Doctoral Thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

A role for CD44 in atherosclerosis? Studies in mice and humans

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A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarises the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted, or in manuscript).

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Abstract

Atherosclerosis is an inflammatory disease that can lead to clinical complications such as myocardial infarction and stroke. Expressed in both vascular and inflammatory cells, adhesion molecule CD44 can be cleaved from the cell surface, and soluble CD44 can be detected in blood. CD44 mediates many inflammatory events, some possibly critical for atherogenesis. However, the role of CD44 in atherosclerosis remains incompletely understood. Therefore, this thesis aimed to investigate the role of CD44 in atherogenesis.

No association between soluble CD44 in serum and atherosclerosis, cardiovascular risk factors, and diabetes was determined, suggesting that soluble CD44 is not a suitable biomarker for atherosclerosis. In contrast, macrophages from patients with subclinical atherosclerosis showed enhanced levels of CD44 compared to healthy controls. CD44 expression associated with increased interleukin-6 secretion, and macrophages treated with interleukin-6 exhibited augmented CD44 expression.

To further examine the potential role of CD44 in atherosclerosis *in vivo*, low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice with or without CD44 expression were used. A bone marrow transplantation in $LDLr^{-/-}$ mice to obtain a mouse model with CD44-deficiency on bone marrow-derived cells was also performed. Surprisingly, and in contrast to published data on CD44 in apolipoprotein-deficient mice, CD44-deficiency in $LDLr^{-/-}$ mice resulted in no or very modest reduction of lesion development. However, both mast cells and T cells, two cell types involved in lesion instability and rupture, decreased due to CD44-deficiency in advanced lesions. Furthermore, altered CD44 expression may influence the extrinsic coagulation cascade and therefore may affect thrombus formation.

Taken together, CD44 expression increased in macrophages from subjects with atherosclerosis. However, its soluble counterpart did not associate with subclinical atherosclerosis and did not hold promise as potential biomarker. Since altered CD44 expression affects cell composition, it may contribute to lesion stability.

List of publications

This thesis is based upon the following papers, referred to in the text by their roman numerals:

Paper I **Circulating soluble CD44 is higher among women than men and is not associated with cardiovascular risk factors or subclinical atherosclerosis**

Sara Sjöberg, Linda Fogelstrand, Johannes Hulthe, Björn Fagerberg, Alexandra Krettek

*Metabolism **2005**. 54: 139-141*

Paper II **Augmented levels of CD44 in macrophages from atherosclerotic subjects: A possible IL-6–CD44 feedback loop?**

Daniel Hägg, Sara Sjöberg, Lillemor Mattsson Hultén, Björn Fagerberg, Olov Wiklund, Annika Rosengren, Lena M.S. Carlsson, Jan Borén, Per-Arne Svensson, Alexandra Krettek

*Atherosclerosis **2007**. 190: 291-297*

Paper III **CD44-deficiency reduces antithrombin, factor X and factor VIII and promotes blood clots with compact structure**

Lars Faxälv, Sara Sjöberg, Marcus Andersson, Anders Sellborn, Tomas L. Lindahl, Alexandra Krettek

Submitted

Paper IV **CD44-deficiency on hematopoietic cells limits T-cell number but does not protect against atherogenesis in LDL receptor-deficient mice**

Sara Sjöberg, Einar E. Eriksson, Åsa Tivesten, Annelie Carlsson, Anna Klasson, Max Levin, Jan Borén, Alexandra Krettek

Under revision

Paper V **CD44-deficiency reduces mast cell content and affects late but not early atherogenesis in LDL receptor-deficient mice**

Sara Sjöberg, Johannes Wikström, Ken Lindstedt, Johan Bourghardt, Jonas Roberts, Åsa Tivesten, Li-Ming Gan, Alexandra Krettek

Manuscript

Abbreviations

ApoE	Apolipoprotein E
CAD	Coronary artery disease
CCR	Chemokine receptor
CD44	Cluster of differentiation 44
CD44s	CD44 standard form
CD44v	CD44 including variant exon
CVD	Cardiovascular disease
EMC	Extracellular matrix
ERM	Ezrin, Radixin, Moesin
GAGs	Glucosaminoglycans
HA	Hyaluronic acid, hyaluronan
H-CAM	Hermes antigen cell adhesion molecule
HDL	High density lipoprotein
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IMT	Intima-media thickness
INFγ	Interferon gamma
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
MCP-1	Monocyte chemoattractant protein
MHC	Major histocompability complex
MMPs	Matrix metalloproteinases
OPN	Osteopontin
Pgp-1	Phagocytic protein 1
TNFα	Tumor necrosis factor alpha
sCD44	Soluble CD44
SCF	Stem cell factor
SMC	Smooth muscle cells
VCAM-1	Vascular adhesion molecule-1
VLDL	Very low-density lipoprotein

Table of contents

“All models are wrong, but some are useful”

George Box
(1919-)

1 Introduction

1.1 Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of mortality worldwide and causes an estimated 30% of all deaths [1]. Atherosclerosis is one of the underlying mechanisms for CVD mortality.

1.1.1 Pathogenesis of atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterised by thickening of the innermost layer of the arterial wall, known as the intima. Such thickenings, i.e., atheromas or atherosclerotic plaques/lesions, develop in large to mid-sized arteries. Atherosclerotic lesions distribute focally to areas such as bifurcations, branch vessels, and curvatures, where alterations in hemodynamic force occur commonly.

1.1.1.1 The healthy vessel wall

The vessel wall consists of three distinct layers; intima, media, and adventitia (Fig. 1). The intima includes the endothelial layer, which is closest to the blood flow in the vessel lumen, and the underlying internal elastic lamina. The proteoglycan layer is closest to the endothelial layer in the internal elastic lamina, followed by the musculoelastic layer, which lies adjacent to the media [2]. The media consists mainly of smooth muscle cells (SMC), elastic fibres, and proteoglycans [3]. The external elastic lamina lies between the media and the adventitia, which consists of fibroblasts, loosely organized SMCs, collagen fibres, and some elastic fibres. Blood vessels that supply nutrients and oxygen to the arterial wall are located in the adventitia.

1.1.1.2 Fatty streak—the early atherosclerotic lesion

Atherosclerosis begins early in life, and its earliest lesions, i.e., fatty streaks, have been identified during fetal development [4]. The fatty streak may either disappear or progress into an advanced lesion. Fatty streaks consist of lipid-laden cells, or foam cells, that localize beneath the endothelium [5]. While most cells in the fatty streak are monocyte-derived macrophages, T cells are also present [6].

The mechanisms that underpin the initiation of atherosclerosis are complex and remain incompletely understood. The most dominant hypothesis today involves the “response to retention” theory [7, 8], which suggests that low density-lipoprotein (LDL) particles penetrate the endothelial layer and move into the internal elastic lamina, where they bind to proteoglycans. Modified by oxidative and enzymatic processes, LDL particles become pro-inflammatory agents [9-11]. Oxidized LDL particles promote increased expression of adhesion molecules, especially vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P-selectin, in endothelial

cells [12-14]. The adhesion molecules participate in the recruitment of monocytes and T cells to the vessel wall. Monocytes differentiate into macrophages in the subendothelial space, [15] and then begin to phagocytose the modified LDL particles via scavenger receptors, resulting in foam cell formation [16-18]. Epitopes from modified lipoproteins are presented to T cells by antigen-presenting cells within the lesion [19]. Macrophages secrete pro-inflammatory cytokines, which may recruit more monocytes and T cells, further increasing the inflammatory process [20].

Although fatty streaks lack complications, they can grow and transform into more advanced atherosclerotic lesions. Such lesion may rupture after decades of development, resulting in clinical symptoms.

1.1.1.3 Advanced lesion

A growing lesion recruits more inflammatory cells and lipids into the vessel wall. Cytokines, chemokines, and growth factors secreted by macrophages stimulate SMC from the media to proliferate and migrate into the intima. Foam cells and lipids accumulate in the centre of the lesion, i.e., the core, where they are surrounded by a cap consisting of SMC and collagen-rich matrix [21]. While macrophages are the dominant cell type for foam cell formation, SMC can store lipid droplets as well [22]. Plaque grows mainly at the shoulder regions, and immune cells concentrate primarily there and also in the interface between the cap and the core [23, 24]. The lesion can become even more complex, when foam cells in the lipid core go into necrosis and cholesterol crystals form. Necrotic cores are surrounded by a fibrous cap of varying thickness [23]. The pro-inflammatory environment stimulates not only macrophages and T cells but also mast cells, dendritic cells, and natural killer T cells [24-26].

Although the lesion grows in the intima, the lumen diameter remains constant due to remodulation of artery's external boundaries [27]. As the lumen narrows, however, the lesion becomes more fibrous due to of the abundance of synthetic, collagen-producing SMC. Very advanced lesions with abundant SMCs and few macrophages can lead to clinical atherosclerosis by flow-limiting stenosis.

1.1.1.4 Plaque rupture

The fibrous cap that surrounds the lipid, or necrotic, core prevents contact between the plaque's prothrombotic material and the blood [6]. However, remodelling of the atheroma can destabilize the fibrous cap and lead to erosion or rupture [28, 29]. The areas most prone to rupture are the shoulder regions of the lesion where the majority of activated T cells, macrophages and mast cells are located [25, 28, 30, 31]. Inflammatory cytokines such as interferon gamma (INFγ) and tumor necrosis factor alpha (TNFα) inhibit SMC proliferation and collagen production meanwhile matrix metalloproteinases (MMPs) degrade the extracellular matrix (EMC) [32-35]. The cap

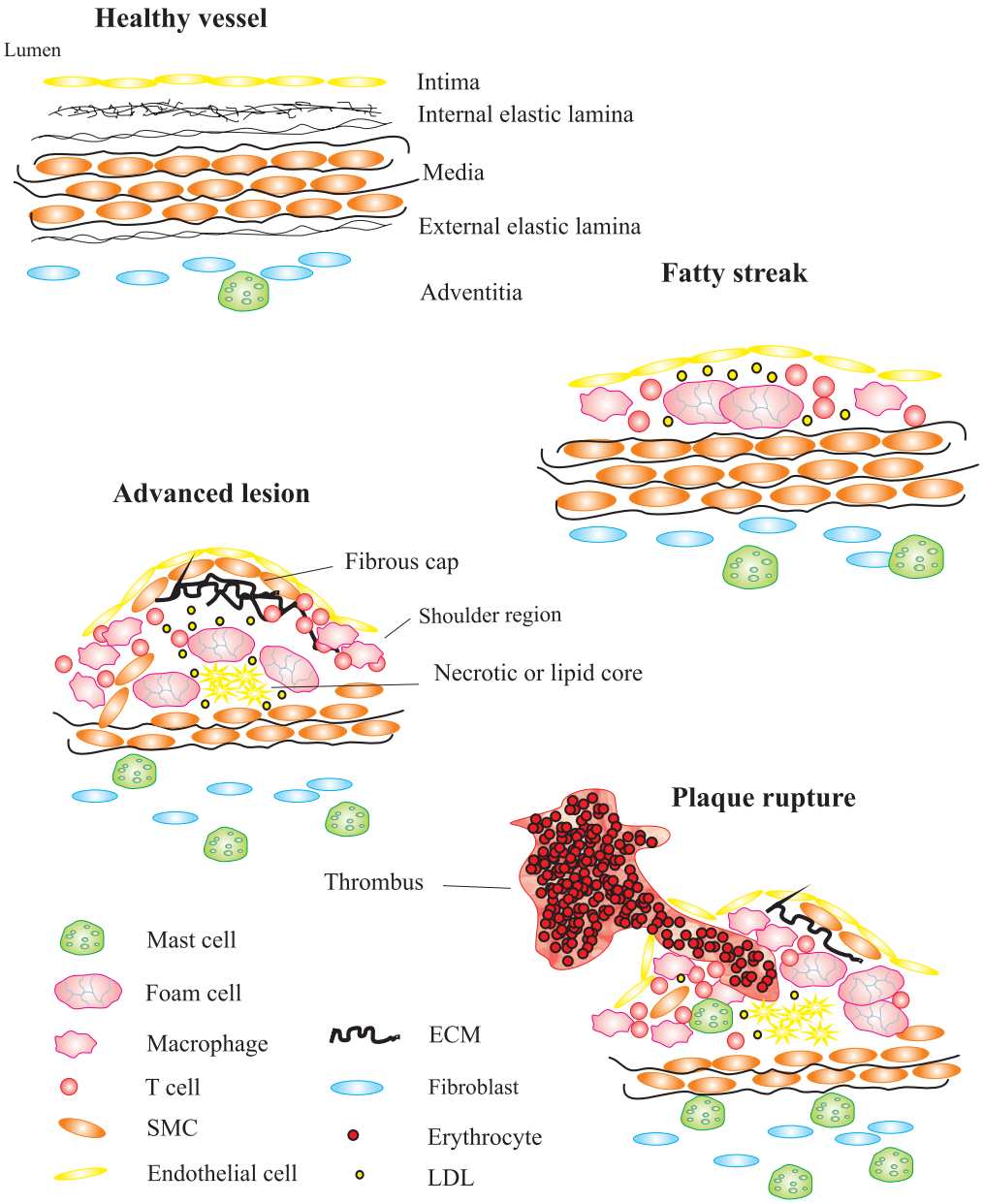

Fig. 1 Developmental stages of atherotic lesions.

becomes weak and prone to rupture. Interestingly in ruptured plaques, increased numbers of macrophages, T cells, and mast cells localise in the adventitia, indicating that inflammatory processes are not restricted to the intima [36].

The most severe clinical complications occur when a lesion ruptures, exposing its prothrombotic content to blood and activating the extrinsic coagulation cascade, thereby causing thrombus formation. A thrombus that forms in arteries supporting the brain can cause ischemic stroke. In the heart, thrombi can cause myocardial infarction and heart failure as well as renal impairment, hypertension, abdominal aortic aneurysms, and critical limb ischemia [21]. However, clinical symptoms only occur if the thrombus causes occlusion. If not, the thrombus is reabsorbed, platelets in the thrombus stimulate SMC proliferation and collagen synthesis [29], and the lesion becomes more fibrous. As the lesion progresses, calcification may occur, leading to advanced calcified plaques [37].

1.1.2 Risk factors for atherosclerosis

Atherosclerosis is caused by multiple factors, both genetic and environmental. The development rate of atherosclerosis varies, as does lesion composition, due to heredity and lifestyle, e.g., diet, exercise, and stress. The most important risk factors for atherosclerosis include hyperlipidemia, smoking, hypertension, diabetes, obesity, and physical inactivity. Low intake of fruits and vegetables and psychosocial conditions also contribute importantly to atherosclerosis [38]. Since immune response participates importantly in atherogenesis, some inflammatory markers, e.g., C-reactive protein, can predict CVD risk [39]. Gender influences CVD as well. European mortality from CVD is higher in women (55%) than men (43%) [40]. However, the head start of the cardiovascular death rate curve in men is earlier than in women but the curves for men and women appears parallel [41].

Certain polymorphisms can increase susceptibility to CVD [42]. However, the impact of most polymorphisms depends on exogenous factors such as smoking and other life-style choices [43]. Single gene mutations lead to atherosclerosis only rarely. The most well-defined mutations include apolipoprotein E (ApoE) and low-density lipoprotein receptor (LDLr), which participate directly in hyperlipidemia through LDL particles that accumulate in the blood.

1.1.3 Cell types in atherosclerosis

Arterial wall and bone marrow-derived cells participate in the development of atherosclerosis. While endothelial cells and SMC dominate in the healthy vessel wall, inflammatory cells, e.g., macrophages, T cells, and mast cell, localise in atherosclerotic plaque.

1.1.3.1 Macrophages

Circulating monocytes enter the plaque in response to cytokines, growth factors, or chemokines, e.g., monocyte chemoattractant protein (MCP)-1, produced by the inflamed intima [44]. Within the lesion, monocytes differentiate into macrophages, increasing pattern-recognition receptors for innate immunity, the germline-encoded part of the immune system that ensures quick response. Pattern-recognition receptors including scavenger receptors and toll-like receptors recognise specific structures on microbes and apoptotic cell fragments [6, 45]. Following ligand binding, toll-like receptors initiate a signalling cascade that results in cell activation. Activated macrophages secrete cytokines and produce proteases, cytotoxic oxygen, and nitrogen radical molecules [46].

The scavenger receptorsmediates fagocytation of for example bacterial endotoxins, apoptotic cell fragments and oxidized LDL-particles [45]. Cholesterol from LDL-particles may accumulate as lipid droplets within the cytosol due to insufficient removal by cholesterol acceptors such as high-density lipoprotein (HDL) [6, 47] Macrophages present antigens from the atherosclerotic lesion, e.g., oxLDL, to T cells via major histocompatibility complex (MHC) molecules, promoting an adaptive immune response [19, 21, 48].

1.1.3.2 T cells

The most dominant T cells in the atherosclerotic lesion are $CD4^+$ T cells [24, 49], and the proportion of activated T cells increases during disease progression [30, 50, 51]. T-cells cluster in areas near MHC class II antigen-expressing cells and undergo clonal expansion [24, 52-54]. Due to their cytokine profile, $CD4^+$ T cells can divide into helper T cell (Th) subpopulations. Th1 is primary in atherosclerotic lesions (reviewed in [6, 21]), and the Th1 response activates macrophages and initiates an inflammatory response characteristic of functions that defend against intracellular pathogens. The Th2 response supports allergic inflammation. The cytokine profile is dominated by INFγ, interleukin-12 (IL-12), IL-15, IL-18 and TNFα, whereas few cells produce IL-4, a Th2-type cytokine. In addition, most oxLDL-reactive $CD4^+$ T cells have a Th1 phenotype [19, 55]. INFγ production augments synthesis of TNFα and IL-1, which increase production of inflammatory and cytotoxic molecules in macrophages and vascular cells [20, 56].

1.1.3.3 Mast cells

Mast cell precursors migrate into tissues in response to chemotactic signals, i.e., chemokine ligand 5 and stem cell factor (SCF) [57, 58]. Different chemokine receptors (CCR), e.g., CCR3, and CCR5, direct such recruitment [59]. These cells then differentiate in response to local growth factors and cytokines such as SCF, interleukin (IL)-3, and IL-6 [60-62]. Two mast cell subsets—connective tissue-type mast cells,

which express both tryptase and chymase, and mucosal-type mast cells, which expresses only tryptase—localize in the arterial wall [63, 64]

Some studies have observed mast cells in the non-diseased arterial wall, mainly in the adventitia [36]. Proliferating mast cells associate with the progression of atherosclerosis [65], and most adventitial mast cells localise in ruptured plaques [36]. In athersclerosis, mast cells also localise in the intima, most frequently in the subendothelial space of the intimal layer [64, 66], possibly due to SCF production of endothelial cells [67, 68]. In addition, mast cells associate with neovascularisation within atherosclerotic lesions [69-71]

Many mouse studies have investigated the functional role of mast cells in atherosclerosis, and mast cell-deficiency reduces atherosclerosis in mice [66, 72]. Furthermore, activated mast cells can induce plaque destabilisation in mice [73]. The intima or media in healthy mice have few or no mast cells. Even in atherosclerotic lesions, the number of mast cells in the intima is low. However, the distance between intima and adventitia in mice is small, and adventitial mast cells may regulate inflammatory processes that occur in the intima [66]. Notably, mediator contents and functions differ between rodent and human mast cells [74].

1.1.4 Adhesion molecules in atherosclerosis

Adhesion molecules are transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions. The adhesion molecule's extracellular component binds to other adhesion molecules or components of the extra cellular matrix, whereas the intracellular domain mediates interactions with the cell's cytoskeleton and intracellular signalling pathway. Depending on their structure and function, adhesion molecules are divided into several families, including integrins, cadherins, the immunoglobulin super family, and selectins. Adhesion molecules that participate importantly in atherosclerosis include P- and E-selectin as well as ICAM-1 and VCAM-1, which belong to the immunoglobulin super family (reviewed in [75]).

Selective focal expression of VCAM-1 in sites prone to atherosclerosis may result from decreased expression of atheroprotective molecules. For example, nitric oxide donors expressed in areas with low susceptibility to atherosclerosis diminish the expression of VCAM-1 by endothelial cells. This protective effect may be mediated via nitric oxide maintenance of the inactive form of nuclear factor-κB (NF-κB), leading to reduced effects of adhesion molecule-inducing cytokines, e.g TNF-α and IL-1β [76, 77].

Leukocyte recruitment is a multi-step process that involves sequential capture, rolling, and adhesion to endothelium, followed by transmigration [78]; all steps include cell

adhesion molecules in both endothelium and leukocytes [79]. Rolling is mediated largely by selectines, particularly P- and E-selectin, whereas the adhesion and transmigration are mediated at least partially by ICAM-1 and VCAM-1 [80]

Extracellular proteases can shed the extracellular domain of adhesion molecules from the cell surface. Although much of the human data that links adhesion molecules to atherogenesis relates to measurement of soluble adhesion molecules, the relationship between plasma levels and cell surface activity remains unclear [81]. Adhesion molecule CD44 can mediate cell recruitment and occurs solubly in blood.

1.2 Adhesion molecule CD44

The cluster of differentiation 44 (CD44) is also known as phagocytic protein 1 (Pgp-1), homing cellular adhesion molecule (HCAM), Hermes antigen, and ECMR III [82, 83]. Recently, CD44 has become the most commonly used term.

CD44 is expressed on the surface of most vertebrate cells, and standard CD44 (CD44s) is the most commonly expressed isoform. During inflammation, CD44 increases in hematopoetic cells and parenchymal cells, and IL-1β induces CD44 expression on vascular SMC [84].

1.2.1 Structure

The CD44 family comprises more than 40 proteins ranging from 80 to 200 kDa, all coded by a single, highly conserved gene [82]. CD44's heterogeneity protein results from both alternative splicing and posttranslational modifications such as glycosylation and sulphatation.

CD44s consists of an extracellular, amino-terminal globular protein domain, a stem structure, a transmembrane, and a cytoplasmic-tail region (Fig.2). These structures are coded by standard exons that form the basis for all CD44 variants. Following alternative splicing of the 10 variant exons, the stem structure can extend to varying lengths, depending on which exon or combination of variating exons are included (for review see [85-87]). In addition, the cytoplasmic tail region of CD44s has two variant exons, one that causes a shorter cytoplasmic tail due to mRNA instability, and a longer variant that occurs more commonly [88, 89]. Cells can express various CD44 isoforms simultaneously, and isoform expression profiles depend on tissue type and stage of differentiation [90-92].

1.2.1.1 Binding site on CD44

CD44's extracellular domains consist of binding sites for several ECM components, e.g., hyaluronan (HA), collagen, laminin, fibronectin, and osteopontin. In addition, CD44 interacts with growth factors, cytokines, chemokines, and MMPs [85, 87]. A link domain within the globular domain consists of binding sites for HA as well as

other glucosaminoglycans (GAGs). GAG affinity depends on the degree of glycosylation of the extracellular CD44 domain, which is regulated by post-translational modifications [93]. Such modifications are specific to cell type and growth condition.

The well-characterised binding site for HA is located on the globular domain of CD44. Insertion of variable exons and glycosylation modificationsleads to modulation of the binding site [93, 94]. Interestingly, CD44 expressed on resting T cells and monocytes is functionally inactive for HA binding but it can be activated by TCR or stimulated by proinflammatory cytokines such as TNFα and INFγ [95]. In monocytes, IL-4 negatively regulates activation of the HA-binding site [96].

CD44 can bind enzymes and substrates on the cell surface and function as a platform for molecules such as growth factors and MMPs. For example, MMP-9 binds to CD44, degrading collagen IV [97] and activating the precursor of transforming growth factor-beta (TGF)-β [98]. Loss of CD44 results in impaired TGF-β activity [99]. Furthermore, a heparin-sulphate proteoglycan isoform of CD44 including variant exon 3 (CD44v3) binds fibroblast growth factor (FGF) [100, 101], vascular endothelial growth factor (VEGF) [101], and heparin-binding epidermal growth factor [101, 102]. Interestingly, CD44v3 is induced during differentiation of monocytes into macrophages [101].

1.2.1.2 Signalling and association with the cytoskeleton

Phosphorylation of the cytoplasmic domain regulates CD44 signalling, and others have suggested a plethora of complex signalling pathways initiated by CD44-ligand interaction. Marhaba and Zöller [103] organized these signalling events into three groups (also reviewed in [85, 104]):

- *Signal transduction directly via CD44* mainly involves signals related to CD44's adhesive functions. CD44's cytoplasmic domain connects with actin in the cytoskeleton via an associated protein, ankyrin, which binds to spectrin, a cytoskeletal protein (Fig. 2A). This association is important for subcellular localisation of CD44. The ezrin/radixin/moesin (ERM) protein complex crosslinks phosphorylated CD44 to actin. Merlin uses the same binding motif as the ERM proteins on dephosphorylated CD44 but does not crosslink actin. Importantly for cell migration and cell shape, changes in phosphorylation might switch the CD44-cytoskeleton association between connection and deconnection.

- *Co-receptor signalling or signalling is guided by the association of CD44 with other tyrosine kinases*. CD44 lacks intrinsic kinase activity and therefore

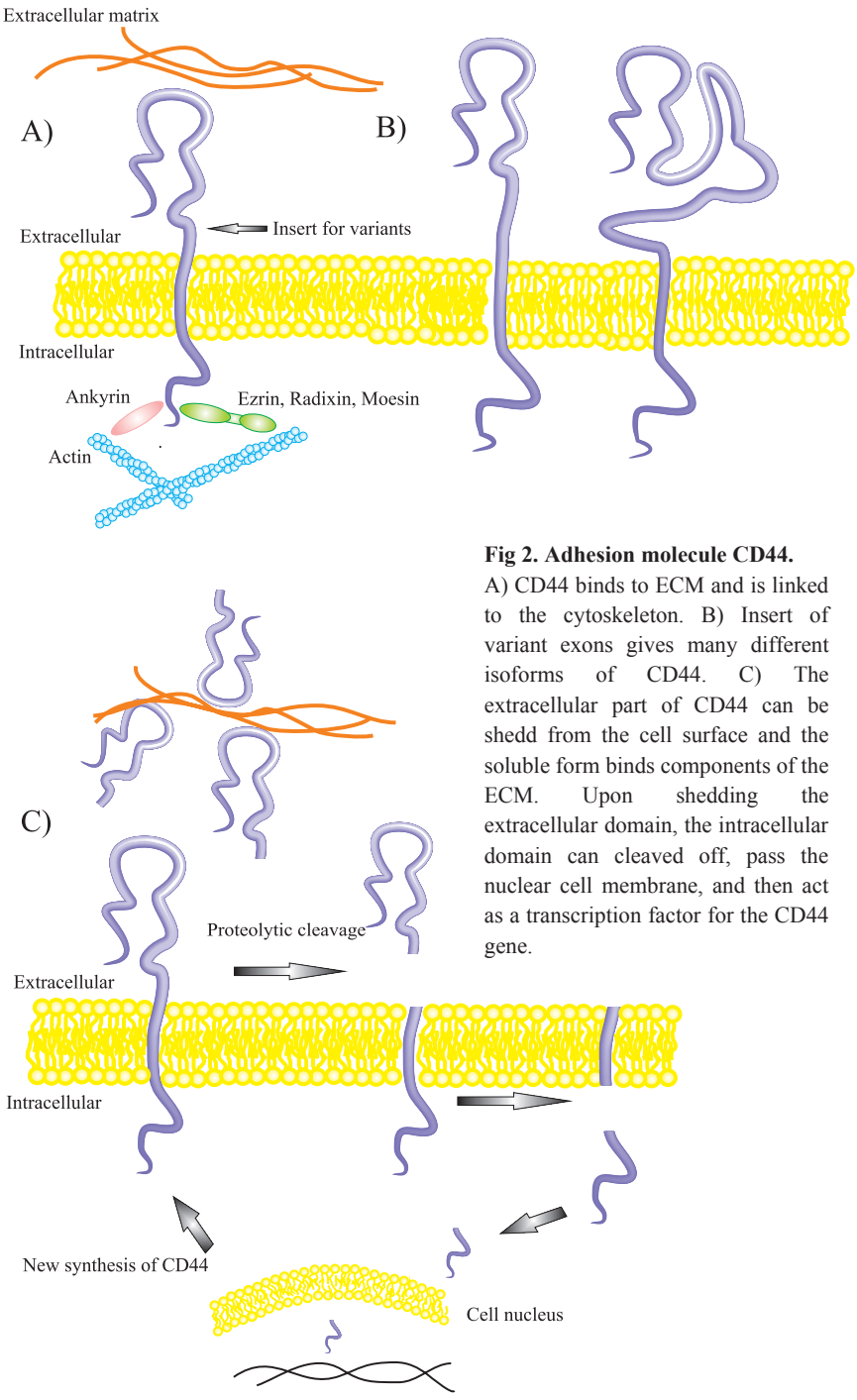

Fig 2. Adhesion molecule CD44.
A) CD44 binds to ECM and is linked to the cytoskeleton. B) Insert of variant exons gives many different isoforms of CD44. C) The extracellular part of CD44 can be shedd from the cell surface and the soluble form binds components of the ECM. Upon shedding the extracellular domain, the intracellular domain can cleaved off, pass the nuclear cell membrane, and then act as a transcription factor for the CD44 gene.

must associate with other receptors to modulate signalling. Rho-family GTPases and Src family members of non-receptor tyrosine kinases form complexes with the intracellular domain of CD44. In addition, CD44 acts as a co-receptor for growth factors and associates with their receptors, e.g., tyrosine kinase receptor ErbB or c-Met [105, 106]

- *CD44 acts as a scaffold* that assists kinases and/or liker protein via apposition, thereby stabilizing or enhancing signalling pathways induced independently of CD44.

1.2.1.3 Soluble CD44

Cell surfaces can release CD44, whose soluble form (sCD44) occurs in serum, lymph, arthritic synovial fluid, and bronchoalveolar lavage [107-109]. Although alternative splicing can result in CD44 isoforms lacking transmembrane and cytoplasmic domains [110], the major mechanism in the production of sCD44 involves shedding membrane-bound CD44 from the cell surface. A putative proteolytic cleavage site is located in the stem-like structure [111]. Proteases such as MMPs and serine proteinases mediate CD44 processing [87, 112]. Upon shedding the extracellular domain (Fig. 2B), the intracellular domain can cleaved off, pass the nuclear cell membrane, and then act as a transcription factor for the CD44 gene [113], thus increasing CD44 turnover, which assists migration [114].

Release of CD44's extracellular domain may affect cellular behaviour and function. Three important functions have been suggested (reviewed in [87]): (i) sCD44 may compete with membrane-bound CD44 for ligand interaction; (ii) cleavage of CD44 disrupts or prevents CD44-dependent cell-cell and cell-matrix adhesion; (iii) cleavage of CD44 participates in cell migration.

1.2.2 Functions of CD44 in inflammation

CD44 has in recent years been intensively studied in both inflammation and cancers. Although major functions involve adhesion and migration, other functions affected by CD44 include leukocyte homing and recruitment, phagocytises, matrix remodelling, proliferation, and apoptosis. Furthermore, CD44 may participate in both arteriogenesis [115] and angiogenesis [116].

Both inflammatory and vascular cells can respond to cytokines with altered CD44 expression including strength of CD44 expression, expression of vCD44, and altered glycosylation [96, 117]. However, CD44 expression may also regulate the release of cytokines. For example, CD44 negatively regulates TLR-mediated cytokine production through a low molecular weight HA-independent mechanism [118]. Furthermore, CD44-deficient mice exhibit prolonged and enhanced inflammation

following heart infarction, including increased expression of IL-1β, TNF-α, IL-6, IL-10, and osteopontin (OPN) after 6 h but not after 24 h[119] In contrast, CD44 activation in macrophages results in a release of TNF and IL-1 [120, 121]

1.2.2.1 CD44 dependent migration and recruitment

CD44 participates importantly in HA adhesion and migration. The mechanism behind CD44-HA migration involves CD44 localization to the leading edge of the cell and lamellipodia, where CD44 links extracellular matrix and the cytoskeleton. Shedding of CD44 at both the leading and the rear edge of the cell, figures importantly in migration capacity. Upon extracellular shedding, release of the intracellular domain induces CD44 synthesis, providing new HA-CD44 interactions [103, 112]. This turnover of CD44 may be the key component for CD44-HA-dependent migration in tumour cells as well as inflammatory cells such as neutrophils [122]. Small HA oligosaccharides, which can occur during pathological conditions such as atherosclerosis, induce CD44 cleavage and increase mobility [123].

Studies with anti-CD44 antibodies reveal a role for CD44 in leukocyte recruitment. For example, anti-CD44 antibodies decrease inflammation by inhibiting leukocyte infiltration and abrogating tissue swelling in murine models of arthritis [124, 125]. In addition, anti-CD44 antibodies prevent inflammation in the central nervous system by directly or indirectly blocking secondary leukocyte recruitment [126]. Similarly, anti-CD44 antibody treatment reduces inflammation in cutaneous delayed-type hypersensitivity response but does not affect leukocyte migration into lymphoid organs [127].

The mechanism supporting CD44-dependent recruitment remains incompletely understood. Integrins participate importantly in T cell adhesion to airway SMC; however, blocking such integrins reveals CD44-dependent adhesion [128]. In addition, CD44 may mediate rolling, and CD44-dependent primary adhesion associates with chronic inflammatory disease activity [129].

1.2.2.2 Cell activation via CD44

CD44 participates in the activation of T cells, natural killer cells [130-132], B-lymphocytes [133], and macrophages[121]. CD44-dependent activation of cytotoxic T cells causes granule exocytosis and kills target cells [134, 135]. The mechanisms that support activation can occur directly via CD44 and its associated proteins or indirectly via cooperation with additional membrane molecules such as TCR-CD3 complex or integrins [136, 137]. Additionally, CD44 can accumulate in the allogenic dendritic cell-T cell immunological synapse and promote dendritic cell-dependent activation of T cells [138].

1.2.2.3 Cell survival and apoptosis

Depending on cell type and condition, CD44 may both promote and inhibit apoptosis and proliferation. Stimulation of CD44 by activating antibodies protects against apopotosis [139]. Antibodies against CD44 induce apoptotic cell death in fibroblasts [140] and inhibit superantigene-induced T cell activation and proliferation by inducing apoptosis [141]. In contrast, CD44 influences apoptosis-induced cell death (AICD) in TCR-activated T cells [142, 143]. In addition, CD44 mediates induction of apoptosis in immature T cells and induction of proliferation in mature T cells. Thus, CD44 might act as a co-stimulating molecule to TCR, exerting its function via apposition of tyrosine kinases [144].

1.2.2.4 CD44-dependent phagocytosis

Binding, internalisation, and intracellular digestion of HA requires CD44 [145]. Lacking CD44, HA may accumulate in the inflamed tissue and thereby modulate the inflammatory response [146, 147]. CD44 also contributes to the inflammatory response by participating in the clearance of microbial pathogens [148] and apoptotic cells [99]. Phagocytosis mediated by CD44 can occur independently of Fcγ-receptors and induces formation of mature phagosomes [149]. Furthermore, CD44 mediates absorption of oxidised LDL in monocytes, possibly enhancing foam cell transformation [150].

1.2.3 CD44 in atherosclerosis

Compared to the healthy vessel wall, the atherosclerotic vessel wall exhibits increased expression of CD44. Whereas the healthy vessel wall expresses only CD44s, the diseased vessel wall displays many isoforms including CD44v3, CD44v4/5, CD44v6, CD44v7/8, and CD44v10. Furthermore, CD44 expression is stronger in macrophage-rich lesions compared to fibrous lesions [117].

The expression pattern of CD44 alters between stable, eroded, and ruptured lesions. In the stable lesion, CD44 localises in inflammatory cells, whereas in eroded lesions, CD44 localises mainly in SMCs in the HA-rich thrombous/plaque interface. Occasional staining is found in platelets and inflammatory cells within the thrombus. In ruptured plaques, CD44 localises in inflammatory cells, mainly those associated with the necrotic core and the thrombus [151].

Many CD44 ligands are expressed in the extracellular matrix of atherosclerotic lesions. Morphological studies have revealed HA in both early and late human atherosclerotic lesions [152], and areas with localised accumulations of HA appear during the formation of atherosclerotic lesion in humans, [153, 154].

Although CD44 and its ligands are expressed in the atherosclerotic lesion, the role of CD44 in atherogenesis is still not yet fully understood. Many of the inflammatory events described above occur in atherosclerotic lesions and are therefore potential target mechanisms through which CD44 may interfere with atherogenesis. In fact, CD44/ApoE-double deficient mice exhibit reduced lesion development, suggesting a role for CD44 in atherogenesis [155]. However, to understand the impact of novel proteins on atherosclerotic development, more than one model must be used.

2 Aim

The general aim of this thesis was to further investigate the role of CD44 in the development of atherosclerosis. The specific aims were to:

- Investigate CD44 as a potential biomarker for atherosclerosis and CVD (**Paper I)**
- Investigate underlying mechanisms behind enhanced CD44 expression in macrophages from subjects with atherosclerosis and the consequence of altered CD44 expression on cytokine production (**Paper II)**
- Investigate if CD44 influences the coagulation cascade (**Paper III)**
- Study the role of CD44 on hematopoietic cells in atherogenesis (**Paper IV)**
- Investigate the role of CD44 in both early and advanced atherogenesis **(Paper V)**

3 Methodological considerations

Scientific research utilizes models to increase understanding of the world around us. To identify the pathogenic mechanisms and determine therapeutic approaches, atherosclerotic research combines analysis of human lesions, clinical investigations, and epidemiological studies with cell culture and animal studies.

This thesis involves studies that measure human material, animal studies that resemble human conditions, and *in vitro* studies. *In vitro* studies are performed in an artificial environment, and their strength lies in their relative simplicity, since there are fewer variables that can act as confounders. On the other hand, *in* vitro studies can only glance at the condition of living (*in vivo*) species. *In vivo* experiments include more conditions that can affect results in real life. However, *in vivo* experiments are more complex to perform and often to interpret, and they include more ethical considerations. How many and what kind of lives are we allowed to affect or end in order to treat another life? It is important to remember the three "Rs"; Reduce the number of animals needed, Refine the methodology used, and Replace the animal models if possible.

Individual papers and their references provide detailed description of material and methods. This section describes methods with specific interest.

3.1 Human studies

3.1.1 Measurement of soluble CD44

We measured circulating sCD44 concentrations with enzyme-linked immunosorbent assays (ELISA) that recognize all soluble isoforms of CD44. We collected serum from subjects participating in two ongoing population-based studies conducted by the same laboratory. The first study included 61-year-old men with and without subclinical atherosclerosis, i.e., carotid artery intima-media thickness (IMT) in tertile 3 as well as atherosclerotic plaques; the control group had IMT in tertile 1 and no atherosclerotic plaques [156]. The second study investigated 64-year-old women with normal glucose tolerance and diabetes mellitus. Exclusion criteria for both studies included clinical atherosclerotic disease, smoking, chronic inflammatory disease, and—for men—diabetes. Because smoking induces CD44 expression [157], both groups excluded smokers.

3.1.2 CD44 in macrophages

3.1.2.1 Microarray of atherosclerotic susceptibility genes

Microarray technology is a useful tool for screening thousands of genes simultaneously and thereby discovering new knowledge about new candidate genes or

regulations of entire pathways under physiological or pathological conditions. Due to multiple testing, the risk of methodological errors and high risk of false-positive findings is low. Microarray technology should be considered a hypothesis generator. All findings require confirmation by other mRNA analysis and/or protein quantification methods.

The Macrophage INTERGENE study used primary macrophages from 15 subjects with subclinical atherosclerosis and 15 matched controls for microarray screening. The study design sought to identify atherosclerosis susceptibility genes by including subjects with a family history of coronary heart disease and at least one atherosclerotic plaque in the carotid or femoral arteries. The study avoided alterations in mRNA expression due to medical treatment by choosing first-degree relatives of patients with coronary heart disease. The control group had no family history of cardiovascular disease and no atherosclerotic plaques at the site of measurement.

3.1.2.2 Primary macrophages

Density gradient centrifugation easily isolated monocytes from peripheral blood. We added a leukocyte concentrate, i.e., buffy coat, to Ficoll-Paque, and density gradient centrifugation yielded mononuclear leukocytes, including monocytes and lymphocytes, at a ratio of approximately 20/80. Monocytic cells adhered to the plastic, and following one week of culture, CD68 staining determined that the purity of the macrophage preparation was >95%. To stimulate the differentiation of monocytes into macrophages, we added granulocyte-macrophage colony stimulating factor (GM-CSF) to the medium. Compared to M-CSF stimulation, which leads to a phagocytic macrophage-like structure, GM-CSF likely generates macrophage populations with dendritic cell-like and antigen-presenting capabilities [158-160]. In addition, GM-CSF differentiates cells into a classical activated macrophage state (M1) that produces proinflammatory cytokines such as TNF, IL-6, and IL-23 following LPS stimulation (rev in [161]).

We used primary macrophages from healthy donors to study the response to IL-6 treatment. Compared with cell lines, primary macrophages offer the advantage of more physiological cells. All cell lines contain genetic modifications that alter the cell cycle into a continuously proliferating state, possibly affecting the gene(s) of interest. However, whereas the cells in cell line experiments are clones of each other, each donor of primary cells has its own genetic profile, yielding large biological variations in cellular response between donors.

3.1.2.3 Investigations of two single nucleotide polymorphism in CD44

Subjects for testing the two SNPs derived from the INTERGENE study [162] that included criteria for coronary artery disease (CAD) in atherosclerotic subjects and no CAD for controls. Manifested CAD in the test group ensured endpoint CAD.

3.2 In vivo studies in mice

3.2.1 CD44 deficient mice models

The CD44-deficient ($CD44^{-/-}$) mice used in this thesis were created by Protin *et al.* through homologous recombination of a reporter cassette directly ligated to the ATG start codon of CD44 [163], resulting in total loss of CD44 expression, including all variants. $CD44^{-/-}$ mice exhibit no overt phenotype, are fertile, and display normal Mendelian inheritance. However, such mice have impaired lymphocyte trafficking [163].

Parallel with Protin *et al.*, Schmits *et al.* created another CD44-deficient mouse by targeting CD44 exons 2 and 3, which also lead to total loss of all CD44 variants[164]. Cuff *et al.* used this model for atherosclerotic studies in ApoE-deficient mice [155]. Since both models target CD44 in a way that depletes all isoforms, they most likely provide similar cellular response.

Due to CD44's multiple roles in cell-cell and cell-matrix interactions as well as migration, the lack of overt phenotype is surprising. In addition, CD44 is expressed at high levels in many tissues during morphogenesis [165-167]. Redundancy by other molecules, e.g., the receptor for hyaluronan-mediated motility (RHAMM), might provide one explanation of the phenotype (rev in [168]). Moveover, less essential CD44 effects during physiological conditions likely become important during pathological conditions [169].

3.2.2 Mouse models in atherosclerosis

Studies of early atherosclerosis in human are scarce. Therefore, studies investigating the development of atherosclerosis rely on animal models. Fortunately, large overlaps in disease development exist between humans and some animal models. Among the most commonly used animals in experimental atherosclerosis is the mouse.

Mice offer many advantages in biological research. They are easy to handle, raise, and feed, and they are cost-effective. In addition, mice can be modified genetically, and inbred strains provide minimal genetic variance. The drawbacks of using mice include small size, which leads to challenging physiological measurements, and dissimilar physiology between mice and humans. A particular disadvantage for atherosclerosis research is that normal mice, or wild-type mice, do not develop atherosclerosis while

consuming a normal diet (chow). Creating a mouse model of atherosclerosis requires either genetically-modified mice or an atherogenic diet.

Even on an atherogenic diet, most mouse strains do not develop atherosclerosis. C57BL/6 is an exception and hence, is used in atherogenic research. Unfortunately, C57BL/6 is difficult to modify genetically. Therefore, modification requires other, more susceptible mouse strains, which are then back-crossed into C57BL/6.

Normal chow diet contains 4%– 6% fat (% weight of fat/weight of diet) and a cholesterol content <0.02%. Mice consuming normal chow exhibit a lipoprotein profile with high levels of HDL and low concentrations of ApoB-containing lipoproteins, i.e., very low-density lipoprotein (VLDL), and LDL (rev in [170]). Paigen diet (15% fat, 1.25% cholesterol, and 0.5% cholic acid) can initiate atherosclerosis in C57BL/6 mice [171]. Cholic acid facilitates cholesterol absorption [172] and suppresses endogenous bile acid synthesis [173], thereby increasing cholesterol in ApoB-containing lipoproteins (rev in [170, 174]).

Atherosclerosis induced by Paigen diet includes fatty streaks in the aortic arch and proximal aorta and does not develop into more advanced plaques in C57BL/6 mice . Experiments on C57BL/6 mice show that dietary cholesterol induces genes of acute inflammation in the liver. Furthermore, cholic acid induces hepatic genes related to fibrosis [175]. Paigen diet has an inflammatory effect in mice by inducing hepatic NFκB activation and expression of acute phase reactants such as serum amyloid A [176].

3.2.2.1 Genetically modified mice models in atherosclerosis

Using genetically modified animals alters the possibilities of studying atherosclerosis in mice. The first models targeted LDLr and its ligand, ApoE, both known to cause familial hyperlipidema. Most genetic modifications in atherogenic mouse models depend on the disruption of lipoprotein regulation and metabolism.

ApoE participates importantly in the clearance of apoB-containing lipoproteins by binding to LDLr, LDLr-related protein, and heparin sulphate proteoglycan [177]. While consuming chow diet, ApoE-deficient ($ApoE^{-/-}$) mice exhibit a 5-fold increase of plasma cholesterol compared to wild-type mice, and most of the cholesterol localises in VLDL and intermediate-density lipoprotein (IDL) particles [178, 179]. Moreover, $ApoE^{-/-}$ mice consuming chow develop lesions throughout the microvasculature [178, 179]. Over time, the lesions develop into more complex lesions than fatty streaks [178]; Western-type diet (21% milk fat and 0.2% cholesterol) accelerates this process [179]. The developed lesions are more lipid-rich than lesions

in mice that consume chow [180]. In addition, ApoE$^{-/-}$ mice exhibit calcification of fibrous tissue [181].

In contrast to VLDL/IDL accumulation in ApoE$^{-/-}$ mice, LDLr$^{-/-}$ mice on chow diet exhibit elevated plasma levels of LDL compared to wild type mice [182]. However, hypercholesterolemia is less severe in LDLr$^{-/-}$ mice compared to both ApoE$^{-/-}$ mice and humans with familial hypercholesterolemia, due to clearance of ApoB48 containing lipoproteins via an ApoE-dependent pathway [183]. In contrast to humans, mice produce B48 particles not only in the intestine but also in the liver [184]. Adding fat and cholesterol to the diet increases LDL levels further as well as VLDL and VLDL remnants, resulting in a lipoprotein profile that resembles the profile found in humans with familial hypercholesterolemia (rev in [170, 174])

LDLr$^{-/-}$ mice consuming chow diet develop no atherosclerosis or very modest lesions in the aortic root and throughout the aorta [182, 185-188]. However, LDLr$^{-/-}$ mice consuming a Western-type diet develop widespread atherosclerosis [189] and advanced, calcified lesions [190]. Due to the possibility of inducing advanced atherosclerosis in LDLr$^{-/-}$ mice with diets, this model is used widely to examine the effect of different sources and levels of fat (reviewed in [170, 174]).

3.2.2.2 Bone marrow transplantation

Bone marrow transplantation (BMT) creates mouse models with one genotype on bone marrow derived-cells and another on all other cells. Since CD44 is expressed in both vascular and inflammatory cells, transplantation of CD44-deficient bone marrow cells provides a model for studying the importance of CD44 in inflammatory cells. The LDLr$^{-/-}$ mouse is a particularly suitable atherosclerosis model for BMT, since the atherogenic effect of LDLr-deficiency is mainly due to reduced lipoprotein clearance. Expression of LDLr on hematopoetic cells does not affect atherogenesis. Therefore, transplanted bone marrow does not have to be LDLr-deficient.

Recipient bone marrow is depleted by lethal total body γ-irradiation and new bone marrow is injected within 24 hours. The total white blood cell count returns to normal levels 8–12 weeks after BMT [191]. Compared to non-BMT LDLr$^{-/-}$ mice, BMT LDLr$^{-/-}$ mice exhibit smaller thoracic aorta lesion areas but larger aortic root lesions. Furthermore, aortic root lesions in BMT mice exhibit lower collagen and increased lipid and macrophage content compared to non-BMT mice. Due to γ-irradiation, increased macrophage content may result from increased endothelial adhesion molecule expression [191-193].

3.2.2.3 Measurement of atherosclerosis

Atherosclerosis has been assessed mainly by cross-sectional analysis of the aortic sinus (Fig. 3A) as well as an *en face* technique (Fig. 3B) that involves cutting the aorta longitudinally to display the lumen [194]. Although the latter method provides information about the distribution of atherosclerosis within the aorta, it provides no information about lesion characteristics. Measuring the aortic sinus requires care that lesion size is measured in sections from the same region because lesions do not distribute uniformly within the whole aortic sinus. If necessary, compensation can be made for oblique sections or different size mice, either by total vessel area, the outer circumference, or maximal diameter.

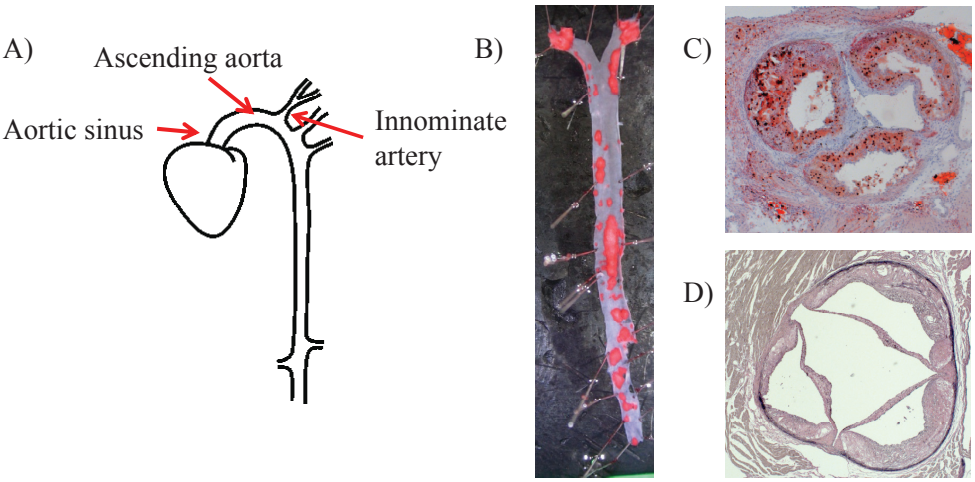

Fig. 3 Measurement of atherosclerosis in mice. A) Site of measurement in the aorta. B) *En face* aorta stained with Sudan IV. Cross sections of aortic sinus from C) frozen and D) paraffin-embedded tissue.

Lesion size in the aortic sinus correlates with the percent of lesion measured by the *en face* technique [195]. However, mice deficient for some molecules, e.g., platelet/endothelial cell adhesion molecule-1(PECAM-1), exhibit site-specific alterations in lesion development [196]. The site-specific response to atherosclerosis by such molecules may result from differing gene expression in the vascular wall caused by flow patterns specific to each site. Therefore, it is important to measure atherosclerosis at more than one site. Furthermore, lesion formation is initiated at different time points in the aortic tree. In mice, the first lesions develop in the aortic sinus [197] and can be used to study early lesion formation shortly after the intervention begins [198]. In contrast, while the innominate artery develops

atherosclerotic lesions at a later time point, it may exhibit a lesion structure that better resembles human advanced atherosclerosis [199]. Thus, the time point for atherosclerosis determination must be chosen with consideration of both the stage of atherogenesis and the site of measurement.

Different lipophilic stains can assess lipid content in atherosclerosis. Sudan IV is used commonly for *en face* arteries, while Oil-Red-O is preferred for sections due to its deep red colour and clear visibility. Since the paraffin-embedding procedure depletes lipids, lipid staining on sections must be performed on frozen tissues. In addition, staining of some epitopes, e.g., CD4 and CD8, can only be accomplished on frozen tissue (Fig. 3C). On the other hand, the structures in paraffin-embedded sections (Fig. 3D) are better conserved, especially after perfusion fixation with formalin.

3.2.2.4 Non invasive atherosclerotic measurement

Measurement of atherosclerosis by ultrasound biomicroscopy offers the possibility of tracing lesion development in living, intact mice, and allows observation of individual lesion development at many time points in the same mouse. The technique is sensitive enough to detect lesions in larger arteries [200] as well as coronary arteries [201, 202]. In paper V, we measured lesion size in the ascending aorta and the proximal innominate artery (Fig. 3A), since lesion size in the aortic sinus is difficult to measure. Although it is possible to distinguish collagen content with ultrasound technique [203], ultrasound measurement provides only limited information regarding lesion characteristics.

4 Results and discussion

Accumulating data suggests an important role for CD44 in inflammatory responses. Due to its widely spread expression pattern and knowledge of its many functions in inflammation, CD44 has many potential mechanisms in atherosclerosis. These mechanisms may be both pro- and anti-atherosclerotic.

4.1 Studies in humans

4.1.1 Soluble CD44 as a biomarker for CVD and atherosclerosis

Soluble adhesion molecules have been proposed as risk markers for CVD and diabetes (reviewed in [204]). Soluble CD44 has been suggested as a potential biomarker for immune activation and inflammation, since the release of CD44 likely associates with enhanced local proteolytic activity and matrix remodelling [87]. Hence, we investigated possible associations between sCD44 and atherosclerosis, cardiovascular risk factors, and diabetes (Paper I).

We determined no association between sCD44 in serum and subclinical carotid atherosclerosis in men or intima-media thickness (IMT) in women. Furthermore, sCD44 did not correlate with known risk factors for CVD, i.e., systolic blood pressure, LDL and HDL cholesterol, triglycerides, or metabolic syndrome. In addition, sCD44 did not associate with diabetes in women.

Since inflammatory responses increase proteolytic cleavage of CD44, the lack of positive correlation between sCD44 and atherosclerosis/IMT surprised us. In addition, proteinases degrade the ECM in atherosclerosis; hence it is likely that CD44 is shed from atherosclerotic lesions. However, atherosclerotic lesions can develop over a long time with only low inflammatory processes. Because all subjects used in this study (Paper I) had no clinical atherosclerotic disease, we cannot eliminate the possibility of stable lesions with low inflammatory activity. In such circumstances, shedding of adhesion molecules is low and a minor release of sCD44 diluted in circulating blood might reach levels detectable by current methods. In addition, little knowledge exists about clearance and catabolism of soluble adhesion molecules. CD44 shed from the cell surface of an atherosclerotic lesion may never reach the circulation, since it may be trapped in the matrix by its ligands, *e.g*. HA, OPN.

Specific isoforms are expressed on activated lymphocytes [205], and antibodies directed against specific CD44 isoforms can prevent the development of colitis [206]. Furthermore, variant isoforms show elevated levels in monocytes during cutaneous inflammation [207]. Krettek *et al* demonstrated increased expression of CD44 variants in atherosclerotic lesion and determined that all investigated isoforms increased.

However, the study was limited to the isoforms CD44s, CD44v3, CD44v4/5, CD44v6, CD44v7/8, and CD44v10 [117]. Since the excess of CD44 isoforms is much larger, it would be interesting to screen for all isoforms and disclose their expression patterns in the atherosclerotic lesion, possibly determining isoforms specific to atherosclerosis or whether specific isoforms increase in patients with vulnerable lesions. If such a specific isoform is shed and released to the circulation it may be possible that it could be used as biomarker. In fact, altered levels of soluble CD44v are found in patients with gastric carcinoma and colorectal cancer and predict disease progression [208, 209].

4.1.2 Enhanced macrophage CD44 expression in atherosclerotic subjects

Susceptibility to atherosclerosis is highly variable, and such differences may result from multiple genetic and environmental variations. We performed microarray analysis of macrophages from atherosclerotic subjects and healthy controls to detect genes with an altered mRNA expression pattern in atherosclerotic subjects (Paper II). Altered protein expression from these genes may increase susceptibility to atherosclerosis, but they also may represent a secondary effect of atherosclerosis, caused by other factors. We found that CD44 is among the strongest of regulated genes and that CD44 mRNA expression increased in macrophages from subjects with atherosclerosis. Protein levels confirmed these data.

4.1.2.1 Potential genetic explanation of enhanced CD44 expression

Genetic regulation within the CD44 gene may indicate a potential mechanism supporting elevated CD44 expression in atherosclerotic subjects. Therefore, to investigate altered CD44 expression as well as a link between CD44 and CAD, we examined two CD44 polymorphisms in the INTERGENE population. However, none of the investigated SNPs associated with CAD.

In addition to the SNPs investigated in Paper II, we conducted a bioinformatic analysis of CD44 in material from the genome-wide association study by The Wellcome Trust Case Control Consortium [210]. Sixty SNPs in and around the locus of CD44 were studied for association with CAD. Because all large materials have problems with multiple testing, we chose a significance level of $p<0.001$ based on a permutation analyse test on 60 SNPs compared to all SNPs on the chromosome. We determined no association between SNPs in the CD44 gene and CAD.

In combination, our data do not support a CD44 genetic variation as explanation for increased CD44 expression in macrophages from atherosclerotic subjects.

4.1.2.2 IL-6 as regulator of CD44 expression

Since atherosclerotic lesion cells express different cytokines that alter expression of different proteins, altered CD44 expression may result from altered cytokine levels. Others have shown that IL-1β augments CD44 in vascular SMC [84] and that IL-1β, TNFα and CD40L augment CD44 in macrophages[117]. Individuals with high risk of atherosclerosis have elevated levels of IL-6 [211]. Thus, we investigated whether IL-6 enhances CD44 expression in macrophages by treating macrophages with IL-6 (Paper II). CD44 increased in cells treated with IL-6 (concentration = 100 pg/mL), a level found in plasma from patients with acute coronary syndromes [212]. Interestingly, cultured macrophages of atherosclerotic subjects showed augmented IL-6 secretion compared with macrophages from non-atherosclerotic subjects. In addition, IL-6 levels in supernatants from cultured macrophages associated with CD44 expression by these cells.

CD44 modulates inflammatory processes and stimulates macrophage cytokine release [120, 121]. To investigate potential cytokine variations due to altered CD44 expression, we performed a cytokine screen from CD44-deficient and wild-type mice. The only cytokine with altered expression was IL-6, which decreased in CD44-deficient mice. However, since we used healthy animals most cytokine levels were low, and often below detection limit. Therefore, it is possible that some of the other cytokines in the screen were affected by CD44 but that this change was not detected. In addition, it may also be possible that CD44 affects cytokine release during pathological but not during healthy conditions.

While studies in other cell types not associated with atherosclerosis indicate a uni-directional association between IL-6 and CD44 [213-216], our data from mice and humans suggest a bi-directional association. Potential intracellular signalling mechanisms involved in this loop may be the transcription factors activating protein 1 (AP-1) and cAMP response element-binding protein which can be activated by CD44. Both transcription factors have binding sites in the IL-6 promoter [215]. In addition, IL-6 signals via STAT3, which has a potential binding site within the CD44 promoter [217]. Thus, we hypothesise that a positive IL-6/CD44 feedback loop may contribute to the development of atherosclerosis.

4.2 Studies in mice

4.2.1 Role of CD44 in atherosclerosis in LDLr$^{-/-}$ mice

Localization of CD44 in atherosclerotic lesions does not disclose the role of CD44 in atherogenesis. Since CD44 participates in many inflammatory processes that may occur in atherosclerotic lesions, an influence by CD44 in the atherosclerotic process is

feasible. Indeed, CD44-ApoE-double-deficient mice ($CD44^{-/-}ApoE^{-/-}$) exhibit reduced atherosclerotic lesion size (50-70%) compared with $CD44^{+/+}ApoE^{-/-}$ [155].

Since ApoE modulates inflammation [218-220]and most potential target processes of CD44 in atherogenesis are inflammatory processes, the role of CD44 requires further examination in a model where ApoE is unmodified. Therefore, we investigated the role of CD44 in atherosclerosis in the $LDLr^{-/-}$ mouse model. Surprisingly, in contrast to $CD44^{-/-}ApoE^{-/-}$ mice, CD44-LDLr-double deficient mice ($CD44^{-/-}LDLr^{-/-}$) exhibited either none or very modest reduction of lesion development compared with $CD44^{+/+}LDLr^{-/-}$ (Paper V). This study includes early as well as more advanced lesions, since we hypothesise that the role of CD44 may change during atherogenesis (see below).

4.2.1.1 Early atherosclerosis

CD44 regulates T cell [221] and macrophage [155] recruitment and thus may affect early atherosclerosis. However, $CD44^{-/-}LDLr^{-/-}$ mice did not exhibit reduced lesion size in the aortic sinus compared with $CD44^{+/+}LDLr^{-/-}$ mice at 4, 6 or 10 weeks of Western-type diet. Mice exhibited small fatty streaks after four weeks of diet, and the 10 week group exhibited lesions that had grown from fatty streak to lesions with an intermediate structure and necrotic cores. therefore, we concluded that CD44 has no major impact on the development of early atherosclerotic lesion formation in $LDLr^{-/-}$ mice.

Two independent studies demonstrated that CD44 increases in the vessel wall of $ApoE^{-/-}$ mice at a very early stage of atherosclerotic development [169, 222]. This is interesting, since CD44 influences gene expression in vascular cells in this early proatherogenic stage in $ApoE^{-/-}$ mice [169]. Therefore, it would be interesting to investigate whether CD44 expression changes in the aortic arch of proatherogenic $LDLr^{-/-}$ mice.

4.2.1.2 Advanced atherosclerosis

Atherosclerotic lesion size measured with ultrasound technique in the ascending aorta and innominate artery showed no differences between $CD44^{-/-}LDLr^{-/-}$ and $CD44^{+/+}LDLr^{-/-}$ mice following 10 or 20 weeks of diet. In addition, we observed no differences in the *en face* whole aorta between $CD44^{-/-}LDLr^{-/-}$ and $CD44^{+/+}LDLr^{-/-}$ mice after 20 weeks of Western diet followed by 10 weeks of chow. However, we observed a minor reduction (~20%) of lesion size in the aortic sinus of male but not female $CD44^{-/-}LDLr^{-/-}$ mice.

Our results suggest only moderate impact of CD44 on atherosclerosis development in $LDLr^{-/-}$ mice. In addition, CD44-deficiency in hematopoetic cells in $LDLr^{-/-}$ mice fed a

cholesterol-rich diet containing cholic acid did not reduce lesion development in either the aortic sinus or the *en face* aorta (Paper IV). Diets with cholesterol and cholic acid increase inflammation, hepatic fibrogenesis, and LDL-fraction [175], leading to a more atherogenic environment compared to Western-type diets. Thus, after six weeks of Paigen diet, BMT mice already exhibited advanced lesions with necrotic cores and fibrous caps in the aortic sinus.

4.2.1.3 CD44 and atherogenesis in ApoE$^{-/-}$ and LDL$^{-/-}$ mice

Potential explanations for the discrepancy in CD44 impact on atherogenesis between the two models include 1) modulated CD44 response due to anti-atherogenic effects of ApoE, and 2) inflammatory response caused by diet.

1) Modulated CD44 response due to anti-atherogenic effects of ApoE

Since low levels of ApoE affect increas CD44 mRNA expression in ApoE-/- mice only modestly, direct regulation of CD44 expression by ApoE is plausible [222]. However, our data do not exclude the possibility that ApoE may modulate activation of CD44 or interfere with the atherogenic effects of CD44. For example, CD44 regulates the production of reactive oxygen species (ROS) in mast cells [223]. Therefore, the antioxidative activity of ApoE [224] may modulate the response to ROS. Whereas CD44 supports both proliferation and apoptosis in T cells by reorganizing membrane receptors and recruiting signaling molecules [144], ApoE inhibits T lymphocyte proliferation by suppressing mitogen-activated proliferation of CD4 and CD8 T cells [218]. In addition, ApoE suppresses production of proinflammatory cytokines by macrophages and T-lymphocytes (rev in [225]). Thus, CD44 depletion in LDLr$^{-/-}$ mice may have a less important role in the development of atherosclerosis due to normal ApoE levels in the LDLr$^{-/-}$ mice.

2) Inflammatory response caused by diet

Adding cholesterol to the diet to induce atherosclerosis in LDLr$^{-/-}$ mice may influence the effects of CD44. Cholesterol induces an acute inflammatory response [175] that may abrogate the contribution of CD44 to lesion development. In addition, both total cholesterol and lipoprotein profile differ between ApoE$^{-/-}$ and LDLr$^{-/-}$ mice. Therefore, inflammatory factors, which have strong impact on lesion development when the influence of atherogenic lipoproteins is moderate, may have less impact when the atherogenic pressure by lipoproteins is increased.

Difference in reduction of atherosclerosis may also result from discrepancies in the developmental stage of atherosclerosis. This is important because different stages in atherogenesis may be influenced by diverse factors that depend on cellular composition and activation. To date, atherosclerosis in CD44$^{-/-}$ mice has been studied at different time points in both early and more advanced lesions in ApoE$^{-/-}$ and LDLr$^{-/-}$

mice. However, a late time-point for lesion stability study in the innominate artery would be an interesting contribution.

4.2.1.4 CD44 influence on cells in the lesion

Although CD44 does not affect lesion size, it may influence the composition of the atherosclerotic plaque. Changed composition may lead to altered lesion stability. Neither total nor hematopoietic CD44-deficiency altered the overall composition of lesions in $LDLr^{-/-}$ mice, measured by SMC, macrophage, and collagen content (Paper IV and V). However, at the late time point (11week) in BMT mice, T cells decreased in CD44-deficient bone marrow. Similarly, Zhao *et al.* recently demonstrated T cell reduction due to CD44 in $ApoE^{-/-}$ mice advanced lesions. Interestingly, reduction of CD44 on either vascular cells or bone marrow-derived cells was sufficient for T cell reduction [221]. In contrast, the earlier time point (6 week) and the 10-week group showed no differences in T cell content.

CD44 participates in the recruitment of lymphocytew to inflammatory sites [124-126] including atherosclerosis in $ApoE^{-/-}$ mice [221]. The reduction in T cells in $LDLr^{-/-}$ mice might therefore be explained by reduced recruitment. However, since the reduction of T cells did not appear in early lesion formation, CD44-dependent recruitment may change due to the degree of inflammation. Another possible explanation is that CD44 stabilises signalling pathways that are important for activation and proliferation of mature T cells [144]. Clarifying the role of CD44 in T cells in atherosclerosis requires further investigation of T cell activation, proliferation, and apoptosis within lesions.

Lymphocytes play an important role in early development of atherosclerosis in $LDLr^{-/-}$ mice. Lymphocyte-deficient $LDLr^{-/-}$ mice fed Western-type diet for 8 weeks exhibit a 54% reduction in lesion size, and reduced lesion size declined during prolonged feeding [226]. Therefore, reduced T cell content in advanced but not early atherosclerosis may not affect lesion size. However, altered T cell content may affect the progression and stability of very advanced lesions.

Mast cells express the HA binding form of CD44 and have previously been described as proatherogenic. We stained for mast cells to investigate CD44 dependency on mast cell infiltration to the vessel wall. Lesions from $CD44^{-/-}LDLr^{-/-}$ mice that consumed 20 weeks of diet exhibited decreased mast cell content compared with $CD44^{+/+}LDLr^{-/-}$ mice. This is interesting since mast cell-deficient mice exhibit reduced T cell content within their lesions[72]. In addition, mast cells can regulate T cell activation and response [227, 228] Furthermore, mast cells associate with the progression of the atherosclerotic disease [65], and mast cell-deficient mice exhibit reduced atherosclerosis [72].

Sun et al. demonstrated that mast cell-deficient mice exhibit reduced atherosclerosis [72]. However, decreased mast cells in our CD44-/- mice were associated with only minor reduction in atherosclerosis in male mice and none in female mice. Thus, a minor proportion of recruited cells may be sufficient for atherogenic effect, or we studied a time point that was too early. Mast cell-deficient mice exhibit reduced atherosclerosis after 26 but not 12 weeks of diet [72]. Since CD44-deficiency leads to reduced by not depleted mast cells, further analyses should investigate mice on longer diet to obtain a model with instable lesion. Furthermore, since activated mast cells induce plaque destabilisation in mice [73], further study could clarify whether mast cell activation is CD44-dependent.

4.2.1.5 A potential role for CD44 in lesion stability and plaque rupture?

We have shown that CD44 affects the presence of mast cells and T cells in the atherosclerotic vessel wall. Both mast cells and T cells are highly present in lesions with unstable features and plaque rupture [30, 36]. CD44 is also present in ruptured lesions [151], where it associates mainly with inflammatory cells.

Plaque rupture causes thrombus formation which is mediated by the extrinsic coagulation cascade. Therefore we studied if coagulation of plasma was affected by CD44 (Paper III). We determined decreased antithrombin levels in $CD44^{-/-}$ plasma, which may promote thrombin accumulation. Interestingly, thrombin generation based on circulating antithrombin distinguishes between acute and stable coronary syndrome where patients with acute syndrome have reduced levels of antithrombin [229]. Furthermore, $CD44^{-/-}$ plasma produced more elastic and compact clots compared to $CD44^{+/+}$ clots. Compact and elastic clots are formed at high thrombin concentration and associate with myocardial infarction in young men [230]. Elevated levels of CD44 in atherosclerosis may therefore be protective if rupture of a lesion occurs. However, understanding the potential role of CD44 in plaque destabilization and thrombus formation requires further analyses.

5 Concluding remarks

Accumulating data suggest an important role for CD44 in many inflammatory processes. These processes also occur during the development of atherosclerosis suggesting a possible role for CD44 in atherogenesis. Altered CD44 expression, may therefore affect the atherosclerotic process. Although atherosclerotic lesions express CD44 and contain proteolytic enzymes, sCD44 did not associate with atherosclerosis. Thus, soluble CD44 may not represent a suitable biomarker for atherosclerosis. In contrast to its soluble form, cell-bound CD44 increased in subjects with subclinical atherosclerosis. Surprisingly, CD44 deficiency had no or only modest influence on atherosclerotic lesion size in the mouse models used in this thesis, and T cell and mast cell content decreased in advanced lesions. Therefore, CD44 may participate in atherosclerosis by affecting the stability of advanced lesions.

5.1 CD44: the chicken or the egg in atherosclerosis?

During my time as a PhD student, we often discussed CD44 in the context of chicken or egg in the development of atherosclerosis. Do changes in CD44 expression alter susceptibility to atherosclerosis, or does CD44 expression change due to factors in the growing atherosclerotic lesion? Does an alternating process between the growing lesion and enhanced CD44 expression stimulate each part, just as chicken and egg developed slowly, over millions of years, from a single cell to a system that requires and depends upon both parts.

In our case, perhaps this is not so simple, since lesion obviously can form without CD44 and stimuli other than atherosclerosis can augment CD44. But then, does CD44 simply increase without doing anything, is it just an innocent bystander? Too much data argue against this, both in T cell recruitment studies and mouse models of atherosclerosis. Therefore, I believe that CD44 is not an innocent bystander in the development of atherosclerosis, just as the cock is not simply a byproduct of the development of the chicken and the egg.

6 Future perspectives

Despite currently accumulating evidence regarding a potential role of CD44 in atherogenesis, our knowledge of its precise function remains scant. Although the study described here determined no association between sCD44 and atherosclerosis, we focussed only on detecting all CD44 isoforms, without distinction. A screening mechanism for individual CD44 isoforms might reveal expression of specific isoforms during different stages of atherosclerosis, thereby determining the potential of CD44 isoforms as biomarkers.

Since sCD44 levels depend on both shedding and release of lesional CD44 in the circulation, measuring cell-bound CD44 in circulating cells may provide an alternative approach. Monocyte-derived macrophages from subclinical atherosclerotic subjects exhibited enhanced CD44 expression (Paper II). Therefore, flow cytometry likely would allow exploration of a possible relationship between CVD and CD44 expression in monocytes, T cells, mast cell precursors, and other inflammatory cells.

Investigating the role of CD44 in advanced, unstable, and rupture-prone lesions might determine CD44's potential for treating atherosclerotic diseases. While $ApoE^{-/-}$ and $LDLr^{-/-}$ mice are good models for studying the hallmarks of lesion instability, e.g., thin fibrous cap, increased levels of macrophages, and low levels of SMC and ECM, examining whether CD44 expression-induced alteration of T cells and mast cells affects plaque rupture will require a different model, such as $ApoE^{-/-}$ and $LDLr^{-/-}ApoE^{-/-}$ mice [231-233]. However, disruptions found in $ApoE^{-/-}$ and $LDLr^{-/-}ApoE^{-/-}$ mice may not resemble human plaque rupture, opening the reliability of these models to debate [199, 234, 235]. Therapeutically, however, the mechanisms underpinning lesion stabilisation are more interesting than the destabilisation process.

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