Key role for VSMCs in vascular remodeling and calcification

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**Key role for VSMCs in
vascular remodeling
and calcification**

Colofon

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Key role for VSMCs in vascular remodeling and calcification

PROEFSCHRIFT

Ter verkrijging van de graad doctor aan de Universiteit Maastricht,
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Volgens het besluit van het College van Decanen,
In het openbaar te verdedigen op
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Voor mijn vrouw Famke,
voor haar onvoorwaardelijke steun

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Chapter 1

General introduction

Cardiovascular disease

The world health organization has reported that cardiovascular disease (CVD) is the number one cause of mortality worldwide ¹. In 2017 an estimated 17.8 million people died from CVD which represents 32% of all global deaths. The majority of CVD related deaths is caused by myocardial infarction and ischemic stroke ². Additionally, most CVDs occur in low- to middle-income countries, where early detection and treatment is not included in primary healthcare programs.

One of the key players in CVD development is arterial remodeling. Continuous stress on the vessel wall results in arterial remodeling initiating mechanisms to repair the vessel wall. Long-term stress induces structural and biological changes that alter characteristics of the vessel wall, leading to chronic vascular remodeling, pathological adaptation and vascular stiffness. This results in increased vessel wall thickness due to hyperplasia and phenotypic switching of vascular smooth muscle cells (VSMCs). Increased arterial stiffness is often accompanied with presence of vascular calcification. In the last decade, vascular calcification was added to the growing list of risk factors for CVD mortality and morbidity, however the precise role of vascular calcification in CVD still needs to be unraveled ^{3,4}.

Arterial remodeling

Arterial remodeling reflects adaptation of the vessel wall to biochemical and biomechanical stimuli ^{5,6}. In the vasculature, there are two types of remodeling, namely outward and inward remodeling. Both types of remodeling coincide with hypertrophy (thickening) or hypotrophy (thinning) of the vessel wall. VSMCs are key in the development of arterial remodeling. In the vessel wall, VSMCs are connected to a fenestrated network of elastin and collagen fibres. The capacity of the vessel wall to elastically distend is important to accommodate the volume ejected with each heartbeat and to limit peripheral pressure pulsations. The active tone and spatial arrangement of VSMCs may influence mechanical load on extracellular matrix (ECM) components and therefore, modulate vessel diameter and stiffness ⁷.

Chronic exposure to high blood pressure increases tensile stress. VSMCs respond by proliferation, resulting in hyperplasia and thickening of the vascular wall. Activation of MMPs facilitate breakdown of elastin and synthetic VSMCs produce collagen, attempting to preserve stiffness homeostasis ⁸. Additional ECM disturbances, such as presence of vascular calcification, have further impact on remodelling and stiffening of the medial vascular layer ^{9,10}. Vascular calcification is defined as pathological mineral deposits in the vessel wall. There are two distinct sites of vascular calcification: medial and intimal vascular calcification, referring to the layer of the vessel wall where calcification is found ¹¹. Arterial remodeling is complex and this thesis aims to address unresolved issues in the interplay between vascular stiffening, vascular calcification and atherosclerosis (Figure 1).

Figure 1. The interplay between arterial remodeling, vascular stiffening, vascular calcification and atherosclerosis. Arterial remodeling processes are initiated by VSMCs who undergo a phenotype switch under stress stimuli, leading to collagen deposition and elastin degradation. The increased collagen deposition combined with lower elasticity lead to arterial stiffness and can form a nidus for the development of vascular calcification. Vascular calcification can be found at two distinct sites: medial and intimal. Both arterial stiffness and medial calcification can initiate atherosclerosis due to flow disturbances and shear stress. Once plaques mature they develop into vulnerable plaques that are prone to rupture. Inside atherosclerotic plaques VSMC phenotype switching plays an important role. Here VSMCs can induce calcification that starts as microcalcification and can develop into large macrocalcifications.

Atherosclerosis

Atherosclerosis is the pathological build-up of plaques in the intimal space thereby thickening the vessel wall and narrowing the lumen. Atherosclerosis is considered an inflammatory disease that is ignited by endothelial stress and lipid accumulation with subsequent leucocyte infiltration^{12,13}. Atherosclerosis development is, in part, driven by factors that are present in the blood compartment. Lipids, high glucose and disturbed hemodynamics, e.g. caused by high blood pressure, can activate the endothelium initiating plaque development¹⁴. More recently, local vascular processes are considered to be important in the development of atherosclerosis^{15,16}. Lineage tracing studies in mice revealed that many cells present in the atherosclerotic plaque are derived from VSMC precursors¹⁷. Moreover, VSMC traces are found in atherosclerotic lesions which often co-localise with macrophage markers¹⁸. This implies that cells in the intima are recruited from the vessel media instead of infiltrating

1 mesenchymal cells from the blood compartment^{18,19}. VSMCs exert multiple roles and functions in atherosclerosis. During plaque development, VSMCs can migrate and form a fibrous cap in which they excrete extracellular matrix proteins (ECMs) that stabilize the plaque. Oppositely, intra-plaque VSMCs can change toward a macrophage-like phenotype^{19,20}. Furthermore, VSMCs undergoing apoptosis with diminished efferocytosis form a nidus for calcification^{21,22}.

Vascular calcification

Intimal calcification often coincides with atherosclerosis as both are pathological processes affecting the intimal layer of the vessel wall and initiate luminal narrowing. Intimal calcification refers to build-up of calcified areas within atherosclerotic lesions. Intimal calcification can exist of two forms: micro- and macrocalcification. Intimal calcification is used as a measure of atherosclerotic burden²³ and large areas of calcification ($>50\text{ }\mu\text{m}$) are considered macrocalcification. Macrocalcification has been recently associated with increased plaque stability²⁴. The plaque stabilising effect of calcification was further adopted since statins, used to lower plasma lipids, have plaque stabilising effects and concomitant increase intimal calcification²⁵⁻²⁷. On the other hand, microcalcification ($<50\text{ }\mu\text{m}$) has been shown to decrease plaque stability and thereby favour thrombus formation²⁸. Clinically, the distinction between micro- and macrocalcification is not possible due to a lack of resolution of computed tomography (CT), being $200\text{ }\mu\text{m}$ ²⁹. Distinguishing vascular calcification based on size is limited and not telling the complete story as density and location of calcification also contribute to the pathogenesis of cardiovascular disease. Coronary artery calcium (CAC) volume is associated with CVD risk whereas CAC density is inversely correlated with CVD risk³⁰. Additionally, the location where calcium deposits reside inside atherosclerotic plaques determines its vulnerability to rupture. Calcium deposits that are located in the necrotic core do not seem to have significant impact on plaque stability whereas calcium deposits that are in the fibrous cap region may lead to plaque rupture³¹.

Medial calcification, also known as Mönckebergsclerosis, is associated with ageing and is regularly found in peripheral arteries^{32,33}. Medial calcification can be found along the entire vascular tree and is often found in patients suffering from diabetes and/or chronic kidney disease (CKD)^{34,35}. Medial calcification is an important confounding factor in the development of vascular stiffening and hypertension, two clinical entities that affect the physiological condition of the vessel wall^{36,37}. Interestingly, early literature reports that medial remodeling or calcification precedes at sites of atherosclerosis³⁸. More recent data suggest an interaction between both medial and intimal calcification, in which both can give impact one another^{39,40}. There are multiple factors and pathways leading to vascular calcification (Figure 2). Below I will summarize the most important.

Figure 2. VSMC phenotype switching. Under physiological condition VSMCs have a contractile phenotype which is necessary to cope with alterations in blood pressure. Upon pathological signals VSMCs respond and initiate a phenotype switch. VSMCs can switch to a synthetic phenotype under increased calcium and inflammation. Vitamin K deficiency, often found in patients on Vitamin K antagonist, induces a VSMC synthetic switch. Synthetic VSMCs are known to induce apoptosis, proliferation, extracellular vesicle release and affect the extracellular matrix. VSMC osteogenic phenotype switch is induced by increased phosphate and lower Fetuin A plasma levels. Osteogenic VSMCs are known to secrete calciprotein particles and increase bone related protein expression. Both synthetic and osteogenic VSMCs are known to cause vascular calcification.

VSMC phenotype switching

VSMCs display phenotypic plasticity during normal development, disease and repair of the vasculature. Under pathological signals such as inflammation or mineral disbalance, VSMCs undergo phenotypic switching. This VSMC phenotype switching is characterized by increased proliferation and migration⁴¹ and extracellular vesicle (EV) release⁴². High levels of calcium and phosphate drive VSMCs towards an osteogenic phenotype which express bone related proteins and deposit mineralized bone-like matrix⁴³.

Tissue damage and inflammation of the vessel wall can initiate calcification by the induction of VSMC apoptosis. Apoptosis is considered to be a central aspect of vascular calcification, due to the shedding of apoptotic bodies that can form a nidus for calcification^{44,45}. Moreover, synthetic VSMCs are able to shed EVs that can serve as nidus for calcification^{42,46}. Furthermore, *in vitro* experiments have demonstrated that apoptosis increases calcification whereas inhibition of apoptosis decreases vascular calcification²¹.

Loss of calcification inhibitors

In order to prevent spontaneous calcification, inhibitors of calcification are present in both the circulation and in the vasculature. Fetuin-A and matrix Gla protein (MGP) are such proteins with strong ion binding capacity^{47,48}. Fetuin-A is a plasma protein, mainly synthesized in liver and plays a critical role in the stabilization of protein-mineral complexes, also termed calciprotein particles (CPPs). The formation of CPPs prevents pathological calcium and phosphate deposition in tissue if they are cleared from the circulation. CPP clearance is attributed to liver Kupffer cells and if not performed correctly leads to particle-induced inflammation and vascular calcification⁴⁹.

MGP is a vitamin K-dependent protein that requires vitamin K to become biologically active⁴⁸. In mice, MGP knock-out⁵⁰ or drug induced knock-down of active MGP^{10,51} results in both intimal and medial vascular calcification. Additionally, in patients, usage of vitamin K antagonists is found to induce vascular calcification⁵²⁻⁵⁴.

Other well-known calcification inhibitors are osteoprotegerin (OPG), pyrophosphate (ENPP1) and osteopontin (OPN). Clinically, high levels of OPG and lower levels of ENPP1 are associated with atherosclerosis and vascular calcification⁵⁵. Furthermore, OPN is found in mineralized tissues such as bone and teeth but also in calcified plaques⁵⁶.

Disease-related calcification

Many diseases are associated with a higher risk for vascular calcification. Type 2 Diabetes Mellitus (T2DM) is associated with increased prevalence of CVD and mortality. T2DM patients appear to have extensive medial calcification which is an independent predictor of cardiovascular mortality⁵⁷.

CKD patients suffer from hyperphosphatemia which is, on its own, a risk factor for increased mortality⁵⁸. Additionally, high phosphate levels are associated with severe progression of vascular calcification⁵⁹. The uremic environment that CKD patients display evokes VSMC osteogenic phenotype switching that results in mineralization of surrounding tissue^{33,60}.

The interaction between inflammation and vascular calcification is not completely understood. Vascular calcification can be found in atherosclerosis, which is considered an inflammatory disease, and many inflammatory markers are associated with vascular calcification⁶¹. On the other hand, medial vascular calcification is less associated with inflammation. Chronic inflammation is known to be associated with atherosclerotic calcification in mice⁶² and has been confirmed by ¹⁸F-FDG positron emission tomography with subsequent CT scanning in patients⁶³.

Pseudoxanthoma elasticum (PXE) is a monogenetic disorder caused by a mutation in the ATP binding cassette subfamily C member 6 (ABCC6) gene. PXE is characterized by dystrophic mineralization of soft connective structures, primarily elastic fibers. PXE manifests in several organs such as the skin, eye and heart⁶⁴. Additionally, CVD is often encountered, primarily reflected by mineralization of mid-sized arteries in

the lower extremities. The onset of PXE is usually rather late and slowly progressing, which clinically manifests as mineralization of the Bruchs' membrane in the eye and or elastic fibers in the arterial blood vessels causing increased morbidity with occasional mortality⁶⁵.

Outline of this thesis

The research described in this thesis addresses arterial remodeling: the interaction between vascular stiffness and vascular calcification both in relation to atherosclerosis. In more detail, I investigated the role of vascular smooth muscle phenotypic switching in vascular remodeling in relation to calcification.

Chapter 2 elaborates on the interactions between vascular remodeling, vascular stiffness and vascular calcification. In this chapter, we discuss the role and phenotypic plasticity of VSMCs in vascular remodeling. We review in detail the latest clinical techniques of measuring vascular remodeling.

Chapter 3 focusses on the role of VSMC calcification in the tunica media in relation to the initiation and development of atherosclerosis. To address this, I used DBA/2 mice that are prone to develop medial vascular calcification. Using a viral LDLr knock-down this allowed me to study the role of medial calcification on the development of atherosclerosis.

Chapter 4 focusses on the role of VSMC apoptosis in the tunica media in relation to the initiation and development of atherosclerosis. I have employed an ApoE^{-/-} mouse model that has knocked in the human diphtheria toxin receptor specifically in VSMCs which allowed me to reduce VSMC number in the vessel wall prior to initiation of atherosclerosis. In *Chapter 4*, I present data on the role and contribution of medial VSMCs in the development of atherosclerosis.

In *chapter 5* I describe a novel approach to measure VSMC calcification. We reason that the biomarker for vascular calcification is not a single one but rather the collection of all plasma components. This paper describes the new cell-based assay for measuring the composite biomarker for vascular calcification.

Chapter 6 elaborates on this first clinical study by focusing on the role of vitamin K-dependent MGP in vascular calcification and whether MGP can be used as biomarker to predict presence of vascular calcification. We measured MGP by different means, ranging from genomic level (MGP SNPs) to expression (RNA levels) and protein excretion (immunohistochemical staining and plasma levels).

In *chapter 7* key findings are summarized and the results from this thesis are discussed, additionally, future research directions are provided.

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Chapter 2

The Role of Vascular Smooth Muscle Cells in Arterial Remodeling: Focus on Calcification-Related Processes

A.M.G. Jaminon, K. Reesink, A.A. Kroon and L.J. Schurgers

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Abstract

Arterial remodeling refers to the structural and functional changes of the vessel wall that occur in response to disease, injury, or aging. Vascular smooth muscle cells (VSMC) play a pivotal role in regulating the remodeling processes of the vessel wall. Phenotypic switching of VSMC involves oxidative stress-induced extracellular vesicle release, driving calcification processes. The VSMC phenotype is relevant to plaque initiation, development and stability, whereas, in the media, the VSMC phenotype is important in maintaining tissue elasticity, wall stress homeostasis and vessel stiffness. Clinically, assessment of arterial remodeling is a challenge; particularly distinguishing intimal and medial involvement, and their contributions to vessel wall remodeling. The limitations pertain to imaging resolution and sensitivity, so methodological development is focused on improving those. Moreover, the integration of data across the microscopic (i.e., cell-tissue) and macroscopic (i.e., vessel-system) scale for correct interpretation is innately challenging, because of the multiple biophysical and biochemical factors involved. In the present review, we describe the arterial remodeling processes that govern arterial stiffening, atherosclerosis and calcification, with a particular focus on VSMC phenotypic switching. Additionally, we review clinically applicable methodologies to assess arterial remodeling and the latest developments in these, seeking to unravel the ubiquitous corroborator of vascular pathology that calcification appears to be.

1. Introduction

The vessel wall consists of multiple cells, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), fibroblasts and pericytes, and structural extracellular matrix (ECM) components, such as elastin and collagen¹. ECs form the innermost layer, being the barrier between the blood-carrying lumen and vessel wall. In the vessel wall, ECs are surrounded by the tunica media, consisting almost entirely of circumferentially oriented VSMCs. Medial VSMCs, facilitating vessel dilation and constriction, are surrounded by interconnected elastic lamellae². The outer layer of the vessel wall is the adventitia and contains ECM, maintaining structural integrity under peak mechanical load, and fibroblasts and progenitor (mesenchymal stem cell-like, MSC-like) cells. The interplay of all components of the vessel wall is needed to preserve vascular health³⁻⁵.

The vessel wall is continuously exposed to biomechanical and biochemical stressors that elicit functional and adaptative responses. For instance, when blood flow is acutely increased, the increased wall shear stress (or friction) is sensed by ECs, which release relaxants. These relaxants (e.g., nitric oxide, NO) lower the active tone of VSMCs, leading to (flow-mediated) vessel dilation to counteract the initial increase in wall shear stress⁶. Long-term changes in wall shear stress provoke diameter adaptation⁷. Wall thickness changes are seen in response to increased wall stress (or wall tension), due to high blood pressure⁸. Such arterial remodeling responses shape early development of the macro-circulation, but are also affected by normal aging processes⁹. A multitude of biological processes contribute to pathological vascular remodeling, such as inflammation, oxidative stress, lipid accumulation, and degradation of the ECM^{10,11}. Due to the heterogeneity of vascular remodeling, it is difficult to pin-point single biological processes responsible for vascular disease. Lessons from genetic disorders such as pseudoxanthoma elasticum (PXE), Marfan's syndrome and Keutel syndrome, have addressed the complexity of arterial remodeling¹². A better understanding of the complexity of arterial remodeling will help unravel its role as a cause or consequence of cardiovascular disease (CVD).

VSMCs in the medial layer of the vessel wall play a key role in arterial remodeling. VSMCs are the most abundant cell type in the arterial vessel wall and are pivotal in maintaining vessel structure and function¹³. VSMCs are considered heterogeneous and display a high degree of plasticity⁵. Under physiological conditions, VSMCs have a contractile phenotype which facilitates the contraction and dilation of the vasculature, which, in smaller resistance arteries, is key to the regulation of blood flow. Upon biological stress signals or vascular injury, VSMCs respond by losing contractility markers and differentiate towards a synthetic VSMC phenotype. Synthetic VSMCs subsequently express proteins involved in proliferation and migration¹⁴. Contractile VSMCs may adapt to stress by differentiating towards synthetic VSMCs which are able to newly synthesize ECM components such as collagen. To remodel the ECM, synthetic VSMCs produce metalloproteinases (MMPs), such as collagenases and elastases, that allow them to migrate to sites of injury¹⁵⁻¹⁷. VSMC-mediated remodeling of ECM within the vessel wall may result in increased arterial stiffness^{13,18}, contributing to systolic hypertension and altered hemodynamic conditions in end-organs such as the brain, kidneys and heart¹⁹.

VSMCs are involved in many vascular diseases, such as atherosclerosis and aneurysm formation. In all these vascular pathologies, vascular calcification is involved

^{5,20,21}. Vascular calcification is defined as the deposition of calcium crystals within the vessel wall, initiated as microcalcification and propagating towards macrocalcifications ²², and, eventually, encroaching entire segments of the vasculature ²³. Vascular calcification is an active process with a key role for VSMCs, including apoptosis ²⁴, osteochondrogenic transdifferentiation ²⁵, extracellular vesicle release ², calcium overload ²⁶, and cellular senescence ²⁷ (Figure 1). The aim of the present review is to provide an overview of the pathways underlying arterial remodeling, with a particular focus on the role of VSMCs and calcification-related processes.

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Figure 1. (a) Vascular smooth muscle cell (VSMC) phenotypic switching. Under physiological conditions, VSMCs display a contractile phenotype that regulates vessel structure and function. Under stress, arterial remodeling will occur, leading to VSMC phenotypic switching. Factors inducing the synthetic phenotype include platelet derived growth factors (PDGF), protease-activated receptors (PARs) and tumor necrosis factor- α (TNF- α). Synthetic VSMCs initiate vessel repair and can switch back to the contractile phenotype, driven by factors such as heparin or laminin. The osteogenic phenotype can be induced by prolonged exposure to BMP-2 or high phosphate. Osteogenic VSMCs shed extracellular vesicles that promote vascular calcification. **Panel (b) Pathways affecting vascular calcification.** Vascular calcification is an active process which can be initiated by several pathways, including: 1. biochemical factors, 2. physical factors, 3. vascular calcification (VC) inhibitors and 4. ECM factors. Biochemical factors, such as raised calcium and phosphate levels and PDGF, are stress molecules that induce VSMCs to switch towards a synthetic or osteogenic phenotype, including an increased release of extracellular vesicles. The ECM of the vessel wall directly influences VSMCs. Changes in collagen and elastin content cause VSMCs to change morphology and phenotype. VSMCs in turn, produce MMPs that induce structural changes in the vessel wall by rearranging collagen and elastin, promoting the migration and proliferation of VSMCs and other cell types. Physical factors such as shear stress and tensile stress affect ECs' NO release, which influences the VSMC phenotype. Shear-induced stress induces a synthetic phenotype by decreasing α -SMA, MYH and smtn expression. Tensile stretch induces VSMCs to produce ECM proteins, such as collagen and fibronectin promoting vessel fibrosis. VC inhibitors, such as OPN, MGP and PP_i , affect the VSMC phenotype by inducing changes in RNA expression patterns. Taken together, all stimuli play a part in an orchestrated VSMC response, ultimately promoting vascular calcification.

2. Biology of Vascular Remodeling

2.1 Arterial Remodeling

Arterial remodeling reflects the adaptation of the vessel wall to biochemical and biomechanical stimuli ^{9,12}. In the vasculature, two types of remodeling can be distinguished: outward and inward remodeling, with respective hypertrophy (thickening) or hypotrophy (thinning) of the vessel wall. Large conduit vessels do not have the ability to constrict in response to stress, and therefore show hypertrophic remodeling. Atherosclerosis is characterized by an increase in vessel diameter, with the thickening of both the media and intima, and classically termed outward hypertrophic remodeling ²⁸. Aneurysm formation is characterized by an increase in vessel diameter, with a thinning of the vessel wall, and termed outward hypotrophic remodeling ²⁹ (Figure 2). Inward remodeling is less frequently seen and is observed in more muscular peripheral arteries, probably reflecting the sustained vasoconstriction of vessels ³⁰.

Figure 2. Vascular remodeling types. There are several types of arterial remodeling: hypotrophic, eutrophic and hypertrophic. Additionally, remodeling can be inward and outward. Hypotrophic remodeling results in a thinner vessel wall, which can be both inward and outward. In both cases, the wall-to-lumen ratio decreases. Hypertrophic remodeling results in the thickening of the vessel wall, that can also be inward and outward. The thickening of the vessel wall results in an increased wall-to-lumen ratio. In the eutrophic situation, wall-to-lumen ratios do not differ, but the size of the vessel can change. Atherosclerosis is characterized by an increased wall-to-lumen ratio, with both thickening of the media and intima and, therefore, is classified as inward or outward hypertrophic remodeling. Aneurysm formation is characterized by an increase in vessel diameter with a thinning of the vessel wall (outward hypotrophic remodeling). Inward remodeling is less frequent and is more associated with muscular peripheral arteries, reflecting sustained vasoconstriction of vessels.

Under low laminar flow, platelet derived growth factors (PDGF) and transforming growth factor- β (TGF- β) promote inward remodeling by increasing VSMC proliferation and collagen deposition³¹. Inward remodeling also contributes to atherosclerosis, but does not lead to an increased vessel size (Figure 2). Atherosclerosis development involves many cellular processes, like the recruitment of inflammatory cells by chemotaxis and infiltration³².

VSMCs are connected to a fenestrated network of elastin and collagen fibers. The capacity of the vessel wall to elastically distend is important to accommodate the volume ejected with each heartbeat and to limit peripheral pressure pulsations. The active tone and spatial arrangement of VSMCs may influence the mechanical load on the ECM components and, therefore, modulate vessel diameter and stiffness¹⁸. Chronic exposure to high blood pressure increases tensile stress⁹ to which VSMCs respond by proliferation, resulting in hyperplasia and thickening of the vascular wall. Concomitant activity of MMPs facilitates the structural breakdown of elastin ECM, and synthetic VSMCs produce collagen ECM, attempting to preserve stiffness homeostasis³³. High blood pressure thus aggravates age-related stiffening of arteries³⁴⁻³⁶. Additional ECM disturbances, such as the presence of calcium crystals, have a further impact on the stiffening of the medial layer^{37,38}.

During aging, it is generally accepted that the number of cells in the vasculature decreases, although the causes of this finding remain to be established³⁴. It has been hypothesized that VSMCs become senescent and that cell division rates decrease³⁹. The recent literature ignores vessel wall cellularity and often refers to cellular processes, such as apoptosis, inflammation, calcification and epigenetic effects, all playing a part in vessel wall aging²⁰. Additionally, with aging, collagen content in major arterial vasculature increases, whereas elastin content decreases and the number of VSMCs declines⁴⁰. As a consequence, remaining VSMCs are embedded in a collagen-enriched ECM with fewer cellular focal adhesions⁴¹⁻⁴³. Within arterial vessels, differences exist in the content of elastin to VSMC ratios⁴⁴. Large arteries close to the heart contain more elastin and are therefore called “elastic” arteries. It is particularly elastic arteries that stiffen with age⁴⁵. Large artery stiffening results in decreased arterial compliance, especially in those aged over 60 years^{19,46,47}. Peripheral vessels contain more VSMCs relative to elastin and are termed “muscular” arteries. In muscular arteries, the relative elastin content increases with age, most likely caused by the decline in the number of VSMCs and decreased collagen content⁴⁴. It should be noted that the absolute amount of ECM proteins in the vasculature decreases with age, but that fat and extracellular material, such as calcium crystals, increase⁴⁸. Taken together, the number of VSMCs within the vasculature strongly correlates with vascular stiffening and the arterial remodeling processes³⁴.

Endothelial function plays an important role in arterial remodeling. Blood flow and normal wall shear stress stimulate ECs to produce NO (Figure 3). NO induces the relaxation of local VSMCs, which leads to dilation of the vessel wall. Endothelium-dependent vasodilation decreases with age, which appears to be associated (perhaps causally or bi-directionally) with a quiescent state of VSMCs and is clearly implicated to be associated with hypertension and CVD^{49,50}. Under pathological conditions, ECs are known to produce cytokines and growth factors⁵¹, which induces VSMCs phenotype switching from contractile to a more synthetic phenotype. An increased wall shear stress increases endothelial derived NO release, further decreasing VSMC proliferation and

increasing VSMC apoptosis⁵², resulting in outward remodeling and stiffened vasculature, due to the formation of the ECM matrix by remaining synthetic VSMCs^{34,53}.

Figure 3. Endothelial cell-smooth muscle cell communication. Endothelial cells (ECs) communicate with vascular smooth muscle cells (VSMCs) in two ways, called endothelial derived hyperpolarization (EDH). EDH has two major pathways: 1. Diffusion of nitric oxide (NO), which is produced by ECs and diffuses through the internal elastic lamina (IEL) to induce the relaxation of VSMCs. 2. Signaling through myoendothelial gap junctions (MEGJ) that connect ECs directly to VSMCs and cross the IEL. This direct communication between ECs and VSMC via MEGJ occurs via so-called endothelial derived hyperpolarizing factors (EDHF). 3. Major factors that stimulate EC NO secretion are flow or shear-stress alterations. Differences in wall shear rates are known to induce endothelial-derived changes in VSMC phenotype.

2.1.1. Inflammation and Arterial Remodeling

Inflammatory processes play a significant role in arterial remodeling and are linked to the development of atherosclerosis. Chronic low-grade inflammation is a key driver of arterial aging. VSMC phenotypic switching and ECM biochemical changes activate inflammatory pathways and oxidative stress signaling⁵⁴. Cytokines determine processes such as endothelial dysfunction, renin angiotensin activation and metalloproteinases release^{55,56}. VSMCs are involved in local vessel wall inflammatory functions.

Proliferation

VSMCs have low turnover rates in healthy vessels, but increase proliferation upon vascular injury to initiate repair⁵⁷. Increased expression of aging genes p16^{INK4a} and p21⁵⁸ have been linked to VSMC senescence and are present in atherosclerotic plaques, indicating lower proliferation and, thus, decreased repair capacity⁵⁹.

Cell Death

Cell death is linked to vascular disease, but absolute rates are difficult to determine. VSMC apoptosis in atherosclerosis has been linked to plaque vulnerability features⁶⁰, and chronic VSMC apoptosis has been shown to promote atherogenesis and plaque progression⁶¹. In vascular aging and medial remodeling, VSMCs cell death does not cause significant inflammation, due to the efficient IL-1 β -mediated clearance of apoptotic bodies by VSMCs⁶².

Platelets and Extracellular Vesicles (EVs)

Platelets have shown to affect VSMC inflammatory phenotypes and injury responses⁶³. Additionally, platelet EVs induce an inflammatory response in VSMC in vitro⁶⁴. Platelet factor 4 (PF4) has a central role in the stimulation of VSMC-mediated cytokine release, which is primarily affected by increased krüppel-like factor 4 (KLF-4) transcription. Experimental atherosclerosis models indicated that PF4 is proatherogenic and it has been found to penetrate deep into the vessel wall, underlining the importance of platelets aside from their effect on the endothelium⁶⁵.

2.2. The Role of VSMC Plasticity in Vascular Remodeling

Under physiological conditions, VSMCs display a contractile phenotype, in which markers such as alpha-smooth muscle actin (α -SMA), smooth muscle 22-alpha (SM22- α), smoothelin A/B (smtn), smooth muscle-myosin heavy chain (MYC11) and Calponin-1 (CNN-1) are highly expressed^{14,66,67} (Table 1). VSMCs are heterogeneous with regard to their expression, are derived from different embryonic origins and have a strong genetic component causing VSMC diversity. Numerous in vivo studies have shown the existence of diverse VSMC populations within the same artery in rats⁶⁸, pigs⁶⁹ and humans⁷⁰. We refer to VSMC plasticity here as the capability of VSMCs to switch phenotype. VSMC phenotypes are changed by environmental cues, such as soluble biochemical compounds, ECM proteins and biophysical conditions^{33,71} (Figure 1). VSMCs exhibit a whole array of phenotypes, ranging from contractile–quiescent to migratory–proliferative–synthetic and osteogenic-, macrophage- or MSC-like⁷²⁻⁷⁴ (Table 1). In the following subsections, we will briefly describe the most relevant factors that affect VSMC phenotype (Figure 1 and Table 2).

Table 1. Proteins that define VSMC phenotype.

Protein	Gene Name	Abbreviation	Expression	
			Contractile	Synthetic
Alpha smooth muscle actin	<i>ACTA-2</i>	α -SMA	+	+
Smooth muscle myosin heavy chain	<i>MYH11</i>	SMMHC	+	–
Smooth muscle 22 alpha	<i>TAGLN</i>	SM22- α	+	–
Smoothelin	<i>SMTN</i>	Smtn	+	–
Calponin	<i>CNN-1</i>	CNN-1	+	–
Tumor necrosis factor alpha	<i>TNFA</i>	TNF- α		+
S100 calcium binding protein A4	<i>S100A4</i>	S100A4	–	+
Monocyte chemoattractant protein 1	<i>CCL2</i>	MCP-1	–	+

+, positive effect; –, negative effect.

Table 2. Factors involved in VSMC phenotype switching.

Factors Involved in VSMC Phenotype Switching	Phenotype	
	Contractile	Synthetic
Biochemical compounds		
PDGF	–	+
TGF- β	+	–
PARs	–	+
TNF- α	+	+
Angiotensin II	+	+
Extracellular matrix components		
Integrin: $\alpha 1\beta 1$, $\alpha 7\beta 1$, $\alpha 8\beta 1$	+	–
Integrin: $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$	–	+
Collagen type I	–	+
Collagen type IV	+	–
Elastin	+	–
Heparin	+	–
Fibronectin	–	+
Laminin	+	–
Physical factors		
Tensile stress	–	+
Shear stress	–	+
Transcription		
KLF4	–	+
Oct4	–	+

Abbreviations: PDGF, platelet derived growth factor; TGF- β , transforming growth factor beta; PARs Protease-activated receptors; TNF- α , tumor necrosis factor alpha; KLF4, krüppel-like factor 4; Oct4, octamer-binding transcription factor 4. +, positive effect; –, negative effect.

2.2.1. Biochemical Compounds

PDGF is an important signaling molecule in the initial phase of VSMC differentiation. During vascular development, PDGF causes mesenchymal cell recruitment and subsequent proliferation⁷⁵. PDGF is known to induce VSMC differentiation towards a synthetic phenotype, as it downregulates the contractile marker α SMA in aortic VSMCs⁷⁶. Additionally, PDGF increases proliferation and migration in pig and human coronary VSMCs^{69,77,78}. Moreover, in vivo studies showed that inhibition of PDGF results in reduced VSMC proliferation and migration after arterial balloon injury^{79,80}.

TGF- β , as opposed to PDGF, induces a contractile and non-proliferative VSMC phenotype. An absence of TGF- β results in severe congenital cardiovascular disease, mainly shown in structural defects in experimental animals⁸¹. In vitro treatment of VSMCs with TGF- β induces expression of α SMA, MHC and CNN1^{69,82,83}.

Activated coagulation proteins also have been shown to affect VSMC phenotype via protease activated receptors (PARs)⁸⁴. Both tumor necrosis factor-alpha (TNF- α) and angiotensin II have been shown to induce the contractile, as well as the synthetic, phenotype of VSMCs. It has been shown that angiotensin II affects VSMC phenotypic plasticity via the induction of ROS and decreased scavenging activity of nitrate reductase (NAD(P)H) oxidases⁸⁵ and subsequently induces aneurysm formation via oxidative stress^{21,86}.

2.2.2. Extracellular Components

The ECM in which VSMCs are embedded also affects VSMC phenotype. VSMC phenotype is modulated via integrin receptors that are present on ECM proteins⁸⁷. ECM consists of structural proteins, such as collagen, elastin and proteoglycans. For instance, the proteoglycan heparin promotes VSMC contractility. In vitro, heparin treatment of VSMCs induces a contractile phenotype and slows down its proliferation^{69,88}. Collagen has pleiotropic effects on VSMC phenotype, depending on the type of collagen. Collagen type-I and fibronectin induce a synthetic VSMC phenotype^{89,90} with proliferation^{91,92}. On the contrary, collagen type-IV and laminin promote a contractile VSMC phenotype^{89,93}. Intact elastin is associated with a contractile VSMC phenotype. The loss of elastin, by a genetic mutation, is associated with increased hypertrophy and hyperplasia of VSMCs^{94,95}. Additionally, elastin remodeling, by the modulation of cathepsins and MMPs, promotes VSMC phenotypic switching that induces calcification^{96,97}.

Not only composition, but also the organization of structural fibers, determines VSMC phenotype. Culturing VSMCs in 3D compared to 2D increases contractile expression markers and induces TGF- β expression^{98,99}. Culturing VSMCs on scaffold templates controls spatial organization and morphological response of VSMCs, indicating that VSMCs react to structural environmental changes to retain vessel function¹⁰⁰.

2.2.3. Biophysical Factors

Hemodynamics at the vessel level (flow and transmural pressure) also affect VSMCs by determining tensile loading and active tone (e.g., by NO). Changes in vessel wall shear stress modulate EC NO release, and influence VSMCs via cell–cell interaction with ECs¹⁰¹. Using co-cultures of ECs and VSMCs, shear stress induces a synthetic VSMC phenotype by decreasing α -SMA, MYH and smtn expression⁶⁹. However, 2D co-culture systems do not mimic the complex vessel wall architecture in vivo. Changes in blood pressure directly transfer into changes in ECM tensile stress, which, as determined by (local) matrix elastic behavior and VSMC stiffness, results in VSMC deformation (i.e., mechanical strain). Alterations in VSMC strain are understood to modulate cellular phenotype³³. Mechanical stretching forces applied directly to VSMCs enhance the expression of ECM proteins, such as collagen and fibronectin¹⁰². Biophysical factors in the physiological range promote VSMCs to maintain or adopt a contractile phenotype, whereas pathological biophysical stimuli promotes VSMCs to switch towards a synthetic phenotype.

2.2.4. Transcriptional Regulators

KLF4 is an important transcriptional regulator defining VSMC phenotype during development¹⁰³ and after vascular injury¹⁰⁴. KLF4 expression is increased in lesions of ApoE^{-/-} mice on a Western type diet¹⁰⁵. Additionally, there is increased binding of KLF4 to α SMA and SM22 α promoters upon vascular injury in mouse carotid arteries¹⁰⁴. The loss of KLF4 has favorable effects, inhibiting plaque pathogenesis and reducing plaque vulnerability¹⁰⁶. KLF4 is critical in the regulation of phenotypic switching of VSMCs, both in vitro and in vascular injury models. KLF4 expression results in profound activation of pluripotency genes such as Oct4 and Sox2, indicating that VSMCs can reactivate its pluripotency network in response to vascular stress or

damage⁵. Moreover, KLF4 activates > 800 pro-inflammatory VSMC genes, of which many are atherosclerosis relevant¹⁰⁶. Additionally, KLF4 knock-out in an atherosclerosis animal model revealed the switch of VSMCs toward a synthetic phenotype, while suppressing the macrophage-like form¹⁰⁶.

3. Clinical Features of Vascular Remodeling

3.1. Hypertension

Hypertension is widely accepted as a risk factor for the development of CVD. However, hypertension was first considered to be a consequence of aging and seen as insurmountable¹⁰⁷. Later, several studies revealed that hypertension was associated with increased cardiovascular mortality^{108,109}. A meta-analysis on blood pressure and cardiovascular disease showed that a rise of 20 mmHg in systolic blood pressure (SBP) and of 10 mmHg in diastolic blood pressure (DBP) was associated with a more than two-fold increase in vascular mortality¹¹⁰. More recently, the CALIBER study revealed that patients with hypertension (defined as > 140/90 mm Hg or those receiving blood pressure-lowering drugs) have a lifetime risk for overall CVD of 63.3% at 30 years of age (compared to 46.1% in patients with normal blood pressure) and develop CVD 5 years earlier¹¹¹. Decreasing the SBP by 20 mmHg was associated with a 39% reduction in cardiovascular (CV) events in the total group and in a 69% reduction in patients between 60 and 69 years of age¹¹². Currently, much of the focus on the primary and secondary prevention of CVD revolves around control of blood pressure¹¹².

The systolic aspect of hypertension is quite strongly determined by age- and disease-related decreases in arterial compliance^{19,113-115}. Therefore, arterial remodeling processes affecting medial elastic properties are directly relevant in risk profiling and as treatment targets.

3.1.1. Cellular Components and Hypertension

High blood pressure is a multifactorial disorder in which genetic alterations, environmental factors and comorbidities interact^{26,116}. ECs play an important role in the development of hypertension. In health, ECs are exposed to physiological shear stress, which is necessary to maintain proper functioning of the endothelium. The NO excreted by ECs regulates vascular tone, and thereby preserves ECM and VSMCs' functioning¹¹⁷. In pathology, blood flow is more oscillatory, with higher peaks and blood stasis during diastole¹¹⁸. Turbulent flow and changes in shear stress, either high or low, affect EC function. Low shear stress areas are considered prone to developing atherosclerosis, inducing pro-inflammatory pathways and lead to dysregulation of the cytoskeleton and junctional proteins of ECs^{117,119,120}. High shear stress induces morphological changes in ECs, as they align in the direction of the flow. Additionally, arterial outward remodeling occurs, leading to increased NO synthesis^{121,122}.

In hypertension, the amplitude and rate of elastic distension of arteries are increased, causing fatigue, damage and degradation of the vessel wall ECM³⁴. Moreover, excessive VSMC strains induce phenotypic switching of these VSMCs to more synthetic types and, consequently, promotes arterial fibrosis, (further) compromising arterial compliance¹²³.

3.1.2. Calcification and Hypertension

Several pathologies are associated with VSMC phenotype switching¹⁴. Synthetic VSMCs produce EVs^{2,124,125}, that have been found in both the intimal and medial layers of the vessel wall¹²⁶. VSMCs are known to release EVs upon phenotypic switching towards synthetic or osteogenic phenotype. EVs derived from VSMCs share similarities with EVs from osteoblasts, having calcium-binding capacities and osteoblast-like ECM production^{2,83}. Recently, it was shown that not all EVs promote calcification¹²⁷. This implies that EV content is variable and dependent on VSMC-mediated biogenesis. A specific subclass of EVs are exosomes, which express tetraspanins (CD9, CD63 and CD81) and differ in expression pattern, and, hence, mineralizing capacity¹²⁷. Furthermore, calcifying conditions *in vitro* increase the multi-vesicular body, forming enzyme sphingomyelin phosphodiesterase 3 (SMPD3) and subsequent exosome genesis⁸³. Consequently, the inhibition of SMPD3 ablates the generation of exosomes and their subsequent calcification. Additionally, sortilin has been suggested as a key player in the VSMC-mediated calcifying EVs genesis and release. Sortilin is a sorting receptor that directs target proteins to a designated location via the secretory or endocytic compartment¹²⁸. Recent findings have identified that sortilin promotes vascular calcification via the trafficking and loading of tissue-nonspecific alkaline phosphatase into EVs¹²⁹. Moreover, sortilin co-localized with calcification in human calcified vessels¹²⁹. Differences between mineralizing and non-mineralizing EVs eventually determine calcification of the ECM in proximity of VSMCs. Hence, better insight into EV composition, such as lipid content and RNA and protein profile, might provide new insights into the mechanism by which VSMC derived EVs contribute to vascular calcification and result in novel targets for treatment.

In response to calcified ECM, neighboring ECs and VSMCs react and induce the production of osteogenic factors, such as bone morphogenetic protein 2 (BMP-2) and 4 (BMP-4)^{130,131}. Besides this expression of bone-associated proteins, the number of VSMCs decreases with age, and an increase of collagen-to-elastin ratio further increases the stiffening of the vessel wall¹³².

In hypertension, calcium handling is disturbed, which is associated with an increased activation of L-type calcium channels and sensitivity of (hypertensive) patients toward calcium channel blockers^{133,134}. Increased intracellular calcium induces the activation of receptors coupled to phospholipase C, leading to the generation of second messengers that trigger cytokines, ROS and miRNAs, and also cellular derived EVs¹³⁵. Additionally, increased intracellular calcium activates the contractile machinery of VSMCs, leading to hyper-contractility. Excessive intracellular calcium rapidly disintegrates both mitochondria and structural components of VSMCs, and results in calcium depositions within elastic fibers¹³⁶. Consequently, elasticity of the vasculature decreases, further contributing to increased blood pressure^{137,138} and VSMC stiffening¹³⁵. Specific calcium antagonists, blocking L-type voltage-dependent calcium channels, reduce calcium internalization and normalize blood pressure^{26,133}. Of note, these calcium channel blockers also prevent calcification of the vasculature^{139,140}.

3.1.3. Consequences of Vascular Calcification

Vascular calcification is associated with arterial stiffness¹⁴¹⁻¹⁴³. In chronic kidney disease, medial calcification of large arteries is directly responsible for the increased stiffness (or reduced distensibility)¹⁴⁴. The presence of vascular calcification

(process) in the vessel wall contributes to ECM degradation and wall thickening^{34,145}. Taken together, vascular calcification contributes directly and indirectly to arterial stiffening and, ultimately, hypertension^{146,147}. Atherosclerosis has also been associated with arterial stiffness and hypertension^{51,148}. Additionally, increased blood pressure and wall shear stress increase vulnerability and, consequently, atherosclerotic plaques become prone to rupture^{149,150}.

3.2. Atherosclerosis

Atherosclerosis is characterized by intimal plaque build-up and often referred to as a thickening of the vessel wall, ultimately narrowing the lumen of the vessel. Atherosclerosis is considered an inflammatory disease, starting with endothelial stress and subsequent monocyte infiltration^{151,152}. Clinically, plaques may rupture, resulting in stroke or myocardial infarction^{32,153}. Besides the classical view that systemic factors are causative for atherosclerosis, recent data suggest that local vascular processes also initiate atherogenesis^{154,155}. Lineage tracing studies revealed that many of the cells present in atherosclerotic plaques are derived from VSMC precursors¹⁰⁶. Additionally, it is now recognized that atherosclerotic plaque macrophages have lineage traces of VSMCs, indicating that cells in the intima originate from the vascular media, and not necessarily from the circulating mesenchymal cells that infiltrate the vessel wall at sites of injury¹⁰⁶.

Arterial remodeling is considered key to the development of atherosclerosis³². Arterial remodeling of the vessel wall can be initiated via many processes, including oxidative stress¹⁵⁶, proliferation^{152,157}, VSMC phenotype switching², cell infiltration, apoptosis and calcification⁶⁰.

3.3. Intimal and Medial Aspects of Vascular Calcification

Vascular calcification can be found at two anatomical locations, the medial and intimal layer. In the 1940s, post-mortem analysis of aortic specimens from CVD patients revealed that atherosclerosis is only present at vascular areas with either medial degradation or medial calcification¹⁵⁸. Histological examination of aortas showed that medial calcification preceded atherosclerotic plaque build-up. Intimal calcification is seen as a hallmark of atherosclerosis and, clinically, is used as a marker for atherosclerotic burden¹⁵⁹⁻¹⁶¹. For a long time, calcification has been considered as a passive degenerative detrimental process, not amendable for intervention. This view has recently changed, since statin treatment is associated with both increased plaque stability and increased plaque calcification¹⁶²⁻¹⁶⁵. However, the apparent protective role of calcification in stabilizing plaque is in contrast with the predictive value of calcification for CVD morbidity and mortality¹⁶⁶. Moreover, medial calcification has been shown to change blood dynamics by inducing arterial remodeling processes and increasing arterial stiffness¹⁶⁷⁻¹⁶⁹. Medial calcification strongly correlates with the phenotypical switching of VSMCs and a rise in blood pressure. Macrocalcifications in patients who underwent a computed tomography (CT) scan were shown to have a three- to four-fold risk of developing fatal cardiovascular events^{164,168}. Additionally, microcalcifications, which are precursors of macrocalcification, have been shown to induce plaque vulnerability¹⁷⁰⁻¹⁷².

Cellular Processes of Vascular Calcification

Specific stimuli, such as elevated calcium or phosphate levels, induce the switching of VSMCs to an osteogenic phenotypic where the cells acquire features of chondrocytes and osteoblasts ¹⁷³. Osteogenic VSMCs show an increased expression of osteogenic markers, such as alkaline phosphatase, BMP-2 and runt-related transcription factor 2 (Runx2), but show decreased calcification-inhibitor protein expression ¹⁷⁴. VSMCs exposed to elevated calcium levels display an intracellular calcium overload that may induce microcalcifications, eventually resulting in macrocalcification, which contributes to vascular stiffness and hypertension ²⁶. The process of calcification, culminating in either micro- or macrocalcification, compromises the structural integrity of the vessel wall and, hence, its functional properties.

Oxidative stress is an important player in the development of vascular calcification. Oxidative stress pathways in VSMCs have been linked to development aortic valve calcification ¹⁷⁵. Increased oxidative stress induces expression of Runx2, a key transcription factor associated with osteoblast differentiation ¹⁷⁶. VSMC-specific Runx2 knockout was shown to decrease vascular calcification and atherosclerosis development ¹⁷⁷. Additionally, increased H₂O₂ levels have been found in the close vicinity of calcification nucleation foci, together with an enhanced expression of oxidases ¹⁷⁸. High phosphate levels, present in chronic kidney disease, are associated with extent of vascular calcification ¹⁷⁹. In vitro, phosphate has been shown to induce mitochondrial-derived free radicals, by enhancing mitochondrial membrane potential ¹⁸⁰. Antioxidants inhibit phosphate-induced ROS production ¹⁸⁰. Increased oxidative stress is also found in diabetic patients ¹⁸¹, leading to an increased accumulation of advanced glycation end products (AGEs). AGEs have been shown to induce vascular calcification via increased oxidative stress of VSMC ^{182,183}.

3.4. Fibrosis

Fibrosis of different organs contributes to up to 50% of worldwide mortality ¹⁸⁴. In the vasculature, myofibroblasts produce ECM proteins such as collagen and fibronectin ¹⁸⁵. Myofibroblasts are derived from MSC-like cells that reside in the vessel wall. The role of these MSC-like cells in the vessel wall is not fully understood, and is believed to play an important role in structural vessel repair. In vascular fibrosis, excessive collagen deposition leads to a reduced compliance of the vessel, which indicates increased arterial stiffness. Furthermore, high blood pressure induces structural changes including hypertrophy of the vessel wall ^{186,187} and cellular ¹⁴ and ECM remodeling ^{188,189}.

Lineage tracing studies have shown that resident Gli1⁺ MSC-like cells are a major contributor of vascular fibrosis in arterial remodeling ¹⁹⁰. Acute vessel wall injury revealed that a significant number of Gli1⁺ cells were detected in the media and neointima, and that they express VSMC markers such as α SMA and CNN1. Additionally, some 50% of the newly formed VSMCs were derived from Gli1⁺ progenitors, indicating the involvement of adventitial cells in arterial remodeling ¹⁹¹. Likewise, angiotensin II induced hypertension was caused by an exuberant production of collagen in the adventitial layer ¹⁹². VSMCs that migrate towards the adventitia start expressing stem cell markers Sca-1 and CD34 ¹⁹³. These “adventitial stem cells” change towards fibroblasts and deposit collagen and other ECM, contributing to adventitial fibrosis ¹⁹⁴.

During atherosclerosis development, fate-tracing studies in mice have shown that MSC-like cells migrate into the media and neo-intima. Also, MSC-like Gli1⁺ cells were a major source of osteoblasts-like cells, significantly contributing to the process of vascular calcification in both vascular media and intima. The genetic ablation of Gli⁺ cells before injury dramatically reduced the severity of vascular calcification¹⁹¹. These data indicate that fibrotic processes are regulated by myofibroblasts and MSC-like cells and that these are important in the development of atherosclerosis and vascular calcification.

4. Assessing Vascular Remodeling and Disease

4.1. Pulse Wave Velocity (PWV), Distensibility Coefficient (DC) and Intima-Media Thickness (IMT)

Carotid-femoral PWV is considered the gold standard for assessing central arterial stiffness^{195,196}. PWV, however, only measures vascular characteristics of the aorta and does not harbor information on any other vessels. Multi-slice CT, measuring calcification, reflects the atherosclerotic burden, and IMT measures atherosclerosis in the carotid arteries. Below, we describe in more detail measures of vascular remodeling and disease.

4.1.1. Pulse Wave Velocity

Measuring arterial stiffness in the clinic is challenging. The methods that are available for measuring arterial stiffness are non-invasive vascular imaging, such as ultrasound and magnetic resonance imaging (MRI), and high-fidelity recording of the pulse wave, performed by tonometry or ultrasound Doppler. Functional measures that can be obtained are pulse wave velocity (PWV) and the distensibility coefficient (DC). In the clinic, carotid-femoral PWV (cfPWV) adds to existing risk scores in cardiovascular risk management¹⁹⁷, while arterial stiffening has been shown to be independently associated with cardiovascular risk and mortality^{198,199}. Brachial-ankle PWV is also used as an alternative to cfPWV, correlating reasonably well²⁰⁰.

The interpretation of arterial stiffness findings in human studies is subject to (1) the stiffness measure/method used and (2) the mechanistic or constitutive focus of the study. Constitutive properties that are often considered to interpret arterial stiffness findings are those related to the ECM or VSMCs¹⁸.

4.1.2. Distensibility Coefficient

Arterial stiffness can be determined by measuring local vessel diameter and distension by ultrasound or MRI¹⁸. The measured data can be used to calculate the local distensibility coefficient (DC) or compliance. PWV, as well as DC, are well known to be pressure dependent and require blood pressure adjustment^{201,202}. Adjustment is valid when considering group analyses, but lacks applicability in the use of individual cases²⁰³.

PWV is largely dependent on the elastic properties of the large arteries. Therefore, PWV should be interpreted with care, since it only reflects the stiffness of the aorta. In conduit arteries, the relative amount of VSMCs are limited and, thus, the contribution of ECM to arterial stiffening may be expected to be more pronounced. In peripheral, more muscular, arteries, age-related stiffening is not as straightforward²⁰⁴.

PWV in the lower extremities can change significantly, which may explain why no evident relation between PWV and age has been reported^{34,205}. Additionally, hypertrophy of VSMCs is commonly observed in hypertension^{206,207}, with the potential to (partially) determine PWV²⁰⁸. Thus, PWV is an important clinical marker for risk assessment, as it correlates with cardiovascular risk, mortality, and organ damage of the heart, kidney and brains²⁰⁹.

Techniques for measuring PWV are developing. cfPWV is most commonly used for aortic stiffness, but is susceptible to local wave reflections and not suitable for obtaining information on carotid arteries²¹⁰. Ultrasound ultrafast imaging techniques are being explored to directly measure the propagation of the pulse wave, with limited success for the common carotid artery^{211,212}. Elastography is a promising technique, as it measures the propagation of shear waves in the tissue²¹³. The propagation of the shear wave is directly proportional to the elastic modulus of the tissue²¹⁴. PWV can also be measured using MRI, which also allows for segmentation. Its drawbacks are costs and long scanning times²¹⁵. Currently, the available PWV toolbox can only be applied to large and medium-sized arteries, but techniques are developing for smaller vessels.

At present, the interpretation of stiffness measurements in terms of arterial wall constitutive properties remains a challenge¹⁸. Medial calcifications are known to co-localize with elastin fragmentation spots, but the mechanical contribution of the calcific deposits to vessel stiffness remains to be established; the elastin degradation alone could be sufficient to explain stiffening³⁵.

4.1.3. Intima-Media Thickness

IMT is used as a measurement for assessing the thickness of the tunica intima and media (estimated from lumen-intima and media-adventitia echo complexes). IMT is often measured using ultrasound and can be readily obtained in superficial arteries. One aspect that is not measured by IMT is adventitial thickness is a significant contributor of pathology development¹⁹². Wall thickness data are important in determining arterial wall material stiffness and wall stress. But, IMT can also be used to detect atherosclerosis, and is used to follow progression of the disease²¹⁶. IMT is shown to be an independent predictor of overall cardiovascular events, including myocardial infarction and stroke²¹⁷. Multiple studies have shown that increases in IMT can be significantly delayed using effective cardiovascular drugs, such as statins^{218,219}. However, further observational studies revealed that this delay did not result in reduced cardiovascular events²²⁰.

To study large populations, non-invasive techniques such as IMT are preferred over invasive measurement tools. As such, IMT provides a reliable tool for assessing vessel wall macro-structural changes. However, some limitations for IMTs exist. There is a lack of standardized protocols for the use of IMT, and attempts to establish them have not reached consensus yet^{221,222}. IMTs of certain segments, such as the common carotid artery, are related to hypertension, blood pressure and vascular hypertrophy²²³, whereas the internal carotid artery correlates with atherosclerotic plaques²²⁴. Taken together, additional risk assessments, such as plaque presence and stenosis severity, will be complementary to the use of IMT alone. A novel technique to determine extra-media thickness may have great potential in combination with IMT to discriminate between the medial and adventitial layer which aids in risk assessment²²⁵. Moreover, other imaging techniques, such as coronary artery calcium (CAC) scanning, have been demonstrated to be superior for predicting CVD events^{226,227}.

4.2. Calcification Imaging by Computed Tomography

Calcification imaging in the clinic is mostly based on CT, which is an angiography technique based on X-ray radiation and makes use of computer processing to create a detailed 3D image. Calcification scores, such as the coronary artery calcium (CAC) score, can be derived from CT scans and, in recent decades, this has been used as a measure of atherosclerotic burden. The Agatston score is the most frequently used method to calculate CAC. The Agatston score is defined as a weighed sum of the calcification area, with density given as Hounsfield Units ^{228,229}. CAC represents an accurate measure to assess atherosclerotic burden ^{159,230}. Additionally, CAC correlates with traditional risk factors for atherosclerosis, such as hypertension, hypercholesterolemia and diabetes ²³¹. CAC also associates with cardiovascular morbidities and mortalities, including cardiac death and stroke ^{232,233}.

CAC is regarded as the best clinical predictor for the accumulation and progression of vascular calcification over time ^{232,233}. CAC can be used to identify high-risk individuals that need immediate medical attention or intervention ²³⁴. Interventions to slow down or even regress calcification, using the supplementation of vitamin K, are currently being conducted ²³⁵⁻²³⁷. High dietary intake of vitamin K is associated with a lower risk of coronary heart disease ²³⁸ and supplementation with vitamin K significantly delays the progression of calcification ²³⁹. Conversely, statin treatment is known for its beneficial cardiovascular effects, however, it is also shown to increase CAC score ²⁴⁰⁻²⁴². This indicates that type of calcification, as well as the volume or density of calcification, determines whether it is plaque-stabilizing or plaque-destabilizing. Indeed, CAC volume is positively and independently associated with CVD, whereas CAC density was inversely correlated with CVD risk ²⁴³.

Measuring CAC as a clinical marker for CVD has some limitations. Firstly, CAC is limited to coronary arteries, which do not represent the calcification of other vessels of the arterial system. CAC does not represent total plaque size, as histopathological studies showed that the area of calcium deposition was much smaller than total plaque volume ²⁴⁴. Additionally, CAC score poorly correlated with luminal narrowing and severity ²⁴⁵. Secondly, distinction between medial and intimal calcification is not possible with current resolutions of CT. Intimal and medial calcification are different pathologies, with different risk factors for developing CVD ^{246,247}. Additionally, microcalcification ($< 15 \mu\text{m}$) cannot be assessed, as the resolution of CT is some $200 \mu\text{m}$ ^{222,248,249}.

Thirdly, evidence suggests that atherosclerotic plaques that have denser calcification have smaller lipid cores, with increased stability of plaques, resulting in lower CVD risk ²⁵⁰. Greater CAC volume, however, is associated with a higher risk of CVD ²⁵¹. This indicates that future research should integrate density and volume into CAC scoring. Finally, CAC involves exposure to radiation, which limits the use of calcium scores as a follow-up on atherosclerosis progression ²⁵².

4.3. ^{18}F -NaF Positron Emission Tomography

^{18}F -NaF PET scanning gained recent attention as a novel imaging modality for vascular calcification ²⁵³. In bone, ^{18}F -NaF is incorporated into newly formed hydroxyapatite crystals by exchanging fluoride with the hydroxyl groups ²⁵⁴. In the vasculature, ^{18}F -NaF PET imaging has been reported to identify active

(micro)calcification with high specificity²⁵⁵. Importantly, CT and echocardiography cannot identify active vascular calcification, which is considered the main driver of disease progression²⁵⁶. Assessing the molecular bioactivity of calcification in atherosclerotic plaques is of additive value to identify vulnerable plaques²⁵⁶⁻²⁵⁸. Additionally, ex vivo histological studies showed that the resolution of ¹⁸F-NaF has the potential to discriminate between micro- and macrocalcification²⁵⁷. Taken together, ¹⁸F-NaF uptake provides complementary information to CT, as it allows for the detection of ongoing active calcification of plaques, which is a major improvement to the prediction of future cardiovascular risk. Limitations of ¹⁸F-NaF PET imaging are the relatively high costs and exposure to radiation, but, also, ¹⁸F-NaF PET imaging lacks the resolution to discriminate between medial and intimal calcification.

5. Conclusions

Arterial remodeling is defined as the adaptation of the vessel wall away from a normal homeostatic state towards pathologic development, triggered by chronic exposure to stress signals. Different arterial remodeling processes have been described, such as inflammation, oxidative stress, lipid accumulation, and degradation of the ECM. Many of these are regulated by VSMCs and affect vessel wall morphology and properties. VSMCs exhibit cellular plasticity and, upon stress stimuli, they may differentiate towards synthetic, macrophage-like and osteogenic VSMCs. As a consequence, VSMCs initiate and support remodeling processes involving ECM synthesis and cell proliferation, migration and contraction, which aid in maintaining tissue functionality. Disruption of VSMC phenotypic plasticity has many pathological consequences, including the development of hypertension, atherosclerosis, aneurysm formation, intimal and medial calcification, and vascular fibrosis, ultimately leading to increased CVD morbidity and mortality. Early detection of vascular remodeling is key in the prevention and prognosis of CVD. Clinically, assessment of calcification-mediated remodeling remains difficult. Assessment tools include measurements of pulse wave velocity, distensibility coefficient and intima-media thickness. These techniques may capture changes in dynamic and geometric vessel wall properties, but cannot directly monitor the underlying arterial remodeling processes. The addition of techniques, such as ¹⁸F-NaF PET and CT imaging may provide further mechanistic insight into vascular calcification development and other VSMC-mediated processes.

Combining expertise in vascular biology, clinical vascular expertise and multi-scale/multi-modality imaging should advance the understanding and identification of critical determinants of vascular remodeling, ultimately resulting in tailor-made diagnoses and treatments.

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Chapter 3

The role of medial vascular calcification on the development and progression of atherosclerosis

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In Preparation

Abstract

Background: Vascular calcification is becoming increasingly important in cardiovascular risk stratification. Vascular calcification is classified as either intimal or medial calcification, although clinically available methods do not allow this distinction yet. Suitable models that can study both medial and intimal calcification are scarce. We developed a novel mouse model to study the role of medial calcification on atherosclerosis development.

Methods: DBA/2 mice were treated with warfarin for either 0, 1, 2 or 4 weeks to induce medial calcification. After warfarin treatment mice received a 12 week Western type diet (WTD) with or without a gain of function PCSK9 virus to induce LDLr knockdown. Before and after WTD, carotid-femoral pulse wave velocity (cfPWV) was measured. ^{18}F -NaF positron emission tomography (PET) spectra of the vasculature were taken. Hearts and the aorta were isolated and stained for Hematoxylin & Eosin, Alizarin red, uncarboxylated matrix Gla protein and Elastica von Gieson.

Results: Warfarin treatment induced time dependently vascular, valvular and ectopic heart calcification. Warfarin induced extensive medial remodeling, such as extracellular matrix production, elastin damage and medial calcification. Additionally, we detected increased vessel stiffness and higher uptake of ^{18}F -NaF after warfarin treatment. Overexpression of PCSK9 in DBA/2 mice resulted in LDL receptor knockdown with a subsequent increase in plasma cholesterol levels. In LDLr knockdown animals, warfarin induced medial calcification resulted in accelerated atherosclerosis and concomitant increased intimal calcification.

Conclusion: To the best of our knowledge we report on the first mouse-model to study the effect of medial calcification on atherosclerosis development. We show that warfarin induces medial remodeling which results in accelerated atherosclerosis development. Our model provides the foundation to further study the impact of vascular remodeling on cardiovascular disease and develop new strategies for treatment.

Introduction

Vascular calcification is associated with an increase in CVD risk and affects about 90% of patients with symptomatic CVD ¹. Moreover, progression of arterial calcification (>15% annually) is associated with a significant increase in risk for myocardial infarction ². It has become evident that atherosclerotic calcification burden is a potent risk marker of cardiovascular events. Cardiovascular calcification is often present in elderly populations or patients with high atherosclerotic risk profile such as diabetes and chronic kidney disease. However, both chronic kidney disease (CKD) and diabetes patients are predominantly characterized by the development of medial calcification ^{3,4 5}. Recent meta-analyses indicated that over 70% of diabetic patients and up to 93% of CKD have arterial medial calcification ^{6,7}. Arterial medial calcification is associated with a 5- to 10-fold increased risk of CVD in CKD and diabetes patients ¹.

Vascular calcification can be classified as intimal and medial calcification, based on the anatomical site where it is detected ^{8,9}. Medial calcification, also known as Monckeberg sclerosis, is formed along the elastic fibers of the tunica media and can be found throughout the entire vascular tree ¹⁰. Medial calcification is linked to vessel stiffness, hypertension and increased pulse wave velocity (PWV) ^{11,12}. Clinical events associated with medial calcification include heart failure, aneurysm formation and stroke through isolated systolic hypertension ¹³⁻¹⁶. Data suggest that medial calcification is a stronger predictor for CVD events than intimal calcification in diabetes ^{17,18} and CKD ^{19,20}.

Since medial calcification has been described to precede occurrence of atherosclerosis ²¹ we aimed to investigate the role of medial calcification on atherosclerosis development in a novel developed experimental animal model.

Methods

Animals

DBA/2 mice were bought from Charles River Laboratories Inc ('s-Hertogenbosch, The Netherlands). Mice were kept in a temperature-controlled environment (20°C) with a regular day & night cycle. All animal experiments were conducted under a protocol approved by the ethics committee for animal experiments of Maastricht University.

Warfarin (a vitamin K antagonist; VKA) diet was prepared as described previously ²². In brief, warfarin (3 mg/g; Sigma, Zwijndrecht, The Netherlands) supplemented with vitamin K1 (1.5 mg/g; Sigma, Zwijndrecht, The Netherlands) was mixed with normal chow diet (Arie Blok diets, Woerden, The Netherlands). Regular Western type diet (WTD) was bought directly from the supplier (0.25% cholesterol; Arie Blok diets, Woerden, The Netherlands). pAAV/D374Y-hPCSK9 (PCSK9) overexpressing virus was obtained from the Viral Core Facility (Charité Universitäts Medizin, Berlin, Germany). PCSK9 virus was diluted in 0.9% Saline and stored at -80°C until use.

Experimental animals (10-12 weeks old males) were weighed, tonometry was performed and subsequently mice were placed on warfarin diet for either 0 week (control), 1, 2 or 4 weeks (Figure 1). After warfarin diet the animals underwent a second tonometry measurement after which they were placed on a WTD for 12 weeks. One day before sacrifice animals received a third and last tonometry measurement. For the pilot

experiment, animals were treated similarly except after warfarin diet, mice were injected IV with PCSK9 virus (5×10^{10} particles/ mouse) after which they were placed on a WTD for 12 weeks.

At sacrifice, blood was collected in 3.2% sodium citrate by vena cava puncture and the aorta was flushed with 100 μ M sodium nitroprusside in phosphate buffered saline (PBS). The aortic arch, its main branches as well as other organs, were excised and fixated in 1% paraformaldehyde overnight and embedded in paraffin. Some mice ($n = 6$) received ^{18}F -NaF intravenously (IV) to image active vascular calcification via micro positron emission tomography - computed tomography (μ PET-CT) scanning. Aortic arch and aortic root were sectioned and stained for hematoxylin and eosin (HE), alizarin red (AR), Elastica von Gieson (EvG) and uncarboxylated matrix Gla-protein (ucMGP).

Plasma cholesterol determination

Total cholesterol (Horiba Medical, Montpellier, France) measurements were performed as described in manufacturer's manual.

μ PET ^{18}F -NaF

Animals were starved 2 hours before ^{18}F -NaF injection. Mice were anaesthetized and ± 10 MBq ^{18}F -NaF was injection IV after which mice were left to wake up. ^{18}F -NaF was circulated for 30 minutes before the animals were sacrificed as described above. Aortic arches were isolated and imaged with μ PET.

Pulse wave velocity

Animals were anaesthetized and the left cervix and groin were shaved. ECG electrodes were connected, and the first tonometer probe was placed on the cervix (left carotid) while the second probe was placed on the groin (left femoral artery). After a stable heartbeat signal was obtained the measurement was started and the distance between the cervix and groin was measured using a caliper.

Histology and histochemistry

5 μ M serial sections were cut from paraffin embedded aortic arches and aortic roots and stained with Alizarin Red (AR), hematoxylin and eosin (HE). AR positivity was determined using FIJI software (v2.0.0-RC-69) and normalized against total aortic area. Atherosclerotic plaque burden was analyzed on HE stains and normalized against total arch area using FIJI software (v2.0.0-RC-69). Total atherosclerotic burden was measured in the aortic arch and its major branching points. Atherosclerotic calcification was determined as AR positivity inside plaques.

Western blotting

Livers were homogenized with glass beads and a bead beater in RIPA buffer (89900, Thermo Scientific) containing MS-SAFE (MSSAFE-5VL, Sigma). Protein concentrations were determined using microBCA assay (23235, Thermo Scientific) and lysates were separated on 7.5% Mini-PROTEAN TGX Precast Protein Gels (456-1024, BioRad). Samples were transferred to a nitrocellulose membrane (BioRad) and incubated overnight with anti-LDLr (3839-100, Biovision) and anti-GAPDH (MAB374, Millipore). Protein was detected using HRP-conjugated secondary antibodies (anti-chicken: ab97135, Abcam; anti-rabbit: 7074S, Cell Signalling) and visualized by

enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific).

Immunohistochemistry

Antigen retrieval was performed by heating samples in target retrieval solution (Dako, Heverlee, Belgium). Subsequently, tissues were blocked and incubated with primary antibody overnight as described: ucMGP (1:400; IDS, Boldon, UK). After overnight incubation, sections were incubated for 1 hour with HRP-conjugated secondary antibody (p0447; Dako) and detected with NovaRED (Vector Labs, Amsterdam, the Netherlands).

Elastin fibers were detected using Elastica von Gieson staining. In brief, tissues were stained in Lawson solution (Klinipath, Olen, Belgium) and differentiated in 70% ethanol. Tissues were counterstained for collagen and connective tissue in von Gieson solution.

Images were captured using a DM400 microscope (Leica, Eindhoven, the Netherlands) and imaging software (LAS V, Eindhoven, the Netherlands).

Statistical analysis

All values are shown as mean $\pm$ SD unless stated differently. Statistical analysis was performed by 1- way ANOVA with Bonferroni post hoc test using PRISM software (GraphPad). Values of $P < 0.05$ were considered statistically significant.

Results

In this study we exposed animals to a VKA antagonist diet (warfarin) to induce time dependently medial calcification (1 week, 2 weeks and 4 weeks warfarin treatment). A schematic overview of the experimental design is displayed in Figure 1A.

Figure 1. Baseline characteristics. (A) Schematic overview of the experimental design of the study. (B) Bodyweights of DBA/2 mice at t=0 and t=12 weeks of WTD diet. (C) Cholesterol levels of DBA/2 mice at t=0 and t=12 weeks of WTD.

Baseline characteristics

Total bodyweights of mice at t=0 and t=12 were not changed by exposure to different durations of warfarin (Figure 1B). As expected, weight of animals increased after WTD (mean 33.3 ± 4.0 gr), as compared to before WTD (mean 26.8 ± 1.9 gr; Figure 1B). Additionally, cholesterol levels after t=0 and t=12 were not altered between control and warfarin treated animals (Figure 1C). Cholesterol levels were significantly higher when comparing the animals before (mean 2.7 mM) and after WTD (mean 5.5 mM; $p<0.0001$; Figure 1C).

Atherosclerosis does normally not develop in rodents ²³, and during warfarin treatment we could not detect atherosclerotic plaques (supplemental Figure 1).

Cardiovascular calcification

Medial calcification of the aorta as quantified by AR staining was significantly increased in the animals in the 4-week warfarin treated group (Figures 2A and 2B) compared to animals treated for 0, 1 and 2 weeks of warfarin (0 and 1 week, $p<0.0001$; 2 weeks, $p<0.001$). Medial calcification was predominantly found in the ascending aorta, close to the aortic root. Furthermore, after WTD medial calcification was significantly increased in animals that received warfarin for only 1 or 2 weeks as compared to no warfarin pretreatment. Additionally, aortic valve calcification was significantly increased in the 4 week warfarin group ($p=0.026$; Figures 2C and 2D). Noticeably, calcification did not occur in the luminal/ leaflet region but was found at the border of the aortic valve area (black arrow, Figure 2C).

DBA/2 mice are also prone to develop calcification of the heart, more specifically dystrophic cardiac calcifications ²⁴. Therefore, dystrophic calcification of the heart was determined before and after WTD. Warfarin treated animals displayed increased calcification of the heart compared to control treated animals ($p=0.002$; Figure 3A).

Figure 2. Heart calcification, pulse wave velocity and ^{18}F -NaF uptake. (A) Heart calcification score of animals at $t=0$ and $t=12$ weeks. A significant increase in heart calcification was found in animals treated with VKA. (B) Delta pulse wave velocity (PWV) comparing $t=0$ with $t=12$ weeks of WTD. All animals on WTD showed a significant increase in pulse wave velocity when challenged with a VKA diets, while animals on a control diet did not increase PWV. (C) ^{18}F -NaF PET scans of aortic arches. (D) Quantification of ^{18}F -NaF signal. There was a significant increase in ^{18}F -NaF uptake in animals treated for 4 weeks with VKA. * $p<0.05$; ** $p<0.01$

Pulse wave velocity and ^{18}F -NaF uptake

Medial calcification is linked to vessel wall stiffness and therefore we measured the carotid-femoral PWV (cfPWV). PWV was assessed before and after WTD and is displayed as delta value ($\Delta\text{PWV} = (\text{PWV at } t = \text{end}) - (\text{PWV at } t = \text{Before WTD})$). Priming the vasculature for 1, 2 and 4 weeks of warfarin showed a significant increase in cfPWV compared to control (no warfarin treatment; $p<0.01$; Figure 3B).

Since warfarin inhibits the activation of vitamin K dependent proteins, and thus induces calcification by impaired calcification inhibition, we measured calcification activity using the PET tracer ^{18}F -NaF. ^{18}F -NaF uptake is measured as an increase in SUVmax. ^{18}F -NaF uptake in the 4-week warfarin treatment was significantly increased as compared to the other treatment groups ($p<0.01$; Figures 3C and 3D).

Figure 3. Heart calcification. (A) Representative images of the aortic arch and stained for alizarin red (AR). (B) Quantification of AR signal. At $t=0$ a significant increase in AR positivity was found in the 4 week VKA groups. At $t=12$ weeks both 2 and 4 weeks of VKA treatment significantly increased AR positivity. (C) Representative images of the aortic root area stained for AR. (D) Quantification of AR positivity in the aortic root area. A significant increase was found in the 4 weeks VKA group. * $p<0.05$; *** $p<0.001$; **** $p<0.0001$

PCSK9 pAAV gain of function to induce atherosclerosis

DBA/2 mice do not develop atherosclerosis. Therefore, to provoke atherosclerosis, we employed PCSK9 overexpressing virus (pAAV) to induce LDL receptor (LDLr) knockdown, followed by 12 weeks of WTD ²⁵. Virus efficacy was determined by protein expression of the LDL receptor in liver lysates. LDLr knockdown was achieved up to 95% compared to control animals, were no virus was injected (Figure 4A and supplemental Figure 2B). Additionally, plasma cholesterol levels significantly increased after PCSK9 overexpression (mean 31.2 ± 9.1 mM) compared to control animals on WTD (mean 5.6 ± 1.2 mM) for the same duration (p<0.0001; Figure 4B and Table 1).

Figure 4. Atherosclerosis induction. (A) LDL receptor expression was significantly decreased in liver lysates of mice that received PCSK9 overexpressing virus (1 and 4 weeks VKA groups). (B) plasma cholesterol levels before and after WTD and with and without the PCSK9 virus. WTD significantly increased the plasma cholesterol levels. PCSK9 overexpression significantly increased plasma cholesterol compared to WTD only. ****p<0.0001

Table 1. Animal weight and Cholesterol levels.

		t=0		t=4		t=8		t=12	
		Average	SD	Average	SD	Average	SD	Average	SD
Weight (g)	1 wk W	24,8	2,4	28,5	3,4	31,4	3,4	31,6	3,3
	4 wk W	28,2	3,7	30,1	5,5	33,2	5,8	34,1	6,0
Cholesterol (mM)	1 wk W			33,8	7,2	39,3	8,3	33,8	10,0
	4 wk W			43,8	4,7	25,9	7,4	28,1	7,3

Atherosclerosis development after medial calcification.

As described above, DBA/2 mice treated with warfarin develop medial calcification without atherosclerosis. After different time durations of warfarin treatment, DBA/2 mice were injected with PCSK9 virus and treated for 12 weeks with WTD. Warfarin treated animals developed extensive medial calcification (Figures 5A and 5B) with simultaneously also atherosclerotic plaque calcification, that was significantly increased compared to control animals (p<0.0001; Figures 5C and 5D).

Additionally, warfarin duration was associated with increased development of intimal calcification ($p<0.01$; Figures 5C and 5D).

Figure 5. Atherosclerosis development after vascular calcification induction. (A) Representative images of aortic arches and stained for AR. Animals were treated for 0, 1 and 4 weeks of VKA (4x magnification). (B) Quantification of AR signal. Both 1 week and 4 weeks of VKA use significantly increased the AR signal. (C) Representative images of atherosclerotic plaques stained for AR. Animals were treated with VKA for 0, 1 and 4 weeks (10x magnification). (D) Quantification of AR signal normalized for plaque area. Both 1 week and 4 weeks of VKA treatment induced a significant increase in plaque calcification. ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

ucMGP and medial remodeling

UcMGP positivity was found in close proximity of intimal calcification (Figure 6A, panel A2 and A3) and at areas of medial calcification (Figure 6A, panel A4 and A5). Warfarin treated animals showed significantly more ucMGP positivity compared to control animals ($p<0.0001$; Figure 6B).

Medial remodeling is often found at shoulder regions of atherosclerotic plaques (Figure 6C, panel C4 and C5). Warfarin treatment induced significantly more elastin damage in the medial layer of the vessel wall compared to control treatment ($p<0.01$; Figure 6D). Elastin fibers underneath atherosclerotic plaques were often thinner (Figure 6C, panel C2) and showed a higher degree of debranching (Figure 6C, panel C5). Furthermore, elastin breaks were found more frequently underneath atherosclerotic plaques (Figure 6C, panel C3) compared to areas without atherosclerotic plaques (Figure 6C, panel C1). Additionally, elastin breaks were found at areas with medial calcification (Figure 6C, panel C6).

Figure 6. ucMGP and elastin staining. (A) Representative images of areas of ucMGP positivity. 1. 0 week VKA animal with no ucMGP positivity. 2. 1 week VKA animal with ucMGP positivity inside the atherosclerotic plaque. 3. 4 week VKA animal with ucMGP positivity inside the atherosclerotic plaque. 4. & 5. Medial calcification show colocalisation with ucMGP positivity. (B) ucMGP positivity score was significantly increased in the 4 week VKA group compared to control $p < 0.0001$. (C) Representative images of Elastin von Gieson staining. 1. Vessel wall without atherosclerotic plaque. 2. Atherosclerotic plaque. 3. Elastin breaks underneath atherosclerotic plaques. 4. Medial remodeling at plaque shoulder regions. 5. Debranching at plaque shoulder regions. 6. Medial calcification. (D) Elastin damage score. A significant increase in elastin damage was found in animals treated with VKA $p < 0.01$.

Discussion

In this paper, we describe the first experimental animal model to investigate effects of vascular medial remodeling and calcification on development of atherosclerosis. We show that short term VKA treatment has major consequences on the long term, both in development of medial calcification, aortic valve calcification and increased vascular stiffness. Additionally, even short term VKA treatment resulted in significantly increased intimal calcification. Our data indicate that VKA induces vascular remodeling that is not visible with current imaging modalities, but which results in increased progression of atherosclerosis and intimal calcification.

We and others have shown that VKA treatment induces development of both medial²² and intimal calcification^{26,27}. Warfarin has been extensively used as inducer for medial calcification²⁸. C57bl/6 mice however, do not develop medial calcification when treated with warfarin. Even 5/6 nephrectomy, a well-established vascular calcification model in rats, did not induce significant vascular calcification in C57bl/6 mice²⁹. Moreover, *Apoe* deficient mice on a C57bl/6 background are known to develop atherosclerosis when fed a western type diet, yet hardly develop significant medial calcification²⁶. DBA/2 mice are known for developing ectopic calcification, which is due to a defect in the *Abcc6* gene and low plasma magnesium levels²⁴. DBA/2 mice develop spontaneous ectopic heart calcification²² which is concurrent with our results. We observed that almost 70% of animals displayed signs of calcification of the heart which was further enhanced by VKA treatment.

Since DBA/2 mice do not develop atherosclerosis, we applied a recently developed LDLr knockdown using PCSK9 gain of function virus ²⁵. In animals on a WTD but with no LDLr knockdown, cholesterol levels only increased slightly compared to chow-fed mouse plasma cholesterol levels. Animals that received the virus displayed high cholesterol plasma levels that remained elevated for the whole duration of the experiment. It has been shown that LDLr knockdown using PCSK9 gain of function results in atherosclerotic calcification, however without signs of medial calcification ³⁰. Therefore, we developed an experimental animal model sensitive to develop both medial calcification and atherosclerosis.

Literature that dates back to the time before non-invasive imaging, reported that atherosclerotic plaques develop in areas of medial degradation or calcification ²¹. With the introduction of non-invasive imaging detailed information about the precise location of calcification is lost. Current multi slice CT cannot differentiate between medial and intimal calcification. Most literature reports on a relation between progression of lesion size and plaque calcification ^{31,32}. In our study we show that medial calcification present prior to atherosclerosis results in progression of atherosclerotic plaque calcification. We hypothesize that vitamin K deficiency can lead to detrimental medial remodeling causing elastin damage forming the nidus for calcification. Moreover, we show that extensive medial remodeling contributes to accelerated atherosclerosis.

¹⁸F-NaF PET scanning has recently been introduced as novel imaging modality for vascular calcification ³³ and is used to detect active calcification with relatively high specificity ³⁴. In our study we examined uptake of ¹⁸F-NaF to confirm active mineralisation. We found a significant increase in ¹⁸F-NaF PET signal in warfarin treated animals, indicative for active calcification. Measurement of active calcification by ¹⁸F-NaF is considered complementary to CT and predicts progression of calcification ^{35,36}. Furthermore, ¹⁸F-NaF detects high risk lesions and future cardiovascular events more accurately ^{37,38}. The presence of both medial and intimal vascular calcification was confirmed by histochemical staining. ¹⁸F-NaF positivity was found to colocalize in areas of calcification positive stain in the aortic arch. It is known that presence of vascular calcification increases the risk for cardiovascular morbidity and mortality ¹. We also noticed that VKA increased aortic valve calcification. Aortic valve calcification is common in the ageing population and has become a substantial disease burden in the Western world ³⁹. The precise pathophysiology of calcific aortic valve stenosis is still unclear but use of VKA is known to accelerate this process ⁴⁰⁻⁴². Moreover, aortic valve calcification is associated with increased cardiovascular events and mortality ⁴³.

Conclusion

Medial calcification represents an underestimated risk factor for cardiovascular disease. In this study we developed a mouse model that develops both medial calcification and atherosclerosis. We show that VKA use, dose dependently increases vascular, valvular and ectopic heart calcification. Additionally, short term VKA treatment increases vessel stiffness and elastic fiber damage resulting in accelerated atherosclerosis. Our research opens new avenues to study the impact of vascular remodeling on atherosclerosis and whether targeting medial calcification results in reduced cardiovascular disease.

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Supplemental material

Supplemental figure 1. Hematoxylin and Eosin (HE) staining. Representative images of aortic arches stained for HE. No plaque development was found after VKA treatment.

Supplemental figure 2. Weight curve and liver LDL receptor knockdown. (A) Weight curve of animals receiving PCSK9 virus injection and a WTd for 12 weeks. (B) Liver LDL receptor protein expression. All animals that received the PCSK9 overexpressing virus were showing knockdown of the LDL receptor.

Chapter 4

Medial vascular smooth muscle cell apoptosis accelerates atherogenesis and promotes features of a vulnerable plaque

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In preparation

Abstract

Background: Vascular smooth muscle cells (VSMCs) are involved in many arterial diseases including atherosclerosis and aneurysm formation. In the vessel wall, VSMCs are key players in vascular remodeling, calcification and apoptosis. In a mouse model of inducible VSMC-specific apoptosis, chronic apoptosis of VSMCs in established plaques has been shown to induce features of plaque instability and accelerated atherogenesis. Based on these findings, we studied the role of medial VSMCs on the initiation and progression of atherosclerotic lesions.

Methods: SM22 α -iDTR/ApoE^{-/-} mice were injected with diphtheria toxin (DT) for 3 weeks to induce medial VSMC apoptosis. After 3 weeks, DT treatment was arrested and some mice were sacrificed (t=0). Remaining mice were fed a Western type diet (WTD, 0.25% cholesterol) and sacrificed after 6 and 18 weeks of WTD, respectively. Before sacrifice mice underwent applanation tonometry to measure vessel stiffness. Next, the aortic arch (including branches) was excised and tissues were stained for Hematoxylin & Eosin, Mac3, Von Kossa, picro Sirius Red and α -smooth muscle actin. Additionally, vessel contractility was determined using a wire myograph.

Results: Some 50% decrease in cellularity was observed in SM22 α -iDTR+/ApoE^{-/-} mice after DT exposure as compared to control. Vessel wall relaxation was deteriorated in SM22 α -iDTR+/ApoE^{-/-} mice which was endothelial cell dependent. Additionally, *in vitro* isolated SM22 α -iDTR+/ApoE^{-/-} VSMCs displayed hypertrophy and increased expression of contractile markers. Atherosclerotic plaque size in SM22 α -iDTR+/ApoE^{-/-} mice was significantly increased as compared to control mice. After respectively 6 and 18 weeks of WTD, atherosclerotic plaques in SM22 α -iDTR+/ApoE^{-/-} mice displayed features of plaque vulnerability.

Conclusion: We show that VSMCs are key in maintaining vessel wall homeostasis and that disruption by DT induces vessel wall remodeling, resulting in declined vessel wall responsiveness. Additionally, VSMCs are pivotal in vessel health as depletion results in accelerated atherosclerosis induction and promotes features of a vulnerable plaque.

Introduction

Cardiovascular disease (CVD) is the number one cause of death in the western world and manifests in various phenotypes including atherosclerosis, stroke, myocardial infarction and aneurysm formation ^{1,2}. Despite the development and application of effective treatment, such as the use of medicines and surgery, there is still a relatively high CVD morbidity and mortality ^{3,4}.

Under physiological conditions, vascular smooth muscle cells (VSMCs) in the tunica media regulate vascular tone and diameter in order to maintain hemodynamic balance ^{5,6}. Besides VSMCs, extracellular matrix (ECM) proteins including elastin, collagen, fibrinogen and endothelial cells (ECs) are involved in maintenance of physiological vascular function. Remodeling of the vascular media is caused by various stress factors which affect the functionality of the vessel wall ^{6,7}. Under physiological conditions, the majority of VSMCs in the vessel wall display a contractile phenotype that supports vascular tone. However, a subset of medial VSMCs has the ability to differentiate into a synthetic phenotype ⁸. This phenotypic plasticity of VSMCs is necessary to deal with varying conditions of the vascular tissue. Synthetic VSMCs are prone to undergo apoptosis or further phenotypic differentiation towards an osteochondrogenic phenotype ⁹. Both apoptosis and osteochondrogenic differentiation of VSMCs are known to contribute to vascular remodeling, which results in adaptation of the vessel wall. Vascular remodeling is related to pathological processes such as inflammation, fibrosis and calcification of arteries. Vascular calcification can lead to increased elastin breaks and further induction of apoptosis of VSMCs ¹⁰. Vascular remodeling has been linked to the development of atherosclerosis and vessel wall occlusion ¹¹⁻¹³. Atherosclerosis is known as a chronic inflammatory disease characterized by endothelial activation and lipid accumulation in the vessel wall ¹⁴. Accumulation of VSMC into the neointima has long been considered a key event in atherosclerosis progression. Here, VSMC promote atherosclerotic plaque stability by depositing extracellular matrix proteins such as collagen to create a thick fibrous cap.

To investigate the role of arterial remodeling on atherosclerosis and features of plaque vulnerability we used SM22 α -iDTR+/ApoE^{-/-} mice that are previously described by Clarke and coworkers ^{15,16}. The authors showed that apoptosis of VSMCs in the vasculature is a key factor in the development of plaque instability in the early and late phase of atherosclerosis ¹⁵. Medial vascular calcification and apoptosis has been shown to affect atherosclerosis formation ¹⁶⁻¹⁸.

In our study, we used SM22 α -iDTR+/ApoE^{-/-} mice to investigate the role of VSMCs in the vascular media on vessel wall properties and the initiation and progression of atherosclerosis. VSMC apoptosis, and consequently depletion of medial VSMCs was induced prior to formation of atherosclerotic plaques. We demonstrate the important contribution of medial VSMCs on vessel wall homeostasis and on atherogenesis and plaque stability.

Methods

Animal protocols

SM22 α -iDTR/ApoE^{-/-} mice were kindly provided by Prof. Dr. M. Bennett ¹⁵. Control animals were diphtheria toxin receptor negative littermates (SM22 α -iDTR/ApoE^{-/-} mice). Mice were kept in a temperature-controlled environment (20°C)

with a regular day & night cycle. All animal experiments were conducted under a protocol approved by the local ethics committee for animal experiments, Maastricht University, The Netherlands.

Purified, recombinant, unnicked human diphtheria toxin (DT) (Quadrachtech Diagnostics, Surrey, United Kingdom) was dissolved in 0.9% saline solution containing 0.2% low endotoxin bovine serum albumin (Sigma, Zwijndrecht, the Netherlands), sterile filtered and stored at -80°C until use.

Experimental animals (12 week old males) were weighed and injected 3 times a week intraperitoneally with 1 ng/g bodyweight of DT for 3 weeks. Next, a 1-week wash-out period was provided. During this period, mice were fed a normal chow diet and water ad libitum.

At sacrifice, blood was collected in 3.2% sodium citrate by vena cava puncture and the aorta was flushed with 100 μ M sodium nitroprusside in phosphate buffered saline (PBS). Bone marrow was collected by flushing the femur with PBS. The aortic arch and its main branches were excised and fixated in 1% paraformaldehyde overnight and embedded in paraffin. First-order mesenteric arteries and carotid artery were used in myograph and two-photon imaging experiments. Besides, aortas were snap-frozen in liquid nitrogen and stored in -80°C after which RNA extraction was performed.

To study atherosclerosis development, mice were fed a WTD containing 0.25% cholesterol, 15% cacao butter and 1% corn oil (AB diets, Woerden, the Netherlands) for either 6 or 18 weeks and were sacrificed using the same procedure.

Plasma cholesterol and triglyceride determination

Total cholesterol (Horiba Medical, Montpellier, France) and triglyceride (Horiba Medical, Montpellier, France) measurements were performed as prescribed.

FACS analysis of blood and bone marrow

The whole blood cell composition was measured using a haematology-analyzer (XP-300, Sysmex, Etten-Leur, the Netherlands). After measurement, erythrocyte lysis was performed and the blood cell composition was further determined using antibodies against CD11b, CD4, Ly6G, B220 (all BD Biosciences, Temse, the Netherlands), Ly6C (Miltenyi Biotec, Leiden, the Netherlands), CD3e, NK1.1 and CD8a (all eBioscience, Vienna, Austria). Bone marrow cell composition was analysed using antibodies versus Ly6G, Ly6C, B220, NK1.1, CD3e, CD11b and CD115 (eBioscience, Vienna, Austria). Flow cytometry was performed using a FACS Canto (BD Biosciences, Temse, the Netherlands) and analysed using FACSDiva software (v6.1.3, BD Biosciences, Temse, the Netherlands).

Wire Myograph

Drugs used: Phenylephrine (PHE), Acetylcholine (ACh), Indomethacin (INDO, cyclooxygenase-inhibitor), Sodium Nitroprusside (SNP) and L-NAME (NO synthase inhibitor) were all purchased from Sigma Aldrich (Zwijndrecht, the Netherlands).

First order mesenteric resistance arterial segments of approximately 2 mm in length were isolated and mounted in wire myographs (Model 610M, DMT, Denmark) for recording of isometric force development. After mounting, the vessel segments were equilibrated for 15 minutes at 37°C in an oxygenated (95%O₂ and 5% CO₂, pH 7.4)

Krebs-Ringer bicarbonate buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃ and 5.5 glucose. Following equilibration, the vessel segments were distended to a diameter corresponding to 90% of the passive diameter at a transmural pressure of 100 mmHg¹⁹.

Contractile responses to KCl (62.5 mM, K⁺) and increasing concentrations of Phenylephrine (0.01 - 30 μ M, PHE) and relaxing responses to increasing concentrations to Acetylcholine (0.01- 10 μ M, ACh) were recorded. The effects of nitric oxide synthase inhibition (100 μ M, L-NAME) and cyclo-oxygenase inhibition (1 μ M, INDO) were evaluated. Endothelium-independent NO-mediated vasorelaxation was measured using increasing concentrations of Sodium Nitroprusside (0.001 - 1 μ M, SNP) during precontraction with PHE (30 μ M).

Two-photon imaging

For imaging of the mounted and pressurized blood vessels, a Leica TCS SP5 (Leica Microsystems GmbH, Wetzlar, Germany) two-photon laser scanning microscope was used with a Ti-Sapphire Chameleon Ultra II (Coherent Inc, Santa Clara, CA, USA) laser. Excitation was at 840 nm using a fs-pulsed laser (maximal power at the sample at 100% of 50 mW). A Leica objective, HCX APO L 20x/1.00 was used. Fluorescence detection was performed using four detectors (1 forward and 3 backward), set according to the emission spectra. For DAPI a setting between 440 and 500 nm (channel 1), for the autofluorescence of Elastin a setting between 500-560 nm (channel 2) was used. For the SHG, both in forward (channel 4) and backward (channel 3) direction, a setting of 400-440 was used. With this choice of settings DAPI appears strongest in channel 1 and 2 and is color-coded Cyan. Elastin appears in all channels, but strongest in channel 2 and is color-coded green. Backward SHG appears exclusively channel 3 and is color-coded Magenta. Forward SHG is color-coded yellow.

Maximum projection images were made via the Imaris software (Oxford instruments, Bitplane, Zürich, Switzerland), looking specifically at channel 2 and separating nuclei from elastin structures using segmentation based on shape. VSMCs were separated from other cell nuclei (fibroblasts and endothelial cells) segmenting on their typical orientation and shape. The segmentation was checked manually and the separated VSMC nuclei were counted in Imaris.

Histology and immunohistochemistry

5 μ M serial sections were cut from paraffin embedded aortic arches and stained with haematoxylin and eosin (H&E). Plaque burden was analysed on these stained sections using ImageJ software (v1.47). Total plaque burden was measured in the aortic arch and major branching points.

Antigen retrieval was performed by heating samples in target retrieval solution (Dako, Heverlee, Belgium). Subsequently, tissues were blocked and incubated with primary antibodies overnight as described: α SMA (1A4, Dako; 1:500); Mac3 (M3/84; Pharmingen, 1:200). After overnight incubation, sections were incubated for 1 hour with HRP-conjugated secondary antibodies and detected with NovaRED (Vector Labs, Amsterdam, the Netherlands). Apoptotic cells were detected using a terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) kit (Roche, Woerden, the Netherlands) as described in the manufacturers protocol. Calcification was detected using Von Kossa and collagen fibers using picro sirius red. Additionally, picro sirius red

stained sections were also used for determination of the necrotic core size (percentage of the plaque) and the thickness of the fibrous cap. Fibrous caps were determined as the collagen rich area overlying the necrotic core. Images were captured using a DM400 microscope (Leica, Eindhoven, the Netherlands) and imaging software (LAS V, Eindhoven, the Netherlands).

RNA extraction, cDNA synthesis and quantitative analysis

Aortic arches were homogenised by glass beads (BioSpec, Bartlesville, USA) in TRI reagent (Sigma). Isolated primary vascular smooth muscle cells (VSMCs) were directly lysed in TRI reagent. After incubation, total RNA was extracted using a phenol-chloroform method as previously described by Chomczynski et al.²⁰. RNA concentration was quantified spectrophotometrically at 260 nm. 1 µg of total RNA was treated with DNase I (Promega) and reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), RNAout, dNTPs, dithiothreitol (all Invitrogen) and an oligo(dT)adapter (Eurogentec) for 1 hour at 37°C. Gene expression levels were quantified by real-time quantitative PCR (qPCR) on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science). Amplification reactions were carried out in a volume of 10 µl including 20 ng of total cDNA, 5 µl QuantiTect SYBR Green PCR Kit (Qiagen) and 2 µM of each primer (supplemental table 3). An initial denaturation step (15 minutes at 95°C) was followed by 50 cycles of amplification (denaturation: 15 seconds at 95°C, annealing: 30 seconds at 57°C, extension: 45 seconds at 72°C). Specificity was analysed by melting curve plots. Fluorescence curves were analysed with LightCycler 480 Software (Version 1.5) and relative quantification by plotting N0 ration.

Statistical analysis

All values are shown as mean ± SEM unless stated differently. Statistical analysis was performed by 1- way ANOVA with Bonferroni post hoc test using PRISM software (GraphPad). Values of $P < 0.05$ were considered statistically significant.

Results

Diphtheria toxin reduces medial VSMC cellularity in SM22α-iDTR+/ApoE^{-/-} mice

A schematic overview of the animal study performed is displayed in figure 1A. Mice were injected 3 times/ week for 3 weeks with diphtheria toxin after which a washout period of one week was maintained. At $t=0$, cellularity of the vessel wall was measured for different vessels (Figure 1B). Aorta nuclei were counted by HE stain and SM22α-iDTR+/ApoE^{-/-} showed some 50% reduction in cell count compared to SM22α-iDTR-/ApoE^{-/-} mice (Figure 1C). First-order mesenteric and carotid artery were imaged by two-photon microscopy and SM22α-iDTR+/ApoE^{-/-} showed some 40% reduction in the number of cells compared to SM22α-iDTR-/ApoE^{-/-} mice (Figures 1 D-F). VSMCs were stained with DAPI to visualize their nuclei (representative images displayed in Figure 1G and H). Using two-photon microscope, 3D reconstruction of the vessel was performed (Figure 1I) and cell number was measured in 3D reconstructed vessel.

Mice fed standard chow diet did not present atherosclerotic plaques in the aortic arch and its main branches of either SM22α-hDTr⁺/ApoE^{-/-} or SM22α-hDTr⁻/ApoE^{-/-} mice at $t=0$, which is consistent with previously reported data^{21,22}.

Figure 1. Characterization of mice exposed to DT. (A) Experimental flow chart and SM22α-iDTR+/ApoE^{-/-} model validation at t=0. (B) Schematic representation of the vessels that were used. (C) Cellularity of the vessel wall (cells/mm²) was determined by quantifying the number of nuclei in the vessel wall after the wash out period. SM22α-iDTR+/ApoE^{-/-} mice showed a 50% reduction in cell count. (D) Representation of an aortic arch of SM22α-iDTR+/ApoE^{-/-} mouse. (E) Representation of an aortic arch of SM22α-iDTR-/ApoE^{-/-} mouse. (F) Cellularity of the vessel wall determined by TP imaging. SM22α-iDTR+/ApoE^{-/-} mice showed a 40% reduction in cell count. (G and H) Representing images of mesenteric artery by TP microscopy of both SM22α-iDTR+/ApoE^{-/-} (G) and SM22α-iDTR-/ApoE^{-/-} (H) mice. (I) 3D reconstruction of the vessel wall. *p<0.05; **p<0.001

Media thickness is increased in SM22α-iDTR+/ApoE^{-/-} mice and *in vitro* isolated iDTR+ VSMCs show increased cell size and signs of a contractile phenotype

VSMCs isolated from SM22α-iDTR+/ApoE^{-/-} mice aorta's showed hypertrophy when comparing to VSMCs isolated from SM22α-iDTR-/ApoE^{-/-} mice (Figures 2A, 2G). Also, medial layer thickness of mesenteric arteries in SM22α-iDTR+/ApoE^{-/-} was significantly increased compared to SM22α-iDTR-/ApoE^{-/-} mice (Figures 2B, 2H). Additionally, mRNA expression of αSMA and CNN-1 was increased in thoracic aorta and VSMCs isolated from SM22α-iDTR+/ApoE^{-/-} (Figures 2 C-F).

Figure 2. *In vitro* isolated VSMCs and media thickness. (A) Mouse VSMC cellular size. A significant increase in VSMC size was found in SM22 α -iDTR+/ApoE $^{-/-}$ mice. (B) Media thickness determined by TP imaging. (C) Mouse VSMC α SMA RNA expression. (D) Mouse VSMC CNN-1 expression. (E) Thoracic aorta α SMA RNA expression. (F) Thoracic aorta CNN-1 RNA expression. (G) Representative images of VSMCs isolated from SM22 α -iDTR+/ApoE $^{-/-}$ mice. (H) TP imaging representation to determine media thickness. ****p<0.0001

SM22 α -iDTR+/ApoE $^{-/-}$ mice show decreased mesenteric artery vessel vasodilation with increased cfPWV

SM22 α -iDTR+/ApoE $^{-/-}$ first-order mesenteric arteries (Figure 3A) displayed no change in K $^{+}$ and phenylephrine (PHE) induced contraction (Figure 3B). To assess endothelium-dependent responses, mesenteric arteries were contracted using PHE and next exposed to increasing concentrations of acetylcholine (ACh). SM22 α -iDTR+/ApoE $^{-/-}$ showed a significant decrease in E $_{\max}$ compared to SM22 α -iDTR-/ApoE $^{-/-}$ mice, while pEC $_{50}$ remained the same (Figure 3C). To assess the contribution of endothelial derived hyperpolarizing factors (EDHF) to ACh induced relaxing responses, arteries were contracted with PHE in the presence of l-NAME (to prevent involvement of NO). Sensitivity and maximal response did not differ significantly between SM22 α -iDTR+/ApoE $^{-/-}$ and SM22 α -iDTR-/ApoE $^{-/-}$ mice. However, SM22 α -iDTR+/ApoE $^{-/-}$ showed a complete ablation of EDHF-related vessel dilation (Figure 3C). To assess maximal vasodilation, Na-nitroprusside (NP) was added and there was no difference in pEC $_{50}$ and E $_{\max}$ between SM22 α -iDTR+/ApoE $^{-/-}$ and SM22 α -iDTR-/ApoE $^{-/-}$ mice (Figure 3D). Carotid-femoral pulse wave velocity (cfPWV) was significantly increased in SM22 α -iDTR-/ApoE $^{-/-}$ compared to SM22 α -iDTR+/ApoE $^{-/-}$ mice.

Figure 3. Vessel contractility properties. (A) Schematic representation of the aorta indicating which vessels were used. Blue boxed vessels used in cPWV, mesenteric arteries (white box) used for myograph measurements. (B) Contractile response to PHE in SM22α-iDTR+/ApoE^{-/-} (closed squares) and SM22α-iDTR-/ApoE^{-/-} (closed circles) vessels. (C) Relaxing responses to ACh in SM22α-iDTR+/ApoE^{-/-} (closed squares) and SM22α-iDTR-/ApoE^{-/-} (closed circles) vessels, in addition with l-NAME in SM22α-iDTR+/ApoE^{-/-} (green squares) and SM22α-iDTR-/ApoE^{-/-} (red circles). (D) Relaxing responses to NP in SM22α-iDTR+/ApoE^{-/-} (closed squares) and SM22α-iDTR-/ApoE^{-/-} (closed circles) vessels. (E) Relaxing responses to ACh in SM22α-iDTR+/ApoE^{-/-} (closed squares) and SM22α-iDTR-/ApoE^{-/-} (closed circles) vessels, in addition with l-NAME in SM22α-iDTR+/ApoE^{-/-} (green squares) and SM22α-iDTR-/ApoE^{-/-} (red circles). (F) cPWV results. A significant decrease in PWV was found in SM22α-iDTR+/ApoE^{-/-} mice. *p<0.05

Baseline characteristics of SM22α-iDTR+/ApoE^{-/-} mice after atherosclerosis induction

We investigated parameters affecting atherosclerosis in both SM22α-iDTR+/ApoE^{-/-} and SM22α-iDTR-/ApoE^{-/-} mice. At time of sacrifice, the observed bodyweights after 6 or 18 weeks of WTD did not significantly differ between SM22α-iDTR+/ApoE^{-/-} and SM22α-iDTR-/ApoE^{-/-} (Table 1). Additionally, blood lipid levels were measured in mice sacrificed after 6 and 18 weeks of WTD. Similarly, there were no significant differences in cholesterol and triglyceride levels between SM22α-iDTR+/ApoE^{-/-} and SM22α-iDTR-/ApoE^{-/-} mice (Table 1). At the time of sacrifice, no organ abnormalities were observed in mice of both strains, as has been shown before¹⁵.

Following treatment with DT, circulating leukocyte profiles had not significantly altered, both in SM22α-iDTR+/ApoE^{-/-} and SM22α-iDTR-/ApoE^{-/-} mice (Supplemental table 1). Analysis of the leukocyte populations in bone marrow did reveal a difference. After 6 weeks of WTD, B-cell levels had significantly increased in SM22α-iDTR+/ApoE^{-/-} mice as compared to SM22α-iDTR-/ApoE^{-/-} mice (Supplemental Table

2). No significant differences were observed between other leukocyte populations in bone marrow from SM22 α -iDTR+/ApoE $^{-/-}$ and SM22 α -iDTR-/ApoE $^{-/-}$ mice.

Table 1. Baseline characteristics of SM22 α -iDTR-/ApoE $^{-/-}$ and SM22 α -iDTR+/ApoE $^{-/-}$ mice. All values are represented as mean $\pm$ SD. Significance was analyzed using a one-way ANOVA with Bonferroni's multiple comparison test.

	6 weeks	6 weeks	18 weeks	18 weeks
	SM22 α -hDTr/ApoE $^{-/-}$	SM22 α -hDTr/ApoE $^{-/-}$	SM22 α -hDTr/ApoE $^{-/-}$	SM22 α -hDTr/ApoE $^{-/-}$
Weight (g)	29,00 $\pm$ 1,30	28,40 $\pm$ 1,80	33,20 $\pm$ 3,60	32,2 $\pm$ 3,60
Cholesterol (mM)	5,77 $\pm$ 0,70	6,21 $\pm$ 1,24	5,21 $\pm$ 0,80	5,66 $\pm$ 0,59
Triglycerides (mM)	1,49 $\pm$ 0,30	1,67 $\pm$ 0,94	1,47 $\pm$ 0,53	1,62 $\pm$ 0,58

DT induced medial VSMC apoptosis accelerates early and late atherogenesis

After DT induction and VSMC ablation mice were fed a WTD for either 6 or 18 weeks (Figure 4A). After 6 weeks of WTD, atherosclerotic plaques were present in both SM22 α -iDTR+/ApoE $^{-/-}$ and SM22 α -iDTR-/ApoE $^{-/-}$ mice (Figures 4B and C). Quantification of plaque size using H&E stained aortic sections showed a 2-fold larger atherosclerotic plaque burden in SM22 α -iDTR+/ApoE $^{-/-}$ mice as compared to SM22 α -iDTR/ApoE $^{-/-}$ littermates. After 18 weeks of WTD (Figures 4B and C), a similar 2-fold significantly increased plaque burden was observed in SM22 α -iDTR+/ApoE $^{-/-}$ mice (Figure 4C).

Figure 4. VSMCs apoptosis accelerates atherosclerosis. (A) Schematic overview of the study indicating the period of pro-atherogenic diet. (B and C) After 6 weeks of WTD, atherosclerotic plaques in SM22 α -iDTR+/ApoE $^{-/-}$ mice are significantly larger as compared to SM22 α -iDTR-/ApoE $^{-/-}$ mice. Representative images of the aortic arch and main branch points are shown (panel B, top left: SM22 α -iDTR-/ApoE $^{-/-}$, top right: SM22 α -iDTR+/ApoE $^{-/-}$). After 18 weeks of WTD, atherosclerotic plaques in DT mice are significantly larger as compared to WT mice. Representative images of the aortic arch and main branch points are shown (panel B, bottom left: SM22 α -iDTR-/ApoE $^{-/-}$, bottom right: SM22 α -iDTR+/ApoE $^{-/-}$). (C) Quantification of atherosclerotic plaque area. Images were taken at 4x magnification. * $p<0.05$; *** $p<0.001$

Reduced medial VSMC number is associated with features of plaque vulnerability

In addition to atherosclerotic plaque burden, atherosclerotic plaques of both SM22 α -iDTR+/ApoE^{-/-} and SM22 α -iDTR-/ApoE^{-/-} mice were analyzed for features of plaque vulnerability. Atherosclerotic plaques of SM22 α -iDTR+/ApoE^{-/-} mice had more Mac3 positive stain after 6 weeks of WTD, indicative of a more inflammatory plaque phenotype, in comparison to SM22 α -iDTR-/ApoE^{-/-} mice (Figures 5A and 5B). After 18 weeks of WTD, the difference in Mac3 disappeared.

Next, we analyzed atherosclerotic plaque calcification in both SM22 α -iDTR+/ApoE^{-/-} and SM22 α -iDTR-/ApoE^{-/-} mice (Figures 6B and 6D). After 6 weeks of WTD, no calcification was detectable in both SM22 α -iDTR+/ApoE^{-/-} or SM22 α -iDTR-/ApoE^{-/-} (data not shown). After 18 weeks of WTD, calcification was significantly present. However, there was no significant difference in relative plaque calcification between SM22 α -iDTR+/ApoE^{-/-} and SM22 α -iDTR-/ApoE^{-/-} mice (Figure 5C and 5D). However, when calculating absolute values, there is an increase in plaque calcification of 72% between SM22 α -iDTR+/ApoE^{-/-} (mean 31123 $\mu\text{m}^2 \pm 28221 \mu\text{m}^2$) and SM22 α -iDTR-/ApoE^{-/-} (mean 18676 $\mu\text{m}^2 \pm 14606 \mu\text{m}^2$) mice ($p=0.081$; Figure 5E).

Figure 5. Plaque macrophage infiltration and calcification. (A) Representative images of Mac3+ IHC staining. (B) Quantification of Mac3+ per plaque, in which a significant increase is found in the SM22 α -iDTR+/ApoE^{-/-} vs. SM22 α -iDTR-/ApoE^{-/-} group at 6 weeks. (C) Representation of Von Kossa staining which was only detected at the later time point of 18 weeks. (D) Quantification of relative Von Kossa positivity in which no differences were found between SM22 α -iDTR+/ApoE^{-/-} and SM22 α -iDTR-/ApoE^{-/-}. (E) Quantification of absolute Von Kossa positivity in which a trend was found between SM22 α -iDTR+/ApoE^{-/-} and SM22 α -iDTR-/ApoE^{-/-}. All images were taken at 4x magnification. * $p<0.05$

VSMCs are key to build the atherosclerotic fibrous cap. SM22 α -iDTR+/ApoE^{-/-} mice displayed significantly thinner fibrous caps as deduced from picro sirius red staining (Figures 6A and 6B) at both 6 and 18 weeks of WTD. Collagen content in atherosclerotic plaques was not significantly different after both 6 and 18 weeks of WTD (Figure 6C and 6D).

After 6 weeks of WTD, no necrotic core could be observed in either SM22 α -iDTR+/ApoE^{-/-} or SM22 α -iDTR-/ApoE^{-/-} mice. After 18 weeks SM22 α -iDTR+/ApoE^{-/-}

mice had significantly larger necrotic cores as compared to SM22 α -iDTR-/ApoE $^{-/-}$ littermates (Figures 6E and 6F).

Subsequently, VSMC content of atherosclerotic plaques in both SM22 α -iDTR+/ApoE $^{-/-}$ and SM22 α -iDTR-/ApoE $^{-/-}$ mice after 6 or 18 weeks of western type diet was analyzed. After 6 weeks, significantly less VSMC were present in plaques of SM22 α -iDTR+/ApoE $^{-/-}$ mice as compared to SM22 α -iDTR-/ApoE $^{-/-}$ mice (Figures 6 G and H). After 18 weeks, the difference in plaque VSMC number disappeared. TUNEL staining revealed no difference in the degree of apoptosis in plaques of either SM22 α -iDTR+/ApoE $^{-/-}$ or SM22 α -iDTR-/ApoE $^{-/-}$ mice after 6 or 18 weeks of western type diet (data not shown).

Figure 6. Plaque vulnerability features. (A) Representation of picro Sirius Red staining (PSR) images that were taken to assess cap thickness. (B) At both time points SM22 α -iDTR+/ApoE $^{-/-}$ mice were showing thinner fibrous caps compared to SM22 α -iDTR-/ApoE $^{-/-}$. (C) Representation of overall PSR staining. (D) No significant differences were found in SM22 α -iDTR+/ApoE $^{-/-}$ vs. SM22 α -iDTR-/ApoE $^{-/-}$ mice when looking at collagen content. (E) Representation of PSR images for necrotic core determination. (F) A significant increase of necrotic core was found in SM22 α -iDTR+/ApoE $^{-/-}$ plaques compared to SM22 α -iDTR-/ApoE $^{-/-}$. (G) Representative images of αSMA positive stained images. (H) A significant decrease in αSMA positivity was found in SM22 α -iDTR+/ApoE $^{-/-}$ vs. SM22 α -iDTR-/ApoE $^{-/-}$ mice after 6 weeks of diet. (C) Images were taken at 4x magnification. (A), (E) and (G) Images were taken at 10x magnification. *p<0.05; **p<0.01

Discussion

In this paper, we studied the role of medial VSMC apoptosis on atherogenesis. VSMC-specific apoptosis of medial VSMCs was induced in SM22 α -iDTR+/ApoE^{-/-} mice to study the contribution of vascular medial remodeling in atherosclerosis. We demonstrate that a loss of medial VSMCs prior to atherosclerosis results in accelerated de novo and on the long term larger atherosclerotic plaques. Additionally, atherosclerotic plaques had features of a more vulnerable phenotype, characterized by a thin fibrous cap, a large necrotic core, increased inflammation and reduced number of VSMCs.

Our observation that reducing VSMCs in the medial layer prior to atherogenesis accelerates plaque growth, extends the findings of Clarke and coworkers who demonstrated that VSMC apoptosis in mice with established atherosclerotic plaques increased atherosclerotic plaque size and calcification including medial degeneration^{15,16}. In our mouse model, the accelerated growth of atherosclerotic plaques might be caused by a number of reasons. Firstly, VSMCs slow down plaque growth by formation of a thick fibrous cap, which inhibits monocyte and macrophage infiltration into the atherosclerotic plaque and reduces plaque inflammation and necrotic core formation¹⁶. Fibrous cap thickness depends on the number of VSMCs in the fibrous cap and the extracellular matrix they deposit^{23,24}. Our data support these observations as atherosclerotic plaques of SM22 α -iDTR+/ApoE^{-/-} mice show significantly less α SMA positive cells and a much thinner fibrous cap.

Secondly, VSMCs are efficient efferocytes and contribute to clearance of apoptotic remnants in their environment²⁵. Due to their high plasticity, VSMCs can switch to a macrophage-like phenotype expressing typical macrophage markers like CD68 and Mac2²⁶⁻²⁸. However, during hyperlipidaemia, defective phagocytosis is induced when VSMCs switch to macrophage-like cells accompanied by IL-1 driven inflammation^{29,30}. Apoptotic cell remnants like apoptotic bodies serve as a nidus for vascular calcification^{10,31}. Additionally, clearance of apoptotic cells in the atherosclerotic plaque is severely impaired, in part due to oxidative stress and cytoplasmic saturation of indigestible components³⁰. Reduced efferocytosis has been demonstrated to promote atherosclerotic plaque growth^{32,33}. Hence, the pro-calcifying environment in atherosclerotic plaques, combined with poor clearance of apoptotic bodies form a strong stimulus for calcification in this situation^{34,35}. Additionally, we need to reconsider the use of therapies aiming to reduce restenosis by inducing VSMC apoptosis as these therapies might have drastic effects on the stability of developing and established atherosclerotic plaques in patients³⁶⁻³⁸.

In our study, apoptosis of medial VSMC is induced prior to atherogenesis. Under physiological conditions, apoptotic cells are rapidly cleared from the vasculature³⁹. In contrast to previous results, we did not observe a difference in atherosclerotic plaque calcification. However, absolute values of calcification were increased in SM22 α -iDTR+/ApoE^{-/-} compared to SM22 α -iDTR-/ApoE^{-/-} mice. Clinically, coronary artery calcification (CAC) volume is positively associated with CVD⁴⁰ and higher risk for rupture⁴¹. Our results support these clinical data that increased of plaque calcification volume results in decreased stability of atherosclerotic plaques⁴².

Aging of the vessel wall is accompanied by vascular remodeling with loss of elasticity and increased deposition of calcium in the media. Moreover, aging is related to medial calcification and thus a higher cardiovascular morbidity and mortality⁴³. For

a long time, it has been demonstrated that calcification and degeneration of the media is an important risk factor for developing atherosclerosis¹⁷. Clarke and coworkers showed that inducing apoptosis of VSMCs in established atherosclerotic plaques induced intimal calcification. We show that reduced medial VSMC number results in accelerated development of atherosclerosis.

Initially, VSMCs were thought to promote atherosclerotic plaque growth by migrating into the plaque and proliferate, causing plaque growth and occlusion of the vessel^{15,16,44}. However, our study demonstrates that medial VSMCs significantly contribute to both inhibition and stability of atherosclerotic plaques. This may occur through several mechanisms, including the interaction between VSMCs and ECs. Here we show that the loss of medial VSMCs is compensated by hypertrophy of the remaining VSMCs. Isolated VSMCs *in vitro* display signs of increased contractility, which might be a response to maintain vessel wall contraction. *Ex vivo* myograph analysis revealed significant differences in vessel dilation, with a clear reduced dilation capability in vessels with hypertrophic VSMCs, an effect caused by a loss of endothelial derived hyperpolarizing factors. It is noteworthy that the communication between VSMCs and ECs is affected without disturbing NO mediated pathways. The exact mechanism by which this decrease in vessel wall dilation properties are caused is not fully understood but might arise from the impaired cellular connections in gap junctions between the ECs and the VSMCs⁴⁵. The reduced capacity in interaction between ECs and VSMCs might lead to EC activation with subsequent VSMC phenotype switching, consequently contributing to the development of atherosclerosis^{46,47}. This corroborates with our findings of a more pro-inflammatory plaque phenotype in mice that had reduced medial VSMCs. Finally, our data revealed decreased cfPWV in mice with reduced medial VSMCs, indicating that vessel compliance is increased.

In conclusion, we show that medial VSMCs are important for maintaining vessel wall integrity and in preventing vascular medial remodeling and atherosclerosis. Our work highlights the need for further studies to unravel the driving forces of medial remodeling as an inducer of accelerated atherosclerosis.

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Supplemental material

Supplemental table 1. Flow cytometric analysis of blood after 6 and 18 weeks of western type diet. All values are represented as mean $\pm$ SD. Significance was analyzed using a one-way ANOVA with Bonferroni's multiple comparison test.

Celltype (% of viable cells)	Subpopulation (% of parent)	6 weeks SM22 α -hDTr ⁻ /ApoE ^{-/-}	6 weeks SM22 α - hDTr ⁺ /ApoE ^{-/-}	18 weeks SM22 α -hDTr ⁻ /ApoE ^{-/-}	18 weeks SM22 α - hDTr ⁺ /ApoE ^{-/-}	P
B-cells		37,8 $\pm$ 11,19	40,13 $\pm$ 21,08	25,31 $\pm$ 13,20	28,59 $\pm$ 9,25	n.s. .
T-cells		10,30 $\pm$ 2,25	12,79 $\pm$ 3,40	8,90 $\pm$ 5,07	7,64 $\pm$ 2,34	n.s. .
	CD4+ T-cells	54,56 $\pm$ 2,61	52,31 $\pm$ 2,87	47,85 $\pm$ 4,30	46,25 $\pm$ 4,22	n.s. .
	CD8+ T-cells	51,35 $\pm$ 3,40	43,68 $\pm$ 6,72	55,96 $\pm$ 7,11	52,28 $\pm$ 6,00	n.s. .
NK-cells		5,62 $\pm$ 1,43	4,10 $\pm$ 1,21	3,55 $\pm$ 1,24	3,15 $\pm$ 0,73	n.s. .
Granulocytes		65,35 $\pm$ 9,12	69,21 $\pm$ 15,86	66,64 $\pm$ 13,30	67,55 $\pm$ 9,241	n.s. .
Monocytes		29,28 $\pm$ 7,55	24,95 $\pm$ 13,12	27,49 $\pm$ 11,36	27,93 $\pm$ 7,95	n.s. .
	Ly6C low	28,78 $\pm$ 13,15	22,04 $\pm$ 7,59	22,28 $\pm$ 9,75	17,43 $\pm$ 6,48	n.s. .
	Ly6C medium	6,03 $\pm$ 1,50	6,71 $\pm$ 2,84	3,15 $\pm$ 1,03	3,47 $\pm$ 0,95	n.s. .
	Ly6C high	53,97 $\pm$ 14,33	55,25 $\pm$ 15,78	59,00 $\pm$ 13,35	67,00 $\pm$ 11,41	n.s.

Supplemental table 2. Flow cytometric analysis of bone marrow after 6 and 18 weeks of western type diet. All values are represented as mean $\pm$ SD. Significance was analyzed using a one-way ANOVA with Bonferroni's multiple comparison test.

Celltype (% of viable cells)	Subpopulation (% of parent)	6 weeks SM22 α -hDTr ⁻ /ApoE ^{-/-}	6 weeks SM22 α -hDTr ⁺ /ApoE ^{-/-}	18 weeks SM22 α -hDTr ⁻ /ApoE ^{-/-}	18 weeks SM22 α -hDTr ⁺ /ApoE ^{-/-}	P
B-cells		6,54 $\pm$ 3,61	10,46 $\pm$ 3,54	4,71 $\pm$ 1,60	5,89 $\pm$ 2,48	0,0009
NK-Cells		0,36 $\pm$ 0,17	0,45 $\pm$ 0,12	0,33 $\pm$ 0,29	0,32 $\pm$ 0,14	n.s.
Granulocytes		53,54 $\pm$ 6,22	54,34 $\pm$ 4,00	50,41 $\pm$ 5,84	48,47 $\pm$ 7,99	n.s.
Monocytes		10,58 $\pm$ 3,44	12,91 $\pm$ 3,68	9,63 $\pm$ 3,91	10,37 $\pm$ 3,06	n.s.
	Ly6C low	8,74 $\pm$ 4,42	11,97 $\pm$ 3,61	10,23 $\pm$ 5,64	14,52 $\pm$ 6,32	n.s.
	Ly6C high	79,10 $\pm$ 15,94	74,13 $\pm$ 14,44	81,46 $\pm$ 11,32	75,88 $\pm$ 10,64	n.s.
T-cells		1,36 $\pm$ 0,57	1,72 $\pm$ 0,71	1,41 $\pm$ 0,70	1,66 $\pm$ 0,51	n.s.

Supplemental table 3. Primer list.

Gene	Forward	Reverse
B-actin	GGCACCACACCTTCTACAATG	GGGGTGTTGAAGGTCTCAAAC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
18s	ACTTTTGGGGCCTTCGTGTC	GCCCAGAGACTCATTTCTTCTTG
CNN1	GCAGTGGACACACGCATTTT	AACAACGGCCCAAGACTC
α SMA	CTGACAGAGGCACCACTGAA	CATCTCCAGAGTCCAGCACA

Chapter 5

Use of primary vascular smooth muscle cells to predict vascular calcification potential of patients: Development of a BioHybrid Assay

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In preparation

Abstract

Background: Vascular calcification is an active process and increases cardiovascular disease (CVD) risk. The current methods to detect vascular calcification are computed tomography and ^{18}F -NaF positron emission tomography. Clinical research into validated biomarkers is making progress but there is still no consensus on an appropriate marker for vascular calcification. We reason that the biomarker for vascular calcification is not a single plasma component but the collection of all plasma components and aimed to develop a cell-based assay to measure calcification (BioHybrid assay).

Methods: Human vascular smooth muscle cells (hVSMCs) were exposed to high calcium conditions and were challenged with different serum and plasma samples of patients susceptible for vascular calcification. Patient sera and plasma were derived from different cohort studies including chronic kidney disease (CKD) stages III, IV, V and VD, pseudoxanthoma elasticum and other CVD disease with high calcification score. Live hVSMC calcifying propensity was measured in a new developed assay using Fetuin A-Alexa 546.

Results: The BioHybrid assay was comparable to the gold-standard O-cresolphthalein method of calcification determination and is able to measure calcification development over time. The BioHybrid assay indicated that serum of dialysis patients before dialysis were more prone to calcify as compared to serum of the same patient after dialysis. Additionally, PXE, oral anticoagulation (OAC) and high CAC score patients showed greater calcification as compared to control.

Conclusion: We demonstrate a novel method for detecting vascular calcification *in vitro*. Using a Fetuin A fluorescent probe we have developed a real-time calcification assay that is able to give a quantifiable readout of calcification development over time in patients vulnerable for vascular calcification.

Introduction

Cardiovascular disease (CVD) is the largest killer in the world and globally CVDs account for approximately 30% of all-cause deaths annually ¹. Currently the majority of medical interventions for CVD occur post-event. Therefore, there is an unmet need of pre-event diagnostics to accurately predict susceptibility to cardiovascular events. Clinical research into validated biomarkers is making progress but is still insufficient to resolve professional consensus on appropriate markers ².

Vascular calcification is an active process that occurs within the vessel wall and increases the risk of cardiovascular disease ³. Moreover, in the clinic coronary artery calcification (CAC) is measured by computed tomography (CT) as marker for cardiovascular burden. Calcification present at any vascular site relates to some 3.5-fold increase for CVD events ⁴. Additionally, vascular calcification progression is associated with a 17-fold increase of risk of myocardial infarction ⁵. Therefore, means to measure vascular calcification amount or progression rate, promise to predict accurately patient risks for cardiovascular events.

The current methods to detect and assess calcification aim to visualize precipitated calcium salts in the vasculature such as CT and ¹⁸F-NaF positron emission tomography (PET). Circulating biomarkers reflecting the patient's propensity to develop and aggravate vascular calcification have not been identified so far. In this paper we reason that the circulating biomarker for vascular calcification is not a single plasma component but the collection of all plasma components. As readout of this composite biomarker we selected *in vitro* calcification mediated by vascular smooth muscle cells, which are considered to be the cellular protagonists in vascular calcification. This paper describes the BioHybrid calcification assay, a cell-based assay for measuring the composite biomarker of vascular calcification. We measured the BioHybrid response for plasma samples of chronic kidney disease (CKD) patients, pseudoxanthoma elastica (PXE) patients and CVD patients of whom the Agatston score of CAC was measured. We show that there is a strong and significant correlation between the Agatston score and the BioHybrid calcification readout. We conclude that the BioHybrid calcification assay is an informative strategy to determine the patient's vascular calcification propensity.

Methods

Human vascular smooth muscle cell culturing and characterization

Human vascular smooth muscle cells (hVSMCs) were isolated from non-atherosclerotic aortas of surgical biopsies in accordance with MUMC+ research and diagnostic procedure. In short, human vascular tissue samples were transported in ice-cold serum free M199 medium containing 1% Penicillin/ Streptomycin (PS) and washed in phosphate buffered saline (PBS). Intima, fat and connective tissue was removed before cutting the vascular tissue into small fragments and placing each into a laminin (#L2020, Sigma) coated 6-well plates, with the intima facing down. The pieces were covered with M199 medium containing 20% fetal bovine serum (FBS), 1% PS and 1% Amphotericin B (#15290-026, Gibco) and placed into an incubator. When outgrowing cells reached confluency, they were passaged to laminin coated T25 flasks and tested for mycoplasma. hVSMCs were cultured in M199 medium (Gibco, Bleiswijk, the Netherlands)

supplemented with 20% FBS and 1% penicillin/streptomycin (P/S) and used between passages 5-10 for our experiments.

To characterize cells (supplemental Figure 2), hVSMCs were fixed in 4% paraformaldehyde before blocking in 2% BSA, 0.1% triton in PBS for one hour. Primary antibodies were incubated overnight at 4°C: alpha-Smooth Muscle Actin (α SMA) 1:200 (DAKO, M0851), phosphorylated-Myosin Light Chain (pMLC) 1:200 (Cell Signaling, 3675S), Calponin (CNN1) 1:200 (Abcam, ab46794), Smooth Muscle protein 22-alpha (SM22a) 1:200 (Abcam, ab14106), S100 calcium-binding protein A4 (S100A4) 1:200 (DAKO, A5114). Cells were incubated with secondary antibodies for one hour at room temperature: anti-mouse FITC 1:250 (Hycult Biotech, HP2001), anti-rabbit fluorescein isothiocyanate (FITC) (DAKO, F0205). Before imaging cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for one minute. Imaging was performed on the Cytation 3 system (BioSPX, Abcoude, The Netherlands).

hVSMC mediated calcification

For calcification experiments, hVSMCs were seeded at a density of 1.3×10^4 cells per cm^2 . After 24h, hVSMCs were cultured in calcification medium (M199, 5% human serum/plasma, 1% P/S and 3.6 mM Ca^{2+}) up to 14 days. Plasma samples were further supplemented with Hirudin to prevent clotting of the samples.

Real-time kinetic assay

For real-time calcification measurement, Fetuin A-Alexa 546 (3 $\mu\text{g/ml}$; kindly provided by Prof. Dr. W. Jannen-Dechent, RWTH Aachen, Aachen, Germany) and Hoechst (1 $\mu\text{g/ml}$) were supplemented at the start of the experiment. At various time points after calcification induction, red fluorescent protein (RFP) and DAPI channel (cell count) were imaged up to 14 days. After calcification was detected, cells were imaged regularly to follow calcification progression. Imaging was done with the Cytation 3 system (BioSPX, Abcoude, The Netherlands) and the Gen5 software (BioTek).

End-point assay

For end-point kinetic measurement the O-cresolphthalein method (Randox, London, United Kingdom) was used. The O-cresolphthalein assay was carried out according to manufacturer's instructions, after solubilising mineral deposits in 0.1M HCl.

Patient serum/plasma preparation

Patient serum and plasma samples were obtained from different cohort studies. Patient samples that were included were: CKD5D, CKD 3-5, PXE, high and low CAC score patients, oral anticoagulation (OAC) patients and aortic valve calcification (AVC) patients that received vitamin K1 supplementation. CKD patients are known to be prone to develop vascular calcification⁶. PXE patients are known to have a monogenetic liver disorder of the ATP Binding Cassette Subfamily C Member 6 (ABCC6), which is characterized by dystrophic mineralisation of soft connective tissues and elastic structures in several organs⁷. Additionally, we used samples from individuals enrolled with AVC that were supplemented with vitamin K1 or placebo⁸. Last, serum and plasma of healthy controls was obtained as negative control. This research was approved by the Ethics Committee of the Klinikum Aachen and Sidney Kimmel Medical College at

Thomas Jefferson University, Philadelphia and conducted in accordance with the Declaration of Helsinki.

Dephosphorylated-uncarboxylated matrix Gla protein (dp-ucMGP) measurement

Plasma dp-ucMGP levels were determined using the commercially available IVD CE-marked chemiluminescent InaKtif MGP assay on the IDS-iSYS system (IDS, Boldon, UK). In brief, patient samples and internal calibrators were incubated with magnetic particles coated with murine monoclonal antibodies against dp-MGP, acridinium-labelled murine monoclonal antibodies against ucMGP, and an assay buffer. The magnetic particles were captured using a magnet and washed to remove any unbound analyte. Trigger reagents were added; the resulting light emitted by the acridinium label was directly proportional to the level of dp-ucMGP in the sample. The within-run and total variations of this assay were 0.8–6.2% and 3.0–8.2%, respectively. The assay measuring range was between 300 and 12,000 pmol/L and was linear up to 11,651 pmol/L⁸. All assays were performed by Coagulation Profile laboratories, department of Biochemistry, Maastricht, the Netherlands.

Data analysis and statistics

Real-time calcification development over time was performed by using Gen5 version 2.9 (BioTek). Area of Alexa 546 signal was determined and normalized against cell count. Data are presented as mean $\pm$ standard deviation (SD). Non-parametric Mann-Whitney U test was performed for comparison between two groups. For more than two groups, significance was determined using one-way analysis of variance (ANOVA) with comparison between groups by Dunn's multiple comparison test. For correlation determination a linear regression model and R^2 was determined. Statistical significance was defined as $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

Results

BioHybrid calcification assay

Thus far, measurement of *in vitro* calcification is only possible via end-point assays using alizarin red or O-cresolphthalein quantification. We designed a novel *in vitro* calcification assay using hVSMCs in which calcification development was followed in time by fluorescently labeled Fetuin A. To test the robustness of our live calcification assay we compared the BioHybrid assay with the O-cresolphthalein method, that measures calcification as calcium/protein ratio. We found a strong correlation ($R^2=0.72$) between Fetuin A-Alexa 546 fluorescence per cell (%RFP/ cell count) and O-cresolphthalein ($\mu\text{g Ca}^{2+}/\mu\text{g protein}$; Figure 1A; representative images Figure 1D).

Variability

A great advantage of using Fetuin A-Alexa 546 is that it can be used in real time, measuring calcification progression *in vitro* over time. The use of Fetuin A-Alexa 546 unfolds novel possibilities that can provide a quantifiable readout of calcification during one assay. We tested a variety of both serum and calcium conditions to establish the optimal parameters for our assay. We found that the use of 5% serum was optimal for a robust increase in calcification (Figure 1B). Under 0.5% and 2.5% FBS conditions,

development of calcification was too rapid (Figure 1B) and when using 10% FBS calcification formation was low (data not shown), as was shown previously⁹. Another parameter that is not uniform in calcification assays is the used concentration of calcium to trigger calcification development. All our assays were performed with 3.6 mM of calcium chloride (CaCl₂). Higher concentrations of CaCl₂ (4.5 mM and 5.4 mM) had a much more rapid development of calcification, comparable to lower serum levels (supplemental Figure 1). Hence, we used 3.6 mM CaCl₂ and 5% serum for our BioHybrid assay.

Since we developed the BioHybrid system to test clinical samples, we investigated whether human serum or plasma could allow for calcification development. Comparing FBS with either a pool of healthy control serum/ plasma or the individual serum/ plasma from each of our healthy controls we did not find differences in hVSMC calcification (Figure 1E). This indicates that serum as well as plasma samples can be used to measure calcification with our assay.

Figure 1. BioHybrid calcification assay. (A) Live calcification assay compared to the O-cresolphthalein method. (B) RFP signal over time, to determine the best calcifying conditions. (C) micro calcification can be determined with the Fetuin A-Alexa546 probe. (D) Representative images of the BioHybrid calcification assay at t=1 and t=7 days. 0.5% FBS concentration give rise to a strong positive signal while 5% FBS only shows mild calcification after 7 days. (E) Calcification assay comparing FBS to serum of healthy individuals and a pooled condition. ****p<0.0001

Calcification propensity of CKD5D serum

We measured the calcifying potential of serum of patients diagnosed with CKD5D. After 3 days there was a distinguishable difference between serum of CKD5D patients and healthy controls (Figure 2A and 2B). After 6 days, the CKD5D sera continued to increase *in vitro* calcification whereas the control population maintained lower levels of calcification (Figure 2A and 2B).

Next, we interrogated whether dialysis would have an effect on the calcification propensity of VSMCs. When analyzing the rate of calcification between two time points (72 hours apart), we show that pre-dialysis serum samples induce a significantly increased rate of VSMC calcification, compared to post-dialysis of the same patients (Figure 2C). Further, the BioHybrid system is notably responsive to dialysis after 3 and 6 days (Figures 2E-G). Moreover, serum from 5 out of the 7 patients had a significantly lower induction of calcification following dialysis treatment (Figure 2D). Serum from one patient had no significant difference and intriguingly one other patient showed a significant increase in *in vitro* VSMC calcification after dialysis (Table 1).

Figure 2. Calcification in CKD5D patients. (A) Serum of CKD5D patients tested in the BioHybrid assay. A significant increase of calcification propensity was found at $t=3$ and $t=6$ days. (B) Representative images of the calcification assay, control vs. CKD5D serum. (C) The effect of dialysis on the calcification rate. Serum of patient before dialysis has a significant higher calcification rate as compared to the same patients after dialysis. (D) Calcification increase from $t=3$ to $t=6$ days in the assay. (E) The effect of dialysis on calcification at $t=3$. As significant increase in calcification was found in serum before dialysis. (F) The effect of dialysis on calcification at $t=6$. As significant increase in calcification was found in serum before dialysis. (G) Representative images of the calcification assay of serum before and after dialysis. * $p<0.05$; *** $p<0.001$

Table 1. Calcification response to dialysis. Positive indicates that serum before dialysis has more calcification compared to serum of the same patient after dialysis.

Response to dialysis	Percentage (%)
Positive	71.4
No change	14.3
Negative	14.3

Calcification propensity of CKD 3-5 serum

We compared hVSMC calcification induction of patient serum from CKD stages 3, 4 and 5 against a pool of healthy donors. As seen figure 3A the healthy control had the lowest rate of calcification development per hour. CKD5 showed the highest calcification rate followed by CKD4 and CKD3 respectively ($p=0.022$; CKD5 vs. Ctrl). We did observe a positive trend in calcification rate of CKD patients, which was statistically significant compared to control ($p=0.039$). By day 7, the most significant *in vitro* calcification was observed in CKD5 patients, followed by CKD4, CKD3, and the lowest in healthy controls (Figures 3B and 3C; not significant).

Figure 3. Calcification in CKD stages III-V. (A) Calcification rate in the BioHybrid calcification assay. A significant increase was found in serum of CKDV patient compared to control. (B) Calcification signal at $t=7$. (C) Representative images at $t=7$ days. $*p<0.05$

Calcification propensity of PXE plasma

PXE is a connective tissue disorder that results in elastic tissue mineralization and calcium deposition. PXE patients display reduced anti-mineralization capacity that results in extensive ectopic calcification of the skin, eyes and cardiovascular system. We found that plasma from PXE patients calcified much faster compared to both a healthy control plasma pool and spouse control plasma (Figure 4A and 4B). This difference was found to be significant between the PXE group and spouse controls (Figure 4A; $p < 0.001$).

Figure 4. Calcification in PXE patients. (A) Calcification of plasma of PXE patients compared to their spouses and healthy controls. A significant increase in calcification was found PXE patients compared to their spouses. (B) Representative images of the calcification. *** $p < 0.001$

Calcification propensity of low and high Agatston score serum

Next, we tested whether our BioHybrid system is able to detect differences in *in vitro* calcification of patients with high and low CAC scores. For this we used serum from patients which were high (>1500) or low (<50) in Agatston score. *In vitro* calcification was significantly higher for serum from patients with a high Agatston scoring compared to serum from patients with a low Agatston score (Figure 5A and 5C; $p = 0.002$). A significant correlation was observed between Agatston score and VSMC calcification in our BioHybrid assay. Dividing patients in high and low Agatston score, those with a high Agatston score displayed a significant correlation with *in vitro* calcification (Figure 5B; $R^2 = 0.68$). No such correlation was observed with patients in the low Agatston group (data not shown).

Figure 5. Calcification in low and high Agatston score patients. (A) Calcification of serum from low vs. high Agatston score patients. A significant increase in calcification was found in serum of patients determined to be high compared to low Agatston score. (B) Correlation of Agatston score to calcification in our BioHybrid assay. Only a significant correlation was found in the high Agatston score group ($R^2=0.68$). (C) Representative images of calcification from patients with low vs. high Agatston score. $^{**}p<0.01$

Calcification propensity of OAC plasma

Both patients and healthy volunteers on vitamin K antagonists (VKAs) displayed increased *in vitro* calcification (Figure 6A and 6B) when compared to no anticoagulation use (normalized INR). Additionally, healthy volunteers supplemented with vitamin K2 (MK7) show decreased calcification (Figure 6B). Complementary, patients that were using VKAs had lower turbidity time, while MK7 supplementation increased this (Figure 6C).

Figure 6. Calcification of plasma of OAC patients. (A) Calcification of plasma of a patient on VKA vs. the same patients with a normalized INR. A significant decrease in calcification was found in plasma with a normalized INR. (B) Calcification of plasma from a healthy volunteer taking VKA or MK7 supplementation. A significant increase in calcification was found when taking VKA while a decrease in calcification was found when taking MK7 supplementation. (C) Turbidity time of plasma from a patient taking VKA or MK7 supplementation. A significant decrease in turbidity time was found when taking VKA while an increase in turbidity time was found with MK7 supplementation. $^{**}p<0.01$; $^{***}p<0.001$

Calcification propensity of AVC serum with vitamin K1 treatment

Finally, we interrogated serum samples from AVC patients that were supplemented with vitamin K for one year⁸. After 12 months of supplementation with vitamin K, serum from patients showed reduced *in vitro* calcification (Figure 7A). Conversely, serum from patients given placebo for one year exhibited increased calcification (Figure 7A and 7E; $p=0.06$). Exogenous supplemented vitamin K to serum from patients in the placebo group had no effect on calcification also when compared to patients treated for one year with vitamin K (Figure 7B).

Finally, patient plasma was used to determine the vitamin K status of AVC patients using dp-ucMGP as biomarker (Figure 7C). Dp-ucMGP was significantly decreased in the patients supplemented with vitamin K1 ($p<0.0001$). Comparing the delta change in Agatston score between baseline and one year treatment, no differences in either carotid artery or aortic valve calcification between vitamin K1 or placebo could be observed (Figure 7D).

Figure 7. Calcification of AVC patients. (A) Calcification effect of 1 year Vitamin K supplementation vs. placebo. 12 months vitamin K supplementation decreased calcification while 1 year placebo supplementation increased calcification (not significant). (B) the effect of exogenous Vitamin K supplementation. Addition of exogenous Vitamin K did not decrease calcification as 1 year vitamin K supplementation did. (C) Dp-ucMGP change after one year of supplementation. Dp-ucMGP levels significantly decreased after 1 year of vitamin K supplementation. (D) Changes in Agatston score after one year of supplementation. (E) Representative images of calcification from patients taking Vitamin K or placebo supplementation. **** $p<0.0001$

Discussion

In vivo determination of cardiovascular burden using vascular calcification is challenging, costly, and exposes patients to radiation. Direct measures for assessing calcification are CT or ^{18}F -NaF Positron Emission Tomography (PET). Detection of vascular remodeling is based upon biomechanical properties of the vessel wall such as pulse wave velocity and intima-media thickness. Both *in vivo* measurements are limited to late stages of vascular calcification and are thus a marker for cardiovascular burden. Detecting vascular calcification at an early stage would allow to intervene before vascular disease has been established. Therefore, utilising serum of patients to detect vascular calcification at an early stage would provide a unique clinical tool. Here we demonstrate the *in vitro* use of fluorescently labeled Fetuin A as imaging tool to follow calcification progression real-time and to predict patient susceptibility for developing

calcification. We verified our assay using serum from patients with vascular calcification progression.

The gold standard for measuring *in vitro* calcification, is a singular time-point read, based on a subjective time-point assessment of abundant calcification being present. The two methods that are predominantly used for determining calcification are Alizarin Red staining and O-cresolphthalein. Additionally, the rate in which serum calcifies, dependent on factors present in serum, can be assessed using the T₅₀ assay that measures the propensity of serum to calcify under non-physiologic conditions¹⁰. T₅₀ results have shown to be associated with cardiovascular and all-cause mortality and aortic stiffening in renal patients¹¹⁻¹³. Yet, this assay is mainly useful for serum of CKD patients, and does not take into account the vascular component. All current methods for calcification determination are end-point assays and there is need of a method capable of quantifying calcification rate in real-time. We found a strong and significant correlation between the O-cresolphthalein method and the BioHybrid assay. More specifically, in the early stages of *in vitro* calcification detection, the Fetuin A probe was superior and showed greater sensitivity. End-point assays require multiple replicates to record calcification development over time. With the BioHybrid assay we exploit live-cell imaging, enabling us to follow calcification development of one sample over time. Severe renal artery, aortic calcification and aortic valve calcification is symptomatic in CKD patients¹⁴. Additionally, up to 93% of CKD patients on dialysis display vascular calcification^{15,16}. Vascular calcification is also found in patients that suffer from PXE. PXE is a monogenetic liver disorder characterized by a loss-of-function mutation in the ABCC6 gene. PXE patients display reduced plasma anti-mineralization capacity due to lower Fetuin A and MGP activity. Additionally, they display extensive ectopic calcification which primarily manifests in the skin, the eye and cardiovascular system. PXE is metabolic disorder and thus ideally suitable for our BioHybrid screening^{7,17}.

We found that *in vitro* calcification was increased for both CKD and PXE patient serum/ plasma. CKD5D serum induced extensive calcification after three days, further progressing after six days whereas calcification progression of serum from CKD 3, 4 and 5 patients was significantly slower. This is in line with the less *in vivo* calcification that is found in the clinical situation with the different stages of CKD¹⁸⁻²⁰. Additionally, pre-dialysis serum was shown to increase calcification development, indicative of altered blood components caused by dialysis. However, not every dialysis patient had a similar response in the Biohybrid assay. One patient did not show a pre- and post-dialysis difference, while one patient showed increased calcifying potential post-dialysis. This indicates that not all patients share the same blood components and respond differently in our assay, while they undergo dialysis and are having CKD. In our assay, PXE serum showed increased calcification when compared to spouse controls. Interestingly the constituents of PXE have been already demonstrated to interfere within ordinary elastic fiber deposition²¹.

Finally, the Biohybrid assay enables us to stratify amongst susceptible patients based on their effect of *in vitro* calcification and could aid in the discovery of dialysable or systemic factors that determine *in vivo* calcification.

CAC score is evaluated as the gold-standard clinical predictor for the accumulation and progression of vascular calcification over time^{5,22}. Additionally, CAC score is used to identify high-risk individuals that need immediate medical attention or

intervention²³. Vitamin K supplementation is regarded as one of the solutions to hold the progression of vascular calcification. In particular, in patients that are on VKA treatment²⁴. Indeed we show that patients as well as healthy volunteers have a high calcifying propensity when using VKAs. Additionally, when supplemented with vitamin K2 there is a decreased calcification tendency. This is confirmed by the T50 measurement that determines turbidity time of patient plasma.

Serum from patients with high CAC scores developed more *in vitro* calcification as compared to low CAC score individuals. We further observed a positive correlation of calcification development within the high CAC score group which was non-existing in the low CAC group. Additionally, serum of patients that received vitamin K supplementation for 12 months showed lower calcification development as compared to placebo controls. Interestingly, although all patients' cardiovascular status continued to decline the placebo group had on average a 25% increase in Agatston scoring during this year, whereas the vitamin K group had only 12% increase. These results indicate that the BioHybrid assay can predict vascular calcification development and is sensitive to distinguish highly susceptible patients. Moreover, serum after one-year treatment with vitamin K resulted in lower *in vitro* calcification which associated with lower progression of Agatston score *in vivo*.

Dp-ucMGP is a biomarker that has been shown to associate with vascular calcification^{4,25}. Vitamin K supplementation decreased dp-ucMGP levels in all patients, yet this was not reflected in the development of vascular calcification. We reason that dp-ucMGP only reflects as one biomarker involved in calcification. Thus, dp-ucMGP reflects the current status of the protein in the plasma pool, while the BioHybrid assay employs the whole blood compartment. We hypothesize that the BioHybrid platform can become an assay for clinical risk assessment of vascular calcification progression.

In conclusion, we demonstrate a novel method for detecting vascular calcification *in vitro*. Using a Fetuin A fluorescent probe we have developed a real-time calcification assay that is able to give a quantifiable readout of calcification development over time. We applied our assay to patients with varying calcification levels and demonstrated its application as an indicative readout based on cardiovascular status. Further the sensitivity of this assay has been demonstrated in response to dialysis, vitamin K treatment, as well as both metabolic and non-metabolic disorders that directly affect cardiovascular status. We propose wide scale application of this assay in larger cohorts to further validate application and efficacy of this tool as well as in application as a low-cost, non-invasive cardiovascular diagnostic.

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Chapter 6

Matrix Gla protein is an independent predictor of both intimal and medial vascular calcification in chronic kidney disease

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Abstract

Matrix Gla protein (MGP) is a potent inhibitor of vascular calcification (VC) and requires carboxylation by vitamin K to exert calcification inhibition. Chronic kidney disease (CKD) patients undergo early vascular aging often involving extensive VC. The present cross-sectional study investigated the association between circulating dp-ucMGP levels, MGP expression in vascular tissue and MGP polymorphisms. In 141 CKD stage 5 patients, CAC score was significantly increased in the highest tertile of dp-ucMGP ($p=0.002$), and a high medial VC score was associated with elevated dp-ucMGP levels. MGP vascular expression was associated with increased circulating dp-ucMGP and CAC scores. MGP SNP analysis revealed that patients homozygous for the C allele of the rs1800801 variant had a higher CAC score (median 15 [range 0-1312]) compared to patients carrying a T allele (median 0 [range 0-966] AU). These results indicate that plasma levels of dp-ucMGP are an independent predictor of increased VC in CKD5 patients and correlate with both higher CAC scores and degree of medial calcification. Additionally, high vascular expression of MGP was associated with higher CAC scores and plasma dp-ucMGP levels. Taken together, our results support that MGP is involved in the pathogenesis of VC.

Introduction

Matrix Gla protein (MGP) is a vitamin K dependent protein (VKDP) that is involved in the inhibition of vascular calcification (VC). MGP is small secretory protein (14 kD) that is primarily secreted by vascular smooth muscle cells (VSMCs) in the arterial wall ¹. MGP contains five Glu residues that require carboxylation to become activated and to fulfill its calcification inhibitory function. This carboxylation step cannot take place in the absence of vitamin K, which has an unequivocal role in driving this post-translational step ^{2,3}. Vitamin K is a co-factor for the enzyme γ -glutamyl carboxylase that converts glutamic acid (Glu) into γ -carboxyglutamic acid (Gla) residues ². This conversion is critical for the activation of MGP. Additionally, there are three serine residues that need phosphorylation ^{4,5}. The exact role of phosphorylation of MGP is still not known, but it is believed to play an important role in the regulation of secretion of the protein ³. Upon activation, MGP binds calcium-salts with high affinity, thereby affecting the calcification processes. The importance of MGP in the inhibition of calcification is illustrated by studies of MGP knockout mice, who die within two months after birth due to severe arterial calcification and rupture of the aorta ¹.

Chronic kidney disease (CKD) patients have an extremely high risk for developing vascular disease ⁴. VC, manifested both as medial and intimal calcification with distinct pathologies, is a common risk factor in CKD ⁵. Additionally, vitamin K deficiency is frequently encountered in CKD, which is associated with increased plasma levels of dephosphorylated uncarboxylated MGP (dp-ucMGP) plasma levels ^{6,7}. Furthermore, increased plasma dp-ucMGP levels associate with VC ^{8,9}, cardiovascular morbidity-mortality ¹⁰ and aortic valve calcification ¹¹. However, the connection between local vessel wall expression of MGP (transcription), production (tissue ucMGP) and excretion (plasma dp-ucMGP) is not well explored in CKD. In addition, the vasculature is exposed to an toxic uremic milieu, which has been shown to affect carboxylase activity causing vascular vitamin K deficiency and increased VC ¹². Additionally, uremia promote VC in a rat model ¹³ and induced bone-specific proteins in cultured VSMCs ¹⁴.

The use of dp-ucMGP has clinical potential as a prognostic biomarker of VC ¹⁵ and might provide complementary information to traditional cardiovascular disease (CVD) risk factors ¹⁶. Furthermore, carboxylated MGP bind, via the negative charge, to active calcification. The inactive form of MGP (dp-ucMGP) is set free in the circulation because of lack of negative charge to bind to calcium crystals and, thus, might be used as biomarker to identify high-risk CVD patients, allowing early intervention ¹⁷. Furthermore, although single nucleotide polymorphisms (SNP) of MGP have been associated with outcomes in diabetes and CKD, its association to VC remains obscure. MGP SNP analysis could help us understand the complex nature of MGP expression and regulation. Other VKDPs, such as osteocalcin (OC) share the carboxylation step to become activated. Similar to MGP, uncarboxylated OC (ucOC) is increased in vitamin K deficiency ¹⁸ and has been reported to have a role in the development of VC ¹⁹.

We investigated the potential association between MGP and VC in CKD stage 5 patients undergoing living donor renal transplantation (RTx). We link MGP genetics (SNPs), transcription (mRNA) and protein data (immunohistochemistry and plasma levels) to clinical vascular phenotype (CAC and histology).

Methods

Patients and study design

Adult ESRD patients undergoing living donor (LD) kidney transplantation (tx) at the Department of Transplantation Surgery at Karolinska University Hospital between March 2009 and October 2016 were invited to participate in the study. The etiologies of CKD were chronic glomerulonephritis (n=52), hypertension and renovascular disease (n=8), diabetic nephropathy (n=9) and others or unknown causes (n=72). The cohort included CKD5 non-dialysis (ND) patients (n=51), prevalent peritoneal dialysis (PD) patients (n=39) and prevalent hemodialysis (HD) patients (n=51). PD patients were treated (median vintage time 11.4 months) with different combinations of biocompatible glucose-based or amino acid-based, or, for the long dwell, icodextrin-based solutions. HD-patients were treated by conventional maintenance HD or other dialytic techniques such as hemodiafiltration (median vintage time 14.4 months). Sixteen (11%) out of 141 patients had diabetes. Twenty-three (15%) of the patients had previously been diagnosed with cerebrovascular, cardiovascular, and/or peripheral vascular disease (grouped as CVD). We measured circulating plasma dp-ucMGP in 141 CKD5 patients including 51 non-dialyzed (CKD5-ND) and 90 CKD5 patients treated by either PD (n=39) or HD (n=51). Patient characteristics are shown in Table 1 and a flow chart of patient inclusion is displayed in supplemental figure 1. Age ranged from 19 to 75 years and patients were recruited from March 2009 to October 2016.

Exclusion criterion was unwillingness to participate. Informed consent was obtained from each patient. The Ethics Committee of the Karolinska Institutet approved study protocols. The studies were conducted in adherence to the Declaration of Helsinki.”

Biochemical assessments

Prior to the LD-RTx, fasting blood samples were drawn and stored in -80°C. Biochemical analyses of plasma cholesterol, triglycerides, HDL-cholesterol, hemoglobin, creatinine, calcium, phosphate, albumin (coefficient of variation, CV, 3–4%), were performed at the Clinical Chemical Laboratory of Karolinska University Hospital, Stockholm, Sweden. LDL was calculated using the Friedewald formula: [(total cholesterol) - (high-density lipoprotein cholesterol) - (triglycerides/5)].

Plasma dp-ucMGP

Plasma dp-ucMGP levels were determined using the commercially available IVD CE-marked chemiluminescent InaKtif MGP assay on the IDS-iSYS system (IDS, Boldon, UK). In brief, patient samples and internal calibrators were incubated with magnetic particles coated with murine monoclonal antibodies against dp-MGP, acridinium-labelled murine monoclonal antibodies against ucMGP, and an assay buffer. The magnetic particles were captured using a magnet and washed to remove any unbound analyte. Trigger reagents were added; the resulting light emitted by the acridinium label was directly proportional to the level of dp-ucMGP in the sample. The within-run and total variations of this assay were 0.8–6.2% and 3.0–8.2%, respectively. The assay measuring range was between 300 and 12,000 pmol/L and was linear up to 11,651 pmol/L. Assays were performed in a single run by Coagulation Profile BV, department of Biochemistry, Maastricht, the Netherlands.

Vascular scoring by histology

Inferior epigastric arteries (n=118) were collected from patients within 20 min from the start of kidney transplantation procedure. After fixation (4% phosphate buffered formalin) and paraffin embedding, 1-2 μ m thick tissue sections were stained with hematoxylin, eosin and von Kossa method before evaluation by experienced pathologists. The extent of medial calcification was assessed by a pathologist in vascular biopsies³⁸ and graded as 0 to 3. Patients graded as 0 and 1 represented no/ minimal vascular calcification, and those graded 2 and 3 represented moderate/ extensive vascular calcification.

ucMGP immunohistochemical staining

After deparaffinization and rehydration of inferior epigastric arteries (n=20), immunostaining for ucMGP was performed as described previously³⁵, using a monoclonal MGP antibody directed against uncarboxylated MGP (1mg/mL, 1:400 diluted) and a goat anti-mouse HRP (60 minutes at room temperature; Dako, Glostrup, Denmark) secondary antibody. Detection was performed using Novared stain (Vector Labs, Burlingame, CA), yielding a red color. The degree of ucMGP staining was measured semi-quantitatively and assessed by independent pathologists and graded as 0 to 3. Patients graded as 0 and 1 represented zero to minimal ucMGP staining, and patients graded 2 and 3 represented moderate to extensive ucMGP positivity.

MGP expression

Inferior epigastric artery samples collected at LD-RTx (n=41) were incubated overnight in AllProtect Tissue Reagent (Qiagen, Hilden, Germany) and subsequently stored at -70 °C. RNA was isolated from arteries using TRIzol Reagent and MGP mRNA levels were analysed with TaqMan chemistry (Thermo Fisher Scientific, Waltham, MA USA) as previously described³⁶.

Genotyping of MGP rs4236, rs1800801 and rs1800802

A subset of 117 CKD5 patients with available peripheral blood samples and 389 controls (anonymous blood donors recruited in Stockholm municipality) underwent genotyping. Genomic DNA was extracted following standard procedures at the Karolinska Biobank. Genotypes for rs4236, rs1800801 and rs1800802 polymorphisms were determined with PCR-based allelic discrimination using TaqMan SNP Genotyping Assays, QuantStudio 5 System (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan Genotyper Software according to manufacturer's protocols.

CAC score

Cardiac computed tomography (CT) scans were performed using a 64-channel detector scanner (Lightspeed VCT; General Electric (GE) Healthcare, Milwaukee, WI). CAC scores were expressed in Agatston units³⁷, the protocol and measurements as described previously in detail³⁸. Total CAC score was calculated as the sum of CAC scores in the left main artery, the left anterior descending artery, the left circumflex artery, and the right coronary artery.

Statistical analyses

Continuous data are expressed as median (10th to 90th percentile) and nominal or ordinal data as percentage. Statistical significance was set at the level of $P < 0.05$. Comparisons between groups were assessed with the non-parametric Wilcoxon test/Kruskal-Wallis ANOVA-test for continuous variables and Chi-square test for nominal variables. Non-parametric Spearman rank correlation analysis was used to determine associations between variables. We performed multiple imputation of missing values for multivariate linear regression and multivariate logistic regression using the function PROC MI, with all variables in the covariate section used to produce the values for imputation. The original n for each variable is given throughout. The results for each imputation were generated using PROC REGRESS and LOGISTIC, and then combined using PROC MIANALYZE. We used 20 imputed datasets for this study to ensure that our effect estimates were not overly inaccurate due to Monte Carlo variability. Multivariate linear regression analyses of dp-ucMGP were used and results were shown as standardized β regression coefficients. We performed multinomial logistic regression analysis to examine factors associated for determinants of vascular calcification 0-1 score vs 2-3. Statistical analyses were performed using statistical software SAS version 9.4 (SAS Campus Drive, Cary, NC, USA) and Stata 15.1 (Stata Corporation, College Station, TX, USA)³⁸. Figures were created using GraphPad Prism version 8.3.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

Results

Clinical and biochemical characteristics

Demographic and clinical characteristics are shown in Table 1 and supplemental Table S1. Patients in the highest tertile of dp-ucMGP (>1491 pM) levels were older and had higher CVD prevalence (25%). Moreover, serum creatinine was higher in the highest tertile of dp-ucMGP compared to the other two tertiles (782 $\mu\text{mol/L}$). Additionally, glu-OC (37.8 ng/mL), mid-OC (90.5 ng/mL) and Osteoprotegerin (7.6 pg/mL) were also elevated in the highest tertile of dp-ucMGP.

Table 1. Baseline clinical and biochemical characteristics in 141 CKD 5 patients in relation to tertiles of dp-ucMGP.

	All (n=141)	1 st tertile (n=46)	2 nd tertile (n=47)	3 rd tertile (n=48)	p-value
Age (years)	47 (24, 63)	43 (22, 63)	49 (23, 62)	50 (32, 68)	0.008
Males, (%)	70	65	68	75	0.57
Diabetes mellitus, (%)	11	7	11	17	0.29
Cardiovascular disease, (%)	16	7	15	25	0.04
BMI, (kg/m ²)*	24.5 (20.8, 29.9)	24.0 (19.8, 29.1)	23.5 (20.8, 30.4)	25.1 (21.8, 30.2)	0.17
Systolic BP, (mmHg)*	140 (117, 169)	140 (116, 162)	140 (116, 167)	144 (121, 181)	0.29
Diastolic BP, (mmHg)*	84 (69, 96)	82 (68, 92)	82 (72, 100)	86 (67, 99)	0.33
Hemoglobin, (g/L)‡	114 (99, 132)	111 (98, 129)	118 (99, 135)	113 (99, 132)	0.09
HbA1c, (mmol/mol)†	33 (23, 40)	35 (26, 40)	32 (25, 40)	32 (21, 41)	0.06
Triglycerides, (mmol/L)	1.3 (0.7, 2.4)	1.2 (0.6, 2.2)	1.2 (0.7, 2.6)	1.4 (0.7, 2.6)	0.58
Total cholesterol, (mmol/L)	4.5 (3.1, 6.2)	4.3 (3.0, 6.1)	4.6 (3.2, 6.0)	4.5 (3.0, 6.4)	0.64
HDL cholesterol, (mmol/L)	1.4 (0.9, 2.1)	1.4 (1.0, 2.1)	1.4 (0.9, 2.0)	1.3 (0.8, 2.2)	0.37
Serum Creatinine, ($\mu\text{mol/L}$)	723 (488, 1022)	622 (388, 985)	743 (517, 1018)	782 (531, 1135)	0.01
Serum Albumin (g/L)*	35 (29, 40)	35 (30, 42)	35 (29, 40)	36 (29, 41)	0.80

Uric Acid, (μmol/L)•	369 (254, 522)	413 (285, 557)	350 (239, 510)	357 (247, 469)	0.02
PEW (SGA>1), n (%)**	25	31	26	18	0.33
Vascular calcification biomarkers					
Coronary artery calcium, (AU) ‡‡	8 (0, 1170)	0 (0, 236)	8 (0, 1392)	34 (0, 1948)	0.002
Calcium, (mmol/L)*	2.3 (2.0, 2.5)	2.3 (2.1, 2.4)	2.3 (2.0, 2.6)	2.3 (1.9, 2.6)	0.67
Phosphate, (mmol/L)*	1.6 (1.0, 2.4)	1.7 (1.0, 2.2)	1.6 (1.1, 2.5)	1.7 (0.9, 2.5)	0.96
Glu-OC, (ng/mL)**	14.0 (1.8, 87.6)	7.5 (1.6, 33.7)	15.5 (1.7, 122.6)	37.8 (2.3, 99.2)	0.001
Gla-OC, (ng/mL)**	33.2 (11.7, 97.6)	26.7 (11.0, 73.5)	33.2 (11.7, 138.0)	42.9 (9.7, 102.2)	0.23
Mid-OC, (ng/mL) ¶¶¶	59.5 (13.5, 259.5)	37.9 (11.6, 178.6)	61.8 (11.3, 218.6)	90.5 (17.7, 395.8)	0.02
Osteoprotegerin, (pg/ml)	6.1 (3.8, 12.0)	5.6 (2.8, 10.1)	5.9 (3.9, 11.1)	7.6 (4.2, 14.0)	0.002
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Data presented as median (10th – 90th percentile) or percentage
Abbreviations: CKD, chronic kidney disease; dp-ucMGP, dephosphorylated uncarboxylated Matrix Gla protein; BMI (body mass index); BP, blood pressure; HbA1c, Hemoglobin A1c; HDL, high-density lipoprotein; PEW, protein energy wasting; SGA, subjective global assessment; AU, Agatston unit; Glu-OC, undercarboxylated osteocalcin; Gla-OC, carboxylated osteocalcin; mid-OC, n-mid osteocalcin *n= 139; **n= 137; ***n= 109; ‡n= 120; ‡‡n= 110; ‡‡‡n= 103; †n= 121; ††n= 99; †††n= 68; ¶n= 106; ¶¶n= 138; ¶¶¶n= 91; •n= 128; ••n= 140; •••n= 114

dp-ucMGP levels were associated with increased CAC and medial calcification score

Patients in the 3rd tertile showed higher CAC score compared to the other two tertiles ranging from a CAC score with a median (10th – 90th percentile) of 0 (0, 236) in the 1st 8 (8, 1392) in the 2nd and 34 (0, 1948) in the 3rd dp-ucMGP tertile (p=0.002) (Figure 1a). Medial VC (scored by pathologists, ranging from 0 - no, 1 – mild, 2 – moderate, to 3 - severe calcification) was associated with an increase in dp-ucMGP levels (median 1193 in group 0-1 and 1458 in group 2-3) (p<0.05) (Figure 1b).

Figure 1. dp-ucMGP levels associate to CAC score and vascular media calcification score. (a) CAC score measured in 110 CKD5 patients in relation to tertiles of dp-ucMGP levels. **(b)** dp-ucMGP levels of 128 CKD5 patients displayed against low (score 0+1) and high (score 2+3) media VC.

Calcification of the vascular media co-localized with ucMGP

Patients that had a medial VC score of 0 showed no staining for ucMGP (Figure 2a). On the contrary, patients that had a medial VC score of 3 showed a strong staining of ucMGP around calcified areas (Figure 2b). Calcified areas are accompanied by a loss of structure and show no cellular components, the arrows indicate calcified areas that stain highly positive for ucMGP (Figure 2b).

Figure 2. Immunohistochemical staining of ucMGP in epigastric artery from CKD5 patients. **(a)** shows ucMGP staining of epigastric artery of a patient with a medial VC score of 0 (left, 4x; right, 20x). **(b)** shows ucMGP staining of an epigastric artery with a medial VC score of 3 (left, 4x; right, 20x).

Multivariate analysis of factors associated with dp-ucMGP

In multivariate linear regression analysis of determinants of dp-ucMGP were bone markers i.e. 1-SD increase of ucOC (est 0.48; $p=0.001$) and OPG (est 0.28; $p=0.02$) after adjusting for 1-SD increase of age and albumin (Table 2).

Table 2. Multiple linear regression models for 1-standard deviation increase of plasma dp-ucMGP in 141 CKD 5 patients.

	Model (coefficient +SE, p-value)
1-SD increase of age, years	0.05+0.12 (0.69)
1-SD increase of ucOC, ng/ml	0.48+0.14 (0.001)
1-SD increase of albumin, g/L	0.13+0.12 (0.27)
1-SD increase of Mid-OC, ng/ml	-0.21+0.15 (0.16)
1-SD increase of osteoprotegerin, pg/ml	0.28+0.12 (0.02)

Abbreviations; 1-SD, one standard deviation; dp-ucMGP, dephosphorylated uncarboxylated Matrix Gla protein; CKD, chronic kidney disease; SE, standard error; ucOC, undercarboxylated osteocalcin; mid-OC, n-mid osteocalcin

Multivariate analysis of factors associated with medial calcification

In a multivariate logistic regression analysis of factors associated with high medial VC (Table 3) (VC score media (0-1) = reference), high medial VC was associated with age ($p=0.006$), sex ($p=0.001$), dp-ucMGP ($p=0.04$) and DM ($p=0.008$) after adjusting for hsCRP and CAC score ($p=0.059$) (Table 3).

Table 3. Multinomial logistic regression analysis of factors associated with high calcification score media (2+3) (n=122, pseudo $r^2=0.22$). Patients with calcification score media 0+1 served as the reference.

	Odds ratio (95%CI)	p-value
1-SD increase of age, years	2.3 (1.3-4.1)	0.006
1-SD increase of dp-ucMGP, pmol/L	1.6 (1.0-2.5)	0.04
Gender (female, male)	7.3 (2.2-25.1)	0.001
1-SD increase of hsCRP, mg/L	1.3 (0.4-1.3)	0.31
Diabetes mellitus (yes, no)	26.0 (2.3-293.2)	0.008
1-SD increase of CAC-score, AU	1.2 (0.5-2.9)	0.59

Abbreviations: 95%CI, 95% confidence interval; 1- SD, one standard deviation; dp-ucMGP, dephosphorylated uncarboxylated Matrix Gla protein; hsCRP, high-sensitivity C-reactive protein; CAC, coronary artery calcium; AU, Agatston unit

High arterial MGP mRNA expression associates with high CAC

We divided patients according to median of MGP mRNA expression. High expression of MGP (n=41) showed higher CAC score with a median (10th – 90th percentile) of 16 (0 - 1804), compared to the low MGP expression group with a median (10th – 90th percentile) of 0 (0 - 255) (p=0.036) (Figure 3a). Additionally, high expression of MGP showed higher dp-ucMGP plasma levels, with a median (10th - 90th percentile) of 1459 (711 - 5313), compared to low MGP expression with a median (10th - 90th percentile) of 1100 (760 - 1653) (p=0.034) (Figure 3b). In a Spearman’s rank (rho) analyses, MGP expression was significantly correlated with CAC score (Rho=0.39) and medial calcification score (Rho=0.37). After adjusting for age and sex in a multivariate linear regression analysis, these significant correlations were lost.

Figure 3. Association between MGP expression and CAC and dp-ucMGP. (a) MGP expression measured in 41 CKD5 patients in relation to CAC score. (b) MGP expression measured in 41 CKD5D patients in relation to dp-ucMGP plasma levels.

MGP polymorphisms - relation to circulating dp-ucMGP, arterial MGP mRNA and VC

Distributions of MGP rs4236, rs1800801, rs1800802 polymorphisms in CKD5 patients and controls are presented in Supplemental Table S2. The genotype frequencies of the rs4236 and rs1800801, but not rs1800802, differed significantly between patients and controls: in patients, the rs4236 T/T and rs1800801 C/C genotypes were more common (44% vs. 39% and 53% vs. 44%, respectively), whereas rs4236 C/C and rs1800801 T/T were less frequent (both 6% vs. 15%) compared to controls. A selection of patient characteristics was investigated in relation to MGP genotypes (Supplemental Table S3). None of the MGP SNPs were associated with age, sex, DM or dp-ucMGP levels. Neither the rs1800801 nor the rs1800802 SNPs associated with MGP mRNA levels but when dividing patients according to rs4236 T-homozygotes vs. C-allele carriers, MGP mRNA levels were found to be higher in patients homozygous for the T allele compared to patients with CC and CT genotype (1.3 vs. 0.9 RQ, $p=0.03$). Whereas patient's CAC scores were not associated with MGP genotypes, media VC score differed significantly according to rs1800801 genotype, with 50% of C homozygotes having a moderate to extensive calcification (i.e. score 2 or 3) compared to 24% and 14%, respectively, in patients with C/T and T/T genotypes ($p=0.01$) (Supplemental Table S3). When comparing C-allele homozygotes to T-allele carriers in relation to either none/minimal calcification (score 0-1) or moderate/extensive calcification (score 2-3) the difference became even more significant (50% vs. 22%, $n=109$; $p=0.005$). Similarly, patients that were homozygous for the rs4236 T-allele had more often moderate-extensive medial VC than patients carrying a C-allele (49% vs. 27%, $p=0.02$).

Discussion

We examined the role of MGP as a potential biomarker for the extent of VC in CKD5 patients and confirmed that an increase in plasma dp-ucMGP levels was significantly associated with an increase in both CAC score and medial arterial VC. CKD patients are predisposed to VC²⁰, an independent risk factor for CVD morbidity and mortality^{4,21}. CAC score determines the extent of CAC and is correlated to VC at other places in the arterial tree²²⁻²⁴. However, as CT of the heart involves radiation and its value to determine microcalcifications is limited, circulating biomarkers that reflect early VC would be cheaper, safer and less invasive. Additionally, to interfere with VC progression, biomarkers that predict initiation phases of VC, such as MGP are of value in prediction and to follow treatment of VC.

MGP is a potent inhibitor of VC produced by VSMCs²⁵, which are pivotal in maintaining MGP homeostasis, as they are involved in both the production and activation of MGP, both of which are important for the subsequent inhibition of VC^{21,25}. Since MGP is an active inhibitor of VC it could be a potential useful biomarker. Indeed, circulating plasma levels of dp-ucMGP correlate with amount of VC^{22,23} and arterial stiffness²⁶. In accordance, we report that patients with the highest dp-ucMGP levels had a higher CAC score and more extensive medial VC.

CKD patients suffer from vitamin K deficiency and are prone to develop VC²⁴. Since vitamin K is necessary for the activation (carboxylation) of MGP in the vessel wall, MGP will be produced in the inactive form when levels of vitamin K are low²⁵. We show that next to circulating levels of dp-ucMGP, highly calcified epigastric arteries also show co-localization with ucMGP. This data supports the hypothesis that circulating

dp-ucMGP reflects local tissue levels of ucMGP which might be caused by a vascular vitamin K deficiency, a risk factor for VC. Although vitamin K intake or plasma levels were not measured in the present study, our findings indirectly imply that vitamin K status is low since also ucOC was significantly increased²⁷. After correction for age and albumin, dp-ucMGP remained significant for bone markers. Moreover, after correction for CAC score and inflammation, dp-ucMGP remained significant for medial calcification score.

In this study, high MGP expression was associated with a CAC score and increased medial calcification (not significant) scored by pathologists. High expression of MGP infers to a high local production of MGP. However, nothing is known about the activation state of this locally produced MGP. As CKD patients are vitamin K deficient²⁴, we hypothesize that most of the MGP is present as inactive MGP. This accord with our results showing a strong association between MGP expression and circulating dp-ucMGP. *In vitro*, low expression of MGP was associated with calcification via upregulation of BMP-2²⁸. Additionally, under specific pro-calcific conditions, such as a high calcium concentration, calcification is increased whereas MGP expression is decreased²⁹. This is contradictory to our results but might be explained by the carboxylation efficiency of MGP. Total MGP in the plasma is the sum of both active (cMGP) and inactive (ucMGP) MGP; thus, high levels of dp-ucMGP do not reflect total level of MGP that is expressed in the tissues. The importance of MGP as inhibitor of VC is demonstrated by MGP *ko* studies in mice that showed increased soft tissue calcification³⁰. Moreover, MGP^{-/-} mice express higher levels of osteogenic proteins, such as osteopontin, OC and Cbfa1³¹. Due to the complex nature of MGP, further studies need to examine the precise relation between local vasculature MGP expression and circulating MGP.

Besides measurement of plasma dp-ucMGP, we genotyped a subgroup of patients for three well-known SNPs (rs4236, rs1800801 and rs1800802) in the MGP gene. In comparison to healthy controls, our subgroup of CKD patients differed in respect to two of the SNPs, rs4236 and rs1800801, with patients displaying clearly lower frequencies of the minor alleles C and T, respectively. As none of the SNPs were associated with plasma dp-ucMGP levels, the biological impact is unclear. However, because the MGP rs1800801 T-allele is associated with lower medial VC score, this allele may be protective. The literature is, however, conflicting regarding the potential protective effect of the T-allele on vascular disease. A meta-analysis by Sheng et al³² showed that the MGP T-allele was associated with a higher risk for VC whereas Taylor et al.³³ found no association between the MGP rs1800801 and CAC score. An explanation for the conflicting evidence might be the differences in ethnic composition between the study populations and limited power.

The present results should be considered in light of some strengths and limitations. The relatively low sample size restricts power of the multivariate analyses and information retrieved from the MGP gene variants. Moreover, the observational nature of the study restricts causal conclusions. Furthermore, results obtained in this study are retrieved from different patient cohorts which means that our study population is heterogenous, for example displaying a population aged from 19-75. Since dp-ucMGP only represents a minor fraction of the total ucMGP content it should be interpreted with care¹⁷. CAC determination by cardiac CT cannot distinguish between medial or intimal calcification. However, we did find a strong association between medial VC in the

epigastric artery and CAC. Finally, expression levels of MGP were only measured in limited number of patients and further studies are needed to examine the role of MGP expression in VC. For the MGP gene variants we need to perform additional in vitro and in vivo experiments to assess its causal role.

As increased dp-ucMGP levels associate with increased CAC and medial VC our results support that dp-ucMGP is an independent predictor of VC and a risk factor for arterial stiffness and cardiovascular mortality³⁴. Alterations in plasma dp-ucMGP correlate with local tissue expression of ucMGP around areas of VC, low general MGP expression and genomic SNP analysis. Although our data indicates a predictive value of MGP as a biomarker for VC, further studies need to confirm whether these finding translate into CVD morbidity and mortality. Additionally, discovering the precise regulation of MGP in VC may provide novel therapeutic approaches with a potential role for vitamin K supplementation.

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Supplemental material

Flow chart of study

Supplemental Figure S1. Flow chart of patient inclusion.

Supplemental Table S1. Other baseline clinical and biochemical characteristics in 141 CKD 5 patients in relation to tertiles of dp-ucMGP.

	All (n=141)	1 st tertile (n=46)	2 nd tertile (n=47)	3 rd tertile (n=48)	p-value
iPTH, (ng/L)	261 (83, 558)	255 (81, 747)	270 (84, 502)	290 (49, 563)	0.72
1,25-OH vitamin, (mmol/L) ††	18 (9, 32)	18 (11, 35)	18 (9, 33)	17 (7, 32)	0.89
25(OH) D-vitamin, (mmol/L) †††	35 (17, 72)	33 (16, 75)	38 (12, 69)	36 (21, 73)	0.64
hsCRP, (mg/L) ••	0.7 (0.2, 4.0)	0.5 (0.2, 2.5)	0.7 (0.2, 3.9)	1.5 (0.3, 10.7)	<0.001
IL-6, (pg/mL) ***	1.3 (0.1, 4.1)	0.9 (0.01, 3.0)	1.6 (0.3, 3.4)	1.4 (0.02, 6.6)	0.06
TNF, (pg/mL) ††††	10.5 (7.6, 16.5)	8.9 (7.3, 12.3)	10.7 (7.3, 16.1)	12.6 (9.1, 20.4)	<0.001
CTX, (ng/ml) •	1.8 (0.5, 5.1)	1.8 (0.4, 4.9)	1.8 (0.6, 5.5)	1.9 (0.5, 4.4)	0.88
FGF-19, (pg/ml) *	117 (41, 331)	133 (39, 439)	86 (35, 263)	135 (53, 343)	0.02
FGF-23, ††† (pg/ml)	4055 (676, 48027)	3668 (350, 32935)	7581 (826, 44887)	6391 (624, 99267)	0.40
Klotho, (pg/ml) ••	389 (213, 669)	366 (188, 763)	382 (212, 671)	414 (257, 585)	0.66
ALP, (U/L)	65.9 (39.8, 118.3)	69.7 (41.7, 118.2)	65.1 (37.4, 122.9)	65.5 (39.4, 121.5)	0.95
BALP, (ug/L)	15,8 (8.6, 41.5)	16.1 (8.9, 40.7)	14.7 (8.6, 41.1)	17.2 (8.3, 43.4)	0.84
Sclerostin, (pg/ml) **	432 (245, 830)	409 (215, 802)	440 (244, 807)	431 (259, 897)	0.55
Medication					
Statins, (%)	37	30	36	44	0.41
β-blockers, (%)	58	44	62	69	0.04
Calcium blocker, (%)	51	54	55	44	0.46
Phosphate binders, (%)	53	54	53	52	0.98

Data presented as median (10th – 90th percentile) or percentage

MGP is an independent predictor of vascular calcification in CKD

Supplemental Table S2. Genotype distributions of *MGP* polymorphisms rs4236, rs1800801 and rs1800802 in 117 CKD5 patients and 389 controls.

		CKD5 patients	Controls	P-value*
MGP rs4236, n (%)	T/T	51 (44)	152 (39)	0.03
	T/C	59 (50)	177 (46)	
	C/C	7 (6)	60 (15)	
MGP rs1800801, n (%)	C/C	62 (53)	171 (44)	0.03
	C/T	48 (41)	161 (41)	
	T/T	7 (6)	57 (15)	
MGP rs1800802, n (%)	A/A	69 (59)	232 (59)	0.89
	A/G	42 (36)	145 (37)	
	G/G	6 (5)	16 (4)	

*Pearson’s Chi-Square test

Supplemental Table S3. Distribution of patient characteristics in relation to *MGP* genotypes in 117 CKD5 patients.

<i>MGP</i> genotypes				P-value
rs4236				
	T/T	T/C	C/C	
Age (years)	47 (24-64)	45 (23-62)	39 (28-66)	0.58
Males (%)	76	63	43	0.11
DM (%)	10	8	14	0.88
dp-ucMGP (pmol/L)	1333 (745-2218)	1193 (661-2526)	1329 (799-1998)	0.65
<i>MGP</i> mRNA (RQ) [‡]	1.2 (0.3-3.4)	0.9 (0.4-1.7)	0.61 (0.5-0.8)	0.15
CAC score (AU) [#]	37 (0-1729)	1 (0-1068)	0 (0-12)	0.09
Media VC score, (%) [□]	0 11	18	29	0.30
	1 40	54	57	
	2 31	20	0	
	3 18	9	14	
rs1800801				
	C/C	C/T	T/T	
Age (years)	47 (25-63)	45 (23-62)	41 (29-63)	0.65
Males (%)	74	63	43	0.15
DM (%)	10	8	14	0.88
dp-ucMGP (pmol/L)	1315 (755-2737)	1191 (650-2478)	1329 (799-1998)	0.57
<i>MGP</i> mRNA (RQ) [‡]	1.2 (0.4-3.1)	0.9 (0.4-1.8)	0.6 (0.5-0.8)	0.16
CAC score (AU) [#]	15 (0-1312)	0 (0-990)	0 (0-12)	0.06
Media VC score, (%) [□]	0 15	15	29	0.05
	1 35	62	57	
	2 33	15	0	
	3 17	9	14	
rs1800802				
	A/A	A/G	G/G	
Age (years)	45 (23-62)	47 (25-64)	49 (36-60)	0.59
Males (%)	70	64	70	0.85
DM (%)	9	12	0	0.62
dp-ucMGP (pmol/L)	1323 (766-2526)	1232 (654-2016)	1121 (848-1884)	0.78
<i>MGP</i> mRNA (RQ) [‡]	1.0 (0.5-2.1)	1.3 (0.3-2.0)	1.3 (0.9-1.6)	0.88
CAC score (AU) [#]	0 (0-1061)	12 (0-1021)	15 (14-1710)	0.27
Media VC score, (%) [□]	0 17	16	0	0.35
	1 54	39	40	
	2 15	34	40	
	3 14	10	20	

Data presented as median (range of 10th–90th percentile) or percentage. Differences between genotype groups were evaluated with Kruskal-Wallis and Pearson’s Chi-square tests. ^{*}n=41; [#]n=91; [□]n=108

Chapter 7

General discussion

General discussion

The vessel wall is continuously exposed to stress, resulting in vascular remodeling. Arterial remodeling involves numerous molecular pathways regulating vascular repair. During long-term stress the vessel wall cannot cope with this resulting in structural and biological changes that modify vessel wall characteristics. This pathological adaptation eventually results in vascular stiffness. One major contributor of arterial remodeling is the development of vascular calcification. Vascular calcification has long been neglected and was considered clinically irrelevant. However, it is nowadays appreciated that vascular calcification is a highly active and regulated process, with a major role of vascular smooth muscle cells (VSMCs).

In this thesis I studied the role of arterial remodeling processes and the downstream development of vascular stiffness and calcification. Additionally, I studied the interaction between arterial medial remodeling and the initiation and progression of atherosclerosis. In particular, I focused on the role of VSMCs in the process of vascular remodeling and calcification.

In chapter 2, I provide an extensive review on the role of VSMCs in arterial remodeling with a focus on calcification-related processes. In chapters 3 and 4 we report on *in vivo* mouse models and employed these to study the effect of medial remodeling on the development of atherosclerosis. In chapter 3 we investigated the effect of medial calcification on the development of atherosclerosis. In chapter 4 we studied the role of medial VSMC apoptosis on the development of atherosclerosis. In Chapter 5 I describe a novel BioHybrid *in vitro* approach to study the cardiovascular risk profile of patient plasma by using human primary VSMCs as biosensor for calcification. In chapter 6 we studied the association between vitamin K-dependent matrix Gla protein (MGP) and vascular calcification, and additionally the role of MGP as a biomarker.

The key findings of my thesis are:

- (I) VKA induced medial calcification accelerates atherosclerosis development (chapter 3)
- (II) VKAs induce extensive arterial remodeling in the medial layer of the vessel
- (III) Apoptosis of medial VSMCs aggravate atherosclerosis and induces features of plaque instability (chapter 4)
- (IV) The BioHybrid is a novel VSMC-based system using serum of patients to study their cardiovascular risk profile (chapter 5)
- (V) Vitamin K-dependent MGP is an independent predictor of vascular calcification in patients with end stage renal disease (chapter 6)

VKA induced development of medial calcification

In my thesis I focus on mechanisms that lead to arterial remodeling. Vascular calcification is considered a late-stage result of arterial remodeling. It has been reported that VKA use is associated with increased vascular calcification ¹⁻³. Isolated medial calcification is predominantly found in CKD ⁴, Mönckeberg Sclerosis ⁵, breast calcification ⁶ and calciphylaxis patients ⁷ but often coincides with atherosclerosis. Additionally, patients susceptible for the development of medial calcification, such as chronic kidney disease and diabetes patients, die regularly from cardiovascular disease (CVD) ^{8,9}. Indeed, some 80 years ago it has been reported that medial calcification or degeneration precedes atherosclerosis ¹⁰.

Germline genetically engineered animals such as LDLr^{-/-} and Apoe^{-/-} mice, are preferred to study atherosclerosis^{11,12}. DBA/2 mice, that are susceptible for the development of ectopic calcification, do not develop atherosclerosis¹³. We designed a novel experimental animal model that develops VKA induced medial vascular calcification¹⁴ with subsequent induction of atherosclerosis by introducing LDL receptor knock-down. We employed the gain-of-function proprotein convertase subtilisin/kexin type 9 (PCSK9), that binds hepatic low-density lipoprotein (LDL) receptors directing them to the lysosomes for degradation¹⁵. Employment of PCSK9 gain of function in DBA/2 mice with medial calcification raised lipid levels to that of LDLr^{-/-} and Apoe^{-/-} mice. Additionally, plaque development was found in the aorta and side-branches. We found that VKA use induces arterial medial remodeling and calcification which results in increased atherosclerosis and intimal calcification. Currently, the model is employed to unravel the mechanisms by which VSMCs are involved in medial remodeling and have an impact on atherosclerosis.

VSMC apoptosis aggravates atherosclerosis progression and induces features of plaque instability

Maintaining vessel homeostasis involves a balance between proliferation and apoptosis of VSMCs. After vessel injury VSMC apoptosis occurs, followed by increased proliferation. The fact that after vessel injury normal medial cellularity is achieved, indicates that VSMC apoptosis triggers repopulation of the vessel¹⁶. Additionally, vascular diseases such as aneurysm¹⁷ and atherosclerosis¹⁸ are associated with increased VSMC apoptosis, resulting in inflammation, endothelial damage and blood flow oscillations. My work confirms that loss of medial VSMCs results in larger atherosclerotic plaques with features of a more vulnerable phenotype. Our observations extend previous findings demonstrating that the induction of VSMC apoptosis in established atherosclerosis increased both plaque size and features of plaque vulnerability^{19,20}. An explanation for the increase in plaque size is the observed thinner fibrous cap, which facilitates increased leukocyte infiltration, plaque inflammation and necrotic core formation. The fibrous cap consists of VSMCs that stabilize the atherosclerotic plaque. Because of medial VSMC apoptosis, it is plausible that migration of medial VSMCs towards the fibrous cap is impaired. Our data are in line with data from Clarke and coworkers who showed that intimal VSMC apoptosis resulted in thinning of the fibrous cap with subsequent necrotic core enlargement. A thin fibrous cap is characteristic of advanced human atherosclerosis, and plaques with thin fibrous caps are associated with increased risk on rupture²¹. Additionally, VSMCs have efficient efferocytosis capacity that can contribute to clearance of debris in atherosclerotic plaques²². The decline in efferocytosis capacity combined with the lower number of VSMCs might further accelerate atherosclerotic plaque growth.

We also found that extensive medial VSMC apoptosis did not affect vessel contractility. This finding suggests that the remaining VSMCs become hypertrophic and compensate for VSMC loss and maintain vessel wall contractility. Indeed, VSMC contractility markers were increased in animals with increased VSMC apoptosis. Vessel wall distensibility however, was compromised and vessel dilation was reduced compared to healthy vessels. These effects likely caused by a loss of endothelial derived hyperpolarizing factors. The exact mechanism by which this decrease in vessel wall

dilation is caused is not fully understood but might arise from the impaired cellular connections in gap junctions between endothelial cells and VSMCs ²³.

Development of the BioHybrid system – using *in vitro* VSMCs to measure cardiovascular risk

To predict risk for cardiovascular disease, circulating biomarkers for vascular calcification are being used. However, this circulating compartment comprises not a single component but the collection of all circulating components. Therefore, we developed an assay that enables us to measure the cardiovascular risk profile of whole patient serum using VSMCs *in vitro* as detection system. The BioHybrid system is able to measure *in vitro* VSMC calcification using serum of patients prone to develop vascular calcification. Current *in vitro* VSMC models for determining calcification are end-point assays that measure calcification at a subjectively chosen time-point. Our BioHybrid assay provides a reproducible, sensitive and human VSMC-based calcification assay that can be monitored in time, and measures the effect of circulating components on the vascular system. Current clinical determination of vascular calcification includes CT and PET imaging while remodeling, a feature of vascular calcification, can be measured via PWV and IMT. All techniques are limited to late stages of vascular calcification development. Thus, we aim for applying the Biohybrid screening system as a method to detect early stages of vascular calcification. Several patient populations, including CKD ²⁴, PXE ²⁵ and diabetes patients ⁸ are known to be prone to develop vascular calcification. In our Biohybrid system we tested serum from CKD and PXE patients and demonstrated its applicability as an indicative readout based on the cardiovascular status of these patients. Indeed, most patient serum/ plasma samples show a large responsiveness in our platform. Patients that display vascular calcification are largely heterogenous in nature which is reflected in our Biohybrid platform. Not all patients measured respond in a similar manner, opposite responsiveness in the Biohybrid system is a powerful tool to screen for factors that determine the calcifying potential and enables us to stratify patients in better way. Furthermore, the sensitivity of the assay has been demonstrated in response to dialysis, vitamin K treatment and both metabolic and genetic disorders.

The role of vitamin K-metabolism on arterial remodeling in the clinical situation

Clinical assessment of arterial remodeling processes is challenging. Assessment tools include measurements that determine vessel characteristics such as pulse wave velocity, distensibility and intima-media thickness, and imaging modalities like computed tomography (CT) and positron emission tomography (PET). Additionally, biomarkers that can predict arterial remodeling or vascular calcification are scarce and not specific. Recently, cellular senescence (measured as CDK2A/p16^{INK4a} by immunohistochemistry and mRNA expression) has been suggested as a marker for biological ageing ²⁶. CKD patients are characterised by accelerated ageing expressed as progressive vascular disease with increased vascular calcification. In CKD the arterial wall appears older than its expected age due to mechanisms that lead to premature ageing, such as inflammation, oxidative stress and cellular senescence. Indeed, high levels of senescence were found in CKD patients and were associated with vascular calcification ²⁷. Several biomarkers for vascular calcification have been reported so far and sclerostin ^{28,29}, Fetuin-A ³⁰ and MGP ³¹ are amongst the most studied. Additionally, in the clinic distinction between medial and intimal calcification is not yet possible. In

this thesis we focused on MGP as it is the only factor that can be modified activity wise. We found that the inactive form, dephosphorylated uncarboxylated MGP (dp-ucMGP), was an independent predictor of both intimal and medial vascular calcification in CKD patients. In accordance with our results, dp-ucMGP has been found to correlate with the amount of vascular calcification³²⁻³⁴. Therewith, we found increased local ucMGP tissue and mRNA MGP expression that associates with both the plasma dp-ucMGP as well as CAC levels. We are the first to show that MGP is an important player in the development of vascular calcification. Not only does the plasma dp-ucMGP level associate with medial and intimal calcification we also found increased gene and local tissue expression. Most literature reports only dp-ucMGP levels to be associated with vascular calcification. We believe MGP to be a potent inhibitor of vascular calcification and a promising target to modulate vascular calcification.

One of the strongest and accepted risk-factors for CVD development are increased blood lipid levels. Statins have proven to be effective in lowering LDL cholesterol and have shown to reduce CVD incidence and progression among different risk categories, in men and woman, and younger and older individuals^{35,36}. However, statin use has also been associated with the development of vascular calcification³⁷⁻³⁹. Statins inhibit the enzymatic activity of UBIAD1, an enzyme important for vitamin K synthesis⁴⁰. Therefore, it is tempting to speculate that patients treated with statins are prone to develop vitamin K deficiency. Indeed, CKD patients have been shown to be severely vitamin K deficient⁴¹. MGP requires vitamin K as cofactor to be posttranslationally activated, and increased dp-ucMGP levels reflect vitamin K-deficiency⁴². We hypothesize that statin use accelerates vascular calcification via inducing vitamin K-deficiency, thereby increasing dp-ucMGP levels. Indeed, literature has shown that in CKD5D patients, statin use was associated with CAC progression and that statins blocked vitamin K synthesis *in vitro*. Moreover, dp-ucMGP plasma levels were associated with increased CAC score and medial vascular calcification⁴³.

Concluding remarks and future perspectives

In my thesis I discussed arterial remodeling from a basic, translational and clinical research point of view. Today, CVD treatment is based on resolving symptoms rather than preventing disease development. This is likely caused by late clinical diagnose which can only detect severe stages of malformations. In this thesis, I discussed the importance of arterial remodeling in relation to development of CVD. In chapters 3 and 4 we report on novel animal models representing arterial medial remodeling, i.e. vascular calcification and apoptosis. These models aid the understanding how vascular remodeling affects atherogenesis thereby resulting in possibly CVD-related processes at an earlier stage, even before CVD is detected. Our work puts forward a key role for VSMC phenotypic switching, which plays a pivotal role in vessel repair. Our work revealed that VSMC apoptosis resulted in phenotype change of remaining VSMCs, increasing expression of contractile markers and becoming hypertrophic. This compensatory mechanism resulted in increased vessel contractility. Additionally, my data indicate a strong correlation between vascular calcification and development and progression of atherosclerosis. Vascular medial remodeling especially affects extracellular matrix (ECM) content with debranching, thinning and breaks of elastic fibers. In chapter 5, I describe the generation of a novel method to assess the cardiovascular risk profile using the *in vitro* BioHybrid assay. The BioHybrid assay is

used to measure *in vitro* calcification that reflects *in vivo* vascular calcification. Our data point towards using the whole circulating compartment, rather than a singular biomarker, to assess the cardiovascular risk profile. Moreover, the Biohybrid platform can be extended incorporating multiple assays and cell-reporters that can provide a risk profile for CVD and beyond. Furthermore, the Biohybrid assay may have the potential to be a prediction tool for detecting vulnerable patients. Although we provide data pointing towards using the whole serum compartment for *in vitro* research, single biomarker assessment is still essential and can be used to reflect deficiencies. As such, in chapter 6 the use of dp-ucMGP as biomarker for vitamin K-status to predict vascular calcification was described. Using MGP as an imaging tool is appealing as it potentially demonstrates processes leading to calcification well before the calcification can be detected. As such, MGP may provide an unique imaging tool that increases resolution and interpretation of vascular calcification. Taken together, the data in my thesis show that arterial remodeling is key in the development of vascular calcification and atherosclerosis and that early detection and intervention is necessary to treat CVD.

Concluding figure. Overview of the chapters in this thesis. Chapter 1 is the general introduction. General mechanisms of arterial remodeling are described in Chapter 2. Briefly, arterial remodeling is initiated by VSMCs, the cell type that is most abundant in the vessel wall. VSMCs display a contractile phenotype to maintain vessel homeostasis. Upon stress signals, VSMCs change towards a synthetic phenotype to repair damage inflicted. Long-term damage leads to pathology development such as atherosclerosis, arterial stiffness and vascular calcification. Clinically, arterial remodeling cannot be measured accurately. Measuring vascular calcification currently includes pulse wave velocity (indirect), computation tomography and positron emission tomography using ^{18}F -NaF. Patients that display vascular calcification are often treated with Vitamin K antagonists (VKAs) that are involved in anticoagulation. We found that VKA use (Chapter 6) increases vascular calcification and the inactive form of matrix Gla protein. To study arterial remodeling processes more detailed we developed two animal models that focus on VSMC phenotypic switching. In Chapter 3 we induce VSMC-specific apoptosis and study its effect on atherogenesis. Chapter 4 describes a model in which we induce vascular calcification in the medial layer using VKAs. Last, we aimed to develop an assay able to measure a patients' propensity to calcify (Chapter 5). We validated this new method in highly susceptible patient populations.

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Chapter 8

Summary

In this thesis the main focus was to investigate the role of vascular smooth muscle cells (VSMCs) in vascular remodeling with a special focus on vascular calcification. In **chapter 1** we delineate the impact of cardiovascular disease (CVD) on society. One of the key players that contributes to CVD development is vascular remodeling. Vascular remodeling reflects the adaptation of the vessel wall to biochemical and biomechanical stimuli. In general, two types of remodeling are discriminated: outward and inward remodeling. Vascular remodeling ultimately results in CVD diseases such as atherosclerosis and vascular calcification and arterial stiffening. The VSMC, being the most abundant cell type in the vessel wall, contributes for a major part to the development and progression of vascular remodeling. Processes such as proliferation, migration, extracellular vesicle (EV) release and apoptosis are biological pathways that are regulated by VSMCs. **Chapter 2** focusses in more detail on vascular remodeling processes and especially the role that VSMCs have in its regulation. VSMC phenotypic switching is the most important factor that contributes to vascular remodeling. Under physiological condition VSMCs display a contractile phenotype characterised by high expression of markers involved in contraction and dilation. Upon stress VSMCs respond by changing their phenotype in order to conduct vessel repair. Environmental cues, such as soluble biochemical compounds, extracellular matrix (ECM) proteins and biophysical conditions interact with VSMCs and their phenotypic state. Additionally, special emphasize is given on clinical features of vascular remodeling. Extensive vascular remodeling can lead to pathologies such as hypertension, vascular calcification, atherosclerosis and fibrosis. These clinical manifestations of vascular remodeling are extensively discussed in chapter 2 with a special emphasize on the role of the VSMCs in these diseases. Last, current clinical detection methodologies of vascular remodeling including their strengths and limitations are discussed.

There is a lack of good models to study vascular remodeling, and most models that currently exist focus on diseases themselves, rather than on arterial remodeling processes. In **chapter 3** we developed a model in which we specifically induced medial calcification by the use of Vitamin K antagonists (VKAs). Medial calcification is a late stage remodeling feature and it is difficult to study without developing vascular calcification at other sites of the vascular tree. We found that VKAs induce cardiovascular calcification, including ectopic heart calcification, valvar calcification and medial calcification of the aorta. Additionally, vessel compliance was decreased and isolated aortas showed increased uptake of ^{18}F -NaF a radionuclide tracer that is reported to image active mineralization. Furthermore, we studied the effect of established medial calcification on subsequent atherosclerosis development, and found that intimal calcification was significantly increased. Moreover, increased uncarboxylated matrix Gla protein (MGP) and features of extensive vascular remodeling were found.

In **chapter 4** we studied the effect of VSMC apoptosis on the development of atherosclerosis. Here we exploited a different model of vascular remodeling, we used SM22 $\square$ -hDTr $^{+}$ /ApoE $^{-/-}$ mice to induce VSMC apoptosis. Upon DT exposure, VSMC cell numbers were decreased up to 50% compared to control littermates. In SM22 $\square$ -hDTr $^{+}$ /ApoE $^{-/-}$ mice we found that the remaining VSMCs showed signs of increased contractility and hypertrophy, both in the thoracic aorta and in ex vivo cultured VSMCs. In hDTr $^{+}$ mice we found that vessel contractility was preserved while vessel dilation capability was impaired. Upon atherosclerosis induction (6 and 18 weeks) SM22 $\square$ -hDTr $^{+}$ /ApoE $^{-/-}$ mice developed significantly larger atherosclerotic plaques, with

increased inflammation (6 weeks), a thinner fibrous cap (6 and 18 weeks), lower intimal VSMCs (6 weeks) and a larger necrotic core (18 weeks). These are all hallmarks of a more vulnerable plaque phenotype.

To gain further insight in the mechanisms of vascular calcification development, we used isolated human primary VSMCs and developed a novel in vitro calcification assay that measures kinetic calcification development. In **chapter 5** we compare this novel assay to existing methods for calcification determination. One major advantage over other assays is the ability to measure calcification development over time. This BioHybrid platform can be used for both human serum and plasma samples. The Biohybrid platform was assessed by the use of samples of patients known to be susceptible for the development of vascular calcification, such as chronic kidney disease (stages III, IV, V and VD; CKD), pseudoxanthoma elasticum, high coronary artery calcium (CAC) score patients, VKA users and aortic valve calcification patients. For most of these patients the BioHybrid platform was able to detect a high calcification propensity compared to controls.

As biomarker is still valuable for clinical research we studied, in **chapter 6**, the role of MPG as a biomarker for vascular calcification in CKD patients. The most inactive form of MGP (dephosphorylated uncarboxylated MGP; dp-ucMGP) was shown to associate with increased CAC and medial calcification score. Additionally, at areas of extensive medial calcification, ucMGP positivity was found. In multivariate analyses dp-ucMGP was associated to bone markers and medial calcification score was associated with age, gender, diabetes mellitus and dp-ucMGP levels. Moreover, MGP mRNA expression was shown to associate high CAC score and with high dp-ucMGP levels in the plasma. Last, we sought to determine the role of MGP single nucleotide polymorphisms in relation to the development of vascular calcification. We could not find clear MGP SNP that associated with vascular calcification.

Chapter 9

Nederlandse samenvatting

Het hoofddoel in mijn proefschrift was het onderzoeken van de rol van vasculaire gladde spiercellen (VSMCs) in vasculaire remodelering met een speciale focus op vasculaire verkalking. In **hoofdstuk 1** besprak ik de impact van hart- en vaatziekten (CVD) op de samenleving. Een van de belangrijkste spelers die bijdragen aan CVD is vasculaire remodelering. Vasculaire remodelering is het aanpassingsvermogen van de vaatwand op biochemische en biomechanische prikkels. In het algemeen zijn er twee typen van remodelering te onderscheiden: buiten- en binnenwaartse remodelering. Omvangrijke vasculaire remodelering leidt uiteindelijk tot CVD, zoals atherosclerose en arteriële stijfheid, die beiden worden gekenmerkt door vasculaire verkalking. VSMCs zijn de meest voorkomende cellen in de vaatwand en dragen voor een groot deel bij aan de ontwikkeling en progressie van vasculaire remodelering. Biologische processen zoals proliferatie, migratie, extracellulair vesicle (EV) afgifte en apoptose worden door VSMCs gereguleerd. **Hoofdstuk 2** gaat meer gedetailleerd in op de rol van VSMCs in vasculaire remodelering. VSMC fenotypische switch is een van de belangrijkste factoren die bijdraagt aan vasculaire remodelering. Onder fysiologische condities hebben VSMCs een contractiel fenotype dat wordt gekarakteriseerd door een hoge expressie van markers die belangrijk zijn bij de contractie en dilatatie van de vaatwand. Bij overmatige stress reageren de VSMCs en veranderen deze hun fenotype om de vaatwand te herstellen. Omgevingsfactoren, zoals oplosbare biochemische stoffen, extracellulaire matrix eiwitten en biofysische condities hebben interactie met de VSMC en hun fenotype. Bovendien, wordt er in hoofdstuk 2 speciale nadruk gelegd op klinische aspecten van vasculaire remodelering. Een grote mate van vasculaire remodelering kan leiden tot ziektebeelden zoals hypertensie, atherosclerose, arteriële stijfheid, vasculaire verkalking en fibrose. Als laatste, wordt de huidige diagnostiek van vasculaire remodelering besproken met daarbij hun sterkte- en zwaktespunten.

Preklinisch is er een gebrek aan goede modellen voor het bestuderen van vasculaire remodelering. In **hoofdstuk 3** hebben wij een diermodel ontwikkeld waarin we mediale verkalking kunnen opwekken door het gebruik van vitamine K antagonisten (VKA). Mediale verkalking is een kenmerk van vasculaire remodelering dat laat tot uitdrukking komt en is moeilijk te bestuderen in onderzoek dat zich focust op atherosclerose. In dit onderzoek lieten wij zien dat VKA cardiovasculaire verkalking opwekken, inclusief ectopische hart verkalking, klep verkalking en mediale verkalking van de aorta. Bovendien, was vaatwand compliantie verlaagd en zagen we een verhoogde opname van ^{18}F -NaF, een radionuclide PET tracer die gebruikt wordt bij het in beeld brengen van actieve verkalking. Verder, hebben wij gebruik gemaakt van het “gain of function” AAV virus dat het PCSK9 eiwit verhoogd en hebben wij het effect van mediale verkalking op de ontwikkeling van atherosclerose bestudeerd. In dit model hebben we laten zien dat het aanwezig zijn mediale verkalking leidt tot een significante toename van zowel atherosclerose als intinale verkalkingen. Bovendien, was er een toename van niet-gecarboxyleerd matrix Gla eiwit (MGP) en vonden wij kenmerken van extensieve vasculaire remodelering.

In **hoofdstuk 4** hebben wij het effect van VSMC apoptose op atherosclerose ontwikkeling bestudeerd. We hebben hier een ander diermodel gebruikt, namelijk SM22 Δ -hDTr+/ApoE $^{-/-}$ muizen waarin we specifiek VSMC in apoptose kunnen brengen. Na het toedienen van DT, verminderde het aantal VSMCs in de vaatwand met 50% t.o.v. controle nestgenoten. In SM22 Δ -hDTr+/ApoE $^{-/-}$ muizen hebben wij laten

zien dat de achtergebleven VSMCs een verhoogde contractiliteit hebben en hypertroof zijn, zowel in de aorta als in ex vivo geïsoleerde cellen. Verder lieten we zien dat SM22 α -hDTr+/ApoE β - muizen eenzelfde vaatwand contractiliteit hadden maar dat vaatwand dilatatie was verminderd. Na het opwekken van atherosclerose (zowel vroege als late atherosclerotische plaques) ontwikkelden SM22 α -hDTr+/ApoE β - muizen significant grotere atherosclerotische plaques, met verhoogde ontsteking (vroeg), een dunnere fibrotische cap (beide vroeg en laat), minder intimale VSMCs (vroeg) en een grotere necrotische kern (laat). Al deze eigenschappen zijn kenmerken van een meer kwetsbaar plaque fenotype.

Om verder inzicht te verschaffen in de mechanismen van de ontwikkeling van vasculaire verkalking, hebben wij een nieuwe kinetische in vitro calcificatie assay ontwikkeld die gebruik maakt van geïsoleerde humane VSMCs. In **hoofdstuk 5** hebben wij deze assay vergeleken met bestaande methoden voor het meten van verkalking. Een groot voordeel van onze assay is dat we verkalking kunnen meten over tijd. Ons “BioHybrid” platform kan worden gebruikt voor zowel humaan serum als plasma. Het BioHybrid platform is gevalideerd door serum en plasma samples van patiënten te gebruiken die klinisch hebben bewezen gevoelig te zijn voor het ontwikkelen van verkalkingen, zoals: chronische nierziekten (stadium III, IV, V en VD; CKD), pseuxanthoma elasticum, hoge coronair arterie calcium (CAC) score patiënten, VKA gebruikers en patiënten met klepverkalking. Over het algemeen was het BioHybrid platform bekwaam om patiënten met een hoge verkalking neiging op te kunnen pikken vergeleken met controles.

Biomarkers blijven een belangrijke rol spelen in klinisch onderzoek en dus hebben we in **hoofdstuk 6** de rol van MGP als biomarker voor verkalking in CKD patiënten onderzocht. De inactieve vorm van MGP (gedefosforyleerd-niet-gecarboxyleerd MGP; dp-ucMGP) werd geassocieerd met een hoge CAC en mediale verkalking score. Verder konden wij geen rol voor MGP single nucleotide polymorfismen vinden met vasculaire verkalking. Echter, bij gebieden die veel mediale verkalkingen vertoonden, was er een grote mate van ucMGP positiviteit. In multivariate analyses vonden wij dat plasma dp-ucMGP was geassocieerd met bot markers en dat mediale verkalking score was geassocieerd met leeftijd, geslacht, diabetes mellitus en plasma dp-ucMGP waarden. Tenslotte werden MGP mRNA levels geassocieerd met een hoge CAC score en met hoge dp-ucMGP waarden in het plasma.

Chapter 10

Valorization

Valorization, the utilization of academic research. Or the process of value creation out of knowledge and make it available for economic or societal purposes. Eventually so that it can be translated into products, service or other processes regarding industry.

In my thesis we studied the vascular smooth muscle cell (VSMC), a pivotal player in vascular health and the development of cardiovascular disease (CVD). In more detail we focused on its action in vascular remodeling processes that lead to the development of CVD and vascular calcification. CVD is still the number one cause of death in the world. And understanding the mechanisms of CVD is important, but does not help society directly. However, it can help in the development of new treatments strategies and earlier detection. Most clinical tools that are currently available diagnose CVD at a too late stage. Especially, vascular calcification is diagnosed overdue, when only treatment is focused around resolving the symptoms rather than curing the disease. The valorization part of my thesis focusses on the effect of medial remodeling processes that affect vascular calcification and CVD.

Chapter 2 elaborates on the role of VSMCs in arterial remodeling processes, with a focus on calcification-related processes. In this chapter the role of VSMCs in CVD is discussed from a fundamental, translational and clinical point of view. Chapter 3 investigates the effect of medial calcification on atherosclerosis development. We believe that medial degradation precedes the pathological build-up of plaques. This concept is already old, but recent literature investigates atherosclerosis mostly from a lipid point of view. The research in **chapter 3** confirms that medial degeneration, induced as medial calcification by vitamin K antagonists (VKAs), leads to extensive extracellular medial remodeling with subsequent atherosclerosis induction. We believe it is important to disseminate this view with clinicians to change the way of treating CVD. Additionally, big pharmaceutical companies should change their focus on CVD drug discovery from relieving symptoms towards disease prevention. Another important finding in chapter 3 is that VKAs induce accelerated vascular calcification. VKAs are regularly prescribed to patients suffering from CVD, but clinicians are often not aware of the detrimental effects VKAs have on established atherosclerotic plaques. For example, numerous studies have shown that chronic kidney disease (CKD) patients display extreme vascular calcification after VKA use.

In **chapter 4** we focused on the role of VSMCs apoptosis on the development of atherosclerosis. Our results indicate that VSMC apoptosis in the vessel media results in larger and more vulnerable atherosclerotic plaques. Furthermore, recent literature indicates that most cells that reside inside atherosclerotic plaques are derived from VSMC origin. These findings might have a great impact on the clinical situation. The most well-known treatments of atherosclerosis involve lipid lowering, by-pass surgery and stenting. It is pivotal to include the VSMC into current treatment, as an important contributor to the development and progression of atherosclerosis. Future treatment strategies should focus more on changing the phenotype from synthetic, excreting and proliferative VSMCs towards a contractile phenotype in which the proliferative and secretory nature is decreased.

Chapter 5 describes a novel method to measure calcification propensity using patient serum or plasma, and its susceptibility to develop vascular calcification. Current methods for calcification determination are unrefined and there is no consensus on how

the calcification assays are performed. With our assay we are able to measure calcification kinetics and evaluate patient specific responses while restricting to optimal calcification conditions. The BioHybrid assay might potentially be used in future academic research as golden standard. Thereby, normalising and improving the current end-point assays that are available. Moreover, the BioHybrid platform can be expanded by more in vitro assays to cover a wide range of biological read-out parameters. This in combination with the use of several primary cell types provides a platform that is currently unique and non-existing, with the potential to not only combat vascular calcification but other diseases as well. Additionally, the BioHybrid platform is expected to be fully translatable into animal and healthy volunteer phase, making it a patentable solution that can be commercialized.

The concept of a biohybrid platform is appealing but should not attenuate research to new biomarkers. In **chapter 6** we investigate the biomarker potential of matrix Gla protein (MGP). We found that MGP is an independent predictor of both intimal and medial vascular calcification in a specific cohort of CKD patients. Biomarker research is valuable for the clinic as they can directly be used to interpret parameters of patients. The most inactive variant of MGP (dephosphorylated-uncarboxylatedMGP; dp-ucMGP) reflects a patient's Vitamin K status. Vitamin K is key in the activation of Vitamin K dependent proteins (VKDP) that are known to be important in the coagulation cascade. However, other VKDP, such as MGP, are involved in the inhibition of mineralization. We found that dp-ucMGP plasma levels associate with local protein and mRNA expression and also medial calcification. Targeting MGP, and especially its activation, might prove a novel therapeutic target as it can inhibit or prevent local tissue mineralization at a much earlier stage of the disease. One strategy to activate MGP is to supplement Vitamin K to patients. Although critical care should be taken when supplementing Vitamin K to patients that require anticoagulation. Currently, several clinical trials are being conducted to study the beneficial effects of Vitamin K in CVD and specifically vascular calcification prevention.

Taken together, the data described in my thesis provide a new angle for the development of therapeutic strategies to target vascular calcification and atherosclerosis. Ideally, treatment shifts towards earlier stages of the disease in which regression or prevention is still possible. Treating patients earlier is advantageous but clinical detection should develop along. To aid detection, we build the BioHybrid platform which is based on a VSMC readout to patients' plasma or serum. Clinically treatment that prevents or regresses calcification would be greatly appreciated as it is a benefit for CVD mortality/morbidity and expensive treatments costs. In conclusion this thesis, covers the whole spectra of research from fundamental to translational and clinical. Thereby some of the findings can be valuable for the interpretation of a different therapeutic approach or can be translated into products and services that can be commercialized.

Chapter 11

Curriculum Vitae

About the author

Armand Mario Gerard Jaminon was born on November 21st 1989 in Heerlen, The Netherlands. He graduated from Sophianum high school in Gulpen in 2008 completing the Science and Health profile.

After graduation he started to study Bio-Pharmaceutical Sciences at the University of Leiden, where he received his Bachelor's degree in August 2011. After completion of the Bachelor program he started a 2 year Master program in Bio-Pharmaceutical Sciences with a specialization in Drug Delivery Technology and Biopharmaceutics. He obtained his Master's degree in August 2013 after completing an internship of 9 months at the department of Biopharmacy at the University of Leiden under the supervision of Prof. Dr. van Eck and an internship of 6 months at the department of Molecular Genetics at the University of Maastricht under the supervision of Dr. Neumann.

In 2013 he started to work as a researcher at the department of Molecular Genetics of the University of Maastricht under the supervision of Dr. Neumann. Here he performed research on the generation of stable knockout cell lines for AMPK subunits. In 2014 he started his PhD program at the department of Biochemistry at the University of Maastricht under the supervision of Prof. Dr. Schurgers and Prof. Dr. Reutelingsperger. During his PhD program he studied the role of vascular smooth muscle cells in vascular remodeling and calcification. During his PhD program he has published his data in peer-reviewed international scientific journals and presented his work at international conferences in Boston and Helsinki.

After his PhD he started to work as PostDoc on the RegMedXB project at the Biochemistry department of Maastricht University. A project that focusses on the regeneration of the human heart.

Over de Auteur

Armand Mario Gerard werd geboren op 21 november 1989 in Heerlen, Nederland. In 2008 behaalde hij zijn VWO diploma met het profiel Natuur en Gezondheid aan het Sophianum te Gulpen.

Na het afstuderen van de middelbare school begon hij aan zijn studie, bio-farmaceutische wetenschappen aan de universiteit van Leiden, waar hij zijn bachelor behaalde in augustus 2011. Na het voltooien van zijn bachelor begon hij met een 2 jaar durende master opleiding bio-farmaceutische wetenschappen met een specialisatie in geneesmiddelen toediening technologie en biofarmacie. Hij behaalde zijn master in augustus 2013, na het voltooien van een stage van 9 maanden bij de afdeling biofarmacie aan de universiteit van Leiden onder supervisie van Prof. Dr. van Eck en een stage van 6 maanden bij de afdeling moleculaire genetica aan de universiteit van Maastricht onder supervisie van dr. Neumann.

In 2013 begon hij als onderzoeker bij de afdeling moleculaire genetica aan de universiteit van Maastricht onder supervisie van Dr. Neumann. Hier deed hij onderzoek naar de generatie van stabiele "knockout" cellijnen van AMPK sub-eenheden. In 2014 begon hij zijn promotie onderzoek bij de afdeling biochemie aan de universiteit van Maastricht onder leiding van Prof. Dr. Schurgers en Prof. Dr. Reutelingsperger. Tijdens zijn PhD bestudeerde hij de rol van gladde spiercellen in vasculaire remodelering en calcificatie. Tijdens zijn promotie traject publiceerde hij zijn data in peer-reviewed

internationale wetenschappelijke tijdschriften en presenteerde hij zijn werk op internationale conferenties in Boston en Helsinki.

Na zijn promotie startte hij als PostDoc in het RegMed XB project op de afdeling biochemie van de universiteit van Maastricht. Het project focust zich op de regeneratie van het humane hart.

Chapter 12

List of publications

Publications

Jaminon AMG, Dai L, Qureshi AR, Evenepoel P, Ripsweden J, Söderberg M, et al. Matrix Gla protein is an independent predictor of both intimal and medial vascular calcification in chronic kidney disease. *Sci Rep. Nature Publishing Group*; 2020 Apr 20;10(1):6586–9.

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Chapter 13

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