Target Validation in Neovascularization

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Target Validation in Neovascularization

Validatie van Inflammatoire Aangrijpingspunten in Vaatnieuwvorming

(met een samenvatting in het Nederlands)

Proefschrift

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PART I

INTRODUCTION

CHAPTER

1

General introduction and thesis outline

GENERAL INTRODUCTION

CHAPTER 1

Cardiovascular disease

A major problem in the Westernized world is the aging population that goes hand in hand with an increasing incidence of patients suffering from cardiovascular disease. The term cardiovascular disease summarizes all conditions characterized by complications of the heart and/or blood vessels. Clinically, the most important subgroup is formed by vascular occlusive diseases, caused by atherosclerosis.[1] Atherosclerosis is a chronic inflammatory disease where endothelial dysfunction predisposes an excessive accumulation of oxidized low-density lipoproteins and inflammatory cells into the arterial wall, resulting in the development of an atherosclerotic plaque. With a progressing atherosclerotic plaque, plaque rupture with acute luminal thrombosis or a gradual decrease in luminal diameter can occur resulting in an acute or chronic lack of blood supply respectively often followed by severe tissue damage. The number of patients suffering from diabetes, obesity and hypertension increases annually which is partly caused by an unhealthy lifestyle such as fat diets, lack of exercise and smoking.[2] The high mortality rate in this population at risk is frequently related to an accelerated development of atherosclerosis associated with an increased incidence of cardiovascular complications. Several blood-flow restoring pharmaceutical and interventional treatments are available. However, not all patients benefit from these existing interventions. Hence, cardiovascular diseases are still the leading cause of death throughout the world.[3] The search for novel therapeutic targets will remain an important research focus in the scientific community.

Neovascularization

One alternative that has gained attention as a potential therapeutic opportunity is the stimulation of new vessel growth. In order to maintain the body's homeostasis, a constant and sufficient amount of oxygen and nutrients has to be delivered via the blood vessels to all tissues. If oxygen is deprived, these tissues become ischemic and new blood vessel formation, also referred to as neovascularization, is stimulated to restore tissue oxygenation. The body has two major repairing mechanisms that increase vessel growth: angiogenesis and arteriogenesis (collateral artery growth).[4] Angiogenesis is characterized by the sprouting of new blood vessels from existing ones, triggered by a hypoxic environment downstream of an occlusion. Arteriogenesis is the remodelling of pre-existing anastomoses into large conducting vessels circumventing the occluded artery (collaterals). In

patients with vascular occlusive disease, therapeutic enhancement of collateral growth has gained most attention, as this process can fully compensate for the occluded artery. Nevertheless, angiogenesis shares many characteristics with arteriogenesis and knowledge obtained in this field can be of tremendous value for the proper understanding of the underlying mechanisms of collateral vessel growth.[5] It is known that in some patients with an arterial occlusion, blood flow is improved by spontaneous development of collaterals.[6] Unfortunately, collateral growth is a time consuming process not capable of rescuing an ischemic organ that suffers from acute ischemia. Stimulating the growth of new vessels in patients with vascular occlusive disease could theoretically restore blood flow to the ischemic tissue or could even prevent an ischemic event.

Therapeutic innovations to stimulate neovascularisation

Potentially effective therapeutic agents have been tested that can stimulate neovascularization in vivo. Most of these pharmaceutical interventions have been based on the principle that a local inflammatory response accelerates vascular growth. Animal studies revealed successful pro-inflammatory interventions that resulted in improved reperfusion following acute tissue ischemia. However, thus far translation of these therapies into man failed.[7] It is disappointing to notice the lack of successful studies that resulted in a new clinical applicable drug for the stimulation of new vessel growth. The search for new targets and interventional strategies is still ongoing. There is strong supportive evidence showing that local inflammation is essential in both angiogenesis and arteriogenesis.[8] In this introduction we will shortly discuss two potential inflammatory pathways that could contribute to improved tissue neovascularisation.

IL-33/ST2 pathway

The IL-33/ST2 axis is a pathway associated with Th2 driven inflammation and has been shown to exert an important role in many inflammatory disorders.[9] ST2, the receptor for IL-33, is a member of the IL-1 receptor family and mediates the biological function of IL-33. Due to alternative splicing, ST2 can be found in a transmembrane form (ST2L) and a soluble form (sST2) that lacks the transmembrane and intracellular domains.[10] Soluble ST2 is a promising new biomarker for adverse cardiovascular events. There is consisting evidence that sST2 is independently predictive for mortality in patients with heart failure or myocardial infarction.[11,12] Signalling of IL-33 through ST2L has been shown cardioprotective in several experimental studies[13–15] and a beneficial role has been implicated in atherosclerosis.[16] On the

other hand, sST2 can prevent this cardioprotective intracellular signalling, as it may capture IL-33 away from the circulation.[17] Besides, various studies have indicated that sST2 possesses immunosuppressive properties related to Toll-like receptor (TLR) mediated inflammation as well.[18–20] Together with the observation that IL-33 holds pro-angiogenic properties in vitro and in vivo,[21] we suggest that interference in this pathway might be of therapeutic value.

Mast cells

Mast cells are inflammatory cells traditionally known for their role in allergy and innate immune responses.[22] Later on it became evident that mast cells exert an important role in several other inflammatory mediated processes. Mast cells are filled with cytoplasmic secretory granules, which they exocytose upon activation. Multiple studies have linked mast cell degranulation or activation to normal and pathological neovascularization.[23] More specifically, several potent angiogenic factors are present in the secretory granules of mast cells.[24] More recently, it was demonstrated that mast cells are also actively involved in the initiation and progression of atherosclerotic disease.[25–27] Together with the observation that mast cells accumulated in neovessel-rich areas of the plaque, this data suggests that mast cells may render the plaque vulnerable by inducing plaque angiogenesis.[28–31] It is unknown to which extend mast cells are involved in arteriogenesis, however, they were found in the wall of growing collaterals.[32] Based on these observations we considered mast cells as a potential target for the therapeutic stimulation of neovascularization.

THESIS OUTLINE

In this thesis two potentially interesting targets, that have been shown to play an important role in inflammation and have been implicated to exert a function in neovascularization, were investigated; the IL-33/ST2 pathway and the mast cell. The aim of this thesis is to provide insights into the role of these pathways in the underlying mechanisms that lead to plaque vascularization and collateral vessel growth.

This thesis has been divided in four parts. The first part describes the role of the IL-33/ST2 pathway and mast cells in atherosclerotic disease and discusses the potential of implementing these pathways in therapeutic neovascularization.

In **Part II** the IL-33/ST2 pathway will be discussed. IL-33 intracellular signalling through its membrane receptor ST2L has been shown cardioprotective.[13–15] Soluble ST2 (sST2), functioning as a decoy receptor for IL-33,[17] is emerging as a novel biomarker for ischemic heart disease. **Chapter 2** provides an overview of the currently available literature on sST2 as a biomarker for adverse cardiovascular events. Furthermore, the possibility of modulating the IL-33/ST2 pathway for new therapeutic options will be discussed. In **Chapter 3** we studied the effect of cardiovascular interventions and established cardiovascular disease risk factors on plasma sST2 expression levels. Various experimental data have implicated a role for sST2 in TLR related immunosuppression. Administration of sST2 inhibited LPS induced cytokie secretion in several cell types.[18–20] Therefore we investigated possible associations between sST2 plasma levels and cell responsiveness in patients that underwent CABG surgery (**Chapter 4**). Soluble ST2 was also associated with the progression of atherosclerotic disease in a mouse model.[16,33] Therefore we addressed the question whether sST2 levels were associated with vulnerable plaque characteristics as plaque angiogenesis in patients with significant carotid stenosis (**Chapter 5**). As follow-up data of these patients was available we also investigated the predictive value of sST2 for future cardiovascular events in cerebral occlusive disease. The role of the IL-33/ST2 pathway in neovascularization is unclear. Therefore we investigated the effects of IL-33 administration in collateral artery growth (arteriogenesis) in a mouse hind limb model (**Chapter 6**).

Part III describes the role of mast cells in plaque angiogenesis. Experimental research suggests that mast cells participate in plaque development.[25–27] Furthermore, mast cells and their components can induce angiogenesis in vitro and in vivo.[24] Plaque angiogenesis is an important characteristic of the rupture-prone plaque.[34] In **Chapter 7**, we investigated to which extent intraplaque mast cells are associated with plaque angiogenesis, compared to other cell types as macrophages and neutrophils. Furthermore, as follow-up data was available, we investigated the association between mast cell numbers and increased risk for future cardiovascular events.

Immunoglobulins are important mediators in mast cell activation. In order to investigate whether immunoglobulins are responsible for mast cell activation and plaque angiogenesis in atherosclerosis we related immunoglobulin levels to several mast cell parameters and plaque vulnerability (**Chapter 8**).

In **Part IV** the data presented in this thesis are summarized and discussed (**Chapter 9**).

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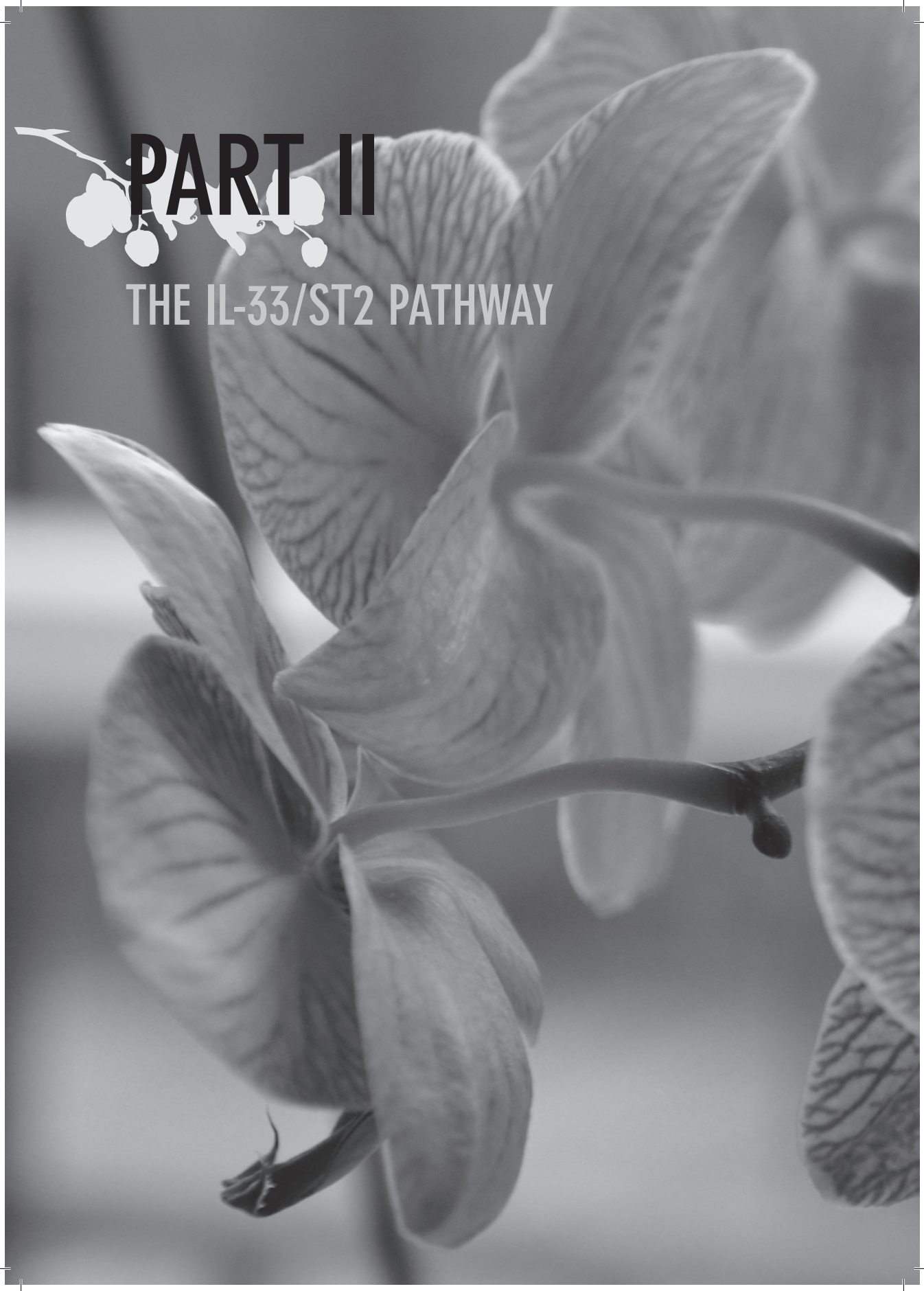

PART II

THE IL-33/ST2 PATHWAY

CHAPTER

2

The role of the interleukin 1 receptor-like 1 (ST2) and Interleukin-33 pathway in cardiovascular disease and cardiovascular risk assessment

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ABSTRACT

There is an ongoing search for biomarkers that can facilitate the diagnosis of subclinical or clinically manifest cardiovascular disease. One of the emerging biomarkers currently under investigation is ST2, which is the receptor of Interleukin-33 (IL-33). ST2 is a member of the Interleukin-1 receptor family and exists in a transmembrane (ST2L) and a soluble form (sST2) due to alternative splicing. Several groups have reported sST2 elevations in serum of cardiovascular disease patients. There is consisting evidence that sST2 is independently predictive for mortality in patients with heart failure or myocardial infarction.

In addition to its potential as a biomarker for adverse cardiovascular events, ST2 is considered to play a causal role in chronic cardiovascular diseases such as atherosclerosis and heart failure. Signalling of IL-33 via ST2 has been shown to be cardioprotective in mouse models of myocardial infarction, heart transplantation and cardiac hypertrophy and fibrosis. Furthermore, treatment with IL-33 reduced the development of plaques in atherosclerotic mice.

In this paper we will review the currently available literature on sST2 as a biomarker for adverse cardiovascular events. In addition, we will elaborate on the potential mechanistic role of the IL-33/ST2 pathway in chronic inflammatory cardiovascular diseases.

INTRODUCTION

Cardiovascular diseases are still a major burden for Western society. Morbidity and mortality numbers are especially high amongst the subgroup of vascular occlusive diseases, caused by atherosclerosis. Atherosclerosis is a chronic inflammatory process, characterized by plaque formation due to a combination of endothelial dysfunction, accumulation and oxidation of low-density lipoproteins and infiltration of inflammatory cells into the arterial wall. Slowly progressing stenosis results in chronic lack of blood supply and may be complicated by plaque rupture and thrombosis followed by myocardial infarction or even death. Many individuals are at intermediate risk to suffer from adverse cardiovascular events in the next 10 years: a group that is rapidly growing with the aging population. There is a need for indicators to identify those patients who would benefit most from therapeutic interventions. In order to cope with this increasing problem, finding novel therapeutic opportunities and identifying the patient at high risk are emerging issues.

In this review we will discuss the potential value of an inflammatory pathway that gained increasing attention in experimental mechanistic and human prediction studies: the IL-33/ST2 pathway. This pathway has been shown to exert an important role in several chronic inflammatory diseases. Signalling of IL-33 through ST2 has been shown to be cardioprotective and has been implicated in atherosclerosis. Moreover, it plays a role in adaptive angiogenesis, suggesting that interference in the IL-33/ST2 pathway might be of therapeutic value. Especially the results for soluble ST2 (sST2) as a biomarker for mortality and heart failure in ischemic cardiovascular disease are promising. This paper discusses the recent developments of sST2 as a biomarker in cardiovascular disease and the role of modulating the IL-33/ST2 signalling cascade as a possible therapeutic intervention.

The IL-33/ST2 pathway

The IL-33/ST2 pathway has been implicated to be involved in many inflammatory disorders and has gained the interest of several research fields. IL-33 is an IL-1 like cytokine merely expressed in the nucleus of healthy endothelial and epithelial cells rather than cells of hematopoietic origin[1,2] and possesses transcriptional regulatory properties.[3,4] As IL-33 lacks a nuclear export sequence and was found to be released upon cell necrosis,[1] it was thought that IL-33 acts as an alarmin to alert the body of cell damage.[5] However, different groups showed that also fibroblasts[6,7] and hematopoietic cells as macrophages and mast cells can

be primed to produce and secrete IL-33.[8,9] Recent data show that IL-33 can be secreted by living cells and is localized in membrane bound cytoplasmic vesicles upon biomechanical stress.[7] After release, IL-33 is biologically active independent of caspase-1 cleavage unlike other members of the IL-1 family.[10] Instead, cleavage by caspase-1 or other apoptotic caspases has been shown to inactivate IL-33.[11-13]

ST2, Interleukin 1 receptor-like 1, is a member of the IL-1 receptor family and mediates the biological function of IL-33.[1] Due to alternative splicing, ST2 can be found in a transmembrane form (ST2L) and a soluble form (sST2) that lacks the transmembrane and intracellular domains.[14] Besides ST2L and sST2 another splice variant has been identified, ST2V[15] however this variant will not be discussed in this review. Expression of membrane bound ST2L was found on hematopoietic cells,[16] in particular Th2-cells[17,18] and mast cells.[19] Immediately after the discovery of selective ST2L expression on Th2 cells, it was revealed that this receptor was important for Th2 mediated inflammation.[17,20] Although signalling through ST2L is not necessary for Th2 cell differentiation,[21] it does play a role in the activation of Th2 cells.[22] IL-33 is also considered to modulate Th2 responses as it can act as a chemoattractant for Th2 cells.[23] More evidence for the importance of IL-33 in Th2 signalling came from the group of McKenzie that generated a ST2 -/- mouse model in which Th2 cytokine production was severely impaired.[24] Subsequently, it was demonstrated that indeed Th2 associated inflammation was abolished in mice where IL-33 signalling was prevented.[25,26]

When it became evident that IL-33 was the ligand for ST2, various in vitro cell assays have been performed to examine the responsiveness of different cell types to IL-33 stimulation.[27] In short, most studies were in line with previous data, showing that IL-33 signalling results in the secretion of Th2 associated cytokines as IL-5 and IL-13 via the NF-κB pathway.[1] Despite contradictory results, revealing lack of association between the ST2 receptor and a Th2 cytokine response,[28] most data points towards a key role for ST2 receptor signalling in Th2 mediated inflammation. This is of importance as atherosclerosis is a Th1 associated inflammatory disease and a shift towards Th2 is regarded as cardioprotective. A schematic overview of the IL-33/ST2 pathway is shown in Figure 1.

Soluble ST2, a biomarker for cardiovascular disease

ST2L and sST2 are found to be differentially expressed in distinct organs.[29] While ST2L is merely detected on the surface of circulating cells, sST2 is suggested to be predominantly produced and secreted by tissue resident cells. Experimental studies revealed that sST2 levels were not increased after whole blood stimulation

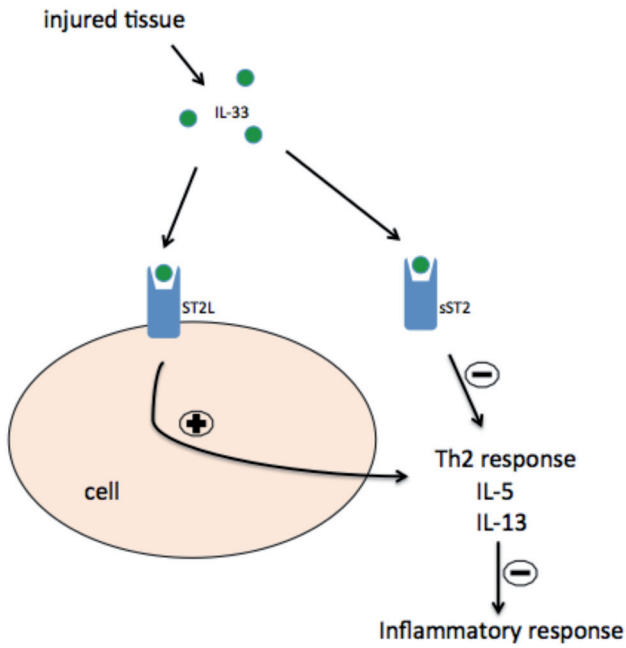

Figure 1 | Schematic overview of the IL-33/ST2 pathway
IL-33 is released upon cell injury. Binding of IL-33 to ST2L induces an intracellular signalling cascade resulting in the production of Th2 associated cytokines. Soluble ST2 can prevent this Th2 associated inflammatory response by capturing IL-33 away from the circulation.

with bacterial ligands,[30,31] indicating that circulating cells are not the main source for serum sST2 levels in human pathology. The hypothesis that sST2 originates from tissue resident cells was strengthened by the observations that sST2 is secreted by serum-stimulated fibroblasts,[32] human endothelial cells,[33] stressed cardiomyocytes[34] and lysophosphatidic acid stimulated epithelial cells.[35]
The observation that sST2 is released following injury of cardiomyocytes led to the hypothesis that sST2 could serve as a biomarker for cardiovascular disease. Indeed, sST2 has recently emerged as a novel biomarker with prognostic value in cardiovascular disease. After the discovery that sST2 levels were increased after myocardial infarction,[34] multiple studies have been performed showing that patients suffering from cardiovascular diseases as chronic heart failure,[36,37] obesity[38] and type 2 diabetes[39] have higher baseline sST2 levels compared to healthy controls. Soluble ST2 levels correlated not only to severity of disease[40,41] but the levels were also found to be independently predictive for mortality and heart failure.[40,42-44] Furthermore sST2 levels were not only raised in diseased patients or patients who experienced an acute event. Also after heart or abdominal surgery,

trauma and peripheral arterial interventions sST2 levels were elevated.[45-48] These studies confirmed that sST2 release is associated with the severity of intervention or surgery.

Results from clinical studies associating sST2 with cardiovascular disease are summarized in Table 1. The published results are not always consistent. For example, some studies could not confirm an association between sST2 and heart failure[49,50] or predict myocardial damage in patients with chest pain.[51] However, the support for evidence that sST2 is a strong independent biomarker with additive value on top of traditional risk factors that predicts death due to cardiovascular disease is increasing. Multimarker prediction studies have demonstrated that the sST2 level has supplemental prognostic value in addition to other biomarkers e.g. (NT-proBNP, Troponin, creatinine,) in acute myocardial infarction[52,53] and heart failure.[54-61] Interestingly, similar results were obtained for baseline sST2 levels measured in patients with chronic manifestations of cardiovascular disease. For example, sST2 levels in a NSTEMI patient population[62] and patients with chronic heart failure[37,57] were independently predictive for the occurrence of cardiovascular death.

Moreover, in other diverse groups of patients presenting with different clinical manifestations, sST2 levels were found to be predictive for heart failure or death. For example, in patients with chest pain[63] or dyspnoea[64-70] sST2 levels were increased at baseline in patients who died during follow up. Death rates were also increased in patients with high sST2 levels who were referred for suspected cardiac ischemia to the hospital for an echocardiogram[71] and an unselected population of patients arriving at the intensive care unit.[72] Another remarkable observation was done in the Framingham study that showed that sST2 levels have predictive value for cardiovascular events in addition to risk factors and other biomarkers in a non-symptomatic healthy population.[73] These studies are of particular interest as they show that sST2 could facilitate risk prediction of cardiovascular events not only for secondary manifestations but also primary manifestations of the disease.

IL-33 and ST2: role in cardiovascular pathophysiology

Currently, efforts are made in experimental research to answer the question if the biomarker association of sST2 with disease severity and death prediction in human cardiovascular disorders is causal or a consequence of reverse causality. Experimental studies have revealed that sST2 captures IL-33 from the circulation,[74] thereby preventing IL-33 induced cardioprotective intracellular signalling. For

Table 1 | Association and longitudinal studies exploring the role and predictive value of soluble ST2 in cardiovascular disease.

Author	N	Disease of study group	Endpoint
Weinberg et al., 2002 [34]	69	Myocardial infarction	Association study
Weinberg et al., 2003 [42]	161	Heart failure	Mortality and transplantation
Shimpo et al., 2004 [44]	810	STEMI	Mortality and heart failure at 30 days
Brown et al., 2007 [51]	348	Chest pain	Major adverse cardiovascular events at 30 days
Januzzi et al., 2007 [69]	593	Dyspnoea	Mortality at 1 year
Bartunek et al., 2008 [37]	121	Congestive cardiomyopathy	Association study
Boisot et al., 2008 [56]	150	Acute heart failure	Mortality at 90 days
Martinez-Rumayor et al., 2008 [65]	231	Acute dyspnoea	Mortality at 1 year
Mueller et al., 2008 [43]	137	Acute heart failure	Mortality at 1 year
Rehman et al., 2008 [40]	346	Acute heart failure	Mortality at 1 year
Rehman et al., 2008 [66]	577	Acute dyspnoea	Mortality at 1 year
Sabatine et al., 2008 [53]	1239	STEMI	Major adverse cardiovascular events at 30 days
Dieplinger et al., 2009 [50]	251	Dyspnoea	Acute heart failure
Pascual-figal et al., 2009 [110]	36	Heart failure	Sudden cardiac death
Shah et al., 2009 [70]	134	Acute dyspnoea	Mortality at 4 years
Bayes-Genis et al., 2010 [58]	48	Heart Failure	Cardiovascular death, heart failure and transplantation at 1 year
Daniels et al., 2010 [71]	588	Patients referred for echocardiogram	Mortality at 1 year
Dieplinger et al., 2010 [64]	251	Dyspnoea	Mortality at 1 year
Eggers et al., 2010 [111]	403	NSTE-ACS	Mortality at 1 year
Januzzi et al., 2010 [68]	517	Acute dyspnoea	Mortality at 4 years
Socrates et al., 2010 [67]	1091	Dyspnoea	Mortality at 30 days and 1 year
Weir et al., 2010 [112]	100	Myocardial infarction	Left ventricle function
Dhillon et al., 2011 [113]	577	NSTEMI	Major adverse cardiovascular events
Ky et al., 2011 [59]	1141	Chronic heart failure	Mortality and transplantation
Manzano-Fernández et al., 2011 [55]	447	Acute heart failure	Mortality at 1 year
Pascual-figal et al., 2011 [54]	107	Acute heart failure	Mortality
Aldous et al., 2012 [63]	995	Chest pain	Mortality and heart failure
Bayes-Genis et al., 2012 [57]	891	Chronic heart failure	Mortality
Broch et al., 2012 [41]	1449	Heart failure	Cardiovascular death, MI and Stroke
Dhillon et al., 2012 [52]	677	Unselected STEMI	Mortality at 30 days and 1 year
Dieplinger et al., 2012 [72]	530	Patients admitted to ICU	Mortality at 90 days
Dworakowski et al., 2012 [114]	41	Aortic valve implantation	Association study
Kohli et al., 2012 [62]	4426	NSTEMI	Mortality and heart failure at 30 days
Manzano-Fernández et al., 2012 [61]	72	Acute heart failure	Mortality
Santhanakrishnan et al., 2012 [49]	101	Reduced ejection fraction	Association study
Wang et al., 2012 [73]	3428	Non-symptomatic participants	Mortality and heart failure
Zhang et al., 2012 [36]	543	Chronic heart failure	Association study
Zilinski et al., 2012 [60]	30	Heart failure	Mortality and transplantation at 90 days

example, administration of IL-33 in different animal models of cardiovascular disease resulted in functional improvements and increased survival rates,[6,75,76] while sST2 actually exacerbated disease.[76] Association studies in the field of genetics support the view that ST2 may be causally related with the disease. Two single nucleotide polymorphisms (SNPs) in the distal promoter region of the ST2 gene were found to be associated with angiographic severity of coronary artery disease.[77]

Rupture of the atherosclerotic plaque is the underlying mechanism of myocardial infarction, which can subsequently give rise to heart failure. A particularly interesting observation was that atherosclerotic ApoE-/- mice on a high fat-diet receiving IL-33 treatment, developed smaller lesions in which less macrophage and T-cell accumulation was observed.[78] Recombinant sST2 treatment resulted in an increased plaque size, although inflammatory cell numbers in plaques remained unaltered. Indications supportive for a role of the IL-33/ST2 pathway in the development of the atherosclerotic plaque was obtained from experiments demonstrating that administration of IL-33 lowered foam cell formation considerably in vitro and in vivo in ApoE-/- mice.[79] Diabetes is a metabolic disease that is closely related to the initiation and progression of atherosclerosis. It is prevalent in obese individuals and one of the main risk factors for vascular occlusive diseases. IL-33 was found to exert protective anti-atherosclerotic effects in obese mice.[80] IL-33 and ST2 protein are both expressed in the atherosclerotic plaque.[81] It deserves consideration that also in experimental studies conflicting results have been reported regarding the role of IL-33/ST2 pathway in arterial occlusive disease. IL-33 stimulation induces the expression of adhesion molecules and activates inflammation in human endothelial cells and the atherosclerotic plaque.

Cardiac hypertrophy and fibrosis are two pathological processes resulting in systolic and/or diastolic heart failure. Subsequent cardiac hypertrophy further aggravates cardiac function. Soluble ST2 and ST2L expression levels are increased in cardiomyocytes[34] and cardiac fibroblasts[6] upon mechanical stress. Administration of IL-33 significantly decreased cardiac fibrosis and hypertrophy in a trans-aortic constrictive mouse model, a phenotype that was not observed in ST2-/- mice.[6] Despite the observed migration of fibroblasts and changed cytokine expression profiles following IL-33 administration in vitro, there was no change in the expression of typical fibrosis markers such as collagen I, collagen III and transforming growth factor-beta.[82] These data suggest that other cell types are

necessary for the decreased fibrosis in vivo. In vitro studies showed that IL-33 mediated protection of cardiomyocytes against hypoxia-induced apoptosis was repressed by sST2.[75] Also in an ischemia/reperfusion rat model, mortality was lower amongst IL-33 treated rats.[75] Less apoptosis of cardiomyocytes was observed corresponding with significantly smaller infarct size and fibrosis volume resulting in improved cardiac function compared to control animals.[75]

Fibrosis of extracellular tissue is a process that reflects tissue damage and remodelling in many different organs. The ST2/IL-33 pathway also plays a role in other diseases with a fibrotic component. IL-33 protein levels were detected in normal and fibrotic liver tissue and isolated liver cells. Similarly to fibrotic heart tissue, IL-33 levels were increased upon stimulation of hepatic stellate cells with inflammatory proteins. Messenger RNA levels of both IL-33 and ST2 were increased in fibrotic livers and levels correlated with collagen expression levels.[83] In agreement with the observations in cardiac fibrosis, sST2 treatment enhanced a Th2 mediated inflammatory response resulting in excessive fibrogenesis in a mouse mode of hepatic injury and fibrosis.[84] Also in other models of liver failure, IL-33 signalling has been reported to exert protective actions[85,86] while ST2 deficient mice developed more severe hepatitis. IL-33 is also expressed in the nucleus of activated pancreatic stellate cells isolated from human and rat pancreatic tissue and increased upon stimulation with inflammatory cytokines.[87] However, in these cells, ST2L levels were low and not responsive to IL-33, suggesting that IL-33 may not play a dominant role in pancreatic fibrosis. In contrast, IL-33 has been shown to worsen skin fibrosis.[88]

Soluble ST2 as an immunosuppressant

Besides capturing IL-33 from the circulation and thereby preventing intracellular IL-33 signalling, various data have indicated that sST2 is an immunosuppressant related to the Toll-like receptor (TLR) pathway. Inflammation is a naturally occurring physiological process activated by exogenous ligands to protect the body from invading pathogens. Also endogenous ligands, secreted upon cell damage, can activate TLR mediated inflammation. When an inflammatory response persists, a negative feedback mechanism is induced to protect the body from excessive inflammatory injury. As the TLR pathway has been shown to exert an important role in almost all inflammatory mediated processes and the IL-33/ST2 pathway has been implicated in TLR regulation, we propose the IL-33/ST2 pathway as a possible target for the development of new strategic therapeutics.

In one of the first studies performed by Sweet and colleagues, it was shown that

sST2 could bind to bone marrow derived macrophages and that this binding was enhanced by lipopolysaccharide (LPS) stimulation, the most potent TLR4 ligand.[89] This binding lowered TLR4 mRNA levels and the cytokine expression levels that were induced by LPS treatment. Also in vivo they showed that administration of sST2 after LPS injection decreased the LPS mediated mortality associated with a reduced inflammatory cytokine profile. In addition, sST2 overexpression in vivo downregulated TLR4, TNF-α and IL-6 gene expression.[90] Furthermore, it was shown that LPS induced cytokine production is suppressed by sST2 in a monocyte cell line[91] and dendritic cells.[92] In addition, macrophages deficient for ST2 produced more cytokines with and without LPS stimulation compared to control cells.[93] This was also reflected in vivo. Wild type mice primed with LPS followed by a lethal dose of LPS survived, while ST2-deficient mice that did not develop endotoxin tolerance died from the challenge, which correlated with increased cytokine levels in the serum samples.[93] In contrast, in mice subjected to bacterial infection, sST2 treatment negatively regulated TLR2 signalling however it was not required for bacterial lipoprotein induced tolerance, as tolerance was still observed in ST2 -/- mice.[94]

These data implicate that sST2 acts as a negative feedback mechanism on the TLR pathway. After an event, TNF and IL-6 levels rise fast, and these factors are also known to induce sST2 expression.[95,96] This suggests that sST2 might have an immunosuppressive role in human pathology as well. However, in humans the TLR response is downregulated within minutes after an exogenous or endogenous challenge while the increase in sST2 levels takes hours. It is therefore not likely that the increased sST2 is mechanistically responsible for early downtoning of the TLR response. We suggest that it reflects a more general, longer lasting immunosuppressive status of the human body after challenge.

IL-33 a possible mediator for collateral formation

Patients with vascular occlusive diseases can profit from the formation of collateral blood vessels to restore the blood flow to ischemic tissue or prevent an ischemic event. Collateral formation (arteriogenesis) is a naturally occurring process of vessel remodelling that shares many mediators with angiogenesis. Although experimental data is scarce, a possible role for the IL-33/ST2 pathway in collateral vessel formation has been implicated. Upon stimulation of angiogenesis, nuclear IL-33 in endothelial cells is rapidly lost.[97] A remarkable observation in this study was that although endothelial cells are the main source for IL-33, it is completely absent in the endothelial cells of tumour vessels. A possible explanation is that

these endothelial cells in tumours are highly angiogenic and that IL-33 is therefore no longer present.[97] Besides, IL-33 was shown to induce permeability, migration and proliferation of endothelial cells in addition to sprouting and tube formation in vitro and in vivo.[98] Unpublished data in our group showed that IL-33 treatment decreased perfusion restoration by almost 50% in a hind limb ischemia model. Taken these data together, the ST2/IL33 pathway is likely to exert an important function in new vessel formation, which provides a therapeutic window for the stimulation of collateral formation in ischemic heart disease.

The IL-33/ST2 pathway in other chronic inflammatory diseases

In contrast to the protective effect observed in cardiovascular diseases and infectious disease, IL33 signalling was found harmful in other inflammatory diseases as arthritis and asthma.[99] IL-33 has been observed to exacerbate airway inflammation[100] in asthma. In addition, treatment with sST2[101] and blocking IL-33 signalling[102] has been shown beneficial in asthmatic inflammation. In arthritic disease, characterized by cartilage and bone destruction, sST2 treatment in a mouse model has been shown to attenuate disease progression.[103] In addition, mice deficient in ST2 developed less collagen-induced arthritis.[104] Conversely treatment of IL-33 in WT mice had a pro-inflammatory function and exacerbated arthritis[105] and inhibiting IL-33 signalling was protective in experimental arthritis.[106] A possible reason that could explain part of the inconsistency in outcome between the different inflammatory diseases in literature is that ST2 can form a heterodimer with either IL-1RAcP[107,108] or SIGIRR[109] resulting in different inflammatory responses.

Conclusions

In conclusion, sST2 levels are associated with inflammatory cardiovascular disease and predict cardiovascular events, specifically cardiovascular death, during follow up. The results for sST2 as a biomarker for cardiovascular disease are very promising. Although it is not clear whether this increase in sST2 is causally related to disease, experimental data imply multiple biological functions for the IL-33/ST2 pathway. However, discrepancies in experimental and clinical data do exist and further research is warranted to elucidate the complex pathway of IL-33 and ST2 in cardiovascular disease. As the complete set of immune disorders seems to be related with the IL-33/ST2 pathway with different outcome, therapeutic strategies should be approached with caution.

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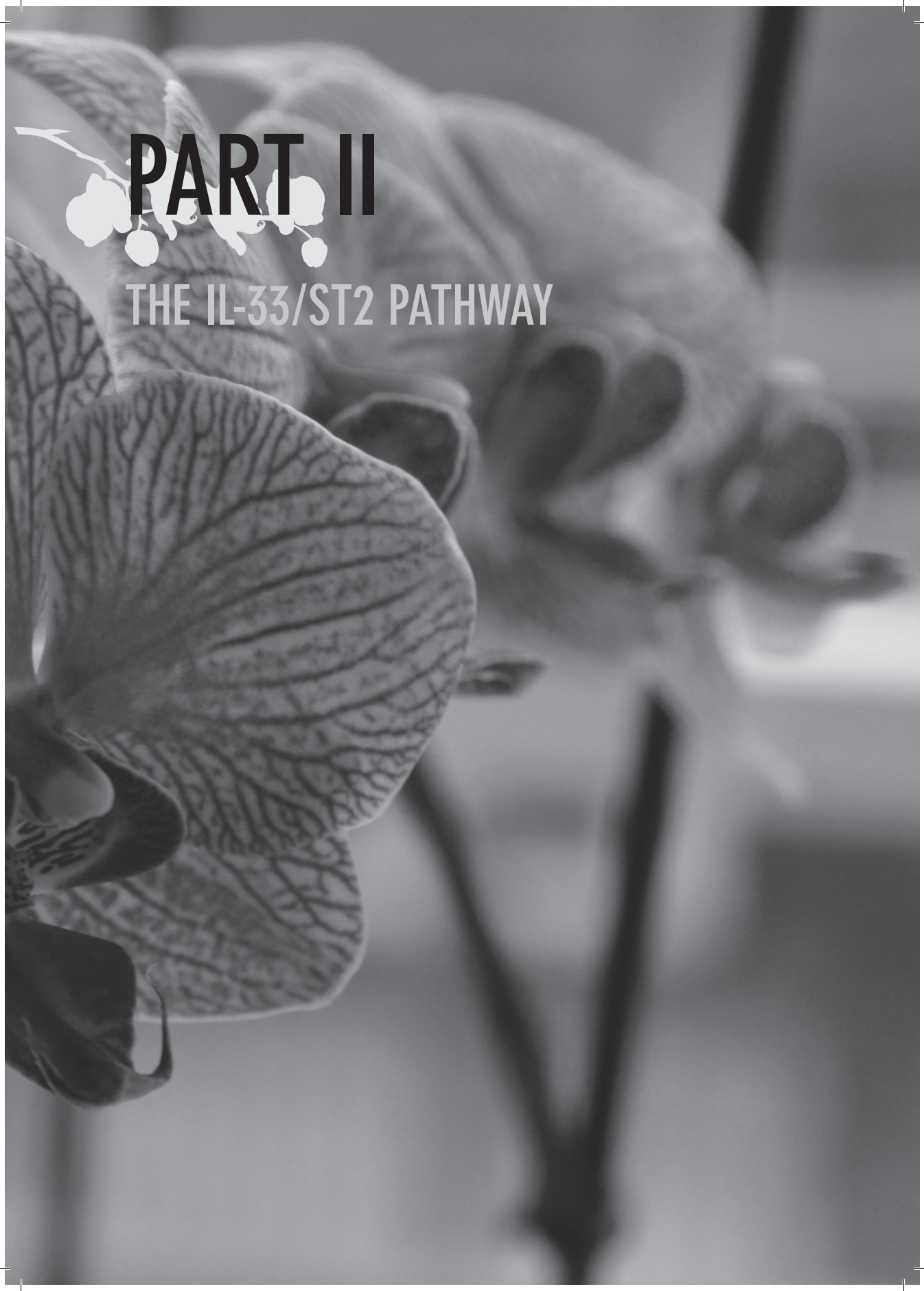

PART II

THE IL-33/ST2 PATHWAY

CHAPTER

3

Temporal changes of soluble ST2 after cardiovascular interventions

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ABSTRACT

Background

Soluble ST2 (sST2), a member of the IL-1 receptor family, has been proposed as a novel biomarker with predictive value for heart failure and mortality in patients suffering from cardiovascular diseases. The influence of clinical characteristics on variability of sST2 levels is relatively unexplored. Here we studied the effect of cardiovascular interventions and clinical characteristics on plasma sST2 expression levels.

Material and methods

In the current study, sST2 levels were assessed in the plasma of patients scheduled for coronary artery bypass grafting (CABG) (n=76), percutaneous coronary intervention (PCI) (n=68) or peripheral vascular surgery (n=27).

Results

Age was the only classical risk factor significantly correlating with sST2 levels. Soluble ST2 levels were significantly increased one hour after CABG (48 [33-70] vs. 61 [42-89] pg/mL, p=0.001) and increased even further after 24 hours (1116 [578-13666] pg/mL, p<0.001). An average three-fold increase in sST2 levels was also observed in patients 24 hours after peripheral interventions (30 [21-41] vs. 98 [48-211] pg/mL, p<0.001). Two months after PCI we found that sST2 levels were significantly higher compared to baseline levels (41 [29-61] vs. 48 [31-80] pg/mL, p=0.007, n=52). In addition, we did not observe an association between sST2 and any inflammatory or cardiac specific markers that were measured in this study.

Conclusions

Soluble ST2 increases significantly following cardiovascular interventions. The notion of a recent cardiovascular intervention is a strong determinant of sST2 levels and therefore needs to be taken into account when exploring sST2 as predictor of future cardiovascular events.

INTRODUCTION

There is an ongoing search for biomarkers that facilitate the diagnosis or prediction of subclinical and clinically manifest cardiovascular disease. An emerging biomarker currently under investigation is ST2. ST2 is a member of the IL-1 receptor family and exists in a transmembrane form (ST2L) and a soluble form (sST2) due to alternative splicing.[1] Several groups have reported that sST2 is elevated in the serum of patients suffering from cardiovascular disorders.[2-4] Subsequently, multiple studies have shown that sST2 levels are positively correlated with severity of cardiovascular disease and, more importantly, that it has predictive value for future adverse cardiovascular events (reviewed by Miller and Liew[5]).

ST2L is expressed on immune cells and is the receptor for the pro-inflammatory Th2 associated cytokine IL-33.[6] Signalling of IL-33 through ST2L has been found cardioprotective in a mouse model of myocardial infarction,[7] heart transplantation[8] and cardiac hypertrophy and fibrosis,[9] with increased survival rates after IL-33 treatment. Furthermore, IL-33 reduced the development of atherosclerotic plaques in ApoE-/- mice on a high fat-diet.[10] The biological role of sST2 is based on capturing IL-33 from the circulation and thereby preventing IL-33 signalling,[11] which might explain the pathophysiological and detrimental role of sST2 in cardiovascular diseases.[8,10]

To gain insight into the specificity of sST2 as biomarker, it is important to assess the association between sST2 levels and baseline patient characteristics. Moreover, changes in sST2 levels in response to cardiovascular interventional treatment should be taken into account to accurately determine its predictive or diagnostic value. In the current study, we measured sST2 protein levels in the plasma of three different patient cohorts suffering from cardiovascular disease. We assessed sST2 levels before and after three different typical cardiovascular interventions: coronary artery bypass grafting (CABG), elective percutaneous coronary intervention (PCI) and peripheral vascular surgery. In addition, we investigated whether sST2 expression in patients was associated with established cardiovascular disease risk factors and other inflammatory markers.

MATERIALS AND METHODS

Study population and design

Adult patients with coronary or peripheral artery disease from three different cohorts treated in Utrecht and Eindhoven, the Netherlands, were included. The local medical ethical boards of the participating hospitals approved the studies. All patients signed a written informed consent prior to inclusion. Exclusion criteria were known history of other chronic inflammatory diseases or acute inflammatory disorders or immunosuppressive drug use. Baseline characteristics, medication use and medical history were gathered from questionnaires and the patient medical records. All interventional procedures were performed routinely according to hospital protocols. Blood samples were collected in lithium-heparin anti-coagulated tubes. The blood was centrifuged and the plasma obtained was stored at -80 degrees. The three patient groups and time points of blood withdrawal are described separately.

Coronary artery bypass grafting

Seventy-six subsequent patients scheduled for on-pump CABG were included.[12] Blood samples were collected before surgery (n=76), within one hour after chest closure (n=76) and 24 hours after surgery (n=73).

Peripheral surgical interventions

In this study cohort, 27 subsequent patients with peripheral artery disease were scheduled for surgical interventions (ring strip cutter n=10, arterial bypass n=6, carotid endarterectomy n=4, femoral endarterectomy n=2, aortic surgery n=2, other n=3). Procedures have been described previously.[13] In short, blood samples were drawn before surgery (n=27), directly after (n=27) and 24 hours after surgery (n=18). In addition, from 9 randomly chosen patients, blood was drawn at three extra time points; 5 minutes after skin incision, directly after arterial incision, and 30 minutes after the first vascular incision.

Percutaneous coronary intervention

In this study a total of 68 subsequent patients scheduled for PCI were included. From 68 patients blood samples were drawn directly after sheet insertion. Eventually, from 52 patients additional blood samples were taken 2 months after the procedure.

Quantification of sST2, cTnI, IL-6, IL-8, IL-10 and TNFα levels in patient plasma

Soluble ST2 levels were measured with an IL-1 R4/ST2 enzyme-linked immunosorbent assay (ELISA, RayBiotech, Norcross, Georgia, USA) according to the manufacturer's instructions. In brief, plasma samples and standards incubated for 2.5 hours in a 96-well plate pre-coated with a capture antibody for human ST2. After the 96-well plate was washed using an automatic washer, a biotin labelled anti-human ST2 antibody was added to the wells. In between washes the HRP antibody was added, followed by administration of the substrate. After 30 minutes the stop solution was added and the luminescence was measured at 450nm with an ELISA reader (Multiskan FC, Thermo Fisher Scientific, Vantaa, Finland). The upper detection limit of the RayBio® human IL-1 R4/ST2 ELISA kit was 1200 pg/mL and the lower limit of detection was 2 pg/mL, with an intra-assay CV<10% and inter-assay CV<12%. The samples above the detection limit were diluted 5 to 40 times to obtain a value within the detection limits.

TNFα, IL-6, IL-8 and IL-10 cytokine levels were measured with a multiplex fluorescent bead immunoassay (FlowCytomix human Th1/Th2 11plex, Bender MedSystems, Mercure group, Vienna, Austria). The other 7 analytes measured in this Th1/Th2 11-plex (IL-1β, IL-2, IL-4, IL-5, IL-12, IFNγ, and TNFβ) revealed many values below the detection limit and were therefore not used for further analyses. According to the manufacturer's instructions, microspheres coated with specific antibodies and biotin-conjugated antibodies against the human cytokines were added to a filter plate. Plasma samples incubated for 2 hours at room temperature on a microplate shaker. Streptavidin-Phycoerythrin (PE) was added to the wells after washing. After one hour the samples were washed again and measured on a flow cytometer (cytomics FC500, Beckman Coulter, Fullerton, CA, USA). In addition, plasma samples were sent to the diagnostics laboratory for the analysis of cardiac Troponin I (cTnI), using an immunoassay method (ADVIA Centaur® System, Bayer HealthCare, Diagnostic Division, Bayer Group, Leverkusen, Germany). For all values below the detection limit, zero was retained as a value.

Statistics and data analysis

IBM SPSS statistics version 20 was used for all analyses (IBM corporation, Armonk, NY, USA). Soluble ST2 was not normally distributed; non-parametrical testing was used to determine differences. The Mann-Whitney U test was used to obtain differences in sST2 levels for all risk factors. The Wilcoxon signed rank test was used to compare differences in sST2 levels before and after operation. The

spearman correlation coefficient was used to determine relations between sST2 and age. Differences were considered significant with a p-value of below 0.05.

RESULTS

Baseline characteristics of the study population

Soluble ST2 was measured in a total of 171 patients scheduled for CABG (76), PCI (68) or peripheral surgical interventions (27). Table 1 depicts baseline clinical characteristics for the three cohorts included in this study. The patient groups describe a relatively typical population of patients with vascular occlusive diseases. The mean age of the patients was around 66, with a male majority ranging between 62-85%.

Table 1 | Baseline characteristics and medication use

Baseline Characteristics	PCI (n=68)	CABG (n=76)	Peripheral interventions (n=27)
sST2 pg/mL, median [IQR]	46 [29-62]	48 [33-70]	30 [21-41]
Age, mean years (sd)	63 (11)	67 (9)	66 (9)
BMI kg/m^2, mean (sd)	28 (4)	27 (3)	27 (3)
Male sex, n (%)	42 (62%)	59 (78%)	23 (85%)
Risk factors			
Current smoker, n (%)	7 (10%)	17 (22%)	10 (37%)
Diabetes mellitus, n (%)	13 (19%)	22 (29%)	7 (26%)
Hypertension, n (%)	35 (51%)	49 (64%)	23 (85%)
History MI, n (%)	18 (26%)	48 (63%)	
History PCI, n (%)	21 (30%)	16 (21%)	
Family history CVD, n (%)	39 (57%)	51 (67%)	13 (54%)
Medication			
Statin use, n (%)	60 (88%)	67 (88%)	20 (74%)
Beta-blocker, n (%)	58 (85%)	69 (91%)	16 (59%)
Calcium antagonist, n (%)	24 (35%)	24 (32%)	7 (26%)
Nitrates, n (%)	26 (38%)	42 (55%)	4 (15%)
ACE-inhibitors, n (%)	21 (31%)	42 (55%)	9 (33%)
Diuretic, n (%)	16 (24%)	10 (13%)	11 (41%)

Data are presented as mean (sd), median [interquartile ranges] or as No. (%) PCI = Percutaneous Coronary Intervention; CABG = Coronary Artery Bypass Grafting; BMI = Body Mass Index; MI = Myocardial Infarction; CVD = Cardio Vascular Disease; ACE = Angiotensin-Converting Enzyme

Relation between sST2 levels and clinically relevant characteristics

Table 2 shows clinically relevant characteristics in relation to baseline sST2 expression levels. In all studies sST2 was associated with age (CABG r=0.240, p=0.037; PCI r=0.291, p=0.016; peripheral interventions r=0.332, p=0.091). For none of the other main cardiovascular risk factors we observed consistent differences in sST2 levels (Table 2).

Table 2 | Baseline soluble ST2 levels (pg/mL) in respect to risk factors

Study	PCI (n=68)	p-value	CABG (n= 76)	p-value	Peripheral interventions (n=27)	p-value
Age	r=0.291	**0.016**	r=0.240	**0.037**	r=0.332	0.091
Sex						
Male	48 [29-64]	0.668	49 [33-70]	0.93	30 [21-41]	0.682
Female	41 [30-56]		43 [34-83]		27 [12-53]	
Current smoker						
Yes	31 [28-61]	0.414	47 [27-61]	0.609	31 [20-34]	0.725
No	47 [30-62]		48 [36-86]		29 [21-63]	
Diabetes mellitus						
Yes	41 [30-74]	0.803	48 [32-110]	0.626	26 [20-66]	0.825
No	46 [29-61]		48 [33-63]		31 [21-40]	
Statin use						
Yes	47 [29-63]	0.446	49 [32-90]	0.635	25 [19-40]	0.086
No	41 [30-47]		42 [37-56]		33 [28-79]	
Hypertension						
Yes	46 [31-63]	0.615	46 [32-55]	0.171	30 [21-37]	0.123
No	42 [28-62]		51 [36-98]		31 [20-55]	
History MI						
Yes	38 [17-51]	0.113	49 [33-70]	0.821		
No	49 [30-64]		46 [34-89]			
History PCI						
Yes	50 [27-63]	0.76	40 [27-50]	0.239		
No	46 [30-61]		50 [36-82]			
BMI > 25						
Yes	42 [29-61]	0.888	47 [33-67]	0.917	30 [21-38]	0.606
No	50 [24-69]		49 [32-87]		30 [16-256]	

Data are presented as Spearman's rank correlation coefficient (r) or median [interquartile range]. PCI = Percutaneous Coronary Intervention; CABG = Coronary Artery Bypass Grafting; MI = Myocardial Infarction; BMI = Body Mass Index

Furthermore, we observed that patients with a BMI>25 had significantly lower sST2 levels at 24 hours after surgery compared to patients with a BMI≤25. This was observed in both the patients scheduled for CABG (1096 [471-10485] vs. 11974 [987-20775], p=0.025) and the patients scheduled for peripheral interventions (87 [48-160] vs. 244 [184-255], p=0.051).

Soluble ST2 levels following a cardiovascular intervention

Directly one hour after CABG a significant increase in sST2 plasma levels was observed (48 [33-70] vs. 61 [42-89] pg/mL, p=0.001). One day after surgery the levels of sST2 were >20 fold increased. This increase was consistent in all patients (1116 [578-13666] pg/mL, p<0.001; Figure 1). An increase in sST2 was also observed after peripheral surgical interventions. This increase was less evident compared to CABG surgery (from 30 [21-41] to 98 [48-211] pg/mL, p<0.001; Figure 2). We measured sST2 levels before and 2 months after PCI in 52 patients in one of the hospitals. In this group, the sST2 levels were significantly higher two months after PCI procedure compared to baseline levels (41 [29-61] vs. 48 [31-80] pg/mL, p=0.007).

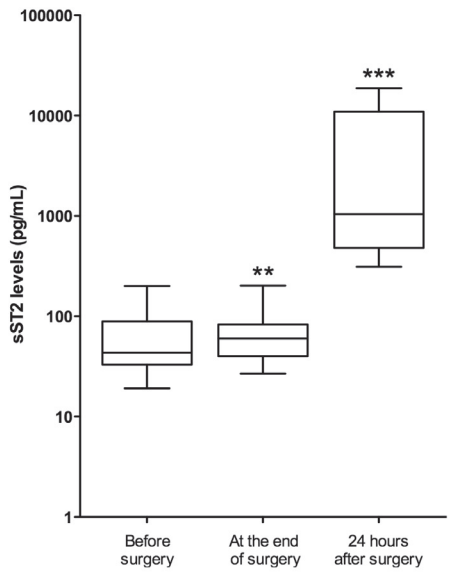

Figure 1 | Box-plot (10-90% range) of sST2 levels (pg/mL) following CABG surgery (** p<0.01 vs. before surgery; *** p<0.001 vs. before surgery)

Relation of sST2 and other established inflammatory and cardiac specific markers

In most patients from the three cohorts, there were no detectable levels of the inflammatory markers IL-6, IL-8, IL-10, TNFα and cTnI before surgery or intervention (data not shown). No correlations have been observed between the patients that did express inflammatory markers and sST2 levels.

In the CABG cohort IL-6, IL-8, IL-10, TNFα and cTnI levels were also measured directly and 24 hours after surgery. Only for IL-6 and cTnI levels we observed an increase in expression after surgery (Figure 3). Cardiac TnI and IL-6 levels increased significantly directly after CABG surgery (1.2 [0.6-1.9] ng/mL and 14 [6-32] pg/mL, respectively) till at least 24 hours (2.68 [1.7-4.6] ng/mL and 18 [11-36] pg/mL, respectively). Levels in IL-8, IL-10 and TNFα did increase after procedure, however only in 30 to 50 percent of the patients. This increase was not associated with sST2 levels. In addition, also for cTnI or IL-6 there was no association observed with sST2 levels for all time points.

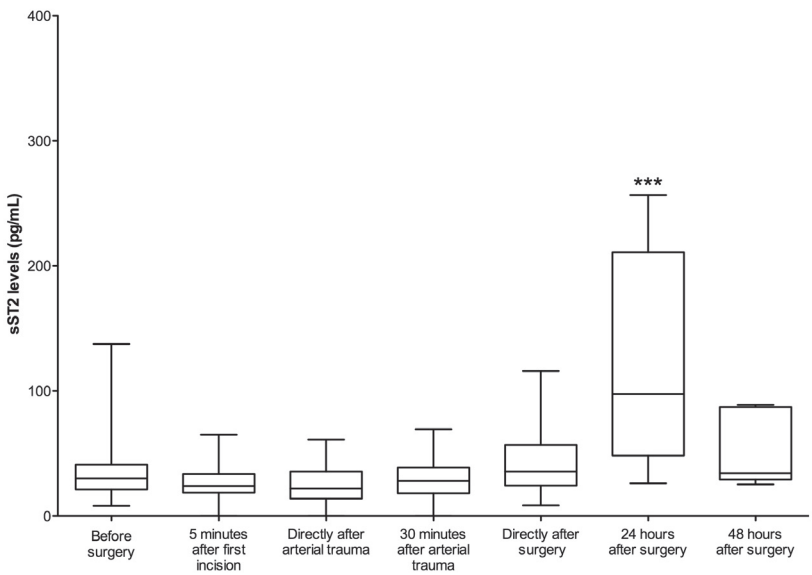

Figure 2 | Box-plot (10-90% range) of sST2 levels (pg/mL) before and after peripheral artery surgery (*** p<0.001 vs. before surgery)

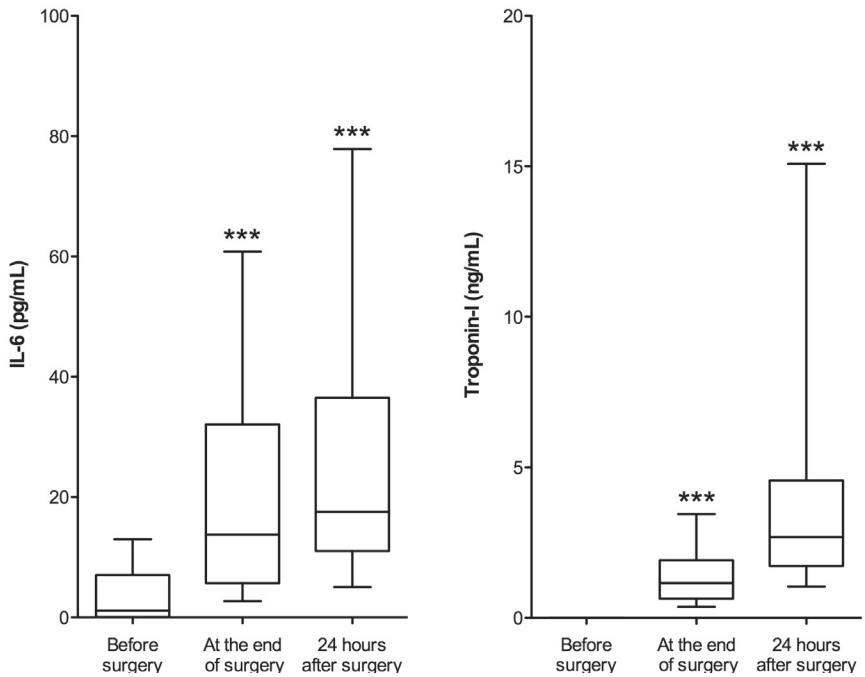

Figure 3 | Box-plot (10-90% range) of IL-6 (pg/mL) and cTnI (ng/mL) levels following CABG surgery. (***p<0.001 vs. before surgery)

DISCUSSION

Soluble ST2 has emerged as a novel biomarker with prognostic value in cardiovascular disease. Multiple studies have shown that sST2 levels are independently predictive for events in patients with cardiovascular disease (reviewed by Miller and Liew[5]). More recent studies showed that the value of sST2 in risk prediction increases even more in combination with other markers (e.g. NT-proBNP, Troponin).[14-18] More importantly a large multimarker study showed that sST2 significantly improved risk classification in addition to 5 other biomarkers.[19] Nevertheless, for any biomarker it is important to assess whether expression levels are influenced by traditional risk factors or clinical procedures that may affect the disease progression. Insight into factors that influence variability in sST2 levels is scarce. Different research groups observed that patients with severe cardiovascular disease had higher levels of sST2 compared to healthy controls.[2,4] Furthermore, an increase in sST2 expression was observed within 24 hours of admission after myocardial infarction[20] or acute coronary syndrome.[21] Here we

show that sST2 levels are not only increased after a cardiovascular event, but also after different types of cardiovascular surgical or catheter interventions. Soluble ST2 levels increased significantly after cardiac surgery and resulted in peak levels 24 hours after CABG surgery and several peripheral interventions. This confirms the observation that after heart or abdominal surgery, sST2 levels are elevated.[22-24] Furthermore, our data in the PCI cohort suggest that an increase might last till at least 2 months after intervention. Since we did not collect blood from these patients 24 hours after coronary intervention we can only speculate about the fluctuations of sST2 expression levels at earlier time points. These data suggest that the time of blood withdrawal, early or late after an event or intervention, will significantly influence sST2. This raises the question at which time point sST2 levels have the highest diagnostic or prognostic value for the patient. For example, most published reports show that only baseline sST2 levels, at the moment of admission, were predictive for clinical outcome. However, in some of the studies, the change in sST2 levels over a few days period was independently predictive for mortality or transplantation.[2,25-27] Unfortunately the event rate in our cohorts was too small to elaborate on this, so further studies are needed to address this question.

We show that the sST2 levels 24 hours after CABG surgery are much higher compared to the levels in patients that underwent peripheral arterial interventions. Based on this and the observation that the baseline sST2 levels in both groups are similar, we suggest that the degree of injury due to the cardiovascular surgery and potential subsequent inflammatory responses is a determinant of sST2 levels after intervention. In our cohorts, we found that age was the only common risk factor associated with sST2 levels. This positive association with age has also been observed in patients with heart failure[14,17,28,29] and acute dyspnoea.[16] However, in other studies, gender and hypertension were also associated with sST2 levels.[21,29] Remarkably, overweight patients (BMI>25) have lower sST2 levels after 24 hours of both CABG and peripheral surgery, compared to patients with a normal body weight. This suggests that sST2 expression levels following arterial injury in overweight patients might be suppressed. Still many discrepancies exist in literature between the risk factors that can influence sST2 levels.

Furthermore, it is important to note that sST2 is not cardio specific. It has been observed that sST2 levels are also systemically increased in several other inflammatory diseases as, amongst others, asthma,[30] rheumatoid arthritis,[31] lupus[32] and sepsis.[22] In our study we show that none of the measured inflammatory

or cardiac specific markers is associated with sST2 levels before, directly after or 24 hours after operation or procedure. It is well established that other inflammatory markers like interleukins and cardiac specific markers increase after surgery, the so-called acute phase proteins. Interestingly, sST2 does follow the same pattern compared to the a-specific inflammatory cytokine IL-6 and the cardiac specific TnI, which is released after heart injury, although an association was not observed. Question remains, what could be the cellular source of sST2? Experimental studies have shown that sST2 levels were not increased after whole blood stimulation with a bacterial solution[33] or LPS,[34] suggesting that not the circulating cells, but probably the tissue resident cells are the main sST2 secreting cells. This hypothesis is strengthened by previous data where an increase in sST2 was observed after stimulation of resting fibroblasts.[35] In addition, also the cardiomyocytes have been considered as a source for sST2 in response to myocardial infarction.[20]

In vitro and in vivo mice studies have shown that sST2 has an immunosuppressive role by inhibiting Toll-like receptor (TLR) responses.[36-38] This immunosuppressive role of sST2 might also apply after a cardiovascular event or intervention. It is well established that a cardiovascular event or intervention induces the secretion of several endogenous ligands that can activate TLR2 and TLR4 pathways (reviewed in[39]). TLR stimulation results in a hyporesponsive state of the white blood cells in order to protect the body from excessive inflammatory injury.[13] This down regulation of the TLR response after PCI takes only minutes and precedes the increase in sST2. It is therefore not likely that the increased sST2 is mechanistically responsible for the downtoning of the quickly induced hyporesponsive state after cardiovascular interventions, but reflects a more general, longer lasting suppressive status of the human body after challenge.

Study limitations

This is a cross-sectional study. Therefore we could not assess the effect of medication use on sST2 levels. In this observational study, however, we did not show any consistent effects of different medication types on sST2 levels in our cohorts. The retrospective and descriptive aspects of this study weaken the observations. Due to the low sample sizes of the cohorts and the incapability to perform a meta-analysis, differences between risk factors might be underpowered. In addition, it has already been shown extensively that sST2 has predictive value for future cardiovascular events, however, the event rates in our studies were

too low to determine whether sST2 levels after cardiovascular surgery were predictive for future events in our cohorts.

In conclusion, we show that sST2 levels are increased after cardiovascular interventions, and that the type of procedure may determine the intensity of this up regulation. In addition, sST2 may act as an acute phase protein but is not associated with any of the other inflammatory markers measured in this study. The aspecific increase in sST2 levels following (cardiovascular) interventions merits careful consideration in future prognostic studies.

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CHAPTER
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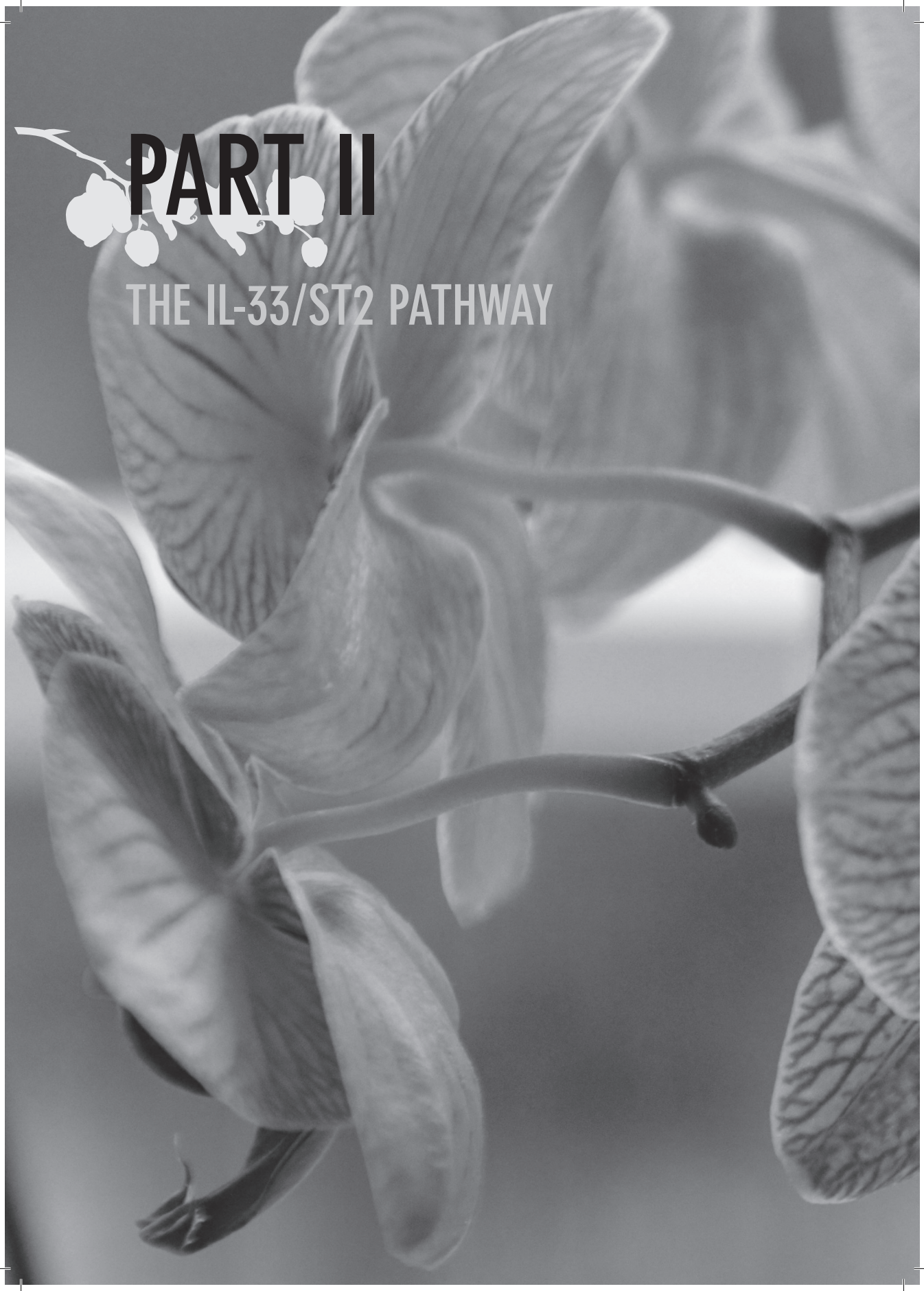

PART II

THE IL-33/ST2 PATHWAY

CHAPTER

4

Soluble ST2 levels are associated with long-term immunoparalysis in patients after CABG procedure

Submitted

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ABSTRACT

Background

Soluble ST2 (sST2), a member of the IL-1 receptor family, has been shown a negative regulator of Toll-like receptor (TLR) responses in vitro. It is unknown whether sST2 is negatively associated with TLR responsiveness in patients.

Objective

This study was designed to assess sST2 expression levels in the plasma of patients during and following heart surgery and relate this score with TLR responsiveness of circulating monocytes.

Methods and results

In the current study we determined soluble ST2 levels in the plasma of patients scheduled for CABG (n=76). Patients were categorized into 2 groups: high (n=40) and low sST2 levels (n=31) at 24 hours after CABG procedure. High sST2 levels were associated with low TLR2 and TLR4 membrane expression levels on monocytes 24 hours after surgery (3.4 [2.9-4.2] vs. 2.7 [2.5-3.5], p=0.009 and 1.6 [1.3-2.4] vs. 1.4 [1.1-1.6], p=0.02, respectively). In addition, we observed a similar association with IL-6 and TNF cytokine secretion by monocytes (141 [71-216] vs. 97 [53-141] pg/mL, p=0.059 and 388 [233-581] vs. 243 [101-418] pg/mL, p=0.009).

Conclusions

Twenty-four hours after heart surgery, sST2 expression levels are negatively associated with monocyte responsiveness.

Soluble ST2, interleukin 1 receptor-like 1, is a member of the TIR domain-containing superfamily of Toll-like receptors (TLRs) and is currently under investigation as a biomarker for cardiovascular disease. Associations have been reported between increased sST2 levels and adverse cardiovascular events following myocardial infarction and heart failure.[1,2] IL-33 intracellular signalling through its membrane receptor ST2L has been shown cardioprotective,[3–5] while sST2 was shown to exacerbate disease.[5] Experimental studies revealed that sST2 can act as a decoy receptor for IL-33,[6] which might explain its association with secondary events. Besides mediating the biological role of IL-33, sST2 has been implicated in the down regulation of TLR mediated immune responses. Soluble ST2 was shown to counteract TLR2 and 4 induced cytokine production in different cell types.[7–10] Furthermore, TLR2 and 4 expression levels were decreased upon sST2 administration.[7,10] It is known that endogenous ligands are released upon cell damage during a cardiovascular event or an intervention, which can initiate a TLR mediated inflammatory reaction. During a persistent inflammatory reaction, a negative feedback mechanism, resulting in a hyporesponsive state of the white blood cells, is induced to protect the body from excessive damage (immunoparalysis).

The primary objective of this study was to examine whether sST2 levels are associated with white blood cell responsiveness in patients undergoing coronary artery bypass grafting (CABG). We compared the responsiveness of blood monocytes between patients who showed high sST2 or low sST2 plasma levels during and after the CABG procedure. Responsiveness was determined by the secretion of IL-6, IL-8, IL-10 and TNF and the expression of TLR2 and 4 on peripheral blood monocytes.

Seventy-six subsequent patients scheduled for on-pump CABG were included.[11] The local medical ethical board of the UMC Utrecht approved the study. All patients signed a written informed consent prior to inclusion. TLR responsiveness was measured as previously reported.[11] In short, blood samples were collected before surgery (n=76), within one hour after chest closure (n=76) and 24 hours after surgery (n=73). Lithium-heparin blood samples were stimulated with 10 ng/mL lipopolysaccharide (LPS) overnight at 37°C and 5% CO^2. After stimulation TLR2 and 4 expression levels were measured by flow cytometry and cytokine levels were measured in supernatant with a multiplex fluorescent bead immunoassay (FlowCytomix human Th1/Th2 11plex, Bender MedSystems, Mercure group, Vienna,

Austria). Soluble ST2 levels were measured with an IL-1R4/ST2 enzyme-linked immunosorbent assay (ELISA, RayBiotech, Norcross, Georgia, USA) according to the manufacturer's instructions.

We have previously shown that sST2 levels were significantly elevated 1 hour after CABG, but raised to exceptionally high levels at 24 hours after surgery.[12] The cohort was divided into two groups: patient with plasma values above the upper detection limit (n=40, high sST2) and those with sST2 levels below the upper limit (n=31, low sST2) at 24 hours after CABG procedure. Twenty-four hours after CABG procedure we observed that high sST2 levels associated with low TLR2 and 4 membrane expression levels on monocytes (3.4 [2.9-4.2] vs. 2.7 [2.5-3.5], p=0.009 and 1.6 [1.3-2.4] vs. 1.4 [1.1-1.6], p=0.02, respectively; Figure 1A, B). In addition, we observed a similar association for IL-6 and TNF cytokine secretion by monocytes (141 [71-216] vs. 97 [53-141] pg/mL, p=0.059 and 388 [233-581] vs. 243 [101-418] pg/mL, p=0.009, respectively; Figure 1C, D). IL-8 and IL-10 secretion was not significantly different between the two groups. The association between sST2 and monocyte TLR surface receptors and cytokine secretion was not observed in blood drawn at baseline or one hour after CABG procedure.

In this study, we observed that cytokine secretion from monocytes after ex vivo LPS stimulation was significantly lower in patients with high sST2 plasma levels compared to patients with low sST2 plasma levels. We have previously shown that sST2 levels do not correlate with cytokine expression levels in patient plasma.[12] In addition, down regulation of the TLR response after intervention takes only minutes[13] and precedes the increase of sST2 plasma levels, which takes hours.[12] This suggests that the immunoparalytic effect of high sST2 levels after 24 hours is not yet reflected in blood cytokine levels, but is only reflected in the responsiveness of the circulating inflammatory cells. Therefore, it would be of interest to see whether this negative association is reflected in blood cytokine levels after a longer period of time. Furthermore, it would be interesting to examine whether sST2 might suppress cytokine levels after a second challenge, known as tolerance, as was observed in mice.[14] In that study, ST2-deficient mice did not develop endotoxin tolerance and died from a second challenge while wild type mice survived the lethal dose of LPS after being primed. However, in another study where mice were subjected to bacterial infection, bacterial lipoprotein induced tolerance was still observed in ST2 -/- mice.[15]

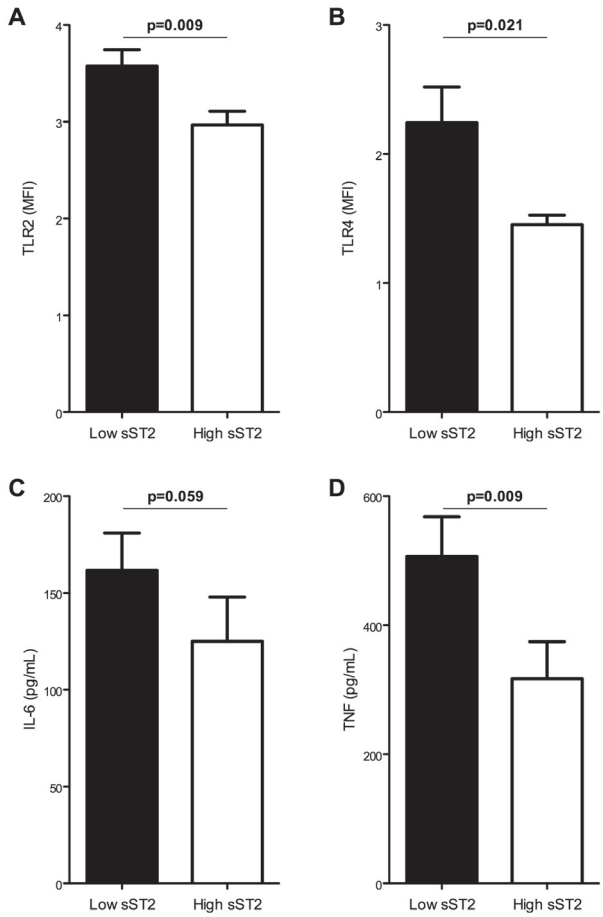

Figure 1 | Soluble ST2 plasma levels in plasma obtained 24 hours after CABG procedure in relation to monocyte responsiveness. Association between sST2 plasma levels and monocyte TLR2 (A) and 4 (B) surface expression levels. Association between sST2 plasma levels and monocyte cytokine secretion of IL-6 (C) and TNF (D).

As our observation is only associative, in vitro cell experiments with human plasma containing different physiological sST2 levels might give additional information whether such mechanism might actually occur in human disease.
It has been shown that very high concentrations of sST2 levels are necessary to counteract the intercellular signalling of IL-33.[4] This might also explain why we did not observe a relation with sST2 levels and the TLR pathway at baseline or 1 hour after CABG procedure but only after 24 hours when patient sST2 plasma levels were extremely high. Nevertheless, our observations can still not explain

why sST2 levels in cardiovascular disease patients, where high levels of sST2 are uncommon, are predictive for future events.[16] This illustrates the need to further investigate the biological role of sST2.

In conclusion, our data show a clear association between sST2 plasma levels and ex vivo cell responsiveness, which might reflect a more general, longer lasting immunoparalytic status of the human body after challenge.

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PART II

THE IL-33/ST2 PATHWAY

CHAPTER

5

Soluble ST2 levels are not associated with secondary cardiovascular events and vulnerable plaque phenotype in patients with carotid artery stenosis

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ABSTRACT

Objective

Soluble ST2 (sST2), a novel biomarker predictive for heart disease, has recently been shown associated with the progression of atherosclerotic disease in a mouse model. The present study was designed to assess sST2 plasma levels in patients scheduled for carotid endarterectomy and relate it with the occurrence of adverse cardiovascular events during follow-up. In addition, sST2 levels were associated to patient clinical data and atherosclerotic plaque characteristics.

Methods and results

Plasma sST2 levels were measured in 391 patients who underwent carotid endarterectomy and were subsequently followed for 3 years. Primary composite endpoint was the occurrence of an adverse cardiovascular event.

At baseline, no differences were observed in sST2 levels between asymptomatic (n=75) and symptomatic (n=316) patients (85 [49-122] vs. 90 [58-137] pg/mL, p=0.263). Soluble ST2 plasma levels did not differ between patients who experienced a secondary manifestation of cardiovascular disease and patients who remained free of symptoms (90 [60-129] vs. 88 [46-140] pg/mL, p=0.519). There was no association between sST2 levels and any of the following plaque characteristics: size of a lipid core, degree of calcification, number of macrophages or smooth muscle cells, amount of collagen and number of microvessels.

Conclusions

Soluble ST2 plasma levels have no predictive value for future cardiovascular events in patients with significant carotid artery stenosis. In addition, we did not observe an association between plasma sST2 levels and the histopathological features of a rupture-prone plaque. This study does not provide supportive evidence that sST2 reflects a progressive state of advanced atherosclerotic disease.

INTRODUCTION

The aging population leads to an increase of cardiovascular morbidity and mortality. Biomarkers may facilitate the identification of patients at high risk who subsequently can undergo preventive treatment. ST2, the receptor for the Th2 associated cytokine interleukin (IL)-33, is a member of the IL-1 receptor family that has recently gained significant interest in the biomarker research field. As a result of alternative splicing, ST2 is expressed in a transmembrane form (ST2L) and a soluble form (sST2).[1] Soluble ST2 has been studied extensively as it was shown to be elevated in patients with cardiovascular disease.[2,3] Moreover, sST2 levels were demonstrated to have predictive value for the occurrence of future cardiovascular events in patients suffering from ischemic heart disease.[4]

ST2L, expressed on the surface of many inflammatory cells,[5] is the receptor through which signalling of IL-33 has been found to exert a cardioprotective role. Administration of IL-33 in animal models of cardiovascular disease resulted in functional improvements and increased survival rates.[6-8] This might explain an unfavourable role of sST2 in cardiovascular disease[6,8,9] since capturing of IL-33 by the soluble form of ST2 from the circulation hampers the beneficial effect of IL-33.[10]

Previous human studies on the predictive value of sST2 for heart failure and mortality mainly focussed on patients with ischemic heart disease.[11] In the present study, we investigated the predictive value of sST2 on future cardiovascular atherosclerotic events in a patient group with significant carotid artery disease: in this patient domain the predictive value of sST2 is still unknown. Soluble ST2 protein levels were assessed in the plasma of patients that underwent carotid endarterectomy and related to the occurrence of adverse cardiovascular events during follow-up.

In patients with coronary and carotid artery disease, acute cardiovascular manifestations are often the result of plaque rupture followed by thrombus formation. Therefore, a particularly interesting observation was that ApoE-/- mice on a high fat-diet receiving IL-33 treatment developed smaller lesions, while sST2 administration resulted in increased plaque size.[9] Experiments showing that administration of IL-33 lowered foam cell formation considerably in vitro and in vivo,[12] further support an important role for the IL-33/ST2 pathway in atherosclerotic plaque development. Therefore, in the current study, associations between sST2 plasma levels and the characteristics of a vulnerable plaque have been investigated.

MATERIALS AND METHODS

Study population and design

A total of 391 patients of the Athero-Express were included in this study. The Athero-express biobank involves patients that underwent carotid endarterectomy (CEA) in two Dutch teaching hospitals in Utrecht and Nieuwegein the Netherlands.[13] Indication for CEA was based on recommendations by the Asymptomatic Carotid Atherosclerosis Study, the North American Symptomatic Carotid Endarterectomy Trial and the European Carotid Surgery Trial.[14-16] Patients were operated between March 2002 and August 2008 and randomly selected among those of whom blood plasma samples were available. The local medical ethical boards of both participating hospitals approved this study. The participating patients signed a written informed consent prior to inclusion. The patient's baseline characteristics and medical history were obtained via questionnaires and the patient medical records.

Follow-up

After CEA, patients were followed up to 3 years by questionnaire. Primary outcome was defined as any cardiovascular event including; (non-) fatal stroke, (non-) fatal myocardial infarction, sudden death and other vascular death and any invasive arterial intervention that had not been planned at the time of inclusion (e.g. carotid surgery or angioplasty, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA), peripheral vascular surgery or angioplasty).

Materials

The carotid plaques used in this study were processed as described previously.[13] In short, after surgical dissection the plaque was cut into segments of 5 mm. The segment with the largest plaque area was fixed in formalin and embedded in paraffin for histology. The two adjacent sections were frozen in liquid nitrogen and used for protein isolation. In addition, blood was drawn prior to CEA procedure and plasma was stored at -80 °C.

Quantification of sST2 levels in patient plasma

Soluble ST2 levels were measured with an IL-1 R4/ST2 enzyme-linked immunosorbent assay (ELISA, RayBiotech, Norcross, Georgia, USA) according to the manufacturer's instructions. In brief, plasma samples 1:1 diluted with dilution

buffer and standards were incubated for 2.5 hours in a 96-well plate pre-coated with a capture antibody for human ST2. After the 96-well plate was washed using an automatic washer, a biotin labelled anti-human ST2 antibody was added to the wells. In between washes, the HRP antibody was added, followed by administration of the substrate. After 30 minutes the stop solution was added and the luminescence was measured at 450nm with an ELISA reader (Multiskan FC, Thermo Fisher Scientific, Vantaa, Finland). The upper detection limit of the RayBio® human IL-1 R4/ST2 ELISA kit was 1200 pg/mL and the lower limit of detection was 2 pg/mL, with an intra-assay CV<10 % and inter-assay CV<12%.

Plaque protein measurements

To investigate whether sST2 levels are associated with the plaque's inflammatory status, we used expression data of pro-and anti-inflammatory cytokines e.g. IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, INF-γ and TNF-α that were available from measurements of a previous study. These expression levels had been assessed in 211 of the carotid atherosclerotic plaques that were included for the current study by Fluorescent Bead Immunoassay 810FF (Bendermed Systems, Vienna, Austria).

Kidney function assessment

Kidney function was determined by calculating the glomerular filtration rate (eGFR) expressed in mL/min/1.73m^2 as previously described.[17] Serum creatinin was measured in blood plasma.

Immunohistochemistry

Consecutive sections were stained for CD68 (macrophages), smooth muscle cells (alpha actin) CD66 (neutrophils), mast cell tryptase (mast cells) and CD34 (endothelial cells). As previously described, image-analysing software (Soft Imaging Solutions GmbH, Münster, Germany) was used to determine positive macrophage and smooth muscle cell staining expressed as a percentage of covered plaque area. Microvessels were counted in three hot-spots and were expressed as average microvessel density per hotspot.[18] The presence of intraplaque haemorrhage was assessed with Haematoxylin and Eosin (H&E) staining and fibrin (Mallory's phosphotungstic acid-haematoxylin).[19] Calcification (H&E) and collagen content (picrosirius red) were scored semi-quantitatively. The size of the extracellular lipid core (atheroma) was assessed by the H&E and picrosirius red stain.[13] Overall plaque phenotype was based on the percentage of confluent lipid areas of the total plaque area that were visually estimated (fibrous: <10% fat; fibroatheromatous: 10-40%; atheromatous: >40% fat).

Statistics and data analysis

IBM SPSS statistics version 20 was used for all analyses (IBM corporation, Armonk, NY, USA). Soluble ST2 levels are not normally distributed; non-parametrical testing was used to determine differences. The Mann-Whitney U test was used to study sST2 levels as a continuous variable for all risk factors. To assess the independent association between sST2 plasma levels and history of cardiovascular events and interventions, a binary logistic regression model was used in which we corrected for the potential confounders age and diabetes. The relation between sST2 and the occurrence of future manifestations during follow-up was examined using the cox-regression survival analysis. Differences were considered significant with a p-value of below 0.05.

RESULTS

Baseline characteristics

Soluble ST2 plasma levels were measured in a total of 391 patients that underwent carotid endarterectomy. In Table 1 the baseline clinical characteristics of the patients are depicted. With a mean age of 67 years and 67% males, the study population reflects a relatively typical population of patients with cerebral vascular occlusive diseases. The majority of patients was symptomatic (81%), hypertensive (87%) and used statins (74%).

Soluble ST2 plasma levels and clinically relevant characteristics

Table 1 summarises the associations between sST2 expression levels and relevant clinical characteristics. Plasma sST2 levels correlated with age (r=0.240, p=0.037). Higher sST2 levels were observed in males compared to females (91.8 [62.3-145.0] vs. 76.7 [47.1-111.2] pg/mL, p=0.002) and in patients with diabetes mellitus (106.4 [62.8-181.6] vs. 88.0 [55.0-125.3] pg/mL, p=0.021). Furthermore, we observed that patients with a history of coronary artery disease that previously experienced a myocardial infarction (MI) or underwent PTCA/CABG had higher sST2 levels (100.8 [69.6-153.4] vs. 84.4 [52.6-126.4] pg/mL; p=0.007). However, in a logistic regression model controlling for age and diabetes, sST2 levels were no longer associated with previous MI or coronary interventions. Soluble ST2 levels were not significantly higher in patients that previously underwent a peripheral artery intervention (95.3 [64.9-160.4] vs. 88.7 [56.1-126.9] pg/mL; p=0.244).

Table 1 | Baseline characteristics of the patients in relation to sST2 plasma levels

		sST2 (pg/mL)	p-value
Age, mean years (sd)	67 (9)	r = -0.119	**0.018**
BMI, mean kg/m^2 (sd)	27 (4)	r = 0.053	0.318
Sex			
Male	263/391 (67%)	91.8 [62.3-145.0]	**0.002**
Female	128/391 (33%)	76.7 [47.1-111.2]	
Current smoker			
Yes	133/383 (35%)	90.5 [53.8-137.8]	0.926
No	250/383 (65%)	89.4 [60.4-128.2]	
Diabetes mellitus			
Yes	88/391 (23%)	106.4 [62.8-181.6]	**0.021**
No	303/391 (77%)	88.0 [55.0-125.3]	
Statin use			
Yes	288/389 (74%)	89.5 [55.6-141.8]	0.591
No	101/389 (26%)	88.3 [58.0-124.4]	
Hypertension			
Yes	340/391 (87%)	88.5 [57.5-130.5]	0.588
No	51/391 (13%)	100.9 [57.2-127.4]	
History peripheral intervention			
Yes	71/391 (18%)	95.3 [64.9-160.4]	0.244
No	320/391 (82%)	88.7 [56.1-126.9]	
History coronary artery disease			
Yes	104/391 (27%)	100.8 [69.6-153.4]	**0.007**
No	285/391 (73%)	84.4 [52.6-126.4]	
Clinical Presentation			
Asymptomatic	75/391 (19%)	85.1 [48.6-121.5]	0.263*
Symptomatic	316/391 (81%)	90.0 [58.2-136.8]	
Amaurosis fugax	56/316 (18%)	89.0 [59.4-164.1]	
TIA	180/316 (57%)	91.8 [55.3-127.1]	
Stroke	80/316 (25%)	87.7 [63.4-155.4]	

Data are presented as No. (%) and median [IQR] unless otherwise indicated; r = Spearman's rank correlation coefficient; sd = standard deviation; IQR = interquartile range; BMI = body mass index; TIA = transient ischemic attack; * p-value represents statistical analysis for asymptomatic patients versus symptomatic patients (composed of amaurosis fugax, TIA and stroke)

Clinical presentation was not associated with sST2 levels: no differences were observed in sST2 levels between asymptomatic patients (n=75) and symptomatic (n=316) patients (85 [49-122] vs. 90 [58-137] pg/mL, p=0.263). Additionally, no association was found between sST2 levels and the delay between surgery and presentation of symptoms.

Relation of sST2 and other biomarkers

A correlation was found between sST2 and the acute phase protein CRP mg/l (r=0.107, p=0.035). Kidney function expressed as eGFR (mL/min/1.73m^2) correlated negatively with sST2 (r=-0.165, p=0.008).

Outcome

A total of 75 out of 391 patients suffered from secondary cardiovascular manifestations during a 3-year follow-up. There was no significant difference in baseline sST2 plasma levels between patients that did and those that did not experience a cardiovascular event (90 [60-129] vs. 88 [46-140] pg/mL, p=0.519). Likewise, when divided into two groups by the median, high sST2 levels did not associate with secondary manifestations of cardiovascular disease (39 [20%] in the low level sST2 group vs. 36 [19%] events in the high sST2 group; Figure 1). Median sST2 levels were higher in patients that experienced a cardiac event (n=10), but this did not reach significance (p=0.324). Soluble ST2 plasma levels were significantly higher in patients with all-cause mortality (88.2 vs. 111.8 pg/mL; n=24, p=0.031). However in a cox regression analysis correcting for age and gender, sST2 was no longer predictive (p=0.134)

Plasma sST2 levels and plaque characteristics

As depicted in Table 2, sST2 levels were not associated with plaque phenotype categorized in three groups: fibrous, fibroatheromatous, atheromatous (88.2 [48.9-126], 85.5 [53.3-130.9], 92 [67.1-139.8] pg/mL respectively; p=0.242). There was no association between sST2 levels and any of the following plaque characteristics: calcification, collagen, smooth muscle cells, macrophages, neutrophils or mast cells (Tables 2 and 3). In addition, sST2 levels were not related to the plaque protein levels of the anti- or pro- inflammatory cytokines (Table 3). Although no association was found between sST2 levels and microvessel density, an increase of sST2 level was observed in patients with plaques that scored positive for intraplaque haemorrhage (92.6 [61.9-144.9] vs. 80.3 [49.4-107.5] pg/mL, p=0.004).

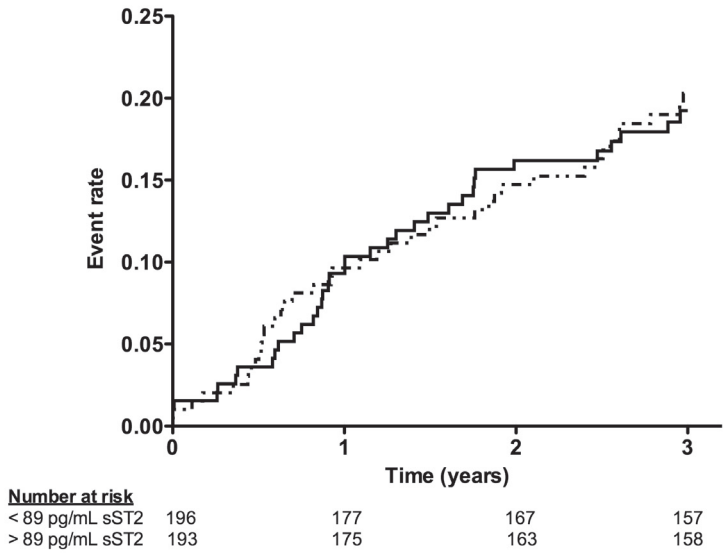

Figure 1 | Kaplan-Meier survival curves for sST2 plasma levels vs. combined cardiovascular outcome after carotid endarterectomy. High sST2 levels (continuous line) vs. low sST2 levels (dashed line) based on the median (89 pg/mL) as a cut-off value (p=0.861). The numbers of patients at risk for cardiovascular events are shown at 0, 1, 2 and 3 years after endarterectomy.

Table 2 | Soluble ST2 plasma levels with respect to the histological parameters of the plaque

		sST2 (pg/mL)	p-value
Plaque phenotype			
Fibrous	125/391 (32%)	88.2 [48.9-126.0]	0.242
Fibroatheromatous	143/391 (37%)	85.5 [53.3-130.9]	
Atheromatous	123/391 (31%)	92.2 [67.1-139.8]	
Intraplaque haemorrhage			
Yes	272/391 (70%)	92.6 [61.9-144.9]	0.004
No	119/391 (30%)	80.3 [49.4-107.5]	
Collagen			
Minor	76/391 (19%)	106.0 [60.4-142.7]	0.402
Moderate	219/391 (56%)	86.5 [56.2-127.3]	
Heavy	96/391 (25%)	88.1 [57.4-132.4]	
Calcification			
No/minor	168/391 (43%)	86.9 [56.6-143.0]	0.619
Moderate/heavy	223/391 (57%)	89.8 [57.4-127.1]	

Data are presented as Spearman's rank correlation coefficient (r) or median [interquartile ranges]

DISCUSSION

Soluble ST2 is emerging as a novel biomarker for prediction of mortality and heart failure in patients with established cardiac disease. Multiple studies showed that sST2 is a biomarker that adds value in risk prediction for development of cardiac disease on top of traditional risk factors and other biomarkers in patients with ischemic heart disease.[11] Stenosis or rupture of the atherosclerotic plaque is the underlying cause for myocardial infarction or heart failure of ischemic aetiology. Experimental studies have shown a possible role for the IL-33/ST2 pathway in progression of the atherosclerotic plaque,[9,12] but studies revealing an association between sST2 and atherosclerotic lesion phenotype in humans is lacking. Therefore, we aimed to investigate whether sST2 levels would have predictive value for adverse cardiovascular events in a patient group with clinically manifest cerebral artery disease. In addition, the dissection of atherosclerotic plaques in this patient group allowed an association study between sST2 levels and atherosclerotic plaque phenotype.

Here we show that baseline sST2 levels have no predictive value for future combined cardiovascular events in a patient group with severe carotid stenosis that underwent an endarterectomy. Our results suggest that sST2 is a less potent biomarker for adverse events in the presence of cerebral ischemic disease compared to patients suffering from cardiac ischemic disease. There are several potential explanations for this observation. In the presence of myocardial infarction and subsequent heart failure, severe tissue damage and remodelling takes place in a relatively short time span whereas in the presence of carotid stenosis tissue damage may be minimal or temporary. Furthermore, a significant number of patients suffered from relative minor complications as transient ischemic attack or amaurosis fugax. Another explanation is that blood was drawn prior to surgery which is executed weeks after the index event whereas in cardiac ischemia the intervention is executed mostly in the acute phase.

No difference in sST2 levels was observed between asymptomatic and symptomatic patients at the moment of admission for surgery. Previous studies have shown that sST2 rises to extreme levels within 24 hours after myocardial infarction or cardiovascular interventions after which they return to baseline levels within a few days.[20-22] This indicates that the time of blood withdrawal is of great importance since the delay following a cardiovascular event strongly influences sST2 levels. Therefore, we examined whether the delay between

Table 3 | Soluble ST2 plasma levels with respect to inflammatory markers in the plaque

	Q1	Q2	Q3	Q4	p-value	Spearman (r)	p-value
sST2 pg/mL	[<57.4]	[57.4-89.4]	[98.5-130.3]	[>130.3]			
Plaque histology							
Patient numbers (n)	98	99	97	97			
Microvessel density	8.3 [5.7-11.3]	8.3 [5.2-11.7]	7.7 [5.0-11.1]	7.7 [4.8-11.9]	0.489	-0.028	0.624
Smooth muscle cells	2.01 [0.74-3.83]	2.65 [0.96-4.12]	1.55 [0.52-3.14]	1.69 [0.55-4.02]	0.088	-0.085	0.094
Macrophages	0.72 [0.18-1.50]	0.75 [0.22-2.08]	0.68 [0.17-1.35]	0.65 [0.18-1.54]	0.376	-0.035	0.491
Plaque protein							
Patient numbers (n)	49	62	51	49			
IL-2 pg/mL	184 [0-357]	229 [104-533]	153 [4-335]	190 [0-364]	0.104	0.004	0.950
IL-4 pg/mL	144 [0-351]	180 [70-372]	126 [0-283]	168 [0-316]	0.329	0.016	0.819
IL-5 pg/mL	126 [22-324]	153 [65-358]	116 [56-231]	123 [0-304]	0.609	-0.037	0.594
IL-6 pg/mL	53 [16-125]	50 [12-139]	38 [18-81]	31 [14-75]	0.667	-0.098	0.158
IL-8 pg/mL	41 [8-164]	48 [7-230]	60 [6-153]	43 [1-151]	0.888	-0.047	0.497
IL-10 pg/mL	18 [0-64]	22 [8-78]	14 [0-40]	20 [0-51]	0.280	-0.011	0.868
TNF-a pg/mL	12 [0-28]	12 [4-39]	8 [0-22]	15 [0-33]	0.621	-0.030	0.662
IFN-y pg/mL	53 [3-248]	90 [29-318]	78 [14-177]	91 [8-235]	0.473	0.023	0.740

Data are presented as Spearman's rank correlation coefficient (r) or median [interquartile ranges]

operation and the presentation of symptoms was associated with sST2 levels. We showed that the delay did not alter the sST2 levels. However, it merits careful consideration that patients were often scheduled about 10 weeks after presenting clinical symptoms, which might explain the normalisation of sST2 levels. Current guidelines have resulted in much shorter delays between the cerebrovascular event and surgery which is relevant before extrapolating results to current practice. In the current follow-up study we could not select sufficient numbers of patients presenting with stroke who were operated within 1-2 weeks to investigate whether sST2 levels were increased within this few day time frame. Interestingly, higher sST2 levels were observed in patients with previous cardiac events or coronary interventions, but not in patients with history of peripheral interventions. However, after adjusting for confounders in a logistic regression model, other risk factors were found to be responsible for this effect. Unfortunately, we were underpowered for analysing risk prediction in the subgroup of patients suffering from adverse cardiac events during follow-up. Nevertheless, in line with previous research, a positive correlation between all-cause mortality and sST2 levels was observed.[23-28] We confirmed the previously reported positive association between CRP and sST2 levels.[3,24,28-30] Furthermore, our data concord with previous studies associating sST2 levels with, age, gender, diabetes mellitus.[23,26,30-33] In addition, in our cohort a positive correlation between sST2 and CKD was observed confirming previous observations.[34] This is of interest as CKD has previously been shown one of the most important determinants for future cardiovascular events.[17]

Another research question we addressed was whether sST2 levels associated with plaque phenotype. Previously, sST2 administration in ApoE-/- mice resulted in the development of larger atherosclerotic plaques, although collagen content and inflammatory and smooth muscle cell numbers were not altered.[9] We studied advanced atherosclerotic disease and therefore our study cannot address the question if sST2 plasma levels are associated with plaque size in humans. However, we could investigate whether sST2 levels were associated with histological markers of plaque vulnerability. In line with the previously reported mouse data, we showed that sST2 plasma levels in patients with carotid artery disease are not related to most of the established characteristics of the rupture-prone atherosclerotic plaques. Nevertheless, we observed an association with intraplaque bleeding which warrants further research since plaque haemorrhage is considered an emerging determinant of plaque destabilisation.

Signalling of IL-33 through ST2L has been found to stimulate Th2 associated cytokine production,[35] which is abolished by sST2 acting as a decoy receptor for IL-33.[10] We therefore investigated whether high levels of sST2 would prevent Th2 associated cytokine production and redirected the balance to the Th1 associated cytokines. In this study we did not find an association between sST2 levels and any of the cytokines related to a Th1 or Th2 inflammatory response in the plaque. Taken together, these data suggest that serum sST2 levels do not influence atherosclerotic plaque progression nor their inflammatory profile. Keeping in mind that sST2 levels increase fast and to extreme levels after trauma or surgical procedures, this might indicate that sST2 is just a plain marker for systemic inflammation.

Conclusions

The associations found between sST2 and traditional risk factors, other biomarkers and all-cause mortality are in line with previous studies. Soluble ST2 levels have not been found associated with any of the histopathological characteristics of a rupture-prone plaque. Together with the observation that sST2 levels are not elevated in patients that develop secondary cardiovascular events, this study suggests that sST2 levels are not related to progression of atherosclerotic disease following cerebrovascular ischemia. This implies that sST2 levels may have added value in risk prediction for cardiac disease in patients with acute manifestations of myocardial ischemia or severe chronic heart failure, but not in a subgroup of patients with cerebrovascular disease.

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PART II

THE IL-33/ST2 PATHWAY

CHAPTER

6

Interleukin-33 inhibits perfusion recovery in the mouse hind limb model

Submitted

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ABSTRACT

Background

Stimulating the growth of collateral arteries (arteriogenesis) in patients with vascular occlusive diseases is a therapeutic option to restore blood flow to the ischemic tissue. A local inflammatory response precedes the process of arteriogenesis. IL-33, a Th2 associated cytokine that signals through the ST2 receptor has been implicated in inflammatory processes including angiogenesis. We studied the effects of IL-33 treatment on perfusion restoration in the mouse hind limb ischemia model.

Methods and results

Perfusion restoration was significantly decreased in animals treated with recombinant IL-33 (n=10) 4 and 7 days after femoral artery ligation compared to NaCl treated control animals (day 4: 23% ± 5 vs. 50% ± 7 perfusion recovery, $p<0.01$, day 7: 52% ± 10 vs. 86% ± 4, $p<0.05$). Histological analysis revealed no difference in T-cell numbers in the perivascular space surrounding the developing collaterals between IL-33 treated and control mice at 4 (2.57 ± 1.00 vs. 2.39 ± 0.65 T-cells/artery, $p=0.773$) or 7 days (2.92 ± 0.59 vs. 1.63 ± 0.38 T-cells/artery, $p=0.117$). Soluble IL-33 receptor (soluble ST2) levels were significantly increased in the first five days after arterial ligation.

Conclusions

The pro-inflammatory cytokine IL-33 inhibits collateral formation resulting in a lower perfusion restoration after femoral artery ligation. Further research is needed to elucidate the underlying mechanism of these findings.

INTRODUCTION

Tissue oxygenation and nutrient delivery via the blood vessels is essential to maintain normal organ function. If oxygen is deprived, tissues become ischemic and severe organ damage can occur. Despite therapeutic interventions to restore tissue oxygenation, morbidity and mortality in this patient population remains increasing. Hence, there is an urgent need to identify new therapies to restore blood flow.

Collateral growth can naturally occur in patients with significant vessel stenosis, thereby protecting the organ against ischemic injury.[1] Pre-existing anastomoses remodel into large conducting vessels that can compensate the functional defect of the occluded artery.[2] Acceleration of collateral development might therefore be a therapeutic option in patients with severe arterial stenosis.

An inflammatory environment has been shown an essential determinant for collateral growth.[3] IL-33 is a Th2 associated cytokine that signals through the transmembrane receptor ST2 (ST2L) and has been implicated to play a role in many inflammatory processes.[4] Previous studies reported an involvement of IL-33 in angiogenesis, a process that shares many characteristics with arteriogenesis.[5] IL-33 was shown abundantly present in the nucleus of endothelial cells in healthy and inflamed tissues as well as in human tumours.[6] In cell-based assays, IL-33 induces migration, proliferation, hyperpermeability and capillary-like network formations of endothelial cells.[7] Furthermore, in vivo matrigel plugs impregnated with IL-33 contained more functional vessels compared to control plugs.[7] On the other hand, another study demonstrated IL-33 expression predominantly in resting endothelial cells in healthy tissue and only limited staining in vessels within tumour tissue.[8] Moreover, this study showed that IL-33 expression was rapidly lost upon angiogenic or pro-inflammatory stimuli.

Previous pro-arteriogenic compounds that were successfully tested in animal models, failed in clinical trials often due to severe side effects. These side effects were frequently related to an accelerated progression of atherosclerotic disease. Interestingly, IL-33 intracellular signalling has been shown cardioprotective in several in vivo models.[9–11] Especially of interest is the finding that IL-33 treatment in atherosclerotic mice actually decreased plaque size.[12] Based on the beneficial effects on atherosclerosis and the pro-angiogenic properties, we hypothesized that IL-33 could be a suitable candidate for therapeutic enhancement of collateral formation if it showed pro-arteriogenic properties. In this study, the effect of systemic IL-33 administration was investigated on collateral growth in the

presence of arterial occlusion. As IL-33 is known to exert its function especially on T-cells (Th2 cells specifically[13,14]) we examined T-cell accumulation near the collaterals. In addition, perfusion recovery was compared between ST2 deficient and WT mice.

MATERIALS AND METHODS

Animal procedures

Age and sex-matched 10-12 weeks old male mice underwent femoral artery ligation to induce hind limb ischemia as described previously.[15] In brief, mice were anesthetized with an intraperitoneal injection containing 5 mg/kg midazolam (Actavis, München, Germany), 0.15 mg/kg medetomidine (Orion Pharma, Espoo, Finland) and 0.02 mg/kg fentanyl (Hameln Pharmaceuticals GmbH, Hameln, Germany). The right femoral artery was exposed and ligated immediately proximal and distal to the inguinal ligament. After wound closure the antagonist containing 1 mg/kg flumazenil (Roche, Basel, Switzerland) and 5 mg/kg atipamizole (Dechra, Bladel, Netherlands) was injected subcutaneously. Blood flow in the paws was measured by laser Doppler (Moor Instruments, Devon, UK) at indicated times. Perfusion recovery is expressed as percentage perfusion of the ischemic vs. the non-ischemic paw. Mice were terminated at designated time points using an intraperitoneal injection with 75 mg/kg ketamine (Vetoquinol, Lure-Cedex, France) and 1 mg/kg medetomide (Orion Pharma, Espoo, Finland) followed by heart puncture. All experiments were approved by the university animal experimental committee following the guide for the care and use of Laboratory Animals published by the US National Institute of Health (NIH Publication No.85-23, revised 1996).

Twenty male C57Bl6/J mice (Harlan, the Netherlands) were divided in two groups (n=10). Mice were injected with 100 µl IL-33 (1 µg; PeproTech, Rocky Hill, NJ, USA) or NaCl as control intraperitoneal at day 0, 2 and 4. Blood flow in the paws was measured before, directly after and subsequently 4 and 7 days after operation. Two mice were excluded from the control group due to technical failure as perfusion increased 15% directly after surgery, resulting in 8 control mice and 10 IL-33 treated mice. Mice were sacrificed 7 days after surgery. Blood was collected by heart puncture and adductor muscles were excised from both hind limbs. Muscle tissues were either fixed in formalin and embedded in paraffin for

histological analysis (n=5) or stored at -80°C for protein and RNA isolation (n=5). An additional 10 mice for both groups received the same procedure but were terminated 4 days after unilateral femoral ligation to obtain additional adductor muscle tissue for histology purposes.

To measure circulating sST2 and IL-33 levels at several time points after hind limb ischemia, 70 male C57Bl6/J mice (Harlan, the Netherlands) were divided into 8 groups. All mice underwent permanent unilateral femoral artery ligation and were terminated 1, 2, 3, 4, 5, 6 and 7 days after operation. Blood was collected by heart puncture with a heparin coated anti-coagulation syringe and centrifuged. The plasma obtained was stored at -80°C. In an additional 14 mice we tested whether the obtained differences were specific for the ligation procedure or just an overall inflammatory reaction due to the surgery. Ten mice underwent unilateral femoral artery ligation and ten mice were sham operated. The surgical procedure was identical, however the femoral artery was not ligated in the sham treated group.

For the knockout experiments, 30 WT Balb/c mice and 30 ST2-/- mice on a Balb/c background (Amsterdam Medical Centre-Aria, Amsterdam, The Netherlands) were kindly provided to us by Andrew NJ McKenzie.[16] Perfusion measurements in the paws were conducted before, directly after and subsequently 4, 7, 14, 21 and 28 days after operation. A timeframe of 28 days was chosen as Balb/c mice have been reported to display a delayed perfusion recovery compared to Bl6 mice.[17]

Quantification of plasma soluble ST2 and IL-33 levels

Soluble ST2 and IL-33 levels were measured in plasma collected from 70 mice terminated at different time points after femoral artery ligation. Soluble ST2 levels were measured with a T1/ST2 enzyme-linked immunosorbent assay (ELISA, MDBioproducts, St. Paul, MN, USA) according to the manufacturer's instructions. In brief, plasma samples and standards were incubated for 1 hour in a 96-well plate pre-coated with a capture antibody for mouse ST2. After the 96-well plate was washed using an automatic washer, conjugate was added to the wells. Between washes, the streptavidin-HRP antibody was added, followed by administration of the substrate. After 15 minutes the stop solution was added and the luminescence was measured at 450nm with an ELISA reader (Multiskan FC, Thermo Fisher Scientific, Vantaa, Finland).

IL-33 levels were measured with a pre-coated mouse IL-33 Quantikine ELISA kit (R&D systems, Wiesbaden, Germany) according to the manufacturer's instructions. In short, plates were washed using an automatic washer prior to a 4°C overnight incubation of plasma and standard samples. Between washes, the IL-33 detection antibody was added and incubated for 1 hour, while shaking. The HRP solution incubated for 30 minutes, followed by substrate administration. After 10 minutes incubation in the dark, the stop solution was added to the wells and absorbance was measured at 450nm with an ELISA reader.

Immunohistochemistry

Presence of T-cells (CD3, 1:100, Dako, Glostrup, Denmark) was assessed in paraffin embedded adductor tissue of the IL-33 and NaCl treated mice, 4 and 7 days after ligation. Sections were counter stained with Haematoxilyn. Sections were analysed using Analysis 2.8 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). All T-cells accumulating in the perivascular space were counted. About 5-6 vessels were photographed per animal in the adductor tissues of both hind limbs. Numbers are expressed as cells/vessel.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Differences in perfusion restoration and T-cell presence were assessed using a Mann Whitney U tests. Soluble ST2 levels were log transformed to obtain a normal distribution. To establish significant differences in sST2 levels between time-points, a one-way ANOVA analysis of variance followed by Bonferoni post-hoc test was performed. Difference in sST2 levels between the sham operated and ligated group was assessed using an unpaired Student's t-tests. P-values below 0.05 were considered significant.

RESULTS

The effect of IL-33 treatment on perfusion recovery

Perfusion recovery was measured to assess collaterals formation in the occluded hind limbs of IL-33 treated (n=10) and control mice (n=8). Perfusion recovery was significantly lower in IL-33 treated mice compared to control mice 4 days (50% ± 7 vs. 23% ± 5, $p<0.01$) and 7 days after surgery (86% ± 4 vs. 52% ± 10, $p<0.05$; Figure 1).

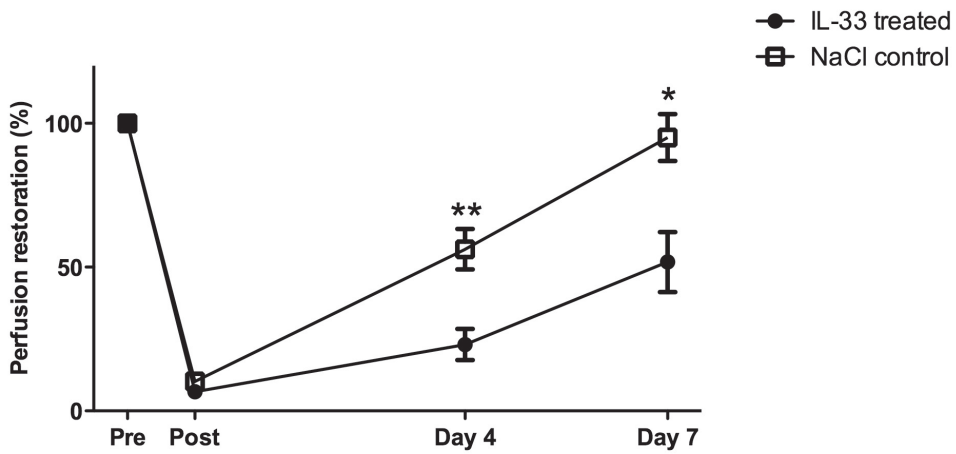

Figure 1 | The effect of IL-33 treatment on perfusion recovery. Percentage perfusion restoration in IL-33 treated and NaCl control mice at 4 and 7 days after ligation. (*p<0.05, **p<0.01)

T-cell extravasation into the perivascular space

T-cells have been shown to play an important role in arteriogenesis. As IL-33 exerts most of its actions via T-cells, we investigated whether the observed difference in perfusion restoration could be explained by a decreased T-cell extravasation into the perivascular space of collateral vessels in the adductor tissue of IL-33 treated mice. The number of T-cells per vessel did not differ between the IL-33 treated and control mice 4 and 7 days after ligation (day 4: 2.57 ± 1.00 vs. 2.39 ± 0.65, p=0.773; day 7: 2.92 ± 0.59 vs. 1.63 ± 0.38, p=0.117; Figure 2).

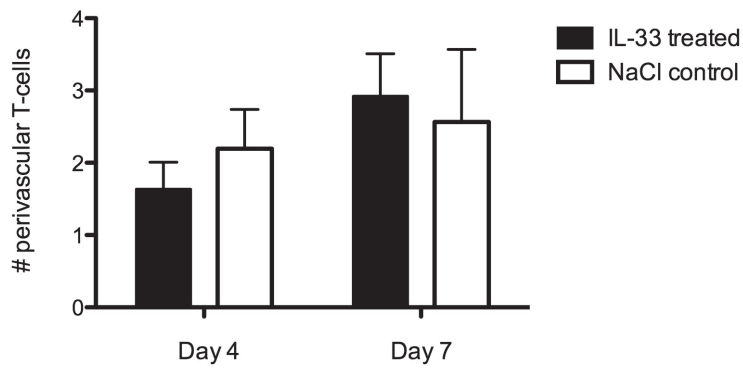

Figure 2 | T-cell numbers in perivascular space after IL-33 treatment at day 4 and day 7

Soluble ST2 plasma levels following hind limb ischemia

Soluble ST2 can inhibit IL-33 intracellular signalling by binding circulating IL-33. Therefore sST2 levels were determined in non-operated (day 0) and operated mice (day 1-7). Mice terminated 1 day after ligation had significantly higher sST2 plasma levels compared to the non operated animals (1163 ± 193 vs. 478 ± 265 pg/mL, $p<0.01$; Figure 3A). This increase lasted till 5 days after operation ($p<0.01$), after which levels returned to baseline. IL-33 cytokine plasma levels could not be detected (data not shown).

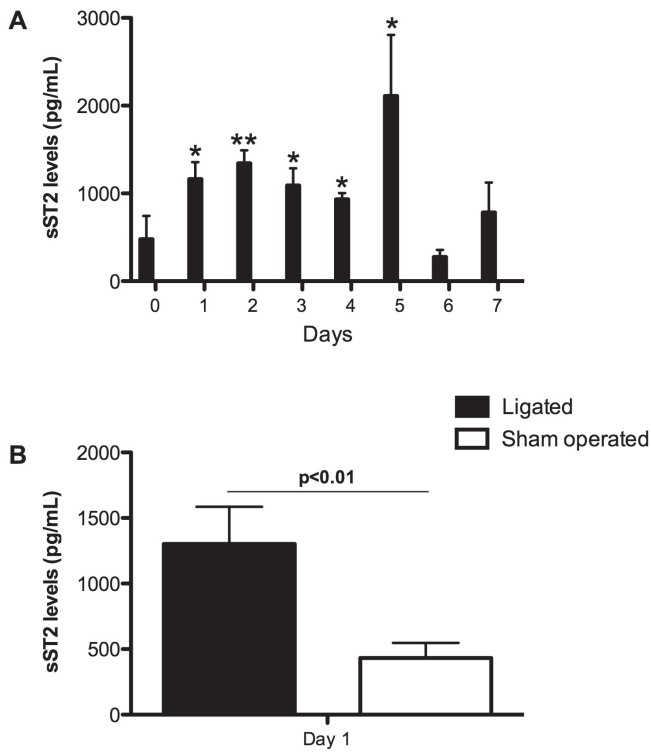

Figure 3 | Soluble ST2 plasma levels following femoral artery ligation. Temporal changes in sST2 plasma levels the first 7 days after ligation (A). Soluble ST2 plasma levels in ligated vs. sham operated mice at day 1 (B). (*$p<0.05$, **$p<0.01$)

Soluble ST2 levels were assessed in two additional groups (n=7), undergoing either normal ligation (ligated) or operation without ligation (sham-operated). Both groups were terminated one day after procedure. Soluble ST2 levels were significantly higher in the ligated mice compared to sham-operated mice (1303 ± 283 vs. 433 ± 115 pg/mL, $p<0.01$; Figure 3B).

Perfusion recovery in ST2 deficient mice

ST2 deficient mice on a Balb/c background and WT Balb/c mice were used to determine whether IL-33 signalling is dependent on ST2L. In both groups, mortality numbers were high. Survival rates were higher in ST2 deficient mice compared to the Balb/c WT mice. Due to insufficient numbers at day 14, days 14, 21 and 28 were excluded from analysis. No significant differences were observed in perfusion restoration rates between ST2 knockout and WT mice at day 4 (11% ± 1 vs. 14% ± 4, p=0.772) or at day 7 (17% ± 2 vs. 15% ± 5, p=0.337; Figure 4).

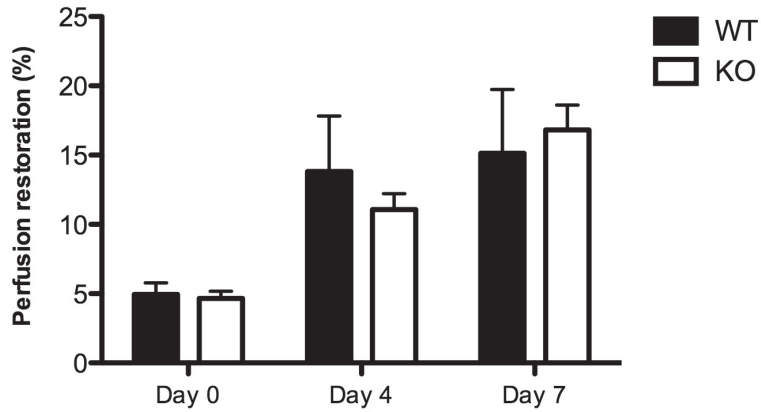

Figure 4 | Percentage perfusion restoration at day 4 and day 7 in WT and ST2-/- mice

DISCUSSION

The IL-33/ST2 pathway has been shown to play an important role in inflammatory diseases. Previous studies have shown that IL-33 signalling is protective in cardiovascular disease.[9–11] It is yet unclear if IL-33 signalling is beneficial or detrimental for the formation of blood vessels. Although IL-33 was reported pro-angiogenic in several *in vitro* and *in vivo* experiments,[7] another study suggested that its absence rather than its expression is associated with angiogenesis.[8] To our surprise, IL-33 inhibited perfusion restoration by 54% at 4 days and by 40% at 7 days after femoral artery ligation compared to control animals, suggesting IL-33 an unlikely candidate for therapeutic acceleration of vessel growth in the presence of ischemic disease. Still, our results indicate that IL-33 significantly influences perfusion recovery, supporting a role for the IL-33/ST2 pathway in arteriogenesis.

Elucidating the mechanism by which IL-33 inhibits perfusion recovery can be of value for understanding the underlying mechanisms of vessel growth. Absence of inflammatory cells in the perivascular space is one of the most important determinants known to hamper the development of collateral vessels.[18] Expression of ST2L, the receptor for IL-33, has been found on T-cells and in particular Th2 cells.[13] T-cells are known to play a key role in matrix degradation and vessel remodelling and are important during arteriogenesis.[19] After femoral artery excision, perfusion restoration was severely retarded in immunodeficient ApoE-/- mice, which lack all forms of T-cells.[20] Furthermore, the absence of CD4+ T-cells was shown to impair the inflammatory response and collateral development, which was reversed by infusion of spleen derived purified CD4+ T-cells.[21] To investigate whether the lower perfusion recovery in IL-33 treated animals could be explained by hampered T-cells extravasation, T-cells in the perivascular space of collateral vessels were counted. We observed no significant differences in T-cell numbers per vessel between IL-33 treated and control mice. Apparently, attraction and extravasation of T-cells is not altered and unlikely responsible for the observed differences in perfusion. However, in the current study we only quantified the general T-cell population. It might therefore be of interest to further discriminate T-cell subtypes in the perivascular space. Another explanation for this observation is a different activation status of the T-cells in IL-33 treated mice.

In another attempt to get more insight into the IL-33/ST2 pathway in collateral formation, we studied perfusion restoration in ST2 deficient mice. In our study we did not observe any difference in perfusion restoration between ST2 deficient and WT mice at 4 and 7 days after surgery. As we expected ST2 deficiency to be beneficial for collateral formation (as no IL-33 signalling would be possible), we chose another background mouse model for the knock out experiments since Balb/c mice have been reported to display a delayed recovery compared to Bl6 mice.[17] Unfortunately, the WT Balb/c mice did not respond very well to the surgery and mostly died before the end of the experiment. Due to these practical difficulties, we were not able to perform a robust statistical analysis for the perfusion measurements beyond day 7. Still, at day 4 and day 7, the ST2 deficient mice did not show the expected increase in perfusion recovery. This suggests that physiological IL-33 plasma levels after femoral artery ligation in WT mice are insufficient to exert any effect on collateral vessel growth. The observation that IL-33 plasma levels after procedure were below the ELISA detection limit supports this hypothesis. This could be explained by a lack of IL33 secretion upon surgery

or by the observed increase in sST2 plasma levels in the first days after ligation. It is known that sST2 levels increase after myocardial infarction[22] or acute coronary syndromes in humans.[23] In addition, sST2 levels increase after cardiovascular surgical or catheter interventions.[24] This increase in sST2 is likely due to cell or tissue damage occurring during a complication or intervention.[25] Although the biological function of sST2 in humans is still unknown, it is thought that sST2 may capture IL-33 away from the circulation thereby preventing intracellular signalling as shown in experimental mice studies.[26] Taken together, these data imply that physiological IL-33 levels are too low to exert an effect on collateral growth, while the increase in sST2 is not sufficient to abolish the inhibitory effect of recombinant IL-33 administration.

In conclusion, our main finding is that we observed a significant decrease in perfusion recovery in IL-33 treated mice. Although multiple efforts were taken to investigate how IL-33 affects perfusion recovery, it remains unclear. Future research has to be conducted to get more insight into the underlying mechanisms.

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PART III

MAST CELLS

CHAPTER

7

Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events

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ABSTRACT

Aims

Human autopsy, animal and cell culture studies together have merged in a concept suggesting participation of mast cells (MCs) in the generation of atherosclerotic plaques. More specifically, these studies have suggested MC-induced intraplaque neovascularization as one mechanism by which MCs may render the plaques vulnerable. The present study was designed to assess the association between MC numbers and neovascularization in human atherosclerotic plaques, and to relate the abundance of plaque MCs to the occurrence of adverse cardiovascular events during follow-up.

Methods and results

Atherosclerotic plaques of 270 patients suffering from carotid artery stenosis were stained for the presence of MCs (MC tryptase). Furthermore, during a follow-up of 3 years, cardiovascular-related endpoints were assessed in 253 patients.

On average a high number of MCs were observed per plaque cross-section (median 108 [55-233] cells per section). Plaques with high MC numbers revealed an unstable lipid-rich inflammatory phenotype and were associated with symptomatic patients. In addition, MC numbers were positively associated with microvessel density ($r=0.416$, $p<0.001$). Patients with high intraplaque MC numbers showed significantly more cardiovascular events during the follow-up (58/142 vs. 31/111 events, $p=0.029$). In a multivariate analysis with correction for the main risk factors of cardiovascular diseases, MCs remained independently associated with adverse cardiovascular events ($p=0.025$).

Conclusions

MCs are highly prevalent in human carotid atherosclerotic lesions and associated with plaque microvessel density. Furthermore, intraplaque MC numbers associate with future cardiovascular events.

INTRODUCTION

Acute clinical manifestations in patients suffering from atherosclerosis are usually caused by plaque rupture followed by intraluminal thrombus formation. Unstable plaques that are prone to rupture are characterized by a large lipid core, a thin fibrous cap, intraplaque neovascularization and a large inflammatory cell infiltrate composed of macrophages, T-cells and mast cells (MCs).[1] MCs are inflammatory cells traditionally known for their role in allergy and in innate immune responses.[2] MCs are filled with cytoplasmic secretory granules, which they exocytose upon activation. Such activated MCs are particularly abundant at the sites of atheromatous erosion or rupture in patients who have died of myocardial infarction.[3] More recently, animal experiments have demonstrated that MCs are also actively involved in the initiation and progression of atherosclerotic disease. For example, more unstable atherosclerotic lesions were observed in ApoE-/- mice treated with MC activators.[4,5] This unfavourable phenotype of plaque destabilization could be prevented by treatment with a MC stabilizer such as sodium cromolyn[4] or tranilast.[6] In line with these data, it was observed that MC deficient mice were demonstrated to develop smaller lesions with a more stable phenotype.[7–9]

One of the proposed mechanisms by which MCs may render the plaques unstable is their ability to induce neovascularization under normal and pathological conditions.[10] Importantly, in autopsy studies it has been observed that MCs accumulate in neovessel-rich areas of atherosclerotic plaques.[11–14] Moreover, it was demonstrated that the MCs situated near the newly formed vessels contained basic fibroblast growth factor (bFGF), a potent pro-angiogenic factor.[15] These data suggest that MCs are involved in the formation of microvessels in the atherosclerotic plaque and so accelerate plaque progression into an unstable plaque phenotype.

We recently showed that intraplaque neovascularization and intraplaque haemorrhage (IPH) in carotid plaques are predictive for cardiovascular events elsewhere in the human body.[16] This underscores the need for improved understanding how intraplaque neovascularization renders atherosclerotic plaques prone to trigger atherothrombotic events in the atherosclerosis-prone segments of the arterial tree. Although it is known that the accumulation of inflammatory cells into the plaque largely contributes to the process of plaque destabilization, the size of the athero-express bank allows the identification of inflammatory cell types that are independently associated with intraplaque neovascularization. In

the present study we thus aimed to examine the association of local MC numbers with local plaque characteristics, such as plaque neovascularization, in a large number of human samples. As an association between local MCs and future cardiovascular events has not been investigated previously, we related plaque MC numbers with the occurrence of future cardiovascular events.

MATERIALS AND METHODS

Study population and design

The Athero-express is an ongoing longitudinal study in two Dutch teaching hospitals in Utrecht and Nieuwegein, the Netherlands.[17] In this study a total of 270 patients of the Athero-express biobank, undergoing carotid endarterectomy (CEA) were included. Indication for CEA was based on recommendations by the Asymptomatic Carotid Atherosclerosis Study and Asymptomatic Carotid Surgery Trial studies for asymptomatic patients and the North American Symptomatic Carotid Endarterectomy Trial and European Carotid Surgery Trial studies for symptomatic patients.[18-20] All indications were reviewed in a multidisciplinary vascular team, and all patients were evaluated by a neurologist before CEA to assess their neurological status and document the preoperative symptoms.

We selected patients who remained healthy and patients who suffered from an event during follow-up in a 2:1 ratio. These patients were randomly selected among those who had been operated between March 2002 and December 2007. The local medical ethical boards of both participating hospitals approved the studies. All participating patients signed a written informed consent prior to inclusion. Of all patients the baseline characteristics, medication use and medical history were obtained via questionnaires and the patients' medical records.

Follow-up

After CEA patients were followed up to 3 years by questionnaire. Primary outcome is defined as any cardiovascular event including; (non-) fatal stroke, (non-) fatal myocardial infarction, sudden death, other vascular death and any arterial vascular intervention that had not been planned at the time of inclusion (e.g. carotid surgery or angioplasty, coronary artery bypass, percutaneous coronary artery intervention, peripheral vascular surgery or angioplasty). Endpoints were validated by two members of the outcome assessment committee. If no consensus was reached, a third observer was consulted for final judgment of the endpoint.

Materials

A total of 270 patients that underwent CEA were randomly selected for the present study with a ratio of 2:1 for control patients vs. patients who suffered from an event during follow-up. Six patients were excluded from the study as the endarterectomy specimens were unsuitable for histological analysis, resulting in a total of 264 patients (events n=89, controls n=175). The carotid plaques used in this study were processed as described previously.[17] In short, after surgical dissection the plaque was cut into segments of 5 mm. The segment with the largest plaque area was fixed in formalin and embedded in paraffin for histology. The two adjacent sections were frozen in liquid nitrogen and used for protein isolation. In addition, blood was drawn prior to CEA procedure and plasma was stored at -80 °C.

Immunohistochemistry

To assess MC numbers in the atherosclerotic specimens, plaque sections were pre-treated with pepsin buffer for 15 min at 37 °C. A monoclonal mouse antibody against MC tryptase (dilution 1:400, Dako-Cytomation, Carpinteria, CA, USA) was used to stain MCs. Powervision poly HRP against mouse IgG (ImmunoLogic, Duiven, The Netherlands) was used as secondary antibody after which the tryptase staining was visualized with diaminobenzidine. Sections were counterstained with Haematoxylin. Total MC numbers were determined by counting all MCs present in a plaque cross-section at x40 magnification. A subset was analysed by another independent researcher to assess intra-observer variability (Spearman correlation coefficient $r=0.947$; $p<0.001$). In 100 randomly chosen plaques the number of degranulating MCs was determined. A degranulating MC was defined by a group of MC tryptase positive extracellular granules in close proximity of each other or in close proximity of a MC. The percentage of degranulating MCs was assessed by counting the first 50 MCs observed by the researcher. This percentage was extrapolated to the total number of degranulating MCs per plaque section. Total plaque area (mm^2) was measured using analySIS 2.8 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) to determine the distribution density of MCs expressed as numbers of MC/mm^2. Consecutive sections were also stained for CD68 (macrophages), CD66 (neutrophils) and CD34 (endothelial cells). Image-analysing software (Soft Imaging Solutions GmbH, Münster, Germany) was used to determine positive macrophage staining expressed as a percentage of covered plaque area[17] and to assess total neutrophil numbers. Microvessels were counted at three sites with most prevalent CD34 staining within the section (hotspots) and were expressed as average microvessel density per hotspot.[16] The

presence of IPH was assessed with Haematoxylin and Eosin (H&E) staining and fibrin (Mallory's phosphotungstic acid-haematoxylin). The size of the extracellular lipid core (atheroma) was assessed by the H&E and picrosirius red stain.[17] Overall plaque phenotype was based on the percentage of confluent lipid areas of the total plaque area that were visually estimated (fibrous: <10% fat; fibroatheromatous: 10-40%; atheromatous: >40% fat).

For the double immunostaining of MCs and vessels pepsin pre-treated sections were incubated with the mouse anti MC tryptase antibody (1:400) followed by Brightvision poly AP-anti-Mouse IgG (Immunologic) incubation and development with Liquid permanent red (DAKO, Glostrup, Denmark). Subsequently sections were boiled in citrate buffer pH6.0, incubated with a monoclonal mouse anti CD34 antibody (1:400; Immunotech, Marseille, France) followed by incubation with Brightvision poly AP-anti-Mouse IgG and developed with alkaline phosphatase substrate kit III (Vector Labs, Burlingame, CA, USA). For the double immunostaining, no nuclear staining was used.

Quantification of mast cell tryptase levels in patient plasma.

Plasma obtained before surgery was available from 135 (events n=52, controls n=83) of the selected patients. Baseline characteristics did not differ between these patients and the total group of studied patients (data not shown). MC tryptase levels were determined in citrate plasma samples using an ImmunoCAP® 250 tryptase assay (Phadia AB, Uppsala, Sweden)

Quantification of RANTES, Eotaxin-1, MCP-1 and TGF-β1.

Protein isolation on the adjacent segments to the culprit lesions was performed by a standardized method. In short: the segments were grinded in liquid nitrogen and dissolved in Tripure or Tris (Roche). Total protein concentration of each sample was quantified. Levels of monocyte chemotactic protein-1 (MCP-1), CCL-5 (RANTES) and CCL-11 (Eotaxin) were measured by Multiplex Immunoassay (Bioplex, Biorad Laboratories, Hercules, USA) in tripure protein isolates. TGF-β1 expression was quantified in Tris isolates by Quantikine Human TGF-β1 Immunoassay (R&D Systems, Wiesbaden, Germany). Protein levels for all four chemoattractants were corrected for total amount of protein within the segment.

Statistics and data analysis

SPSS 20 was used for all analyses (SPSS Inc, Chicago, IL, USA). MC numbers are not normally distributed; non-parametrical testing was used to determine differences.

The Mann-Whitney U test was used to obtain differences in MC numbers as a continuous variable for all common risk factors. For multiple groups testing the Kruskal-Wallis test was used. The Spearman correlation coefficient was used to calculate relations of (degranulating) MC/mm^2 or tryptase plasma levels with all continuous variables in this study. Differences were considered significant with a two-sided p-value of below 0.05. The receiver operating characteristic (ROC) curve was used to determine the optimal cut-off value to divide the patients in two groups with low and high MC/mm^2. To determine the prognostic value of intraplaque MC/mm^2, the Cox-regression model was used. A multivariate analysis was performed to adjust for confounders. Based on previous reports,[4,6] we expected the number of MCs to associate with plaque vulnerability and increased risk for future cardiovascular events.

RESULTS

Baseline characteristics

Table 1 depicts baseline clinical characteristics for the patients included in this study. The patient population represents a typical population of patients with vascular occlusive diseases. The mean age of the patients was 68, with a preponderance of males (71%). Moreover, the majority of patients was symptomatic (81%), suffered from hypertension (88%) and used statins (73%).

Mast cells and plaque characteristics

MCs were found to be distributed throughout the entire plaque. They were more abundantly present in the areas where also microvessels were present (Figure 1C), i.e. in the deep layers of the plaque at the interface of the media (Figure 1A). In more unstable lesions, MC accumulations were also found in the shoulders of the plaques (Figure 1B). The median number of total MCs present was 108 [55-233] cells per plaque section (Table 2). Total MC numbers were associated with plaque phenotype categorized in three groups: fibrous, fibroatheromatous and atheromatous (79 [37-165], 124 [64-237], 139 [59-243] MC per plaque section, respectively; p=0.008). In addition, high MC numbers were associated with the presence of IPH: 70 [37-137] MCs in plaques without IPH vs. 130 [65-241] MCs in plaques with IPH (p=0.001). Moreover, MCs did correlate positively with the percentage of plaque area covered with macrophages (r=0.156, p=0.011) and with the number of counted neutrophils (r=0.380, p<0.001). Degranulating MCs

Table 1 | Baseline characteristics of the patients in relation to MC numbers in carotid plaques

		MC/mm^2	p-value
Age, mean years (sd)	68 (9)	r=-0.099	0.11
BMI, mean kg/m^2 (sd)	26 (4)	r=0.037	0.554
Sex			
Male	188/264 (71%)	3.34 [1.64-5.68]	0.073
Female	76/264 (29%)	2.56 [1.25-5.05]	
Current smoker			
Yes	101/260 (39%)	3.22 [1.78-4.98]	0.955
No	159/260 (61%)	3.05 [1.51-6.10]	
Diabetes mellitus			
Yes	59/264 (22%)	3.52 [1.65-5.87]	0.237
No	205/264 (78%)	2.97 [1.53-5.28]	
Statin use			
Yes	194/264 (73%)	3.01 [1.55-5.01]	0.196
No	70/264 (27%)	3.52 [1.71-7.41]	
Hypertension			
Yes	233/264 (88%)	2.97 [1.56-5.69]	0.923
No	31/264 (22%)	3.44 [2.01-5.09]	
History myocardial infarction			
Yes	55/262 (21%)	3.61 [1.59-6.80]	0.158
No	207/262 (79%)	2.97 [1.56-5.25]	
History vascular intervention			
Yes	164/264 (62%)	3.17 [1.45-5.61]	0.976
No	100/264 (38%)	3.05 [1.62-5.56]	
Clinical Presentation			
Asymptomatic	49/264 (19%)	2.12 [1.23-4.23]	0.016*
Symptomatic	215/264 (81%)	3.34 [1.75-5.87]	
Amaurosis fugax	41/264 (15%)	2.81 [1.61-5.28]	
TIA	117/264 (44%)	3.61 [1.76-6.62]	
Stroke	57/264 (22%)	3.22 [1.60-6.31]	

Data are presented as No. (%) and median [IQR] unless otherwise indicated; r = Spearman's rank correlation coefficient; sd = standard deviation; IQR = interquartile range; BMI = body mass index; TIA = transient ischemic attack; * p-value represents statistical analysis for asymptomatic patients versus symptomatic patients (composed of amaurosis fugax, TIA and stroke)

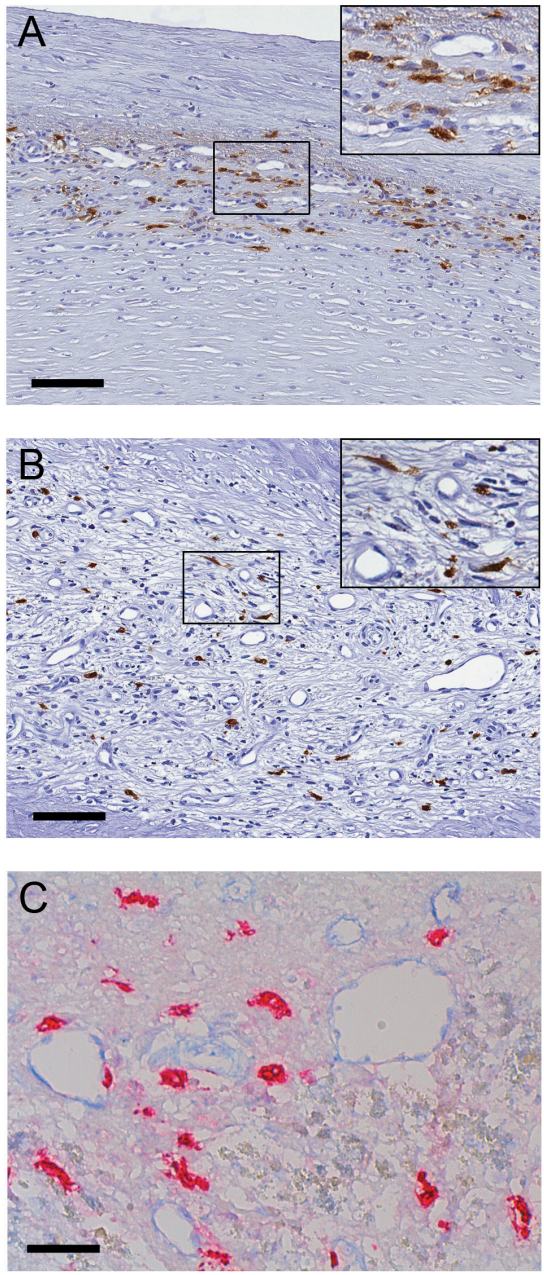

Figure 1 | Immunohistochemical detection of MCs in carotid atherosclerotic plaque.
An immunohistochemical MC tryptase staining showing MCs (in brown) in an atherosclerotic plaque surrounding microvessels at the interface to the media (A) and around microvessels in the shoulder of an atherosclerotic plaque (B). Bar = 100 µm. Inlay with magnification of indicated area. C. Double immunostaining showing MCs (in red) around microvessels of which the endothelium is stained blue (no nuclear staining). Bar = 400 µm

Table 2 | MC numbers in carotid plaques with respect to the histological parameters of the plaques

Plaque characteristics	MCs (n)	p-value
	108 [55-233]	
Plaque phenotype		
Fibrous	79 [37-165]	p=0.008
Fibroatheromatous	124 [64-237]	
Atheromatous	139 [59-243]	
Intraplaque haemorrhage		
Yes	130 [65-241]	p=0.001
No	70 [37-137]	
Macrophages	r=0.156	p=0.011
Neutrophils	r=0.380	p<0.001
Microvessel density	r=0.416	p<0.001

Data are presented as Spearman's rank correlation coefficient (r) or median [interquartile ranges]; MCs are presented as total number of MCs per cross-section (n)

were also abundantly present in the plaques. Their numbers correlated with the percentage of plaque area covered macrophages (r=0.310, p=0.002), but did not associate with any other characteristic of the rupture-prone plaque.

Mast cells and intraplaque neovascularization

A strong significant correlation between MCs and intraplaque microvessel density was observed. A 2.4 fold increase in MC/mm^2 was observed comparing the first and fourth quartile of counted plaque microvessels (Figure 2). In addition, also on a continuous scale, microvessel density correlated significantly with total MC numbers (r=0.416, p<0.001; Table 2). A positive correlation with microvessel density was also observed for neutrophils and macrophages (r=0.128, p=0.045 and r=0.133, p<0.001, respectively; data not shown). Therefore, we examined whether a more general pan-inflammatory process would explain the observed association between MCs and microvessel density. In Figure 3, the average microvessel density is provided for 4 groups based on high or low amounts of MCs and macrophages (Panel A) or MC and neutrophil numbers (Panel B) using the median as a cut-off value. Plaques with high microvessel density were characterized by high MC numbers. The number of microvessels did not differ strongly between

macrophage rich and macrophage poor cross-sections when controlled for MC numbers. Plaques characterized by high abundance of both cell types did show a higher microvessel density. Similar results were observed for neutrophils. Plasma tryptase levels correlated with the number of MCs and degranulating MC/mm^2 plaque (r=0.243, p=0.004 and r=0.363, p=0.001, respectively), but were not associated with microvessel density. Furthermore, no correlation was found between the number of degranulating MC/mm^2 and microvessel density. In addition, no association was observed for MCs and plaque protein levels of the MC chemoattractants RANTES (r=-0.041, p=0.597), Eotaxin (r=0.126, p=0.100), TGF-β1 (r=0.089, p=0.239) or MCP-1 (r=0.044, p=0.564). In contrast, a positive correlation was observed between macrophage numbers and MCP-1 or RANTES plaque protein levels (r=0.138, p=0.001 and r=0.107, p=0.008, respectively).

Mast cells and clinically relevant characteristics

Patients entering the study with symptoms as amaurosis fugax, TIA and stroke contained significantly more MCs in their plaques compared with asymptomatic patients (3.34 [1.75-5.87] vs. 2.12 [1.23-4.23] MC/mm^2, p=0.016; Table 1). In addition, we observed a trend that males have more MCs in their carotid plaque compared with females (3.34 [1.64-5.68] vs. 2.56 [1.25-5.05] MC/mm^2, p=0.073). None of the other risk factors was associated with MC numbers.

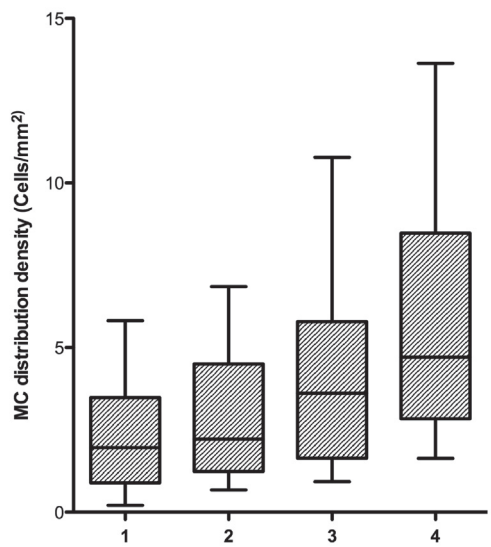

Figure 2 | Vertical bar plot of the association between MC/mm^2 and microvessel density. MC/mm^2 are presented against the quartiles of average microvessel density (p<0.001)

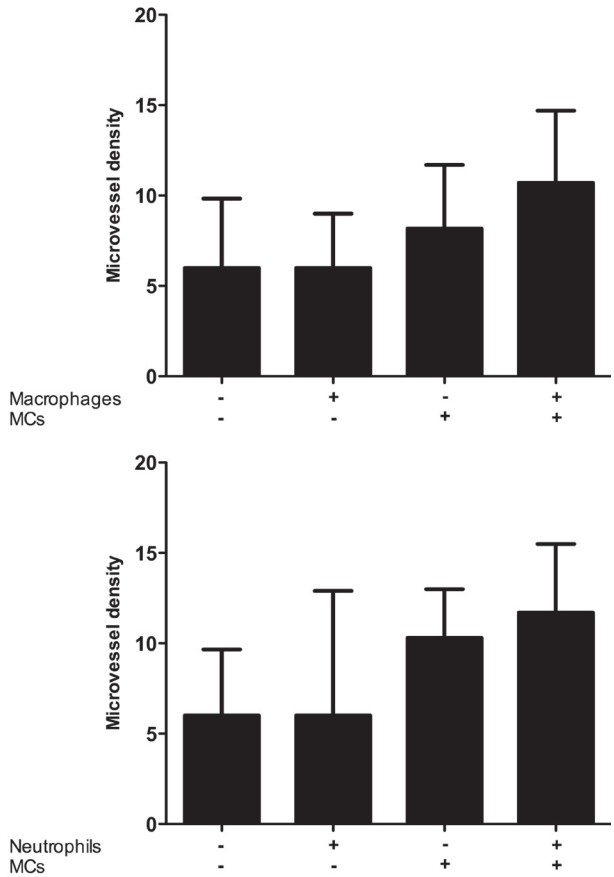

Figure 3 | Number of microvessels for plaques was categorized in 4 groups based on the amount of macrophages (upper panel) or neutrophils (lower panel) and MCs. + indicates all cross-sections with a value the same as or above the median; – indicates a value below the median. The number of sections per group for macrophages (n=65, n=65, n=36, n=95, respectively) and for neutrophils (n=21, n=16, n=21, n=29, respectively)

Outcome

Eleven patients were excluded for lack of relevant clinical data resulting in 253 patients of whom in 89 we observed secondary cardiovascular events. In 164 patients without any future events, a median MC/mm^2 of 2.83 [1.48-5.45] was observed. Events are sub-divided in (non-) fatal stroke (3.33 [1.76-7.01], n=32), (non-) fatal myocardial infarction (3.68 [1.41-6.80], n=19), sudden death and other vascular death (3.38 [1.84-7.54], n=20) and peripheral (3.30 [1.65-5.38], n=34) and coronary interventions (3.61 [2.40-5.32], n=19) that had not been planned at the time of inclusion.

The receiver operating characteristic (ROC) curve analysis was used to reveal the optimal cut-off value of 2.75 MC/mm^2 plaque to classify the patients for future cardiovascular events (>2.75 MC/mm^2, n=142; <2.75 MC/mm^2, n=111). Patients with a value >2.75 MC/mm^2 were associated with more cardiovascular events during follow-up (58 [41%] vs. 31 [28%] events, hazard ratio (HR) = 1.62, 95%CI [1.05-2.51], p=0.029). To check for confounders, we added MC/mm^2 as a linear variable in a multivariate analysis with the most prominent risk factors for cardiovascular disease, i.e. gender, age, hypertension, smoking, diabetes, body mass index (BMI) and statin use. MCs remained significantly associated with adverse events (HR per sd = 1.23, 95%CI [1.03-1.47], p=0.025). Also other clinical factors that could potentially influence MC numbers, as other inflammatory co-morbidities and anti-inflammatory drug use, did not affect the relation of MCs and primary outcome.

In addition, higher tryptase plasma levels were observed in patients that had a secondary event (52 events out of 135 patients, 5.3 [4.2-6.9] vs. 4.5 [3.7-5.8] µg/mL, p=0.046, Figure 4).

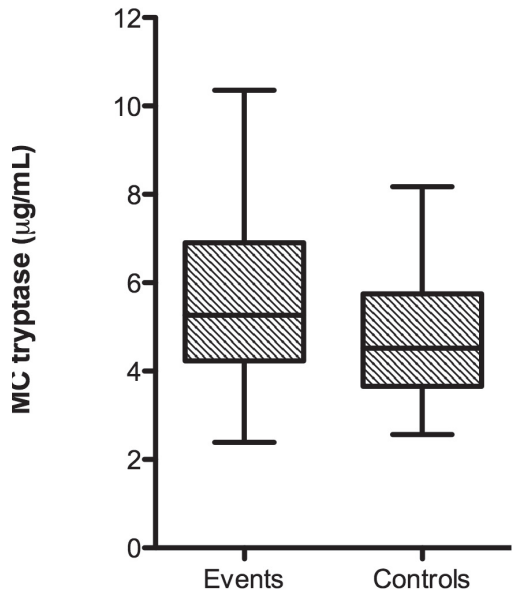

Figure 4 | Plasma tryptase levels (µg/mL) in patients with and without cardiovascular events during follow up (p=0.046)

DISCUSSION

Atherosclerosis is a chronic inflammatory disorder where numerous inflammatory cell types are known to be involved. Such cells can enter the plaque from the circulation through the endothelium and create an inflammatory environment in the vessel wall. A characteristic of a rupture-prone plaque that has gained attention of the scientific community is the presence of intraplaque vessels. In this study we show that MCs associate with microvessel density in the plaque. It is known that not only MCs, but also other inflammatory cells, are capable of inducing new vessel formation,[21] suggesting that an overall inflammatory environment is responsible for the formation of the neovessels in the plaque. We also observed that, besides MCs, macrophages and neutrophils are associated with microvessel density. However, this study provides a sufficient sample size to show that the association between microvessel density and MCs is significant and independent of the presence of other inflammatory cell types. Particularly interesting, is the finding that high MC numbers in the plaque were associated with a higher average microvessel density independent of the amount of macrophages and neutrophils. These data suggest that MCs might be important for plaque neovascularization. Nevertheless, when all cell types are present an even higher microvessel density is observed. A possible explanation might be that, as the plaque progresses, the number of inflammatory cells increases due to increased extravasation of the cells into the plaque via the newly formed microvessels. Of note, in this association study it remains an open question whether the MC numbers are a cause or a consequence of vessel formation, i.e. whether MCs are responsible for the induction of the new vessels or whether the MCs and other inflammatory cells enter the plaque via the vessels after their formation. Probably both mechanisms occur, as low numbers of MCs are already present in the normal arterial wall[13] and increase with plaque progression.[15,22] In this study, we did not observe a correlation between known MC chemoattractants and MC numbers within the plaque, while for monocytes and their attractors a positive association was observed. This could be explained by the life span of MCs cells, which is long compared with other inflammatory cell types. Subsequently, chemokine levels at the moment of plaque excision may not reflect earlier MC recruitment. Additional research is needed to elucidate the underlying mechanism of MC migration towards the atherosclerotic plaque.

Necrotic areas in atherosclerotic plaques did not contain MCs. Large plaques with avascular fatty areas would result in an undervalued number of MCs when expressed per mm^2. Therefore, in the histological analyses we expressed the number of MCs/plaque. Here, we show that plaques with high MC numbers show signs of increased plaque vulnerability. Previous studies demonstrated that this observation is also reflected in the association with clinical outcome, since higher MC numbers were observed in the plaques of patients with unstable angina or symptomatic carotid artery disease.[23] We confirm these observations in our patient cohort where we also noticed more MC in the plaques of symptomatic patients compared with asymptomatic patients.

The impact of MCs on atherosclerotic plaque progression has long been underestimated, as it was considered that MCs were only present in low numbers. Therefore another important finding in this study is that MCs are highly prevalent in the atherosclerotic lesion: in some of the plaques total MC numbers of about 800 cells per section were detected, with an overall median of just above 100 cells per section. Plaques often showed confluency of CD68-positive foam cells. The percentage of macrophages is therefore depicted as percentage of covered area, because it was not always possible to quantify the individual cells. This makes comparison of absolute macrophage and MC numbers difficult in our cohort. It is though acknowledged that macrophages by far outnumber all other cell types in the advanced atherosclerotic plaques.[23] Nevertheless, together with experimental data in literature, our data suggests that the MC is a prominent inflammatory cell type accumulating in the atherosclerotic plaque during plaque progression.

We considered that particularly degranulating MCs would be responsible for inducing intraplaque neovascularization as they are frequently observed near the microvessels.[11,15,24,25] However, we could not observe an association between degranulating MC numbers and microvessel density. Also, no association was observed between degranulating MCs and any of the other rupture-prone characteristics or future events. This might suggest that the induction of new vessel formation is more related to a regulated non-exocytotic release of pro-angiogenic growth factors rather than to the extent of an exocytotic release of countable granules, i.e. degranulation of activated MCs.[26]

In this study we show that MC presence is associated with thrombus formation and IPH. It was hypothesized that MC components can induce hyperpermeability or erosion of endothelial cells of the microvessels in the plaques eventually leading

to IPH or thrombus formation.[3,27] This is in accordance with the most important finding in our study, that plaque MC numbers associated independently with future cardiovascular events. In contrast, in the study of Hellings et al., showing that microvessel density is also predictive for future events, neither macrophage nor neutrophil numbers were found to associate with the occurrence of secondary manifestations.[16]

Interestingly, we also observed higher plasma tryptase levels in patients that experienced a secondary event. The tryptase levels correlated positively with the number of (degranulating) MCs in the plaque, implying that the presence of degranulating, i.e. tryptase-secreting MCs in a plaque is not only responsible for the local matrix degradation, but also representative for the systemic changes that might be responsible for the secondary manifestations. The correlation of intraplaque MC numbers with future events has never been reported previously, however, MC tryptase plasma levels have been tested as a possible biomarker for cardiovascular disease. Thus, in agreement with our data, elevated tryptase levels were observed in patients with substantial coronary heart disease with the highest levels in the subgroup with acute myocardial infarction.[28] In addition, higher MC tryptase levels were observed in patients with significant coronary artery disease (CAD) defined by stenosis of over 50%.[29] Moreover, in another study of the group of Deliargyris tryptase levels were elevated in patients with CAD.[30] Conversely, no differences in MC tryptase were observed in patients with acute coronary syndrome.[31,32] Besides tryptase, several other MC derived components have also been associated with disease severity, and, in general, most studies underline the importance of MC mediators in inflammatory diseases.

Taken together, we show here for the first time that intraplaque MC numbers and plasma MC tryptase associate with future cardiovascular events. However, the results of this clinical proof-of-concept study are not sufficient to suggest utilization of the above observations in a regular clinical setting for prediction studies. Nevertheless, the data do strengthen the hypothesis that the presence of MCs in advanced carotid plaques increases risk for secondary cardiovascular manifestations, possibly by inducing intraplaque neovascularization and via matrix degradation, which may together increase the incidence of IPH and thrombus formation. However, extensive research is necessary before MC stabilizing agents can be considered as a possible therapeutic opportunity preventing clinical manifestations by plaque stabilization in the future. For example, animal

experiments inhibiting MC activation by stabilizing agents should be performed to proof causality between plaque MCs and neovascularization.

In conclusion, we show that MC numbers in the carotid atherosclerotic plaque associate with future cardiovascular events. These data correspond with the strong association found between MC numbers and intraplaque neovascularisation, now evolving as an important characteristic of rupture-prone atherosclerotic lesions, which may trigger acute atherothrombotic complications in the vulnerable patients.

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PART III

MAST CELLS

CHAPTER

8

Circulating immunoglobulins are not associated with intraplaque mast cell number and other vulnerable plaque characteristics in patients with carotid artery stenosis

Submitted

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ABSTRACT

Background

Recently, we have shown that intraplaque mast cell numbers are associated with atherosclerotic plaque vulnerability and with future cardiovascular events, which renders inhibition of mast cell activation of interest for future therapeutic interventions. However, the endogenous triggers of mast cell activation during the progression and destabilization of atherosclerosis remain unidentified. Mast cells can be activated by immunoglobulins and in the present study, we aimed to establish whether specific immunoglobulins in plasma of patients scheduled for carotid endarterectomy were related to intraplaque (degranulating) mast cell numbers and plasma tryptase levels. In addition, the levels were related to other vulnerable plaque characteristics and baseline clinical data.

Methods and results

ACPA-IgG, oxLDL-IgG, total IgG and total IgE levels were measured in 135 patients who underwent carotid endarterectomy. No associations were observed between the tested plasma immunoglobulin levels and (degranulating) mast cell numbers in atherosclerotic plaques. Furthermore, no associations were found between IgG levels and the following plaque characteristics: size of a lipid core, degree of calcification, number of macrophages or smooth muscle cells, amount of collagen and number of microvessels. Statin use was negatively associated with all immunoglobulins except total IgG.

Conclusions

In patients suffering from carotid artery disease, total and specific IgE and IgG levels do not associate with plaque mast cell numbers or other vulnerable plaque histopathological characteristics. This study thus does not provide supportive evidence that the immunoglobulins tested in our cohort play a role in mast cell activation or progression of the atherosclerotic plaque.

INTRODUCTION

The incidence of atherosclerotic disease is increasing by the aging population and the unhealthy life style in the Western world. The mast cell, a prominent inflammatory cell type and a major effector cell in allergy and asthma, has been shown to accumulate both in the rupture-prone shoulder region of human atheromas[1,2] and in the perivascular tissue during atherosclerotic lesion progression.[3] Recently, we have shown that intraplaque mast cell numbers are associated with plaque vulnerability and interestingly, with future cardiovascular events.[4] In that study, mast cell numbers associated with vulnerable plaque characteristics such as lipid size, intraplaque haemorrhage, microvessel density and inflammatory cell accumulation, suggesting that mast cells actively contribute to atherosclerotic plaque progression and destabilization. Inhibition of mast cell activation may therefore be of interest for future therapeutic interventions. However, the mechanism of mast cell activation during the development of atherosclerosis remains up to date unresolved. Previously, we and others have established that mast cells in the vessel wall can be activated by for example neuropeptides,[5] complement factors[6] and lipid mediators[7] in animal models of atherosclerosis. Furthermore, the mast cell expresses the high-affinity IgE receptor (FcεR1) and the IgG receptor (FcγR).[8,9] Mast cells can be activated via IgE mediated crosslinking of the FcεR, after which mast cell release granules into the surrounding area. IgE levels have been shown to be elevated in patients with unstable angina pectoris[10] and intriguingly, also higher in dyslipidemic men as compared to control subjects.[11] Furthermore, Lappalainen et al. demonstrated that specific oxLDL-IgG immune complexes were able to induce mast cell activation.[12] Circulating specific IgE and IgG antibodies or lipid-immunoglobulin immune complexes, which exert their effects through the FcεR and FcγRs, are known to play a role in several immune responses[9] and may thus also be involved in mast cell activation within the atherosclerotic plaque, thereby affecting plaque stability. Based on these observations, we hypothesise that circulating Igs may activate mast cells and thereby accelerate the destabilization of the atherosclerotic plaque. This study was designed to assess the presence of associations between Igs expression and mast cell numbers in plaques from patients with carotid stenosis. Hence, we assessed total and specific IgG and IgE plasma levels and related their numbers to several mast cell parameters and established vulnerable plaque characteristics. In additions, Ig levels were related to clinical characteristics.

MATERIALS AND METHODS

Study population and design

A total of 135 patients of the Athero-Express were included in this study. The Athero-express biobank involves patients that underwent carotid endarterectomy (CEA) in two Dutch teaching hospitals in Utrecht and Nieuwegein, the Netherlands.[13] Indication for CEA was based on recommendations by the Asymptomatic Carotid Atherosclerosis Study, the North American Symptomatic Carotid Endarterectomy Trial and the European Carotid Surgery Trial.[14–16] Patients were operated between March 2002 and August 2008 of which intraplaque MC numbers were available.[4] In that study, patients were selected who remained healthy and patients who suffered from an event during follow-up in a 2:1 ratio. Of 135 patients blood plasma samples were available. Total MC numbers and baseline characteristics did not differ between the patients of which plasma was and was not available. The local medical ethical boards of both participating hospitals approved this study. The participating patients signed a written informed consent prior to inclusion. The patient's baseline characteristics and medical history were obtained via questionnaires and the patient medical records.

Materials

The carotid plaques used in this study were processed as described previously.[13] In short, after surgical dissection the plaque was cut into segments of 5 mm. The segment with the largest plaque area was fixed in formalin and embedded in paraffin for histology. The two adjacent sections were frozen in liquid nitrogen and used for protein isolation. In addition, blood was drawn prior to CEA procedure and plasma was stored at -80 °C.

Quantification of immunoglobulin and MC tryptase levels in patient plasma

Plasma total IgE and IgG levels were measured using a human IgG and IgE ELISA according to manufacturer's protocol (Bethyl Laboratories, Montgomery, TX). Plasma IgG-oxLDL levels were measured by coating cupper oxidized human LDL in PBS (pH=9,0) on MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) overnight at 4˚C. Diluted Samples and standards (Biomedica, Wien) were added and incubated for 2 hours at 37˚C. Supernatants were discarded and plates were washed thoroughly. Anti-human IgG-HRP (Bethyl Laboratories, Montgomery, TX) was added as detection antibody for 1 hour at 37˚C. Bound oxLDL-IgG was visualized by using 2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma). Colour

was measured at an optical density of 415nm using a conventional ELISA reader. Between each ELISA step plates were washed with PBS containing 0.05% Tween20. Plasma IgG-ACPA levels were detected by binding of CCP2 peptide to a streptavidin coated MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) in PBS/0.1%BSA for 1 hour at room temperature. Samples and standard (pooled ACPA+ sera) were added tot the wells and incubated for 1 hour at 37˚C. Rabbit anti-human IgG-HRP (Dako, Denmark) was added to detect ACPA-IgG. Bound oxLDL-IgG was visualized by using 2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma). Colour was measured at an optical density of 415nm using a conventional ELISA reader. Between each ELISA step plates were washed with PBS containing 0.05% Tween20. MC tryptase levels were determined in plasma samples using an ImmunoCAP® 250 tryptase assay (Phadia AB, Uppsala, Sweden)

Immunohistochemistry

Sections were stained for mast cell tryptase (mast cells), CD68 (macrophages), smooth muscle cells (alpha actin), and CD34 (endothelial cells) as previously described. Total MC numbers were determined by counting all (degranulating) MCs present in a plaque cross-section at x40 magnification.[4] A degranulating MC was defined by a group of MC tryptase positive extracellular granules in close proximity of each other or in close proximity of a MC. The total plaque area (mm^2) was measured using the analySIS 2.8 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) to determine the distribution density of MCs expressed as numbers of MC/mm^2. Image-analyzing software was used to determine positive macrophage and smooth muscle cell staining expressed as a percentage of covered plaque area.[13] Microvessels were counted in three hot-spots and were expressed as average microvessel density per hotspots.[17] Collagen content (picrosirius red) was scored semi-quantitatively. The size of the extracellular lipid core (atheroma) was assessed by the H&E and picrosirius red stain.[13]

Statistics and data analysis

IBM SPSS statistics version 20 was used for all analyses (IBM corporation, Armonk, NY, USA). Immunoglobulin levels are not normally distributed; non-parametrical testing was used to determine differences. The Mann-Whitney U test was used to study immunoglobulin levels as a continuous variable for all risk factors. The Spearman correlation coefficient was calculated to assess associations between immunoglobulin levels and all continuous variables in this study. Differences were considered significant with a p-value of below 0.05.

RESULTS

Baseline patient characteristics

Total IgE, total IgG, ACPA-IgG and oxLDL-IgG plasma levels were measured in a total of 135 patients that underwent carotid endarterectomy. Baseline clinical characteristics of the 135 patients are provided in Table 1. The studied patient population with a mean age of 67 and a male prevalence (71%) reflects a relatively typical population of patients with cerebral vascular occlusive diseases. The majority of patients was symptomatic (74%), hypertensive (86%) and used statins (69%).

Table 1 | Baseline characteristics of the patients in relation to MC numbers in carotid plaques

		Total IgG	p-value	ACPA-IgG	p-value	oxLDL-IgG	p-value	Total IgE	p-value
Age, mean years (sd)	67 (9)	r = -0.082	0.347	r = 0.007	0.934	r = 0.031	0.724	r = 0.097	0.265
BMI, mean kg/m^2 (sd)	27 (4)	r= -0.115	0.19	r = -0.040	0.647	r = 0.001	0.995	r = -0.126	0.15
Gender									
Male	96/135 (71%)	16.8 [11.7-23.5]	0.694	20.6 [15.7-37.8]	0.326	336 [239-499]	0.074	131.1 [57.5-317.4]	0.169
Female	39/135 (29%)	17.2 [12.6-23.1]		19.5 [14.0-35.8]		282 [233-358]		76.7 [31.4-293.4]	
Current smoker									
Yes	55/134 (41%)	16.6 [10.3-23.3]	0.635	19.5 [15.2-30.2]	0.235	303 [252-481]	0.427	173.4 [65.5-449.8]	**0.032**
No	79/134 (59%)	17.1 [12.3-23.2]		22.6 [15.4-40.5]		306 [219-502]		91.5 [41.5-203.0]	
Diabetes mellitus									
Yes	25/135 (19%)	15.4 [12.1-22.9]	0.63	20.6 [16.9-38.2]	0.618	288 [241-391]	0.554	79.8 [41.9-288.5]	0.329
No	110/135 (81%)	17.0 [12.3-23.7]		20.3 [15.4-37.0]		320 [238-489]		125.0 [57.5-312.1]	
Statin use									
Yes	93/135 (69%)	17.1 [12.3-22.8]	0.72	19.1 [15.0-30.4]	**0.009**	288 [224-406]	**0.004**	97.5 [43.1-276.8]	**0.012**
No	42/135 (31%)	16.8 [12.3-25.0]		24.8 [18.3-53.6]		399 [282-584]		157.3 [75.2-545.4]	
Hypertension									
Yes	116/135 (86%)	17.2 [12.5-23.2]	0.633	19.9 [15.3-37.5]	0.786	302 [233-480]	0.207	113.0 [49.1-304.8]	0.265
No	19/135 (14%)	15.0 [10.4-26.0]		23.4 [15.7-36.9]		341 [260-537]		183.3 [68.8-594.0]	
Hypersensitive									
Yes	27/132 (20%)	17.1 [12.6-22.2]	0.906	19.5 [17.1-46.1]	0.522	305 [223-412]	0.539	148.1 [44.6-449.8]	0.401
No	105/132 (80%)	16.6 [11.6-23.9]		20.6 [15.3-34.6]		303 [237-489]		115.6 [50.5-304.7]	
History VI									
Yes	54/135 (40%)	18.5 [12.6-23.4]	0.39	18.3 [14.7-26.5]	**0.035**	297 [216-483]	0.391	86.2 [47.7-274.4]	0.239
No	81/135 (60%)	15.4 [11.9-23.5]		23.4 [16.8-40.3]		306 [254-484]		135.4 [54.2-358.9]	
History MI									
Yes	30/134 (22%)	19.0 [12.7-22.7]	0.673	18.2 [14.0-26.5]	0.087	321 [222-428]	0.62	85.6 [51.7-282.9]	0.601
No	104/134 (88%)	16.5 [12.2-23.5]		22.3 [16.0-38.7]		304 [239-508]		129.7 [49.1-310-8]	
Clinical presentation									
Asymptomatic	35/135 (26%)	15.8 [12.3-23.2]	0.419*	20.0 [16.0-35.2]	0.833*	314 [239-505]	0.377*	113.0 [47.0-304.9]	0.431*
Symptomatic	100/135 (74%)	18.1 [12.1-24.1]		21.9 [14.2-41.3]		293 [224-399]		144.3 [68.8-356.7]	
Amaurosis fugax	22/135 (16%)	16.6 [12.5-24.9]		19.4 [15.6-46.6]		296 [240-424]		120.9 [46.3-327.8]	
TIA	51/135 (38%)	16.2 [11.5-23.2]		20.9 [16.9-36-9]		300 [223-512]		106.3 [32.9-281.6]	
Stroke	27/135 (20%)	15.4 [12.3-22.7]		19.8 [15.0-25.2]		352 [257-515]		135.4 [66.6-319.9]	

Data are presented as No. (%) and median [IQR] unless otherwise indicated; r = Spearman's rank correlation coefficient; sd = standard deviation; IQR = interquartile range; BMI = body mass index; TIA = transient ischemic attack; * p-value represents statistical analysis for asymptomatic patients versus symptomatic patients (composed of amaurosis fugax, TIA and stroke)

Plasma immunoglobulin levels and mast cell parameters

ACPA IgG plasma levels correlated positively with oxLDL-IgG (r=0.231, p=<0.01) and total IgE plasma levels (r=0.229, p=<0.01). Between total IgE and oxLDL-IgG a correlation was found borderline significant (r=0.169, p=0.051). Total IgG did not correlate to any of the other immunoglobulins measured. No association was found between total IgE, total IgG, ACPA-IgG and oxLDL-IgG plasma levels and total mast cell numbers or total degranulating mast cells (Table 2). In addition we did not observe any association between the four immunoglobulins and tryptase plasma levels.

Table 2 | Immunoglobulin plasma levels with respect to mast cell parameters

	Total IgG	p-value	ACPA-IgG	p-value	oxLDL-IgG	p-value	Total IgE	p-value
Total mast cells	r = -0.038	0.664	r = -0.080	0.358	r = 0.137	0.114	r = -0.038	0.664
MCs/mm^2	r = -0.104	0.23	r = -0.073	0.403	r = 0.115	0.186	r = -0.014	0.872
Degranulating MCs/mm^2	r = -0.074	0.519	r = -0.163	0.152	r = 0.008	0.946	r = -0.076	0.506
Plasma tryptase	r = -0.076	0.378	r = 0.049	0.575	r= 0.040	0.643	r = 0.064	0.457

Data are presented as Spearman's rank correlation coefficient (r)

Plasma immunoglobulin levels and vulnerable plaque characteristics

As depicted in Figure 1, no consistent associations were found between immunoglobulin levels and measures of vulnerable plaque phenotype. There was no association between immunoglobulin levels and any of the following plaque characteristics: fat deposition, collagen, smooth muscle cells, macrophages, microvessel density.

Plasma immunoglobulin levels and clinically relevant characteristics

Table 1 provides an overview of the associations between the four immunoglobulin expression levels and relevant clinical characteristics. A negative association was observed between statin use and total IgE (97.5 [43.1-276.8] vs. 157.3 [75.2-545.4] ng/mL, p=0.012), ACPA-IgG (19.1 [15.0-30.4] vs. 24.8 [18.3-53.6] U/mL, p=0.009) and oxLDL-IgG (288 [224-406] vs. 399 [282-584] mU/mL, p=0.004). Higher total IgE levels were observed in smokers compared to none smokers (91.8 [62.3-145.0] vs. 76.7 [47.1-111.2] ng/mL, p=0.002). Clinical presentation was not associated immunoglobulin levels: no differences were observed in expression levels for all four immunoglobulins between asymptomatic patients (n=35) and symptomatic (n=100) patients. Additionally, no association was found between immunoglobulin levels and the delay between surgery and presentation of symptoms. ACPA-IgG

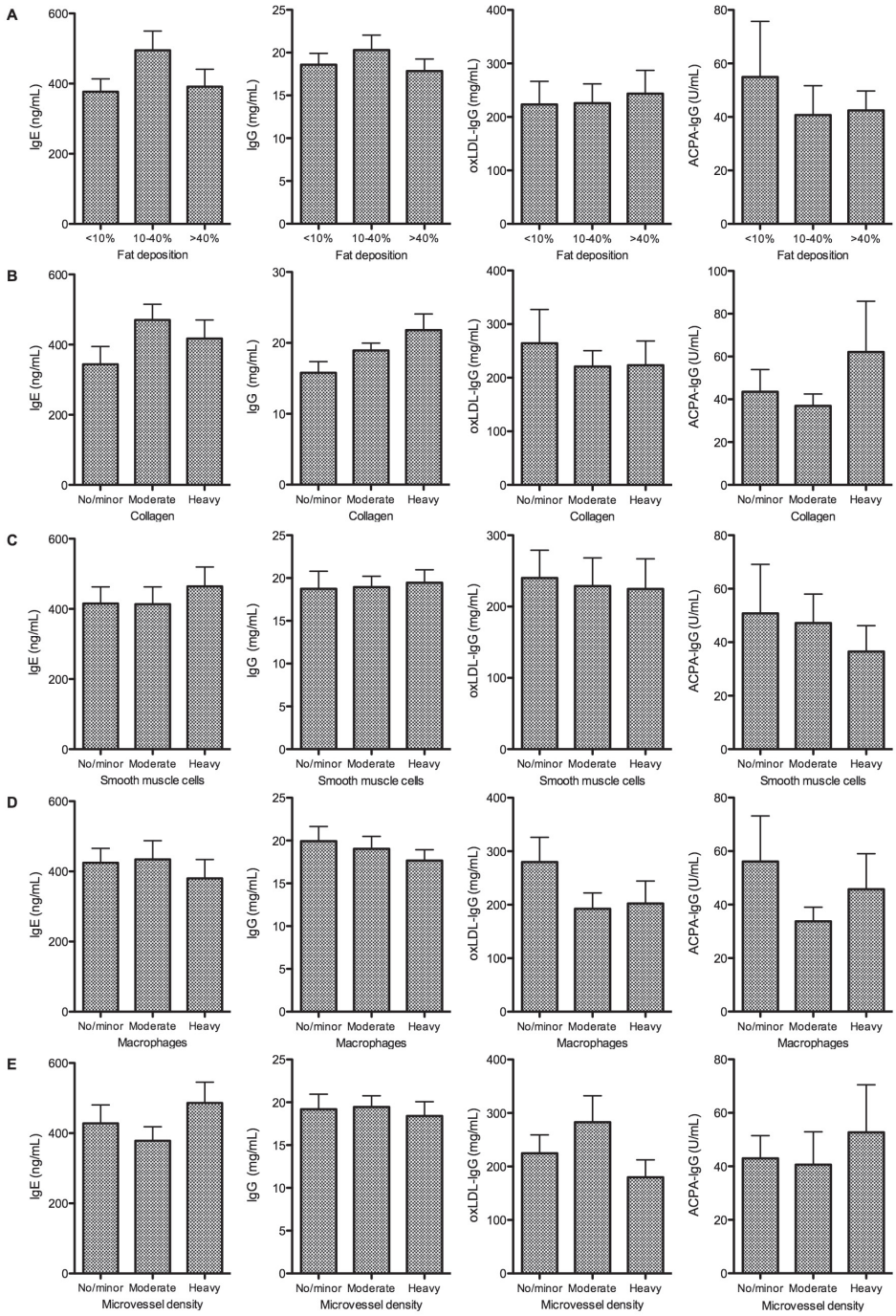

Figure 1 | Immunoglobulin plasma levels with respect to histological parameters of the plaque. Immunoglobulin plasma levels in relation to fat deposition (A), collagen (B), smooth muscle cells (C), macrophages (D) and microvessel density (E).

levels tended to be lower in patients that had a previous vascular intervention (p=0.035) or myocardial infarction (p=0.087).

DISCUSSION

Progression of an atherosclerotic plaque is often characterized by measures of plaque vulnerability. A vulnerable plaque is prone to rupture resulting in severe cardiovascular complications. Experimental studies have shown that mast cell activation results in progression and destabilization of the atherosclerotic plaque. In addition, in human plaques, mast cells correlated with vulnerable plaque characteristics and appeared associated with future combined cardiovascular events.[4] It has been suggested that activated mast cells induce intraplaque neoangiogenesis, thereby making the plaque more susceptible for rupture. For therapeutic intervention, identification of the endogenous mast cell activators during the progression and destabilization is of great value. In allergy and asthma, IgE is the main trigger for mast cell degranulation via crosslinking of the FcεR, while specific IgG immune complexes can exert mast cell activation resulting in cytokine release via binding to various Fcγ receptors. Previous studies have demonstrated that plasma IgE levels may be linked to the presence of cardiovascular diseases[11] and mice lacking the FcεR displayed reduced atherogenesis.[18] Furthermore, oxLDL specific IgG molecules that can form immune complexes with oxLDL have been detected in human and rabbit atherosclerotic plaques[19] and these immune complexes were able to induce TNFα and IL-8 release from human mast cells.[20] Taken together, these data suggest that IgE and specific IgGs may be important mast cell activators in cardiovascular disease.

It is however still unknown whether circulating Igs are capable of activating mast cells in human atherosclerosis. Here we show that circulating IgG, IgE, ACPA-IgG and oxLDL-IgG are not associated with mast cell determinants in a patient cohort with severe carotid stenosis that underwent an endarterectomy. No association was observed between any of the four immunoglobulins measured in this study and total plaque MC numbers or MC tryptase plasma levels. In addition, we did not observe any correlation with intraplaque degranulating MC numbers. Our results do not provide supportive evidence that increased Ig expression induces activation of mast cells in advanced human atherosclerotic plaques.

Previously, IgE was shown an independent marker for cardiovascular disease in men.[21] Therefore we explored the possibility of an association between the four

Igs and future adverse events in patients with established cardiovascular disease. We did not find any association between Ig levels and future cardiovascular events, however, we were underpowered for analysing risk prediction for the Igs in our cohort. Nevertheless, no association was found with histological markers of plaque vulnerability, one of the most important determinants for future cardiovascular complications.

Serum oxLDL specific IgG antibodies have previously been linked to the presence and destabilization of the atherosclerotic plaque.[22] As mentioned above, specific IgGs have been observed within the atherosclerotic plaque. In the current study, we did not detect any correlation between plasma oxLDL-IgG levels and mast cell activation or plaque phenotype. However, systemic oxLDL-IgG levels may not be reflective for the local oxLDL-IgG immune complexes that may actually activate the mast cell within the atherosclerotic plaque. Histological analysis of immune complexes, and with that also local IgE content, and colocalization with mast cells within the plaque may provide more information on the mechanisms of mast cell activation in atherosclerosis.

Antibodies against anti-citrullinated proteins (APCA) have been found to be increased in patients with rheumatoid arthritis (RA), and as atherosclerosis and RA have been previously found to share homologies in inflammatory response, we determined ACPA-IgG levels in our patients. Interestingly, a relatively large percentage of patients suffering from atherosclerosis (24 out of 135) appeared positive (>50 aU/mL) for the presence of these ACPA-IgG antibodies, while only one patient actually suffered from RA.

Interestingly, lower IgE, ACPA-IgG and oxLDL-IgG levels were observed in patients that used statins. When we differentiated between the patients on statins and the patients not on statins it does not affect the outcome of the associations observed between Ig levels and mast cell parameters or plaque vulnerability (data not shown). The inhibitory effects of statins on oxLDL-IgG antibody levels have been previously described,[22] however reduced levels of IgE and ACPA-IgE after statin treatment has to our knowledge not been reported before. These data thus identify a novel effect of statin treatment in addition to lipid-lowering.

In conclusion, no associations were found for total IgE, total IgG, ACPA-IgG and oxLDL-IgG and the presence of total mast cell numbers or the number of degranulating mast cells in atherosclerotic plaques. Furthermore, the Igs were not related to most of the established characteristics of the rupture-prone atherosclerotic plaques. Taken together, this study does not provide supportive

evidence that the four investigated circulating immunoglobulins activate mast cells during the progression of atherosclerotic disease. We can however not exclude that the mast cells may be activated by other specific Igs, or that local factors within the vessel wall are more predominant determinants of mast cell activation in the atherosclerotic plaque. Future research on the local environmental specific IgE and IgG levels within the plaque may thus shed more light on mechanism of mast cell activation in atherosclerosis.

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PART IV

SUMMARY

CHAPTER

9

Summary, general discussion and future perspectives

SUMMARY AND GENERAL DISCUSSION

According the latest factsheets of the world health organization, cardiovascular disease remains a major burden for western society.[1] Due to an aging population and an unhealthy lifestyle, cardiovascular disease morbidity numbers are still increasing. The incidence is especially high amongst patients with vascular occlusive disease, secondary to atherosclerosis. Due to atherosclerotic plaque progression (stenosis or plaque rupture) blood flow to organs downstream of the occlusion is reduced to such extent that organ failure or even death might follow. Consequently, finding novel therapeutic opportunities is still of interest. One possible alternative is the stimulation of new vessel growth also referred to as neovascularization. The two most prominent processes of neovascularization are angiogenesis and arteriogenesis (collateral vessel growth).[2] An inflammatory environment has been shown essential for the growth of new vessels.[3] This explains why previous targets found to stimulate neovascularization were mainly inflammatory mediators. The emphasis of this thesis is put on the search for new targets. In the first part we focused on different aspects of the IL-33/ST2 pathway, a fairly new pathway involved in many inflammatory processes. In the second part we concentrated on the mast cell and its role in intraplaque angiogenesis. Mast cells are particularly known for their role in allergy, but are also implicated in many inflammatory processes, amongst others atherosclerosis.

The IL-33/ST2 pathway

In order to cope with the increasing incidence of cardiovascular disease, there is a need to identify those patients at risk for future cardiovascular complications on top of drug development. Soluble ST2 is an emerging biomarker that gained substantial interest for the purpose of risk stratification in cardiovascular disease. Since the finding that sST2 levels were elevated in serum of patients that experienced a myocardial infarction,[4] numerous groups have reported that sST2 has predictive value independently of other risk factors for heart failure and mortality in patients with ischemic heart disease (**chapter 2**). For all biomarkers it is important to assess factors that can influence expression levels to optimize the predictive and diagnostic value for future events. In **chapter 3** we studied the effects of cardiovascular interventions and clinical risk factors on sST2 expression levels in patients with vascular occlusive disease. An increase in plasma sST2 expression level was observed following cardiovascular interventions. Levels

peaked at 24 hours after intervention, and were substantially higher in CABG patients compared to peripheral vascular surgery. These data suggest that tissue damage independent of tissue origin and the degree of injury are important determinants of sST2 expression levels. This should be taken into account in future prediction studies. Another research question we tried to assess in this study was whether the increase in sST2 expression level had any biological relevance. Worse outcome in patients with high sST2 levels might be explained by the decoy function of sST2;[5] it captures IL-33 from the circulation and thereby prevents cardioprotective intracellular signaling.[6,7] However, another possibility is that sST2 expression levels are up regulated in an attempt to protect the human body from excessive injury. With a persistent inflammatory response, a negative feedback mechanism has to be activated to prevent further damage. In different cell types it was observed that sST2 administration downregulated LPS induced cytokine production independently of IL-33.[8–10] In **chapter 3** we observed a large increase in sST2 plasma levels in patients 24 hours after CABG. Furthermore we observed differences is sST2 levels between patients, therefore we hypothesized that patients with high sST2 levels would present lower Toll-like receptor (TLR) cytokine plasma levels than the patients with low sST2 levels (**chapter 4**). Unfortunately we did not see a negative association between sST2 and TLR cytokines in the plasma. However, we did observe that cytokine levels secreted by blood monocytes of patients with high levels of sST2 after ex vivo LPS stimulation were significantly lower compared to the cytokine levels secreted by blood monocytes of patients with low sST2 levels. This suggest that the immunoparalytic effect of high sST2 levels after 24 hours is not yet reflected in blood cytokine levels in comparison to patients with low sST2 plasma levels, but is reflected in the responsiveness of the circulating inflammatory cells. We acknowledge that this observation is only associative. In this perspective it would be interesting to see whether this negative association will be reflected in blood cytokine levels after a longer period of time. Furthermore, in vitro cell experiments with human plasma containing different sST2 levels might give additional information whether this mechanism occurs in human disease.

In an atherosclerotic mouse model (ApoE-/-), it was shown that IL-33 administration resulted in smaller plaques accompanied with less inflammatory cell accumulation.[7] In contrast, they show that an increased plaque size was observed in atherosclerotic mice receiving sST2 treatment, although plaque phenotype remained unaltered. The relation between sST2 expression levels and

vulnerable plaque characteristics in humans was yet to be explored. To investigate this, sST2 levels were measured in patients of the Athero-Express biobank. In this biobank patients undergoing carotid endarterectomy are included and followed up to three years, making the database suitable for studying plaque vulnerability and risk prediction.[11] Therefore, we investigated whether sST2 expression levels were associated with plaque phenotype and future cardiovascular events in a patient population with cerebrovascular disease (**chapter 5**). In this study no relations between sST2 plasma levels and histopathological characteristics of a rupture-prone plaque, as plaque angiogenesis, were shown. Yet, an association with intraplaque bleeding was observed and as plaque haemorrhage is a strong determinant of plaque destabilization this merits further research.[12,13] Nevertheless, no association between sST2 plasma levels and future cardiovascular events were observed in this patient cohort. An interesting observation, as this implies that sST2 levels do not relate to progression of atherosclerotic disease but probably only reflect a state of tissue damage as occurs to a higher extent in patients with severe acute ischemia. This might explain why sST2 levels may have additional value in risk prediction for cardiac disease in patients with acute manifestations of myocardial ischemia[14] or severe chronic heart failure,[15] but not in a subgroup of patients with slowly progressing cerebrovascular disease. Future investigations are necessary to investigate this hypothesis.

In the final chapter of this part we investigated the effect of IL-33 on collateral growth in the mouse hind limb model. The main sources for IL-33 are healthy endothelial cells.[16] In endothelial cells of tumour vessels IL-33 is completely absent.[17] IL-33 was shown to possess several angiogenic properties in vitro and in vivo.[18] However another study showed that nuclear IL-33 disappeared upon angiogenic stimulation.[19] Together with the finding that IL-33 intracellular signaling was protective in atherosclerotic disease,[7] we hypothesized that this pathway might be an interesting candidate for the stimulation of arteriogenesis. The effects of IL-33 administration on arteriogenesis were not yet investigated. We show that systemic injection of IL-33 actually inhibits perfusion recovery (**chapter 6**). Control mice injected with NaCl have about 50% more perfusion restoration compared to IL-33 treated mice. IL-33 was shown to exert most of its functions through T-cells.[20,21] T-cells are known to play a key role in matrix degradation and vessel remodelling, both critical processes for collateral growth.[22–25] However, the hampered perfusion recovery could not be explained by a difference in T-cell numbers accumulating in the perivascular space. Furthermore, we show

that there is no difference in perfusion recovery between WT mice and ST2-/- mice. We expected better perfusion recovery in ST2-/- mice, as no intracellular signaling of IL-33 would be possible. A reasonable explanation for this observation could be that physiological IL-33 levels are too low in the WT mice and therefore insufficient to exert an inhibitory effect on perfusion recovery. In this case, it makes perfect sense that no improved perfusion recovery was observed in ST2-/- mice. Although ample aspects of this pathway remain unclear, IL-33 was shown unfit for the use of therapeutic enhancement of arteriogenesis. Nevertheless, our results together with existing literature do imply an important role for the IL-33/ST2 pathway in new vessel growth. Elucidating the underlying mechanism might eventually provide knowledge necessary for future drug development.

Mast cells

In this part we investigated mast cells and their components as possible targets for the stimulation of new vessel growth. Mast cells are major effector cells in allergy and innate immunity.[26] Experimental research has established a role for activated mast cells in the initiation and progression of atherosclerotic disease.[27–29] Post mortem studies showed that mast cells with angiogenic factors were predisposed near the microvessels in atherosclerotic lesions.[30–33] In **chapter 7** we sought to find additional proof that mast cell might render the plaque vulnerable by the inducing angiogenesis. This research question was addressed by using patients with carotid stenosis included in the Athero-Express biobank.[11] As hypothesized, mast cells associated with plaque vulnerability. A high correlation between mast cells and intraplaque angiogenesis was observed. This association was independent of macrophage and neutrophil presence, underlining the prominent role of the mast cell in plaque progression. Nonetheless, being a merely associative study, it remains to be elucidated whether intraplaque mast cell presence is a cause or consequence of microvessel density.

Furthermore, mast cells associated with intraplaque haemorrhage and thrombus formation, two important determinants of cardiovascular manifestations. In line with this observation we found that intraplaque mast cells and tryptase plasma levels associated with the occurrence of adverse cardiovascular events. Important to note is that this was not observed for macrophage or neutrophil numbers in the plaque.[34,35] Taken together this data indicates that mast cells are more important in atherosclerotic disease than was originally thought. Further experiments are necessary to provide additional proof that indeed activated mast cells are responsible for plaque angiogenesis.

A question that remains is how mast cells are activated to stimulate plaque neovascularization. It was hypothesized that specific antibodies might activate mast cells, as occurs during allergy or innate immune responses. Receptors for IgG and IgE are situated on the surface of mast cells.[36,37] In patients with severe atherosclerosis, antibodies against oxLDL have been found in the circulation.[38] Also total IgE and IgG levels have been implicated in atherosclerotic disease. Therefore we investigated whether circulating immunoglobulin levels associated with (degranulating) mast cell numbers (**chapter 8**). No relation was observed between the four investigated immunoglobulins IgG, IgE, ACPA-IgG and oxLDL-IgG and intraplaque (degranulating) mast cell numbers or tryptase plasma levels. In line with this data we show that there is no relation between the immunoglobulins and plaque vulnerability. This data does not imply that immunoglobulins do not exert any role in atherosclerosis; though levels do not reflect disease severity. Numerous other factors are described in literature that can activate mast cells, including IL-33.[39,40] However based on our own data we do not suggest that this is the case in vessel formation. Future research is needed to identify which factors are responsible for mast cell activation. This knowledge could result in better understanding of the complete process and ultimately in a new therapeutic solution for patients with occlusive disease.

FUTURE PERSPECTIVES

The ultimate goal of this thesis was to validate and characterize new targets for the therapeutic enhancement of neovascularization. Our data implies that interference in the IL-33/ST2 pathway is not likely to contribute to the improvement of tissue neovascularization. In contrast of what we expected, we found that perfusion restoration after femoral artery ligation was inhibited in IL-33 treated mice. This suggests that inhibition of IL-33 intracellular signaling might be used to enhance neovascularization. However, the observation that we did not observe any difference in perfusion restoration between WT and ST2 deficient mice, suggests that physiologic IL-33 levels do not affect perfusion restoration. Therefore administration of an agonist of IL-33 or sST2 recombinant will not likely result in improved collateral growth. Although this makes the pathway unsuitable for therapeutic enhancement of neovascularization, the effect of IL-33 administration on collateral growth was evident. Furthermore, we also observed a negative association between sST2 levels 24 hours after CABG procedure and

TLR expression levels and cytokine secretion. As TLR mediated inflammation has been shown important in almost all inflammatory processes, including new vessel formation, our data implies that investigating the IL-33/ST2 pathway in more depth, might provide additional knowledge important for unravelling the mechanism of vessel growth.

The contribution of mast cells to new vessel growth has been studied extensively. Mast cells and their expressed biological components have been shown to possess angiogenic properties in normal and pathological conditions. Our data shows that mast cells are highly associated with intraplaque angiogenesis. In addition to previous research, we show that this association appears to be independent of other inflammatory cell types as macrophages that are also known to stimulate angiogenesis. Furthermore, the number of mast cells associated with intraplaque haemorrhage. The observation that mast cells are the only reported inflammatory cell type in plaques that associated with future combined cardiovascular events, supports the view that mast cells are more important than was originally considered. Our findings together with existing literature provides a strong indication that further investigation of mast cells in the process of new vessel growth might be of value for therapeutic purposes. We suggest that elucidating the precise mechanism how mast cells are involved in the induction of neovascularization might eventually result in the development of new therapeutic strategies to accelerate collateral vessel formation.

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PART IV

SUMMARY

CHAPTER

10

Nederlandse samenvatting

Vandaag de dag vormen hart- en vaatziekten de belangrijkste doodsoorzaak in de Westerse wereld. Naar verwachting zal het aantal mensen dat lijdt aan deze ziekten verder stijgen mede als gevolg van een ongezonde levensstijl en een toename van de levensverwachting. In veel gevallen is de oorzaak van deze ziekten gelegen in een verstoring die optreedt in de wanden van de bloedvaten. Deze verstoring wordt aangeduid met de term aderverkalking of atherosclerose. Het gaat hierbij om een chronisch ontstekingsproces waarbij cholesterol en ontstekingscellen zich ophopen in de vaatwand. Zo'n ophoping wordt een plaque genoemd. Wanneer een dergelijke plaque blijft groeien (stenose) of scheurt (ruptuur) kan een bloedvat geheel of gedeeltelijk worden afgesloten met als gevolg dat de toevoer van bloed naar organen en weefsels die van dat bloedvat afhankelijk zijn stopt of sterk verminderd. Een constante bloedtoevoer is essentieel om organen en weefsels van voldoende voedingsstoffen en zuurstof te voorzien. Gebeurt dit niet of onvoldoende dan zal er orgaanschade optreden. In ernstige gevallen kan dit leiden tot orgaanfalen of zelfs de dood. Het is daarom van belang om adequate therapeutische oplossingen te vinden voor het probleem van aderverkalking. Een mogelijke oplossing is het stimuleren van de groei van nieuwe bloedvaten. De vorming van nieuwe bloedvaten wordt neovascularisatie genoemd. De twee voornaamste processen van neovascularisatie zijn angiogenese en arteriogenese (collaterale vaatgroei). Het is bekend dat voor de groei van nieuwe vaten een zogenaamde inflammatoire omgeving van groot belang is. Dat wil zeggen dat de juiste ontstekingsmediatoren aanwezig moeten zijn in het gebied waar de groei van de nieuwe bloedvaten moet plaatsvinden. In dit proefschrift hebben we twee componenten van het ontstekingssysteem, te weten de IL-33/ST2 signaleringsroute en de mestcel, onderzocht op hun geschiktheid als therapeutisch aangrijpingspunt met betrekking tot het stimuleren van vaatgroei tijdens aderverkalking.

De IL-33/ST2 signaleringsroute

De IL-33/ST2 signaleringsroute speelt een belangrijke rol bij verschillende inflammatoire ziekten. De receptor van IL-33, ST2, komt voor als de membraangebonden receptor ST2L en de vrij oplosbare receptor soluble ST2 (sST2). Verschillende studies hebben laten zien dat de intracellulaire signaleringsroute die wordt aangezet door binding van IL-33 aan ST2L een positief effect heeft op hart- en vaatziekten, inclusief aderverkalking. Soluble ST2 kan dit positieve effect van IL-33 tenietdoen door het te binden en zo te voorkomen dat het aan ST2L kan binden. Behalve dat het belangrijk is om nieuwe therapeutische oplossingen te vinden voor hart- en vaatziekten is het van groot belang om mensen op te

sporen die een verhoogd risico lopen op de complicaties die gepaard gaan met deze ziekten. Soluble ST2 is een potentiële biomarker welke voorspellende waarde heeft voor toekomstig hartfalen en mortaliteit in patiënten met ischemische hartziekten (**hoofdstuk 2**). Voor alle biomarkers is het van belang dat wordt uitgezocht door welke factoren hun waarden worden beïnvloed. In **hoofdstuk 3** hebben we onderzocht of chirurgische cardiovasculaire interventies van invloed zijn op de bloedwaarden van sST2 in patiënten met ernstig vaatlijden. Dit bleek inderdaad het geval te zijn. Ons onderzoek laat zien dat sST2 bloedwaarden significant gestegen zijn na een cardiovasculaire ingreep. We denken dat deze stijging in sST2 bloedwaarden veroorzaakt wordt door de vaatschade die optreedt bij een dergelijke interventie en dat de ernst van de beschadiging mede bepalend is voor de mate van de stijging. Bij toekomstig onderzoek waarin sST2 als biomarker wordt getoetst zal rekening moeten worden gehouden met deze bevindingen.

Een andere vraag die we geprobeerd hebben te beantwoorden is of de stijging van de sST2 bloedwaarde ook daadwerkelijk een biologische functie heeft bij de mens. Er is namelijk ontdekt dat behalve het wegvangen van IL-33, sST2 een negatief effect heeft op de Toll-like receptor (TLR) response. De TLR response is nodig om een inflammatoire omgeving te induceren die dodelijk is voor bacteriën en virussen die het lichaam zijn binnengedrongen. Bij een ontsteking komen tal van chemische factoren (cytokines) vrij die helpen deze indringers uit te schakelen. Uit onderzoek is gebleken dat tijdens een aanhoudende inflammatoire response een negatieve terugkoppelingscascade ingang wordt gezet om onnodige lichaamsschade te voorkomen. Het is ook bekend dat deze cascade binnen enkele minuten wordt geactiveerd na het optreden van vaatschade zoals bijvoorbeeld tijdens een operatie. In een groep van patiënten die een hartoperatie hebben ondergaan hebben we onderzocht of sST2 mogelijk verantwoordelijk is voor deze negatieve terugkoppeling. We hebben dit gedaan door te kijken naar de reactiestaat van de bloedcellen (**hoofdstuk 4**). De bloedwaarden van sST2 voor en na de operatie werden onderzocht op mogelijke associaties met bepaalde cytokines en TLR receptoren. We vonden dat alleen de sST2 bloedwaarden van 24 uur na operatie negatief geassocieerd waren met die van de onderzochte cytokines en TLR receptoren. Dit doet vermoeden dat sST2 geen rol speelt bij de snelle negatieve terugkoppeling, maar mogelijk wel bij een langdurig verminderde immuun response.

Zoals eerder vermeld heeft IL-33 een gunstig effect op aderverkalking. In experimenten met muizen is aangetoond dat het toedienen van IL-33 resulteerde in kleinere plaques met minder ontstekingscellen, terwijl het toedienen van

sST2 juist leidde tot grotere plaques. Het was echter nog niet bekend of de sST2 bloedwaarden misschien ook geassocieerd zouden kunnen zijn met de progressie van aderverkalking of het plaque fenotype bij de mens. Deze mogelijkheid hebben we in **hoofdstuk 5** onderzocht bij patiënten met ernstige aderverkalking van de halsslagaders. Bij deze patiënten wordt de plaque operationeel verwijderd en in het laboratorium onderzocht op een tal van eigenschappen ter bepaling van het fenotype. Uit ons onderzoek bleek echter dat er geen associatie was tussen de sST2 bloedwaarden en het fenotype van de plaque. Nadat deze patiënten zijn geopereerd worden ze nog 3 jaar gevolgd en eventuele complicaties gerelateerd aan hart- en vaatziekten worden gedocumenteerd. Hier zagen we geen voorspellende waarde van de sST2 bloedwaarden met betrekking tot het optreden van secondaire complicaties. Op basis van onze onderzoeksresultaten denken wij dat sST2 bloedwaarden zeer waarschijnlijk niet gerelateerd zijn aan de progressie van aderverkalking, maar eerder een reflectie zijn van weefselschade zoals optreedt bij een acuut zuurstoftekort. Verder onderzoek zal moeten uitwijzen of deze veronderstelling juist is.

In het laatste hoofdstuk van dit deel van het proefschrift hebben we gekeken of manipulatie van de IL-33/ST2 signaleringsroute daadwerkelijk een effect heeft op vaatgroei. In eerdere studies was reeds een verband gevonden tussen de IL-33/ST2 signaleringsroute en angiogenese. Samen met de ontdekking dat IL-33 een gunstig effect heeft op aderverkalking leek deze signaleringsroute een geschikte kandidaat voor een mogelijke therapeutische oplossing. Experimentele resultaten zijn schaars en ook het effect van IL-33 op collaterale vaatgroei was nog niet onderzocht. In **hoofdstuk 6** beschrijven we dat het toedienen van IL-33 een negatief effect had op het perfusie herstel in een muismodel waarbij de beenslagader ter hoogte van de lies wordt afgebonden. We laten zien dat dit negatieve effect van IL-33 niet werd veroorzaakt door een verandering van het aantal T-cellen, waarvan bekend is dat ze door dit cytokine beïnvloed worden en dat ze een belangrijke rol spelen bij het proces van collaterale vaatgroei. Op grond van bovenstaand resultaat hadden we verwacht dat het perfusieherstel na het afbinden van de beenslagader in ST2 deficiënte muizen juist versneld zou plaatsvinden, aangezien er geen IL-33 intracellulaire signalering mogelijk is. We vonden echter geen verschil in de snelheid van herstel tussen de wild type en ST2 deficiënte muizen. Een mogelijke verklaring zou kunnen zijn dat de IL-33 waarden die in de wild type muis bereikt worden zo laag zijn dat zij geen invloed hebben op het herstel van perfusie. Hoewel er nog veel onderzoek naar de IL-33/ST2 signaleringsroute gedaan moet worden maakt onze bevinding deze ongeschikt

voor een eventuele medische toepassing waar het gaat om het stimuleren van vaatgroei. Het negatieve effect van IL-33 was echter significant, daarom kan meer onderzoek naar deze signaleringsroute mogelijk wel helpen om een beter inzicht te krijgen in de onderliggende mechanismen van vaatgroei. Deze kennis kan van groot belang zijn voor de ontwikkeling van een medicijn.

De mestcel

Mestcellen zijn ontstekingscellen die een belangrijke rol spelen bij allergische reacties en immuunresponsen. Experimenteel onderzoek heeft aangetoond dat geactiveerde mestcellen betrokken zijn bij het ontstaan en de progressie van aderverkalking en dat vaatgroei hier mogelijk een rol bij speelt. Plaque angiogenese is een van de kenmerken van een kwetsbare plaque. In plaques die postmortaal werden verkregen bleken mestcellen in de buurt van bloedvaatjes aanwezig te zijn. Het is al langer bekend dat deze cellen of componenten in hun granules angiogenese kunnen stimuleren. Hetzelfde is echter ook aangetoond voor macrofagen en andere ontstekingscellen. In **hoofdstuk 7** laten we zien dat er een zeer hoge associatie bestaat tussen de aanwezigheid van mestcellen en plaque angiogenese en dat deze associatie onafhankelijk lijkt van andere ontstekingscellen. Dit zou mogelijk kunnen beteken dat mestcellen belangrijker zijn voor de vorming van nieuwe bloedvaten dan bijvoorbeeld macrofagen. Deze studie was echter puur associatief en het blijft de vraag of de aanwezigheid van mestcellen een oorzaak of een gevolg is van de nieuwe vaatgroei. Een interessante bevinding uit deze studie was verder dat de aanwezigheid van mestcellen geassocieerd bleek te zijn met het optreden van bloedingen in de plaque en de vorming van bloedstolsel (trombus), twee belangrijke processen die vaak gepaard gaan met ernstige complicaties. In goede overeenkomst met dit resultaat vonden we dat mestcellen ook geassocieerd waren met secundaire manifestaties als gevolg van hart- en vaatziekten. Hierbij is van belang dat dit nooit eerder is aangetoond voor andere ontstekingscellen. Samenvattend wijzen onze data erop dat mestcellen belangrijker zijn dan oorspronkelijk werd gedacht. Verder onderzoek zal moeten uitwijzen of geactiveerde mestcellen inderdaad verantwoordelijk zijn voor plaque angiogenese.

Als we ervan uitgaan dat mestellen inderdaad verantwoordelijk zijn voor plaque angiogenese dan rest de vraag hoe deze cel geactiveerd wordt. In **hoofdstuk 8** hebben we de mogelijke rol van (specifieke) antilichamen onderzocht. Het is namelijk bekend dat patiënten met ernstige aderverkalking antilichamen maken tegen specifieke vetdeeltjes. Ook van andere antilichamen is aangetoond dat

ze een rol spelen bij aderverkalking. Uit onze studie kwam echter naar voren dat geen van de onderzochte antilichamen een associatie vertoonde met de hoeveelheid mestcellen in de plaque en/of de activatiegraad van deze cellen. Overeenkomstig met dit resultaat werd evenmin een associatie gevonden tussen deze antilichamen en het fenotype van de plaques. De literatuur kent nog tal van andere factoren die mestcellen kunnen activeren en er is dus meer onderzoek nodig om te achterhalen welke van deze factoren verantwoordelijk zijn voor de activatie van de in de plaque aanwezige mestcellen. Dit onderzoek zal niet alleen leiden tot een volledig beter begrip van het totale proces, maar wij verwachten ook dat het uiteindelijk kan resulteren in het gebruik van mestcellen als therapeutisch aangrijpingspunt bij aderverkalking.

APPENDIX

Dankwoord

List of publications

Editorial

DANKWOORD

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*Both authors contributed equally. *Submitted*

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EDITORIAL

Are mast cells the real culprit in atherosclerosis?

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This editorial refers to 'Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events', by S. Willems *et al.*, doi:10.1093/eurheartj/eht186

Atherosclerosis in the presence of hyperlipidaemia is considered today as an inflammatory disease of the arterial wall. The dominant inflammatory cell is the macrophage; however, interaction with other inflammatory cells and red cells may be critical in the outcome of the plaque, i.e. whether plaques remain stable or become unstable. It has been repeatedly shown that macrophage interaction with T cells and dendritic cells is critical for the progression of plaques and for maintaining and sustaining inflammation. The role of mast cells in atherosclerosis has only been studied sporadically, and that too mostly in the aortic and coronary beds. Willems *et al.*[1] now show for the first time not only that severe carotid atherosclerotic disease is associated with a high number of mast cells but that the instability of the plaque may be influenced by the number of mast cells and their state of activation, which probably contribute to angiogenesis and plaque haemorrhage. Also, patients with high intraplaque mast cells had significantly greater cardiovascular events during a 3-year follow-up. However, the basic mechanisms involved will need further study in both animal models and man.

Paul Ehrlich's doctoral thesis was a milestone in the study of mast cells, and he noted that they had similar staining characteristics to basophils, demonstrating metachromatic staining with aniline dyes.[2] Mast cell development depends on the expression of KIT ligand, also known as mast/stem cell growth factor (SCF), receptor from the pluripotent haematopoietic progenitor cells (HPCs) that express $CD34^+$, $CD117^+$, $CD13^+$, $Fc\varepsilon RI^-$, maturing in the target tissues, such as skin, mucosal surfaces, and probably also in the arterial wall, although other factors are necessary.[3] The knowledge that anaphylaxis was linked to release of histamine, a component of mast cells, was the beginning of the understanding of the role of mast cells in immunological reactions especially in anaphylaxis and innate and adaptive immunity. Mast cells exert physiological functions when activated by stimuli such as IgE and IgG, FcR signalling by bacterial antigens, complement components, and endogenous inflammatory factors, which result in the immediate release of mast cell cytoplasmic granules. The mast cells either produce or release proinflammatory cytokines [interleukin-6 (IL-6), interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and others], chemokines [monocyte chemotactic protein-1 (MCP-1), IL-8, RANTES (regulated upon activation, normal T-cell expressed and secreted), eotaxin, and leukotrines], proteases [tryptase, chymase, angiotensin-converting enzyme (ACE), carboxypeptidase, and cathepsin G], growth factors [fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF)], histamine, heparin, and chondroitin sulfate proteoglycan. The proteases also exert their influence on LDL- and HDL-cholesterol. Mast cell tryptase and chymase promote macrophage LDL uptake and also result in functional changes in HDL_3 components, apolipoprotein (apo) E, apoA-I, and apoA-IV, that results in inability of foamy macrophages to promote cholesterol efflux.[4] (*Figure 1*)

Constantinides in 1953/54 first noted the presence of mast cells in atherosclerotic lesions in rabbits and man.[5] A higher prevalence of mast cells was shown predominantly in the adventitia of both aortic and coronary atherosclerotic lesions (raised lesions—fatty streak, fibrofatty plaque, and fibrous plaque) compared with those without any lesions in subjects 15–34 years of age dying of non-cardiac causes.[6] In the aorta, the number of mast cells was significantly greater in the dorsal (lesion 'prone') as compared with the ventral (lesion 'resistant') half of the wall. Also, the number of mast cells was 10 times greater in the adventitia in atherosclerotic lesions as compared with the intima.[6] By tryptase staining, mast cells have been demonstrated to be located in the endothelium and subendothelium, as well as in the shoulder regions of both early and more advancing plaques.[5] Degranulated mast cells have been found at the site of rupture and those mast cells can activate matrix metalloproteinase (MMP)-1, -2, and -9 through release of proteases leading to weakening of the fibrous cap, which makes them a powerful participant in the conversion of a stable lesion into a unstable plaque even if their numbers are far fewer than macrophages.[3] We have also shown that mast cell numbers were increased in a young man with transient vasospasm and minimal disease in the left anterior descending coronary artery.[7] Histamine, a powerful vasoconstrictor, has been shown to be increased in the coronary arterial wall of patients dying from coronary artery disease.[8] The most compelling data of the involvement of mast cell in the generation of unstable lesions come from the laboratory of Biessen who showed in ApoE−/− mice that activation of mast cells resulted in larger plaque area, an increase in

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