Detection of apoptosis in patients with coronary artery disease

Assessment of temporal patterns and potential sources

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In loving memory of my mother and father
Your legacy lives on

*"Do not go gentle into that good night.
Rage, rage against the dying of the light."*
- Dylan Thomas

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LIST OF PAPERS

This thesis is based on the following original publications, which will be referred to by their Roman numerals

I. Wei Li , Caroline Lidebjer, Xi-Ming Yuan, **Aleksander Szymanowski**, Karin Backteman, Jan Ernerudh, Per Leanderson, Lennart Nilsson, Eva Swahn, Lena Jonasson. NK cell apoptosis in coronary artery disease: relation to oxidative stress. *Atherosclerosis*. 2008 Jul;199(1):65-72.

II. **Aleksander Szymanowski**, Wei Li, Anna Lundberg, Chamilly Evaldsson, Lennart Nilsson, Karin Backteman, Jan Ernerudh, Lena Jonasson. Soluble Fas ligand is associated with natural killer cell dynamics in coronary artery disease. *Atherosclerosis*. 2014 Apr;233(2):616-22.

III. Lennart Nilsson, **Aleksander Szymanowski**, Eva Swahn, Lena Jonasson. Soluble TNF receptors are associated with infarct size and ventricular dysfunction in ST-Elevation Myocardial Infarction. *PLoS One*. 2013; 8(2): e55477.

IV. **Aleksander Szymanowski**, Joakim Alfredsson, Magnus Janzon, Tomas L. Lindahl, Eva Swahn, Lena Jonasson, Lennart Nilsson. Soluble markers of apoptosis in myocardial infarction patients during acute phase and 6-month follow-up.
Submitted

ABSTRACT

The atherosclerotic process and its consequences are considered driven by an imbalance between pro- and anti-inflammatory actions. One contributing factor in this scenario is an altered regulation of apoptosis, which affects both immune, vascular and myocardial cells. The general aim of this thesis was to measure soluble markers of apoptosis in peripheral venous blood, in various clinical stages of coronary artery disease (CAD) and to further identify possible sources with specific focus on natural killer (NK) cell apoptosis and myocardial ischemia-reperfusion (IR)-injury.

There was evidence of an increased apoptosis of NK cells, but not T cells, in the circulation of CAD patients. Spontaneous NK cell apoptosis and the cells' sensitivity to oxidative stress in the form of oxidized lipids *ex vivo*, were increased. Findings were thus suggestive of an enhanced apoptosis contributing to the reduced NK cell activity seen in CAD. However, we could not verify that oxidative stress in the circulation was a driving force behind this loss.

Soluble forms of the cell surface bound receptors of apoptosis include soluble (s) Fas and sFas ligand (L). They are detected in plasma and used as surrogate markers of apoptosis. Here we investigated the relationship between these markers and NK cell apoptosis and NK cell levels, in a 12 month longitudinal study on CAD patients. Plasma levels of sFasL correlated with increased susceptibility to NK cell apoptosis *ex vivo* but also with the levels of NK cells in the circulation after a coronary event. NK cells undergoing apoptosis *ex vivo* were also found to be a major source of sFasL themselves, indicating potential usefulness of sFasL in monitoring changes in NK cell levels.

Apoptosis is suggested to be a key event in IR-injury, resulting in increased infarct size, left ventricular (LV) dysfunction, remodeling and heart failure. We investigated soluble markers of apoptosis in relation to these parameters in a ST-elevation myocardial infarction (STEMI) population. In addition to sFas and sFasL, we also measured tumor necrosis factor (TNF) receptor (R) I and II in this study. Acute phase levels of sTNFRI and sTNFRII, but not sFas or sFasL, correlated to cardiac MR (CMR) measures of infarct size and LV-dysfunction at 4 months after the ischemic event. Also, the soluble markers of apoptosis were correlated with matrix metalloproteinase (MMP)-2, a mechanistic trigger for cardiomyocyte apoptosis, further strengthening the role of apoptosis in IR-injury.

Finally we explored the temporal patterns of soluble markers of apoptosis after an MI and, furthermore, investigated possible differences between patients presenting with a non(N)-STEMI versus STEMI. The sTNFRI/II and the sFas/sFasL pathways of apoptosis showed different temporal changes indicating diverse roles of these two systems. NSTEMI and STEMI patients however, shared these temporal patterns pointing to apoptosis as equally involved in either infarct type. Furthermore sTNFRs, but not sFas/sFasL correlated to levels of cytokine interleukin (IL)-6 illustrating the overlapping role TNF signaling in inflammation and apoptosis, while again suggesting differences between the TNF and the Fas/FasL systems during myocardial IR-injury.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vid åderförfettning (ateroskleros) ansamlas kolesterol och vita blodkroppar i kärlväggen, där de över tid bildar inflammatoriska härdar (plack). En vanlig plats för denna process är i kranskärlen, där placket kan expandera och orsaka en försnävning av kärlets inre diameter, vilket kan ge upphov till kärlkramp. Plackets egenskaper förändras över tid och risken finns att härden plötsligt spricker med blodproppsbildning och hjärtinfarkt som följd. Konsekvenserna av en hjärtinfarkt, såsom hjärtsvikt, är i stor utsträckning relaterade till hur stor del av hjärtmuskeln som tagit skada. Vid en stor hotande hjärtinfarkt är prio ett därför att omedelbart öppna det tilltäppta kärlet med ballongvidgningsteknik och på så vis begränsa hjärtskadans omfattning. Det plötsliga återflödet av blod till hjärtmuskeln (reperfusion) innebär dock potentiella risker, då det kan starta upp processer som ytterligare skadar hjärtmuskeln och ökar hjärtinfarktens storlek.

En bidragande faktor både till aterosklerosprocessen själv och till de beskrivna konsekvenserna tros vara en rubbad programmerad celldöd, s. k. apoptos, av ett flertal celltyper. Syftet med denna avhandling var att hos kranskärlssjuka mäta markörer för apoptos i blodet och dels relatera dessa till vita blodkroppar, fr a NK-celler, samt undersöka eventuella kopplingar mellan apoptosmarkörer och graden av skada vid hjärtinfarkt.

Tydliga skillnader sågs mellan kranskärlssjuka patienter och friska individer, såsom en ökad apoptos av NK-celler i blodet. Benägenheten hos NK-celler att gå i apoptos bekräftades både då de lades i neutralt odlingsmedium samt vid påverkan från oxidativ stress, dvs cellulär påverkan från reaktiva syreföreningar, en komponent i aterosklerosprocessen. Bedömningen blev att en ökad apoptos som orsak till ett sänkt NK-cellsantal föreligger vid koronarsjukdom. Dock kunde en oxidativ stress i cirkulationen som strikt orsak till denna cellförlust ej verifieras.

Den ökade benägenheten hos NK-celler att gå i apoptos korrelerade med halter av lösliga apoptosmarkörer i blodet och nivåerna av en av markörerna, sFasL följde också *antalet* NK-celler i blodet vid återhämtningsfasen efter en hjärtinfarkt. NK-cellerna visade sig också själva kunna frisätta sFasL. Sammantaget påvisades en ökad omsättning av NK-celler efter hjärtinfarkt och kopplingen till sFasL tyder på att denna skulle kunna utgöra en surrogatmarkör för förändringar av antalet NK-celler i blodet.

Apoptos bedöms bidra till den reperfusionsskada som kan uppstå då blodflödet plötsligt återställs vid behandlingen av en hjärtinfarkt. Vi fann i en sådan patientgrupp samband mellan de lösliga apoptosmarkörerna sTNFRI och sTNFRII i blodet och den slutliga infarktstorleken samt graden av nedsatt pumpförmåga i hjärtat, mätt med magnetkameraundersökning, 4 månader efter hjärtinfarkten. Detta pekar mot att konsekvenserna av behandlingen i akutskedet, med en ökad apoptos, kvarstår långt in i återhämtningsfasen.

Slutligen undersöktes fördelningsmönstret av lösliga apoptosmarkörer över tid hos patienter med olika typer av hjärtinfarkt. Mönstret visade sig vara likartat och pekade på att apoptos kan ha en viktig roll oberoende av infarkttyp. Dock tycks de olika apoptotiska signalsystemen ha skilda betydelser i dessa processer, då fördelningsmönstret för TNFR- respektive Fas/FasL-systemen var helt olika över tid.

Sammanfattningsvis stöder våra fynd att apoptos på flera sätt spelar en viktig roll vid koronarsjukdom. Att förhindra apoptos i situationer där ökad apoptos bidrar till sjukdomsprogress och därmed försämrad prognos torde medföra positiva konsekvenser.

ABBREVIATIONS

7-AAD	7 aminoactinomycin D
ACS	Acute coronary syndrome
ACVD	Atherosclerotic cardiovascular disease
ADCC	Antibody-dependent cell-mediated cytotoxicity
AP	Angina pectoris
APC	Antigen presenting cell
7 β OH	7 β hydroxycholesterol
CAD	Coronary artery disease
CCS	Canadian cardiovascular society functional class
CD	Cluster of differentiation
CHF	Congestive heart failure
CMR	Cardiac magnetic resonance
CVD	Cardiovascular disease
DC	Dendritic cell
δ EDVI	Change in end-diastolic volume index
δ ESVI	Change in end-systolic volume index
DISC	Death inducing signaling complex
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FasL	Fas Ligand
FOXP3	Forkhead box P3
GIK	Glucose-insulin-potassium
hsTnT	High sensitivity troponin T
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IR	Ischemia-reperfusion
KIR	Killer cell immunoglobulin-like receptors
LGE	Late gadolinium enhancement
LVEF	Left ventricular ejection fraction
MACE	Major adverse cardiac events
MHC	Major histocompatibility complex
MI	Myocardial infarction
MPO	Myeloperoxidase
MPTP	Mitochondrial permeability transition pore
MMP	Matrix metalloproteinase
NF- κ B	Nuclear factor κ B

NK cell	Natural killer cell
NO	Nitric oxide
NSTE-ACS	Non ST-elevation acute coronary syndrome
NSTEMI	Non ST-elevation myocardial infarction
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCI	Percutaneous coronary intervention
PI	Propidium iodide
PMT	Photo multiplier tube
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
STEMI	ST-elevation myocardial infarction
STNFR	Soluble tumor necrosis factor receptor
TBARS	Thiobarbituric acid reactive substances
TGF	Transforming growth factor
Th1	T helper 1
TIMI	Thrombolysis in myocardial infarction
TNF	Tumor necrosis factor
TLR	Toll like receptor
T reg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

BACKGROUND

Coronary artery disease

The spectrum of atherosclerotic cardiovascular diseases (ACVD), traditionally comprised of cerebrovascular, coronary artery and peripheral vascular disease, are responsible for a dominating 30 % of all deaths worldwide, with acute myocardial infarction (MI) as a complication of coronary artery disease being the single most frequent cause of death [1, 2]. In Sweden, as in most countries in Western Europe and North America, a steep decline in both the incidence and the mortality rate of acute MI has occurred over the last decades. This is due, both to primary and secondary preventive measures, as well as state of the art treatment in the acute setting. The opposite can be observed in many developing countries where an adaptation to a western-like lifestyle without appropriate medical resources has brought about an increasing ACVD burden with largely unchanged mortality rates [3, 4].

According to the INTERHEART trial, 9 out of 10 MIs can be explained by known, and to a large extent modifiable risk factors. In this case-control study comprising nearly 30000 subjects, a raised apoB/apoA1 ratio, smoking, psychosocial factors, diabetes, hypertension, abdominal obesity, no regular alcohol consumption, lack of regular physical activity and low daily intake of fruits and vegetables all stood out in men and women, young and old alike, in all regions of the world [5]. Adding factors entails increased danger and even though absolute risk assessment in a single individual is difficult, validated score systems help in risk management [6]. Many of these risk factors have been associated with low-grade chronic inflammation and since the inception of coronary artery disease (CAD) being an inflammation driven condition, which will be covered further below, large prospective studies have investigated the predictive value of circulating inflammatory markers such as C reactive protein, interleukin (IL)-6 and tumor necrosis factor (TNF) and found them to be independent markers of CAD risk [7].

The clinical manifestations of CAD are commonly divided into stable CAD and acute coronary syndromes (ACS). Typical of stable CAD are reversible episodes of myocardial ischemia due to an oxygen supply/demand mismatch most often due to a narrowing of a coronary artery by an atherosclerotic plaque. These transient events are often induced by exercise or emotional stress and the occurrence of ischemia is generally symptomatic in the form of chest pain, angina pectoris (AP) but nausea, dyspnea and general uneasiness is also common. CAD

patients may be asymptomatic or their characteristic symptoms unworsened for a considerable time, stable AP [8]. However, a plaque, previously significant or not, may destabilize and erode or rupture resulting in thrombus formation and the clinical manifestation of an ACS. This condition, if left untreated, carries significant mortality and morbidity and includes unstable AP and MI. Unstable AP is defined as rapid worsening of known angina, i.e with less provocation/at rest or new onset of severe angina pectoris. An ECG recording not uncommonly reveals ST segment depression, but there is no or negligible signs of myocardial damage. This is however the case, when the deficit of oxygen and nutrients causes myocardial cell death, i.e an MI [9].

Myocardial infarction is divided into ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI) based on the type of changes observed in the ST segment of the ECG, with the former usually indicating an occluded epicardial coronary artery while in the latter, a less severe and more limited ischemia occurs. In STEMI, the time from onset of symptoms to initiation of treatment is of great essence. Urgent re-opening of the occluded infarct-related artery by percutaneous coronary intervention (PCI) is the treatment of choice to limit ischemic injury [10]. The infarct size, determined by measurement of myocardial markers in the circulation (such as troponins) or by cardiac magnetic resonance imaging, is strongly correlated to future morbidity and mortality [11-14].

In NSTEMI, stabilization by antiplatelet/anticoagulation medication with a coronary angiography (with possible revascularization) within a few days, is sufficient in the majority of cases [15]. Finally, in stable AP patients where treatment mainly is focused on symptom control, revascularization is considered when conservative measures, i.e optimal medical therapy, has proven insufficient [16].

Atherosclerosis

For much of the last century, most people considered atherosclerosis to be a cholesterol storage disease characterized by the collection of cholesterol and thrombotic debris in the artery wall. The modern era of the cell biology of atherosclerosis in the 1960s and 1970s focused on the proliferation of smooth muscle cells as starting point of atherosclerotic plaques. However, in the mid 80s the concept that atherosclerosis is a chronic, low grade inflammatory disease emerged [17], a notion since then supported by a vast amount of experimental research increasing our understanding of this, the most complex of pathologies.

At the same time, knowledge gained, has as previously mentioned greatly improved patient care, thus forming a true archetype for translational research. Experimental studies point to that the inflammation in the arterial wall is initiated by the uptake and retention of low density lipoproteins (LDL) just below the innermost layer of the artery, the tunica intima [18]. There it binds to proteoglycans of the extracellular matrix and is prone to oxidative modifications caused by enzymatic attack by myeloperoxidase (MPO) and lipoxygenases or by reactive oxygen species (ROS), creating oxidized LDL (oxLDL). The constituents of the oxLDL locally activate the endothelial cells into expressing adhesion molecules which in synergy with chemokines from vascular cells, attract different blood borne immune cells and promote their transmigration into the vessel wall [19,20]. Among these, monocytes transform into macrophages accompanied by the upregulation of their scavenger receptors. The internalization of the oxLDL and the ensuing cholesterol accumulation eventually turns these macrophages into the foam cells thus forming the basis for the early plaque. Antigenpresenting cells such as dendritic cells, activate T-cells which have the ability both to release signaling molecules known as cytokines that can enhance the inflammatory response further, and to kill neighbouring cells not displaying the correct recognition receptors [20]. A proinflammatory milieu is created with ingrowth of smooth muscle cells and an increasing lipid core also containing necrotic or apoptotic cells on the inside covered by the fibrous cap. As the disease progresses the phagocytic ability of foam cells to engulf cellular debris, oxLDL as well as other apoptotic cells (efferocytosis), becomes impaired, giving rise to a necrotic core [21]. The size of the necrotic core in relation to the other constituents of the plaque is one of the determinants if the plaque is stable, i.e. resistant to rupture or not. A large lipid rich, acellular core with a present increased proteolytic activity by matrix metalloproteinases (MMPs) degrading the extracellular matrix as well as the fibrous cap which is thinned out, are hallmarks of an unstable, or vulnerable plaque. When plaque rupture occurs, prothrombotic factors in the vessel wall, including tissue factor, collagen and von Willebrand factor are exposed to the blood, activating both the coagulation cascade and platelets. A local thrombus is formed, which, depending on the extent of myocardium that is supplied and the balance between pro- and anticoagulant properties at the moment, will elicit a clinically evident ACS or not. Also, plaques can heal but with progressive risk for future ruptures as well as development of significant lumen stenosis [22].

Immune cells in atherosclerosis

The functions of the immune system are organized into innate immunity, forming the primary protection against infections and the adaptive immunity, which requires maturation into a more specialized defense. When bacteria and other microorganisms pass through the bodies natural barriers, e.g mucosal membranes, the pathogen-associated molecular patterns (PAMPs) they display, are recognized by pattern recognition receptors (PRRs) expressed by innate immune cells. This initiates the defense process where focus lies on killing off the invading organism through either induction of programmed cell death, apoptosis or by phagocytosis. The same cells augment the immune response, by activation of nearby effector cells through secretion of inflammatory mediators, such as cytokines and chemokines. The latter also induce upregulation of leukocyte adhesion molecules and facilitate cell migration to the site of infection. Cells pertaining to innate immunity are neutrophils, monocytes/macrophages, natural killer (NK) cells, mast cells and dendritic cells (DCs) [23].

In contrast to the direct actions of innate immunity, adaptive immunity requires a learning process initiated by antigen presenting cells (APCs), often DCs, which by displaying antigens to T cells promotes their expansion and differentiation to first effector – and then memory cells. The T cell effector mechanisms are similar to those in innate immunity. Importantly, a subset of T cells, the T helper cells also aid in the activation of B cells, the central player in the defense against extracellular pathogens through antibody production [23].

Memory T- and B cells have the ability to stay dormant for decades but can be activated by the pathogen that once generated them, through a secondary immune response, significantly more rapid than the primary.

Peripheral blood mononuclear cells (PBMCs), i.e. T cells, B cells, NK cells, monocytes and DCs, together with neutrophils (which constitute > 90 % of the polymorphonuclear cells), comprise the heterogenic bulk of immune cells that carry out the innate and adaptive immune responses in the atherosclerotic process. In the following section several of these cells will be described more in detail but with emphasis on cells of certain interest for this thesis.

T cells

T cells (~40% of PBMCs) appear early in the atherosclerotic plaque. Their presence, visualized in 1986, as expression of MHC class II and CD3+ was the first evidence pointing to a role of adaptive immunity in atherosclerosis [17]. The majority of T cells in human atherosclerotic plaques are CD4⁺ T-helper (Th) cells, expressing the $\alpha\beta$ T-cell receptor (TCR). On engagement of their TCR with the antigen–MHC complex (typical antigens being oxLDL and heat shock protein (hsp) 60) displayed on the surface of an APC, naïve CD4⁺ T lymphocytes differentiate into various effector or regulatory subsets, not least depending on the maturity state of the dendritic cells functioning as APC. The resulting cells elicit distinct functions and display specific profiles of cytokine production [24].

A polarization towards a Th1 phenotype carry proatherogenic features including the production of the hallmark cytokines interferon (IFN)- γ and TNF- α , which can exert diverse proatherogenic actions. IFN- γ activates macrophages and DCs, which improves the efficiency of antigen presentation, promotes further Th1 polarization and is associated with both disease progression and plaque rupture. The other type of polarization, Th2, primarily involved in allergic diseases such as atopy and asthma, was long believed to have purely antiatherogenic properties balancing, or even neutralizing the Th1 response. Recent research on murine knock-out models has provided a more complex picture with the type of interleukin produced being one of the determinants of the effects of Th2 weighted responses [25].

More definitely balancing the Th1 response are CD4⁺T regulatory (Treg) cells a subpopulation of T cells specialized in maintaining immune homeostasis and self-tolerance by suppressing pathogenic immune responses. Treg cells are subdivided schematically into two major subsets: naïve and memory Treg cells. After development in the thymus, naïve Treg cells recognize specific self-antigens. Cells are characterized by expression of the surface receptors CD25, CD4, and the transcription factor Forkhead box P3 (FOXP3). Treg cells can inhibit effector T_H cells in several ways: they induce cell-contact-dependent suppression of cell proliferation and activation, downregulate the availability of growth factors to effector T cells by enhanced consumption of IL-2, and inhibit effector T_H cell functions through secretion of the anti-inflammatory cytokines transforming growth factor (TGF)- β and IL-10 [26]. In addition to the suppression of pathogenic T_H-cell proliferation and cytokine production, Treg cells can also inhibit APCs to present antigen, and are, thus, suppressors of both the initiation and the continuation of T cell responses.

IL-10 and TGF- β , the two cytokines responsible for most of the effects mediated by Treg cells, have been shown to have potent antiatherogenic activities.

Genetic inactivation or blockade of IL-10 or TGF- β with neutralizing antibodies accelerated lesion development and aggravated vascular inflammation in mouse

models of atherosclerosis. In patients with ACS, decreased levels of Treg cells have been reported [27, 28] and low numbers Treg cells are associated with increased risk of having an MI independently of other risk factors [29].

B cells

Both cellular mechanisms and antibody production point to a more important role of B cells in atherosclerosis than previously believed.

In humans, levels of natural IgM antibodies, produced by B-1 cells against oxLDL are inversely related to atherosclerotic plaque size [30] and to ischemic cardiovascular events and thus considered anti-atherogenic. [31].

B-2 cells also respond to atherosclerosis-associated antigens and produce immunoglobulin (Ig) G antibodies and even though most studies point to a pro-atherogenic effect [32], IgG antibodies against certain oxLDL-epitopes seem to be protective.

Neutrophils

About 50% of circulating leukocytes are polymorphonuclear neutrophils, or commonly neutrophils. Neutrophils play an essential role in innate immunity and is one of the first players at sites of inflammation. Recognition of pathogens are through previously mentioned PAMPs by its PRRs and the response involves both phagocytosis and release of proinflammatory cytokines such as IL-1 β . However, neutrophils also exert anti-inflammatory effects e.g through TGF β thus contributing to the regulation of the inflammatory response [33].

A known fact since quite some time, is the positive correlation between the neutrophil count in the circulation, the extent of atherosclerosis on a coronary angiography [34] and the development of ACS [35]. Only quite recently however, with the recognition of CD66b, a specific neutrophil marker, neutrophils have been found in rupture-prone plaque areas [36] as well as in coronary lesions from patients with MI and unstable AP but not in those with stable AP. This would imply a role of neutrophils primarily in the destabilization phase of plaques. However, in mice, hypercholesterolemia-induced neutrophilia was positively correlated with the extent of early atherosclerotic lesion formation and when rendered neutropenic, reduced plaque sizes were recorded, providing evidence for the contribution of neutrophils in atherosclerosis development [37]. Neutrophils are also the prime source of MMP-9 in the circulation of patients with stable AP, lending further evidence for an active role of neutrophils in plaque development [38].

Monocytes/macrophages

The hallmark cell of innate immunity functions by non-specific host protection against foreign pathogens via their prompt elimination. The mode is by use of non antigen-specific pattern recognition receptors such as Toll-like receptors (TLRs), CD14 and scavenger receptors, detecting highly conserved components and neutralizing them by subsequent phagocytosis. [39]. Receptors can also be stimulated, causing a activation response by ligands originating from the host itself, examples including the well known scavenger receptor recognizing oxLDL [40].

Two ways of subclassifying monocytes/macrophages exist, the first involving the monocytes is based on differential expression of CD 14 and CD 16 respectively. "Classical" monocytes (CD14++CD16-) representing about 80% of circulating monocytes are regarded as inflammatory and dominate in atherosclerotic lesions. The "non-classical" monocytes, (~5%), with highly expressed CD16 (CD14+CD16++) control the integrity of the vascular endothelium and respond quickly to pathogens. Finally an intermediate type (~10%), CD14++CD16+ has quite recently been described [41]. The subdivision has shown some promise regarding risk stratification in that elevated levels of classical monocytes predicted cardiovascular events in a general population independent of classical risk factors [42] and the intermediate group stood out as independent risk factor for major cardiovascular events in 951 patients undergoing elective coronary angiography [43].

When leaving the circulation, monocytes differentiate into macrophages, for which the second classification adhere. Parallelling Th1 and Th2 type of responses, there are M1 and M2 macrophages with M1 macrophages being found mainly in active lesions producing proinflammatory cytokines like IFN γ , TNF α and IL-1 β . M2 macrophages are involved in wound healing, tend to be anti-inflammatory and are correlated with plaque shrinkage [39, 44]. Macrophages participate in the atherosclerotic process from its inception to plaque rupture in the most diverse ways, including powerful release of not only mentioned cytokines but also tissue-degrading MMPs which actions include thinning of the fibrous cap, proinflammatory mediators like ROS and vascular endothelial growth factor (VEGF) promoting intraplaque angiogenesis, inducing further plaque growth and instability.

NK cells

General aspects

Human NK cells comprise approximately 15-20% of all peripheral blood lymphocytes and have a turnover of about two weeks. Originally described as large granular lymphocytes with natural cytotoxicity against tumor cells, knowledge of their diverse roles has expanded and includes the combat against microbial infections as well as in controlling inflammatory and autoimmune disorders [45]. NK cells are characterized phenotypically by the presence of the surface marker CD56 and the lack of CD3. Two distinct subsets exist, identified by the cell surface density of CD56. The majority (about 90%) are CD56^{dim}, the remaining CD56^{bright}. CD56^{dim} cells are primary cytotoxic while CD56^{bright} cells produce more cytokines but greatly gain cytotoxic activity when activated [46, 47]. Cytotoxicity is initiated through several possible mechanisms, the end result being induction of programmed cell death, apoptosis, or lysis of the cell in question [48]:

1. a. The death receptor pathway, mediated by membrane-bound or soluble factors belonging to the TNF superfamily binding to apoptosis-inducing ligands leading to caspase activation and death.

b. The granule exocytosis pathway, dependent on the pore-forming protein perforin, released by the NK cell and which allows passage of serine proteases (granzymes) into the cytoplasm of target cells where granzymes induce cell death either through caspase activation or mitochondrial injury.
2. Antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies bound to infected cells are recognized by surface receptor CD16 on NK cells resulting in activation and subsequent induction of apoptosis.

Cytokines are crucial in NK cell activation. They include IL-12, IL-15, and IL-2 and are released by e.g viral infected cells. When activated, NK cells secrete proinflammatory cytokines, including IFN γ and TNF α which in turn induce both macrophage phagocytosis and cytotoxic T cells to clear pathogen. Cytokine release also upregulates neighbouring NK cells own cytotoxic capacity.

Powerful cytotoxicity and cytokine-producing effector functions need robust mechanisms that control the initiation of the cytolytic processes and avoid unintentional tissue damage. NK cells express two types of receptors for this purpose, killer *activating* receptors such as NKG2D which detect ligands on

cells in "distress", and *inhibitory* receptors e.g killer cell immunoglobulin-like receptors (KIRs). In the latter, without the recognition of MHC class I receptors, susceptible target cells are considered "non-self" and the NK cell lose its inhibitory signals provided by KIRs, allowing toxicity [49]. The same mechanism applies to both true non-self cells as to infected/damaged own cells, which often lose/downregulate their MHC class I receptor in the process.

In the end, the intensity and the quality of NK cell cytotoxic and cytokine responses is the sum of a dynamic equilibrium involving the cytokine environment, the expression of mentioned receptors as well as on the interactions with other cells of the immune system, primarily T cells, DCs and macrophages.

NK cells in atherosclerosis

The exact significance of NK cells in the different stages of CAD still remains to be clarified. This is partly due to that unlike research on other immune cells active in the atherosclerotic process, animal studies on NK cells have proven difficult (and thus performed to a limited extent) since mice lack CD56 and the heterogenicity of other recognition receptors make knockout models challenging to fabricate. Nonetheless, the mounting evidence gathered, point to an active role [49, 50]. One is the reciprocal pattern of activation - by cell-cell contact and cytokines - between NK cells and proinflammatory monocytes [51]. Assisted by T helper cells, NK cells can activate monocytes both in the circulation, facilitating their transmigration, as well as their differentiation into macrophages in the vessel wall. NK cells can further promote the proinflammatory milieu of the vessel wall by its local release of IFN γ . Recently, more direct effects on the atherosclerotic process have been shown such as the production of perforin and granzyme B which facilitates the expansion of necrotic cores representing vulnerable lesions prone to rupture as in acute MI [52].

Despite these potentially proatherogenic effects, NK cells also exhibit immunoregulatory properties that might be antiinflammatory and antiatherogenic [46,47,53]. Studies have shown that depletion of NK cells can strongly exacerbate disease in mouse models of chronic inflammation [54, 55]. Furthermore, the NK cell immunoregulatory activity was perforin-dependent, indicating that NK cell cytolytic activity plays an important role in vivo [56]. Studies in patients with multiple sclerosis have provided further support to the existence of an immunoregulatory pathway wherein activated NK cells mediate elimination of activated autologous CD4+ and CD8+ T-cells [57, 58].

Loss of natural NK cell activity has been demonstrated in patients with CAD as

compared to healthy controls [59]. The reduced NK cell activity was explained foremost by a decreased number of NK cells. A similar reduction in NK cell number and function has been found in several autoimmune diseases, suggesting a protective role of NK cells in autoimmunity [60]. A low NK cell number is a predictor for both morbidity and mortality in the elderly and in certain malignancies, whereas a restoration of NK cell function indicates an improved prognosis [61, 62]. However, the clinical significance of impaired NK cell function in CAD is still uncertain.

Also, the mechanism behind the lowered NK cell count in CAD is not fully elucidated, but increased apoptosis is one possibility, as NK cells have been shown to be sensitive both to spontaneous and oxidatively induced apoptosis [63]. In that context, much points to that the NK cells need to be in an activated or “primed” state to undergo apoptosis [64]. In support of this, NK cells from CAD patients were more prone to activation after stimulation with IL-2 ex vivo [58].

Modes of cell death

General aspects

There are three modes for a cell to end its life cycle: necrosis, autophagy and apoptosis.

Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma leading to the unregulated digestion of cell components, the end result being the loss of cell membrane integrity and an uncontrolled release of lysosomal enzymes into the extracellular space. This initiates in the surrounding tissue an inflammatory response which prevents nearby phagocytes from locating and eliminating the dead cells by phagocytosis macroscopically rendering necrotic tissue [65].

Autophagy (autophagocytosis) is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. Regarded as one of the modes for programmed cell death, the *primary* function of autophagy is conferring survival, typically occurring as an adaptation to starvation or stress [66]. This involves the formation of a double membrane around cytoplasmic substrates resulting in an autophagosome. Through fusion with a lysosome, the contents of the autophagosome are degraded via acidic lysosomal hydrolases. Death of the whole cell occurs when this action does not suffice or the significance of organelles involved is too central.

Apoptosis

General aspects

Apoptosis, the prototypic mode of programmed cell death, is considered a physiological, homeostatic and vital component of various processes, during development and aging. It is involved in normal cell turnover, proper evolvment and functioning of the immune system and embryonic development. Apoptosis also occurs as a defense mechanism such as a response to internal and external stressors, including oxidative stress, immune reactions and DNA damage [67]. Despite the wide variety of both physiological and pathological stimuli that *can* trigger apoptosis, not all cells will necessarily die in response to the same stimulus, this depending on a complex set of conditions for the cell in question, including the strength of the stimulus, the status of the cell and its surroundings. The same factors also play a central role in determining if a cell will die through necrosis or apoptosis [68].

The various apoptotic pathways which will be described below, all induce the same morphological changes in the cell as observed in a light microscopy. These are cell shrinkage, with increased density of the cytoplasm, chromatin condensation (pyknosis), and later, destructive fragmentation (karyorrhexis) of the nucleus. Blebbing of the plasma membrane occurs followed by separation of cell fragments into apoptotic bodies, during a *budding* process where the plasma membrane is kept intact. Alterations during the process however, include upregulation of surface receptors and the display of cell membrane components, including phosphatidylserine, providing "eat-me" signals for scavenger receptors on macrophages who subsequently phagocytose the apoptotic bodies and degrade them within phagolysosomes. In the early phases of apoptosis, Annexin V, a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues can be used for the detection of apoptosis. The cell is eventually lost but with virtually no inflammatory reaction associated with the process, in contrast to what is seen during necrosis [69].

Classically, two main apoptotic pathways have been described: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway, even though the perforin-granzyme dependent induction of apoptosis mediated by eg NK-cells described above, is by some considered a separate pathway. Importantly, the extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway [70], also, the pathways are linked and molecules in one pathway can influence the other [71]. This thesis deals

primarily with mechanisms involving the extrinsic pathway why apoptosis from hereon refers to that, but first a brief summary of the intrinsic pathway.

The *intrinsic* pathway is activated by non-receptor-mediated stimuli, as diverse as radiation, toxins, hypoxia, viral infections and free radicals. They produce intracellular signals that act directly on targets within the cell and which are mitochondrial-initiated events. Stimuli cause changes in the inner mitochondrial membrane which increases permeability and is followed by release of pro-apoptotic proteins e.g cytochrome C and Smac/DIABLO into the cytosol [72]. These proteins activate caspases, the central proteases involved in apoptosis. Caspases cleave the active or proform of proteins responsible for the different steps in the apoptotic pathway, thereby promoting or inhibiting further reactions. These, belonging to the Bcl-2 family of proteins in turn control and regulate the apoptotic mitochondrial events and their special significance is that they can determine if the cell commits to apoptosis or aborts the process [73].

The *extrinsic* pathway of apoptosis involves surface bound receptors attributed to the TNFR family, of which the portal figures are TNFR1 and TNFR2 [74]. Another member, Fas, initiates apoptosis by binding to FasL. Members of the TNFR family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain” [75]. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. The sequence of events that defines the extrinsic phase of apoptosis are best characterized with the Fas/FasL and TNF- α /TNFR1 models. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. For example does the binding of Fas ligand to Fas result in the binding of the adapter protein FADD and the binding of TNFR1 ligand (TNF α) to the TNFR1 receptor promotes the binding of the adapter protein TRADD with recruitment of FADD and RIP [76, 77].

FADD associates with procaspase-8 and a death-inducing signaling complex (DISC) is formed, which activates procaspase-8 into caspase-8. Once caspase-8 is activated, the execution phase of apoptosis is triggered, however, the death receptor-mediated apoptosis can be inhibited at several steps [78, 79]. Central in limiting the apoptotic rate is downregulation of the transmembrane receptor and shedding of its extracellular part, which through binding to circulating ligands can limit ligand-transmembrane receptor interaction [80].

These soluble forms, sFas, sFasL, sTNFR1 and sTNFR2 can be quantified in plasma and might be used as surrogate markers of apoptosis [81,82]. The same receptor-ligand binding triggering the apoptotic demise of a cell, *can* also initiate a different, *survival* pathway, through involvement of other adapter

proteins instead rendering signals of cell survival and proliferation [83]. The pivotal player here is NF- κ B, a transcription factor for an array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors. The fate of the individual cell, dying or surviving, is eventually the resultant of the multiple death and survival signals [84].

Apoptosis in cardiovascular disease

Apoptosis has to be tightly regulated since too little or too much cell death may lead to pathology, including developmental defects, autoimmune diseases, neurodegeneration, or malignancies. In atherosclerotic lesions apoptosis can affect all cells present. It is triggered by inflammatory processes, both via cell-cell contact, by cytokines and oxidized lipids.

The role and consequences of apoptosis in atherogenesis are dual and seem strongly dependent on the stage of the plaque and the cell type involved: In early stages, apoptotic death of smooth muscle cells - and inflammatory cells, such as lymphocytes and macrophages, may delay the atherosclerotic process. However, once the plaque is formed, apoptosis may lead to plaque rupture and thrombosis [85]. A typical example is apoptotic loss of vascular smooth muscle cells (VSMC) which decreases the synthesis of extracellular matrix (ECM) components thus weakening the fibrous cap [86].

The central point here is the balance – or imbalance between proinflammatory pathways and events promoting resolution of inflammation. The most studied example is the monocyte/macrophage in which continuous intracellular accumulation of lipids (including cholesterol, oxysterols and other fatty acids) induces endoplasmic reticulum stress triggering foam cell apoptosis [87]. However, aberrations in foam cells, such as deficiency of pro-apoptotic factors (e.g. Bax and p53), prevent cell apoptosis and contribute to atherosclerosis progression, especially in above mentioned early lesions [88]. In advanced atherosclerosis the sources of apoptotic cells instead overwhelm the efferocytic program. Such defective efferocytosis allows apoptotic cells to undergo secondary necrosis, thereby feeding the necrotic core and a constant flow of proinflammatory mediators that override existing pro-resolution signals [89]. Also typical of proinflammation is the continuous recruitment of inflammatory cells from the circulation into the plaque [90]. In early lesions, apoptosis of macrophages means a net decrease in homing substrates expressed as chemokines for proinflammatory leukocytes, including additional monocytes, neutrophils and CD4⁺ T cells.

In the myocardium, apoptosis of cardiomyocytes occurs as a result of myocardial damage of both ischemic and non-ischemic origin and its contribution to the progressive loss of cardiomyocytes is actively present through all stages of heart failure [91].

Soluble markers of apoptosis in cardiovascular disease

As previously mentioned, the extracellular part of the receptors pertaining to the TNF receptor super family, can as part of the apoptotic process be shedded and measured in the circulation. They are the most widely used surrogate markers for assessing apoptosis and increased or decreased plasma levels have, as will be described, been shown to correlate to different manifestations of CVD, levels being significantly different compared to healthy controls. The exact origin of the soluble markers of apoptosis detected in the circulation is impossible to determine as many cell types of the cardiovascular system, including cardiomyocytes and cells of the atherosclerotic plaque share these receptor types. It therefore seems appropriate to point out that measurements performed should be connected to a specific disease state – and phase – of interest, to increase specificity. Other markers of apoptosis measurable in plasma have quite recently been introduced and include caspase-3, one of the very downstream effector caspases in both the intrinsic and extrinsic pathways. In a large health survey, plasma levels of caspase-3 correlated positively with coronary artery calcium, aortic wall thickness and aortic compliance [92].

The soluble markers of apoptosis, though originally stemming of basic research on apoptosis have emerged as possible risk markers in CVD. The rational for this will now be discussed shortly.

After MI, despite best-available treatment, the cumulative incidence of mortality and congestive heart failure (CHF) increases time dependently [93]. The introduction of natriuretic peptides has improved risk prediction in CHF [94], as has scoring systems regarding risk for ischemic events [6, 7]. However, because of the complexity of the conditions, a combination of risk markers enhances the probability of adequate risk prediction on an individual basis [95]. As previously described, mechanistically apoptosis plays a central role in the development of an atherosclerotic plaque, but also, as will be pointed out, in ACS and congestive heart failure with lowered left ventricular ejection fraction (LVEF). Several studies lend support for soluble markers of apoptosis as independent predictors for both morbidity and mortality in patients with CVD. Increased sFas seems to convey an increased risk, being more elevated in patients with acute MI with hemodynamic complications than in stable subjects [96]. In patients at high cardiovascular risk, i.e with diabetes mellitus, hypertension or the metabolic syndrome, levels of sFas were increased and sFasL lowered compared with CVD patients without these factors. Interestingly, aggressive Atorvastatin treatment diminished sFas levels pointing to the connection of apoptosis with inflammation [97]. Elevated sFas is also linked to the extent of coronary artery disease in patients with renal failure [98]. sFasL on the other hand, has emerged as a possible atheroprotective marker [99]. Blanco-Colio et al [100] showed a positive correlation between sFasL levels and

forearm reactive hyperemia in patients with CAD speculating that normal endothelial cells contributed to the circulatory levels of sFasL. In clinical studies, sTNFRI is elevated in patients with acute myocardial infarction with some evidence of being both a predictor of the development of CHF and of overall mortality, independently of other well established risk markers such as NT-BNP [101, 102].

To summarize, apoptosis plays an active role in all stages of atherosclerosis as well as in myocardial damage in a wide range of clinical settings, ranging from acute MI to end-stage CHF. In particular, apoptosis has been found to be involved in irreversible myocardial injury during STEMI, which will be discussed in detail next.

Ischemia-reperfusion injury

General aspects

As mentioned in the beginning, urgent re-opening of the occluded infarct-related artery in STEMI patients is the treatment of choice to limit ischemic injury [10]. However, the sudden re-initiation of blood flow can lead to a local acute inflammatory response with further endothelial and myocardial damage, so-called ischemia-reperfusion (IR) injury [103, 104]. This seemingly paradoxical phenomenon involves a wide range of pathological processes that contribute to reversible and irreversible tissue damage. These include vascular leakage, transcriptional programming, no reflow, activation of the innate and adaptive immune responses as well as cell death programs [105, 106]. Animal studies demonstrate that IR-injury may account for up to 50 % of the final infarct size, indicating its potential importance for the prognosis of STEMI patients [103]. IR-injury is a heterogenic condition which causes different degrees of injury depending on the severity and longevity of the ischemic episode preceding the reperfusion. Fundamentally, IR-injury brings about four types of dysfunction in the heart:

1. *Myocardial stunning*, the mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow. No matter how severe or prolonged, this is a fully reversible abnormality within days to weeks of the ischemic event [107].
2. The *no-reflow phenomenon* involves the breakdown of, or obstruction to the coronary microvasculature during reperfusion and which can markedly reduce blood flow to the infarct zone [108]. A combination of tissue edema,

endothelial disruption, inflammation, vasoconstriction and microthrombi, the latter arising from embolisation of plaque components during PCI, all contribute to the process [109, 110]. It may manifest at the time of PCI as persistent ST-elevation and coronary 'no-reflow' i.e TIMI flow ≤ 2 as defined by the Thrombolysis in Myocardial Infarction (TIMI) blood flow grade scale, used to evaluate the quality of coronary flow during coronary angiography [111, 112]. The presence of the no-reflow phenomenon indicates a microvascular obstruction and its presence in reperfused STEMI patients, as visualised eg by cardiac magnetic resonance imaging (CMR), is associated with larger infarct size, worse LV ejection fraction, adverse LV remodelling as well as unfavorable short and long term outcomes [113-116].

3. Experimental animal studies from the 1980s were among the first to describe ventricular arrhythmias specifically induced by reperfusion. In reperfused-STEMI patients, the most commonly encountered *reperfusion arrhythmias* are idioventricular rhythm, ventricular tachycardia and ventricular fibrillation. In most cases arrhythmias appear within the first 12-24 h of reperfusion and are easily dealt with but delayed reperfusion with concomitant large infarcts and depressed LV function carry worse prognosis also in this regard [117,118].

4. The concept of *lethal IR-injury* as an independent mediator of cardiomyocyte death, distinct from ischemic injury, has been debated; some researchers have suggested that reperfusion only exacerbates the cellular injury that was sustained during the ischemic period [119]. The uncertainty relates to the inability to in situ accurately assess the progress of necrosis during the transition from myocardial ischemia to reperfusion [120]. As a result, the most convincing means of showing the existence of lethal reperfusion injury as a distinct mediator of cardiomyocyte death is to show that the size of a myocardial infarct can be reduced by an intervention used at the beginning of myocardial reperfusion [121]. In 2005, Staat et al. provided the first clinical evidence that lethal IR-injury actually exists in man. They demonstrated a 36% reduction in MI size in reperfused-STEMI patients randomized to receive ischaemic post-conditioning (four-30 s inflations/ deflations of the angioplasty balloon following stent deployment) [122]. The fact that a therapeutic intervention, applied at the onset of myocardial reperfusion, could reduce MI size has provided evidence that lethal IR-injury exists in man and is a viable target for cardioprotection [90]. Several potential mediators of lethal IR-injury have been described and here, the ones that have drawn the most attention, including therapeutic attempts will be discussed briefly. Whether preventing lethal IR-injury in reperfused-STEMI patients can actually reduce major adverse cardiac events (MACE) remains to be demonstrated.

a. The *oxygen paradox* refers to reperfusion of ischemic myocardium, which generates oxidative stress, itself potentially mediating myocardial injury. Unfortunately, both animal and clinical studies examining the cardioprotective potential of antioxidant reperfusion therapy have been inconclusive [123,124].

Oxidative stress also reduces the bioavailability of nitric oxide (NO) at reperfusion hereby removing its cardioprotective effects and the administration of NO donors is cardioprotective [125]. However, the NO donor, Nicorandil, did not limit MI size when administered to reperfused-STEMI patients [126].

b. At the time of myocardial reperfusion, there is an abrupt increase in intracellular Ca^{2+} , secondary to sarcolemmal membrane damage and oxidative stress-induced dysfunction of the sarcoplasmic reticulum. These two forms of injury overwhelm the normal mechanisms that regulate Ca^{2+} in the cardiomyocyte, a phenomenon termed the *calcium paradox* [127]. The result is intracellular and mitochondrial Ca^{2+} overload, inducing cardiomyocyte death by causing hypercontracture of the heart cells. Experimental animal studies have demonstrated that pharmacologically inhibiting intracellular and mitochondrial calcium overload at the onset of myocardial reperfusion [128,129] can reduce MI size by 40–50%. However, clinical studies investigating this therapeutic approach have been negative [130-132].

c. The mechanisms and consequences of the oxygen and calcium paradoxes converge on the *mitochondrial permeability transition pore* (MPTP). A non-selective channel of the inner mitochondrial membrane, during myocardial ischemia, the MPTP remains closed, only to open within the first few minutes after myocardial reperfusion, in response to eg mitochondrial Ca^{2+} overload and oxidative stress. Opening the channel collapses the mitochondrial membrane potential and uncouples oxidative phosphorylation, resulting in ATP depletion and cell death [133,134]. Under experimental conditions, inhibition of MPTP opening by Cyclosporin A at the time of reperfusion has shown reductions in MI size by 40-50% [135-137], an effect partly confirmed in a clinical STEMI study [138].

d. The *inflammatory* response to IR-injury is required for healing and scar formation in the infarcted area. However, the release of chemoattractants and ROS from injured endothelial cells and cardiomyocytes draw inflammatory leukocytes such as neutrophils into the infarct zone where they cause vascular plugging and release proteolytic enzymes and reactive oxygen species amplifying tissue damage [139]. Whether this acute inflammatory response results in cardiomyocyte death or is simply a response to the infarct is unclear, but experimental animal studies have reported reductions in MI size by up to 50% with several interventions, eg administering leukocyte-depleted blood

[140] or antibodies against cell-adhesion molecules such as p-selectin and ICAM-1[141,142]. However, clinical studies with antiinflammatory agents have failed to demonstrate any impact on clinical outcomes in reperfused-STEMI patients [143,144].

e. Experimental animal studies have shown that modulating the *metabolism* of the myocardium by promoting the use of glucose as energy source by means of glucose-insulin-potassium (GIK), during acute myocardial ischaemia is beneficial for the heart [145]. GIK-therapy has been clinically tested with mixed results where positive outcomes were related to GIK given early, as in the prehospital setting, when the myocardium was still acutely ischemic [146,147].

Apoptosis in IR-injury

While cell death contributing to infarction usually displays the pathological features of necrosis, there is increasing evidence that also apoptosis contributes to the total cell loss, during ischemia and/or reperfusion. In myocardial infarction apoptotic myocytes have been detected especially in the border zone between viable and necrotic myocardium [148, 149]. In experimental animal models of ischemia-reperfusion, detection of fragmented DNA, by TUNEL technique, pathognomonic for apoptosis, was increased and apoptosis was thus thought to be accelerated by the process of reperfusion [150]. Much point to that both the intrinsic and extrinsic pathways are active in this process [151].

From the point of soluble markers, several experimental studies have demonstrated that TNF, the ligand for both TNFRI and TNFRII, has a role in myocyte apoptosis during IR-injury, leading to LV-dysfunction and CHF [152,153]. However, the results have been conflicting. Depending on animal model used, the intensity of the TNF signaling, choice of cell type being stimulated and the extent to which TNFRI and TNFRII were activated selectively, either a proapoptotic or an antiapoptotic effect has been found [154-157].

Since the apoptotic component of cell death may be particularly relevant at reperfusion, both physical and biological pathways have been explored as targets for intervention to limit lethal IR-injury. Physical is foremost referring to ischemic preconditioning, meaning short repeated periods of ischemia preceding a longer subsequent period of ischemia, followed by reperfusion. Such manoeuvres in experimental animals were associated with reduced amounts of apoptotic myocytes and smaller infarcts [158,159]. Furthermore, the effect was related to an attenuation of proapoptotic factors such as TNF release and Caspase-3 activity as well as upregulation of survival signals [160, 161].

Growth factor signaling including Transforming growth factor β (TGF- β 1) [162], Insulin [163], Insulin-like growth factor (IGF)[164] and Cardiotrophin-1 (CT-1) [165] all induce anti-apoptotic actions through either inhibition of proapoptotic signals or promotion of survival signals. In mouse models several of these confer protection against irreversible IR-injury but data on the possible effects on humans are largely lacking.

AIMS OF THE STUDY

General aims

The overall objective of this thesis was to measure soluble markers of apoptosis in various clinical stages of CAD, and to assess their temporal patterns and possible sources, such as lymphocyte apoptosis in peripheral blood and myocardial injury.

Specific aims

1. To investigate lymphocyte apoptosis, in particular NK cell apoptosis, in stable CAD as well as in ACS patients.
2. To study the associations between NK cell apoptosis and soluble markers of apoptosis in these same populations.
3. To evaluate the role of apoptosis in IR-injury by correlating levels of soluble markers of apoptosis with the extent of myocardial injury in STEMI patients.
4. To study the temporal changes in soluble markers of apoptosis during the first 6 months after NSTEMI and STEMI, respectively.

METHODOLOGICAL CONSIDERATIONS

Study populations and designs

All studies were approved by the ethical review board of Linköping and conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained from all study participants (paper I-IV). A summary of the study participants included in the studies is depicted in table 1.

Patients taking part in study I, II and IV were recruited at the Department of Cardiology, Linköping University Hospital, while participants for study III were recruited from 10 international sites as part of the F.I.R.E. trial

(<http://clinicaltrials.gov>, NCT00326976) [166]. Patients were excluded if they had severe heart failure, immunologic disorders, neoplastic disease, evidence of acute or recent (<2 months) infection, had undergone recent trauma, surgery or revascularization procedure or treatment with immunosuppressive/anti-inflammatory agents (except low-dose aspirin) or dietary supplementation.

Patients in study III were subjugated to additional exclusion criteria, which are described in the section of that study. All control subjects were randomly selected from a population-based register representing the hospital recruitment area. For all of the studies, control subjects were included if they were anamnesticallly healthy, taking no medication and with normal routine laboratory tests. However, statin treatment was allowed for primary prevention. In addition, in paper I and paper II, respectively, 11 and 5 anonymous blood donors were included from the Blood bank at Linköping University Hospital. They had no symptoms of disease, had normal blood pressure, were on no prescribed medication and were seronegative for HIV, hepatitis B and C and syphilis. Unless stated otherwise, blood samples were always drawn in the morning after an overnight fast.

Table 1. Summary of study participants in paper I-IV.

		Paper I	Paper II	Paper III	Paper IV
NSTE-ACS, n			31		40
Age, years			69(50-83)		66(±11)
Females, n (%)			8 (26)		30(12)
SA, n		28	34		
Age, years		61 (56-65)	63(44-77)		
Females, n (%)		5 (18)	6 (18)		
STEMI, n				48	61
Age, years				61 (±11)	66(±13)
Females, n (%)				16 (35)	34(21)
Blood donors, n		11	5		
Age, years		31 (24-37)	29 (27-32)		
Healthy controls, n		30	37		
Age, years		60 (55-73)	63 (45-77)		
Females, n (%)		4 (13)	9 (24)		
Study participants, n		69	107	48	101

For paper I and II values are given as median (25th-75th percentile) and in paper III and IV as mean (±SD). NSTE-ACS, Non-ST-segment elevation Acute Coronary Syndrome; SA, Stable Angina; STEMI, ST-elevation myocardial infarction.

Study I

Initially, assays on isolated NK cells from 11 blood donors were performed, where possible time- and dosedependent effects of oxidized lipids on apoptosis and ROS production in NK cells and T cells were investigated (Fig. 1.).

In total, 28 patients with angiographically verified CAD and stable AP (SA) in accordance with the Canadian Cardiovascular Society functional class (CCS) I and II, and 30 age and sex matched controls, were recruited in 2005-2006. All patients were on long-term (>2 months) statin treatment.

In 12 of the patients and 14 of the controls, NK cell apoptosis *in vivo* was investigated. These patients constituted a subgroup of Cohort I described below.

In 16 individually matched pairs of patients and controls (also described below in Study II), spontaneous and oxidation-induced NK cell apoptosis *ex vivo*, oxidative stress in plasma as well as antioxidants (carotenoids) in plasma were measured.

Fig. 1. Study design in **study I**

Study II

In paper II, the study design of which is summarized in Figure 2 and 3, two independent cohorts of CAD patients, Cohort I and II, were analyzed. Cohort I, which was recruited between 2006-2010, involved 31 patients with non-ST-elevation acute coronary syndrome (NSTEMI), 34 patients with SA and 37 controls. NSTEMI patients were included if they had a diagnosis of non-ST elevation myocardial infarction (NSTEMI) based on ST-T segment depression and/or T-wave inversion on the ECG and elevated troponins. SA patients were included if they had CCS class II or III effort angina and objective signs of ischemia obtained by exercise testing or myocardial scintigraphy. All patients in Cohort I underwent coronary angiography. Index blood samples (day 1) were collected before coronary angiography and in the NSTEMI patients within 24 hours of admission. Longitudinal measures were available in 15 NSTEMI patients and 28 SA patients, blood samples being collected at 3 and 12 months.

NK cell and T cell apoptosis *in vivo* was investigated as well as levels of the soluble markers of apoptosis, sFas and sFasL and interleukin (IL)-6 in plasma.

Cohort II, recruited between 2005-2008 consisted of 16 CAD patients who 3-6 months earlier had suffered a coronary event (NSTEMI-ACS and/or coronary revascularization process). They were individually matched against 16 controls and in addition to spontaneous NK cell and T cell apoptosis *ex vivo*, levels of sFas and sFasL in plasma were measured. Cohort II is the same cohort that is described in Study I.

In vitro assays of cytokine-induced NK cell and T cell apoptosis and release of sFas and sFasL were carried out, in 5 blood donors.

Fig. 2. Study design in **study II**.

Fig.3. Study design in **study II** (continued).

Study III

The study was a pre-specified substudy of the F.I.R.E. trial [166]. 48 subjects with ST-elevation myocardial infarction (STEMI) were enrolled at a total of 10 participating sites between 2006-2008. STEMI was defined as ≥ 2 mm ST-elevation in at least 3 leads on the ECG. Inclusion demanded a first (known) myocardial infarction with percutaneous coronary intervention (PCI) indicated as standard care and with presentation within 6 hours of symptom onset. Patients with prolonged ischemic symptoms, cardiogenic shock, peripheral vascular disease and history of kidney (serum creatinine >250 $\mu\text{mol/l}$) or liver dysfunction were excluded. Venous blood was drawn before PCI and at 24 h. Analyses performed included sTNFRI and sTNFRII, sFas, sFasL and MMP-2 in plasma.

A cardiac magnetic resonance (CMR) scan was performed at 5 days and 4 months to evaluate infarct size, left ventricular (LV) dysfunction (expressed as LV ejection fraction), and measures of LV remodelling (Fig.4).

Blood work	Cardiac MR
sTNFRI, sTNFRII,sFas, sFasL (ELISA)	Infarct size Late Gadolinium Enhancement (LGE) zone.
MMP-2 (ELISA)	LV-dysfunction (LVEF)
	LV remodeling (δ EDVI and δ ESVI at 5 d vs 4 months)

Fig.4. Study design in **study III**.

Study IV

The study was a pre-specified substudy of the APACHE (Assessing Platelet Activity in Coronary HEart disease) trial [167] which between 2009 and 2011 recruited 125 patients, 73 with STEMI and 52 with NSTEMI, scheduled for coronary angiography. STEMI and NSTEMI inclusion criterias were defined according to Global definition of myocardial infarction [168]. Exclusion criteria were: Participation in an intervention study, treatment with warfarin before admission, short life expectancy (less than 6 months) and unwillingness to participate. Blood sample sets for the present substudy were obtained from 61 STEMI and 40 NSTEMI patients and blood was drawn before Clopidogrel loading, at 6-8 hours, at 3 days and at 6 months. Analyses performed (Fig.5) included sTNFRI, sTNFRII, sFas, sFasL, IL-6 and high sensitivity troponin T (hsTnT) in plasma. A transthoracic echocardiography was performed on day 2-3 for evaluation of systolic LV function.

Blood sample→ Plasma→ sTNFRI, sTNFRII, sFas, sFasL. Interleukin-6 (IL-6).

*High sensitivity troponin T.

Fig.5. Study design in **study IV**.

Laboratory methods

The laboratory methods used in this thesis are described in detail in the Material and methods section of each separate paper. Here the central aspects of the most prominent assays and methods are summarized.

Collection and handling of blood samples

In paper I and II, venous blood from all study participants was collected in vacutainer tubes using sodium heparin as anticoagulant and eventual analyses on whole blood were carried out within 60 minutes. Remaining samples were centrifuged within 30 minutes at 2500 rpm for 15 minutes, to separate plasma and was then stored immediately at -70° C at the Biobank of the Department of Cardiology, University hospital Linköping, until analyzed. In paper III and IV venous blood from all study participants was collected in vacutainer tubes using sodium heparin as anticoagulant. Samples were centrifuged for 15 minutes at 3000 rpm to separate plasma and was then immediately stored at -20°C and within a week at -70°C at the Biobank of the Department of Cardiology, University hospital Linköping, until analyzed. All blood withdrawal and centrifugation procedures were at room temperature (18-24°C) to minimize the risk of platelet activation.

Isolation of NK cells and T cells

(Study I and II)

In order to perform assays *ex vivo* on NK cells and T cells, they had to be isolated. Whole blood was mixed with phosphate buffered saline and layered onto Lymphoprep (Axis-Shield PoC AS, Oslo, Norway), a density gradient medium for the isolation of peripheral blood mononuclear cells (PBMC). Granulocytes and erythrocytes have a higher density than PBMC and therefore sediment through the Lymphoprep layer during centrifugation, here 400x g for 30 min. After washing and resuspension of the PBMC, NK cell and T cell Negative Isolation Kits (DynaL Biotech ASA, Oslo, Norway) were used to separate the NK cells and the T cells, respectively from the PBMC. The technique involves incubation of PBMC with an antibody mix directed against either non-NK (CD3+CD56-) or against non T cells (CD3-) cells. Here 20µl/10⁶ PBMC for 10 min at 4°C. Dynabeads, superparamagnetic beads coated with an IgG antibody directed for the antibodies of the antibody mix were used. The now bound cells

were removed with a magnet, leaving only NK cells or T cells, respectively which was verified by flow cytometry (FACSCanto, BD, Biosciences).

Assays of spontaneous and induced NK cell and T cell apoptosis *ex vivo*

(Study I and II)

Isolated NK cells and T cells were cultured in RPMI culture medium, 10 % Fetal Bovine Serum (FBS), L-glutamine, 100µg/mL penicillin G and 100 µg/mL streptomycin and put in a humidified atmosphere at 37°C for 18 h. After washing, cells were stained with Annexin V-FITC (Annexin-fluorescein isothiocyanate) and propidium iodide (PI, in this setting used as marker of necrosis), (Roche Diagnostics GmBbH, Mannheim, Germany). Samples were analyzed on a Becton-Dickinson LSR II Flow cytometer using CellQuest software (10000 cells in each sample) determining the number of spontaneously apoptotic or necrotic cells. The reagents mentioned are later described in the section on Flow cytometry.

To determine the apoptosis-inducing effect of oxidized lipids on NK cells and T cells, cells were exposed to 50µg/ml oxLDL and 30µM 7β-hydrocholesterol for 18 h, with native LDL, cholesterol and ethanol as controls. The number of apoptotic or necrotic cells were determined in the same way as for the spontaneous assay.

Assay of spontaneous NK cell and T cell apoptosis *in vivo*

(Study I and II)

The NK cells (CD3-CD56+) and T cells (CD3+) in peripheral blood were identified by adding monoclonal antibodies directed against CD3 (marked with FITC) and CD56 (marked with allophycocyanin) (BD Biosciences), 3,5 µl of each to 50µl of whole blood and incubated in the dark for 15 min. Red blood cells were lysed (increases the following flow cytometry accuracy for lymphocytes) using 500 µl of Pharmlyse[®] lysing buffer (BD Biosciences). After washing, cells were resuspended in 100µl of Annexin V binding buffer, 3,5 µl Annexin V conjugated with phycoerythrin and 3,5µl of 7-amino-actinomycin D (7-AAD, a marker of necrosis) at room temperature for 15 min. Finally, flow cytometry followed for analysis of apoptotic or necrotic cells.

Assay of cytokine-induced apoptosis of NK cells and T cells *in vitro* (Study II)

NK cells and T cells were isolated as previously described. Cells were plated at 100000 cells/well in 96-well plates and cultured at 37°C. They were subsequently either left unstimulated, stimulated with IL-15 alone (15 ng/ml) or with both IL-15 and IL-12 (3ng/ml) (Both interleukins from R&D systems, UK). After 48 h of stimulation, supernatants (described below) were collected for soluble markers of apoptosis and cell apoptosis determined by flow cytometry as previously described.

Flow cytometry (Study I and II)

Flow cytometry is a technology that simultaneously measures and then analyzes cells, as they labelled with fluorescent antibodies flow in a fluid stream passing a beam of light. Thousands of cells can be analyzed each second for relative size, granularity and fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell scatters incident laser light and emits fluorescence. The scattered and fluorescent light is collected by appropriately positioned lenses and is thereafter steered into filters isolating specific wavelength bands which are detected by photomultiplier tubes (PMTs). The PMTs produce electronic signals proportional to the optical signals striking them and which are further digitalized for computer analyses. Data are then sorted manually and displayed in either histograms or dot-plots.

Cells of interest can be labelled by fluorochromes by either binding to cell surface receptors (as in the above mentioned characterization of cells) or to intracellular molecules. The latter technique was used in *study I*, where intracellular reactive oxygen species (ROS) was assayed by flow cytometry following dihydroethidine staining. Another important application of the method in this thesis was the above mentioned assay on apoptosis and necrosis of NK cells and T cells. Annexin V (or Annexin A5) is an intracellular protein that binds to phosphatidylserine (PS) on the inner leaflet of the plasma membrane. During early apoptosis, membrane asymmetry is lost and PS translocates to the external leaflet where fluorochrome-labeled (eg FITC) Annexin V can be used to specifically target and identify apoptotic cells. The cell membrane of live and early apoptotic cells does not permeate 7-amino-actinomycin D (7-AAD) and propidium iodide (PI) – both of which bind strongly to DNA - while a late apoptotic or necrotic cell does, why fluoresceinconjugated 7-AAD and PI indicate dead cells.

Fig. 6. Summary of the general principles of flow cytometry

Enzyme-linked immunosorbent assay (ELISA)

(Study I-IV)

The ELISA technique is a quantitative sandwich enzyme immunoassay using the principle of antigen-antibody binding to detect antigens or antibodies in e.g. plasma or cell supernatants. Microtiter plates are coated with a capture antibody after which the sample with the antigen of interest is added. Antigen is bound to the antibody and any unbound sample is then washed away. A detection antibody conjugated with an enzyme is added leaving the antigen between (hence the term sandwich) the two antibodies. Finally a substrate is added which is cleaved by the enzyme which brings a change in color. The color intensity measured by a spectrometer is proportional to the amount of antigen bound in each well and a numeric quantification is done using a standard curve for the specific antigen in question.

In study I, ELISA was used in assays of LDL protein oxidation and lipid peroxidation as previously described [169,170]. In study II it was used to determine sFas, sFasL in plasma and in cell supernatants after in vitro stimulation (Quantikine[®] immunoassay R&D systems, Minneapolis Minnesota, USA) as well as to quantify IL-6 in plasma (Quantiglo[®] Chemiluminescent ELISA, R&D systems). In study III, ELISA was utilized for the analysis of sTNFRI, sTNFRII, sFas, sFasL, MMP-2, MMP-8, tissue inhibitors of metalloproteinases (TIMP) 1 and 2 and myeloperoxidase (MPO). In study IV, the same analyses were done as in study III except for the MMPs, TIMPs and MPO. The limits of detection and the coefficients of variation are given in each study in the Methods section but in general the coefficient of variation was under 7% in all studies.

Statistics

Continuous variables with gaussian distribution are presented as mean with standard deviation. For these variables parametric tests are used. Differences between more than two unrelated groups are tested by one-way ANOVA, whereas differences between two groups are tested by unpaired or paired T-test, as appropriate. One-way repeated measures ANOVA is used to analyze changes in variables over time.

Continuous variables with non-gaussian distribution are presented as median with interquartile range (25th-75th percentiles). For these variables non-parametric tests are used. Differences between more than two unrelated groups

are tested by Kruskal-Wallis test, whereas differences between two groups are tested by Mann-Whitney U-test or Wilcoxon signed rank test, as appropriate. Friedman's test is used to analyze changes in variables over time.

Proportions are given as number and percent. Pearson's chi-square test is used to test for differences in proportions. Bivariate correlations are analyzed by Spearman's rank correlation coefficient.

Statistical significances are set at a two-tailed p-value of <0.05. All statistical analyses were performed using IBM SPSS version 17-21.

RESULTS AND DISCUSSION

Study I

NK cell apoptosis in coronary artery disease

Relation to oxidative stress

In a subgroup of Cohort I (12 stable CAD patients and 12 controls), the percentages of apoptotic NK cells (Annexin V+/7AAD-) *in vivo*, were significantly increased in patients compared to controls, while the number of necrotic cells (AnnexinV+/7AAD+) were similar and very low in both patients and controls. A decreased NK cell-mediated cytotoxic capacity in CAD patients due to a quantitative defect has previously been described [59] and an increased apoptotic rate of these cells as a considerable contributing factor was suspected. The findings *in vivo* were therefore extended *ex vivo* where NK cells from CAD patients incubated in medium only for 18 h, underwent apoptosis significantly more than did NK cells from controls. In previous studies, NK cells have shown to be susceptible to oxidative stress, induced by oxidized lipids, like oxLDL which inhibited NK-cell mediated cytotoxicity [171-173] as well as undergoing apoptosis when exposed to hydrogen peroxide (H₂O₂) [174]. The notion that oxidized lipids is a driving force behind the loss of NK-cells in CAD patients gave rise to experiments, first in blood donors, where NK cells were exposed to, either oxLDL or 7βOH, a cholesterol oxidation product, in different concentrations for 6, 24 and 48h. Both oxLDL and 7βOH induced a dose and time-dependent apoptotic NK cell death, the highest amount of apoptotic cells with 100μg/ml oxLDL for 24 h and with 30μM 7βOH for 24 h. However, the longest exposure times (48h) resulted in mainly necrotic cells (Fig.6 and Fig.7).

The set-up was repeated on NK cells from CAD patients and matched controls. NK cells from patients exposed to 30 μ M 7 β OH for 18h yielded a significantly larger amount of apoptotic NK cells compared with controls. NK cell apoptosis induced by 50 μ g/ml oxLDL was more noticeable in patients but the difference did not reach statistic significance (Table 2).

Fig.6. Dose- and time-dependent apoptotic/necrotic cell death induced by oxLDL.

(A) Representative dot plots of annexin V/PI stained cells. The upper panels and lower left two panels are results from cells treated with control medium and different concentrations of oxLDL (24 h), respectively. The lower right panel shows 48 h results (50 μ g/ml oxLDL). The values for the upper right, lower right and lower left squares of each dot plot in the upper left corner represent percentages of necrotic (Annexin V+/PI+), apoptotic (Annexin V+/PI-) and viable cells (Annexin V-/PI-), respectively. (B) Quantification of cell viability as detected by exposure of phosphatidylserine (AnnexinV/PI staining). ^a $p < 0.001$ vs. control. ^b $p < 0.001$ vs. 6 h oxLDL treated cells.

Fig. 7. Dose- and time-dependent death of NK-cells, induced by 7βOH.

Black columns: Exposure by 7βOH for 6 h. **White columns:** Exposure by 7βOH for 24 h.
^ap<0.001 vs. control and ^bp < 0.001 vs. 20 and 30 μM 7βOH treated cells.

Table 2.

Apoptosis of NK cells in CAD patients and controls after stimulation with oxLDL or 7βOH

	Patients	Controls	<i>p</i>
Medium only	2.5 (1.8–3.4)	2.0 (1.4–2.8)	<0.01
Native LDL 50 μg/ml	3.3 (2.1–4.3)	2.6 (1.7–3.2)	NS
OxLDL 50 μg/ml	16.0 (7.3–22.5)	13.6 (6.3–14.6)	NS
7βOH 30 μM	6.9 (6.0–12.1)	3.7 (3.1–6.4)	<0.01

Cells were exposed to medium (spontaneous apoptosis), native LDL, oxLDL or 7βOH for 18 h. Data are given as fold increase related to baseline percentages of apoptotic cells.

The reason for the apparent discrepancy in apoptotic-inducing effect between oxLDL and 7 β OH is not clear, both being potent inducers of apoptosis [175-177] but one explanation could be methodological, in that oxLDL is prepared by UV-radiating LDL at the lab [178], a procedure that is difficult to standardize with potential different results from batch to batch, while 7 β OH is a commercially available compound (and thereby its concentration is more easy to standardize) [179,180].

In order to verify the oxidation induced stress, the production of ROS in NK cells was measured. In the healthy, drug-free blood donors, cellular ROS production increased in a time- and dose-dependent manner strongly pointing to the oxidation-induced effect of oxLDL and 7 β OH (Fig. 8). In CAD patients, ROS production tended to be higher than in controls, however without significant difference.

Fig.8. ROS production induced by oxLDL and 7 β OH.

(A) Cells from blood donors were treated with oxLDL (50 μ g/ml) for 6 and 24 h. a = $p < 0.001$ vs. control and 6 h oxLDL treated cells. b = $p < 0.001$ vs. control. (B) Cells from blood donors were treated with different concentrations of 7 β OH for 6 and 24 h. a = $p < 0.001$ vs. control and 20 and 30 μ M 7 β OH treated cells. b = $p < 0.001$ vs. control and 20 μ M 7 β OH and $p < 0.01$ vs. 30 μ M 7 β OH treated cells. c = $p < 0.01$ vs. control. Lower panel; **Black** columns: Exposure by 7 β OH for 6 h. **White** columns: Exposure by 7 β OH for 24 h

The increased number of apoptotic NK cells in the circulation and the sensitivity of NK cells to oxidized lipids displayed in the *ex vivo* studies promoted the idea of oxidative stress as a driving force of NK cell death in CAD patients. In support of this, several studies have shown increased levels of oxidative biomarkers, such as oxLDL and TBARS in CAD patients [181-183]. However, measurements of oxidative stress *in vivo* assessed as levels of lipid peroxidation products, like carbonyl and TBARS in plasma, did not differ between patients and controls.

A possible explanation for the lack of increased oxidative stress in our patient sample is that all patients were on statin therapy. Statins have antioxidative properties [184] as well as *in vitro* documented effects on apoptosis [185,186] which could have attenuated the differences between patients and controls. However, there may also be methodological explanations since the measurement of circulating oxidative biomarkers is a challenge, not likely to reflect the oxidative stress at the cellular level. The intermediate and endproducts of lipid peroxidation commonly measured, of which TBARS is one, are unstable and heterogenic and their levels in the circulation are therefore considered less reliable compared with measurements in tissue specimens e.g carotid artery plaques [187]. An alternative analytical method might have rendered different results. In contrast to the lipid peroxidation products used in this study, F2-Isoprostanes, prostaglandin-like compounds and end-products of lipid peroxidation, are chemically *stable* and detectable in all human tissues and fluids, in particular urine. However, levels of F2-isoprostanes in plasma were found to be a marker of CAD after adjusting for traditional risk factors [188] and also proved useful in risk stratification for major cardiac events (MACE) in patients presenting with ACS [189].

In order to further evaluate the oxidative stress *in vivo*, carotenoids were measured in plasma. Carotenoids in plasma have been strongly associated with oxidative stress in several previous studies [190, 191]. We found that carotenoid levels were significantly lower in CAD patients than controls, which is supported by studies showing negative correlations between plasma levels of these antioxidants and CAD [192, 193]. Furthermore, concentrations of α -carotene and β -carotene were inversely correlated with the apoptotic rate of NK cells ($r=-0,38$, $p<0,05$ for both correlations). Findings are in line with previous studies showing that levels of oxygenated carotenoids were associated with proportions of NK cells in the blood [194]. Furthermore, β -carotene supplementation has been shown to enhance NK-cell activity in elderly men [195]. In the present study however, no correlation between levels of carotenoids and ROS production was seen, neither with the other markers of oxidative stress in the circulation.

To conclude, the study demonstrated an increased apoptotic rate of NK cells in the circulation of CAD patients and also, that oxidized lipids induced apoptosis and ROS production in a dose- and time-dependent manner in human NK cells. Findings are suggestive of enhanced apoptosis as an important contributing factor to the lowered NK cell levels in seen in CAD. However, based on our chosen methods, we could not find evidence that the NK cell loss *in vivo* depended exclusively on oxidative stress.

Study II

Soluble Fas ligand is associated with natural killer cell dynamics in coronary artery disease

In study I, we found that one plausible reason for the loss of NK cells in CAD patients was an increased apoptotic rate. However, the reduction of NK cells does not necessarily have to be a static phenomenon. The levels of NK cells have been shown to be restored in CAD patients during a 12 month follow-up after a coronary event [196]. We hypothesized that the variations could be due to changes in the apoptotic rate. Soluble forms of Fas and FasL have been shown to be positively associated with NK cell levels in NK cell proliferative disorders [197,62] as well as with circulating levels of apoptotic NK and T cells in autoimmune and neoplastic disorders [198-200]. In CVD, as mentioned above, high levels of sFas point to an increased risk whereas increased sFasL seems to represent the opposite [96-100]. In most of the referred studies however, blood samples were drawn only at one occasion making verdicts on correlation changes over time impossible. Here we wanted to clarify the relationship between sFas, sFasL and NK cell apoptosis in both stable and acute phases of CAD investigating possible temporal patterns in NK cell status during 3 and 12 month follow-up. Moreover, we speculated that we might be able to identify a surrogate marker of peripheral NK cell changes.

Forty-three patients in Cohort I (15 NSTEMI-ACS and 28 SA) participated in the longitudinal study. Over 12 months, in all 43 patients, FasL showed a relative increase by 15 (0,0-32) % and levels of NK cells a relative increase of 31 ((-4,0)-57) %, $p < 0,001$ and $p < 0,05$ respectively (Table 3). Positive correlations were seen between sFasL and proportions of NK cells during follow-up, both at 3 months (Fig.9) and at 12 months. Similar correlations were seen between sFasL and absolute numbers of NK cells as well as between the relative increases of sFasL and NK cells.

There were no correlations between either sFas or sFasL and apoptotic NK or T cells in the circulation at any time point. Nor were there any correlation between sFasL and the number of T cells at any time point.

Table 3. Follow-up changes of sFas, sFasL, IL-6 and NK and T cell subsets

Plasma levels of sFas, sFasL and IL-6, proportions of NK and T cells (% of lymphocytes) and proportions of early apoptotic NK cells and T cells (% of NK or T cells) in a longitudinal analysis of CAD patients with a coronary event; day 1 (prior to coronary angiography), 3 months and 12 months (Cohort I, n = 43, 15 NSTE-ACS, 28 SA).

	Day 1	3 Months	12 Months	p ^a
sFas, pg/ml	7275 (6332–8279)	7849 (6451–8790)	7746 (6848–8869)	0.689
sFasL, pg/ml	51 (37–60)	55 (42–63)	57 (43–70)	< 0.001
IL-6, pg/ml	3.8 (2.1–6.5)	2.2 (1.2–3.1)	2.2 (1.3–3.1)	0.002
NK cells, %	12 (8.6–16)	14 (9.7–20)	16 (11–20)	0.011
NK cells/ μ l	250 (142–371)	291 (200–375)	300 (237–448)	< 0.001
T cells, %	76 (69–80)	75 (69–80)	74 (68–78)	0.064
T cells/ μ l	1700 (990–2128)	1415 (995–2144)	1590 (1070–2115)	0.159
Apoptotic NK cells, %	0.5 (0.2–1.3)	1.1 (0.5–2.2)	0.9 (0.5–1.2)	0.687
Apoptotic T cells, %	1.0 (0.5–1.6)	1.4 (0.6–2.5)	0.7 (0.4–1.4)	0.146

Soluble, s; Fas L, Fas ligand; interleukin-6, IL-6; NK cell, natural killer cell. Ordinal data are presented as median (inter-quartile range).

^a The differences over time were analyzed by Friedman's test.

Fig.9. The correlation between sFasL levels and proportions of NK cells

CAD patients **3 months** after a coronary event (Cohort I) $r=0,40$, $p<0,01$.

We also assessed the concentrations of sFas and sFasL in patients in Cohort II, i.e. the same cohort that is described in Study I. These patients were recruited 3–6 months after a coronary event, i.e. being in a similar clinical stage as patients in Cohort I were during follow-up. Levels of sFas or sFasL did not differ between patients and controls (Table 4). However, in the patients, levels of sFasL were positively and significantly correlated with NK cell apoptosis but not so in controls (Fig. 10). No correlation was found between plasma levels of the soluble markers of apoptosis and NK cell apoptosis induced by oxLDL or 7 β OH *ex vivo*.

Table 4.

Clinical characteristics, plasma levels of sFas and sFasL and *ex vivo* apoptosis of NK cells and T cells in 16 matched pairs of CAD patients 3–6 months after a coronary event (Cohort II) and clinically healthy controls.

	Patients <i>n</i> = 16	Controls <i>n</i> = 16	<i>p</i> ^a
Age, years	60 (58–65)	60 (55–62)	0.210
Female, <i>n</i> (%)	2 (14)	2 (14)	1.000
Smokers, <i>n</i> (%)	2 (14)	2 (14)	1.000
Body mass index, kg/m ²	25 (24–30)	25 (24–38)	0.569
sFas, pg/ml	8087 (6932–8845)	7187 (6261–8035)	0.216
sFasL, pg/ml	55 (48–66)	57 (48–63)	0.926
NK cell apoptosis	2.52 (1.75–3.37)	2.00 (1.35–2.77)	0.024
T cell apoptosis	0.87 (0.79–1.05)	0.65 (0.55–0.97)	0.220

NSTE-ACS, non-ST-elevation acute coronary syndrome; SA, stable angina; soluble, s; Fas L, Fas ligand; NK cell, natural killer cell. Ordinal data are presented as median (inter-quartile range). *Ex vivo* apoptosis of NK cells and T cells is presented as fold increase compared to baseline.

^a Mann–Whitney U-test was used for comparison between the groups.

Fig. 10. The correlation between sFasL levels and NK cell apoptosis *ex vivo* (fold increase compared to baseline) in CAD patients 3-6 months after a coronary event (Cohort II) $r=0,54$, $p<0,05$.

The association between sFasL and apoptosis was further investigated in that isolated NK and T cells from blood donors were stimulated in medium only, with IL-15 or IL-15+IL-12, for 48 h, after which an assessment of their propensity to undergo activation-induced apoptosis was done. Supernatants from the stimulated cells were examined, measuring the sFasL release induced by the different stimuli.

After stimulation with IL-15 only, NK cells showed increased size, granularity and CD56 expression (i.e. indicators of activation) but also increased apoptosis compared with unstimulated cells, while the combination of IL-15 and IL-12 yielded more apoptotic cells than with IL-15 alone. Supernatants of NK cells showed significantly increased sFasL levels from IL-15 stimulated cells with augmented concentrations when using both IL-15 and IL-12 (Fig. 11). Compared to NK cells, T cells were both less prone to undergo apoptosis regardless of stimuli and supernatants contained markedly lower levels of sFasL.

Fig. 11. Release of FasL by isolated NK cells and T cells (CD3+) derived from blood donors. Culture in medium only (-), IL-15 or IL-15+IL-12.

When analysing a larger sample than in Study I, we could not confirm that the levels of apoptotic NK cells were increased in patients compared with controls. However, this may not be so surprising considering the fact that apoptotic cells should be rapidly cleared from the circulation. A swift clearance of apoptotic cells is a physiological phenomenon preventing secondary necrosis of apoptotic cells as well as unnecessary antigen-antibody presentation distant to the target organ, factors both promoting systemic inflammation. Studies in gastric cancer as well as in systemic lupus erythematosus (SLE) have shown higher levels of apoptotic cells [198-200] in the circulation but these are different disease states than CAD. Also, the tumor burden and the extent of organs affected by inflammation in the SLE population respectively, were not stated which make eventual comparisons difficult regarding the residual clearance capacity of apoptotic cells in the circulation. Furthermore, apoptosis is a time-dependent process and NK cells are sensitive to spontaneous apoptosis *ex vivo*. Trying to prevent false positive results, the time limit from blood sampling to flow cytometry was one hour, which could have accentuated differences

with other studies. Hence, low levels of apoptotic cells *in vivo* does not translate to low apoptotic rates but merely a functioning clearing system, as apoptosis of NK cells *ex vivo* was increased in patients and not in controls and positively correlated to sFasL.

We believe that the increase of NK cells in CAD patients during follow-up [196] may stand for an increased proliferative rate. Our *in vitro* data support that FasL is not only a marker of apoptosis but also of proliferation – i.e survival - signaling, if the cell status and its environment at that given time promotes it. This has also been suggested by others [201-203]. Furthermore, it was demonstrated in NK cell lymphomas where plasma levels of sFasL correlated to disease activity, with levels declining in parallel with normalized NK cell counts after successful chemotherapy [62].

Interestingly, the release of FasL may also antagonize apoptosis in that sFasL binds to sFas, thereby inhibiting sFas-mFasL (membrane bound) interaction. Also, since sFasL is 1000-fold less active than its membrane-bound counterpart [202], sFasL has even shown to counteract membrane-bound FasL by competitive binding to Fas *without* causing apoptosis [204]. As previously mentioned sFasL has been suggested being a atheroprotective marker, since it positively correlated with improved endothelial cell function in CAD patients [100]. Also, low levels of sFasL are over-represented in CAD patients and in those with high cardiovascular risk [99, 205]. In addition to NK cells themselves being a source of sFasL the above mentioned release of sFasL from endothelial cells could imply an improved endothelial function, a sign usually pointing to a more antiinflammatory milieu. As has been shown recently, inflammation is clearly linked to a restrained NK cell development and shortened NK cell life span [196, 206]. In this study increasing levels of sFasL during the follow-up after a coronary event were connected to increased numbers of NK cells, thus linking a possibly atheroprotective marker to the NK cell status, pointing to a favorable roll for NK cells in CAD.

Study III

Soluble TNF receptors are associated with infarct size and ventricular dysfunction in ST-elevation myocardial infarction

Mean age of the study group was 61 years and about a third was women. Time from symptom onset to PCI was on average 2,5 hours and after PCI 100% of patients had TIMI III flow compared to 98% with TIMI 0 prior to intervention. CMR was performed in all patients at day 4, while 9 did not undergo the 4 month examination. Of the soluble markers of apoptosis that were measured, sTNFRI, sTNFRII and sFas all increased from baseline to 24 h whereas sFasL decreased. As previously stated, three central correlations between markers in plasma and CMR measures were investigated:

1. *Infarct size*, measured as the late gadolinium enhancement zone (LGE zone). STNFR1 at 24 h tended to correlate and correlated positively, with infarct size at 5 days and 4 months, respectively. The relative change in sTNFR1 between 0 and 24 h showed strong positive correlations with the infarct size at both time points, a pattern which TNFR2 shared but with weaker correlations. sFas and sFasL on the other hand showed a tendency towards negative correlations, however non-significant (Table 5).

Table 5. Correlations between soluble markers of apoptosis and infarct size.

		Total LGE at 5 days	Total LGE at 4 months
sTNFR1	0 h	−0.014	0.037
	24 h	0.231	0.358*
	% change	0.412*	0.385*
sTNFR2	0 h	0.005	0.034
	24 h	0.138	0.203
	% change	0.356*	0.369*
sFAS	0 h	−0.150	−0.039
	24 h	−0.304	−0.099
	% change	−0.242	−0.097
sFASL	0 h	−0.236	−0.192
	24 h	−0.210	−0.174
	% change	0.036	−0.021

Values are given as rho-values from Spearman test.
 *p<0.05.
 sTNFR1, soluble tumour necrosis factor receptor 1; sTNFR2, soluble tumour necrosis factor receptor 2; sFAS, soluble FAS; sFASL, soluble FAS ligand; LGE, late gadolinium enhancement zone.

2. *Left ventricular dysfunction*, measured as LVEF. Both levels at 24 h and the relative 0 h - 24 h change of sTNFR1 were significantly and *negatively* correlated with LVEF at 4 months. Similar trends were found for the other markers without reaching statistical significance (Table 6).

3. *Left ventricular remodeling*, at CMR, and expressed as the change in end diastolic and end systolic volume indices between 5 days and 4 months. No correlations with any of the markers (Table 6).

Table 6. Correlations between soluble markers of apoptosis and measures of left ventricular dysfunction and remodeling.

		LVEF% at 5 days	LVEF% at 4 months	dEDVI	dESVI
sTNFR1	0 h	−0.048	−0.177	−0.070	0.097
	24 h	−0.200	−0.354*	−0.237	−0.067
	% change	−0.274	−0.375*	−0.285	−0.253
sTNFR2	0 h	−0.017	−0.155	−0.119	0.071
	24 h	−0.078	−0.253	−0.160	−0.014
	% change	−0.125	−0.192	−0.045	−0.159
sFAS	0 h	−0.022	−0.075	0.061	0.210
	24 h	−0.264	−0.258	0.171	0.196
	% change	0.048	−0.078	0.173	−0.005
sFASL	0 h	−0.257	−0.228	0.035	0.175
	24 h	−0.249	−0.168	−0.106	0.057
	% change	−0.016	0.087	−0.202	−0.194

Values are given as rho-values from Spearman test.

*p<0.05.

sTNFR1, soluble tumour necrosis factor receptor 1; sTNFR2, soluble tumour necrosis factor receptor 2; sFAS, soluble FAS; sFASL, soluble FAS ligand; LVEF, left ventricular ejection fraction; dEDVI, change in end-diastolic volume index from 5 days to 4 months; dESVI, change in end-systolic volume index from 5 days to 4 months.

A strong positive correlation was found between MMP-2 and all the soluble markers of apoptosis. Starting with MMP-2, experimental data have demonstrated that intracellular MMP-2 activation in response to oxidative stress might be an important mechanism of triggering myocardiocyte apoptosis [207]. It also highlights the broader role of MMPs, as regulating enzymes, far beyond the scope of the traditional role as degradative enzymes of extracellular matrix. TNF has been found to evoke myocardiocyte apoptosis through both the intrinsic and extrinsic pathways [208]. MMP-2 in turn has shown to contribute to TNF-induced apoptosis in cultured myocardiocytes in that inhibiting MMP-2 prior to TNF insult decreased myocardial MMP-2 activity and reduced the percentage of resulting apoptotic cells [209]. Though MMP-2 has been shown to

play an important role in *early* myocardial IR-injury by for example degrading Troponin I [210], later effects have also been described as in a transgenic mouse model with overexpression of TNF, where an increase in MMP activity was recorded during LV-remodeling [211]. Finally, the F.I.R.E trial, showed strong positive correlations between MMP-2 in plasma and infarct size and left ventricular dysfunction [212]. As previously stated, a large number of studies have demonstrated a role for TNF signaling in the process of IR-injury leading to LV dysfunction and negative remodeling followed by congestive heart failure [152].

Following the IR-injury event, there is also a sustained increase of TNF, both locally in the heart as well as in circulating levels in blood pointing to a persistent augmentation potentially counteracting the repair process [213, 214]. However, animal experiments regarding mechanisms and outcomes show quite conflicting results. While high doses of exogenous TNF renders both ischemic pre- and postconditioning less efficient, pre-ischemic neutralization of TNF reduces infarct size in rabbits and local delivery of sTNFR1 gene reduces infarct size following IR-injury in rats [155-157]. Other studies have demonstrated the protective role of TNF signaling on myocardial cell apoptosis. In a TNFR I and II receptor knockout mouse model exposed to IR-injury, resulting infarcts were larger than in mice with one or both receptors intact [154, 215]. A cardioprotective mechanism however seems to be conveyed by the TNFR II receptor when binding to TNF [216] This highlights the complexity of TNF signaling and its pro- vs anti-apoptotic properties which physiologically are, in part, due to the intricate crosstalk between intracellular apoptotic and survival signals [76, 217, 218]. Other potential reasons for the disharmonious outcomes in studies are the different degree of TNF overexpression in the animal models used, with either a preponderance towards TNFRI or TNFRII and the actual species of the laboratory animal itself. Furthermore, some researchers chose to examine TNF overexpression in myocytes only, while others allowed wider expressions including endothelial cells, both models potentially leading to different total effects on apoptosis during IR-injury as well as difficulties in discriminating the contribution to the tissue injury by each cell type[151].

Both Fas and FasL expression are increased in myocytes exposed to hypoxia [219] and activation of the Fas pathway has been shown to induce apoptosis in cardiac myocytes [220]. There is also evidence suggesting that Fas-mediated apoptosis is associated with myocardial reperfusion injury [221], and post-infarction ventricular remodeling [222]. However, Gomez et al. [223] reported that mice lacking functional Fas had no difference in infarct size and apoptosis after IR-injury, suggesting that Fas plays a minor role in this setting. Similarly, Li et al. [222] found that mice lacking Fas ligand had similar infarct sizes 2 days after myocardial infarction. Interestingly though, infecting hearts with an adenovirus encoding soluble Fas, a competitive inhibitor of FasL, improved

cardiac function and survival 3 days after a myocardial infarction. This would suggest that the Fas pathway contributes to cell death in the later stages of an infarction. In one of the few human studies in a real-life acute MI setting examining sFas and sFasL, no correlation was found with infarct size but sFas did correlate to hemodynamic measures of heart failure. In our study, including a homogenous STEMI population, sFas and sFasL did not show any consistent correlations with infarct size, LV-dysfunction or measures of remodeling. However, this does not exclude a role of the Fas/FasL pathway in IR-injury of the myocardium, one reason being that the time points for analysis of sFas and sFasL in the present study (at baseline and 24 hours) might not be the optimal in reflecting activation of the Fas/FasL pathway. The main findings in this study were consistent and significant correlations between 24 h levels of plasma TNFRI and outcome measures of infarct size and LV-dysfunction. These are in line with previous clinical studies on acute MI demonstrating a role of sTNFRI in predicting mortality and new-onset heart failure [101, 102]. Here, however, for the first time, a homogenous STEMI population receiving state of the art treatment was studied in this context. In addition to 24 h levels of sTNFRI, the relative changes of sTNFRI and sTNFRII during the first 24 h are associated with the final infarct size, 4 months later, strengthening the association between apoptosis and the extent of IR-injury.

Study IV

Soluble markers of apoptosis in myocardial infarction patients during acute phase and 6-month follow-up

In study III, the correlations between plasma levels of sTNFRI and sTNFRII taken in the acute phase of STEMI, with outcomes of infarct size and LV dysfunction at follow-up, reinforced apoptosis as an important contributor to IR-injury. This is in line with findings in myocardial biopsies from both humans and laboratory animals which show that the process of apoptosis continues for days to weeks after an ischemic event [148,149, 224]. A lower but still increased apoptosis can persist for months, thus contributing to negative remodeling and subsequent heart failure [152, 225]. However, reports on serial biopsies from the same subjects examining apoptosis over time are lacking. The same is true for markers of apoptosis. Also it was not known whether STEMI and NSTEMI patients differ in regard to these markers. This encouraged further investigations of the temporal patterns of apoptotic markers in patients with ACS, more specifically in the acute phase and at 6 months follow-up in patients with STEMI and NSTEMI, respectively.

The age and gender distribution of the 61 STEMI and 40 NSTEMI patients included in the study, 66 years with about one third being women, was in parallel with many studies on patients with ACS undergoing invasive therapy. A history of previous coronary revascularization and hypertension were more prevalent in the NSTEMI group as was the use of low molecular weight heparin, with iv Glycoprotein IIb/IIIa inhibitors being dominant in the STEMI population, as part of standard care in that patient group at the time of the study. A coronary angiography was performed in 100% of patients in both groups, taking place immediately in the STEMI group and within 72 hours (according to practice in medically stable patients) in the NSTEMI group. Of note, a majority of patients in both groups had no, or only mild LV-dysfunction at echocardiography on day 3 (Table 7.).

For all the analyzed markers, temporal patterns and degrees of changes were similar in the STEMI and the NSTEMI groups. A significant increase of sTNFR I and sTNFR II was seen between baseline and day 3 in both groups. At 6 months sTNFR I had returned to baseline values while sTNFR II remained increased. Plasma sFas and sFasL both increased from day 3 to 6 months and overall from baseline to 6 months while IL-6 increased in both groups during the first three days and had normalized at 6 months (Fig. 12).

Table 7. Baseline characteristics, medications and procedures of the study population.

	STEMI (n=61)	NSTEMI (n=40)	p-value
Baseline characteristics			
Age, years (mean, SD)	66 (13)	66 (11)	0.453
Female, % (n)	34 (21)	30 (12)	0.180
Previous myocardial infarction, % (n)	11 (7)	27 (11)	0.061
Previous PCI, % (n)	2 (1)	22 (9)	0.001
Previous CABG, % (n)	2 (1)	15 (6)	0.015
Previous stroke, % (n)	0 (0)	3 (1)	0.396
Diabetes, % (n)	10 (6)	15 (6)	0.416
Hypertension, % (n)	26 (16)	47 (19)	0.034
Smoking, % (n)			
-Present	21	6	0.097
-Previous	18	15	
Medications			
Aspirin, % (n)	100 (61)	100 (40)	1.000
Clopidogrel, % (n)	100 (61)	100 (40)	1.000
Betablockers, % (n)	97 (59)	88 (35)	0.110
ACE-inhibitors/ARBs, % (n)	80 (49)	78 (31)	0.804
Statin, % (n)	95 (58)	100 (40)	0.515
Fondaparinux, % (n)	2 (1)	78 (31)	<0.001
Glycoprotein IIb/IIIa, % (n)	92 (56)	10 (4)	<0.001
Procedures			
Coronary angiography, % (n)	100 (61)	98 (39)	0.396
PCI, % (n)	93 (57)	83 (33)	0.299
CABG, % (n)	2 (1)	8 (3)	0.066
Systolic LV-function by echocardiography			
Normal, %(n)	29 (18)	70 (28)	0.003
Mildly reduced, %(n)	54 (33)	25 (10)	
Moderately reduced, %(n)	15 (9)	5 (2)	
Severely reduced, %(n)	2 (1)	0 (0)	

SD, standard deviation; PCI, percutaneous coronary intervention; CABG, coronary artery by-pass grafting; LV, left ventricular; STEMI, ST-elevation myocardial infarction; NSTEMI, non-ST-elevation myocardial infarction.

Fig.12. Plasma levels of soluble markers of apoptosis and IL-6 in (A) STEMI and (B) NSTEMI patients.

Data are presented as mean (SD) for sTNFR1, sTNFR2, sFas and sFasL, and as median (25th, 75th percentile) for IL-6. *p<0.05. sTNFR1, soluble tumour necrosis factor receptor 1; sTNFR2, soluble tumour necrosis factor receptor 2; sFas, soluble FAS; sFasL, soluble Fas ligand; IL-6, interleukin-6; STEMI, ST-elevation myocardial infarction; NSTEMI, non-ST-elevation myocardial infarction.

Significant positive correlations were found between hsTnT taken 6-8 h after admission and sTNFR1 and sTNFR2 at day 3, as well as between hsTnT and the absolute changes in the mentioned markers between baseline and day 3. The elevated levels of IL-6 recorded at day 3 correlated noticeably with sTNFR1 and sTNFR2 at all time points and in addition, IL-6 at baseline and at day 3 showed positive correlations with hsTnT. sFas at day 3 correlated inversely with hsTnT and no correlations between sFas, sFasL and IL-6 were found at all. As depicted in table 8, levels of sTNFR1 and sTNFR2 were significantly higher in patients with reduced LV-function compared to subjects with preserved LV-function. No correlations regarding the echo-measures were found for sFas or sFasL.

Table 8. Soluble markers of apoptosis in relation to systolic LV-function.

		Systolic LV function			p-value
		Normal n=46	Mildly Reduced n=43	Moderately or severely Reduced n=12	
sTNFR1 (pg/mL)	baseline	1152(308)	1462(668) ^γ	1180(397)	0.017
	3 days	1326(355)	1647(609) ^γ	1537(511) ^φ	0.015
	6 months	1127(420)	1485(588) ^γ	1409(471) ^φ	0.029
sTNFR2 (pg/mL)	baseline	2306(620)	2676(790) ^γ	2457(680)	0.061
	3 days	2642(642)	3059(812) ^γ	2939(747) ^φ	0.034
	6 months	2663(686)	3020(777) ^γ	2982(864) ^φ	0.036
sFas (pg/mL)	baseline	9218(2058)	9002(2161)	7992(2633)	0.224
	3 days	9793(1967)	9301(2213)	8687(2787)	0.251
	6 months	10271(2224)	10517(3000)	9505(2648)	0.504
sFasL (pg/mL)	baseline	37.7 (15.3)	38.3(15.3)	38.3(13.6)	0.942
	3 days	37.9 (14.8)	38.5(13.6)	39.0(15.2)	0.969
	6 months	44.4 (17.1)	45.9(16.2)	49.6(20.1)	0.641

Values are given as mean (SD). P-values are shown for ANOVA. ^γ p<0.05 comparing normal and mildly reduced LV-function, ^φ p<0.05 comparing normal and moderately or severely reduced LV-function. LV, left ventricular; sTNFR1, soluble tumour necrosis factor receptor 1; sTNFR2, soluble tumour necrosis factor receptor 2; sFas, soluble Fas; sFasL, soluble Fas ligand.

A majority of studies on IR-injury have been in a STEMI setting where ischemia and reperfusion phases are usually well-defined and apoptosis considered an important contributor to myocardiocyte death [227,148,149,224]. Myocardial cell death caused by IR-injury in NSTEMI is, as described by CMR, less extensive and also harder to define clearly [226]. The temporal patterns of soluble markers of apoptosis in this study were found to be very similar in STEMI and NSTEMI, indicating that apoptosis plays an equally active role in either infarct type. Increases of sTNFRs in the acute phase, here between baseline and day 3 are in line with findings in other studies on patients with different MI manifestations and further strengthens the role for TNFRI-mediated activation of apoptosis during an acute MI [101,102]. Furthermore, sTNFRI and sTNFRII on day 3 correlated positively both with levels of hsTnT as well as with the degree of LV-dysfunction on echocardiography. Both these findings touch with the results from study III.

In contrast to the longitudinal changes of the sTNFRs, plasma levels of sFas and sFasL showed a somewhat opposite pattern suggesting differential roles for the two extrinsic pathways of apoptosis in this context. To what extent the sFas/sFasL pathway plays a pro- or antiapoptotic role here is unclear but no correlation to either LV-dysfunction, or any robust ditto when it came to hsTnT, confirms the findings from study III where correlations with both infarct size, LV-dysfunction as well as measures of remodelling were lacking. It is also in line with studies on AMI patients which failed to show correlations between sFas/sFasL and the size of the infarct [96].

Interestingly, we observed significantly lower levels of sFas and sFasL at baseline and at 3 days compared to 6 months. We speculate that plasma levels of these markers are lowered in the acute phase of an MI and then returns to normal during long-term follow-up. This is supported by the fact that levels of sFas at 3 days are negatively correlated with troponin levels, ie the larger infarct the lower levels of sFas. Also, several studies measuring sFas and sFasL during the acute phase response to various diseases have found these markers to be unaltered or even reduced [228, 229]. In this context it is noteworthy, that sFas/sFasL did *not* correlate with IL-6. That was however the case for the sTNFRs, perhaps pointing to a link between TNF-mediated apoptosis and inflammation in IR-injury. There is some evidence for that sTNFRs, especially sTNFRI, could carry prognostic information thus forming a clinical tool in risk management [101, 102] However, in those studies soluble markers were drawn only at the index event and not at follow-up. Also, they were not compared to more novel and established biomarkers such as troponins and brain natriuretic peptides. The current study included 101 patients presenting with either STEMI or NSTEMI, all undergoing coronary angiography in addition to receiving all

other measures of state of the art medical care. Cardiac events during follow-up were appropriately low. The answer to if sTNFRs provide additional prognostic value in these patient groups, requires larger-scale studies.

CONCLUDING REMARKS

Apoptosis is a fundamental and important physiological process. An altered apoptosis can be observed in several significant pathologies, such as CAD. An increased apoptotic rate can take place in numerous cell types, both locally in the plaque, as well as in the circulation. Apoptosis is an ongoing phenomenon but its relative importance varies through the different phases of the atherosclerotic process. In the event of plaque rupture and an impending myocardial infarction, the myocardium is subjected to a varying degree of IR-injury. In this setting and with eventual subsequent congestive heart failure, an increased apoptosis of foremost cardiomyocytes is anticipated. Future therapeutic interventions directed towards apoptosis, requires knowledge about the temporal and spatial profile of this process.

With regard to the mentioned temporal and spatial aspects, *in vivo* measurements of apoptosis in man, is associated with distinct challenges. Collecting peripheral venous blood and analyzing apoptosis of circulating cells *ex vivo* as well as measuring soluble markers of apoptosis in plasma is comparatively simple, if not performed routinely. The obvious limitation involves how to determine the link between the site of interest and in which cell type(s) the increased apoptosis took place.

In the light of these pros and cons we have used peripheral venous blood to study apoptosis in different courses of events and phases of CAD in humans, one focus being on NK cells and soluble markers of apoptosis. We find that patients with CAD have an increased rate of both spontaneous and oxidized lipid-induced NK cell apoptosis. In the follow-up period after a coronary event, there is an increase of the number of circulating NK cells which could be a prognostically favourable sign. Plasma levels of sFasL correlate with the recovery of the number of NK cells as well as with the sensitivity of NK cells to undergo apoptosis *ex vivo*. Our data also show that NK cells themselves are a prominent source of sFasL. Other studies have been suggestive of the latter being an atheroprotective marker. Our findings link NK cell status to sFasL thus supporting a beneficial role for NK cells in CAD. Furthermore, our data indicate that sFasL can be used as a surrogate marker of NK cell changes.

In patients with an acute MI, plasma levels of sFasL and sFas were found to be reduced in the acute phase while recovering during follow-up.

The significance of this is unclear but could imply that the Fas/FasL system carries importance for repair and compensation processes after an MI. The levels of sFas and sFasL show no correlation with measures of infarct size or LV-function after a STEMI, correlations lacking also with LV-function after a NSTEMI. In contrast to this, a clear increase in plasma levels of sTNFR1 and sTNFR2 was recorded in the acute phase of both STEMI and NSTEMI patients and which correlated to both infarct size and LV-function. This is likely to be reflective of an increased apoptosis, partly responsible for the IR-injury and the LV-dysfunction. The similar temporal patterns of sTNFR1 and sTNFR2 in STEMI and NSTEMI is somewhat surprising but indicate that apoptosis has implications also in NSTEMI, in spite of the IR-injury here being less well defined and not as pronounced.

In summary, our findings provide further support for a dynamic pattern of apoptosis in CAD. Also, the data highlights the complexity of the apoptotic pathways, with potentially differentiated roles of the TNFR- and Fas/FasL-systems.

FUTURE DIRECTIONS

Data from numerous experimental and clinical studies demonstrate a role for apoptosis in every stage of CAD, from plaque progression to terminal ischemic CHF. However, many contradictory findings exist with conflicting results from animal studies and clinical trials in humans. The complexity of the apoptotic and antiapoptotic pathways is striking and there is still limited knowledge on the absolute and relative importance of apoptosis in different cell types during the various stages of CAD and its clinical complications. As a result, interventional trials trying to inhibit apoptosis in eg post-MI patients have so far been unsuccessful in improving clinical outcomes. Further basic and translational research is needed to clarify the picture.

The use of soluble markers of apoptosis to monitor the apoptotic process during stages of CAD and to evaluate potential interventions is promising. Easily accessible and easily analyzed, these markers might prove to be clinically useful. However, more data is needed on the correlation between apoptotic rate within the tissue of interest and plasma levels of soluble markers of apoptosis from a peripheral venous blood sample. Also, prospective studies are needed to demonstrate if these markers carry any novel prognostic information when added to traditional cardiac biomarkers.

The final goal would be to intervene and inhibit apoptosis in clinical situations where an enhanced apoptosis contributes to disease progression and a poor prognosis. Such an intervention would need to target specific pathways, in specific cell types, within a specific time frame. Any off-target effects could have serious consequences, since a balanced and controlled apoptosis is essential to uphold homeostasis in most tissues. Thus, further clinical research is needed to improve knowledge on target specificity and how to achieve it.

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APPENDIX (papers I-IV)

Papers

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