Neutrophil mediators and their receptors in the pathogenesis and prognosis of cardiovascular disease

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Neutrophil mediators and their receptors in the pathogenesis and prognosis of cardiovascular disease

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DOCTORAL DISSERTATION

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Abstract

S100A8/A9 and S100A12 are neutrophil released alarmins previously associated with coronary artery disease. S100A8/A9 and S100A12 bind to TLR4 (toll-like receptor 4), RAGE (receptor for advanced glycation end products), and EMMPRIN (extracellular matrix metalloproteinase inducer) leading to cell activation and inflammatory cytokine production. RAGE and EMMPRIN also exist in soluble forms (sRAGE and sEMMPRIN), detectable in the circulation. sRAGE acts as a decoy for RAGE ligands, thus having putative anti-inflammatory and atheroprotective properties. Psychological stress is thought to be an important trigger of cardiovascular events, possibly through inflammatory pathways, but the involved mechanisms and mediators are largely unknown. The overall aim of this thesis is to explore the links between S100A8/A9 and S100A12, sRAGE, sEMMPRIN and atherosclerosis progression, cardiovascular (CV) risk and post-ACS complications.

In **Paper I**, we investigated the associations between plasma sRAGE, sEMMPRIN, and incident cardiovascular disease and mortality in 4612 CV disease (CVD)-free individuals from the CV arm of the population-based Malmö Diet and Cancer study (MDC-CV). The biomarkers were measured at study inclusion, while carotid intima media thickness (IMT) progression and incident coronary events and mortality were assessed during follow-up (median follow-up time was 16.5 years for IMT and 21.5 years for clinical events). We found that high sRAGE was associated with slower IMT progression and decreased major adverse coronary events (MACE) and mortality.

In **Paper II** we investigated the potential links between genetic determinants of sRAGE levels in plasma and CVD development and CV event risk in the general population. We performed a genome-wide association study (GWAS) in 4192 individuals from the MDC-CV cohort. We found that the minor alleles of two single nucleotide polymorphisms (SNPs), rs2070600 and rs204993, were independently associated with lower plasma sRAGE. In 29245 individuals from the entire MDC-cohort, we found an independent association between the presence of the minor (T vs C) allele of rs2070600 and increased risk for first time MACE. Rs204993 was not associated with MACE. Neither SNP was associated to IMT progression.

In **Paper III** we measured sRAGE and S100A12 at baseline in 524 ACS patients, and again 6 weeks later in a subgroup of 114 individuals that also completed a follow-up echocardiography at 1 year. Incidence of recurrent coronary events and heart failure were recorded during a median follow-up of 26 months. High baseline S100A12 and sRAGE were associated with increased risk of recurrent MACE, low or deteriorating left ventricular function and an increased incidence of heart failure. In contrast, patients with increasing sRAGE at 6 weeks compared to baseline had lower incidence of recurrent ACS, possibly suggesting a protective role.

In **Paper IV** we examined the S100A8/A9 response to stress in CAD patients compared to healthy controls. S100A8/A9 was measured before and immediately after a psychological stress test in 60 CAD patients, and at 24 h after the stress test in 27 CAD patients and 28 controls. Saliva cortisol was measured before and 20 min after the stress test in all participants. Acute psychological stress induced a rapid release of S100A8/A9, and plasma S100A8/A9 remained elevated for at least 24h. The sustained S100A8/A9 response at 24h was associated with a poor cortisol response to stress in patients, but not in controls.

Key words S100A12, S100A8/A9, sRAGE, IMT, MACE, post-ACS prognosis, phychological stress

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1. **Grauen Larsen H**, Marinkovic G, Nilsson PM, Nilsson J, Engström G, Melander O, Orho-Melander M, Schiopu A. High plasma sRAGE (soluble receptor for advanced glycation end products) is associated with slower carotid intima media thickness progression and lower risk for first-time coronary events and mortality. This is a non-final version of an article published in final form in *Arterioscler Thromb Vasc Biol. 2019;39:925-933.* DOI: 10.1161/ATVBAHA.118.312319.
2. **Grauen Larsen H**, Sjögren M, Engström G, Nilsson PM, Orho-Melander M, Nilsson J, Mellander O, Schiopu A. The Gly82Ser polymorphism in the receptor for advanced glycation end products is associated with increased risk for coronary events in the population. *Submitted manuscript.*
3. **Grauen Larsen H**, Yndigegn Y, Marinkovic G, Grufman G, Mares RG, Nilsson J, Goncalves I, Schiopu A. The soluble receptor for advanced glycation end-products (sRAGE) has a dual phase-dependent association with residual cardiovascular risk after an acute coronary event. *Atherosclerosis. 2019;287:16-23.* DOI: 10.1016/j.atherosclerosis.2019.05.020. Original article included.
4. Jonasson L, **Grauen Larsen H**, Lundberg AK, Gullstrand B, Bengtsson AA, Schiopu A. Stress-induced release of the S100A8/A9 alarmin is elevated in coronary artery disease patients with impaired cortisol response. *Sci Rep. 2017;7:17545.* DOI: 10.1038/s41598-017-17586-6. Original article included.

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Marinkovic G, **Grauen Larsen H**, Yndigegn T, Szabo IA, Mares RG, de Camp L, Weiland M, Tomas L, Goncalves I, Nilsson J, Jovinge S, Schiopu A. Inhibition of pro-inflammatory myeloid cell responses by short-term S100A9 blockade improves cardiac function after myocardial infarction. *Eur Heart J. 2019;40:2713-2723.* DOI: 10.1093/eurheartj/ehz461

Abbreviations

CVD	Cardiovascular disease
CAD	Coronary artery disease
PAD	Peripheral artery disease
PCI	Percutaneous coronary intervention
CABG	Coronary artery by-pass graft
LDL	Low density lipoproteins
AIT	Adaptive intimal thickening
MMP	Matrix metalloproteinase
MI	Myocardial infarction
DAMP	Danger-associated molecular patterns
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
STEMI	ST-elevation myocardial infarction
NSTEMI	Non-ST-elevation myocardial infarction
ESC	European Society of Cardiology
LVEF	Left ventricular ejection fraction
ECG	Electrocardiogram
NT-proBNP	N-terminal pro-B type natriuretic peptide
hsCRP	High sensitive C-reactive protein
MPO	Myeloperoxidase
HPA axis	Hypothalamus-pituitary-adrenal axis
NETs	Neutrophil extracellular traps
EN-RAGE	Extracellular newly-identified receptor for advanced glycation end-products binding protein
IMT	Intima media thickness
CV	Cardiovascular
RAGE	Receptor for advanced glycation end products
TLR 4	Toll like receptor 4

AGEs	Advanced glycation end products
HMGB1	High mobility group box 1 protein
EMMPRIN	Extracellular matrix metalloproteinase inducer
sRAGE	soluble RAGE
sEMMPRIN	soluble EMMPRIN
MDC	The Malmö Diet and Cancer study
MDC-CV	The Malmö Diet and Cancer study - cardiovascular sub-cohort
GWAS	Genome-wide association study
MACE	Major adverse coronary event (Paper I and II)
	Major adverse cardiovascular event (Paper III)
ACS	Acute coronary syndrome
SNP	Single nucleotide peptide
LD	Linkage disequilibrium
LVESV	Left ventricle end systolic volume
LVEDV	Left ventricle end diastolic volume
eGFR	Estimated glomerular filtration rate
MDRD	Modification of diet in renal disease formula
BMI	Body mass index
TG	Triglycerides
HDL	High density lipoprotein
MAF	Minor allele frequency

Introduction

Inflammation in cardiovascular disease

Cardiovascular disease

Cardiovascular disease (CVD) is a broad concept that includes the diseases of the heart and the blood vessels. CVD includes a wide range of diseases such as acute and chronic coronary artery disease (CAD), stroke, aortic disease, peripheral artery disease (PAD), arrhythmias, heart failure, cardiomyopathies, cardiac valvular disease and thromboembolic disease (Figure 1).

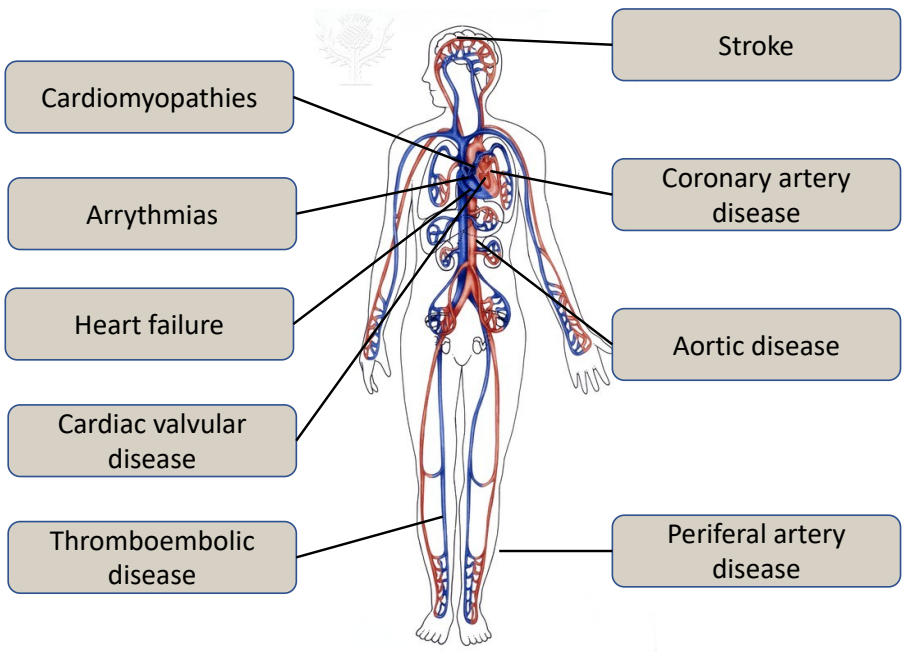

Figure 1. Cardivascular disease – a wide spectrum of diseases

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The vast majority of CVD mortality is caused by CAD and stroke, together responsible for 26.7 % of all deaths worldwide[1]. CVD pathogenesis is heterogeneous, involving a wide spread of underlying pathological processes. However, the most common underlying pathology of CVD is atherosclerosis, that can cause several of the diseases in the CVD spectra, such as CAD, aortic disease and PAD, often simultaneously in the same individual. This thesis will focus on atherosclerotic CVD, with a special focus on CAD.

CAD, also called ischemic heart disease or coronary heart disease, refers to heart conditions that are caused by insufficient oxygen supply to the heart muscle, the myocardium, because of narrowed or occluded coronary arteries leading to myocardial ischemia[2](Figure 2).

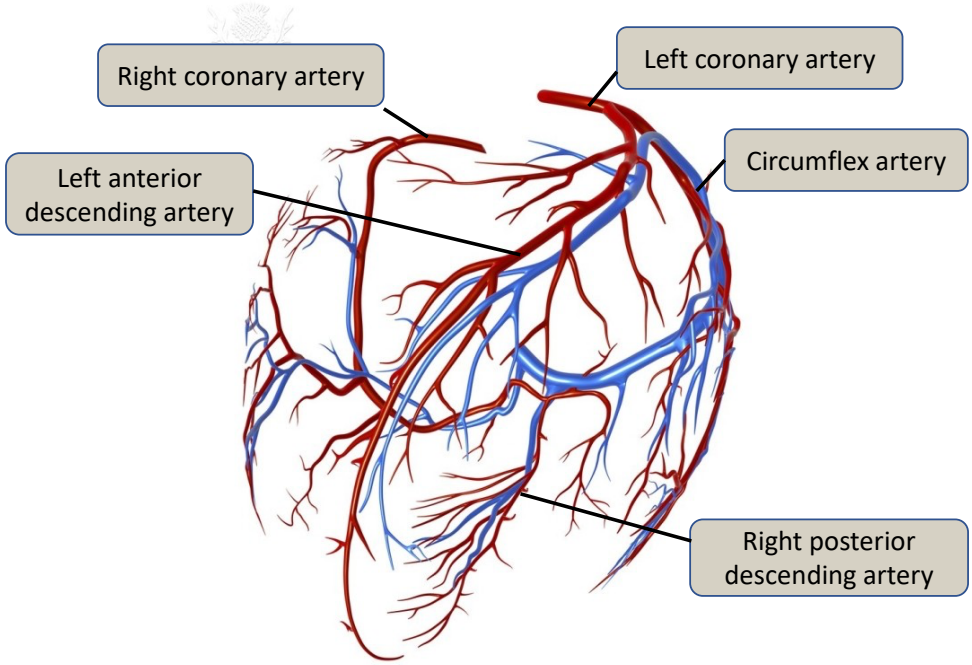

Figure 2. The coronary vasculature

The vessels that supply the heart muscle, the myocardium, with blood. Red vessels are arteries and blue vessels are veins. Modiified and reproduced with permision from Britannica Image Quest, PASIEKA ©Science Photo Library

CAD is the leading cause of death globally in both men and women[3], responsible for 16% of all deaths worldwide[1], causing one death every 4th seconds. Atherosclerosis is the most common underlying cause of CAD, and myocardial infarction (MI) is the most frequent clinical presentation[4]. CAD incidence and consequences has decreased during the last 30 years due to improved primary

prophylaxis with better risk factor management, better treatments such as percutaneous coronary intervention (PCI) and improved coronary by-pass surgery (CABG) techniques, and better secondary prophylaxis treatments such as improved platelet inhibitor medications[5]. In spite of this, CAD still represent a considerable cause of morbidity and mortality. CAD is responsible for 19 % of all deaths in men and 20 % in women in the European population, causing 17 % of all premature deaths before age of 75, and 14 % of deaths before age 65[6]. This makes CAD a highly relevant research area.

Atherosclerosis and inflammation

The normal arterial wall consists of three layers (Figure 3). The innermost layer, the tunica intima, contains under normal condition only one single layer of endothelial cells, connective tissue and the internal elastic membrane. The tunica media mainly contains smooth muscle cells and extracellular matrix components such as collagen and elastin fibres. The outer layer, the tunica adventitia, contains the external elastic membrane, nerve endings, small vessels (vasa vasorum) supplying blood to the outer part of the tunica media, and mast cells.

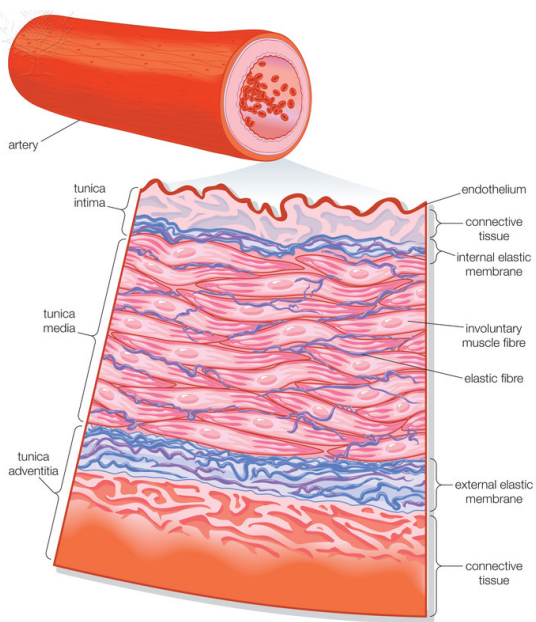

Figure 3. The normal arterial wall

Schematic picture of the three layers of the normal vessel wall. The innermost layer, tunica intima, contains one layer of endothelial cells and connective tissue. The midle layer, tunica media, mainly contains smooth muscle cells and extracellullar matrix components such as collagen and elastin fibres. The outer layer, the tunica adventitia, contains nerve endings, small vessels supplying blood to the outer part of the tunica media (vasa vasorum) and mast cells. Reproduced with permission from Britannica Image Quest, ©Encyclopaedia Britannica

The atherosclerotic process starts with accumulation of low-density lipoproteins (LDL) inside the intima (Figure 4). Here, LDL can undergo oxidation and other modifications[7]. These modifications make the lipoproteins pro-inflammatory and immunogenic, leading to activation of leukocytes and endothelial cells. The endothelial cells upregulate adhesion molecules that bind and slow down circulating immune cells (monocytes, neutrophils, and T-lymphocytes) on the inside surface of the vessel[8]. The immune cells receive chemoattractant signals from the endothelial cells and other cellular components and enter the intima[7, 8]. Inside the intima, monocytes mature into macrophages and express scavenger receptors (Figure 4). The macrophages recognize the modified lipoproteins accumulated inside the intima and begin a clean-up process. The scavenger receptors bind to the modified lipoproteins, leading to phagocytosis and creating foam cells. The majority of the lipid-laden foam cells stem from mononuclear phagocytes, but endothelial cells and smooth muscle cells can also accumulate lipids[9]. The accumulated immune and inflammatory cells produce and release inflammatory mediators in the vessel wall, further activating the endothelial cells and leading to increased endothelial permeability, production of more pro-inflammatory mediators and platelet aggregation[8]. A positive feed-back loop of chronic, low grade inflammation inside the vessel wall is created, leading to continuous recruitment of inflammatory cells and increasing the inflammatory activity.

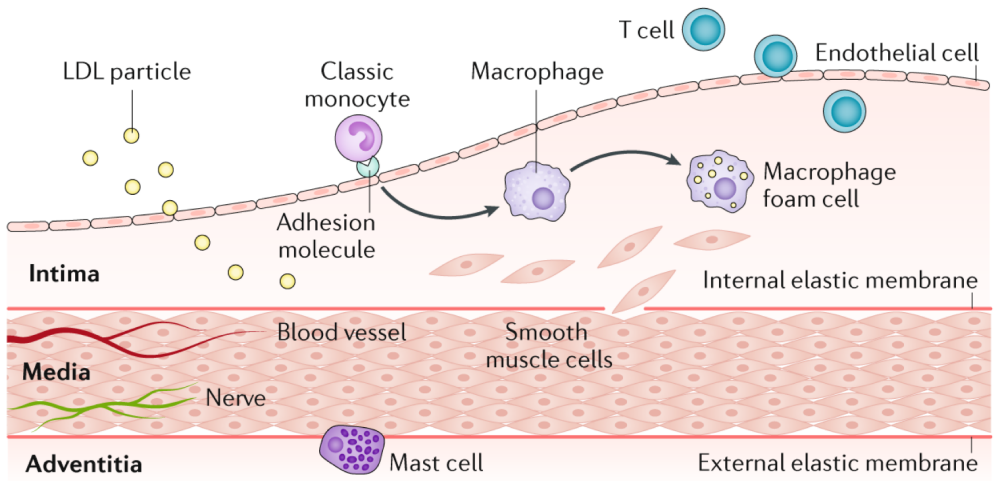

Figure 4. Initiation and progression of ahterosclerosis

The atherosclerotic plaque forms in the tunica intima. LDL particles accumulate and undergo oxidation and other modifications. LDL becomes pro-inflammatory and immunogenic and induces an activation of the endothelial cells. The activated endothelial cells express pro-inflammatory mediators, which attract immune cells such as monocytes, T-lymfocytes and neutrophils into the intima. Here, the monocytes mature into macrophages, which start to engulf LDL by phagocytosis and become lipid-laden foam cells. The smooth muscle cells enter the intima from the tunica media, attracted by the pro-inflammatory signals, lose their contractility and transform into myofibroblasts focused on collagen fiber production. Reproduced with permission from Libby et al.[7] ©Springer Nature.

Atherosclerotic lesions are characterized by progressive thickening of the intimal layer, started by an accumulation of foam cells and inflammatory cells in the intima. The accumulated foam cells can be seen macroscopically as fatty streaks in early atherosclerotic lesions (Figure 5). This is followed by migration of smooth muscle cells from the tunica media in response to pro-inflammatory mediators[7] (Figure 4 and 5). The smooth muscle cells alter their function when entering the intima, losing their contractility and transforming into myofibroblasts focused on extracellular matrix production[10]. The accumulation of lipids, cells and extracellular matrix in the intima leads to adaptive intimal thickening (AIT).

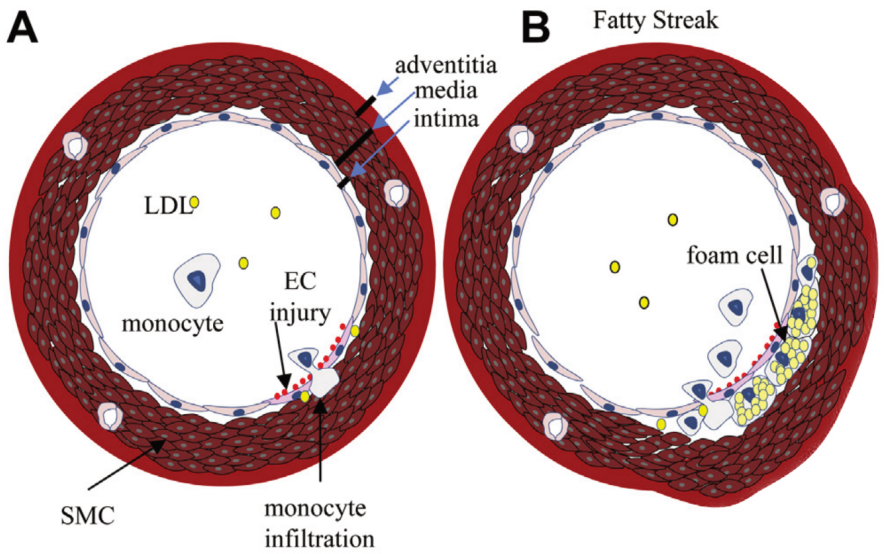

Figure 5. The initiation of an atherosclerotic lesion

The atherosclerotic process starts with accumulation of LDL in the tunica intima, where they undergo oxidation and other modifications. The activated endothelial cells increase their permeability and start attracting myeloid cells, such as neutrophils and monocytes (A). Inside the intima, the monocytes mature into macrophages, start expressing scavenger receptors that recognize the LDL particles and begin to engulf them by phagocytosis. The accumulated foam cells can be seen macroscopically as fatty streaks (B). These changes can regress if the triggering factors are removed. LDL, low density lipoproteins; EC, endothelial cells; SMC, smooth muscle cells. Reproduced with permission from Zeadin et al.[11] ©Elsevier.

Foam cells eventually undergo programmed cell death, called apoptosis. The apoptotic cells will be engulfed by other mononuclear phagocytes, in attempt to remove them before their cell membrane integrity is broken, thereby preventing the cytoplasmic content to leak out into the tissue[9, 12]. This process is called efferocytosis and is vital to the cell clearance process. Efferocytosis has been found to be defective in atherosclerotic plaques[12, 13], especially in more advanced lesions. As the lesion progresses, the removal of apoptotic cell becomes less efficient, and

the apoptotic inflammatory cells cannot be engulfed before they lose the cell membrane integrity, resulting in leakage of pro-inflammatory and proteolytic substances into the matrix. The dying macrophages and smooth muscle cells release pro-inflammatory mediators and pro-coagulant tissue factor into their surroundings, which increases the inflammatory drive and has the potential to induce the formation of a thrombus in case the plaque ruptures[8, 9]. Accumulation of extracellular lipoproteins and cellular debris in the growing atherosclerotic plaque begins to form a necrotic core (Figure 6). The necrotic core is covered to the luminal side by a fibrous cap containing smooth muscle cells and collagen fibres, which stabilizes the lesion[14].

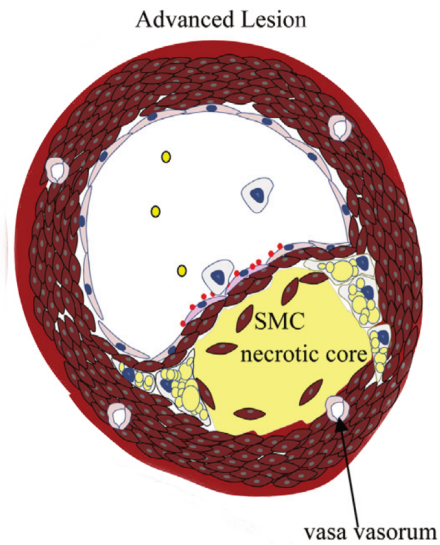

Figure 6. The advanced atherosclerotic lesion

The accumulation of lipoproteins and cellular debris forms a necrotic core inside the lesion. A fibrous cap containing smooth muscle cells and collagen fibers covers the necrotic core, stabilizing it and preventing it from rupturing into the vessel lumen. SMC, smooth muscle cells. Reproduced with permission from Zeadin et al.[11] ©Elsevier.

The macrophages in the atherosclerotic lesion produce enzymes, matrix metalloproteinases (MMPs), that can break down collagen fibres. Increased MMP production will weaken the fibrous cap and increase the risk of the plaque to rupture[8]. Thus, increased inflammatory activity will increase the risk of plaque rupture. T-lymphocytes are also accumulating in the lesion, however in much lower numbers than monocytes/macrophages. T-cells play a pathogenic or a regulatory role of the inflammatory activity in the plaque[8].

In summary, atherosclerosis is characterised by a chronic, low grade inflammation in the arterial wall, initiated by lipid accumulation and amplified by inflammatory

cells and their mediators[8]. There are several potential triggers of this process. Atherosclerosis often starts in places where the arteries are dividing, which creates a turbulent flow. The turbulence can activate the endothelial cells, inducing them to produce proinflammatory mediators, recruit leukocytes and increase the permeability of the endothelium. The lipid accumulation inside the vessel wall increases proportionally to the systemic LDL concentration, and circulating LDL levels are a strong predictive risk factor for atherosclerosis progression and event risk[15]. Other classical risk factors for CVD are hypertension, visceral obesity, smoking and diabetes mellitus. These factors increase atherosclerosis progression and event risk through mechanisms that are intensely studied but not entirely elucidated. They have all been shown to activate inflammatory pathways, increasing the number of inflammatory cells and/or pro-inflammatory mediators in the circulation[7, 16]. This inflammatory reaction can further activate the endothelial cells and increase the inflammatory activity in pre-existing atherosclerotic plaques[7] (Figure 7).

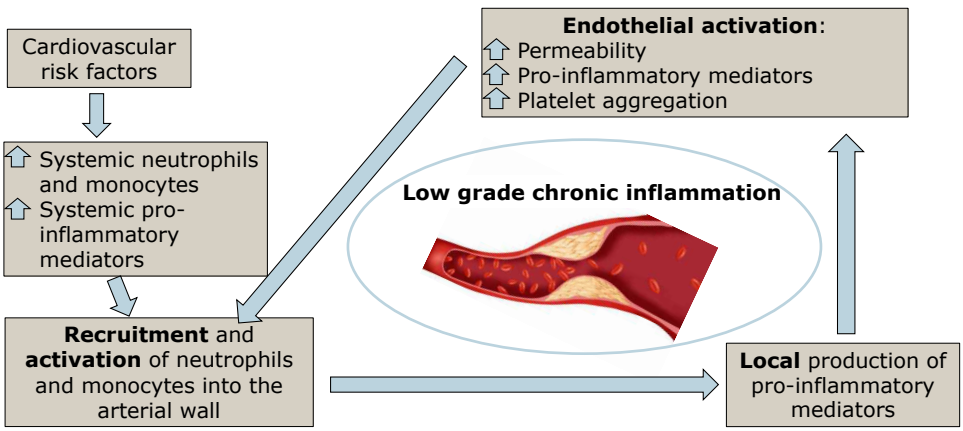

Figure 7. Cardiovascular risk factors and atherosclerosis

A schematic figure of the positive feed-back loop of inflammation in atherosclerosis, initiated by cardiovascular risk factors. The illustration of the artery is used with permission från Britannica Image Quest, ©Photo researchers.

Genetic predisposition is also an important risk factor in CVD. Genetic studies have revealed that any mutation leading to lower LDL levels also lowers the risk of atherosclerotic CVD[15]. The attempts to establish a genetic risk score for CVD prediction have been hampered because of the difficulty of reaching sufficient predictive power, due to the large number of mutations and the relatively low impact of each of the individual mutations. Broader approaches using polygenetic risk scores have been attempted [17]. The criticism that has been raised questions how the result of such a genetic risk score would impact clinical management, and whether

it would demotivate individuals with a non-risk genetic profile to maintain a healthy lifestyle.

As the atherosclerotic lesion progresses, the growing plaque narrows the lumen of the artery, diminishing the blood flow. The tissue irrigated by the insufficient artery will progressively suffer from lack of oxygen and nutrients. In the heart, narrowing of the coronary arteries causes hypoxia in the myocardium (Figure 8). This will classically be experienced as chest pain, which can be associated with dyspnoea, and radiation of the pain to arms, jaws, back or neck and nausea. This symptom panel is called angina pectoris. It is often initiated during physical activity, when the oxygen demand in the myocardium increases above the maximum blood supply provided by the narrowed artery, and disappears at rest. This is called stable angina pectoris. However, if the obstruction is pronounced or complicated by a thrombus the symptoms can also occur at rest, termed unstable angina pectoris.

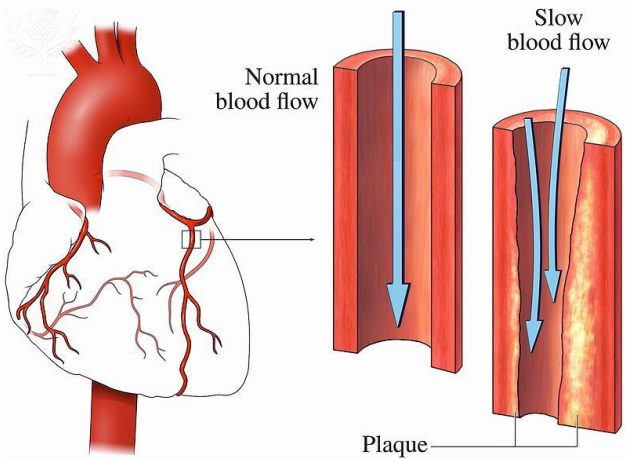

Figure 8. Narrowed lumen in a coronary artery

As the atherosclerotic plaque grows, the lumen of the artery narrows and decreases the blood flow, which induces angina pectoris or myocardial infarction. Reproduced with permission from ©Science Photo Library.

As the necrotic core of the lesion grows, it becomes more unstable. Increased inflammatory activity weakens the protective fibrotic cap, which may induce a plaque rupture. Following plaque rupture, the content of the necrotic core gets in contact with the blood inside the vessel lumen. The high concentration of tissue factor and other inflammatory mediators in the tissue induces platelet aggregation and thrombosis (Figure 9). The thrombus causes an acute obstruction of coronary blood flow, leading to MI. The size of the infarction, i.e. how much of the myocardium is damaged, depends on whether the vessel is completely or

incompletely occluded, and how peripheral in the coronary tree the obstructed vessel is located.

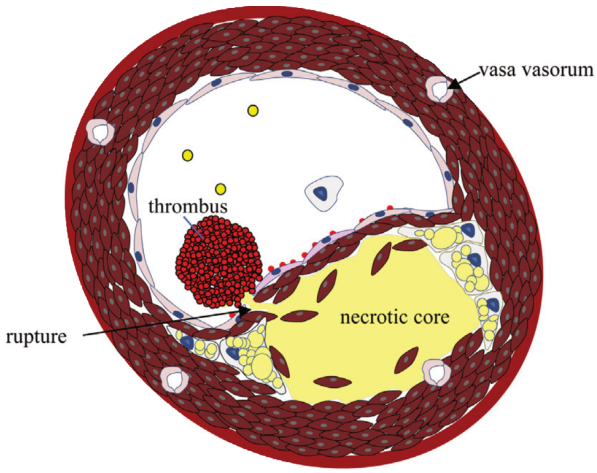

Figure 9. Atherosclerotic plaque rupture

As the nectrotic core grows, it becomes more instable. The increased inflammatory activity will weaken the cap and may cause the plaque to rupture. When the necrotic core content gets in contact with the blood inside the vessel lumen, the inflammatory mediators in the necrotic core will induce formation of a thrombosis, further narrowing the lumen or occluding it completely, causing an MI. Reproduced with permission from Zeadin et al.[11] ©Elsevier.

Myocardial infarction and inflammation

Myocardial infarction (MI) constitutes the necrotic damage to the cardiac muscle due to permanent or temporary ischemia secondary to restriction of coronary blood flow. The angina pectoris triggered by an MI is often more intense and the symptoms are often not terminating without medication if the occlusion persists. Myocardial ischemia causes necrosis of the cardiomyocytes, with the following loss of function in the respective area. It also impairs endothelial cell integrity and increases vessel wall permeability, thereby promoting leukocyte infiltration[18]. The necrotic and ischemic cardiomyocytes and the damaged intercellular matrix will passively release substances signalling tissue damage, so called danger-associated molecular patterns (DAMPs) or alarmins. DAMPs quickly trigger recruitment of a first wave of inflammatory leukocytes, primarily neutrophils. The infiltrating leukocytes secrete more DAMPs, amplifying the inflammatory response. DAMPs bind to pattern recognition receptors (PRRs) on infiltrating leukocytes and endothelial cells, further activating the production of pro-inflammatory mediators such as cytokines, chemokines and adhesion molecules. In this manner, myocardial

ischemia and necrosis induce potent innate immune responses, involving rapid leukocyte recruitment and activation. The inflammatory reaction further increases myocardial damage (Figure 10).

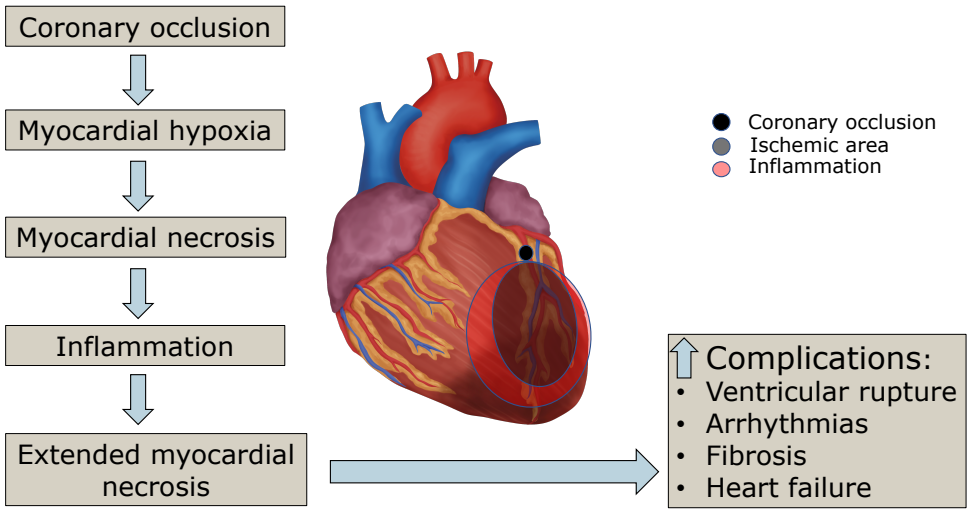

Figure 10. Myocardial infarction

Schematic flow chart of the inflammatory response after a myocardial infarction, increasing the risk of complications. The illustration of the heart: Frida Nilsson, Media-Tryck.

The standard treatment today is reperfusion therapy, most commonly in the form of PCI, and sometimes CABG surgery. PCI implies opening the vessel with an endovascular technique involving balloon angioplasty and often stenting. CABG is open surgery, where the coronary circulation is re-established by using a vessel graft from another part of the body, redirecting the blood flow to the coronary artery below the narrowed or occluded part. According to guidelines, in cases where large distances or lack of resources do not permit these interventions inside a time window of maximum 2 hours, fibrinolytic treatment is performed to resolve the thrombosis medically. Importantly, re-establishing the blood flow after a period of closure may further damage the cardiac tissue via the reperfusion injury mediated by rapid release of reactive oxygen species (ROS) that further increase the myocardial damage and inflammation[18].

The magnitude of the myocardial damage and the subsequent inflammatory responses impact the complications following an MI. Pronounced myocardial necrosis will lead to loss of function in the necrotic area of the myocardium, leading to post-ischemic heart failure and increased risk of post-ischemic arrhythmias. The potent immune response creates an inflammatory echo in other pre-existing atherosclerotic plaques in the coronary tree[9]. It has been shown that the

inflammatory monocytes that infiltrate the heart in large numbers after an MI also increase the vulnerability of non-culprit (not the lesion causing the current coronary event) coronary atheromas, promoting plaque rupture and recurrent ischemic events[19]. Thus, an MI increases the risk of recurrent MIs through immune and inflammatory activation (Figure 11).

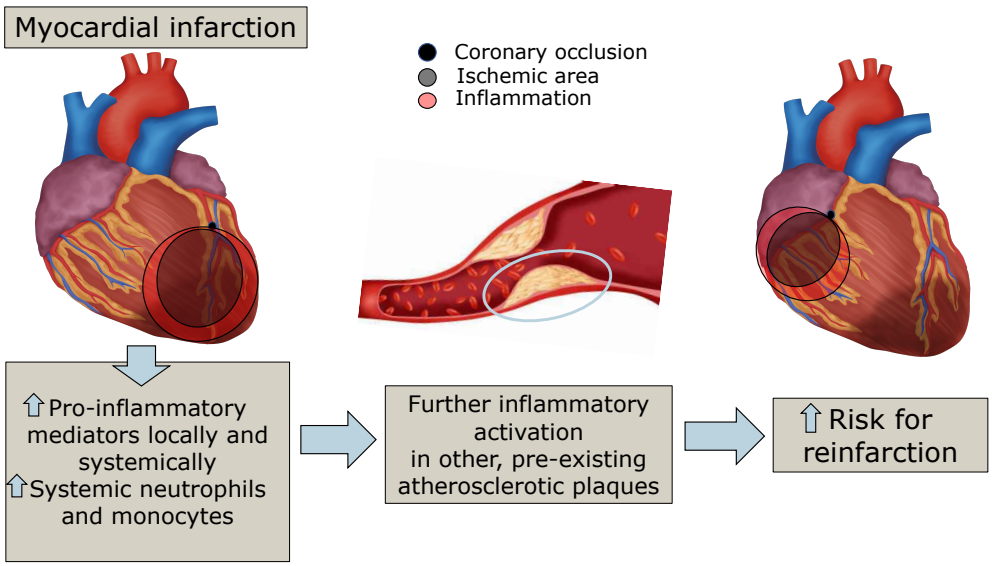

Figure 11. Schematic picture of the mechanisms by which an MI increases the risk for recurrent infarctions

The coronary occlusion leads to ischemia in the myocardial region supplied by the respective artery. Ischemia induces necrosis and a potent inflammatory reaction, leading to increased levels of leukocytes and pro-inflammatory mediators in blood. This creates an inflammatory echo in the coronary tree, leading to inflammation in other pre-existing plaques, and thereby increasing the risk of recurrent infarctions. The illustration of the artery is used with permission från Britannica Image Quest, ©Photo researchers. The illustration of the heart: Frida Nilsson, Media-Tryck.

Post-myocardial infarction prognosis

The size of the necrotic area in the myocardium is crucial for the development of complications after an MI. The larger the area of necrotic tissue, the higher the risk to develop complications such as heart failure, arrhythmias and sudden cardiac death. As mentioned, MI also increases the risk of recurrent MI and stroke, due to increased systemic inflammatory drive, that increases the inflammatory activity in pre-existing plaques at other sites of the artery tree[19]. The short-term (30 days) mortality by any cause after an MI lays around 5%, with some variability depending on the type of infarction[20]. The short term mortality for the transmural, ST-segment elevation myocardial infarction (STEMI) has been found to be as high as 13%, as it induces a larger damage on the myocardium[21]. The risk of sudden cardiac death, i.e. unexpected death caused by sudden cardiac arrest, is highest the first years after the

index event, with especially high risk during the first three months[22, 23]. Sudden cardiac death is most commonly due to a recurrent MI, acute arrhythmias or heart failure. The risk of life-threatening arrhythmias such as ventricular fibrillation or ventricular tachycardia is highest during the first hours after the infarction and declines thereafter, but remains elevated for months to years, if not indefinitely[24, 25]. A large study of in-hospital complications in 24097 non-STEMI (NSTEMI) patients, showed that acute heart failure or shock affected about 10% of the survivors and recurrent MI affected 2.4% during the index hospital stay[26]. In total, 19.1% of the NSTEMI patients had an in-hospital adverse event, defined as reinfarction, arrhythmia, congestive heart failure, major bleeding, stroke or death[26].

However, mortality after MI has declined over the last 30 years, both in the short-term and long-term perspective, due to reperfusion treatment strategies and improved secondary preventive treatments such as betablockers, statins and novel platelet inhibitors[27] (Figure 12).

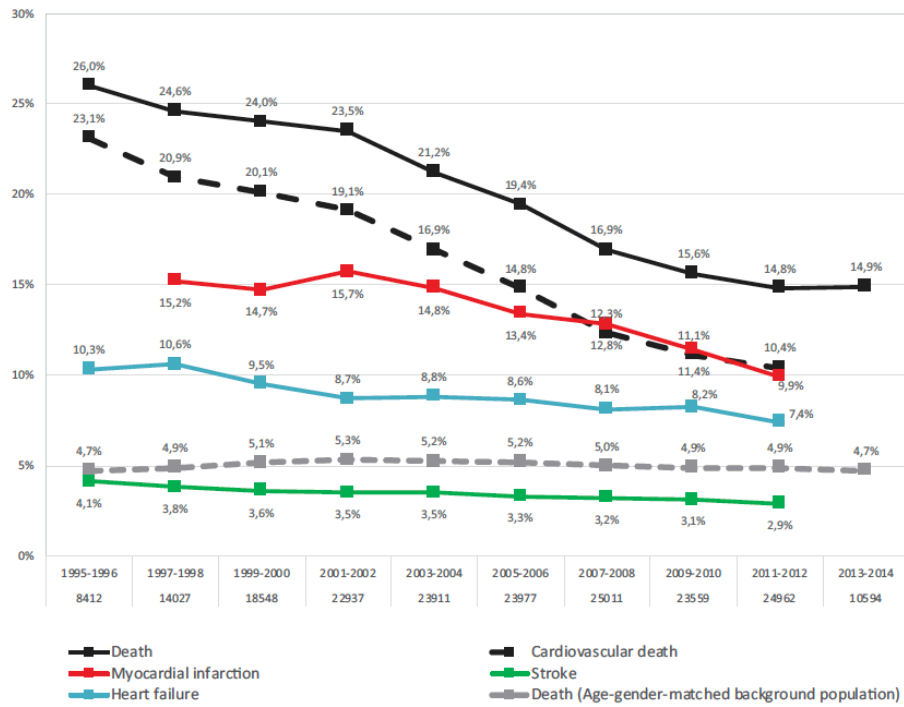

Figure 12. 1 year outcomes post-STEMI 1995-2014

Reproduced from Szummer et al.[27] with permission ©Oxford University Press.

Still, MI survivors are at an increased risk for both death and reinfarction compared to the general population[28]. The risk of death by any cause is 2 times higher for first time MI survivors compared to general population, and 3 times higher for recurrent MI survivors[28]. The risk of a recurrent MI has been found to be highest during the first year after the infarction, with a cumulative risk at 1 year of 5.6% for men and 7.2% for women, and at 7 years of 13.9% and 16.2% for men and women respectively[28]. The risk of stroke has been found to be 1-2 % during the first 3 years after a PCI-treated STEMI [29].

Another long-term complication that causes increased morbidity and mortality after MI is heart failure. About 18% of MI patients suffer from post-MI congestive heart failure[30]. The diagnostic criteria of heart failure according to the European Society of Cardiology (ESC) guidelines are described below[31] (Figure 13). The left ventricular ejection fraction (LVEF), i.e. the volumetric fraction of blood ejected from the left ventricle with each heartbeat contraction, measured by echocardiography, is a very important measurement in heart failure grading.

Type of HF	Criteria 1	2	3
HFrEF	Symptoms and signs	LVEF <40%	-
HFmrEF	Symptoms and signs	LVEF 40-49%	1. Elevated NT-proBNP 2. At least one of below: - Structural heart disease (LVH and/or LAE) - Diastolic dysfunction
HFpEF	Symptoms and signs	LVEF ≥50%	1. Elevated NT-proBNP 2. At least one of below: - Structural heart disease (LVH and/or LAE) - Diastolic dysfunction

Figure 13. The simplyfied diagnostic criteria of heart failure from the European Society of Cardiology, ESC[31]

HF, heart failure; HFrEF, heart failure with reduced ejection fraction; HFmrEF, heart failure with mid-range ejection fraction; HFpEF, heart failure with perserved ejection fraction.
LVEF, left ventricular ejection fraction, i.e. the volumetric fraction of blood ejected from the left ventricle with each heartbeat contraction.
LVH, left ventricular hypertrophy; LAE, left atrial enlargement.
NT-proBNP, N-terminal pro-B type natriuretic peptide.

Heart failure increases the mortality risk after MI, especially in patients with LVEF <35%[22, 23]. In a large study from 2005 including >14000 patients with post-MI heart failure, 7% suffered sudden cardiac death during a median follow-up of 180 days, and 18 % of these were resuscitated. The risk was highest in the first 30 days after infarction, with a risk of sudden cardiac death of 1.4% per month[32]. The rate of arrhythmic death during the first 6 months after an MI was found to be 8% for

patients with LVEF <40% or frequent ventricular ectopy[33], i.e. premature ventricular depolarization initiated in the ventricles rather than from the sinus node.

In order to reduce the risk of post-MI complications, it is vital to identify the patients with high risk to have a poor prognosis, and to improve prognosis by treating known risk factors. This is called secondary prevention, i.e. preventive treatment post-MI with the aim to prevent recurrent infarction, heart failure, arrythmias and, ultimately, death. Different methods are used to stratify residual risk in MI patients. One method is echocardiography, i.e. ultrasound examination of the heart, which all patients are recommended to undergo during the hospital stay. This examination measures how the myocardium and the heart valves are functioning. The definition and classification of heart failure are based on echocardiography, clinical symptoms and signs, and on the biomarker NT-proBNP (or BNP) measured in the circulation (Figure 13). Heart failure is divided into three categories, with different treatment managements. Heart failure with reduced and mid-range LVEF (HFrEF and HFmEF) can be a result of MI, and benefits from classical heart failure medication, initiated during the hospital stay and titrated to target doses after discharge. Heart failure with preserved LVEF (HFpEF) is a condition characterized by decreased elasticity of the myocardium, leading to increased filling pressure of the ventricle during diastole (the filling phase of the heartbeat cycle). This is usually not a result of the MI, but most commonly due to a long-term insufficiently regulated hypertension. Patients with confirmed post-MI heart failure with a LVEF<35% during the hospital stay are recommended heart failure medication and an echocardiographic re-examination after about 3 months. The aim of the re-examination is to find the patients that in spite of optimized heart failure treatment show persistently reduced LVEF <35%, since these patients are at higher risk of sudden cardiac death and are recommended to receive an implantable cardioverter defibrillator (ICD).

Another method used to aid secondary prevention is the measurement of biomarkers. They are used both to evaluate prognosis and in the management of known CVD risk factors. A biomarker used for risk factor management is LDL. High LDL concentration in the systemic circulation has a strong association to CVD progression and event risk[15], and post-MI patients are recommended aggressive lipid lowering treatment[34]. Blood glucose and glycosylated haemoglobin (HbA1c, used to reflect average blood glucose the past three months) are used to find patients with undetected diabetes mellitus and optimize the antidiabetic treatment in post-MI patients with diabetes, since diabetes and poor blood glucose control are associated with CVD risk[35, 36]. Other risk factors measured and addressed within the secondary preventive aim are blood pressure, overweight, sedentarism and smoking.

In spite of risk factor management, a subgroup of MI patients still have a poor prognosis. It is very important to identify these patients, in order to be able to optimize treatment and improve their outcome. The resources are not sufficient to be able to follow-up all post-MI patients with an echocardiography, or even for all

patients to see a cardiologist. Most patients are followed by a nurse at the cardiology ambulatory during the first year after the MI, and hereafter the patient is transferred to the general practitioner. Only the patients with an in-hospital LVEF<35% are followed with a new echocardiography, and only the patients with symptoms or poor risk factor management are seen by a cardiologist at the initiative of the nurse. However, there are patients that develop clinical heart failure symptoms in spite of an in-hospital LVEF above 35% and adequate treatment, or suffer new infarctions in spite of good risk factor management. The vital question is how to prevent this from happening.

All patients take new blood samples 6 weeks after admission from the hospital, to control blood lipids and glucose metabolism. Unfortunately, we lack a specific biomarker that could be measured at this time point and give indications about which patients are at high risk of reinfarctions and heart failure. This would help us initiate a closer follow-up and more intensive risk factor control for these patients.

Biomarkers – definition and importance

A biomarker is a measurable indicator of a state or condition, measured to evaluate normal biological processes, pathogenic processes or pharmacologic responses to therapeutic intervention. Basically, a biomarker is a variable measurable in a biological fluid, to evaluate an ongoing process inside the body. A biomarker can be an active player in the process, a consequence of the process, or a bystander variable that is influenced by a certain process. A biomarker can also be an inactive biproduct of active events, for example a degradation product of an active substance.

A perfect biomarker for atherosclerosis should be able to detect disease activity and progression, and to detect the risk for events before any event has occurred. However, no such biomarker exists at the moment. The biomarkers used in the clinic upon presentation with an MI are cardiac biomarkers, measuring the extent of cardiac injury. A prefect biomarker should have high sensitivity and specificity (Figure 14). The sensitivity is the true positive rate, i.e. the ability of the biomarker to find all the individuals with the disease. The specificity is the true negative rate, i.e. the ability of the biomarker to correctly identify healthy people as not having the disease. When establishing the correct cut-off of what values are considered as inside and outside the reference interval, it is necessary to find the best balance between the sensitivity and the specificity.

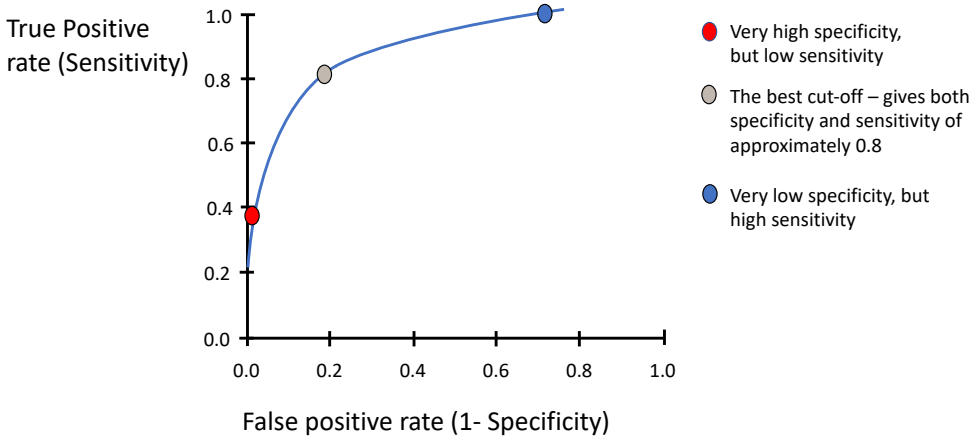

Figure 14. A ROC-curve visualizing the relationship between sensitivity and specificity

Choosing a cut-off for the reference interval for a biomarker depends on the balance between sensitivity and specificity. A cut off with a high sensitivity, i.e. the ability to find all the individuals with the disease, will also give many false positive results, i.e. a low specificity. On the other hand, a cut-off with a high specificity, i.e. the ability to only indicate disease in individuals with the disease, will miss many individuals with the disease, i.e. give a low sensitivity.

A sensitive cardiac biomarker should be able to detect even a small damage of the myocardium. It should also be specific for myocardium damage, and not be increased by damage of skeletal muscle or other organs, and should not be increased in patients without myocardial damage. It should preferably give information of the severity of the infarction and of the prognosis, show the reperfusion therapy result and distinguish between reversible and irreversible damage. It should be usable both in early and late diagnosis of myocardial injury, and it should be easy to measure, fast, cheap and stable enough to be stored[37, 38].

Cardiac biomarkers are needed in all stages of CAD – in predicting the risk of CAD development in healthy individuals, in the diagnosis process of MI and in the secondary prevention management after MI. A biomarker evaluating the risk of CVD development later in life in apparently healthy individuals could be useful for motivating a healthy lifestyle and timely treating CVD risk factors in order to try to prevent the disease from occurring. CAD is developing during many years and is often already manifest when the risk factors are detected and treated. When the first coronary event has happened, it is vital to find the patients with high risk of a poor prognosis, such as recurrent infarction, heart failure and sudden cardiac death, as previously discussed. Cardiac function development can be followed with echocardiography. Since the complication risk after an MI is elevated for several years, the optimal follow-up procedure would be to repeat the echocardiography at 1-year post-MI as a minimum. However, CAD is a common disease, affecting a substantial proportion of the population, and there are not enough resources to do this in all patients. In addition, there are no currently used biomarkers able to

measure myocardial disease progression or the inflammatory activity in the coronary vessels. Therefore, new biomarkers are needed to identify the patients with high risk of developing complications.

Clinical predictors of coronary events and cardiac prognosis

The measurement of the carotid intima media thickness (IMT) has been found to predict the incidence of coronary events in the population[39]. IMT is an ultrasonographic measurement of the thickness of tunica intima and tunica media in the far (deeper from the skin) wall of the artery[40], using high-resolution ultrasound (Figure 15).

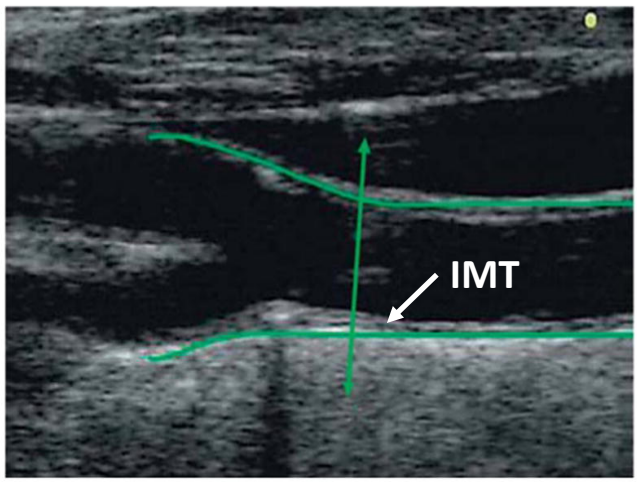

Figure 15. IMT measurement in the common carotid artery

Longitudinal view of the common carotid artery and bifurcation. The green double arrow marks the end of the common carotid artery and the beginning of the internal and external carotid arteries. The IMT is marked with a white arrow. Reproduced from Touboul et. al[41] with permission ©Karger Publishers

Subclinical alterations in the vessel wall proceed the clinical event and are visible with this technique in large arteries close to the skin. The carotid artery is the recommended site of IMT measurement according to the consensus statement[41]. The method is non-invasive, simple and widely available, and has been used as a surrogate marker for vascular disease in many clinical trials[42, 43]. Carotid IMT is a strong predictor of stroke, but has also been found to be a strong predictor of MI[39, 44, 45]. A meta-analysis including 8 large studies showed a relative risk of MI of 1.15 per 0.10-mm common carotid artery IMT difference, independent of age and sex [39]. However, IMT is not only a measure of early atherosclerosis, but also of smooth muscle hypertrophy/hyperplasia and the association between IMT and cardiac

events is non-linear. Currently, IMT measurement is not a method recommended for atherosclerosis screening in the clinic[41, 46, 47], and is mainly used in research.

In primary prevention, i.e. prevention of the first coronary event, the biomarkers used are also risk factors in themselves, such as LDL, high density lipoprotein (HDL), triglycerides (TG) and blood glucose. A risk score built on the combination of risk factors such as hyperlipidaemia, diabetes, hypertension, overweight and smoking habits is currently used in primary prevention practice[34, 47, 48], aiming to identify the individuals at a high risk to develop CVD (Figure 16). However, there is no currently available biomarker able to specifically reflect the extent of atherosclerosis in the coronaries and other vascular territories.

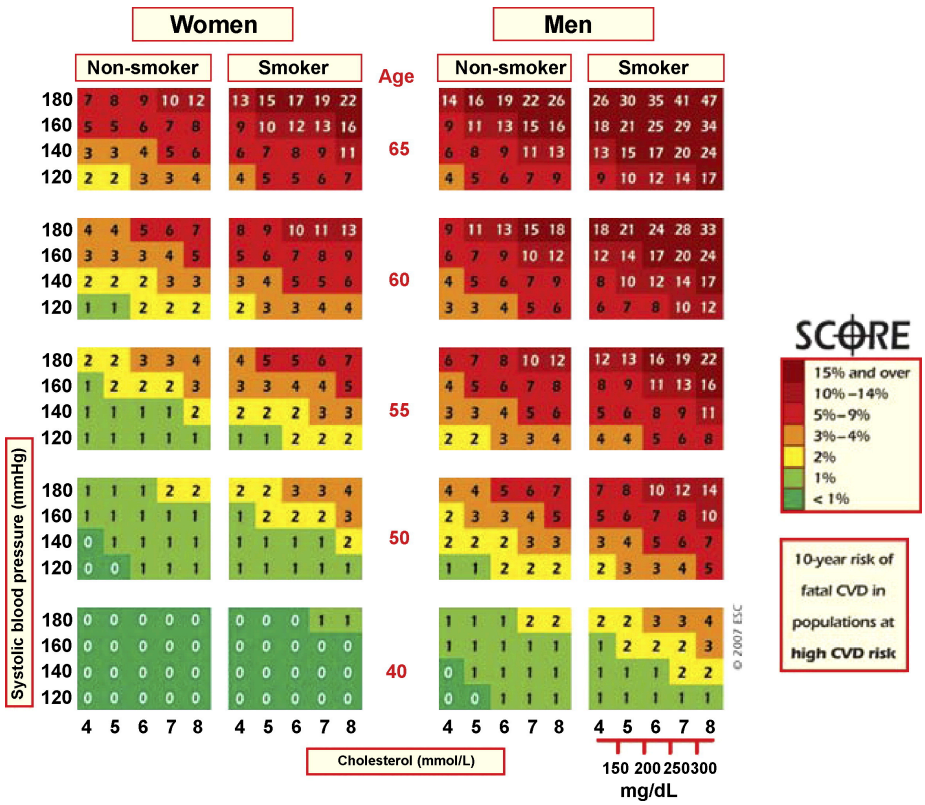

Figure 16. CVD risk score chart from ESC guidelines

Score of the 10-year risk of a fatal CVD based on risk factors[48]. Reproduced with permission from Graham et al.[37] ©Elsevier.

Coronary ischemia has several presentations – stable angina pectoris, unstable angina pectoris, non-ST elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI). The differential diagnosis of these is important for the decision of how to treat the patient. The three latter presentations (unstable angina, NSTEMI and STEMI) are all pathological entities included in the acute coronary syndrome (ACS), where the patient should be admitted to the hospital for expedited examination and treatment. Unstable angina implies an unstable situation in the coronary vessel, but without myocardial injury and release of cardiac biomarkers, whereas NSTEMI and STEMI both implicate an injury to the cardiac muscle. To meet the diagnostic criteria for MI, the patient must have elevation of cardiac biomarkers and at least two of the following: typical symptoms of myocardial ischemia, a characteristic electrocardiogram (ECG) alteration, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality[49] (Figure 17). Thus, cardiac biomarkers are essential to differ both between angina and MI, and between coronary ischemia and other differential diagnoses such as aortic dissection, incipient pneumonia, oesophageal reflux/gastritis and muscle pains.

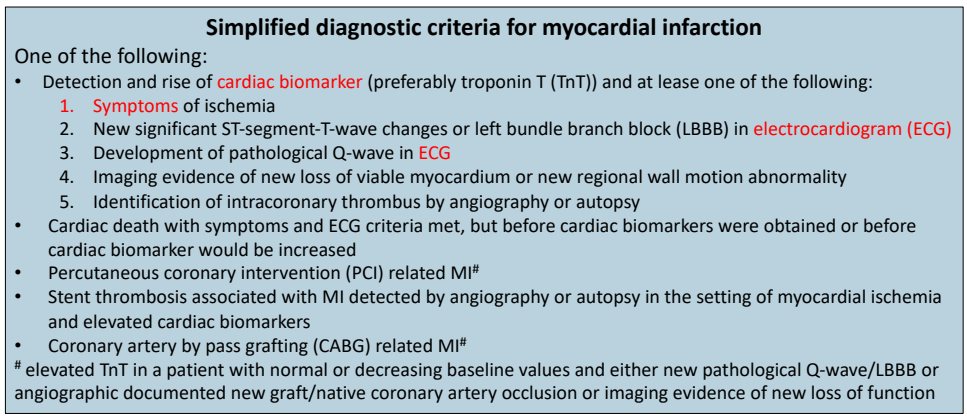

Figure 17. Diagnostic criteria of MI

Simplified diagnostic criteria according to the universal definition of myocardial infarction[49].

The cardiac biomarker preferred today are troponin-T (TnT) or troponin I (TnI), cardiac proteins present in and released from necrotic cardiac muscle cells[50]. Elevated systemic troponin indicate myocardial cell damage and the high content of troponins in the cardiomyocytes makes this biomarker very sensitive to myocardial injury[50]. However, cardiac troponins reveal nothing about the underlying pathophysiology or the aetiology of the cardiomyocyte damage, and can also be elevated by other conditions than MI, even though the sensitivity and specificity are significantly higher in detecting coronary ischemia[50, 51]. The magnitude of the

elevation gives information about the size of the infarction and can thereby indicate prognosis. High acute troponins have been found to be associated with poor post-infarction outcome, characterized by higher post-MI reinfarction rates and mortality[51, 52]. However, troponins give no information about the regeneration process and the ability of the myocardium to heal. In some individuals, the myocardium shows a spectacular ability to regenerate and recovers in spite of a large damage. In other individuals, a rather small damage can cause heart failure development, which may progress even after hospital discharge. In addition, troponins give no information on the progression of atherosclerotic plaques in the rest of the coronary vasculature. Consequently, cardiac troponins are not ideal biomarkers of prognosis in MI.

Many other biomarkers have been studied and considered for MI diagnosis and post-MI risk stratification. No other biomarker has yet outperformed cardiac troponins as a diagnostic biomarker for MI[51]. Several other biomarkers have been explored as indicators of post-MI prognosis. For example, the N-terminal pro-brain natriuretic peptide (NT-proBNP), used in the diagnosis of heart failure. ProBNP is a precursor of the brain natriuretic peptide (BNP), which is synthesized in cardiomyocytes and released upon distension[53]. BNP has both renal and vascular effects, aiming to decrease the strain of the heart. When released, proBNP is cleaved in the physiologically active BNP and the inactive fragment NT-proBNP[53]. NT-proBNP is more stable in plasma compared to BNP, and is the preferred biomarker for this process. BNP and NT-proBNP are produced proportionally to the increased cardiac overload and increased pressure inside the cardiac ventricles. The most common cause of this overload is heart failure, but it can also be due to tachycardias, anaemia, pulmonary embolism and valvular disease. High concentrations of NT-proBNP at the time of an MI have been found to be associated with a poor post-MI prognosis, characterised by increased cardiac event rates and increased mortality[52]. However, since NT-proBNP is signalling cardiomyocyte distension, and not specifically ischemia or necrosis, it is not a specific biomarker for post-MI-prognosis[52].

Further, increased concentration of the inflammatory biomarker high sensitivity C-reactive protein (hsCRP) in MI survivors has been found to be associated both with recurrent CV event rate and with increased mortality[52, 54]. Elevated levels of hsCRP in healthy individuals have also been associated with increased CV event risk[55, 56]. hsCRP reveals the magnitude of ongoing inflammation, but it does not provide information about where the inflammation is located or its mechanisms. An elevated hsCRP can be caused by an ongoing infection, relapse of a rheumatic disease or inflammatory bowel disease, an active cancer or a wide spectrum of other pathophysiological mechanisms. This makes hsCRP a poor biomarker for atherosclerosis progression and event risk, both in primary and in secondary prevention.

White blood cell count has in several studies been correlated to MI incidence and mortality, both in CAD patients and the general population[57-61]. High neutrophil

percentage and high neutrophil/lymphocyte ratio have been proven to outperform white blood cell count as independent predictors of MI incidence and CV mortality in both patients with high risk for CAD[58, 62] and in the general population[57]. Further, neutrophil percentage and neutrophil/lymphocyte ratio at admission have also been correlated with the inability to re-establish coronary blood flow despite PCI in patients with STEMI[63], with long term mortality in MI survivors [64-66] and in CAD patients after PCI[67, 68]. Thus, leukocyte count in general and neutrophils count in particular, are associated with coronary event risk and post-MI outcome, but have the same low specificity problems as potential biomarkers as hsCRP.

Circulating levels of inflammatory mediators have also been linked to CAD progression and prognosis, and efforts have also been made to find inflammatory biomarkers that can identify CVD free individuals at higher risk to develop first-time CAD[69]. Myeloperoxidase (MPO) is a leukocyte-derived enzyme[70], mainly stored and expressed in neutrophils[71] and an important neutrophil bactericidal protein[72]. MPO is linked to inflammation and oxidative stress[71], and causes tissue damage during inflammation by catalysing the formation of several reactive oxidant spieces[70]. High baseline MPO in ACS survivors has been associated with increased risk for short-term recurrent ACS and long-term mortality[73]. The neutrophil-released proteolytic enzyme matrix metalloproteinase 9 (MMP-9) has also been found to be associated with CAD severity in patients with premature CAD[74]. Other cardiac biomarkers that have been considered for potential future use are sST2[75], Galectin-3[76] and GDF-15[77]. They have, however, not entered the clinical practice yet.

In summary, the only biomarkers with sufficient evidence to be used in clinical practice at present are troponin-I/T and NT-proBNP. However, these biomarkers do not have a sufficiently high sensitivity and specificity to identify the patients that are at high risk to develop heart failure or suffer new infarctions due to high inflammatory activity in the plaques. Currently, there is no biomarker able to measure plaque-specific inflammatory activity.

Psychological stress, inflammation and coronary risk

Psychological stress, both chronic and acute, has also been identified as a risk factor for CVD[78]. Population studies have shown associations between acute stress and MI, with an increased risk of CVD events the first hour after an anger outburst[79]. The risk of suffering an MI in the first 2 h after an episode of anger is more than twice as high than before than anger outburst.[80]

The stress response of the human organism is a cooperation between the central nervous system, the endocrine system and the immune system[78]. The main systems that are being activated by psychological stressors and mediate the stress response are the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic

adrenomedullary system, which both activate the renin-angiotensin-aldosterone system in response to different stressors[78]. The activation of these systems results in increased heart rate and blood pressure, endothelial activation and damage, which in turn lead to leukocyte recruitment, adhesion and transmigration of inflammatory cells across the endothelial layer, promoting atherosclerotic plaque formation[78]. Acute psychological stress has been found to trigger leucocytosis and the release of inflammatory mediators such as interleukin(IL)-6, IL-1β, and MPO in humans[81-83], and may thereby increase the risk for CV events[19, 78, 84] (Figure 18). However, the mechanistic links between psychological stress and CVD progression and risk are not yet fully understood[85].

Glucocorticoids are steroid hormones secreted by the adrenal cortex, and cortisol is the most important glucocorticoid. They affect cells by binding to the glucocorticoid receptor, located in the cytosol in most cells, affecting both transcription in the nucleus and transcription factors in the cytosol[86]. Glucocorticoids regulate immune and inflammatory pathways and are essential for dampening the inflammatory response associated with acute stress[87, 88] (Figure 18). Chronic inflammation in pathological conditions, such as autoimmune diseases and depression, has been linked to increased cortisol secretion. A chronic exposure to elevated levels of cortisol induces a cortisol resistance, which reduces the anti-inflammatory actions of cortisol and increases the concentration of pro-inflammatory mediators[89]. This is associated with an impaired HPA axis function and reduced sensitization of the HPA axis to new stressors[90, 91], leading to inadequate cortisol response to stress. This is also proposed as a potential mechanism that favours the chronic inflammation present in CAD[85].

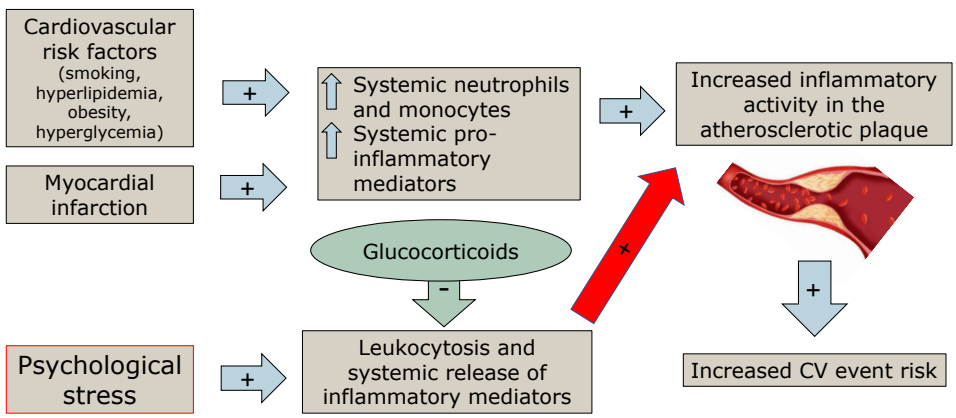

Figure 18. Schematic figure of the possible mechanistic links between psychological stress and cardiovascular risk

CV risk factors and MI increase the risk of reinfarction by increasing systemic inflammation. Psychological stress also induces leukocytosis and increases the release of systemic inflammatory mediators and thereby the CV risk. Under physiological conditions, glucocorticoids dampen the inflammatory stress response. The illustration of the artery is used with permission from Britannica Image Quest, ©Photo researchers.

Compared to healthy controls, CAD patients have a flatter diurnal cortisol rhythm, with high evening cortisol levels, and a blunted cortisol response to acute stress[92]. This cortisol pattern is associated with elevated levels of pro-inflammatory markers[92], higher prevalence of coronary calcifications [93], and with increased risk of coronary events[94]. Consistent to this, CAD patients have been reported to have a more potent increase of inflammatory mediators in response to stress compared to healthy individuals[92, 95]. An imbalance between pro- and anti-inflammatory pathways in CAD patients is generally believed to promote disease progression and to lead to worse clinical outcome[96]. Acute stress causes an immediate increase of the circulating number of neutrophils, monocytes and lymphocytes, which has been seen to increase the inflammatory activity in pre-existing atherosclerotic plaques[19]. However, the exact underlying mechanism for how psychological stress increases the risk for cardiovascular events has not yet been completely elucidated.

Neutrophil mediators in cardiovascular disease

The role of neutrophils in atherosclerosis and myocardial infarction

Neutrophils are the most abundant type of white blood cells. They are effector cells in the innate immune system, the first line of defence of the organism. Neutrophils are part of the polymorphonuclear cell family together with eosinophils and basophils, being characterised by a multilobulated shaped nucleus (Figure 19). Neutrophils are highly mobile cells and are the first responders at sites of inflammation, present within minutes following trauma or cell damage[97]. They originate from myeloid stem cells in the bone marrow, but the matured neutrophils are normally found in the blood stream[97, 98]. Here, the inactive neutrophils have a spherical form. However, when activated, they change shape and become more amorphous and amoebalike, and marginate, i.e. position themselves adjacent to the blood vessel endothelium, ready to migrate into the tissue[98, 99]. Through amoeboid movements neutrophils migrate toward sites of infection and inflammation, a process called chemotaxis. Neutrophils are short lived cells with a life span of between 5 hours and 5 days, in the shorter end when activated[98, 100]. Neutrophils express a variety of cell surface receptors, recognizing ligands such as complement, cytokines and chemokines, which regulate chemotaxis direction and cell function[101]. They have three different strategies for clearing micro-organisms and dead cells. The first is phagocytosis, where they engulf invaders coated with antibodies or complement, but also damaged cells or cell debris. Each phagocytic event results in a phagosome, into which reactive oxygen species and hydrolytic enzymes are secreted. This oxygen consuming process is called respiratory burst[99]. The second strategy is degranulation, i.e. the secretion of granule content with pro-inflammatory, protein-destroying and bactericidal proteins and enzymes[102]. The

third is to form web-like structures containing nuclear contents together with cytoplasmatic and granular proteins[103, 104]. These structures are termed neutrophil extracellular traps (NETs) and are capable to trap and kill pathogens.

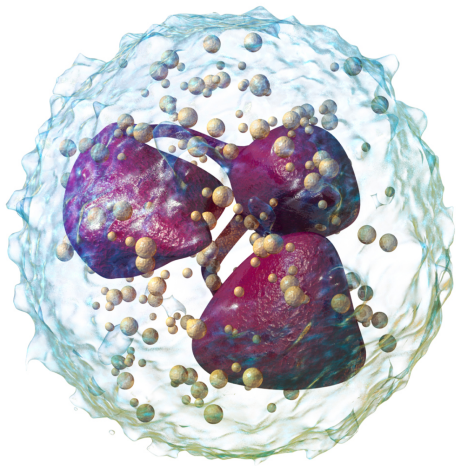

Figure 19. Neutrophil

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Neutrophils were for a long time considered not to have an important role in atherosclerosis pathogenesis[103]. However, neutrophils have been detected in human and mouse atherosclerotic plaques, both in fatty streaks and in more advanced lesions[104]. They represent about 2% of the leukocytes in mouse atherosclerotic plaques, and accumulate in regions with high monocyte density[104]. Dyslipidaemia and peripheral inflammatory signals induce neutrophil recruitment to the lesions, and the number of circulating neutrophils correlate closely with plaque size[105, 106]. Neutrophil depletion has shown to decrease monocyte recruitment into mouse plaques, and to reduce early plaque size[105, 107, 108]. Oxidized LDL might induce neutrophil transmigration and degranulation, which possibly triggers further rapid monocyte recruitment[104]. Neutrophils rapidly become apoptotic after activation, releasing danger signals in the intima. These are sensed by scavenger receptors, resulting in recruitment of monocytes/macrophages and phagocytosis of the apoptotic neutrophils[104]. Neutrophil-derived NETs have also been found in human and mice atherosclerotic lesions and arterial trombi[103]. NETs have been shown to activate endothelial cells, antigen-presenting cells and platelets, leading to a pro-inflammatory immune response that has been suggested to promote atherosclerotic plaque formation and arterial thrombosis[103]. Neutrophils also express proteases and reactive oxygen species that contribute to plaque destabilization[103].

In response to an MI, neutrophils are the first myeloid cells present at site of the infarction[9], responding to alarmins, cytokines, chemokines and endogenous lipid mediators (for example prostaglandin E_2)[18]. They arrive within minutes, rapidly releasing pro-inflammatory mediators such as TNFα, IL-1b and S100 proteins that activate the endothelium and further recruit pro-inflammatory cells[9, 16]. Neutrophils amplify the inflammatory response and are the dominating leukocytes in the heart during the first day after ischemia. Infiltration of neutrophils is predominantly localized in the border zone of the ischemic area and is accelerated by reperfusion[18]. The activated endothelium captures circulating neutrophils by adhesion receptors on the luminal surface. The neutrophils start rolling onto the endothelial layer, sensing chemokines bound to the endothelial cell surface that mediate arrest and firmer binding. They transmigrate through endothelial junctions and migrate into the ischemic tissue, where they release proteolytic enzymes and contribute to the clearance of dead cells and matrix debris[18]. Neutrophils also promote the transition from the inflammatory to the reparatory phase in the myocardium by several mechanisms. One of these mechanisms is the expression of decoy cytokine receptors on apoptotic neutrophils, which induce a depletion of inflammatory mediators in the tissue, and thereby dampen the inflammation[18]. Neutrophils also promote the development of reparatory macrophages in the post-ischemic myocardium, through secretion of neutrophil gelatinase-associated lipocalin (NGAL)[109]. NGAL is a key inducer of macrophage polarization from pro-inflammatory to reparatory macrophages with high efferocytosis capacity. Efferocytosis, i.e. the clearance of apoptotic cells from the infarction area, is crucial to the healing process. Consequently, neutrophil depletion in mice with induced MI has been found to lead to a dysregulated cardiac healing response characterised by increased LVESD, decreased LVEF and increased fibrosis in the infarction area 7-14 days post-MI[109].

The neutrophil mediators S100A12 and S100A8/A9 and their receptors.

S100A12 is a neutrophil-released pro-inflammatory protein that has emerged in recent years as an important mediator in CVD[110] (Figure 20). S100A12, also called the extracellular newly identified receptor for advanced glycation end-products binding protein (EN-RAGE), is a member of the calcium binding S100 calgranulin family, and functions as a DAMP or alarmin, i.e. an endogenous molecule that signals cell and/or tissue damage. Activated or dying neutrophils are the most important source of S100A12[111], and S100A12 represents approximately 5% of all cytosolic proteins in these cells[112]. Mice lack the gene for S100A12, but studies on ApoE-deficient mice transgenic for S100A12 have shown that S100A12 expression leads to atherosclerosis progression, aortic calcification and plaque vulnerability[113]. Conversely, treatment with a specific S100A12 blocker reversed the phenotype and

reduced plaque vulnerability[114]. In humans, S100A12 is released from ruptured atherosclerotic plaques in MI, and from the site of atheroma disruption by PCI in stable CAD[115, 116]. Plasma S100A12 is higher both in CAD patients compared to controls[115] and in ACS patients compared to patients with stable angina pectoris[117]. S100A12 levels correlate with coronary lesion complexity measured by coronary angiography, in both stable angina pectoris patients and in ACS patients[118]. Furthermore, high S100A12 is associated with increased long-term incidence of coronary events in both chronic CAD patients and in individuals without known CAD[119, 120]. However, the association between plasma S100A12 levels and post-ACS prognosis has not previously been explored.

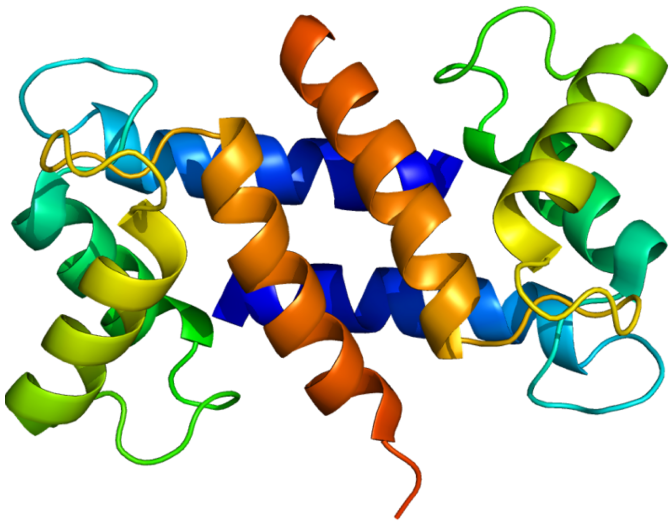

Figure 20. The protein structure of S100A12

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The S100 proteins A8 and A9, also called myeloid-related protein 8 and 14, are also alarmins belonging to the S100 calcium-binding protein family. They are expressed in neutrophils, monocytes, thrombocytes and dendritic cells but also in activated macrophages, vascular endothelial cells and fibroblasts[16, 121]. S100A8 and S100A9 exist as homodimers, but preferably form the heterodimer S100A8/A9, also known as calprotectin (Figure 21). S100A8/A9 constitutes approximately 45 % of all cytosolic proteins in neutrophils and about 1% in monocytes[122], and neutrophils are by far the main source of extracellular S100A8/A9[123]. Systemic levels of S100A8/A9 correlate with established markers of systemic inflammation in patients with stable CAD[124], and with the severity of CAD in type 1 and type 2 diabetic patients[16]. Further, S100A8/A9 is elevated in vulnerable human carotid plaques[125] and is produced locally at the site of the coronary occlusion in MI[126]. High levels of

S100A8/A9 in plasma in the general population are associated with higher neutrophil counts, larger IMT in the common carotid artery and higher incidence of coronary events and cardiovascular (CV) death[123]. In ACS patients, S100A8/A9 levels are increased in blood and at the site of coronary occlusion, and recovered thrombi from coronary vessels contain S100A8/A9 positive cells[126]. These findings suggest that S100A8/A9 is released locally at the site of plaque rupture and diffuses into the systemic circulation. Moreover, the magnitude of S100A8/A9 response has prognostic value, as ACS patients with persistently elevated levels of S100A8/A9 30 days after the acute event have a significantly higher risk to suffer recurrent CV events[127].

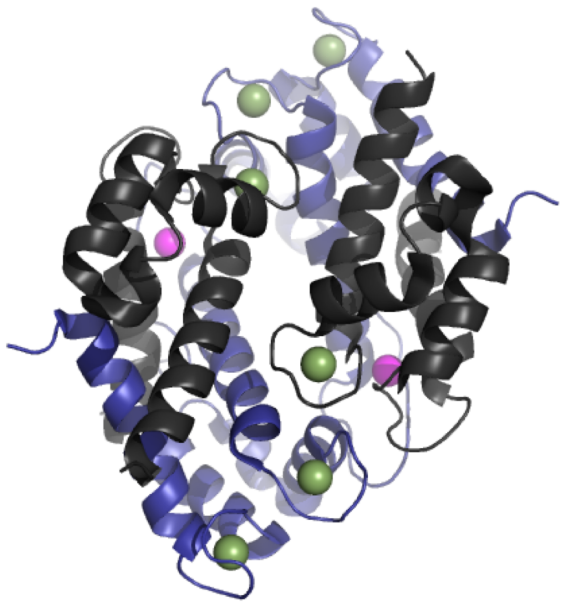

Figure 21. The protein structure of S100A8/A9

The grey chains represent S100A8, the blue chains represent S100A9, the purple spheres represent Mn^{2+} and the green spheres represent Ca^{2+}. By Czeer - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=38618765

S100A8/A9 and S100A12 exert their effects through the pattern recognition receptors (PRRs) RAGE (receptor for advanced glycation end products) and TLR4 (toll like receptor 4)[128, 129]. RAGE is a multiligand innate immune receptor[130] (Figure 22), expressed on the surface of various cell populations, including neutrophils, mononuclear phagocytes, lymphocytes, endothelial cells and smooth muscle cells[128]. RAGE is present at low levels in most tissues but is extensively upregulated in inflammation and in vascular injury[131, 132]. The most well-known RAGE ligands are the S100/calgranulin family proteins, AGEs (advanced glycation end products) and HMGB1 (High mobility group box 1 protein)[128].

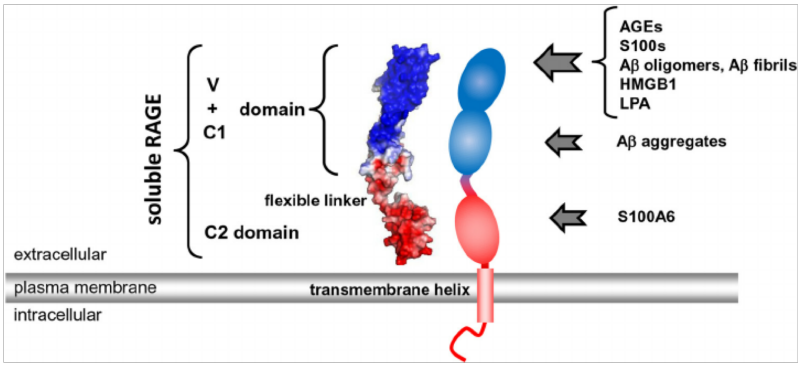

Figure 22. Schematic figure of RAGE and sRAGE structures

The extracellular part of RAGE is composed of three Ig domains: V, C1 and C2. The receptor is achored in the plasma membrane by a transmembrane helix which continues in the cytoplasm as a short unstructured tail. On the left side, a structural model of sRAGE is shown, on the right, a schematic depiction of RAGE. The surface is colored according to the electrostatic charge; areas with positive charge are presented in blue, and areas with negative charge in red. V and C1 domains form a structural unit and carry a positive surface, wheras the C2 domain is negatively charged. Most RAGE ligands bind to the V domain. Reproduced with permission from Kierdorf et al.[133], ©John Wiley and Sons.

Engagement of cellular-bound RAGE by its ligands leads to cell activation and production of pro-inflammatory cytokines, cell migration and tissue infiltration [110, 128, 132, 134] (Figure 23). RAGE-mediated mechanisms have been shown to be involved in atherosclerosis and in post-ischemic heart failure[131, 135, 136]. Mouse studies have shown that RAGE depletion in ApoE$^{-/-}$ mice causes a reduction in both the number of atherosclerotic lesions and the size of lesion areas[137, 138].

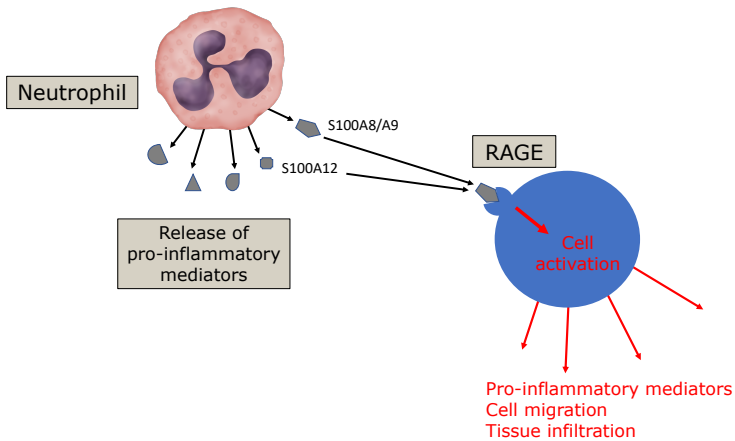

Figure 23. Ligand binding to RAGE

Ligand binding to RAGE leads to cellactivation, production of pro-inflammatory mediators, cellmigration and tissue infiltration.

S100A8/A9 also activates the receptor extracellular matrix metalloproteinase inducer (EMMPRIN), also called CD147 or basigin[139] (Figure 24). EMMPRIN is expressed on leukocytes, platelets, cardiac fibroblasts and endothelial cells[140], and is upregulated on activated T-cells and monocytes during development to macrophages[139, 141]. S100A8/A9 binding to EMMPRIN mediates monocyte recruitment, monocyte and platelet activation and secretion of pro-inflammatory cytokines[139, 142]. EMMPRIN is present in human atherosclerotic plaques, especially in macrophage rich areas, and is thought to be involved in the pathogenesis of MI[139].

Figure 24. Structure of EMMPRIN

The multi functional glycoprotein EMMPRIN has two Ig-like domains in its extracellular portion. Based on PyMOL rendering of PDB 3B5H. By Pleiotrope, reproduced with permission from Wikipedia.

While TLR4 is exclusively cell bound, RAGE and EMMPRIN also exist in a soluble form due to enzymatic cleavage from the cell surface or alternative splicing[139, 143-147]. The majority of sRAGE is generated by proteolytic cleavage from the cellular membrane by the proteases MMP-9 (Matrix Metalloproteinase-9)[146] and ADAM10 (A Disintegrin And Metalloproteinases domain-containing protein 10)[143]. Ligand binding to RAGE has been shown to promote RAGE cleavage from the cell surface[143] (Figure 25). Only a small proportion of the total amount of circulating sRAGE is formed by alternate splicing and secretion[143]. Alternative splicing of mRNA is a process that enables synthesis of different protein variants or isoforms by rearranging the pattern of introns and exon elements joined by splicing during transcription, hereby altering the mRNA coding sequence. The different isoforms can have different functions or properties, since the alternative splicing can introduce or remove important protein domains, affecting the ligand binding properties, activity or stability[147, 148]. The alternative splicing of intron 9 and the

removal of exon 10 results in a isoform lacking the transmembrane and intracellular domain of RAGE creating an alternatively spliced sRAGE form[147]. sRAGE serves as a decoy receptor for RAGE ligands, binding the ligands without activating the signalling pathways, thereby acting in an anti-inflammatory manner[110, 149, 150].

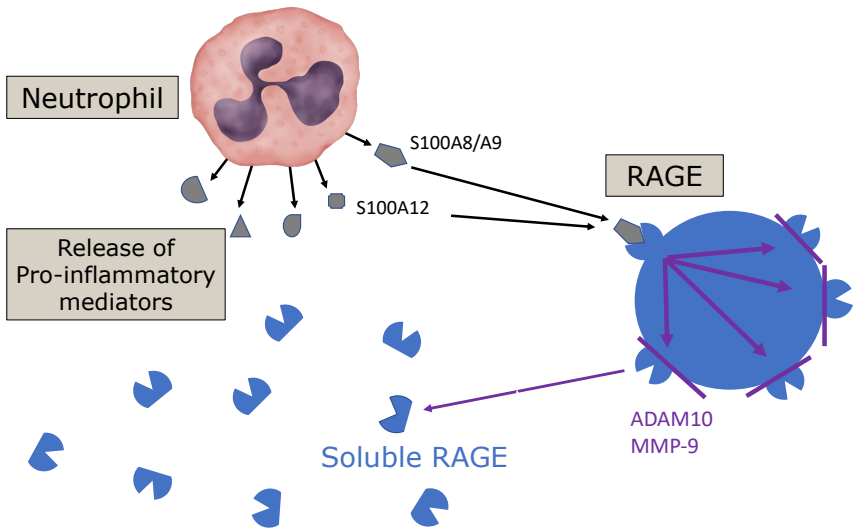

Figure 25. RAGE clevage from the cell surface

The vast majority of sRAGE is generated by proteolytic cleavage of RAGE from the cell surface by the proteases MMP-9 (Matrix Metalloproteinase-9)[146] and ADAM10 (A Disintegrin And Metalloproteinases domain-containing protein 10)[143].

sRAGE treatment has shown to reduce atherosclerosis progression in diabetic mice independently of glycemia and lipids[136, 151] and to reduce vascular medial calcification in mice with chronic kidney disease[152]. In human cross-sectional studies, systemic sRAGE was negatively associated with coronary artery calcium score in the population-based Dallas Heart Study cohort[153] and with non-calcified coronary plaque burden in CAD patients[154]. In 100 patients with pre-mature CAD and 40 CAD-free controls, plasma sRAGE was significantly lower in CAD patients and was an independent predictor of CAD severity[155]. In non-diabetic males, subjects with sRAGE levels within the lowest quartile had an almost seven times higher odds ratio for prevalent CAD compared to the highest quartile[156]. In another study, sRAGE was lower in CAD patients with concomitant PAD compared to patients with only CAD[157]. In type 2 diabetic patients, low levels of sRAGE combined with high S100A12 were strongly associated with increased CV risk[158]. sRAGE has also been studied in experimental models of MI, where intracoronary and intraperitoneal injection of sRAGE during the first 48h after induced MI resulted in significantly decreased infarction size, reduced fibrosis and preserved

left ventricular end diastolic volume (LVEDV), left ventricle end systolic volume (LVESV) and LVEF[159-161]. These findings suggest a potential protective role of sRAGE in CVD, and also that sRAGE has the potential to be a biomarker of atherosclerotic disease progression and post-ACS prognosis. It has also been proposed as a potential treatment for CVD[149, 150]. Similar to sRAGE, sEMMPRIN has been showed to be cleaved from the cell surface by the Membrane type 1 matrix metalloproteinase (MT1-MMP/MMP-14)[145]. Whether sEMMPRIN has similar protective properties against CVD is still unknown.

Study aims

Overall aim

To explore the potential importance of the neutrophil mediators S100A12 and S100A8/A9, and of their soluble receptors sRAGE and sEMMPRIN for CVD development and prognosis in the general population and in ACS patients.

Paper I

The aim of this study was to explore the associations between circulating sRAGE, sEMMPRIN, atherosclerosis progression and CVD risk in previously CVD free individuals. We hypothesized that high levels of the soluble receptors might associate with a positive prognosis, due to their putative anti-inflammatory roles.

Paper II

The aim of this study was to assess whether genetic determinants of sRAGE levels in plasma are linked to CVD risk in the general population. We hypothesized that genetic determinants of lower sRAGE, would be associated with a faster atherosclerosis progression and a higher risk to suffer coronary events and premature death.

Paper III

Here, we aimed to explore the links between plasma S100A12, sRAGE and long-term prognosis post-ACS. We hypothesized that high plasma S100A12 and low plasma sRAGE would be associated to a poor post-ACS prognosis.

Paper IV

In this study we explored the potential links between psychological stress and S100A8/A9 release in chronic CAD patients. We hypothesized that S100A8/A9 release is an integral part of the inflammatory response to stress, and that this response may be exacerbated in CAD patients with a dysregulated cortisol metabolism.

Material and methods

Study populations

Paper I and II

In Paper I we have used the cardiovascular sub-cohort of the prospective population-based Malmö Diet and Cancer study including 6103 individuals (MDC-CV), and in Paper II we used both the sub-cohort (MDC-CV) and the entire MDC cohort including 30447 subjects. The flowcharts describing the inclusion and exclusion criteria in both MDC-CV and MDC are presented in Figures 26 and 27. The MDC study included a random sample of individuals from Malmö, Sweden, born between 1926 and 1945 and included between March 1991 and September 1996[162]. All participants filled in a self-administered questionnaire and went through a clinical examination and blood sample collection at baseline. Information about smoking habits and current medication was collected from the questionnaire. Diabetes mellitus at baseline was defined as self-reported history of a diabetes diagnosis made by a physician or diabetes medication in the questionnaire or fasting whole blood glucose >6.0 mmol/L. Between October 1991 and February 1994 a randomly selected subgroup of MDC was included into a study focusing on carotid artery disease epidemiology (MDC-CV)[163], and underwent ultrasonographic measurement of the IMT in the right common carotid artery. In addition, a plasma aliquot from MDC-CV participants was sent for analysis of biomarkers, including sRAGE and sEMMPRIN. The biomarker analysis was successfully performed in 4742 subjects with a full set of clinical data and available fasting blood samples. A successful genome-wide association study (GWAS) analysis was performed in 4192 of these individuals, and the data was used to identify single nucleotide polymorphisms (SNPs) with significant associations with plasma sRAGE levels.

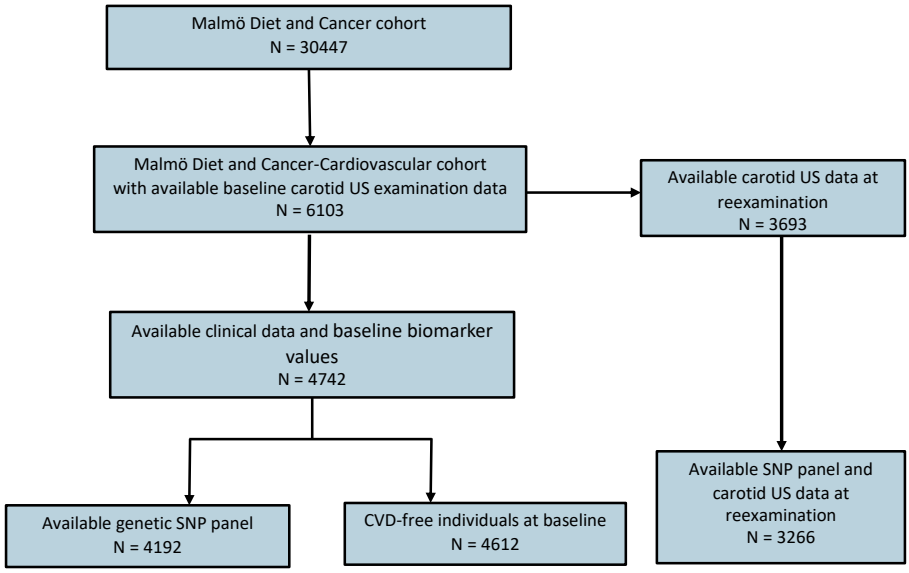

Figure 26. The Malmö Diet and Cancer Cardiovascular cohort (MDC-CV)

Flow diagram of subject inclusion and exclusion from the study. US, Ultrasound; SNP, single nucleotide polymorphism; CVD, Cardiovascular disease

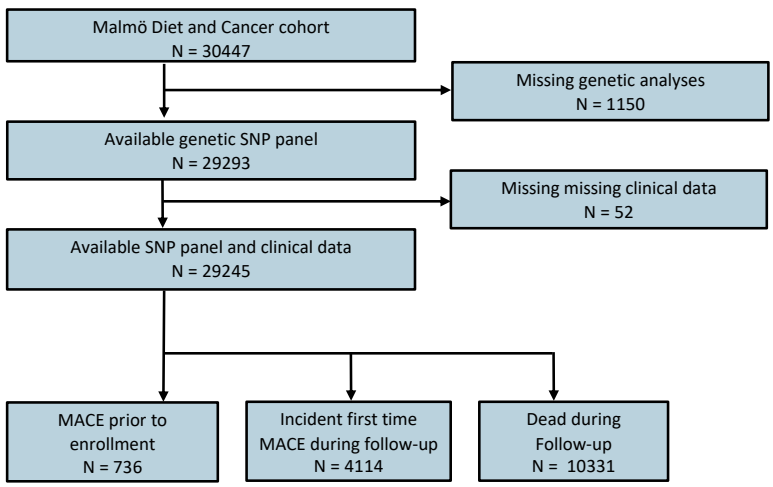

Figure 27. The complete Malmö Diet and Cancer cohort (MDC)

Flow chart diagram of inclusion, exclusion and clinical outcome in the MDC study. SNP, single nucleotide polymorphism; MACE, major adverse coronary event.

The participants were invited to undergo a carotid artery IMT re-examination between 2007 and 2012, completed by 3693 individuals in total, and by 3266 participants with a full set of genetic data[164] (Figure 26). The median time from baseline to IMT re-examination was 16.5 (15.5-17.8) years.

Information on prevalent CVD was obtained from the national Hospital Discharge registry, the Stroke register of Malmö and the regional MI register[44]. Based on the information in the questionnaire and in the registers, a further 130 subjects with previously known CVD were excluded from the analyses in Paper I, since the outcome in this paper was first time major adverse coronary event (MACE). Prevalent CVD was characterized as previous acute MI, stroke, CABG or PCI. This left 4612 subjects for analysis in Paper I, out of which 4581 had available baseline data on ultrasonographic measurement of IMT in the right common carotid artery. In Paper II, the entire MDC cohort was used to analyze the association between SNPs and clinical outcome. GWAS and clinical data were available in 29 245 participants (Figure 27).

All participants gave informed consent. The ethics committee at Lund University approved the study, and it was conducted in accordance with the Declaration of Helsinki.

Paper III

Initially, 605 patients admitted for suspected ACS to the Coronary Care Unit of Skåne University Hospital Malmö were consecutively included in the study between October 2008 and December 2012. ACS was defined as unstable angina or MI, diagnosed according to the universal definition of MI[49]. Fifty patients did not fulfil the diagnostic criteria for ACS and were excluded. Thirty-one patients were further excluded due to missing samples, leaving a final study population of 524 individuals. The national Swedish Web-based system for Enhancement and Development of Evidence-based care in Heart disease Evaluated According to Recommended Therapies (SWEDEHEART), was used to collect information on smoking, diabetes, hypertension, and previous history of heart failure and ACS.

In order to address the current knowledge gap regarding post-ACS evolution of cardiac function and prognosis in the elderly, patients that were 75 years of age or older at inclusion were invited to a more detailed follow-up. The follow-up consisted in collection of a new plasma sample at 6 weeks after the index event and a follow-up echocardiographic examination at 1 year. This follow-up protocol of elderly patients was predefined before the start of the study. Plasma samples were collected in EDTA-coated tubes within 24 hours after admission from all patients, and again after 6 weeks in the detailed follow-up group.

In order to be included into the study, the patients had to be able to provide a written informed consent. No other exclusion criteria were applied. The study has been

approved by the Regional ethics committee in Lund, Sweden and was conducted according to the ethical guidelines of the Declaration of Helsinki.

Paper VI

Two cohorts of CAD patients have been used in this study. In **Cohort I**, we included 60 CAD patients at 6-12 months after a coronary event. The patients had been diagnosed in accordance with the consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee[165]. Forty-four patients had suffered an ASC (24 NSTEMI, 20 STEMI), and 16 had undergone PCI due to stable angina. The exclusion criteria were age >75 years, on-going infection, worsened or recurrent angina or readmission to hospital within the previous 10 weeks, moderate to severe heart failure, chronic inflammatory or immunologic disorders, neoplasm, continuous treatment with immunosuppressive/anti-inflammatory agents, drug or alcohol abuse or poor mental function.

In **Cohort II**, we included 30 patients at 12-14 weeks after a first-time ACS and 30 healthy controls with equal age and gender distribution, randomly selected from the population register. Eleven of the patients had been diagnosed with NSTEMI, and 19 with STEMI. Twenty-eight patients had been treated with thrombolysis and/or PCI, and two had an acute CABG. The controls were all clinically healthy, with neither history nor clinical signs of CAD or other CVD. The exclusion criteria were age >70 years, but otherwise similar to Cohort I. In controls, antihypertensive drugs and statins as primary preventive treatments were allowed. A complete set of blood samples was available from 27 patients and 28 controls in Cohort II.

Written informed consent was obtained from all study participants, the Ethical Review Board of Linköping University approved the research protocol, and the study was conducted according to the ethical guidelines of the Declaration of Helsinki.

Clinical outcomes

Paper I and II

For incident clinical events, the participants were followed from baseline until the first incident MACE, emigration from Sweden or death, or until December 31st, 2014. To obtain information about events we linked the personal identification code of each subject to the national Hospital Discharge registry[166], the National Cause of death Registry and the Swedish Coronary Angiography and Angioplasty Registry (SCAAR)[167]. Incident MACE was defined as an acute coronary event or CABG

surgery or PCI without an acute event beforehand. An acute coronary event was defined as a fatal or non-fatal MI on the base of International Classification of Diseases, ninth revision (ICD-9) code 410 and International Classification of Diseases, Tenth revision (ICD-10) code I21 or death attributable to ischemic heart disease (ICD-9 codes 412 and 414 or ICD-10 codes I22, I23 and I25). Coronary revascularizations were based on the SCAAR procedure codes 3065, 3066, 3068, 3080, 3092, 3105, 3127 or 3158 in the Op6 system or as procedure code FN in the KKÅ97 system. The secondary outcome was total mortality.

Paper III

Adverse events were recorded prospectively during follow-up. The primary outcome was recurrent MACE, defined as major adverse cardiovascular event, a composite of hospitalization with an ACS diagnosis or CV death. The secondary outcomes were hospitalization for heart failure and total mortality. Events were identified by using data from the Swedish Hospital Discharge Register and the Swedish Cause of death Register. The last follow-up date for all participants was 31st of December 2012 for recurrent MI, unstable angina, heart failure and CV death, and 31st of December 2013 for mortality by any cause. MI was defined by ICD 10 codes I21 and I22, unstable angina by code I20, and heart failure by code I50. CV death was defined as death due to MI, ischemic heart disease, cardiac arrest, atrial fibrillation, heart failure, ischemic or haemorrhagic stroke, defined by the ICD 10 codes I20-25, I46, I48, I50 and I60-69.

Carotid IMT measurement by ultrasound

In Paper I, we studied the correlations between baseline sRAGE, sEMMPRIN and carotid artery IMT measured at baseline, in a cross-sectional analysis. Prospectively, we then examined the associations between baseline sRAGE and sEMMPRIN and carotid IMT at re-examination, total carotid IMT progression from baseline to re-examination, and the average carotid IMT progression per year during follow-up. In Paper II, we studied the associations between genetic determinants of plasma sRAGE levels and these IMT variables.

The IMT was measured with a B-mode ultrasonography system (Acuson XP/4 for the baseline examination and Acuson Sequoia for the follow-up examination). The right common carotid artery was scanned in systole, at the top of the R-wave on ECG. The bifurcation area was scanned within a predefined window covering 3 cm of the distal common carotid artery, the bulb and 1 cm of the internal and external carotid artery. With use of a specially designed computer-assisted analysing system, the IMT of the far wall of the right common carotid artery was measured according

to the leading-edge principle. IMT was then determined off-line as the mean wall thickness measured within a 1 cm-long window directly proximal to the bifurcation (Figure 28). All examinations were performed by trained, certified sonographers. To minimize observer bias, each image was analysed without knowledge of subject identification code. Quality control of the methods has been previously published[44].

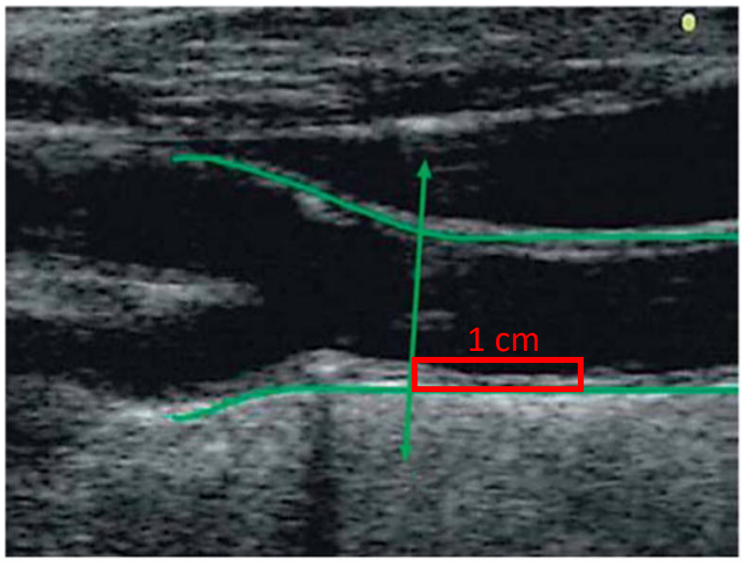

Figure 28. IMT measurement in the common carotid artery

Longitudinal view of the common carotid artery and bifurcation. IMT was determined off-line as the mean wall thickness measured within a 1 cm-long window directly proximal to the bifurcation (the red square). The green double arrow marks the end of the common carotid artery and the beginning of the internal and external carotid arteries. Reproduced from Touboul et al.[41] with permission ©Karger Publishers

Echocardiography

In Paper III, patients 75 years and older underwent a baseline echocardiography examination during the index hospital stay and were invited for a follow-up echocardiography 1 year after inclusion, as prespecified in the project protocol. The echocardiographic follow-up was completed in 113 patients. Experienced sonographers performed all echocardiography examinations, which were analysed using the Xcelera software (Philips) by a single examiner blinded to the clinical data. LVEF was measured according to Simpson´s biplane method in the apical 4-chamber and 2-chamber views. Due to poor image quality and missing frames, we were able to perform measurements of acute LVEF in 76 patients, of LVEF at 1-year post-ACS in 99 patients, and of the LVESV and LVEDV at 1 year in 108

patients. Delta value of LVEF was calculated as the value of LVEF at 1 year minus the LVEF value at inclusion.

Psychological stress test and sample collection

Both cohorts in Paper IV were subjected to a combined psychological stress test. The participants came to the hospital in a fasting state and were instructed to take prescribed drugs as usual. Testing always started between 07.30 – 08.00 a.m. Baseline blood pressure and heart rate were recorded with subjects comfortably lying down on a bed. Saliva samples for baseline cortisol were taken before venepuncture and collection of baseline blood samples.

The psychological stress test included two parts with different stressors, an anger recall test and an arithmetic test, with a pause of 2 minutes in-between[82, 92]. In the first part, the participant was instructed to recall an event that made him/her angry, frustrated or upset, and to discuss for 6 min what had happened and how that made him/her feel. In the second part, the participants were instructed to count backwards from 700 minus 7 as quickly and accurately as possible, and to reach zero within 4 min. Blood pressure and pulse rate were measured before stress and every 2 min until the end of the test.

In Cohort I, the second blood sample was collected at 34 min after the start of the first stressor, i.e. 20 min after the completion of the second stressor. In Cohort II, the second blood sample was collected at 24h after the stress test. In both cohorts, saliva samples were collected at baseline and at 20 min after stress. For measurement of diurnal cortisol variation, additional saliva samples were collected from Cohort I participants twice daily on three consecutive days, 30 minutes after awakening and in the evening before going to bed. Saliva collection was performed by placing salivette cotton swabs (Sarstedt, Nürnbrecht, Germany) under the tongue for 2 min. The swabs were then immediately frozen at -20°C.

Biological sample analyses

Blood sample analyses

The blood was centrifuged at 3000 g for 10 minutes, and plasma was aliquoted and stored at -80°C before analysis. A plasma aliquot from the MDC cohort in Paper I and II, and the ACS cohort in Paper III, was sent to the certified clinical laboratory of Skåne University Hospital Malmö for analysis of fasting whole blood glucose, plasma lipids, creatinine, hsCRP, TnT and cystatin C. Estimated glomerular

filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula in Paper I and II, and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was used to calculate eGFR based on cystatin C, age and sex in Paper III.[168].

Biomarker analyses

Another aliquot from the MDC cohort in Paper I and II, and the ACS-cohort in Paper III, was sent for analysis of S100A12, sRAGE, sEMMPRIN, NT-proBNP and IL-6 at the Science for Life Laboratory, Uppsala, Sweden, by the Proximity Extension Assay technique[169]. Oligonucleotide-labelled antibody pairs were allowed to bind to their respective targets in the plasma samples. By adding a DNA polymerase that induced extension and joining of the two oligonucleotides, a PCR template was formed and pre-amplified with universal primers. By using specific primers, the individual DNA sequence for each target protein was detected and quantified. The reactions were run on a microfluidic real-time quantitative PCR chip (96.96, Dynamic Array IFC, Fluidig, Biomark) and read on a Biomark HD instrument. For S100A12, the within-run coefficient of variation was 8% and the between-run coefficient of variation was 22%. For sRAGE, the within-run coefficient of variation was 9% and the between-run coefficient of variation was 11%, which is comparable with those obtained with commercially available ELISA kits. For EMMPRIN, the within-run coefficient of variation was 4% and the between-run coefficient of variation was 19%. Considering the large number of subjects included and the fact that cases and controls were randomly distributed among the different assay runs, the relatively high inter-assay variability of the S100A12 and sEMMPRIN analyses is unlikely to significantly impact the final results of the study, although we cannot exclude this possibility with certainty. Data analysis was performed by a pre-processing normalization procedure using Olink Wizard for GenEx (Multid Analyses, Sweden). All data for S100A12, sRAGE and EMMPRIN are presented in arbitrary units (au).

Genome-wide association study

A genome-wide association study (GWAS) is a method to study a genome-wide set of genetic variants in different individuals, with the aim to explore whether any variant is associated with a particular trait of interest. The genetic variants studied here are single-nucleotide polymorphisms (SNPs).

The GWAS analysis was run on the whole MDC cohort using the Illumina GWAS chip (GSA v1 array). During the quality control procedures (QC) we removed individuals having a call rate of <0.95, and inbreeding coefficient of >3 SD away from the mean, discordance between inferred and reported gender, duplicate samples, unexpected high proportion of identity by decent sharing, first and second

degree relatives, or deviating from the common population structure. SNPs were filtered out if they were monomorphic or had a call rate of <0.95, extreme deviation from Hardy-Weinberg equilibrium ($P<1x10^{-7}$) or minor allele frequency <1%. The dataset was then imputed using the 1000 Genomes Phase 3 reference panel.

S100A8/A9 ELISA

S100A8/A9 was measured by using a previously described ELISA technique[170]. Microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated at 4°C overnight with 5 μg/ml monoclonal antibody against S100A8/A9 (27E10, BMA Biomedicals AB, August, Switzerland) diluted in PBS pH 7.2, followed by a blocking step using 150 μl of 1% BSA (ICN Biomedicals Inc., Aurora, OH, USA). A buffer containing 0.15M NaCl, 10mM HEPES (Invitrogen, Carlsbad, CA, USA), 1 mM $CaCl_2$, 0.02 mM $ZnCl_2$, 0.05% Tween 20 and 0.1% BSA was used for dilution of serum samples, detection antibody and streptavidin. Serum samples diluted 1/100 were added to the microtiter plates and incubated for 2h. The wells were thereafter washed three times with PBS containing 0.05% Tween 20, followed by overnight incubation with biotinylated polyclonal antibodies against S100A8/A9 (chicken polyclonal antibody MRP8/14, Abcam, Cambridge, UK) diluted 1/2000. After washing, the antibodies were labelled with ALP-bound streptavidin (Dako, Glostrup, Denmark) diluted 1/1000. Bound streptavidin was visualized with disodium-p-nitrophenyl phosphate (Sigma, St Louis, MO, USA) 1 mg/ml dissolved in 10% (w/v) diethanolamine pH 9.8 containing 50 mM $MgCl_2$. The absorbance was measured at 405 nm. All samples were run in duplicate. Optical density values of uncoated wells were used as background and subtracted from the values of the samples. Titration curves obtained from one serum with known concentration were used to calculate the S100A8/A9 concentrations. The lower detection level was 3 ng/mL.

Saliva cortisol

Measurement of free cortisol levels in saliva was performed at the accredited Clinical Chemical Laboratory at Karolinska University Hospital, Sweden, using a commercial radioimmunoassay assay, CORT-CT2 (Cisbio, Bioanalyser, Codolet, France), with a limit of detection of 3.0 nmol/L and limit of quantitation of 100 nmol/L. The interassay coefficient of variance was less than 10% according to repeatedly performed quality assessments.

Statistical analyses

Paper I

The differences in baseline characteristics between the different outcomes were analysed using the Mann Whitney test for continuous variables and the chi-square test for dichotomous variables. Associations between IMT data and biomarker levels were assessed by Pearson´s correlation tests and by multivariable linear regression analysis including 3 different models correcting for potential confounders. Model 1 was adjusted for age and sex; Model 2 additionally included body mass index (BMI), current smoking, diabetes, TG, HDL, LDL, eGFR, systolic blood pressure, blood pressure medication, and statin medication; and Model 3 was adjusted for the same variables as Model 2 with the addition of hsCRP. Skewed variables were logarithmically transformed before analysis. The associations between biomarker levels and outcome were explored through a Kaplan Meier survival analysis with log rank test, followed by multivariable Cox proportional hazards analyses adjusted for the same potential confounders mentioned above.

Paper II

Associations between SNPs and plasma sRAGE at baseline were analysed in a multivariable linear regression adjusted for age and sex. A genome-wide significant p-value $<5 \times 10^{-8}$ was used to select SNPs with a significant association with sRAGE. Spearman correlations between all identified sRAGE-associated SNPs were used to test for linkage disequilibrium (LD). All SNPs with a squared correlation coefficient (r^2) >0.8 were considered to be in LD, and the SNP with the strongest association (i.e. lowest P-value) to sRAGE within each cluster were selected for further analyses. The median baseline sRAGE concentration between individuals within the different allele groups of the selected SNPs were compared in a Kruskal Wallis analysis. The percentage of ln(sRAGE) variance explained by a certain SNP was estimated by calculating the square of the partial correlations coefficient (R^2) derived from a multivariable linear regression adjusted for sex and age at inclusion. The SNPs with true independent association to sRAGE were identified in an age and sex-adjusted multivariable linear regression with sRAGE as dependent variable and the selected SNPs from each of the four identified clusters as independent variables, forced into the same model. The SNPs that maintained a significant association with sRAGE were considered true independent determinants of sRAGE and were chosen for further analyses.

The associations between SNPs and carotid IMT were explored in multivariable linear regression analyses with 2 different models of adjustment for potential confounders. Model 1 was adjusted for age and sex and Model 2 additionally

included BMI, current smoking, diabetes, TG, HDL, LDL, eGFR, systolic blood pressure, blood pressure medication, and statin medication. Skewed variables were logarithmically transformed before analysis. We analysed the associations between the SNPs and carotid artery IMT at baseline, at re-examination, absolute IMT progression from baseline to re-examination, and the average IMT increase per year during follow-up.

To examine the differences in baseline characteristics between event-free individuals and the subjects that suffered an incident event during follow-up (MACE or mortality), we used the Mann Whitney test for continuous variables and the chi-square test for dichotomous variables. The association between sRAGE associated SNPs and the incidence of MACE were explored both from birth and from study inclusion. The risk to develop MACE from birth to the end of follow-up, i.e. first MACE, death or 31 December 2014, was evaluated in logistic regression analyses adjusted for sex. The prospective associations between the SNPs and time from study baseline to incident first-time MACE, death or end of follow-up were examined in Cox regression analyses adjusted for age and sex (Model 1); and age, sex, BMI, current smoking, diabetes, ApoB, ApoA1, eGFR, systolic blood pressure, blood pressure medication, and statin medication (Model 2). The associations between the identified SNPs and incident mortality from baseline to the end of follow-up were explored in a similarly adjusted Cox proportional hazard analyses. A p-value of <0.05 was considered significant. The statistical analyses were performed with the SPSS 25.0 (IBM software, Armonk NY).

Paper III

We compared patient characteristics between the different outcome groups by using the Mann Whitney test for continuous variables and the chi-square test for dichotomous variables. Multivariable Cox proportional hazards analyses were used to assess the associations between biomarkers and outcomes. We used 3 different adjustment models: Model 1 included age and sex; Model 2 included age, sex, hypertension, diabetes mellitus, smoking, eGFR, previous heart failure and/or ACS; Model 3 included the same variables as Model 2, with the addition of the prognostic biomarkers TnT, NT-proBNP and hsCRP. Skewed variables were logarithmically transformed before analysis. The correlations between biomarkers and echocardiographic parameters were analysed with the Spearman rank test. The Wilcoxon signed ranks test was used to evaluate the differences between biomarker levels at baseline and at 6 weeks in the same patient subgroup. P-values under 0.05 were considered significant. All calculations were made using SPSS 23.0 (IBM software, Armonk NY).

Paper IV

We used the Student's t-test to compare the values of normally distributed continuous variables between groups, and the Mann-Whitney U-test for non-normally distributed continuous variables. Differences in categorical variables were assessed with the chi-square test or Fischer's exact test, as appropriate. Variations in values between different time-points when serial measurements were performed in the same subjects were examined by the Wilcoxon paired test for non-normally distributed variables. Spearman's rank test and a multivariable linear regression analysis model were used to test associations between percentage S100A8/A9 increase relative to baseline and other clinical and biochemical parameters in the population. Non-normally distributed continuous variables were logarithmically transformed before being introduced into the linear regression model. A *p*-value under 0.05 was considered to be statistically significant. Values are presented as mean (SD) if normally distributed and median (inter-quartile range) if not normally distributed. All calculations were made using SPSS 22.0 (IBM software, Armonk, NY).

Results

Paper I

In this study, we included 4612 CVD free individuals from the cardiovascular sub-cohort of the population-based MDC cohort (MDC-CV) to examine the associations between sRAGE, sEMMPRIN and the progression of IMT, first time MACE and total mortality. The median follow-up time to event was 21.5 (18.6-22.3) years for MACE and 21.7 (20.9-22.4) years for total mortality.

Relationships between sRAGE, sEMMPRIN and traditional CV risk factors at baseline

The baseline characteristics of the study population are presented in Table 1. As expected, there was a greater proportion of men among the participants with incident MACE, and the individuals in this group were significantly older, had higher BMI, higher prevalence of dyslipidaemia, diabetes, smoking, hypertension and higher use of both lipid-lowering and blood pressure lowering medication. The MACE group also had significantly lower baseline sRAGE, while there was no difference in the sEMMPRIN concentration (Table 1A). We found similar differences in baseline characteristics between individuals that died during follow-up compared to survivors (Table 1B).

Table 1A. Baseline characteristics in the participants with incident MACE compared to MACE free individuals

Characteristics	Whole cohort N=4612	Incident MACE N=546	No incident MACE N=4066	*P**
Age, (years)	58 (52-63)	61 (55-64)	57 (52-62)	<0.001
Male sex, n (%)	1797 (39.0)	322 (59.0)	1475 (36.3)	<0.001
BMI, (kg/m^2)	25.1 (22.9-27.7)	26.0 (23.8-28.7)	24.9 (22.7-27.6)	<0.001
Diabetes, n (%)	340 (7.4)	88 (16.1)	252 (6.2)	<0.001
Current smoking, n (%)	995 (21.6)	144 (26.4)	851 (20.9)	0.004
Medication use				
Statins, n (%)	44 (0.01)	13 (2.4)	31 (0.8)	<0.001
Blood pressure medication, n (%)	677 (14.7)	119 (21.8)	558 (13.7)	<0.001
Lipids				
LDL (mmol/L)	4.1 (3.5-4.8)	4.4 (3.8-5.0)	4.1 (3.5-4.7)	<0.001
HDL (mmol/L)	1.4 (1.1-1.6)	1.2 (1.0-1.4)	1.4 (1.2-1.6)	<0.001
TG (mmol/L)	1.14 (0.9-1.6)	1.3 (1.0-1.9)	1.1 (0.9-1.6)	<0.001
Blood pressure				
Systolic (mmHg)	140 (128-150)	148 (136-160)	140 (125-150)	<0.001
Diastolic (mmHg)	86 (80-92)	90 (82-96)	85 (80-92)	<0.001
eGFR	73.5 (65.2-82.8)	74.2 (65.4-84.3)	73.5 (65.1-82.6)	n.s.
sRAGE (au)	19.8 (16.2-24.1)	19.3 (15.2-23.1)	19.9 (16.3-24.2)	0.001
EMMPRIN (au)	70.2 (61.8-80.4)	70.4 (61.9-80.4)	70.2 (61.7-80.4)	n.s.

*Comparing individuals with and without MACE, Chi square on dichotomous variables and Mann Whitney on continuous variables

Table 1B. Baseline characteristics in the participants that died compared to survivors

Characteristics	Whole cohort N=4612	Dead N=1205	Alive N=3370	*P***
Age, (years)	58 (52-63)	62 (57-65)	56 (51-61)	<0.001
Male sex, n (%)	1797 (39.0)	580 (48.1)	1200 (35.6)	<0.001
BMI, (kg/m^2)	25.1 (22.9-27.7)	25.8 (23.1-28.4)	24.8 (22.8-27.5)	<0.001
Diabetes, n (%)	340 (7.4)	158 (13.1)	182 (5.4)	<0.001
Current smoking, n (%)	995 (21.6)	355 (29.5)	630 (18.8)	<0.001
Medication use				
Statins, n (%)	44 (0.01)	18 (1.5%)	26 (0.7 %)	n.s.
Blood pressure medication, n (%)	677 (14.7)	246 (20.4)	425 (12.6)	<0.001
Lipids				
LDL (mmol/L)	4.1 (3.5-4.8)	4.2 (3.5-4.8)	4.1 (3.5-4.8)	0.017
HDL (mmol/L)	1.4 (1.1-1.6)	1.3 (1.1-1.5)	1.4 (1.2-1.6)	<0.001
TG (mmol/L)	1.14 (0.9-1.6)	1.2 (0.9-1.7)	1.1 (0.8-1.5)	<0.001
Blood pressure				
Systolic (mmHg)	140 (128-150)	142 (130-160)	140 (125-150)	<0.001
Diastolic (mmHg)	86 (80-92)	88 (80-95)	85 (80-90)	<0.001
eGFR	73.5 (65.2-82.8)	73.6 (64.4-83.9)	73.5 (65.3-82.4)	n.s.
sRAGE (au)	19.8 (16.2-24.1)	19.4 (15.6-23.2)	20.0 (16.4-24.3)	<0.001
EMMPRIN (au)	70.2 (61.8-80.4)	70.0 (61.5-80.1)	70.4 (61.9-80.4)	n.s.

**Comparing individuals that died with survivors, Chi square on dichotomous variables and Mann Whitney on continuous variables

The associations between biomarkers and CV risk factors are presented in Table 2. High sRAGE was significantly associated with low BMI, low diastolic blood pressure, higher LDL and higher triglycerides (Table 2). In turn, high EMMPRIN was associated with high BMI, higher LDL, HDL and triglycerides, high systolic blood pressure and ongoing blood pressure medication. Although sRAGE was not independently correlated with the presence of diabetes in this multivariable regression analysis, plasma sRAGE was significantly lower in patients with diabetes compared to diabetes-free controls [median (IQR) 18.4 (15.2-22.4) vs 19.9 (16.2-24.2); *P*<0.001].

Table 2. Association between biomarkers at baseline and CV risk factors

	sRAGE			EMMPRIN		
CV risk factors	ß*	CI	*P*	ß*	CI	*P*
Age	0.003	-0.002-0.009	n.s.	0.003	-0.003-0.008	n.s.
Sex	0.22	0.16-0.29	<0.001	0.28	0.21-0.35	<0.001
BMI	-0.04	-0.05-(-0.03)	<0.001	0.04	0.03-0.05	<0.001
Diabetes mellitus	-0.03	-0.15-0.08	n.s.	-0.05	-0.17-0.07	n.s.
Smoking	-0.005	-0.08-0.07	n.s.	-0.06	-0.13-0.01	n.s.
HDL	-0.06	-0.16-0.36	n.s.	0.34	0.24-0.44	<0.001
LDL	0.034	0.003-0.065	0.033	0.06	0.03-0.09	0.001
Triglycerides	0.10	0.02-0.18	0.016	0.09	0.003-0.17	0.042
eGFR	-0.002	-0.004-0.001	n.s.	0.001	-0.001-0.003	n.s.
Systolic blood pressure	0.000	-0.002-0.002	n.s.	0.005	0.003-0.007	<0.001
Diastolic blood pressure	-0.006	-0.01- (-0.001)	0.012	-0.005	-0.009-(-0.000)	0.042
Statin medication	0.28	-0.02-0.57	n.s.	0.16	-0.13-0.45	n.s.
Blood pressure lowering medication	-0.06	-0.14-0.03	n.s.	0.13	-0.21-(-0.04)	0.04

*Multivariable linear regression calculated per 1 SD increase in biomarker concentration

In Spearman analyses of the correlations between the soluble receptors and established biomarkers of systemic inflammation, baseline sRAGE had a weak inverse correlation with total blood leukocyte counts (r = -0.087, *P* <0.001) and with plasma hsCRP (r = -0.080, *P* <0.001).

Correlations between sRAGE and IMT progression

In a Pearson correlation analysis, baseline sRAGE showed a weak but significant inverse correlation with IMT at re-examination, total IMT increase, and yearly IMT increase rate from baseline to follow-up (Table 3, columns 3 and 4). We confirmed these associations in multivariable linear regression analyses adjusted for age and sex (Table 3, Model 1, columns 5-7). The relationships remained significant after further adjustment for BMI, smoking, diabetes, blood lipids, eGFR, hypertension, statin use and hsCRP (Table 3, Models 2 and 3). No correlations were seen between IMT and sEMMPRIN (Table 3).

Table 3. Association between biomarkers and IMT.

				sRAGE		
IMT		r*	*P*	ß**	CI	*P*
Baseline	1	-0.02	n.s.	-0.004	-0.03-0.02	n.s.
	2			0.01	-0.02-0.04	n.s.
	3			0.01	0.02-0.03	n.s.
Follow-up#	1	-0.06	<0.001	-0.06	-0.09 — -0.02	0.002
	2			-0.05	-0.08 — -0.01	0.009
	3			-0.05	-0.08 — -0.01	0.007
Absolute IMT increase‡	1	-0.06	<0.001	-0.06	-0.10 — -0.03	0.001
	2			-0.07	-0.11 — -0.03	<0.001
	3			-0.07	-0.11— -0.03	<0.001
Yearly IMT increaseϕ	1	-0.06	0.001	-0.06	-0.10 — -0.02	0.002
	2			-0.06	-0.10 — -0.03	0.001
	3			-0.07	-0.10 — -0.03	0.001
				sEMMPRIN		
IMT		r*	*P*	ß**	CI	*P*
Baseline	1	0.01	n.s.	0.01	-0.01-0.04	n.s.
	2			0.000	-0.03-0.03	n.s.
	3			0.003	-0.02-0.03	n.s.
Follow-up#	1	-0.001	n.s.	-0.01	-0.04-0.03	n.s.
	2			-0.01	-0.05-0.02	n.s.
	3			-0.01	-0.05-0.03	n.s.
Absolute IMT increase‡	1	-0.02	n.s.	-0.02	-0.05-0.02	n.s.
	2			-0.01	-0.05-0.03	n.s.
	3			-0.01	-0.05-0.03	n.s.
Yearly IMT increaseϕ	1	-0.007	n.s.	-0.006	-0.04-0.03	n.s.
	2			-0.002	-0.04-0.04	n.s.
	3			-0.002	-0.04-0.04	n.s.

All N=4612
*Pearson correlation; **Multivariable linear regression calculated per 1 SD IMT increase and per 1 SD increase in biomarker concentration
†Adjustment models: Model 1: age and sex; Model 2: age, sex, BMI, current smoking, diabetes, LDL, HDL, TG, eGFR, systolic blood pressure, blood pressure lowering medication and statin medication use; Model 3: age, sex, BMI, current smoking, diabetes, LDL, HDL, TG, eGFR, systolic blood pressure, blood pressure lowering medication, statin medication use and hsCRP

Associations between sRAGE, MACE and total mortality

MACE occurred in 546 of the participants during follow-up (12%), and 1205 died (26%). Acute fatal or non-fatal coronary events occurred in 428 of the 546 participants with MACE, and a further 118 cases underwent CABG or PCI revascularization without having suffered an acute event beforehand. Among the participants who died during follow-up, 341 deaths were due to CVD.

In a Kaplan-Meier survival analysis with log rank test, participants with sRAGE in the highest tertile (tertile 3) had a significantly lower incidence of MACE and total

mortality compared to participants in the lowest tertile (Figure 29A-B). There were no associations between sEMMPRIN and incident events (not shown).

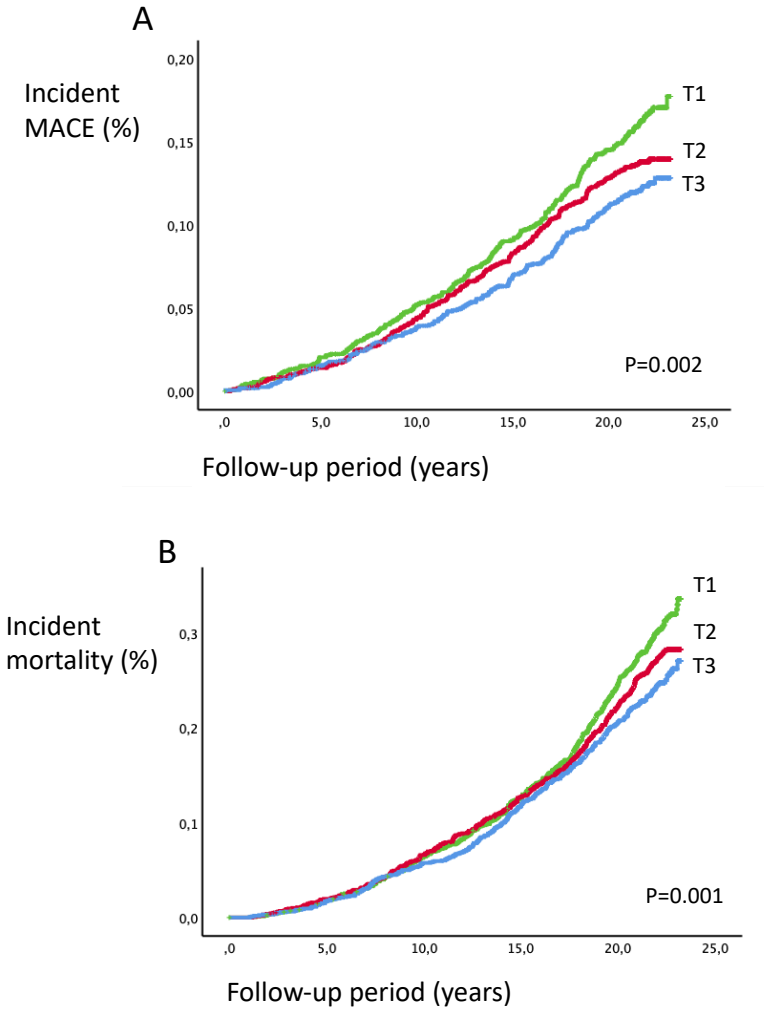

Figure 29. Kaplan-Meier diagrams of incident MACE and total mortality during follow-up

Survival curves of incident MACE (A) and total mortality (B) in the entire cohort (n=4612), by tertiles of sRAGE at baseline. The P-values refer to the difference in incident MACE/mortality between the lowest tertile (T1) and the highest tertile (T3), calculated with an unadjusted log-rank test.

We used multivariable Cox proportional-hazards analyses adjusted for age and sex to prospectively investigate the associations between plasma levels of sRAGE and sEMMPRIN at baseline and the incidence of MACE and total mortality during

follow-up. We found a significant negative association between sRAGE and incident MACE (Table 4, Model 1).

The significance was independent of BMI, smoking, diabetes, blood lipids, eGFR, hypertension and statin use (Table 4, Model 2), but was lost after further adjustment for hsCRP (Table 4, Models 3). We also found a significant negative association between baseline sRAGE and total mortality, which was independent of all considered confounders (Table 4, Models 1-3). No significant associations were seen between sEMMPRIN and the outcomes (Table 4).

Table 4. Associations between biomarkers at baseline and outcome

Biomarker		**sRAGE**			**sEMMPRIN**		
	†	HR*	CI	P	HR*	CI	P
MACE							
n=546	1	0.90	0.82-0.97	0.009	1.07	0.98-1.17	n.s.
	2	0.92	0.85-1.00	0.050	1.06	0.97-1.16	n.s.
	3	0.93	0.85-1.01	n.s.	1.03	0.94-1.13	n.s.
Total mortality							
n=1205	1	0.93	0.88-0.98	0.011	0.98	0.93-1.04	n.s.
	2	0.94	0.88-0.99	0.025	0.97	0.92-1.03	n.s.
	3	0.93	0.88-0.99	0.019	0.98	0.92-1.04	n.s.

All N=4612
*Multivariable Cox proportional hazard analyses calculated per 1 SD biomarker increase.
† Adjustment models: Model 1: age and sex; Model 2: age, sex, BMI, current smoking, diabetes, LDL, HDL, TG, eGFR, systolic blood pressure and blood pressure medication; Model 3: age, sex, BMI, current smoking, diabetes, LDL, HDL, TG, eGFR, systolic blood pressure, blood pressure medication and hsCRP

In receiver operating curve (ROC) prediction models for incident MACE, adding sRAGE to a model based on age and sex only led to a marginal increase in c-statistics (0.678 vs 0.665, n.s.). Similarly, we found only a modest increase in c-statistics for mortality prediction (0.725 vs 0.722, n.s.). Addition of sRAGE to a prediction model including all CV risk factors considered in Model 2 of the Cox regression analysis did not further increase the c-statistics for either MACE or mortality (0.732 vs 0.734, n.s. for MACE; and 0.756 vs 0.756, n.s for total mortality; Figure 30).

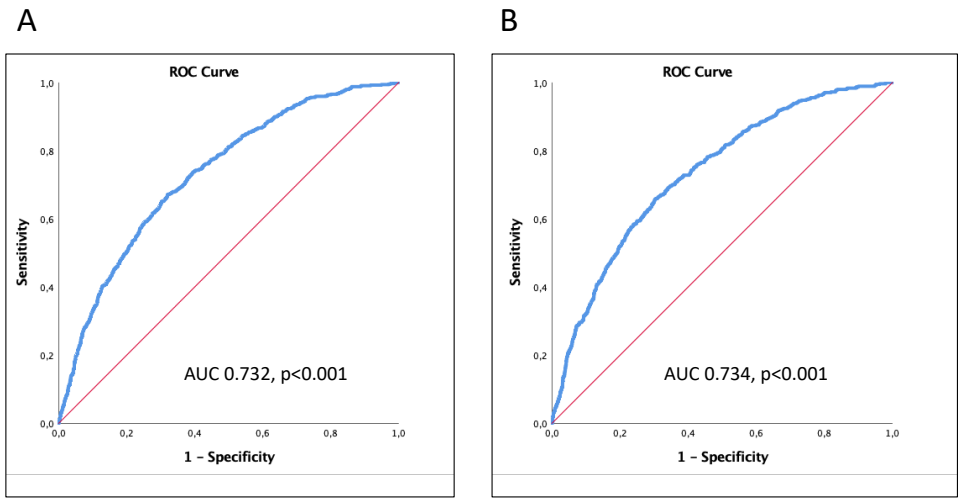

Figure 30. Prediction models

ROC curves for MACE prediction by age, sex and traditional cardiovascular risk factors (A), and age, sex, traditional cardiovascular risk factors and sRAGE (B). AUC, area under the curve.

Paper II

In this study, we performed a GWAS in 4192 individuals from the MDC-CV in order to identify gene variants that influence sRAGE levels. Further, we explored the associations between the identified sRAGE-related SNPs, baseline IMT and IMT progression in the common carotid artery during a median follow-up of 16.5 years. Prospectively, we analysed the relationships between the sRAGE-associated SNPs and total MACE incidence from birth, as well as incident first-time MACE and mortality from baseline in the entire MDC cohort of 29245 individuals. The median follow-up time from inclusion to event was 21.2 (15.6-23.1) years for MACE and 21.6 (18.4-23.3) years for total mortality (time to death or end of follow-up).

sRAGE-associated SNPs

The baseline characteristics of the study participants with valid sRAGE and SNP measurements in the MDC-CV cohort (N=4192) are presented in Table 5.

Table 5. Baseline characteristics of the MDC-CV cohort

Characteristics	N=4192
Age, (years)	58 (52-63)
Male sex, n (%)	1635 (39.0)
BMI, (kg/m^2)	25.1 (22.9-27.7)
Diabetes, n (%)	315 (7.5)
Current smoking, n (%)	902 (21.6)
Medication use	
Statins, n (%)	58 (0.01)
Blood pressure medication, n (%)	647 (15.4)
Lipids	
LDL (mmol/L)	4.1 (3.5-4.8)
HDL (mmol/L)	1.4 (1.1-1.6)
TG (mmol/L)	1.1 (0.9-1.6)
Blood pressure	
Systolic (mmHg)	140 (128-150)
Diastolic (mmHg)	86 (80-92)
eGFR	73.5 (65.2-82.5)
sRAGE (au)	19.8 (16.2-24.1)
IMT	
Baseline (mm)	0.74 (0.66-0.84)
Follow-up (mm)	0.89 (0.78-1.02)
Increase (mm)	0.16 (0.07-0.26)
Increase yearly (mm)	0.009 (0.004-0.017)

The data are presented as median (interquartile range) for the continuous variables.

We found 15 SNPs with GWAS-level associations ($p<5x10^{-8}$) with sRAGE levels at baseline. All SNPs were located in chromosome 6, in a locus in the vicinity of the RAGE-encoding gene (*AGER*). The presence of the minor allele of all the identified SNPs was associated with lower sRAGE levels. We divided the SNPs in four clusters, where the SNPs within each cluster were in close LD to each other (Table 6). The SNP with the strongest association to sRAGE in the first cluster was rs2070600, located within the *AGER* gene and known to induce the missense Gly82Ser mutation in RAGE[171]. A second cluster included rs2854050, a SNP previously shown to be associated with plasma sRAGE levels in other cohorts[172]. The SNPs with the most significant associations to sRAGE levels within the third and fourth clusters, rs206015 and rs204993, were also selected for further analysis (Table 6).

Table 6. Genome-wide significant loci associated with sRAGE levels in plasma

SNPs		*	Basepair	A1/A2	B†	P	MAF	r² ‡
1st cluster	rs2070600	6	32151443	T/C	-0.108	8.94e-13	0.051	Reference
	rs35502919	6	31604355	A/C	-0.090	5.36e-9	0.047	0.77
	rs805284	6	31682029	A/G	-0.089	7.64e-9	0.048	0.77
	rs2844456	6	31864674	C/T	-0.088	8.90e-9	0.048	0.81
	rs34562262	6	32020961	C/G	-0.099	2.27e-10	0.046	0.86
2nd cluster	rs2854050	6	32185605	A/G	-0.085	1.11e-9	0.059	Reference
	rs2022059	6	32156489	C/G	-0.088	1.52e-9	0.053	0.88
	rs2856437	6	32157364	A/G	-0.091	3.52e-10	0.053	0.88
	rs8192575	6	32166384	G/C	-0.082	5.06e-9	0.059	1
3rd cluster	rs206015	6	32182759	A/G	-0.067	4.83e-9	0.089	Reference
	rs206019	6	32180241	T/C	-0.065	2.15e-8	0.087	0.94
	rs206016	6	32181182	A/G	-0.066	1.72e-8	0.087	0.94
4th cluster	rs204993	6	32155581	G/A	-0.055	8.01e-13	0.259	Reference
	rs915895	6	32190217	G/T	-0.044	1.38e-9	0.306	0.73
	rs443198	6	32190406	G/A	-0.043	1.71e-9	0.332	0.64

*Chromosome number
A1= minor allele; A2= major allele
†Beta coefficient derived from a multivariable linear regression with sRAGE as dependent variable, adjusted for age and sex
‡The square of the Spearman correlation coefficient with the reference SNP
MAF= minor allele frequency

The minor allele of all four selected SNPs was associated with significantly lower sRAGE levels (Table 7). The largest decrease in circulating sRAGE was found for rs2070600, with approximately 10% lowering of median sRAGE per minor allele.

Table 7. sRAGE levels according to allele frequency for the four main sRAGE-determinant SNPs identified by GWAS

	sRAGE (au)			
SNPs	Homozygote Major allele	Heterozygote	Homozygote Minor allele	P*
rs 2070600	20.0 (16.4-24.3)	18.2 (14.5-22.0)	16.3 (12.1-19.5)	<0.001
rs 204993	20.5 (16.6-24.7)	19.1 (15.6-23.1)	19.0 (15.3-23.0)	<0.001
rs206015	20.0 (16.4-24.3)	18.8 (15.1-22.7)	18.3 (14.3-23.5)	<0.001
rs 2854050	20.0 (16.3-24.2)	18.5 (14.8-22.4)	17.0 (12.7-20.8)	<0.001

*Kruskal Wallis Test

In a multivariable linear regression analysis of the association between sRAGE and the four SNPs, where we forced all four SNPs into the same model, we found that only rs2070600 and rs204993 maintained a significant association with sRAGE, independently of each other (Table 8, column 6 and 7). This implied that rs2854050 and rs206015, but not rs204993, were in LD with rs2070600, and that the only SNPs that could be considered to be independent determinants for sRAGE were rs2070600 and rs204993. These two SNPs were therefore selected for further examination. In separate multivariable linear regression analyses adjusted for age and sex, we found that both rs2070600 and rs204993 determine approximately 1.2% of the variation in sRAGE (Table 8, column 8).

Table 8. Mutually-adjusted associations between the identified SNPs and plasma sRAGE

SNPs	rs2070600*	rs2854050*	rs206015*	rs204993*	β**	p	$R^{2\#}$
rs2070600	1	0.67	0.48	0.11	-0.092	0.001	0.012
rs2854050	0.67	1	0.62	0.13	0.015	0.603	0.009
rs206015	0.48	0.62	1	0.17	-0.001	0.952	0.008
rs204993	0.11	0.13	0.17	1	-0.041	<0.001	0.012

* r^2 coefficient in a Spearman correlation analysis showing the association between the four selected SNPs.
**Multivariable linear regression analysis of the association between sRAGE and SNPs, adjusted for age, sex and the other SNPs, identifying the SNPs with independent association with sRAGE.
#The square of the partial correlation coefficient between the SNPs and sRAGE in separate multivariable linear regressions adjusted for age and sex, showing the proportion of sRAGE variance explained by each SNP.

Association between sRAGE-associated SNPs and carotid IMT

In the MDC-CV cohort, we analysed the associations between the two identified SNPs and IMT at baseline, at follow-up and IMT progression from baseline to follow-up. We found no significant associations between rs2070600, rs204993 and any of the IMT variables in any of the multivariable linear regression models adjusted for age, sex, CV risk factors and medication (Table 9).

Table 9. Associations between sRAGE genetic variants and carotid IMT

		SNPs			
		rs2070600		rs204993	
IMT	†	ß (CI)	p	ß (CI)	p
Baseline	1	-0.005 (-0.09-0.08)	n.s.	0.03 (-0.02-0.07)	n.s.
	2	-0.02 (-0.10-0.06)	n.s.	0.04 (-0.004-0.08)	n.s.
Follow-up#	1	0.03 (-0.07-0.14)	n.s.	0.006 (-0.05-0.06)	n.s.
	2	0.02 (-0.09-0.13)	n.s.	0.01 (-0.05-0.07)	n.s.
Absolute IMT Increase‡	1	0.01 (-0.10-0.12)	n.s.	-0.03 (-0.08-0.03)	n.s.
	2	0.001 (-0.12-0.12)	n.s.	-0.03 (-0.09-0.03)	n.s.
Yearly IMT Increase	1	0.005 (-0.10-0.11)	n.s.	-0.03 (-0.08-0.03)	n.s.
	2	-0.003 (-0.12-0.12)	n.s.	-0.03 (-0.09-0.03)	n.s.

Multivariable linear regression calculated per 1 SD IMT increase
†Adjustment models: Model 1: age and sex; Model 2: age, sex, BMI, current smoking, diabetes, LDL, HDL, TG, eGFR, systolic blood pressure, blood pressure lowering medication and statin medication

Prospective associations between sRAGE genetic variants, MACE and mortality

The baseline characteristics of the entire MDC study cohort were similar to those of the MDC-CV (Paper II, Supplementary table 1). There was a larger proportion of men among the individuals that suffered a MACE event during follow-up compared to MACE-free individuals, and they were significantly older, had a higher prevalence of hypertension, dyslipidaemia, overweight, diabetes and smoking (Paper II, Supplementary table 2). The use of blood pressure- and lipid lowering medication was also higher (Paper II, Supplementary table 2). Similar differences in baseline characteristics were found between the subjects that died during follow-up and those who survived (Paper II, Supplementary table 1).

Total MACE, defined as prevalent MACE before study inclusion or incident MACE during follow-up, occurred in a total of 4850 individuals (16.6%). Incident first time MACE after study inclusion occurred in 4114 (14%) participants without a history of prevalent coronary heart disease, and 10331 (35%) individuals died during follow-up (Figure 2). In a logistic regression analysis adjusted for sex, we found a positive association between the presence of the minor allele (T vs C) of rs2070600 and occurrence of total MACE (Table 10, adjustment model 1). The T-allele of rs2070600 was also associated with increased risk for first time MACE during follow-up in Cox regression analyses adjusted for age, sex, cardiovascular risk factors and medication at baseline (Table 10, adjustment models 2-3). We found no associations between rs204993 and MACE, and neither SNP was related to total mortality (Table 10).

Table 10. Associations between sRAGE genetic determinants and clinical outcomes

SNPs	†	Incident total MACE[a]		Incident first-time MACE[b]		Mortality	
		OR[*] (CI)	p	HR[ϕ] (CI)	p	HR[ϕ] (CI)	p
Rs2070600	1	1.16 (1.05-1.27)	0.003				
	2			1.12 (1.02-1.23)	0.014	1.05 (0.99-1.11)	n.s.
	3			1.12 (1.02-1.23)	0.023	1.05 (0.98-1.12)	n.s.
Rs204993	1	0.98 (0.94-1.04)	n.s.				
	2			0.98 (0.93-1.03)	n.s.	1.01 (0.98-1.05)	n.s.
	3			1.00 (0.95-1.05)	n.s.	1.02 (0.99-1.05)	n.s.

[a] Incident MACE from birth to the end of follow-up.
[b] Incident first-time MACE from study inclusion to the end of follow-up. Subjects with prevalent MACE have been excluded from this analysis.
† Adjustment models: Model 1: sex; Model 2: age at study inclusion and sex; Model 3: age, sex, BMI, current smoking, diabetes mellitus, ApoA1, ApoB, systolic blood pressure, blood pressure lowering medication and lipid lowering medication
[*] Logistic regression analysis
[ϕ] Cox proportional hazard analysis. Time to event measured from study inclusion.

Paper III

In this prospective cohort study, we examined the associations between plasma S100A12 and sRAGE at the time of an ACS event and the incidence of recurrent MI, heart failure and mortality in a population of 524 ACS patients. Further, in a subgroup of 114 of these patients, we investigated whether the dynamics of S100A12 and sRAGE in plasma during the first 6 weeks post-ACS were associated with residual CV risk. The median follow-up time to event was 25.7 months (IQR 13.3-38.3) for MACE, and 39.5 months (IQR 27.4-52.2) for total mortality.

Baseline characteristics

The baseline characteristics of the study population are presented in Table 11. The patients that suffered a recurrent MACE event during follow-up were older, had a higher prevalence of hypertension, previously known ACS or heart failure, lower eGFR, and higher hsCRP, and NT-proBNP at the time of the index event (Table 11).

Table 11. Baseline differences in clinical characteristics between patients with and without recurrent MACE during follow-up

Characteristics	All patients N= 524	Patients with recurrent MACE N=87	Patients without recurrent MACE N=437	*p*
Age (years)	67 (59-77)	77 (66-85)	66 (58-74)	<0.001
Male sex, n (%)	384 (73.3%)	61 (70.1%)	322 (73.7%)	0.493
Hypertension, n (%)	285 (54.4%)	56(64.4%)	229 (52.4%)	0.041
Smoking, n (%)	132 (25.2%)	18 (20.7%)	114 (26.1%)	0.290
Diabetes, n (%)	126 (24.0%)	24 (27.6%)	102 (23.3%)	0.397
BMI (kg/m$^{2)}$	26.9 (24.3-29.8)	26.0 (23.7-29.7)	27.1 (24.4-29.8)	0.196
eGFR (mL/min/1.73m^2)	72.0 (53.1-94.6)	54.9 (34.5-75.8)	74.7 (57.2-96.5)	<0.001
Index cardiac event				
STEMI	179 (34.2%)	27 (31.0%)	152 (34.8%)	0.501
NSTEMI	295 (56.3%)	56 (64.4%)	239 (54.7%)	0.097
UA	50 (9.5%)	4 (4.6%)	46 (10.5%)	0.086
Previous cardiac event				
HF, n (%)	54 (10.3%)	18 (20.7%)	36 (8.2%)	<0.001
ACS, n (%)	151 (28.8%)	39 (44.8%)	112 (25.6%)	<0.001
TnT (ng/L)	365 (62-1291)	379 (96-1290)	364 (55-1297)	0.337
hsCRP (mg/L)	6.7 (2.9-17.8)	8.9 (3.7-29.6)	6.3 (2.6-16.4)	0.005
NT-proBNP (au)	81.0 (49.5-103.6)	107.6 (83.9-121.9)	75.6 (45.7-97.0)	<0.001
S100A12 (au)	3.7 (3.0-4.8)	4.6 (3.6-6.1)	3.4 (2.9-4.5)	<0.001
sRAGE (au)	25.5 (20.1-34.8)	34.1 (24.6-45.9)	24.8 (19.2-31.8)	<0.001

ACS, acute coronary syndrome; BMI, body mass index; eGFR, estimated glomerular filtration rate; HF, heart failure; hsCRP, high sensitivity C-reactive protein; MACE, major adverse cardiovascular event; NSTEMI, non ST-elevation myocardial infarction; NT-proBNP, N-terminal pro-brain natriuretic peptide; sRAGE, soluble receptor for advanced glycation endproducts; STEMI, ST elevation myocardial infarction; TnT, Troponin T; UA, unstable angina.

Additionally, patients who died had a significantly higher prevalence of diabetes and a lower prevalence of smoking (Paper III, Supplementary table 3). Baseline S100A12 and sRAGE were significantly higher in patients with recurrent MACE (Table 11), ACS (Paper III, Supplementary table 4), or heart failure (Paper III, Supplementary table 5) during follow up, and in patients who died (Paper III, Supplementary table 3).

The patients in the subgroup with detailed follow-up were older compared to the whole cohort, since the inclusion criterion for this subgroup was age >75 years. Additionally, they had lower BMI, lower eGFR, and higher plasma concentration of NT-proBNP at baseline compared to the rest of the cohort (Paper III, Supplementary table 6). The prevalence of smoking was lower, and hypertension was more frequent in this group (Paper III, Supplementary table 6). Baseline S100A12 and sRAGE levels were also higher in the elderly subgroup that underwent detailed follow-up compared to the whole cohort (Paper III, Supplementary table 6).

Associations between biomarkers at the time of the ACS and recurrent coronary events and incident heart failure during follow-up

In the acute phase, S100A12 was directly correlated with hsCRP ($r = 0.281$, $P < 0.001$) and IL-6 ($r = 0.312$, $P < 0.001$). Similar but weaker correlations were observed between sRAGE and hsCRP ($r = 0.091$, $P = 0.038$) and IL-6 ($r = 0.288$, $P < 0.001$). In crude Kaplan-Meier survival analyses with log-rank tests, patients with baseline S100A12 and sRAGE in the highest tertile (T3) had a significantly higher incidence of recurrent MACE compared to patients in tertiles 1 and 2 (Figure 31). The biomarkers were measured within 24h after admission.

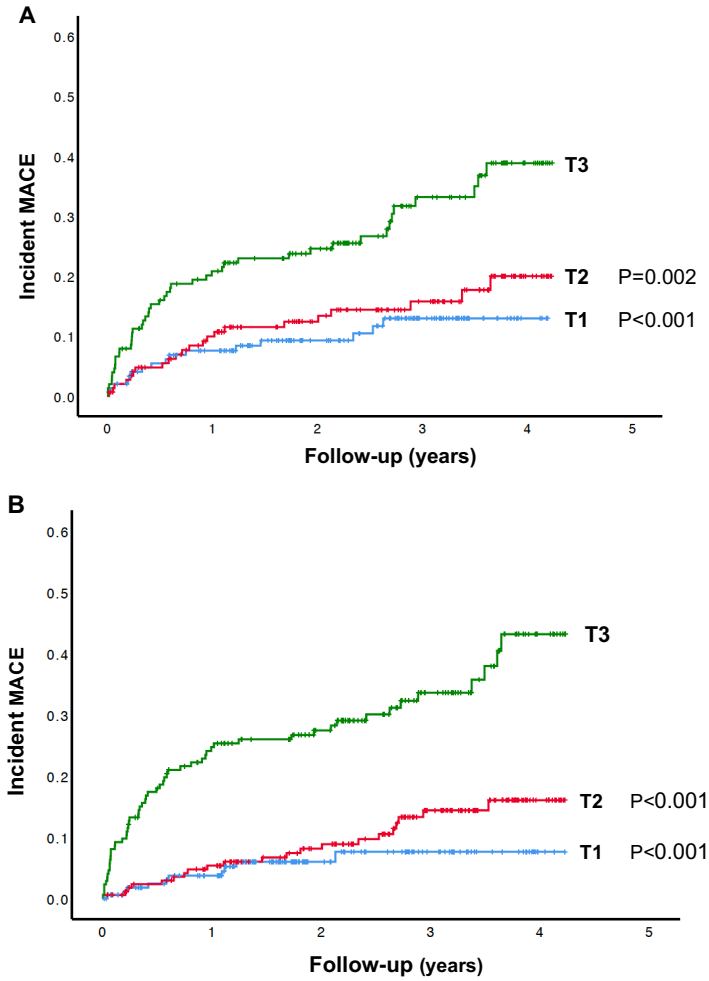

Figure 31. Kaplan Meyer diagrams of incident MACE after the index acute coronary event

Survival curves of incident MACE in the entire cohort (n=524), by tertiles of S100A12 (A) and sRAGE (B) at baseline. The P-values refer to the difference in incident MACE between the respective tertile and the highest tertile (T3), calculated with an unadjusted log-rank test. Reproduced with permission from the original article ©Elsevier.

We used multivariable Cox proportional hazard analyses to investigate the associations between plasma levels of S100A12 and sRAGE at baseline, and the incidence of recurrent MACE, recurrent ACS (fatal or non-fatal) and total mortality in the cohort. The associations between S100A12, sRAGE and MACE remained significant after adjustment for age, sex, and potential clinical confounders. A significantly higher number of events occurred in the highest compared to the lowest tertile (Table 12, tertile T3 vs T1, Models 1-2).

Table 12. Correlations between biomarkers at baseline and outcome

Biomarker at inclusion			MACE (N=87)			ACS (N=76)			Total mortality (N=62)		
			HR	CI	*p*	HR	CI	*p*	HR	CI	*p*
S100A12	1[a]	T2 vs T1	1.3	0.7-2.6	0.377	1.4	0.7-2.8	0.331	1.7	0.8-3.7	0.186
		T3 vs T1	2.1	1.2-3.8	0.011	2.2	1.2-4.1	0.014	2.1	1.0-4.3	0.045
	2	T2 vs T1	1.3	0.6-2.5	0.497	1.2	0.6-2.5	0.583	1.5	0.6-3.5	0.340
		T3 vs T1	2.0	1.1-3.7	0.029	1.9	1.0-3.7	0.046	1.9	0.9-4.1	0.114
	3	T2 vs T1	1.2	0.6-2.4	0.609	1.2	0.6-2.4	0.687	1.8	0.7-4.3	0.203
		T3 vs T1	1.8	1.0-3.4	0.071	1.8	0.9-3.5	0.098	2.1	0.9-4.9	0.086
sRAGE	1	T2 vs T1	1.5	0.7-3.3	0.273	1.3	0.6-2.8	0.527	1.1	0.5-2.3	0.875
		T3 vs T1	4.1	2.1-8.1	<0.001	3.8	1.9-7.5	<0.001	1.8	0.9-3.6	0.099
	2	T2 vs T1	1.4	0.7-3.1	0.358	1.3	0.6-2.8	0.564	0.9	0.4-2.0	0.773
		T3 vs T1	3.6	1.8-7.3	<0.001	3.4	1.6-6.8	0.001	1.3	0.6-2.8	0.453
	3	T2 vs T1	1.3	0.6-2.9	0.486	1.2	0.5-2.6	0.688	1.0	0.4-2.3	0.970
		T3 vs T1	3.2	1.5-6.5	0.002	3.0	1.4-6.2	0.003	1.6	0.7-3.6	0.269

Multivariable Cox proportional hazard analyses of the relationship between biomarker tertiles at inclusion, recurrent CV events and mortality.
Number of events per S100A12 tertile (T1/T2/T3): MACE (16/22/46); ACS (14/20/36); Total mortality (10/17/35)
Number of events per sRAGE tertile (T1/T2/T3): MACE (10/21/56); ACS (10/17/49); Total mortality (11/16/36)
[a]Adjustment models: Model 1: age and sex; Model 2: age, sex and CV risk factors (hypertension, smoking, diabetes mellitus, eGFR, previous heart failure and/or ACS); Model 3: age, sex, CV risk factors and biomarkers (hsCRP, NT-proBNP and TnT)

The association between sRAGE and MACE was also independent of the established clinical biomarkers TnT, hsCRP, and NT-proBNP, but the relationship between S100A12 and MACE lost significance (Table 12, Model 3). Similar results were recorded for recurrent ACS (Table 12). S100A12 also showed a weak association with total mortality in Model 1, which was lost after further correction for clinical confounders. We found no associations between baseline sRAGE and total mortality.

Further, we investigated the relationships between the biomarkers and rehospitalization for heart failure after the index event, as well as with echocardiographic parameters of left ventricle function and remodelling during follow-up. We found that high baseline levels of sRAGE and S100A12 were associated with an increased frequency of heart failure hospitalization (Table 13a, Model 1). In line with these findings, high baseline levels of S100A12 were correlated with low LVEF and large LVESV at 1 year (Table 13b). High sRAGE was also correlated with a low LVEF at 1 year and also with LVEF deterioration during the first year post-ACS, from baseline to follow-up (Table 13b). sRAGE had a stronger association with heart failure hospitalization compared to S100A12, but both associations lost statistical significance when potential confounders and other established biomarkers were considered (Table 13a, Models 2 and 3).

Table 13a. Correlation between biomarkers at baseline and incident heart failure

Biomarker	Models	Heart Failure (N=41)[a]					
		T2 vs T1			T3 vs T1		
		HR[b]	CI for HR	*p*	HR[b]	CI for HR	*p*
S100A12	1	1.8	0.7-5.0	0.229	2.4	1.0-6.1	0.056
	2	1.3	0.5-3.9	0.584	1.4	0.5-3.9	0.507
	3	1.0	0.3-3.1	0.993	0.9	0.3-2.5	0.766
sRAGE	1	1.9	0.6-6.2	0.266	4.6	1.6-13.2	0.005
	2	1.6	0.5-5.1	0.449	2.4	0.8-7.5	0.126
	3	1.4	0.4-4.5	0.606	2.0	0.6-6.6	0.252

[a] Incident hospitalization with a clinical diagnosis of heart failure during follow-up.
[b] MultivariableCox proportional hazards analyses of the relationship between biomarker tertiles at inclusion and incident heart failure.
Number of events per S100A12 tertile (T1/T2/T3): 6/11/22; and per sRAGE tertile (T1/T2/T3): 4/10/27
Adjustment models: Model 1: age and sex; Model 2: age, sex and CV risk factors (hypertension, smoking, diabetes mellitus, eGFR, previous heart failure and/or ACS); Model 3: age, sex, CV risk factors and biomarkers (hsCRP, NT-proBNP and TnT)

Table 13b. Correlation between biomarkers at baselineand cardiac function at 1 year post-ACS

Biomarker	LVEF 1 year post-ACS		LVEF change[a]		LVESV 1 year post-ACS		LVEDV 1 year post-ACS	
	r[b]	*p*	r[b]	*p*	r[b]	*p*	r[b]	*p*
S100A12	-0.254	0.019	0.031	0.816	0.235	0.023	0.114	0.275
sRAGE	-0.196	0.054	-0.282	0.021	0.168	0.086	0.055	0.577

[a] Left ventricle ejection fraction at 1 year follow-up minus ejection fraction at inclusion.
[b] Spearman correlation

Associations between biomarker dynamics in the first 6 weeks post-ACS and prognosis

At 6 weeks post-ACS, high levels of S100A12 were significantly correlated with high hsCRP ($r = 0.372$, $P < 0.001$) and high IL-6 ($r = 0.386$, $P < 0.001$), witnessing an increased systemic pro-inflammatory state. In contrast, there were no associations between sRAGE, hsCRP and IL-6 at this time point. These data suggest that S100A12 might reflect similar pro-inflammatory pathways as hsCRP and IL-6, while sRAGE has different dynamics and possibly a different role during the recovery phase. We investigated whether the dynamics of the biomarkers, i.e. the increase or decrease from the ACS to the 6 weeks follow-up, could better identify patients at high residual risk. The change in biomarker levels was calculated as the value at 6 weeks minus the value at inclusion. Within the detailed follow-up subgroup, the patients who suffered a recurrent MACE were older and had a higher prevalence of previous heart failure at baseline (Paper III, Supplementary table 7).

The levels of TnT, hsCRP and NT-proBNP were lower at 6 weeks compared to baseline, but S100A12 did not change significantly between the two time points. In contrast, plasma sRAGE increased from baseline to 6 weeks [28.4 (21.6 – 37.5) vs 32.6 (26.7 – 41.0), $p<0.001$], and delta sRAGE was significantly higher in patients who remained MACE-free during follow-up (Paper III, Supplementary table 7). The

associations between biomarker change and recurrent MACE, recurrent ACS and total mortality were examined in multivariable Cox proportional hazard analyses using the adjustment models described in the Methods section. The patients were divided into tertiles according to the change in biomarker levels. Tertile 3 included patients with an absolute increase in S100A12 and sRAGE, whereas patients within tertile 1 had decreased levels in plasma at 6 weeks compared to baseline (Table 14).

Table 14: Change in biomarkers from index ACS to the 6-weeks follow-up time point, divided by tertiles

Biomarker change[a]	Tertile 1[b]	Tertile 2[b]	Tertile 3[b]
S100A12 (au)	-1.13 (-2.38 – -0.84)	0.14 (-0.12 – 0.34)	2.52 (1.21 – 8.01)
sRAGE (au)	-6.82 (-12.54 – -3.04)	1.78 (0.83 – 4.90)	13.07 (10.19 – 17.09)

[a] Calculated as biomarker concentration at 6 weeks after the index event minus biomarker concentration at the index event.
[b] Median and interquartile range (IQT)

We found that patients with increasing levels of sRAGE during follow-up had a significantly lower risk for recurrent ACS compared to patients with decreasing sRAGE (Tertile 3 vs Tertile 1, Table 15, Model 1). The association was independent of age, sex, hypertension, smoking, diabetes mellitus, eGFR, previous heart failure and/or ACS, and circulating TnT, NT-proBNP, and hsCRP at 6 weeks (Table 15, Models 2 and 3). The association between sRAGE and incident MACE followed a similar pattern but was only significant in Model 2, suggesting that it is mostly recurrent ACS events that drive the association. We found no significant correlations between the change in S100A12 and outcome. The variation in neither biomarker were associated with mortality.

Table 15. Correlations between the change in biomarker levels from ACS to the 6-week follow-up and outcome

Biomarker change [a]			MACE (N = 30)			ACS (N = 25)			Total mortality (N = 19)		
			HR	CI	*p*	HR	CI	*p*	HR	CI	*p*
S100A12	1[b]	T2 vs T1	1.5	0.6-3.9	0.394	1.5	0.5-4.4	0.456	0.5	0.1-1.7	0.261
		T3 vs T1	1.4	0.5-3.5	0.501	1.7	0.6-4.8	0.332	1.1	0.4-3.0	0.875
	2	T2 vs T1	1.6	0.6-4.7	0.381	1.5	0.4-4.8	0.540	0.5	0.1-2.2	0.401
		T3 vs T1	1.1	0.4-3.5	0.837	1.3	0.4-4.5	0.660	1.1	0.3-3.6	0.872
	3	T2 vs T1	2.1	0.7-6.5	0.240	1.7	0.5-6.0	0.394	0.8	0.2-3.4	0.752
		T3 vs T1	0.8	0.2-2.6	0.646	0.9	0.3-3.3	0.862	1.0	0.3-3.5	0.999
sRAGE	1	T2 vs T1	0.8	0.4-1.8	0.581	0.9	0.4-2.1	0.815	0.7	0.2-1.8	0.437
		T3 vs T1	0.4	0.1-1.1	0.088	0.2	0.0-0.8	0.027	0.4	0.1-1.6	0.198
	2	T2 vs T1	0.6	0.2-1.5	0.254	0.7	0.3-1.8	0.454	0.5	0.2-1.6	0.264
		T3 vs T1	0.3	0.1-1.0	0.040	0.1	0.0-0.7	0.013	0.5	0.1-1.8	0.280
	3	T2 vs T1	0.8	0.3-2.2	0.653	0.9	0.3-2.7	0.864	0.7	0.2-2.2	0.516
		T3 vs T1	0.4	0.1-1.2	0.085	0.2	0.0-0.8	0.026	0.5	0.1-2.1	0.352

Multivariable Cox proportional hazard analyses of the relationships between biomarker change from inclusion to the 6-week follow-up time point, incident CV events and mortality
Number of events per S100A12 tertile (T1/T2/T3): MACE (8/10/10); ACS (6/8/9); Total mortality (7/4/8)
Number of events per sRAGE tertile (T1/T2/T3): MACE (14/11/5); ACS (12/11/2); Total mortality (9/7/3)
[a] The change in biomarker was calculated as the value at 6 weeks minus the value at the time of the ACS
[b] Adjustment Models: Model 1: age and sex; Model 2: age, sex and CV risk factors (hypertension, smoking, diabetes mellitus, eGFR, previous heart failure and/or ACS); Model 3: age, sex, CV risk factors and biomarkers (hsCRP, NT-proBNP and TnT)

In a prediction model of the risk for recurrent MACE, adding sRAGE to traditional CV risk factors improved the prediction model compared to the corresponding models with hsCRP and NT-proBNP. Adding delta sRAGE further improved the model (Table 16).

Table 16. Comparison of risk prediction models in ACS patioents for MACE based on CV risk factors at baseline, with the addition of biomarkers

All patients (n = 524)				**Follow-up sub-cohort (n = 114)**			
Model	AUC	95% CI	P[#]	Model	AUC	95% CI	P
CV risk factors[*]	0.731	0.669 – 0.792	<0.001	CV risk factors	0.632	0.515 – 0.749	0.033
+ sRAGE	0.767	0.711 – 0.823	<0.001	+ sRAGE	0.682	0.576 – 0.788	0.003
+ hsCRP	0.741	0.680 – 0.802	<0.001	+ hsCRP	0.652	0.534 – 0.769	0.014
+ NTproBNP	0.747	0.687 – 0.807	<0.001	+ NTproBNP	0.629	0.516 – 0.741	0.037
				+ sRAGE and delta sRAGE	0.759	0.688 – 0.869	<0.001

AUC – Area under the receiver operating characteristic (ROC) curves of binary logistic regression models for MACE risk discrimination.
[*] Model based on the CV risk factors that differed significantly between patients who suffered MACE during follow-up and patients who remained event-free: age, sex, hypertension, eGFR, previous ACS, previously known heart failure.
[#] Refers to the difference between the respective model and the reference line.
All biomarkers were considered at baseline. Delta sRAGE was calculated as the difference between sRAGE values at 6 weeks after the index event, minus sRAGE at the time of the event.

Paper VI

In this study, we investigated the S100A8/A9 response to acute psychological stress in two different cohorts of CAD patients and related the findings to cortisol reactivity as well as to diurnal cortisol rhythm.

Rapid S100A8/A9 response to acute psychological stress

In Cohort I, we measured the early changes in systemic S100A8/A9 levels induced by acute psychological stress in 60 CAD patients. The clinical and biochemical characteristics of the study group are presented in Table 17.

Table 17. Clinical and biochemical parameters of the CAD patients and controls included in studies I and II

	CAD patients Study I (N=60)	**CAD patients Study II** (N=27)	**Controls Study II** (N=28)	**P* Study II**
Age (years)	65.1 (8.9)	61 (6.0)	61 (6.0)	n.s.
Male sex, n (%)	50 (85.0)	22 (81.4)	23 (82.1)	n.s.
Smoking, n (%)	5 (8.3)	13 (48.1)	9 (32.1)	n.s.
Diabetes, n (%)	11 (18.3)	1 (3.7)	1 (3.6)	n.s.
Systolic blood pressure (mmHg)	138 (15)	136 (21)	144 (16)	n.s.
Diastolic blood pressure (mmHg)	79 (9)	80 (10)	85 (8)	0.012
Heart rate (beats/min)	60 (10)	64 (11)	71 (13)	0.041
BMI (kg/m^2)	27.4 (3.3)	27 (3)	27 (3)	n.s.
Plasma lipids (mmoL/L)				
Total cholesterol	3.76 (0.78)	4.44 (0.78)	5.89 (0.92)	<0.001
LDL	1.99 (0.55)	2.40 (0.63)	3.80 (0.92)	<0.001
HDL	1.17 (0.35)	1.27 (0.33)	1.38 (0.31)	n.s.
TG	1.39 (0.70)	1.72 (0.75)	1.75 (0.91)	n.s.
Circulating cell populations (million/dL)				
Leukocytes	6.6 (2.1)	6.4 (1.3)	6.6 (1.5)	n.s.
Neutrophils	3.7 (1.4)	3.7 (1.1)	3.7 (1.1)	n.s.
Monocytes	0.5 (0.2)	0.7 (0.3)	0.5 (0.2)	n.s.
Lymphocytes	2.1 (0.9)	1.9 (0.6)	2.2 (0.6)	n.s.
Medication				
Statin, n (%)	59 (98.3)	25 (93.6)	3 (10.7)	<0.001
ACE/ARB, n (%)	42 (70.0)	13 (48.1)	3 (10.7)	0.003
Betablocker, n (%)	46 (76.7)	24 (88.9)	5 (17.9)	<0.001
Calcium channel blockers, n (%)	21 (35.0)	4 (14.8)	3 (10.7)	n.s.

Continuous variables are presented as mean (SD).
*Comparison between CAD patients and controls in Cohort II. Student's T-test was used for continuous variables and the chi-square test for categorical variables.

Psychological stress induced a significant elevation in plasma S100A8/A9 measured at 20 min after test completion compared to baseline [median (IQR) 750 (615-1190) ng/mL pre-stress vs 910 (720-1452) ng/mL post-stress; $p = 1.8 \times 10^{-8}$] (Figure 32).

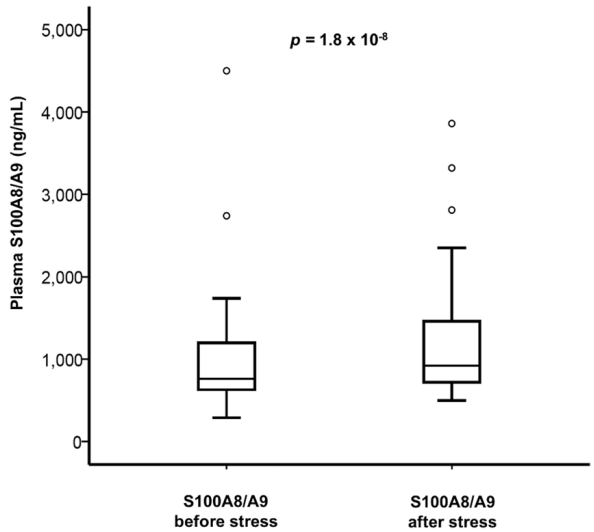

Figure 32. Circulating S100A8/A9 before and after acute psychological stress (Cohort I)

Plasma S100A8/A9 increases significantly in CAD patients (n=60) subjected to acute psychological stress. Plasma samples were collected before and at 20 minutes after the completion of a psychological stress test. The difference between the groups was calculated using the paired Wilcoxon Signed Ranks test. ° denotes outliers. Reproduced with permission from the original article ©the Authors, Springer Nature (http://creativecommons.org/licenses/by/4.0/)

There was a strong positive association between baseline and post-stress S100A8/A9 levels ($r = 0.880$, $p = 1.9 \times 10^{-20}$) (Figure 33A), and both correlated positively with blood neutrophil counts ($r = 0.301$, $p = 0.019$ and $r = 0.302$, $p = 0.019$, respectively). However, the relative S100A8/A9 increase expressed as percent of baseline levels did not correlate with pre-stress S100A8/A9 ($r = -0.114$, $p = 0.385$) (Figure 33B) or with the number of circulating neutrophils (Table 18), suggesting that the individual reactivity to psychological stress was independent of the habitual S100A8/A9 levels and of the neutrophil counts in blood.

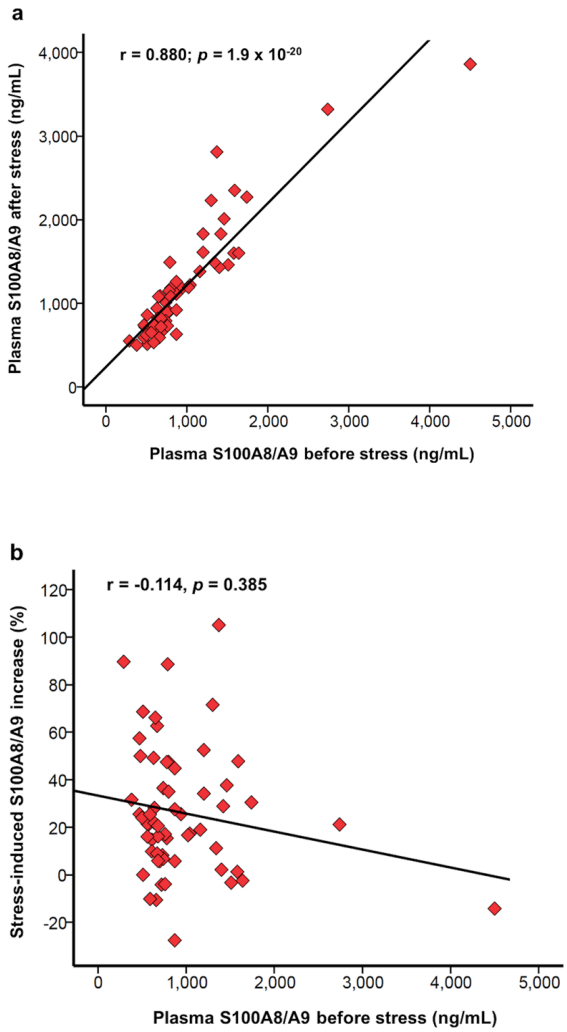

Figure 33. Correlation between baseline S100A8/A9 and the rapid S100A8/A9 response induced by acute psychological stress (Cohort I)

A) Correlation between S100A8/A9 in plasma before and 20 minutes after the end of the psychological stress test. B) Correlation between the S100A8/A9 in plasma before the test and the percentage S100A8/A9 increase induced by acute psychological stress. The correlations were examined using the Spearman test. Reproduced with permission from the original article ©the Authors, Springer Nature (http://creativecommons.org/licenses/by/4.0/)

We also found a positive correlation between the relative S100A8/A9 increase and systolic blood pressure at 10 minutes after the test ($r = 0.287$, $p = 0.016$), suggesting that individuals with a stronger S100A8/A9 response had an impaired systolic blood pressure recovery after the stressor. There were no correlations between the S100A8/A9 response and diastolic blood pressure or heart rate at any time point.

Table 18. Potential determinants of the rapid S100A8/A9 response induced by acute psychological stress in CAD patients (Cohort I)

	Spearman correlation		**Multivariable linear regression**	
	r	P	Beta coefficient*	P
Age	0.029	n.s.	-0.100	n.s.
Sex	–	–	0.098	n.s.
BMI	-0.148	n.s.	-0.274	n.s.
Diabetes	–	–	-0.068	n.s.
Hypertension	–	–	-0.138	n.s.
Smoking	–	–	-0.029	n.s.
Plasma lipids				
Total cholesterol	0.033	n.s.	–	–
LDL	-0.087	n.s.	-0.211	n.s.
HDL	-0.061	n.s.	-0.124	n.s.
TG	-0.100	n.s.	-0.050	n.s.
Circulating cell populations				
Leukocytes	0.113	n.s.	–	–
Neutrophils	0.154	n.s.	0.106	n.s.
Monocytes	0.048	n.s.	-0.041	n.s.
Lymphocytes	0.011	n.s.	0.101	n.s.
Saliva cortisol				
Morning	-0.006	n.s.	0.165	n.s.
Evening	0.315	0.016	0.635	0.004
Cortisol response to stress#	0.032	n.s.	0.218	n.s.
Medication				
Statin	–	–	-0.049	n.s.
Betablocker	–	–	0.082	n.s.
ACE/ARB	–	–	0.205	n.s.
Calcium-channel blockers	–	–	-0.038	n.s.

* Multivartiable linear regression with stress-induced S100A8/A9 increase, expressed as percentage of baseline, as dependent variable.
# Percent cortisol increase at 20 minutes after the stress test compared to baseline

Further, we assessed whether the rapid stress-induced increase of S100A8/A9 in CAD patients was associated with the presence of CV risk factors, blood immune cell counts and parameters of cortisol homeostasis (Table 18). In a bivariate Spearman correlation analysis, the relative S100A8/A9 increase was not associated with any of the considered CV risk factors or cell counts and did not correlate with the rapid cortisol response to psychological stress. However, the S100A8/A9 release strongly correlated with evening saliva cortisol (Table 18). After adjustment for age, sex, BMI, diabetes, hypertension, smoking, plasma lipids, blood immune cell counts, morning saliva cortisol, cortisol response to the stress test and medication in

a multivariable linear regression analysis, evening saliva cortisol remained a potent determinant of stress-induced S100A8/A9 increase (Table 18).

Sustained S100A8/A9 response to acute psychological stress

In Cohort II, we measured the changes in systemic S100A8/A9 levels 24 hours after the psychological stress test in 27 CAD patients and 28 healthy controls. The clinical and biochemical characteristics of the participants are shown in Table 17. The patients had significantly lower levels of total cholesterol and LDL cholesterol, as well as lower diastolic blood pressure and heart rate compared to controls, likely due to medication. However, both patients and controls reacted to the psychological stress test with significant increases in blood pressure and heart rate. When all subjects were considered, there were no significant differences between patients and controls regarding the stress-induced changes in S100A8/A9 levels relative to baseline [median (IQR) 7 (-34 – 106) % vs 33 (-19 – 62) %, $p = 0.960$]. However, there was a large variability in the relative change in S100A8/A9 from baseline to 24h within both groups, indicating that the S100A8/A9 response to stress was highly heterogeneous among individuals. A sustained S100A8/A9 response, defined as an increase of at least 50 % at 24h compared to baseline, was seen in 11 (40 %) of the patients and 10 (36 %) of the controls. Among these subjects, patients tended to have a slightly stronger S100A8/A9 response compared to controls [median (IQR) 155 (88-268) % vs 69 (57-205) *%,* $p = 0.049$].

The acute cortisol response to stress, defined as percentage cortisol increase at 20 minutes after the test relative to baseline values, was significantly lower in CAD patients compared to controls [median (IQR) 25 (0.7-31) % vs. 41 (17-88) %, $p = 0.029$]. The variation in cortisol levels correlated negatively with the S100A8/A9 response at 24h after stress in CAD patients ($r = -0.486$, $p = 0.016$) (Figure 34A), but no such correlation was present in healthy controls ($r = -0.154$, $p = 0.442$) (Figure 34B). The data suggest that CAD patients with a poor cortisol reaction to acute psychological stress maintain a pro-inflammatory status characterized by elevated levels of S100A8/A9 that persist for at least 24 hours after the stressor.

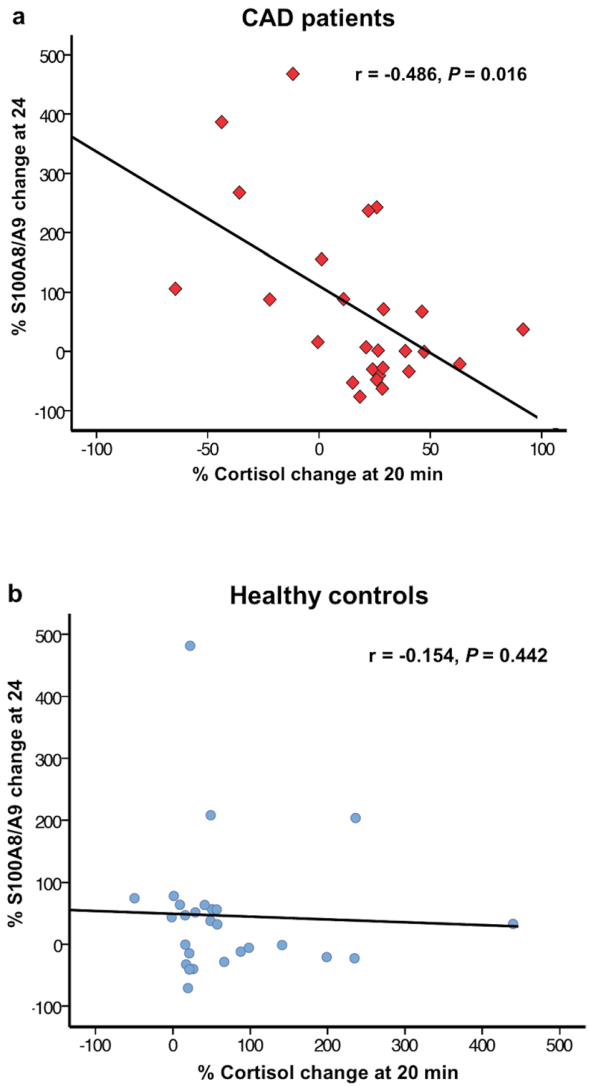

Figure 34. Correlation between the rapid cortisol response at 20 min after the psychological stress test and the sustained S100A8/A9 response at 24 hours after the test in CAD patients (A) and healthy controls (B) (Cohort II)

The S100A8/A9 increase and cortisol increase both expressed as percentage increase relative to baseline.

Discussion

Summary of results

In this thesis we investigated the role of the pro-inflammatory alarmins S100A8/A9 and S100A12, mainly secreted by neutrophils, and of their soluble receptors sRAGE and sEMMPRIN as biomarkers and potential mediators of CVD.

In **Paper I**, we found that high sRAGE levels are associated with slower long-term carotid IMT progression and lower incidence of MACE and mortality in the general population, independently of traditional CV risk factors and kidney function. These human data suggest a potential protective role of sRAGE against CVD development and support the findings of previous experimental studies demonstrating a direct anti-atherogenic role of sRAGE[136, 151, 152, 173, 174]. Although EMMPRIN shares similar ligands with RAGE, with the potential to act as anti-inflammatory decoy receptor, we found no relationships between sEMMPRIN, CV-disease and mortality.

In a GWAS analysis in the same cohort, in **Paper II** we identified that carriers of the minor allele of the SNPs rs2070600 and rs204993 have lower plasma sRAGE. While rs204993 is silent, rs2070600 is a missense mutation inducing structural changes of the RAGE protein. Further, we found that the minor T-allele of rs2070600 is associated with a 16% higher lifetime risk for MACE, independently of traditional CV-risk factors. We found no such association between the silent rs204993 mutation and MACE, suggesting that the relationship between rs2070600 and MACE is probably due to altered RAGE function rather than to its RAGE-lowering effect. This is the first study to demonstrate a relationship between a genetic determinant of RAGE and CVD, supporting a direct involvement of RAGE in the pathogenesis of the disease in humans.

In **Paper III** we found that high plasma levels of both S100A12 and sRAGE at the time of an acute coronary event are associated with increased risk of recurrent MI, cardiac dysfunction and incident heart failure during follow-up. In contrast, patients with increasing sRAGE at 6 weeks after the infarction compared to the acute phase had a very low risk for recurrent MI, suggesting a phase-dependent role for sRAGE in ACS. While in the acute ACS phase sRAGE might serve as a biomarker to reflect the magnitude of the damaging immune and inflammatory activation, the seemingly protective role of sRAGE in the recovery phase post-ACS mirrors the negative association between sRAGE and CVD risk found in Paper I.

In the last study, presented in **Paper IV**, we investigated whether stable CAD patients have a disproportional inflammatory response to stress, measured by the magnitude of S100A8/A9 release, and whether this is related to a dysfunctional activity of the HPA axis. In stable post-ACS patients, we found that acute psychological stress induced a rapid S100A8/A9 increase, associated with a flat diurnal cortisol curve characterized by high evening cortisol. Further, in a case-control study we found that the S100A8/A9 elevation was still present 24 hours after stress in both CAD patients and healthy controls, indicating that the release of S100A8/A9 is not restricted to patients. There was a tendency towards a higher S100A8/A9 response at 24 h in patients, which correlated inversely with the ability to mount an adequate rapid cortisol response to stress. No such correlation was seen in the controls.

S100 proteins and their receptors as biomarkers

sRAGE as a biomarker in the general population

Earlier clinical studies, both in population-based cohorts and CAD cohorts, have indicated that sRAGE could be a possible biomarker for atherosclerotic disease progression[153-157]. Our study presented in Paper I showed a significant inverse association between sRAGE and both IMT and CV event risk. These data are in line with earlier prospective studies investigating the value of sRAGE as a CVD risk biomarker in general population cohorts. In 1201 CVD-free individuals with normal kidney function selected from the population-based Atherosclerosis Risk in Communities (ARIC) study, high sRAGE was associated with a lower incidence of diabetes, coronary events and mortality[175]. Additionally, sRAGE has also been found to be negatively associated with all-cause mortality in an exploratory analysis of 323 individuals from the Framingham Heart Study[176]. Here, we also show that subjects with low plasma sRAGE have a higher systemic inflammatory state, characterized by elevated circulating white blood cells and hsCRP. These findings support sRAGE as a potential biomarker for CVD disease progression and event risk. However, sRAGE did not provide substantial additional predictive information for MACE and mortality on top of age, sex and traditional CV risk factors, as evaluated in ROC prediction models.

This implies that sRAGE has a rather limited clinical use as a biomarker for atherosclerosis progression and event risk in the general population and does not fulfil the criteria of a good biomarker in a primary prevention.

sRAGE and S100A12 as biomarkers in acute coronary syndrome

Previous clinical studies have shown that sRAGE levels are increased in ACS patients at admission compared to healthy controls[177, 178]. Elevated sRAGE at admission in these patients has been associated with worse in-hospital prognosis[179] and with lower LVEF at 7 months[180]. This is consistent with the results of our study, where we found that individuals with acute-phase plasma sRAGE in the highest tertile compared to the lowest tertile have 4.1 higher risk for recurrent MACE and 4.6 higher risk to develop heart failure. As described in the introduction, tissue necrosis caused by ischemia activates the innate immune system and induces a strong inflammatory response in the myocardium[18]. Pro-inflammatory signalling triggered by the activation of membrane-bound RAGE plays an important role in this process[18, 110, 135]. RAGE cleavage from the cell surface has been shown to be triggered by binding of pro-inflammatory ligands such as HMGB1 to RAGE on the cell surface[143]. Similar to S100A12 and S100A8/A9, the alarmin HMGB1 is one of the most important activators of innate immunity and is highly increased during MI[181, 182]. Although this has not been yet studied, binding of S100A12 and S100A8/A9 to RAGE might theoretically lead to a similar cleavage effect. Accordingly, the increased plasma sRAGE in the acute phase of MI might be the result of RAGE shedding from activated leukocytes to counteract the excessive inflammatory activation, and sRAGE levels might reflect the intensity of the immune and inflammatory response (Figure 35). This hypothesis is supported by our data showing that plasma sRAGE at this stage correlates well with the levels of the classic inflammatory biomarker IL-6.

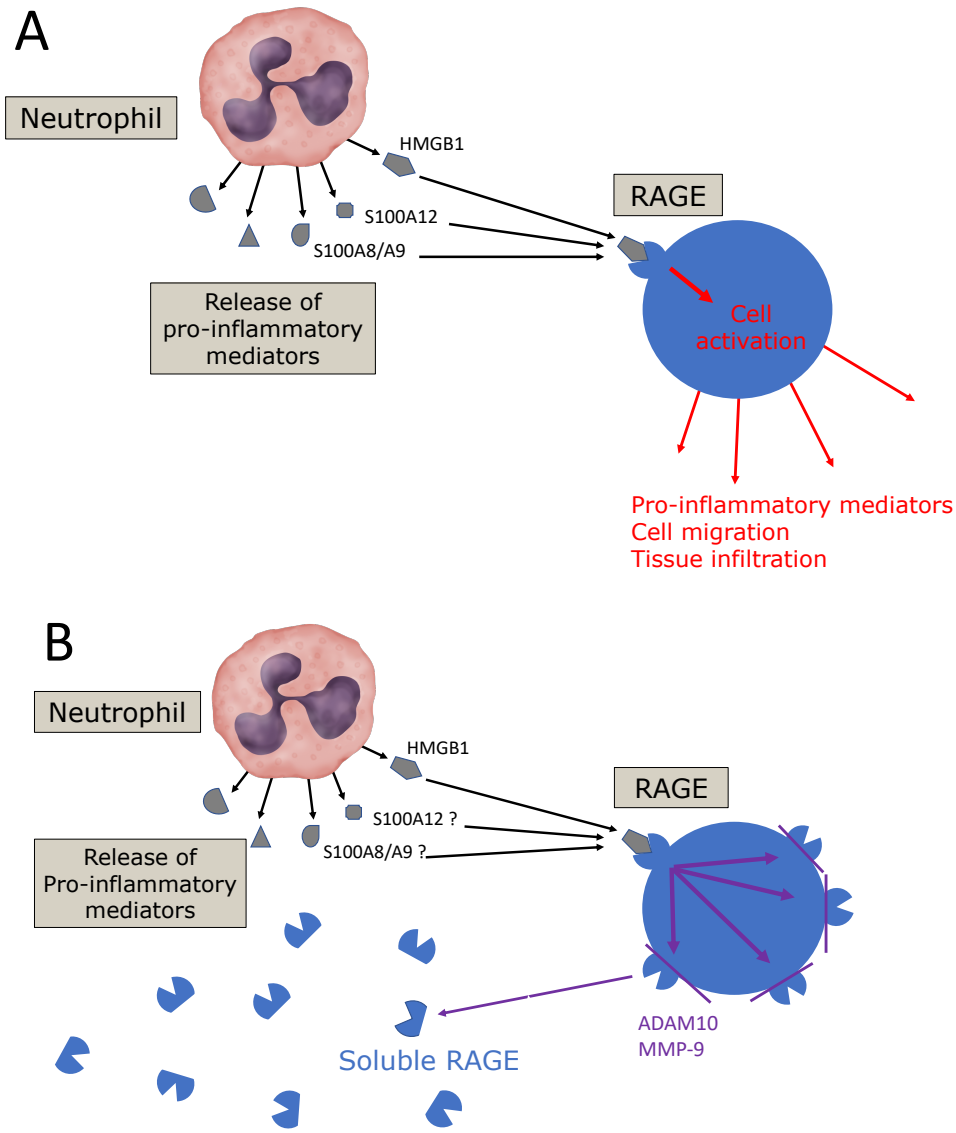

Figure 35. Inflammation during MI

(A) The neutrophils infiltrating the damaged myocardium release RAGE ligands, such as HMGB1, S100A8/A9 and S100A12 leading to cell activation. (B) Ligand binding to RAGE triggers RAGE cleavage of ADAM10 and MMP-9. The pro-inflammatory cues lead to further upregulation of cellbound RAGE, RAGE clevage and increased concentration of sRAGE in plasma.

Our results linking elevated S100A12 with a negative prognosis in ACS patients support the previously published data demonstrating an important role for S100A12 as a pathogenic and prognostic factor in CV disease. The role of S100A12 as an

active mediator in CV disease has been confirmed in animal studies[110, 113, 114], and it has been shown that S100A12 is released to the circulation from ruptured atherosclerotic plaques in ACS and from the site of atheroma disruption by PCI in stable CAD[115, 116]. In ACS patients, S100A12 is increased compared to patients with stable angina pectoris and correlates with coronary lesion complexity[117, 118]. Our results show that individuals with S100A12 in the highest tertile had about 2 times higher risk for recurrent MACE or heart failure development compared to individuals with S100A12 in the lowest tertile. However, the associations were not as strong as for sRAGE.

Our data suggests that high sRAGE and S100A12 in the acute phase of ACS are signals for an exaggerated and aggressive inflammatory response, which could explain the association with the poor prognosis. It has previously been shown that the circulating number of white blood cells are associated with a poor post-ACS prognosis[183-185] and that the circulating number of neutrophils and the neutrophil/leukocyte ratio outperform white blood cell count as a prognostic biomarker post-ACS[63-66]. Since the two RAGE ligands S100A12 and S100A8/A9 are released by activated and dying neutrophils, it is possible that the circulating levels of S100A12 and sRAGE in the acute ACS phase are reflecting the degree of neutrophil and RAGE-positive target cell activation. The associations were strongest for sRAGE, which seems to be a more promising biomarker than S100A12.

The initial myocardial inflammation is followed by a recovery phase, characterized by resolution of inflammation and fibrous scar formation. This phase is predominantly mediated by reparatory macrophages and fibroblasts, and involves production of anti-inflammatory cytokines and profibrotic factors[18]. In our ACS-patient cohort, increasing sRAGE from baseline to 6 weeks identified patients at very low risk to subsequently develop recurrent ACS, independently of all considered predictors. Individuals in the tertile with the most prominent sRAGE increase had one fifth of the risk for a recurrent ACS compared to individuals in the tertile with decreasing sRAGE, independent of age, sex, CV risk factors and the established prognostic biomarkers hsCRP, NT-proBNP and TnT. We found no such associations between the dynamics of S100A12 concentration and recurrent infarction. The association between delta sRAGE and recurrent ACS cannot prove causality but suggests that sRAGE might have a favourable role in the recovery phase by binding soluble alarmins, dampening the inflammatory response and helping to stabilize potentially vulnerable plaques (Figure 36). Shedding of RAGE from the cell surface might also lead to decreased propensity for immune cell activation. Dutta *et al.* have previously shown that inflammatory monocytes that infiltrate the heart in large numbers after an MI increase the vulnerability of non-culprit coronary atheromas, promoting plaque rupture and recurrent ischemic events[19] (Figure 11). As RAGE-mediated signals are important activators of myeloid cell responses, scavenging of RAGE ligands by sRAGE might have an anti-

inflammatory effect actively contributing to CV protection (Figure 36). Experimental support for this hypothesis is provided by animal studies, which have consistently shown a protective effect of sRAGE in CV disease[159-161]

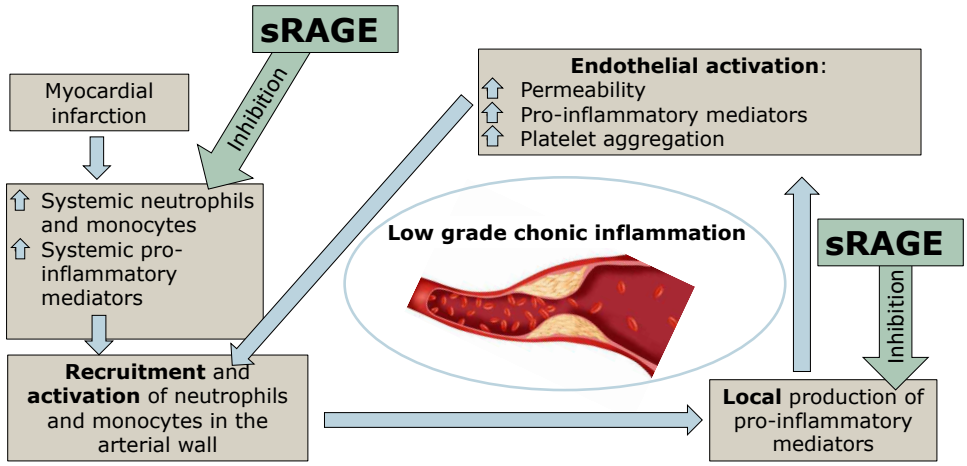

Figure 36. How does sRAGE lower the risk of MACE?

By binding potential RAGE ligands and thereby preventing them from binding to the cellbound receptor, sRAGE has the potential to dampen the inflammatory activity and thereby the risk of MACE. The illustration of the artery is used with permission från Britannica Image Quest, ©Photo researchers.

Adding sRAGE to a prediction model based on traditional CV risk factors improved the model compared to the addition of hsCRP and NT-proBNP. The model improved further when adding delta sRAGE. These data suggest acute sRAGE and post-ACS sRAGE dynamics as potential biomarkers for post-ACS prognosis. A practical clinical application of the data could be that all patients with high sRAGE levels at the time of the ACS should be followed with a new echocardiography 1 year later, regardless of the in-hospital LVEF. Further, patients with decreasing plasma sRAGE at 6 weeks post-ACS should be considered to be at high risk and should benefit from a closer follow-up and more aggressive risk factor control.

However, our results should be interpreted with due caution, as the 1-year echocardiography follow-up data and the 6 weeks follow-up blood samples were only available in the small sub-cohort of 114 older patients. The results should be reproduced in larger cohorts before the value of sRAGE as prognostic biomarker in post-ACS can be established.

S100 proteins and their receptors as potential active mediators in coronary artery disease

The potential involvement of S100 alarmins and sRAGE as active mediators in CVD has been explored in Papers II and IV.

sRAGE as a mediator of CAD in the general population

In Paper II, we aimed to examine whether the relationship between sRAGE and CAD development is genetically determined, as an indicator for a potential causal relationship. We found two genetic variants that lower sRAGE, the minor alleles of rs2070600 and rs204993. However, only rs2070600 showed association to MACE. While rs204993 is a silent mutation in a non-coding DNA region, rs2070600 is a previously described missense mutation located in the exon 3 in the *AGER*, the gene coding for RAGE[186]. The T-allele of rs2070600 induces a shift from Glycine to Serine in the amino acid position 82 of the protein, leading to alteration in the structure and function of the ligand-binding V-domain of RAGE[186-188]. Consistent to our findings, the presence of the T-allele of rs2070600 has previously been found to be associated with lower sRAGE levels in both Dutch and Korean populations, and has been related to a pro-inflammatory state characterized by higher levels of TNFα and hsCRP in plasma[189, 190]. How the Gly82Ser polymorphism leads to reduced circulatory sRAGE remains however to be determined. A previous study of the functional impact of the Gly82Ser polymorphism in humans has shown that mononuclear phagocytes isolated from homozygous 82G/82G or heterozygous 82G/82S subjects express similar levels of RAGE, but that ligand binding to RAGE in the 82G/82S phagocytes induced a 4.5-fold increase in phosphorylated MEK1/2, compared to only 2.6-fold increase in 82G/82G phagocytes. This leads to enhanced pro-inflammatory cytokine and MMP production in response to RAGE ligand binding in rs2070600 T-allele carriers[191], i.e. the presence of this allele enhances the propensity of RAGE for activation. RAGE activation leads to receptor internalization[133, 192] and thereby reduced availability for RAGE cleavage from the cell surface, which might explain the lower sRAGE levels in plasma of T-allele carriers. In theory, the lower systemic sRAGE concentration could also be due to increased resistance of RAGE to cleavage. However, considering that the Gly82Ser mutation occurs in the ligand-binding V-domain, which is located far away from the single transmembrane helix anchor of the receptor, this hypothesis is less plausible (Figure 22).

We are the first to show an independent link between a genetic sRAGE determinant and CV risk. An earlier population-based study including 9017 whites and 2871 blacks also identified rs2070600 as the strongest genetic determinant of lower sRAGE in the white population, but not in blacks[172]. The HR for CAD risk per minor

rs207060 allele was equal to the one in our study, but the association was not significant. The association between rs2070600 polymorphisms and CVD has also been explored in three previous meta-analyses[193-195], where a significant association between rs2070600 alleles and CVD risk could not be found. Common for the studies included in these meta-analyses is that they are based on study cohorts considerably smaller than ours (n=29245). The first one included a total subject size of 2741 CVD patients and 4336 controls[194] from 10 studies, the second 2145 CAD patients and 4966 controls from 14 studies[193], and the third 4402 cases and 6081 controls[195]. Interestingly, all these meta-analyses found an overall HR of 1.12 for the carriers of the Serine-encoding allele of rs2070600 to develop CAD, but neither reached significance. The minor allele frequency (MAF) of rs2070600 is very small, only 5.1% in our population, and the SNP is only responsible for a small percentage of the sRAGE variation. Taking this into consideration, it is likely that these previous studies have been underpowered to detect a significant association.

Considering the lack of association for rs204993, our results suggest that the links between rs2070600 and MACE are probably due to functional changes of the receptor, rather than to the sRAGE-lowering effect itself. However, rs2070600 was only responsible for 1.2% of sRAGE variation in plasma in our population, so the majority of sRAGE concentration is not genetically determined. Thus, we cannot exclude the possibility that sRAGE lowering by environmental factors contributes to increased CV risk by reducing the sRAGE capacity to bind and inactivate RAGE ligands. Supporting the environmental factor hypothesis, an interesting study by Quade-Lyssy *et al.* has shown in an experimental setting that statins stimulate sRAGE shedding. By lowering cholesterol, statins in clinically relevant concentrations induce increased RAGE cleavage by ADAM10, hereby increasing sRAGE levels[196]. This mechanism could contribute to the documented anti-inflammatory effects of statins.

S100A8/A9 as a potential inflammatory mediator in CAD patients

Finally, we investigated whether CAD patients have an elevated S100A8/A9 release in response to psychological stress. We found that psychological stress does induce a rapid S100A8/A9 release in CAD patients, which can already be detected at 20 minutes after the end of the stressor. Such a rapid response is potentially indicative of a heightened propensity for neutrophil activation, as only neutrophils contain large amounts of S100A8/A9 that can be released upon activation during such a short timeframe. Of all the investigated factors, the only significant enhancer of S100A8/A9 release was high evening cortisol. However, elevated S100A8/A9 levels were also registered at 24h after the stressor, suggesting the presence of a sustained response. The sustained S100A8/A9 response was not restricted to CAD patients but was also seen in a substantial fraction of the controls. However, the magnitude of the S100A8/A9 release was related to the inability to mount an

adequate rapid cortisol response to stress only in CAD patients. Consistent with our results, CAD patients have previously been found to have a flatter cortisol rhythm with high evening cortisol levels compared to controls[92]. As earlier mentioned, this is a cortisol pattern also seen in other chronic inflammation conditions, linked to increased cortisol secretion[89]. It reflects an impaired HPA axis function characterised by de-sensitization to new stressors, leading to a blunted cortisol response to stress[90, 91]. CAD patients have previously been found to have a more pronounced increase of pro-inflammatory mediators in response to stress[92], which also is in line with our results, showing an association between a potent sustained S100A8/A9 release and an inadequate rapid cortisol release in response to stress (Figure 37). Additionally, it has been previously seen that patients that reported emotional stress immediately before the onset of an MI were characterized by a significant increase of monocyte-platelet and neutrophil-platelet aggregation in response to a mental stress test, compared with patients with no emotional trigger[197]. Further, the patients with an emotional trigger also presented delayed recovery of the systolic blood pressure after stress[197], which is in line with our findings of a correlation between a strong S100A8/A9 response and elevated systolic blood pressure levels 10 minutes after stress (Cohort I, Paper IV). This suggests that individuals with a stronger S100A8/A9 response also had an impaired systolic blood pressure recovery after stress, and possibly that these individuals might be at a higher risk for recurrent coronary events triggered by psychological stress.

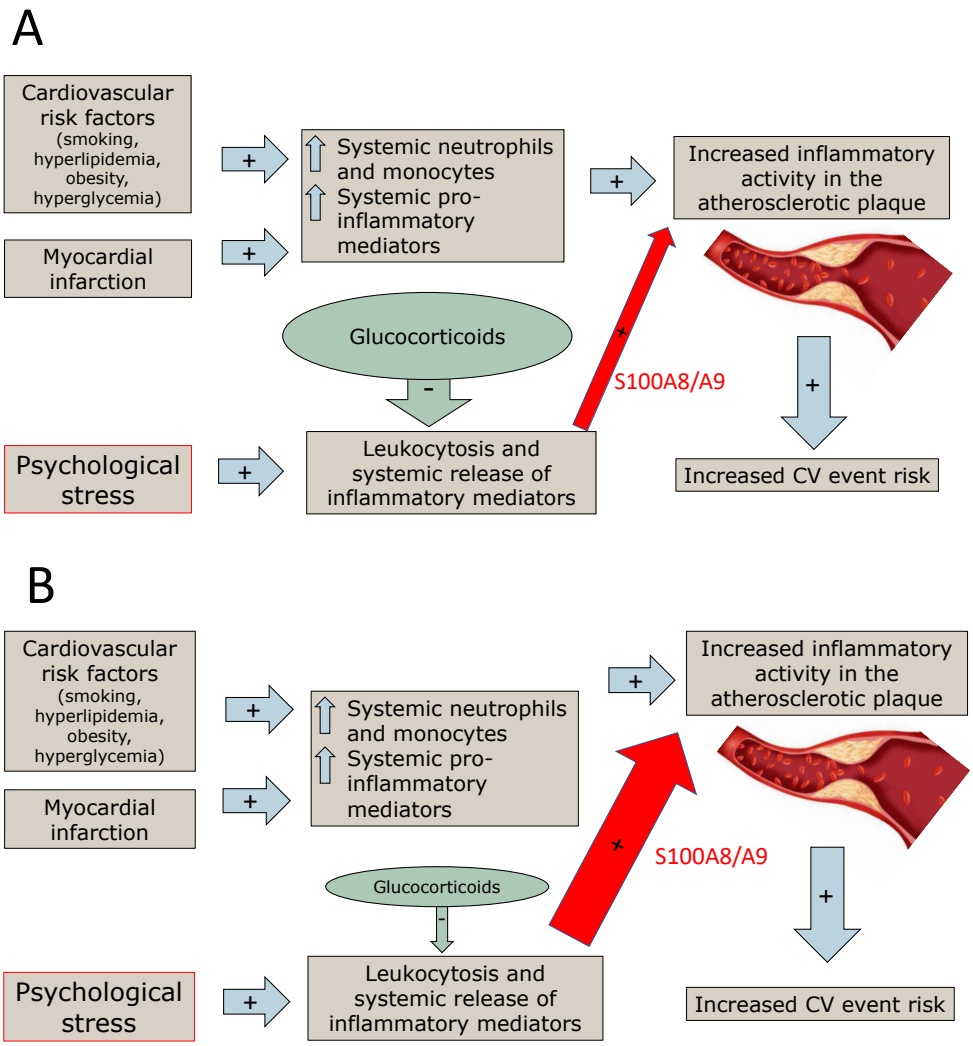

Figure 37. The stress-induced release of S100A8/A9 in controls (A) and in CAD patients (B)

We found that CAD patients release S100A8/A9 in response to psychological stress, and this release was associated to a dysregulated cortisol function characterised by elevated evening cortisol levels. A chronic exposure to elevated levels of cortisol induces a cortisol resistance, which reduces the anti-inflammatory actions and increases the concentration of pro-inflammatory mediators[89]. This leads to an inadequate cortisol response to stress and consequently an increased release of pro-inflammatory mediators. The illustration of the artery is used with permission från Britannica Image Quest, ©Photo researchers.

Unfortunately, we do not have outcome data in these cohorts, which makes us unable to know whether these patients actually have increased risk for recurrent events. In support of the pathogenic hypothesis of S100A8/A9 in ACS, previous studies by us and others have shown that elevated S100A8/A9 is associated with increased incidence of heart failure[198] and recurrent coronary events[127] in ACS patients. Moreover, we have also shown that short-term S100A9 blockade in mice with induced MI leads to highly improved cardiac function, which is a direct evidence of a detrimental role of the protein in MI[198]. These experimental findings have been reproduced in recently published papers by other groups[199, 200].

Limitations

Our studies have some important limitations that need to be considered.

Firstly, since these are association studies, we cannot draw mechanistic conclusions. However, as discussed above, our results support and extend previous findings in both humans and laboratory animals, and the findings can potentially be explained by pathogenic mechanisms already demonstrated in experimental settings.

Secondly, as the biomarkers are measured in arbitrary units in our analysis method in Papers I and III, we cannot determine concrete cut-off values for sRAGE and S100A12 as predictive biomarkers, and we cannot compare the absolute plasma concentrations found in our cohort with those found in other studies.

Further, in Paper I, we do not have detailed clinical data and plasma samples available at carotid IMT follow-up and at the time of the incident clinical events. Therefore, it is unclear how changes in CV risk profile and medication during follow-up might have impacted plasma levels of the biomarkers and their relationship to outcomes.

In Paper II, the SNP rs2070600 was identified by examining genetic variants related to sRAGE in plasma. Thus, other mutations affecting RAGE function but without impact on sRAGE levels could not be detected by our method. Further, the IMT data is only available in the MDC-CV cohort, but not in the large MDC population. Thus, the analyses of the associations between rs2070600 and IMT might be underpowered, since the MAF of rs2070600 is low (5.1%). We do not have information about CV risk factors before study inclusion, so it has not been possible to adjust for CV risk factors in the logistic regression analysis of total MACE.

In Paper III, follow-up blood samples and echocardiographic data were only collected from patients older that 75 years of age, as predefined in the initial study design. Thus, the results obtained in this small sub-population have to be interpreted with caution and cannot be directly extrapolated to the entire study cohort due to significant differences in clinical parameters between the whole cohort and the

follow-up group. Due to the relatively low number of participants and the selection criteria used in this sub-cohort, the data generated in this sub-group should be only considered to be hypothesis-generating. Studies in larger, independent cohorts are necessary to confirm our results.

In Paper IV, the first cohort lacks a control group, so we cannot say anything about the rapid S100A8/A9 response to stress in CAD-free individuals, or how this is correlated to the diurnal cortisol rhythm. We could not conclude whether the participants with higher early increase in S100A8/A9 also maintained higher S100A8/A9 levels over a 24h period, since early and late measurements of stress induced S100A8/A9 were performed in different individuals. The study was not designed to follow up clinical outcome, so we do not have prospective information about incidence of recurrent MACE in the study participants. Consequently, we cannot analyse the potential association between stress-released S100A8/A9 levels, cortisol rhythm and MACE risk.

Conclusions

Neutrophils are myeloid cells that play an important role in CVD. Neutrophil-secreted mediators have attracted increasing attention in recent years as potential therapeutic targets or biomarkers of CVD. In this work, we have studied the relationships between a particular family of neutrophil-secreted mediators, the S100 alarmins and their soluble receptors, and the development of CAD. In particular, we focused on S100A12, also called EN-RAGE, S100A8/A9, also known as calprotectin, and the soluble form of their receptors, sRAGE and sEMMPRIN.

We evaluated the relationships between these mediators and the development of CVD and the incidence of first-time coronary events in the general population, as well as the risk for recurrent coronary events and heart failure in patients with ACS. Additionally, we studied the genetic background of the relationship between the soluble receptor sRAGE and CVD, and we evaluated whether an increased release of S100A8/A9 during stress may contribute to an inflammatory environment in chronic CAD patients.

We found that high sRAGE is associated with slower atherosclerosis progression and decreased MACE risk in the general population. However, the associations were too weak to promote sRAGE as a good biomarker for evaluating CVD risk in CVD-free individuals. No associations were seen between sEMMPRIN and the outcomes. Also, we identified two genetic variants related to lower sRAGE in plasma, but only one of them, rs2070600, was associated to higher MACE risk. The minor allele of rs2070600 is known to enhance the function of the cell bound RAGE receptor, leading to increased pro-inflammatory activity following ligand binding. We believe that this is the main mechanism driving the association between rs2070600

and MACE. How these genetic variants lead to lower sRAGE is not yet known, and the relative contribution of the sRAGE-lowering effect to the increased MACE risk is unclear. The individual SNPs had a very small impact on plasma sRAGE. This indicates that the majority of sRAGE variation is not genetically determined, pointing to the existence of environmental factors that contribute to CVD by lowering plasma sRAGE and its ability to buffer pro-inflammatory RAGE ligands.

Further, we show that high S100A12 and high sRAGE at the time of an ACS are associated with increased risk of recurrent MACE, cardiac dysfunction and incident heart failure during follow-up. Both sRAGE and S100A12 showed promising biomarker properties as predictors of post-ACS prognosis, but sRAGE had a much stronger association to the outcomes. Intriguingly, increasing sRAGE concentration in plasma during the first 6 weeks after ACS was associated with a very low risk for recurrent ACS, suggesting that sRAGE might be involved in beneficial processes during the repair and recovery phase. This hypothesis warrants further exploring.

Previously, both we and others have shown that another alarmin, S100A8/A9, is associated to a poor post-MI prognosis. In study IV, we found that psychological stress induces a rapid S100A8/A9 release in stable CAD patients, and that this release is associated with a dysregulated cortisol rhythm. The S100A8/A9 elevation was still detectable 24 h after stress in both CAD patients and controls, but the magnitude of the release was only associated to impaired cortisol release to stress in patients, not in controls. Whether this mechanism contributes to a higher incidence of recurrent coronary events in the strong stress responders remains to be investigated.

Future perspective

Inflammation is a central process in both atherosclerosis and in MI, impacting atherosclerotic disease progression and post-MI prognosis[7, 18, 96]. It is important to be able to identify patients with sustained vascular and cardiac inflammation, which leads to a poor prognosis. However, there are no clinically used biomarkers able to specifically reflect the extent of residual inflammation post-MI.

In this thesis we identified a promising biomarker, sRAGE, with the potential to identify patients with elevated MI-related inflammation, and thereby at high risk for recurrent MACE and heart failure. In order to determine whether sRAGE could be of value for secondary prevention surveillance in the clinic, we will have to confirm the results in a larger cohort of unselected ACS patients, with echocardiographic data and plasma samples available both at baseline and during follow-up. sRAGE measurements should be performed by ELISA or other techniques able to express the levels in international units. If our results will be confirmed, patients with high sRAGE levels at the time of the ACS and low sRAGE at the 6-weeks follow-up

should be considered to be at high risk and should benefit from a closer follow-up and more aggressive risk factor control. Additionally, sRAGE-treatment post-MI has been shown to improve cardiac function in animal studies[159-161], but whether this treatment would have similar effects in a clinical setting is unclear. Would MI patients also benefit from such a treatment, in spite of already high sRAGE? Or would sRAGE administration reduce residual risk in post-MI patients with low systemic sRAGE in the recovery phase? These questions remain to be addressed.

We also show that another pro-inflammatory alarmin, S100A8/A9, has an elevated release in response to stress in CAD patients with a dysregulated cortisol rhythm. High systemic S100A8/A9 levels have previously been associated to increased CV event risk, both in healthy population and in CAD patients[123, 127]. In addition, we recently found that treatment with an S100A9-blocker during the inflammatory phase after an induced MI in mice potently improved cardiac function compared with controls[198]. The intriguing question that remains to be answered is whether this treatment can be efficient in humans as well. In addition, if CAD patients with a strong S100A8/A9 release in response to stress are subjected to frequent stressful situations, will they have an increased incidence of recurrent events due to the extended S100A8/A9 exposure? To be able to address this question, the stress tests performed in Paper IV would have to be repeated in a CAD cohort with available prospective information on clinical outcome. If CAD patients with a high S100A8/A9 release in response to stress have an increased risk of CV events, would medicating with an S100A9 blocker decrease this risk? These are questions that remain to be answered in the future.

Another question that remains unanswered is whether genetic determinants of S100A12 and S100A8/A9 are associated with atherosclerosis progression and increased CVD risk in the general population. Such an investigation would have the potential to determine whether there is a causal relationship between these alarmins and CVD, which would warrant further development of potential primary prevention treatments targeting S100A12 or S100A8/A9.

Populärvetenskaplig sammanfattning på svenska

Hjärtinfarkt är den största orsaken till sjuklighet och död i världen, vilket gör forskning och utveckling inom detta område angeläget. Bakgrunden till sjukdomen är åderförkalkning, s.k. ateroskleros, i hjärtats blodkärl, vilket orsakar förträngningar och ibland totalt stopp i blodflödet till delar av hjärtmuskeln. Ateroskleros innebär en kronisk inflammation i kärlväggen, som primärt drivs av kolesterolinlagring i kärlväggen. Riskfaktorer för hjärt-kärlsjukdom (rökning, övervikt, högt blodtryck, diabetes och höga blodfetter) leder till en ökad inflammation i kroppen med ökat antal vita blodkroppar och ökad koncentration av inflammationstriggande ämnen i blodet. Detta ökade inflammatoriska pådrag triggar även inflammationen i kärlväggen, och leder till lokal ökning av samma inflammationstriggande faktorer. Därmed drivs uppbyggandet av aterosklerotiska plack i kärlväggen på, leder till försvävning av blodkärlet och slutligen till att placket brister, vilket triggar blodproppsbildning i kärlet som riskerar att stoppa blodflödet helt. Den syrebrist som uppstår i hjärtmuskeln när blodet inte når fram orsakar att den värst drabbade delen av hjärtmuskeln dör. Detta är vad som kallas för en hjärtinfarkt.

Den döda hjärtmuskeln orsakar i sin tur en våldsam inflammatorisk reaktion i hjärtmuskeln. Vita blodkroppar vandrar in i området och frisätter proteiner som eldar på inflammationen ytterligare, attraherar fler vita blodkroppar som frisätter ännu mer inflammationstriggande proteiner, vilket skapar en ond spiral. Inflammationens uppgift är att rensa upp de döda cellerna och påbörja reparation av skadorna. Tyvärr leder inflammationen också till ytterligare hjärtmuskeldöd. Den som överlever sin hjärtinfarkt riskerar därför att utveckla hjärtsvikt, det vill säga att hjärtat pumpar dåligt, och leder därmed till sämre livskvalitet och ökad dödlighet. Efter få dagar börjar anti-inflammatoriska ämnen utsöndras, vilket bromsar inflammationen och utgör startskottet för återuppbyggnadsfasen. Balansen mellan inflammation och motinflammation är viktig för återuppbyggnaden av hjärtmuskeln, och är individuellt mycket olika. Man har hittills inte kunnat urskilja vem som kommer att reagera med överdriven inflammation efter en hjärtinfarkt, med medföljande ökad risk för att utveckla hjärtsvikt, och vem som klarar sig bra. Dessutom har man kunnat visa att den som en gång drabbats av en hjärtinfarkt har högre risk för att drabbas igen relaterat till att det inflammatoriska pådraget vid

hjärtinfarkten leder till aktivering av andra plack i hjärtats kärlträd. Således, ju mer inflammation, desto större risk för nya hjärtinfarkter. Fördelen med att kunna identifiera och behandla dessa individer är uppenbar, men de inflammationsmarkörer som finns idag är alltför trubbiga och ospecifika, och ingen behandling finns ännu.

Man har de senaste 25 åren förbättrat både förebyggande behandling och diagnosticerings- och behandlingsmetoder vid hjärtinfarkt för att snabbt kunna öppna de drabbade blodkärlen i hjärtat, vilket har minskat dödligheten och sjukligheten vid hjärtinfarkt betydligt. Likväl, en människa dör i hjärtinfarkt eller dess sviter var 4:e sekund i världen. Därför behövs nya och bättre mätbara markörer i blodet för att kunna identifiera dessa individer. Man har också sett att psykisk stress verkar vara en viktig trigger av hjärtinfarkt. Man vet att psykisk stress orsakar ett ökat inflammatoriskt pådrag i kroppen och man vet att ett sådant pådrag ökar risken för hjärtinfarkt, men mekanismerna som kopplar psykisk stress och hjärtinfarkt är inte är helt kartlagda.

De två proteinerna som vi har studerat, S100A12 och S100A8/A9 frisätts framförallt av de vita blodkroppar som heter neutrofila granulocyter. S100A12 och S100A8/A9 har en pro-inflammatoriskt roll, och hög koncentration i blodet hos frisk befolkning har visat sig vara associerat med högre risk för insjuknande i hjärtinfarkt och stroke. Dessutom har hög koncentration av S100A8/A9 i blodet hos hjärtinfarktpatienter visat sig vara kopplat till dålig prognos, i form av ökad risk för nya hjärtinfarkter och hjärtsvikt. Dessa två proteiner utövar sin verkan genom att binda till samma cellbundna receptor, RAGE (receptor for advanced glycation end products), en viktig receptor i inflammationsprocessen. När en ligand (det protein som binder till receptorn) binder till RAGE, sker en aktivering av den RAGE bärande cellen, som börjar arbeta för att öka inflammationen. RAGE kan även klyvas från cellytan, och förkommer därmed även i löslig form i blodet. Fritt RAGE (soluble RAGE eller sRAGE) fungerar som en bluffreceptor för de inflammatoriska proteinerna – binder proteinerna, men utan cellaktivering, och förhindrar därmed proteinerna att binda till en cell-bunden receptor. Därmed skulle sRAGE kunna ha anti-inflammatoriska egenskaper, och forskning har visat att sRAGE verkar ha en skyddande effekt mot ateroskleros.

Syftet med denna avhandling är att undersöka kopplingen mellan S100A12, S100A8/A9, sRAGE och aterosklerosprogress, risk för hjärtinfarkt och prognos efter hjärtinfarkt.

I **delprojekt I** (Paper I), undersöker vi kopplingen mellan sRAGE, aterosklerosprogress och risk för hjärtinfarkt hos frisk befolkning. Vi mätte sRAGE i blodet på 4612 individer ur en befolkningsbaserad kohort och följde upp dem under 21,5 år, där det registrerades om studiedeltagarna fick hjärtinfarkt eller dog. Dessutom genomfördes ett ultraljud av deltagarnas högra halspulsåder för att mäta intima media tjockleken (IMT), vilket anses vara ett mått på framtida utveckling av

kärlsjukdom. Denna undersökning upprepades efter 16,5 år. Resultaten visade att hög koncentration av sRAGE i blodet var kopplat till en lägre progressionshastighet av kärlsjukdom, och till en minskad risk för hjärtinfarkt och död senare i livet. Dessa resultat backar upp teorin om att sRAGE har skyddande egenskaper mot ateroskleros.

I **delprojekt II** (Paper II) undersökte vi kopplingen mellan genetiska determinanter av sRAGE och risk för insjuknande i hjärtinfarkt hos friska individer. I en genomtäckande associationsstudie (genome-wide association study, GWAS) på 4192 friska individer, fann vi två genetiska varianter (single nucleotide polymorphisms eller SNPar), rs2070600 och rs204993, som var kopplade till sRAGE koncentrationen i blodet. För bägge SNParna var den minst vanliga varianten kopplad till en lägre sRAGE koncentration. När vi analyserade kopplingen mellan SNParna och risken för hjärtinfarkt hos 29 245 individer ur befolkningen, hittade vi att den sRAGE sänkande varianten av rs2070600 medförde en 16% ökad risk för hjärtinfarkt genom livet. Däremot hittade vi ingen koppling mellan rs204993 och hjärtinfarkt. Tidigare forskning har visat att den minst vanliga varianten av rs2070600 orsakar en ändring av strukturen och funktionen av den cellbundna receptorn RAGE, som medför en ökning av pro-inflammatoriska effekter vid ligand bindning till receptorn. Våra resultat kunde tyda på att det snarare är denna funktionsändring, än den sRAGE sänkande effekten i sig själv, som ger en ökad risk för hjärtinfarkt, eftersom den andra sRAGE sänkande genetiska varianten inte hade samma koppling till hjärtinfarkt.

I **delprojekt III** (Paper III) mätte vi S100A12 och sRAGE hos 524 patienter med hjärtinfarkt, och följde dem härefter under 26 månader. Ultraljud av hjärtat gjordes på alla deltagare innan utskrivning från sjukhuset. I en subgrupp på 114 patienter togs nya blodprover 6 veckor efter hjärtinfarkten och ett nytt ultraljud av hjärtat gjordes efter ett år. Resultaten visade att personer med hög koncentration av sRAGE och S100A12 vid hjärtinfarkten hade ökad risk för att få en ny hjärtinfarkt eller utveckla hjärtsvikt de kommande åren. Mest tydligt var detta för sRAGE, där individer med hög koncentration också visade tecken till uttalad inflammation i blodet och hade 4 gånger så hög risk för att få en ny hjärtinfarkt de kommande åren jämfört med de med låg koncentration. Intressant nog hade de med en stigande koncentration av sRAGE de första 6 veckorna efter hjärtinfarkten 5 gånger mindre risk att få en ny hjärtinfarkt jämfört med de med sjunkande koncentration.

Detta kunde förklaras av att uttalad inflammation ger en våldsam ökning av antal cellbunden RAGE och därmed också ökad koncentration av sRAGE. Således står en hög sRAGE-koncentration i blodet i samband med hjärtinfarkt för en uttalad inflammation i hjärtat, vilket i sin tur förklarar den dåliga prognosen. Däremot verkar hög sRAGE i stabil fas stå för något annat. Här visar våra resultat att stigande sRAGE är kopplat till lägre risk för ny hjärtinfarkt, och därmed verkar ha en skyddande effekt. Detta backar upp teorin om en anti-inflammatorisk effekt för sRAGE. Det betyder också att sRAGE kan identifiera både personer med

omfattande inflammation i hjärtat i akutfasen vid hjärtinfarkten och personer som efter 6 veckor har en otillräcklig inflammationsdämpning. Bägge grupper har ökad risk för nya hjärtinfarkter och hjärtsvikt, och har därmed behov för utökad uppföljning.

I **delprojekt IV** (Paper IV) undersökte vi om S100A8/A9 frisätts vid akut psykologisk stress hos 60 patienter i stabil fas efter hjärtinfarkt. S100A8/A9 mättes i blodet före och 20 minuter efter en psykologisk stresstest. I en annan kohort mättes S100A8/A9 24 timmar efter stresstesten hos 27 patienter som haft hjärtinfarkt tidigare och 28 friska kontrollpersoner. Vi såg att akut psykologisk stress medförde en snabb frisättning av S100A8/A9 i blodet hos patienterna i första kohorten, och att S100A8/A9 koncentrationen fortfarande var stegrad hos en andel av individerna i andra kohorten, både hos patienter och friska kontroller.

Kortisol är ett hormon som frisätts bland annat vid stress och är ett viktigt sätt för kroppen att klara stress. Det är tidigare känt att hjärtinfarktpatienter har sämre kortisolfrisättning vid stress än friska. Här mättes kortisolkoncentrationen i saliven hos samtliga deltagare före och 20 minuter efter stresstesten, samt morgon och kväll i tre dagar. Resultaten visade att den ihållande koncentrationshöjningen av S100A8/A9 24 timmar efter stress var kopplat till dålig kortisolfrisättning vid stress hos patienterna, men inte hos friska. Vi vet från tidigare studier att hög koncentration av S100A8/A9 är kopplat till dålig prognos för hjärtinfarktpatienter. Vår studie stödjer därför att S100A8/A9 skulle kunna utgöra ett viktigt led i kopplingen mellan psykisk stress, inflammation och ökad risk för hjärtinfarkt.

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Supplementary material

Paper II

Supplementary table 1. Baseline characteristics depending on mortality status in the MDC cohort

Characteristics	Whole cohort N=29245	Dead N=10331	Alive N=18914	*P**
Age, (years)	58 (51-64)	64 (58-68)	54 (50-61)	<0.001
Male sex, n (%)	11625 (39.8)	5234 (50.6)	6283 (33.2)	<0.001
BMI, (kg/m^2)	25.4 (23.0-28.1)	25.9 (23.4-28.7)	25.1 (22.9-27.7)	<0.001
Diabetes, n (%)	1334 (4.6)	810 (7.8)	518 (2.7)	<0.001
Current smoking, n (%)	7746 (26.5)	3216 (31.1)	4464 (23.6)	<0.001
Medication use				
Lipid lowering medication, n (%)	887 (3.0)	475 (4.6)	409 (2.2)	<0.001
Blood pressure medication, n (%)	5053 (17.3)	2631 (25.5)	2393 (12.7)	<0.001
Lipids				
Apo A1 (mg/dL)	154 (137-174)	150 (134-170)	156 (139-175)	<0.001
ApoB (mg/dL)	105 (89-123)	110 (94-127)	103 (87-121)	<0.001
Blood pressure				
Systolic (mmHg)	140 (126-154)	148 (132-160)	136 (122-150)	<0.001
Diastolic (mmHg)	85 (80-90)	88 (80-95)	84 (78-90)	<0.001

*Comparing individuals that died with survivors, Chi square on dichotomous variables and Mann Whitney on continuous variables

MACE, major adverse coronary event; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride.

Supplementary table 2. Baseline characteristics in subjects with incident first-time MACE compared to MACE-free subjects in the MDC cohort

Characteristics	Incident MACE N=4114	No incident MACE N=24395	*P*[*]
Age, (years)	61 (55-66)	57 (51-63)	<0.001
Male sex, n (%)	2449 (59.5)	8599 (35.2)	<0.001
BMI, (kg/m^2)	26.2 (23.9-28.9)	25.2 (22.9-27.8)	<0.001
Diabetes, n (%)	378 (9.2)	853 (3.4)	<0.001
Current smoking, n (%)	1282 (33.5)	6320 (27.5)	<0.001
Medication use			
Lipid lowering medication, n (%)	187 (4.5)	424 (1.7)	<0.001
Blood pressure medication, n (%)	1024 (24.9)	3532 (14.5%)	<0.001
Lipids			
Apo A1 (mg/dL)	146 (130-165)	156 (139-175)	<0.001
ApoB (mg/dL)	115 (99-133)	103 (87-121)	<0.001
Blood pressure			
Systolic (mmHg)	148 (134-160)	138 (125-150)	<0.001
Diastolic (mmHg)	88 (80-95)	85 (80-90)	<0.001

[*]Comparing individuals with and without incident first-time MACE, Chi square test on dichotomous variables and Mann Whitney test on continuous variables

MACE, major adverse coronary event; sRAGE, BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride.

Paper III

Supplementary table 3. Baseline differences in clinical characteristics between patients who died during follow-up and survivors

Characteristics	All patients N= 524	Dead N=63	Alive N=461	*P*
Age (years)	67 (59-77)	82 (75-86)	66 (58-74)	<0.001
Male sex, n (%)	384 (73.3%)	45 (71.4%)	339 (73.5%)	n.s.
Hypertension, n (%)	285 (54.4%)	47 (74.6%)	238 (51.6%)	0.001
Smoking, n (%)	132 (25.2%)	9 (14.2%)	123 (26.6%)	0.034
Diabetes, n (%)	126 (24.0%)	22 (34.9%)	104 (22.6%)	0.030
BMI (kg/m$^{2)}$	26.9 (24.3-29.8)	25.7 (23.5-29.8)	27.1 (24.4-29.8)	n.s.
eGFR (mL/min/1.73m^2)	72.0 (53.1-94.6)	49.8 (34.2-68.9)	74.0 (56.9-96.5)	<0.001
Index cardiac event				
STEMI	180 (34.4%)	15 (23.8%)	165 (35.8%)	n.s.
NSTEMI	295 (56.3%)	43 (68.2%)	252 (54.6%)	0.040
UA	50 (9.5%)	5 (7.9%)	45 (9.8%)	n.s.
Previous cardiac event				
HF, n (%)	54 (10.3%)	17 (26.9%)	37 (8.0%)	<0.001
ACS, n (%)	152 (29.0%)	32 (50.7%)	120 (26.0%)	<0.001
TnT (ng/L)	365 (62-1291)	331 (74-1225)	374 (58-1306)	n.s.
hsCRP (mg/L)	6.7 (2.9-17.8)	10.9 (4.4-29.3)	6.3 (2.6-16.6)	0.002
NT-proBNP (au)	81.0 (49.5-103.6)	106.2 (81.8-126.9)	77.2 (47.8-99.7)	<0.001
S100A12 (au)	3.7 (3.0-4.8)	4.72 (3.7-6.9)	3.5 (2.9-4.6)	<0.001
sRAGE (au)	25.5 (20.1-34.8)	33.6 (24.1-45.9)	25.0 (19.5-33.1)	<0.001

ACS, acute coronary syndrome; BMI, body mass index; eGFR, estimated glomerular filtration rate; HF, heart failure; hsCRP, high sensitivity c-reactive protein; NSTEMI, non-ST-elevation coronary infarction; NT-proBNP, N-terminal pro-brain natriuretic peptide; sRAGE, soluble receptor for advanced glycation endproducts; STEMI, ST elevation myocardial infarction; TnT, Troponin T; UA, unstable angina.

Supplementary table 4. Baseline differences in clinical characteristics between patients with and without incident ACS during follow-up

Characteristics	All patients N= 524	Patients with incident ACS N=76	Patients without incident ACS N=448	*P*
Age (years)	67 (59-77)	77 (63-83)	66 (58-75)	<0.001
Male sex, n (%)	384 (73.3%)	53 (69.7%)	331 (73.9%)	n.s.
Hypertension, n (%)	285 (54.4%)	47(61.8%)	238 (53.1%)	n.s.
Smoking, n (%)	132 (25.2%)	14 (18.4%)	118 (26.3%)	n.s.
Diabetes, n (%)	126 (24.0%)	21 (27.6%)	105 (23.4%)	n.s.
BMI (kg/m$^{2)}$	26.9 (24.3-29.8)	26.3 (23.9-30.4)	27.0 (24.4-29.7)	n.s.
eGFR (mL/min/1.73m^2)	72.0 (53.1-94.6)	54.2 (34.8-76.6)	73.9 (56.9-96.3)	<0.001
Index cardiac event				
STEMI	180 (34.4%)	24 (31.6%)	156 (34.8%)	n.s.
NSTEMI	295 (56.3%)	48 (63.2%)	247 (55.1%)	n.s.
UA	50 (9.5%)	4 (5.2%)	46 (10.3%)	n.s.
Previous cardiac event				
HF, n (%)	54 (10.3%)	15 (19.7%)	39 (8.7%)	0.003
ACS, n (%)	152 (29.0%)	34 (44.7%)	118 (26.3%)	0.005
TnT (ng/L)	365 (62-1291)	411 (110-1276)	358 (55-1297)	n.s.
hsCRP (mg/L)	6.7 (2.9-17.8)	8.9 (4.0-31.9)	6.2 (2.6-16.6)	0.005
NT-proBNP (au)	81.0 (49.5-103.6)	105.8 (81.2-121.7)	76.6 (46.4-98.4)	<0.001
S100A12 (au)	3.7 (3.0-4.8)	4.5 (3.5-5.6)	3.5 (2.9-4.6)	<0.001
sRAGE (au)	25.5 (20.1-34.8)	34.1 (24.2-45.5)	24.9 (19.4-32.2)	<0.001

ACS, acute coronary syndrome; BMI, body mass index; eGFR, estimated glomerular filtration rate; HF, heart failure; hsCRP, high sensitivity c-reactive protein; NSTEMI, non-ST-elevation coronary infarction; NT-proBNP, N-terminal pro-brain natriuretic peptide; sRAGE, soluble receptor for advanced glycation endproducts; STEMI, ST elevation myocardial infarction; TnT, Troponin T; UA, unstable angina.

Supplementary table 5. Baseline differences in clinical characteristics in between patients with and without incident heart failure during follow-up

Characteristics	All patients N= 524	Patients with incident HF N=41	Patients without incident HF N=483	*P*
Age (years)	67 (59-77)	77 (72-85)	66 (58-76)	<0.001
Male sex, n (%)	384 (73.3%)	28 (68.3%)	356 (73.6%)	n.s.
Hypertension, n (%)	285 (54.4%)	34 (82.9%)	251 (51.9%)	<0.001
Smoking, n (%)	132 (25.2%)	10 (24.4%)	122 (25.2%)	n.s.
Diabetes, n (%)	126 (24.0%)	20 (48.8%)	106 (21.9%)	<0.001
BMI (kg/m^2)	26.9 (24.3-29.8)	27.2 (23.9-29.8)	26.9 (24.3-29.8)	n.s.
eGFR (mL/min/1.73m^2)	72.0 (53.1-94.6)	46.0 (31.6-64.2)	73.9 (55.8-96.3)	<0.001
Index cardiac event				
STEMI	180 (34.4%)	11 (26.8%)	169 (34.9%)	n.s.
NSTEMI	295 (56.3%)	24 (58.5%)	271 (56.0%)	n.s.
UA	50 (9.5%)	6 (14.63%)	44 (9.1%)	n.s.
Previous cardiac event				
HF, n (%)	54 (10.3%)	15 (36.6%)	39 (8.0 %)	<0.001
ACS, n (%)	152 (29.0%)	19 (46.3%)	133 (27.5%)	<0.001
TnT (ng/L)	365 (62-1291)	496 (94-1248)	356 (54-1300)	n.s.
hsCRP (mg/L)	6.7 (2.9-17.8)	14.7 (5.5-44.0)	6.3 (2.6-16.5)	<0.001
NT-proBNP (au)	81.0 (49.5-103.6)	108.4 (91.1-119.4)	77.2 (46.9-99.7)	<0.001
S100A12 (au)	3.7 (3.0-4.8)	4.6 (3.3-7.1)	3.6 (2.9-4.7)	0.002
sRAGE (au)	25.5 (20.1-34.8)	40.8 (25.8-54.0)	24.9 (19.7-33.1)	<0.001

ACS, acute coronary syndrome; BMI, body mass index; eGFR, estimated glomerular filtration rate; HF, heart failure; hsCRP, high sensitivity c-reactive protein; NSTEMI, non-ST-elevation coronary infarction; NT-proBNP, N-terminal pro-brain natriuretic peptide; sRAGE, soluble receptor for advanced glycation endproducts; STEMI, ST elevation myocardial infarction; TnT, Troponin T; UA, unstable angina.

Supplementary table 6. Baseline differences in clinical characteristics between patients with and without detailed follow-up

Characteristics	All patients N= 524	Patients with detailed follow-up N=114	Patients without detailed follow-up N=410	*P*
Age (years)	67 (59-77)	79 (77-83)	63 (57-72)	<0.001
Male sex, n (%)	384 (73.3%)	76 (66.7%)	308 (75.1%)	n.s.
Hypertension, n (%)	285 (54.4%)	74 (64.9%)	211 (51.5%)	0.014
Smoking, n (%)	132 (25.2%)	9 (7.9%)	123 (30.0%)	<0.001
Diabetes, n (%)	126 (24.0%)	24 (21.1%)	102 (24.9%)	n.s.
BMI ($kg/m^{2)}$	26.9 (24.3-29.8)	26.2 (23.7-29.1)	27.2 (24.5-30.0)	0.008
eGFR ($mL/min/1.73m^2$)	72.0 (53.1-94.6)	54.4 (40.7-69.0)	77.7 (59.0-98.5)	<0.001
Index cardiac event				
STEMI	180 (34.3%)	29 (25.4%)	151 (36.8%)	0.020
NSTEMI	295 (56.3%)	82 (71.9%)	213 (52.0%)	<0.001
UA	50 (9.5%)	4 (3.5%)	46 (11.2%)	0.012
Previous cardiac event				
HF, n (%)	54 (10.3%)	15 (13.2%)	39 (9.5%)	n.s.
ACS, n (%)	152 (29.0%)	37 (32.4%)	116 (28.3%)	n.s.
TnT (ng/L)	365 (62-1291)	430 (67-1290)	356 (55-1294)	n.s.
hsCRP (mg/L)	6.7 (2.9-17.8)	7.8 (3.0-16.0)	6.5 (2.8-18.1)	n.s.
NT-proBNP (au)	81.0 (49.5-103.6)	95.7 (75.6-113.0)	75.1(41.5-98.7)	<0.001
S100A12 (au)	3.7 (3.0-4.8)	4.2 (3.1-5.5)	3.5 (2.9-4.7)	0.008
sRAGE (au)	25.5 (20.1-34.8)	30.1 (23.4-42.8)	24.7 (19.3-33.1)	<0.001

ACS, acute coronary syndrome; BMI, body mass index; eGFR, estimated glomerular filtration rate; HF, heart failure; hsCRP, high sensitivity c-reactive protein; NSTEMI, non-ST-elevation coronary infarction; NT-proBNP, N-terminal pro-brain natriuretic peptide; sRAGE, soluble receptor for advanced glycation endproducts; STEMI, ST elevation myocardial infarction; TnT, Troponin T; UA, unstable angina.

Supplementary table 7. Clinical characteristics of the patients in the detailed follow-up subgroup with and without incident MACE.

Characteristics		All follow-up patients N=114	Patients with incident MACE N=30	Patients without incident MACE N=84	*P*
Age (years)		79 (77-83)	82 (78-85)	79 (76-83)	0.010
Male sex, n (%)		75 (65.8%)	20 (66.7%)	55 (65.5%)	n.s.
Hypertension, n (%)		74 (64.9%)	21(70.0%)	53 (63.1%)	n.s.
Smoking, n (%)		9 (7.9%)	3 (10.0%)	6 (7.1%)	n.s.
Diabetes, n (%)		24 (21.1%)	8 (26.7%)	16 (19.0%)	n.s.
BMI (kg/m^2)		26.2 (23.7-29.1)	25.2 (23.2-28.9)	26.2 (23.8-29.1)	n.s.
eGFR (mL/min/1.73m^2)	acute	54.4 (40.7-69.0)	54.2 (32.4-65.9)	54.4 (40.8-71.2)	n.s.
	6 weeks	47.7 (36.2-57.1)	42.5 (32.8-54.6)	49.2 (37.8-57.4)	n.s.
Index cardiac event					
STEMI		28 (24.6%)	5 (16.7%)	23 (27.4%)	n.s.
NSTEMI		82 (71.9%)	24 (80.0%)	58 (69.0%)	n.s.
UA		4 (3.5%)	1 (3.3%)	3 (3.6%)	n.s.
Previous cardiac event					
HF, n (%)		15 (13.2%)	8 (26.7%)	7 (8.3%)	0.011
ACS, n (%)		36 (31.6%)	10 (33.3%)	26 (30.9%)	n.s.
TnT (ng/L)	acute	430 (67-1290)	317 (101-1092)	482 (66-1750)	n.s.
	6 weeks	23 (13-40)	27 (14-49)	21 (12-36)	n.s.
hsCRP (mg/L)	acute	7.8 (3.0-16.1)	8.8 (4.1-20.6)	7.2 (2.6-15.1)	n.s.
	6 weeks	2.11 (0.9-6.1)	2.0 (0.9-6.7)	2.1 (0.9-5.8)	n.s.
NT-proBNP (au)	acute	95.7 (75.6-113.0)	105.4 (92.7-124.7)	91.5 (67.8-108.2)	0.002
	6 weeks	79.3 (59.7-95.0)	88.7 (76.6-98.9)	73.8 (55.3-93.5)	0.015
S100A12 (au)	acute	4.3 (3.1-5.5)	4.6 (3.9-5.8)	3.9 (3.0-5.5)	0.044
	6 weeks	4.0 (3.1-6.3)	4.7 (3.1-6.2)	3.6 (3.1-6.5)	n.s.
	delta	0.1 (-0.9-1.3)	0.15 (-0.8-1.8)	0.14 (-1.0-1.4)	n.s.
sRAGE (au)	acute	30.1 (23.4-42.8)	34.3 (24.9-48.5)	28.4 (21.6-37.5)	0.011
	6 weeks	33.4 (27.3-42.1)	35.4 (27.9-45.4)	32.6 (26.7-41.0)	n.s.
	delta	1.8 (-3.1-10.2)	0.0 (-8.6-3.3)	4.9 (-1.5-11.6)	0.009

ACS, acute coronary syndrome; BMI, body mass index; eGFR, estimated glomerular filtration rate; HF, heart failure; hsCRP, high sensitivity c-reactive protein; NSTEMI, non-ST-elevation coronary infarction; NT-proBNP, N-terminal pro-brain natriuretic peptide; sRAGE, soluble receptor for advanced glycation endproducts; STEMI, ST elevation myocardial infarction; TnT, Troponin T; UA, unstable angina.

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