMC-mediated calcification. Exogenous stimulation of VSMCs with BMP2 in osteogenic medium induced osteoblastic reprograming of the cells without resulting to matrix mineralisation. On the contrary, administration of both BMP2 and OMD in VSMCs, under high phosphate conditions, resulted to matrix calcification to the same extent as phosphate medium alone, indicating that OMD, by interacting with BMP2, may override the effects of either OMD or BMP2 alone and positively regulate the calcification process (Figure 20B). These results suggest that OMD and BMP2 interaction deploys synergistic effects not only in bone ossification [213] but also in arterial mineralisation.

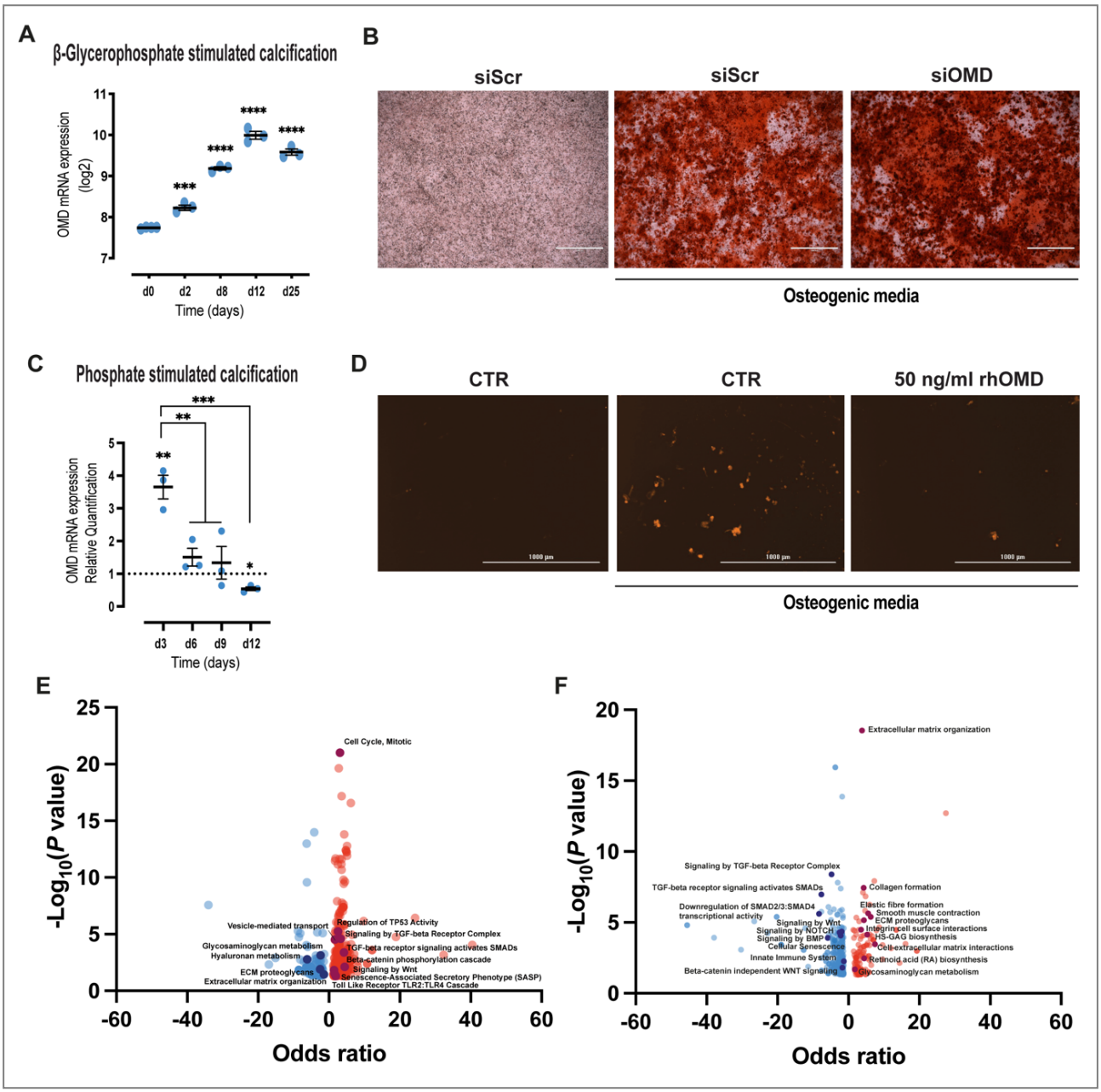

*Figure 19. **A)** OMD mRNA expression levels in HCoSMCs treated with osteogenic medium consisting of 0.1 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone. **B)** Representative images of the calcification assay where HCoSMCs treated with siRNA for OMD or scramble control in osteogenic medium consisting of 0.1 mM l-ascorbate phosphate, 10 mM β-glycerophosphate and 10 nM dexamethasone for 14 days, visualised by Alizarin Red staining. **C)** OMD mRNA expression levels in HAoSMCs treated with 2.6 mM Pi for up to 12 days. **D)** Representative images of the calcification assay where HAoSMCs treated with 2.6 mM Pi for 12 days in the absence or presence of 50 ng/ml human recombinant OMD (rhOMD) protein, visualised by an Alexa Fluor 546 coupled fetuin-A probe. **E)** Volcano plot showing the top significantly downregulated (blue) and upregulated (red) Reactome pathways comparing HCoSMCs treated with siOMD vs. control (siScr) in osteogenic medium for 14 days. **F)** Volcano plot showing the top significantly downregulated (blue) and upregulated (red) Reactome pathways comparing HAoSMCs treated with rhOMD vs. control in osteogenic medium for 6 days.*

Overall, *Studies II* and *III* suggest a consistent association of both plasma and tissue OMD levels with cardiovascular calcification and decreased risk for future CV events, bringing to the frontline the potential use of OMD as a clinical biomarker. Moreover, OMD expression was induced by pro-inflammatory and pro-osteogenic stimuli, while its presence in

extracellular milieu attenuated VSMC calcification. Lastly, worth mentioning that vascular calcification and endochondral ossification may share pathophysiological mechanisms.

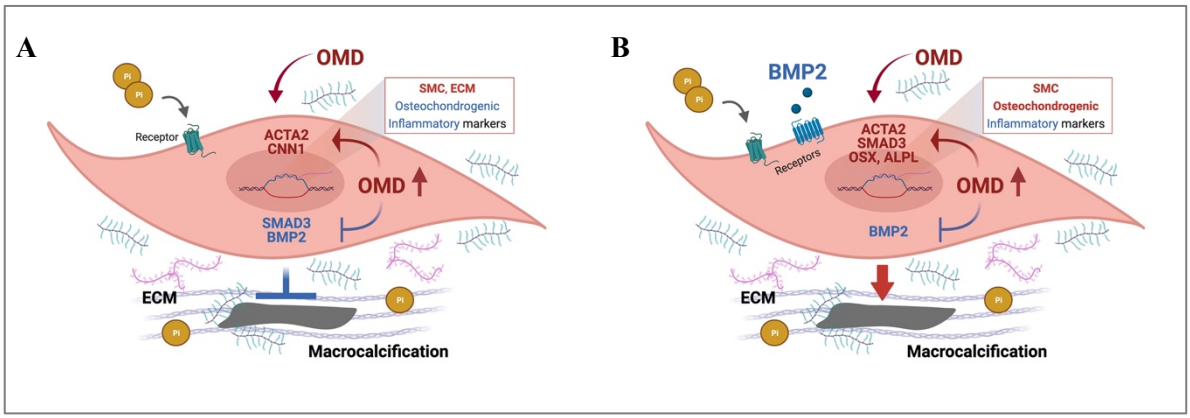

Figure 20. Schematic representation of the mechanisms by which exogenous administration of OMD attenuated ECM calcification (A) whereas its synergistic effect with BMP2 resulted to matrix calcification.

4.4 MAST CELLS INVERSELY ASSOCIATE WITH ATHEROSCLEROTIC PLAQUE CALCIFICATION

In *Study IV*, an integrative systems biology approach was followed from the large human BiKE biobank to explore the link between macrocalcification and MC activation in atherosclerotic plaques. Patients undergoing CEA were enrolled in the study based on symptomatology and medication (Figure 21). Diagnostic pre-operative CTA scans were

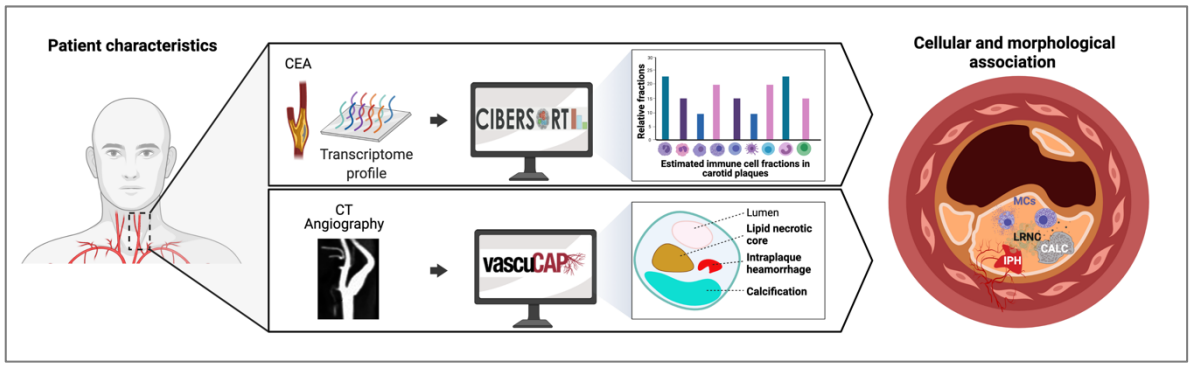

Figure 21. Workflow of the deconvolution methodology applied in the study.

analysed using vascuCAP software for the extent of CALC, LRNC, IPH and other plaque morphological features (Figure 22A). In addition, carotid endarterectomies from the same patients were analysed using global gene expression by microarrays [27,244] and immune cell fractions were estimated from these bulk profiles using deconvolution by the CIBERSORT software [309], based on a "cell-type signature matrix" of 22 well characterised immune cell populations [127]. The analysis revealed an abundance of resting MCs being present in high-calcified plaques, whereas, low-calcified plaques contained relatively more activated MCs. Multivariable correlation analysis between activated MCs and CALC, LRNC, IPH revealed that activated MC fraction was independently negatively correlated with calcification (Figure

22B). Staining of tissue microarrays with the MC-specific tryptase and chymase markers confirmed the presence of MCs in both states (resting and activated) in atheromatous lesions and that both the activated MCs and the total number of MCs normalised by tissue area (mm^2) correlated negatively with calcification which was quantified by Alizarin Red staining. Stratifying the results according to patient medical records showed that the number of activated MCs was elevated in both S and AS patients, with them being detectable in the lesions of patients presenting symptoms of plaque instability, such as AFX, TIA and MS. Exploration of BiKE microarray dataset resulted from calcified plaques for a specific gene signature related to MC activation [253,363,364], revealed that genes related to MC degranulation were enriched in low-calcified plaques, particularly them of S patients (Figure 22C). This result comes in line with previous studies showing an abundance of activated MCs at the sites of atheromatous erosion or rupture in patients who have died of myocardial infarction [256]. Besides this, patients with CV events exhibit elevated plasma concentration of tryptase [262,365].

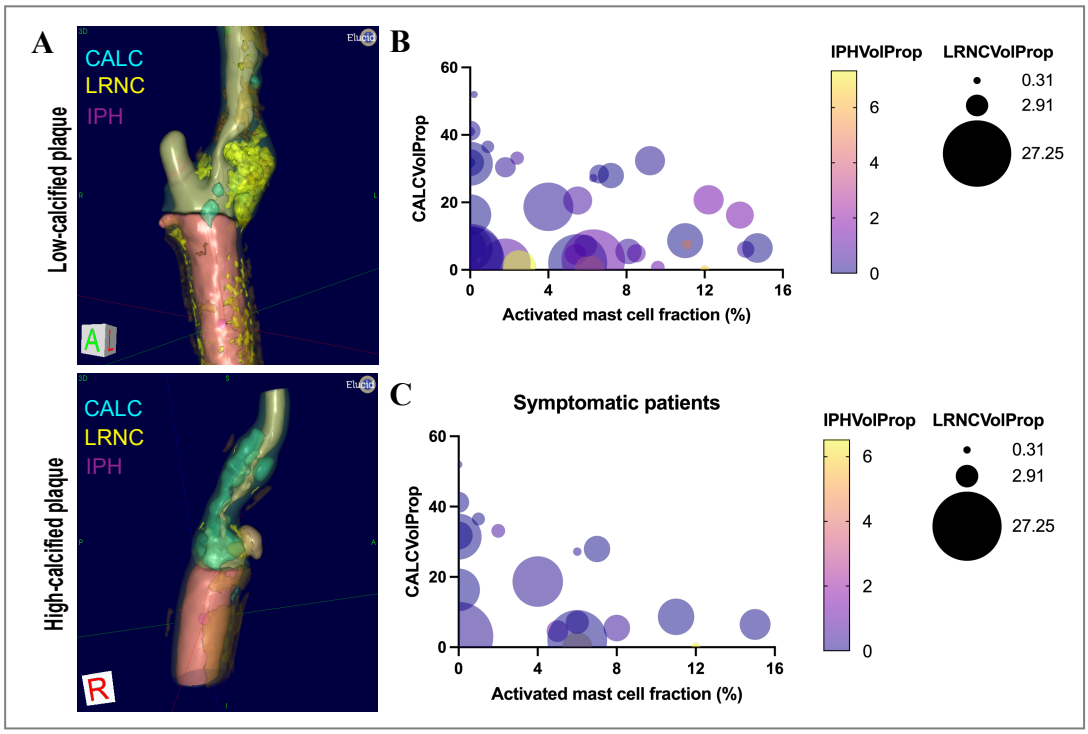

*Figure 22. **A)** 3D reconstructive images with vascuCAP software of low- and high-calcified plaques. **B)** Multiple linear regression analysis between activated MC fraction and CALC, LRNC and IPH as estimated by vascuCAP analysis in the whole cohort and **C)** in S patients.*

4.5 MAST CELLS PARTICIPATE IN PLAQUE CALCIFICATION *VIA* VSMC REPROGRAMMING

To further dissect the plaque cellular microenvironment surrounding MCs and examine their potential cross-talk with other cell types; resting and activated MC fractions were correlated with the leukocyte subtypes from the deconvoluted plaques [309]. Analysis revealed that the activated MC fraction correlated positively with activated NK cells, T cells and resting

dendritic cells, but negatively with B cells, activated CD4 memory cells and resting MCs and NK cells. Indeed, staining of calcified plaques showed the presence of Tryptase^{+} activated MCs in fibrous cap and necrotic core regions, where activated NK cells (CD56^{+}), T (CD3^{+}, CD4^{+}) cells and eosinophils (Siglec8^{+}) were also identified in MC proximity. Here, the followed *in-silico* modeling methodology proposes potential functional immune cell synergies in human atherosclerotic plaques, which may represent a relevant mechanism for atheroprogression to be exploited for future therapeutic interventions [366].

Considering that activated MCs were enriched in low-calcified plaques, *ex vivo* BiKE plaques and *in vitro* cell cultures were employed to investigate their functional role in VSMC-mediated calcification. In confirmation of bioinformatics analysis, *in vitro* evaluation showed that both supernatants of calcified plaques and VSMCs inhibited MC activation, while supernatants from plaques with large LRNC induced MC degranulation. In reverse, both MC fractions triggered VSMC osteoblastic transition as observed by the upregulation of the osteochondrogenic markers (SOX9, SMAD3 and BMP2). This VSMC dedifferentiation resulted in increased matrix calcification independently of MC phenotype.

Overall, *Study IV* conveys in a systematic way that activated MC associate inversely with calcification, but positively with plaque vulnerability features, other immune cells and clinical symptoms. This study proposes that the late-stage, already calcified plaque VSMCs have a feedback effect on dampening MC activation, which finally outweighs the effects of MCs on inducing VSMCs calcification that likely happens earlier during the atheroprogression (Figure 23).

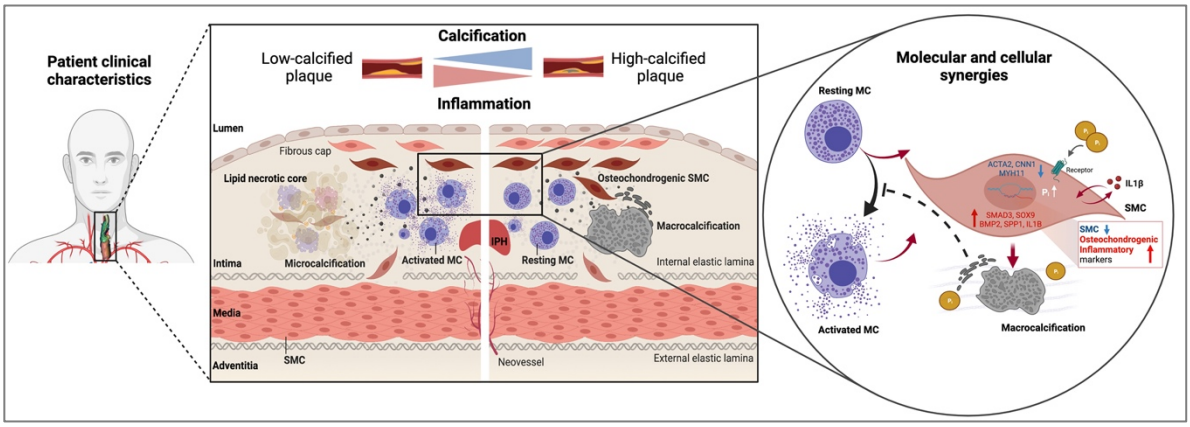

Figure 23. . Schematic representation of the mechanism by which MCs mediate calcification formation in human plaques via VSMCs reprogramming as well as the feedback mechanism by which calcification attenuates MC activation.

In conclusion, integration of clinical and molecular tools were able to identify new molecular networks in CAVD and to comprehensively explore the role of proteoglycan OMD in cardiovascular calcification as well as to connect OMD expression with calcification and future CV events. Moreover, it proposes a new link between MCs and calcification in

calcified plaques. Ultimately, the findings presented in the current thesis not only indicate novel insight into the context of pathophysiological mechanisms of cardiovascular calcification, but also suggest promising biomarkers and therapeutic targets, underlying their translational potential.

5 CONCLUDING REMARKS

Despite the cumulative knowledge about CVD, its burden is growing globally. This indicates an urgent need for implementation of an alternative research strategy. The studies presented in the current thesis aimed to expedite our understanding of the key features of plaque vulnerability, by exploring the interplay between inflammation and calcification. Large-scale omics analysis and cellular and molecular assays in combination with patient medical records and routine clinical imaging techniques were employed in order to form a translational perspective into this thesis.

Study I demonstrates that the reuse and integration of publicly available multi-omic datasets can be a powerful tool to reveal novel pathways in CAVD. The reanalysed data propose an undescribed network in CAVD closely related to mechanisms in AD. Moreover, the analysis pinpoints the significance of the interrelated pathways coagulation and complement and platelet activation and degranulation in the pathophysiology of CAVD. The identification of a novel molecular network provides a rationale for future mechanistic studies to better understand the pathophysiology of CAVD and as such, pave the way for the development of new therapeutic strategies based on network medicine research.

Studies II and ***III*** identify OMD as a novel molecule widely upregulated in the plasma and local tissue in association with cardiovascular calcification. High OMD levels were associated with a decreased risk for future CV events and cardiovascular death. Notably, both studies porpose OMD as an important early modulator of the cardiovascular calcification processes, since it was enriched in calcified tissues. Mechanistically, exogenous OMD attenuated VSMC osteoblastic transition and ECM calcification, result that surpassed by combined administration of OMD with BMP2. These findings establish a role of OMD in cellular transdifferentiation and vascular ECM mineralisation and highlight OMD for potential biomarker and therapeutic evaluation in cardiovascular calcification.

Study IV underpins the crucial role of MCs in atherosclerotic intimal calcification, despite them being a minor cell fraction in the plaque. Interestingly, it shows that activated MCs are abundant in low-calcified plaques with a large necrotic core that give rise to symptoms in patients, while resting MCs are abundant in high-calcified plaques. Additionally, the findings establish possible functional cellular associations between activated MCs with NK cells and other immune cells found in the plaque milieu. Mechanistic insights indicate that MC

phenotypes induce VSMC transition towards a pro-inflammatory- and osteochondrocyte-like phenotype, whereas calcified plaque and VSMCs present an opposing effect by dampening MC activation at late-stages. Additional mechanistic studies deciphering the interplay between MC and VSMC in atherosclerotic calcification will offer new therapeutic avenues for exploration.

Collectively, the results obtained in the current thesis shed light on the pathophysiological mechanisms that drive calcification in patients with atherosclerosis and CAVD. Importantly, the findings presented lay plans for future exploration of new pharmacological targets and biomarkers with a specific focus on cardiovascular calcification.

6 FUTURE DIRECTIONS OF CARDIOVASCULAR RESEARCH

Currently, interventions for atherosclerosis and CAVD are primarily focused to therapeutic management of comorbidities *via* medications or surgical removal of the diseased tissue. In the majority of cases, patient history, evaluation of classical risk factors and general systemic biomarkers as well as pre-operative imaging methods are the only tools for diagnosis, patient stratification and disease prognosis. During the past years, impressive advances in imaging techniques for disease diagnosis and severity have developed. Coupling of standard methodologies with advanced computantional analysis provides valuable knowledge about the qualitative characteristics of atherosclerotic plaques or valve stenosis. Implementation of such methodologies in clinical routines would improve patient outcome. Besides this, it is equally important to understand the cellular and molecular mechanisms that underlying disease pathology. To this end, multiple omics and spatial integrative technologies could tackle the complexity of disease and identify the individual dysregulated phenotype of each patient. Such techniques are capable to delineate in high resolution cellular and morphological aspects that heavily impact disease progression. In addition, combination of multi-omics integration and network-based medicine could help to the direction of identifing new disease-specific biomarkers and therapeutic targeted molecules or networks. Mechanistic *in vitro* and *in vivo* studies are of great value to further explore the potential link between the newly discovered molecules or pathways and the underlying pathophysiology. Once revealed, they set the floor for their manipulation, opening new avenues for disease regression. In order to fully understand the CVDs complexity, a multidisciplinary, integrative and translational experimental strategy is required that can better model the disease and characterise its complex molecular processes with an ultimate goal to improve the classification and management of the patients in risk.

7 SOCIAL IMPACT

CVDs remain the leading cause of death globally, while their burden is increasing rapidly in low- and middle-income countries. In 2019, 17.9 million people died from CVDs, whereas the same year around 7 million premature deaths (under the age of 70) caused by CVDs. It is therefore important to detect disease pathology as early as possible. While clinical procedures have been improved to ameliorate patient symptomatology, early disease diagnosis, prediction and therapy are by far lacking. At the same time, tissue molecular and clinical diversity in combination with population heterogeneity in CVD, undermine the efforts made to uncover significant disease-associated molecules. Innovative methodologies in the context of personalised computantional multi-omics and imaging analyses can increase the power towards discovering novel molecules and pathways, used as early disease-specific diagnostic biomarkers and therapeutic targets. In this thesis, bioinformatic integration of publicly available omics datasets revealed a network-based pathway enrichment, which is related to platelet activation. The observed amyloid structures in calcified valve leaflets suggest a common pathophysiological mechanism between CAVD and AD. At a molecular level, investigation of different tissues from human multi-biobanks and pre-clinical animal models elucidated the role of proteoglycan OMD in cardiovascular calcification. The positive correlation of OMD with calcification and fewer CV events and its presence in stable lesion phenotype highlight its potential function as both an early plasma biomarker and therapeutic target of cardiovascular calcification. Lastly, integration of transcriptomic data and advanced computantional imaging analysis identified a negative correlation between activated MCs and calcification in atherosclerotic plaques, while a positive association with clinical and morphological characteristics of plaque vulnerability. Interestingly, the established cellular associations between MCs and VSMCs try to aid towards the development of new therapeutic strategies.

In conclusion, this thesis aimed in a systematic and comprehensive way to pinpoint the underlying processes associated with vulnerable plaque formation and cardiovascular calcification *via* the computantionally integration of multi-omics and advanced imaging data. Moreover, it established novel preventive, diagnostic, and therapeutic tools which ultimately, accelerate the translation of basic research findings into clinical practice.

8 ACKNOWLEDGEMENTS

To me, this academic journey was a long one (almost six years), full of adventures, challenges and discoveries both personal and scientific. I consider myself very fortunate that I walked through this path which has shaped not only the researcher but also the person I have become.

Despite that the current book represents only a small work, yet of fruitfull collaborations over the last three years, I am lucky that I have worked in several countries (France, Sweden and the Netherlands). And that, because I was given the opportunity to meet excellent people, make friends and "families". Everyone I met, inspired and encouraged me throughout this once-in-a-lifetime experience.

Over the years, this journey has been transformed to an exciting one as a result of many wonderful, experienced and willing people. To start with, **Hildur Arnardottir**, who stepped into as my main supervisor and supported me to continue this academic path. Thank you for providing me with the knowledge and assistance during the times I was struggling. During the time I was working next to you in the lab, I was impressed by your energy, your experimental organisation skills. I am also thankful that you went through the massive administrative work so to make this work happen. Many thanks to my co-supervisor, **Ljubica Matic**, a true young leader, who inspired me with her enthusiasm for research in order to continue this path. Dealing with difficult situations with professionalism and humanity, really embodies to the fullest a role model who strives to find realistic solutions. Sharing our experiences was very relieving for me. I am grateful for your commitment to exceed our expectations not only to publish but also elevate any project of this thesis. Thanks for trusting me and giving me the space to evolve as an independent researcher. Your advices for everyday tasks, your presentation perfectionism and the honest and clear discussions that we had related to science and beyond, will all follow me for the years to come. Before continuing to the rest of my supervisors, I would like to express my honest gratefulness to **Ulf Hedin**, for his trustfulness as well as the way he embraced me and the positive welcome to me when I joined his lab. Besides this, I am thankful for his approach to sensitive issues and his unique excellent way to convey complex clinical and molecular knowledge. Many thanks to **Erik Biessen**, who welcomed me to his lab in Maastricht University during my long secondment and giving me the opportunity to utilise the lab resources. I am always impressed by his outstanding level of knowledge and his "always" active mind to connect dots and increase the impact of all projects where he is getting involved. To **Chris Reutelingsperger**, an entrepreneur, who was always willing to provide his valuable expertise and his capability to broaden my horizons by combining science and business in a unique way.

Special thanks to my mentor, **Angela Silveira**, who supported me throughout the years. My gratitude for the honest discussions we had related to science, career and most importantly life. Thanks for your vital assistance over the years with your excellent administrative skills; you are always one thought and step ahead. Lastly, thanks for your nice vegan fikas we shared together!

To all members of the **Vascular Surgery** group, the ones I have met and those I haven't (clinicians, nurses, administrative personnel) but with one way or another impacted my research. Mostly and above all, a big thank to the truly invisible patients and healthy subjects who have participated in the studies included in the current thesis. The **Vascular Surgery** group is an excellent place for flourishing and genuine interaction between clinicians and basic researchers. Thanks for allowing me to join and meet beautiful people. Starting from **Siw Frebelius**, thanks for your key role in the lab by organising everything that comes across your path. Thanks not only for the administrative work but also for the discussions we had together for concerts and Swedish countryside. You are really an inspiring person; your motivation and energy for work are unbeatable. **Malin Kronqvist** and **Mariette (Mette) Lengquist**, the pillars of the lab, thanks for being there and always willing to help and provide your excellent technical knowledge and contribution. Thanks for the techniques and lab routines you taught me and mostly for being always there. Beyond these, I truly thank you for the great fikas; Malin's delicious carrot and coconut cakes and Mette's hospitality and enjoyable dinner! My "green salads" were not always enough to lower my raised blood sugar levels after your fikas. **Suvi Mäkinen**, I am grateful to have met you and for working with you even for a short time in the lab. Thanks for your hospitality by "lenting" me your house so to explore the beautiful covered-by-

snow Finnish countryside! I honestly hope all the best in your personal and professional life. What is science without basic researchers? **Urszula (Ula) Rykaczewska** thanks for being there and sharing your insightful experimental and scientific competence. I am truly amazed by your positive attitude and the honest discussions in cell culture and elsewhere. I am also astonished by the way you had one shot of tequila after another in one of our first outings! Thanks for your time on sharing with me career and life related thoughts. **Bianca Suur**, thanks for your help in almost everyday lab life as you know-literaly-where everthing is located in the lab. Thanks for giving me the opportunity to know you better by sharing your experiences and for the warm conversations we had along with a glass of Riesling. **Till Seime**, my alter ego in the INTRICARE consortium. A bone biology researcher who conquered the vascular field as well. Thanks for sharing with me all your knowledge about calcification. Grateful that I had the chance to work next to you and share not only experiences, knowledge and concerns, but also the same room in our consortium trips. A truly passionate researcher with great ideas and future who turned his hobby to professionalism. **Glykeria Karadimou**, what to say about you and from where to start. I had contacted you even before I met you many years back, on 2016, to assist me with the KI admission seminar. All those years you have always been there to help me and discuss with me all the raised issues, chatting and sharing with me thoughts and concerns for science, career and life. Honestly, I feel blessed for you being one of the first people I met during this academic journey and you have still remained by my side. **Sampath Narayanan**, thanks for sitting next to me and sharing thoughts and concerns, and for giving us the chance to attend, even virtually, an Indian wedding. It was such a pleasure to get related with this cultural diversity. **Melody Chemaly**, thanks for the trust and all the things I learned from you. I hope I returned those back by giving you my plant to take care of and by the little things I might have taught you about calcification. Glad for the time and thoughts we shared in and out of the lab and hope you forgive me for my statement: “Princess cake is supposed to be green not white”, and that you will remember it with smile. **Hong Jin**, I am happy I met such a skillful person like you. You were always there with a kind and positive attitude. Thanks for giving me the opportunity to participate in your studies. **Otto Bergman**, I firstly met you during my short time in Per’s group, sitting next to you. I was happy that you afterwards joined and positively impacted our whole lab. **Xiang (Bob) Zhang**, thanks for helping me with data analysis and correlogram graphs. You were always there willing to assist me. **Andrew Buckler**, I feel blessed that you joined the lab and you introducing me into the computational biology field, broadening thus my horizons with notions that I had never thought before such as “virtual transcriptomics”. Your presence, your expertise and the software you have developed impacted positively the majority of my studies and I am honored to having met you. **Colin Willems**, **Sofjia Vuckovic** and **Xinran (Aurora) Dong**, you are the newest additions in the lab who brought fresh air, ambitions and young willingness for doing experiments. It was always good to interact with you and made me feel younger!

Clinicians’ turn! **Joy Roy**, **Anton Razuvaev** and **Rebecka Hultgren**, thank each and every one of you for collaborating with me on the studies, sharing comments and celebrating with every achievement. Even if it was always difficult to find you, it was a pleasure to discuss with you for science and life. The next generations of vascular surgeons have a great future next to you. **Eva Karlöf**, thanks for the great collaboration we had together in the projects and for being there to answer all my questions. Also, for inviting me in your glamorous disputation party! What to say for **Katarina Wadén**, a multi-talented and always smiling person. Thanks for the time we spent together supporting each other and chatting about gym, crossfit and weightlifting. I truly hope all the best in your bright career. **Samuel Röhl** and **Moritz Lindquist Liljeqvist**, it was a pleasure to meet you and discuss with you. Sam thanks for training me in rat surgeries, I really appreciate it. **Antti Siika** and **Marko Bogdanovic**, I really appreciate the time sharing with you, chatting for several things and having lots of laugh and joy during your research period.

Besides my KI lab colleagues, I would like to express my gratefulness to all members of the **Experimental Vascular Pathology lab at Maastricht University**, who embraced and welcomed me during my long secondment there. First of all, special thanks to **Olivia Waring**, this great macrophage scientist who took me under her wings and trained me during my time there. It was a real pleasure working by your side and experiencing your energy and multi-tasking daily routine. It was so nice to meeting you and I truly believe you will have a brilliant path ahead. **Pieter Goossens**, thanks for introducing me to the high-resolution multi-spectral imaging, trusting me with your precious

handmade UV box and spending time with me in the microscope room. Beyond these, thanks for the thoughts and moments we shared together. I am also grateful to **Mat Rousch** for making my everyday life easier as he is the person that every lab needs. Thanks for having been always willing and available to help me. Many thanks to other lab members, including **Marjo Donners**, **Lieve Temmerman**, **Renée Tillie**, **Valeria Saar-Kovrov** who welcomed me in the lab and for the nice lunches and talks we had together.

The projects described in this thesis are a result of international collaborations in and out of INTRICARE. To start with those in the consortium, many thanks to **Claudia Goettsch** and **Marina Heuschkel** for approaching me during the the *Study I*, that apart from challenging turned out to be a very nice collaboration. Having succeeded once, why not more? Thanks for our fruitful second collaboration in *Study II*. **Marina**, I remember the time we spent together trying to initiate this project and taking small steps to perform the bioinformatic analysis. I am truly grateful for sharing all those moments together, chatting in person and online for several things related to science and life challenges and career. It was always a pleasure to discuss with you. A true friend! **Anna Witasp** and **Peter Stenvinkel**, thanks for opening your lab to me and sharing with me your precious data. Moreover, I want to thank the other members **Karolina Kublickiene, Thomas Ebert, Samsul Arefin and Sam Hobson** for the pleasant conversations and the common lunches we had together when being shortly under the "same roof". Special thanks to **Lu Dai**, you are a kind soul, a calm power and an amazing young scientist. I am thankful for the nice conversations we had during the INTRICARE meetings but also on the train. You are always beating any challenge with a big smile! I am also grateful to the whole **Department of Biochemistry at Maastricht University** and particularly to **Leon Schurgers** and his group, that hosted me during my time in Maastricht. I learned so many things at your lab, gained from your expertise and the unique resources and techniques that indeed elevated my studies. Special thanks to **Grzegorz (Greg) Wasilewski**, for introducing me to Leon's lab, dealing with my ignorance and being always one WhatApp's reply away of my questions. Furthermore, I want to thank the rest members of the Biochemistry Department including **Petra Lux**, **Armand Jaminon**, **Niko Rapp**, **Cengiz Akbulut,** who oriented me in the lab and made my daily routine easier. I would like to personally thank **Eva Bengtsson** and **Isabel Gonçalves** from Lund University as well as **Ilze Bot** from Leiden University for the fruitful and enjoyable collaboration we had. You all have a great competence in the field and you were always being willing to help me. Also, thanks to all the co-authors of the studies for the great collaboration we had.

Being part of **INTRICARE European Union's program**, I would like to express my gratitude to the project manager **Tara de Koster,** who did a great job by always supporting us, as well as to the great selection of exceptional scientists belonging to this family. Starting from the program co-ordinator **Tilman Hackeng**, to **Rory Koenen**, **Joachim Jankowski**, **Eline Kooi**, **Felix Mottaghy**, **Wilhelm Jahnen-Dechent**, **Johan Frostegard**, **Sébastien Foulquier** and **Jürgen Floege**. Many thanks to the rest of Early Stage Researchers, **Dawid Kaczor**, **Anouk Gentier**, **Mueez Aizaz**, **Alexandru Florea**, **Robert Dzhanaev**, **Shalesh Kumar Samal**, **Stefan Reinhold** and **Maurice Halder**, for the great times we had all together in the meetings, the scientific and extracurricular fun. Special thanks go to **Shruti Bhargava** for the nice moments we had together, the nice discussions and your positive attitude. Each and every one has taught me something different and I wish you all the best in the future.

Furthermore, since this journey started in Nancy, France, I would like to thank all those people and friends who I made during my time in this country and supported me both in professional as well as personal issues. Je tiens à remercier **Patrick Lacolley**, **Véronique Regnault** pour m' avoir accuelli dans leur laboratoire et m'avoir introduit au domaine du vieillissement vasculaire et de la génération de la thrombine, ainsi que les autres membres du laboratoire pour leur hospitalité. Un merci particulier à **John Pirault** pour avoir été mon superviseur pendant mon séjour et le soutien qu'il m'a apporté et **Mélusine Didelot** pour le soutien mutuel pendant les moments difficiles que nous avons traversés. Merci à tous les deux pour les bons moments que nous avons passés ensemble en dehors du laboratoire. **Pasquale Cocchiaro**, molte grazie a te per aver condiviso momenti piacevoli e conversazioni insieme.

I could not stand without mentioning and expressing my greatest gratitude to my beloved "**French family**". **Simon Thornton** and **Marie-Dominique Even**, vous connaissez mes sentiments. Je suis heureux de vous avoir recontré. Vous avez été ma deuxième famille, car vous m'avez pris sous vos ailes et m'avez ouvert votre maison dès mon premier jour à Nancy. Vous avez incarné la véritable signification de "Zeus Xenios". Tout au long de cette année avec vous, j'ai eu la chance de rencontrer vos enfants et d'apprendre tant de choses sur la culture française et néo-zélandaise pendant nos conversations illimitées. Je me souviens toujours du bonheur de nos tables autour d'un verre de vin et du fromage français. J'espère vous rencontrer bientôt.

Thank all the current and previous members of the **Cardiovascular Medicine Division**, especially **Gabrielle Paulsson-Berne**, **Daniel Ketelhuth**, **Lars Maegdefessel**, **Maria Forteza de los Reyes**, **Roland Baumgartner**, **Silke Thul**, **Sven-Christian Pawelzik**, **Ilona Kareinen**, **Martin Berg**, **Konstantinos Polyzos**, **Anton Gisterå**, **Xintong (Sophie) Jiang**, **Stephen Malin**, **Monica Centa**, **Sanna Hellberg**, **Vladimir Shavva**, **Alessandro Gallina**, **April Caravaca**, **Alice Maestri**, **Ruby Schipper**, **Fabiana Baganha**, **Anna Ioannidou, Carolina Hagberg**, **Olivera Werngren**, **Ekaterina Chernogubova**, **Greg Winski** for the nice interactions we had together spontaneously, during lunches or coffee-breaks, your kindness and the practical help. Special thanks to **Per Eriksson** who accommodated me in his lab during my lab transition, his calmness and the nice talks we had. Many thanks to **Anneli Olsson** and **Linda Haglund** for those techniques you taught me and for the nice discussions we had together. You were always there to help and answer all my questions. **Aastha Arora**, **Nancy Simon** and **Shirin Pourteymour**, thanks for the support, your company during our lunches and because I was always having a person to speak. I would like to personally thank **Michael Fored** for the endless assistance throughout the years.

Thanks to all the members of the **Division of Cardiology** and especially **Marita Wallin**, **Ali Mahdi, Tong Jiao** and **Aida Collado Sánchez** for the great interaction and the lunch company.

Thanks to all the staff of **BioClinicum J8**, that are not mentioned previously, for creating a nice working environment. **Alex Addinsall** thanks for the nice discussions during our lunches or late-evenings.

Ράνια σε ευχαριστώ πάρα πολύ που όλα αυτά τα χρόνια ήσουν πάντα δίπλα μου. Αισθάνομαι ευτυχισμένος που σε γνώρισα μαζί με την **Γλυκερία** απο την αρχή αυτού του μακρινού ταξιδιού και που παραμείνατε δίπλα μου. Ευχαριστώ για όλη την υποστήριξη και για τις ωραίες συναντήσεις μας. Ήσασταν για μένα μια πηγή όασης. **Πέτρο** σε ευχαριστώ για όλα...από την πρώτη μου μέρα στην Στοκχόλμη που με φιλοξένησες στο μαγευτικό «τορτελίνι» (Norrtälje) μέχρι τώρα. Είσαι πάντα εκεί δίπλα να με στηρίζεις και να με ταΐζεις φυσικά. **Στέλιο** και **Μαριάννα** σας ευχαριστώ για τις όμορφες στιγμές στο σπίτι σας με τα ωραία bbqs και τα παιχνίδια με τους υπέροχους γιού σας. **Ναταλί** ευχαριστώ πολύ για την παρέα, τα απερολ και τις συζητήσεις μας.

Ευχαριστώ όλους τους φίλους και συγγενείς μου που με στήριξαν όλα αυτά τα χρόνια και μπόρεσα να μοιραστώ μαζί τους εμπειρίες, αγωνίες και όνειρα.

Πιο πολύ ευχαριστώ την οικογένεια μου, την μητέρα μου **Σοφία**, τον πάτερα μου **Δημήτρη** και τα πολυαγαπημένα μου αδέρφια **Φωτεινή** και **Βάιο** για την αγάπη σας. Σας ευχαριστώ για την κατανόηση, τη στήριξη και την εμπιστοσύνη όλα αυτά τα χρόνια, για τις δύσκολες και ευχάριστες στιγμές που περάσαμε μαζί, που είστε πάντα εκεί. Τέλος ευχαριστώ πολύ τον σύντροφο μου στη ζωή, **Γιάννη**, που ήταν δίπλα μου όλα αυτά τα χρόνια, με βοήθησες να στηριχτώ στα πόδια μου και να ξεπεράσουμε μαζί κάθε εμπόδιο. Σε ευχαριστώ που ομορφαίνεις τη ζωή μου.

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10 CURRICULUM VITAE

Nikolaos-Taxiarchis (Nikos) Skenteris was born on November 3rd, 1990 in Lamia, Greece. Nikos obtained a BSc degree in Biology with a specialisation in Molecular Biology, Genetics and Biotechnology from Aristotle University of Thessaloniki, Greece in 2013, and a MSc degree in the "Molecular Basis of Human Disease" from University of Crete, Greece in 2016. Then, he moved to Nancy, France where he jointed Professor Patrick Lacolley's group at INSERM – U1116 and the University of Lorraine, in a project focusing on the effect of omega 3 PUFAs and pro-resolving lipid mediators on thrombin generation and arterial stiffness. In 2016, he was accepted for doctoral studies at Karolinska Institute, Stockholm, Sweden as part of a European Union's Horizon 2020 research and innovation program under the *International Network for Training on Risks of Vascular Intimal Calcification and roads to Regression of Cardiovascular Disease* (INTRICARE) Marie Skłodowska-Curie grant agreement (No 722609). His project was conducted under the supervision of Ass. Prof. Hildur Arnardottir, Department of Medicine and Assoc. Prof. Ljubica Matic, Department of Molecular Medicine and Surgery, at Karolinska Institute as well as Prof. Erik A.L. Biessen, Department of Pathology and Prof. Chris Reutelingsperger, Department of Biochemistry, at Maastricht University and Cardiovascular Research Institute Maastricht (CARIM). Nikos' main research interest was to provide novel translational insight into the context of cardiovascular calcification by applying complex integrative systems approaches, which combine human multi-biobanks, *in-silico* analysis, rodent *in vivo* models of vascular calcification and cellular *in vitro* cultures for functional and mechanistic investigations. His research resulted in this double PhD degree between Karolinska Institute and Maastricht University, summarised in the presented doctoral thesis entitled "Interplay between inflammation and calcification in cardiovascular diseases".

His research has been presented at several international scientific conferences:

2022 2nd European Society for Vascular Surgery-Translational Meeting (Poster)

2022 28th Scandinavian Atherosclerosis Conference (Poster)

2021 89th European Atherosclerosis Society Congress (Poster)

2021 17th International Society of Applied Cardiovascular Biology Meeting (Oral)

2020 Society for Vascular Surgery-Vascular Research Initiatives Conference (Oral)

2020 ATVB Vascular Discovery: From Genes to Medicine (Oral)

2020 21st International Vascular Biology Meeting (Oral)

2020 1st European Society for Vascular Surgery-Translational Meeting (Oral)

2020 86th German Cardiac Society Meeting (Oral)

2019 87th European Atherosclerosis Society Congress (Oral)

His research has been distinguished with several grants and fellowships:

2022 Hjärt-Lungfonden (HLF) Travel Grant, Sweden

2020 Young Investigator Fellowship from European Atherosclerosis Society

2020 Hjärt-Lungfonden (HLF) funding for research (co-applicant), Sweden

2019 Swedish Society for Medical Research (SSMF) Travel Grant for preclinical medical research, Sweden

2019 Karolinska Institutet Travel Grant for long-term research visit, Sweden

2019 Fellowship from "Onassis Foundation" for attending Lectures in Biology and Chemistry, Greece

2016 Fellowship from the "Gazi – Triantafyllopoulou" Foundation, Greece

11 JOURNALS PERMISSION

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- Heuschkel MA, Skenteris NT, Hutcheson JD, van der Valk DD, Bremer J, Goody P, Hjortnaes J, Jansen F, Bouten CVC, van den Bogaerdt A, Matic L, Marx N, Goettsch C. Integrative Multi-Omics Analysis in Calcific Aortic Valve Disease Reveals a Link to the Formation of Amyloid-Like Deposits. *Cells*. 2020;

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- Gonçalves I, Oduor L, Matthes F, Rakem N, Meryn J, Skenteris NT, Aspberg A, Orho-Melander M, Nilsson J, Matic L, Edsfeldt A, Sun J, Bengtsson E. Osteomodulin Gene Expression Is Associated With Plaque Calcification, Stability, and Fewer Cardiovascular Events in the CPIP Cohort. Stroke. 2022

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- Waring OJ, Skenteris NT, Biessen EAL, Donners MMPC. Two-faced Janus: The dual role of macrophages in atherosclerotic calcification. Cardiovasc Res. 2021 Sep 22:cvab301. doi: 10.1093/cvr/cvab301

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