Non-invasive markers to investigate vascular damage in systemic disease

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CHAPTER **1**

General introduction and aims

INTRODUCTION

The prevalence of chronic diseases is rising globally as a result of improved medical care and a higher overall life-expectancy. 'Chronic disease' is an umbrella term used to describe long-standing conditions that require ongoing medical attention[1]. No formal criteria exist for defining a condition as a chronic disease, but frequently mentioned conditions include diabetes mellitus, HIV, chronic kidney disease, and rheumatoid arthritis. Chronic diseases are nowadays the major cause of death and disability worldwide, which is partially driven by an increase in cardiovascular complications[2].

Cardiovascular disease (CVD) includes conditions that affect the heart or blood vessels, such as coronary artery disease, heart failure, cerebrovascular disease, and aneurysms. A common feature of these conditions is that vascular wall injury leads to an impaired vascular function, which in turn contributes to the risk of clinically relevant disease.

In many chronic diseases, although involving different organs and systems, vascular wall injury persists and progresses over time. Even when the primary disease is treated adequately, vascular damage accumulates with time. For example, in diabetes mellitus micro- and macrovascular damage occurs despite adequate glycemic control and in giant cell arteritis subclinical vascular inflammation can persist despite clinical remission, thereby increasing the risk for cardiovascular complications. Vascular complications usually have a significant effect on patients' quality of life and survival. Therefore, detection and control of vascular damage in a pre-clinical stage are essential.

In this thesis, a number of markers suitable for the detection of vascular injury are investigated and discussed. These markers have the potential to help in advancing our understanding of vascular injury related processes, which is the first step towards a better risk assessment and disease control. This general introduction provides background information on the pathophysiology of arterial damage and the markers we investigated.

Artery structure and function

Blood vessels involved in CVD are usually the arteries. Arteries transport oxygen from the heart to the body. Besides, nutrients and immune cells are transported to the periphery through the arterial system. Arteries are classified as elastic arteries

and muscular arteries[3]. The elastic arteries, consisting of the aorta and its branches, directly receive the output of the left ventricle. The elastic arteries distend during the systole and retract during the diastole, thereby pushing the blood forward to the periphery. Muscular arteries are medium-sized arteries that control the distribution of blood throughout the body by vasoconstriction, and dilatation.

The wall of each artery is composed of three layers. The innermost layer is called the tunica intima and consists of a monolayer of endothelial cells and an elastic lamina. A healthy endothelium has a 'vascular protective' function and actively regulates platelet and monocyte adhesion, coagulation and vasoconstriction.

The middle layer of the artery wall, the tunica media, consists of concentric layers of smooth muscle cells, collagen fibers, and elastic fibers. Elastic arteries contain more elastin, while muscular arteries contain relatively more smooth muscle cells. The outer layer, the tunica adventitia, is mainly composed of collagen tissue. Large arteries contain vasa vasorum ('vessels of the vessels') to supply the outer part of the vessel wall. The inner parts receive oxygen and nutrients by diffusion from the vessel lumen.

Arterial disease: inflammation is a key process

A key process in the initiation and development of arterial pathology is inflammation. In vasculitis as well as atherosclerosis development and plaque rupture, local activation of immune cells drives the progression towards clinical relevant disease[4,5]. Atherosclerosis is a very common, slowly progressive condition, while vasculitis is a rare disease in which arterial damage generally develops in days to weeks. In atherosclerosis, inflammation is generally found in the tunica intima, but the tunica media can be involved as well. In vasculitis, the whole vessel wall, from tunica intima to adventitia, can be involved[5]. However, in both conditions local inflammatory responses contribute to injury and dysfunction. Without treatment the clinical complications are usually serious.

Pathophysiology of atherosclerosis

Endothelial dysfunction is the earliest detectable arterial change in the development of atherosclerosis. All classical cardiovascular risk factors, namely smoking, hyperglycemia, dyslipidemia, hypertension and diabetes mellitus are associated with endothelial dysfunction[6]. As a consequence of endothelial dysfunction a complex pathologic response occurs, which involves the accumulation of cholesterol-containing lipoproteins and white blood cells, mainly monocytes, in the tunica intima[7].

The monocytes differentiate into macrophages, which take up oxidized lipids. Lipid-loaded macrophages, called foam cells, actively form inflammatory mediators and are involved in tissue remodeling. Foam cells are the hallmark of the first visible atherosclerotic lesions, the so-called 'fatty streaks'[8]. Most fatty streaks will develop to advanced stages of atherosclerosis during lifetime. Advanced atherosclerotic plaques are characterized by a large lipid-rich/necrotic core (LR/NC), presence of intraplaque hemorrhages (IPH) and a thin fibrous cap[9].

Pathophysiology of vasculitis

Vasculitis forms a spectrum of disease characterized by the presence of inflammatory cells in the vessel wall and reactive vascular damage[10]. Vasculitis can be a primary disease or occur secondary to an infection, autoimmune disease or malignancy. Primary vasculitis is classified based on the size of the predominantly affected vessel. Major categories are large-, medium-, and small-vessel vasculitis. The innate and adaptive immune system contributes to the pathogenesis of vasculitis. Most secondary forms of vasculitis are immune complex-mediated, which means that an antigen-antibody complex triggers vascular inflammation. In primary vasculitis cell-mediated, immune complex-mediated and ANCA-mediated pathways have been described, but underlying mechanisms are largely unknown. An infectious or environmental trigger is thought to be the initiating event in individuals with a genetic predisposition. The contribution of the various inflammatory cell types and cytokines differ per form of vasculitis and is outside the scope of the thesis. Various forms of systemic vasculitis are associated with an accelerated development of atherosclerosis[11]. It has been suggested that endothelial damage due to vasculitis stimulates atherosclerosis development.

Arterial inflammation is related to arterial calcification

Vascular inflammation is closely related to vascular calcification[12]. Vascular calcification particles are composed of various calcium salts, but hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$) forms the largest component, as in bone[13]. Inflammatory factors are thought to promote osteogenesis within the vessel wall. In turn, the presence of calcium deposits itself might also initiate or promote vascular inflammation[14].

Calcification is thought to be the consequence of active bone formation by osteoblast-like cells[15]. These cells might derive from differentiated vascular smooth muscle cells (VSMC) or from stem cells from the circulating blood. Various local and circulating calcification promoting and inhibiting proteins have been identified that influence the

severity and progression of vascular calcification. However, the interaction between the players involved in vascular calcification is incompletely understood. Calcification develops in the intimal, but also in the medial layer of the vascular wall.

Intimal calcification

Intimal calcification is related to the atherosclerotic burden. The process of calcification starts in the early stages of atherosclerosis development and accelerates during disease progression. Therefore, intimal calcium is an important marker of atherosclerosis. The exact impact of calcium on the atherosclerotic plaque is unsure. In recent years, it has been discovered that the size of the calcium particles relates to plaque stability. Small calcification particles, called microcalcifications, seem to destabilize the atherosclerotic plaque, while larger areas of calcification, macrocalcification, might increase plaque stability[16].

Medial calcification

Calcification of the medial layer of the arterial wall is a condition also known as 'Mönckeberg's arterioslerosis'[17]. Calcium deposits accumulate in the elastic fibers of the tunica media. Medial arterial calcification (MAC) typically develops in the peripheral muscular arteries and the aorta. Medial arterial calcification and intimal calcification frequently co-exist, but medial arterial calcification can exist on its own[18]. In contrast to atherosclerosis, MAC does not obstruct the arterial lumen. MAC is associated with aging, diabetes mellitus and chronic kidney disease.

Clinical consequences of arterial damage

Arterial damage can lead to clinical symptoms due to narrowing or occlusion of the lumen, increased stiffness of the vessel wall or weakening of the vessel wall. In atherosclerosis, luminal narrowing or occlusion occurs when intimal thickening impairs the arterial blood flow or when an arterial thrombus is formed. In vasculitis, inflammation can lead to severe arterial wall thickening and consequently narrowing or occlusion of the lumen. The clinical presentation of ischemia is similar between patients with vasculitis and atherosclerosis, although the affected organs can be different.

An increased arterial stiffness occurs in MAC. The arteries lose their elasticity due to fragmentation of the elastic fibers and deposition of collagen and calcifications. An increased arterial stiffness leads to various hemodynamic changes, including a rise in systolic blood pressure and a subsequent increase in cardiac afterload, which causes ventricular hypertrophy[17]. Besides, a decrease in the diastolic blood pressure leads

to a decreased coronary perfusion. In diabetes mellitus and chronic kidney disease, MAC is an independent predictor of future cardiovascular events and mortality[19-22].

A localized weakening in the arterial wall of the arteries can result in the formation of an aneurysm. This occurs in atherosclerosis as well as in vasculitis as a consequence of severe inflammatory injury[23]. Aneurysms usually develop somewhere along the aorta, but also occur in the intracranial vessels. An aneurysm is asymptomatic until its rupture causes an internal bleeding. In some forms of vasculitis bleedings occur from damaged, permeable small vessels, visible as small hemorrhagic spots on the skin.

Vulnerable plaque hypothesis

How the presence of an atherosclerotic plaque eventually can lead to the formation of a superimposed thrombus is not completely understood. Disruption of the endothelial layer of the vessel wall is thought to trigger platelet activation and thrombin generation. Therefore, plaque rupture, which exposes the subendothelial matrix to the circulating blood, is seen as a key event in the pathophysiology of acute CVD[24].

Table 1. Morphological plaque characteristics associated with vulnerability

Plaque thickness
Thin fibrous cap
Intraplaque hemorrhage
Lipid-rich necrotic core
Microcalcification
Active inflammation

The vulnerable plaque hypothesis is based on the assumption that some plaques are more prone to rupture than others. Plaques that are prone to rupture are called vulnerable plaques or high-risk plaques. In order to improve risk prediction, identification of the vulnerable plaque has become a major focus in cardiovascular research. Due to advances in imaging techniques plaque composition and plaque metabolic activity can be visualized, rather than only the plaque thickness. As consequence, several features of vulnerable plaques have been identified (table 1)[9,25].

Detecting arterial damage

During the past decades, various non-invasive markers have become available that give an impression of the state of the arteries for diagnostic, prognostic or research purposes. Those markers can visualize the structure of the artery wall, inform on inflammatory processes involved in arterial damage or reflect local pathology. The

markers that we have evaluated in this thesis are described in more detail in the following paragraph.

Imaging markers

^{18}F-FDG PET/CT

Positron emission tomography/computed tomography (PET/CT) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is a sensitive method for the detection of inflammation in the vascular wall. ^{18}F-FDG is a radiolabeled analogue of glucose that accumulates in activated inflammatory cells[26]. Pathologic studies showed that especially macrophages and neutrophils have a high ^{18}F-FDG-uptake.

In clinical practice, ^{18}F-FDG PET/CT has been proven an accurate tool for the early diagnosis of large vessel vasculitis [27]. In this condition, the pattern of ^{18}F-FDG uptake is diffuse and involves the aorta and its main branches. In contrast, a locally high ^{18}F-FDG uptake can be found in atherosclerotic plaques[28]. The extent of atherosclerotic ^{18}F-FDG uptake has been associated with the risk of future CVD[29].

^{18}F-NAF PET/CT

Another tracer used in PET imaging is ^{18}F-sodium fluoride (^{18}F-NaF). This tracer identifies areas of calcification and has been used in routine clinical care for bone imaging. However, growing evidence suggests a role for ^{18}F-NaF in the identification of atherosclerotic calcification as well[30]. ^{18}F-NaF binds to the surface area of calcium particles[31]. Because a group of small calcium particles has a larger total surface area than one large calcium particle, an intense ^{18}F-NaF signal is especially seen in plaque regions with microcalcifications. In contrast, CT imaging shows only larger calcium particles. ^{18}F-NaF PET imaging offers new opportunities to study the process of atherosclerotic calcification and to identify the vulnerable plaque.

Ultrasound

Ultrasound investigation of the carotid arteries allows for the detection of early atherosclerotic lesions by measuring the intima-media thickness (IMT). The IMT is the distance between the lumen-intimal border and the medial-adventitial border, which are both easily recognized with ultrasonography. An increased wall thickness is a hallmark of atherosclerosis. In research, carotid artery IMT has been used to evaluate the effect of treatment, for example statins, on atherosclerosis development.

Carotid artery IMT is also an indicator of generalized atherosclerosis and is associated with the development of CVD[32].

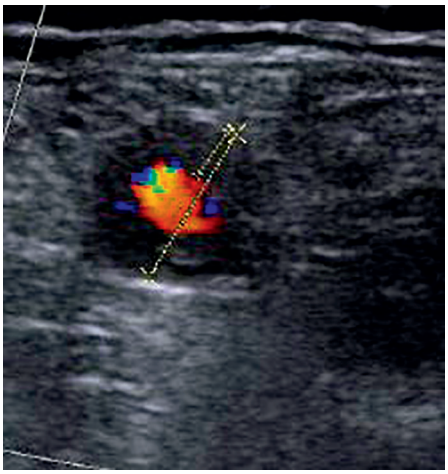

Figure 1. Concentric hypoechoic vessel wall thickening around the arterial lumen; the so-called 'halo sign'.

Arterial wall thickening also occurs in vasculitis. In large vessel vasculitis, a dark shadow around the vessel lumen can be seen with ultrasound. This shadow, the so-called 'halo', is thought to be caused by edema in the vessel wall and surrounding tissue due to inflammation[33]. In giant cell arteritis (GCA), a form of large vessel vasculitis, the presence of a halo in the temporal arteries is frequently found. In a relatively large, prospective, multicenter study, ultrasound showed a better sensitivity for the clinical diagnosis of GCA than temporal artery biopsy, which was considered the gold standard for confirming artery wall inflammation[34].

Magnetic resonance imaging

High-resolution magnetic resonance imaging (MRI) can visualize wall thickening of large and smaller vessels, such as cranial and intracranial arteries. Therefore, MRI may be used for the detection of luminal changes in atherosclerosis or in some forms of vasculitis[35]. The great strength of MRI is the ability is to visualize the phenotype of atherosclerotic plaques, rather than only the narrowing of the vessel lumen. By combining multiple pre- and post-contrast weightings different plaque components can be identified with a high accuracy when compared to histology[36,37].

Non-imaging markers

Ankle-brachial index

The ankle-brachial index (ABI) is the ratio between the systolic blood pressure at the ankles and the arms. The ABI is a widely accepted test to diagnose peripheral arterial disease[38]. A high, as well as a low ABI, are related to vascular pathology. A high ABI points to increased vascular stiffness, caused by calcification of the tunica media[17]. A high ABI is the consequence of the increased pressure that is needed to compress the

relatively stiff arteries of the legs. In contrast, a low ABI is found when the peripheral blood flow is reduced due to atherosclerotic luminal obstruction. Besides being a marker of local pathology, the ABI is a strong predictor of adverse cardiovascular outcomes in high-risk individuals as well as the general population[39-42].

Calcium propensity – T50 test.

The T_{50} test is a recently developed assay that measures the calcification propensity of serum *in vitro*[43]. The T_{50} test measures the time of the transformation from primary calciprotein particles (CPP) to secondary CCPs in serum that is supersaturated with calcium and phosphate. CPPs are complexes of calcium and phosphate ions with serum proteins, such as fetuin-A and albumin. When blood contains increased amounts of calcium and phosphate ions, CPPs are formed in order to prevent ectopic precipitation of calcium-phosphate. The initially formed CPPs, called primary CPPs, are amorphous. After time primary CPPs will transform to secondary CPPs, at least in vitro. Secondary CPPs are crystalline and contain hydroxyapatite. Secondary CPPs are thought to induce inflammation, oxidative stress and calcification in susceptible cells[43].

A long T_{50} time reflects a high resistance to ectopic calcification and *vice versa* a short T_{50} time reflects an increased calcification propensity. In patients with chronic kidney disease, who are at increased risk of vascular calcification, a shorter T_{50} time has been related to all-cause mortality[44,45]. Besides, T_{50} time is associated with cardiovascular mortality in kidney transplant recipient[46,47].

Arterial damage in high-risk groups

Low-grade vascular inflammation occurs with ageing and contributes to phenotypic and functional arterial changes[48]. However, in some chronic conditions, vascular inflammation occurs abundantly or at a younger age. Makers of arterial damage can be used to identify those individuals. Lessons learned from these patient groups might provide insight into the complex, multifactorial pathology of CVD.

Giant cell arteritis

Giant cell arteritis (GCA) is the most common form of vasculitis in adults from Western countries[49]. GCA typically affects individuals aged over 50 years, with a peak incidence at the age of 70 - 80 years. Diagnosing GCA is challenging because the disease can present with a wide range of symptoms. The classical symptoms of GCA are related to inflammation of the cranial arteries and include headache, jaw claudication and visual disturbances. However, the large body arteries can be

affected as well. This is called large vessel vasculitis or extra-cranial GCA. Patients with predominantly extra-cranial involvement often present with non-specific symptoms such as weight loss, fever, malaise and muscle weakness.

Early treatment of GCA with corticosteroids is important to prevent serious vascular complications. However, diagnosing GCA can be challenging due to the heterogeneous presentation of the disease. Various clinical symptoms and laboratory values help to predict the likelihood of GCA, but no formal diagnostic criteria for diagnosing GCA exist. Confirmation of arterial wall inflammation is therefore highly recommended[50].

Temporal artery biopsy (TAB), which is an invasive procedure, has long been seen as the gold standard for assessing vascular inflammation. However, this is changing now that various imaging techniques have become available for the assessment of vascular wall inflammation. Recently, colour duplex ultrasonography (CDU) has been recommended as the first imaging modality in patients with predominantly cranial symptoms[50]. CDU is safe, patient-friendly and usually quickly available. Besides the temporal arteries, the axillary arteries, which are frequently involved in GCA, can also be easily scanned with CDU. However, the diagnostic value of CDU in extra-cranial GCA is still unsure. Currently, ^{18}F-FDG PET/CT scanning is frequently used as the gold standard for extra-cranial involvement. This method is accurate, but also expensive and not always immediately available.

Diabetes mellitus type 1

In type 1 diabetes mellitus (T1DM) an autoimmune-mediated destruction of insulin-producing cells in the pancreas leads to an absolute insulin deficiency[51]. Over the last decades, the lifespan of patients T1DM has rapidly increased due to improvement in glycemic control. Nevertheless, morbidity and mortality from CVD is still a concern in this population. A large prospective trial shows that with the aggressive treatment of cardiovascular risk factors, the risk for mortality approaches that of the general population[52]. However, the risk for coronary artery disease still stays higher than in controls, even when all risk factors are on target. The pathophysiologic mechanisms are largely unknown.

Hemophilia

Hemophilia is a rare bleeding disorder caused by the lack of functional clotting factor VIII (hemophilia A) or clotting factor IX (hemophilia B)[53]. In the Netherlands, around 1600 persons are living with hemophilia. Hemophilia is an X-linked genetic disorder.

The symptoms of hemophilia depend on the degree of clotting factor deficiency. In severe deficiency (< 1% of normal clotting factor) spontaneous bleeds occur, mainly in the joints and muscles.

The treatment of hemophilia consists of replacement of clotting factor VIII or IX. In the early seventies, plasma-derived factors concentrates were used that were not adequately treated to destroy active viruses[54]. Hemophilia patients became infected with HIV and hepatitis and many of them eventually died. Since that tragic time, the treatment of hemophilia has enormously improved. Safe replacement therapy has become available and prophylactic treatment has become standard care in severe hemophilia in Western countries. Due to the improvements in medical care the life expectancy of persons with hemophilia now approaches that of the normal population.

With the increasing age of the hemophilia population, the prevalence of age-related comorbidities is rising as well. Because of their hypocoagulable state persons with hemophilia were historically considered to be protected against acute CVD[55]. It was hypothesized that an arterial thrombus could not be formed. However, we now know that persons with hemophilia are not protected against atherosclerosis and that CVD does occur[56,57]. Cardiovascular risk factors are highly prevalent. Hypertension is even more frequently found in persons with hemophilia than in the general population for unknown reasons[58,59].

The increasing incidence of CVD in hemophilia raises two issues. First, treatment of CVD in hemophilia is challenging, because antiplatelet therapy further increases the risk for bleeding. Second, the exact relation between the coagulation system and atherosclerosis is still poorly understood. We know that the extent of atherosclerosis, as measured with carotid artery IMT, is similar between PWH and controls[56,60]. However, studies among elderly PWH are lacking and the plaque morphology is unknown. It is possible that a higher prevalence of vulnerable plaques neutralizes the potential protective effect of being less thrombogenic. However, this has not been investigated before.

CONTENT OF THIS THESIS

As stated, vascular diseases are common in many chronic conditions. Vascular diseases are characterized by pathological alternations in the vascular wall, which contribute to an increased risk of clinically apparent CVD. A better understanding of the mechanisms and meaning of these vascular changes is a necessary, first step in the development of accurate risk assessment tools and therapeutic interventions. In this thesis, several imaging and non-imaging markers of arterial wall damage are evaluated in a new clinical context. The overall aim is to improve our understanding of the processes involved in arterial disease.

In **chapter 2** we studied ^{18}F-NaF as a marker of microcalcification and plaque vulnerability. We compared *in vitro* ^{18}F-NaF uptake between culprit carotid plaques (plaques that caused a stroke) and non-culprit carotid plaques of patients scheduled for carotid artery surgery. Furthermore, we analyzed to what extent the pattern of ^{18}F -NaF uptake on microPET differed from the pattern of calcification on microCT.

Chapter 3 describes the association between serum T_{50}, as a measure of overall calcification propensity, and other parameters of increased mineral stress in persons with type 1 diabetes mellitus. Vascular calcification progresses faster in diabetes mellitus than in the general population. Disturbances in mineral metabolism could be one of the factors that predispose them to calcification. The association between T_{50} and micro- and macrovascular complications was evaluated as well.

Chapter 4 and **chapter 5** address the relationship between hypocoagulability, as seen in persons with hemophilia, and atherosclerosis. Chapter 4 describes two cross-sectional studies on the ankle-brachial index (ABI) in persons with hemophilia. The first study showed an unexpectedly high prevalence of high ABI, which points to an increased vascular stiffness due to medial arterial calcification. A high ABI is rare in the general population. Therefore, we hypothesized that hemophilia predisposes to a high ABI and that high ABI relates to hypertension, which has a high prevalence in hemophilia. In the second study, we aimed to confirm our previous findings in unselected persons with hemophilia and age-matched controls. In chapter 5, we compared MRI assessed plaques components between persons with hemophilia and controls without bleeding disorder. We hypothesized that an increased plaque rupture due to high-risk morphological features explains the occurrence of acute CVD despite the lifelong hypocoagulability.

In **chapter 6** we evaluated the performance of color duplex ultrasonography (CDU) of the axillary arteries in patients with extra-cranial GCA. First, we compared axillary CDU findings with axillary ^{18}F-FDG PET/CT findings, which we considered the gold standard for large vessel inflammation. Second, we compared the diagnostic value of temporal and axillary artery CDU, to temporal artery CDU only.

In **chapter 7** the results of this thesis are summarized and discussed.

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