Summary and general discussion
INTRODUCTION

In this thesis, we studied the interplay between inflammation and cardiovascular disease. In several mouse and human studies we not only studied the process of atherosclerosis, but also evaluated the role of inflammation in the cardiac microvasculature and atherothrombosis by using a variety of techniques, including (immuno)histochemistry, enzyme-linked immunosorbent assays, positron emission tomography, computed tomography and cardiac magnetic resonance imaging.

In Chapter 2 we described the inhibitory effect of an anti-inflammatory fish oil constituent, docosahexaenoic acid, on the accumulation of the pro-inflammatory advance glycation end product (AGE) N(ε)-(carboxymethyl)lysine (CML), in the microvasculature of the heart. In Chapters 3-8, we studied the effect of three different types of inflammatory responses, following orthopedic surgery, infection and acute myocardial infarction (AMI) on atherosclerotic plaques. In Chapter 9 and 10, we assessed the inflammatory cell composition of coronary thrombi in relation to thrombus age and the occurrence of microvascular injury.

This chapter will put the previous chapters in mutual context. To visually support these paragraphs, we have included an overview of our findings. First, the rationale for the main hypothesis will be shortly discussed, then we will summarize the main findings of the Chapters, address methodological issues and present directions for further research. We will end the general discussion with an overall conclusion.

RATIONALE

Observational studies show a peak incidence of cardiovascular events early after clinical conditions accompanied with severe systemic inflammation. For example, respiratory tract infections are associated with an approximate fivefold risk for early myocardial infarction. Not only infections, but also major surgery is associated with a marked systemic inflammatory response. As was shown in a recent nationwide cohort study indicated that the risk of myocardial infarction in patients undergoing total hip or knee replacement is increased by no less than 25-fold, again predominantly during the first days to two weeks. Furthermore a landmark paper on the role of acute systemic inflammation in atherosclerosis showed increased plaque area and intra-plaque inflammation in mice briefly after acute myocardial infarction, highlighting another form of acute systemic inflammation. The acuteness of the increased risk in the observational studies suggests plaque vulnerability, which is closely correlated with intra-plaque inflammation.
and plaque instability\textsuperscript{6,7}. Indeed, in the aforementioned mouse study intra-plaque macrophage density was increased after a burst of systemic inflammation caused by AMI\textsuperscript{5}. Furthermore, several murine studies have shown pro-atherogenic effects of serum amyloid A peptide, an important\textsuperscript{8} marker and mediator of acute inflammation in mice\textsuperscript{9-12}, suggesting the direct effect of inflammation on the vessel wall.

We hypothesized that systemic inflammation induces vessel wall inflammation with subsequent plaque instability and addressed this hypothesis in the cardiac microvasculature and aortic roots of mice and in the atherosclerotic coronary plaques and coronary thrombi of patients using three different models for acute systemic inflammatory activation: (1) major orthopedic surgery induced tissue damage, (2) systemic infection or (3) acute myocardial infarction itself.

**MAIN FINDINGS, METHODOLOGICAL CONSIDERATIONS AND DIRECTIONS FOR FURTHER RESEARCH**

N(ε)-(carboxymethyl)lysine (CML) is one of the major AGEs and its formation is enhanced in the presence of inflammation\textsuperscript{13}. CML is believed to play a role in the development and progression of atherosclerosis\textsuperscript{13-15} and its accumulation in atherosclerotic blood vessels, increases with age. Although age-related CML accumulation was found in atherosclerotic vessels\textsuperscript{16}, it remained unknown whether age-related CML accumulation also occurs in the non-atherosclerotic microvasculature of the brain and the heart. In Chapter 2 we found age-related CML accumulation in the non-atherosclerotic microvasculature of the heart and the brain of atherosclerotic mice (Fig. 1D). Furthermore, we found that the omega-3 (n-3) long-chain polyunsaturated fatty acid, docosahexaenoic acid (DHA) polyunsaturated fatty acid DHA, the fish oil constituent known for its anti-inflammatory actions, inhibited this age-related microvascular CML accumulation in the heart only. Intra-myocardial blood vessels are more important in the induction of acute events than previously thought and potentially identify new therapeutic targets in the treatment of AMI\textsuperscript{17}. Therefore our finding of increased CML in these vessels is of great interest. The positive effects of our study should be evaluated/validated in a large scale systematic future trial. Of note, recently published trials in patients with coronary artery disease or after AMI did not show an effect of omega-3 fatty acids on major cardiovascular endpoints (e.g. infarct induction), possibly due to state of the art concomitant drug treatment\textsuperscript{18}. However, as suggested by the current guidelines, omega-3 fatty acids supplementation in patients with CAD or after MI and possibly in those with heart failure remains to be encouraged\textsuperscript{18}. We add with our study a potential substrate/pathway for this encouragement.
Figure 1 Intensity score of CML in 40,70 and 90 weeks old mice
Overview of the most important findings of this thesis. ND: not determined; CML: N(ε)-(carboxymethyl)lysine; DHA: docosahexaenoic acid.
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### Atherosclerotic plaque in aorta

- **1. Surgery**
  - Inflammation: 
    - Plaque area: ↑
    - Plaque instability: nd
    - Necrotic core area: ↑
    - Macrophages: ↓
    - Neutrophils: =
    - Lymphocytes: ↓
    - Mast cells: =

- **2. Surgery**
  - Plaque area: =
  - Plaque instability: =
  - Necrotic core area: =
  - Macrophages: =
  - Neutrophils: =
  - Lymphocytes: =
  - Mast cells: =

- **3. LPS**
  - Plaque area: =
  - Plaque instability: =
  - Necrotic core area: =
  - Macrophages: =
  - Neutrophils: =
  - Lymphocytes: =
  - Mast cells: nd

### Atherosclerotic plaque in coronary arteries

- **1. AMI**
  - Plaque area: nd
  - Plaque instability:thing
  - Necrotic core area: =
  - Macrophages: =
  - Neutrophils: =
  - Lymphocytes: =
  - Mast cells: =

- **2. Infection / Sepsis**
  - Plaque area: nd
  - Plaque instability: =
  - Necrotic core area: =
  - Macrophages: =
  - Neutrophils: =
  - Lymphocytes: =
  - Mast cells: =

### Coronary thrombus

- **1. Thrombus age**
  - Macrophages: ↓
  - Neutrophils: ↑
  - Lymphocytes: =
  - Mast cells: nd

- **2. Patient age**
  - Macrophages: ↓
  - Neutrophils: ↓
  - Lymphocytes: =
  - Mast cells: nd

- **3. MVI**
  - Macrophages: =
  - Neutrophils: =
  - Lymphocytes: =
  - Mast cells: nd

### Intramyocardial vessels

- **1. Advancing age**
  - CML: ↑
  - DHA: ↓

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In Chapter 3, we studied the effect of major orthopedic surgery on atherosclerotic plaques. Major orthopedic surgery in ApoE -/- mice causes acute systemic inflammation. At 15 days post-surgery, we observed a significant increase of plaque area, mainly due to necrotic core enlargement (Fig.A1). Plaque necrosis is a characteristic hallmark of atherosclerotic lesions that causes acute atherothrombotic vascular disease. Thus, our finding of a post-surgery increase of necrotic core area may reflect increased plaque vulnerability and could, at least in part, explain the increased incidence of cardiovascular events after major orthopedic surgery. In Chapter 4 we evaluated in a similar approach the effects of a lipopolysaccharide (LPS) evoked acute inflammatory response on atherosclerosis. LPS is part of the outer membrane of Gram-negative bacteria, and elicits strong immune responses in both animals and humans via the Toll-like Receptor 4 (TLR4), an important route in the human inflammatory response/sepsis. We chose this model over an actual infection model, such as the colon ligature and puncture model that in theory could more closely resemble the pathophysiological process of sepsis than the LPS model, since actual infection models are very hard to reproduce and there are issues with controlling the magnitude of the septic challenge. In general one could say that sepsis is one of the most difficult clinical conditions to model in animals as is also shown by the fact that several promising therapeutic agents that were effective in animal studies failed to demonstrate a similar benefit in human clinical trials. In order to validate our model, we measured serum amyloid A and observed indeed a marked systemic inflammatory response, similar to the orthopedic surgery model. However, in contrast to the orthopedic surgery model, we did not observe effects on plaque area, plaque severity and inflammatory cell density in the atherosclerotic lesions of the ApoE3Leiden mice at both t=3 and t=15 days after LPS injection (Fig A2).

Although these two clinical conditions (orthopedic surgery and a LPS stimulated immune response) share the presence of an acute systemic inflammatory response, they also have many differences. Obviously the triggers of inflammation (surgery induced tissue damage vs. LPS stimulation) and therefore the profile of the inflammatory cascade differ. Furthermore, surgery, in contrast to LPS injection, was accompanied by anesthesia, pain, temporarily immobilization, and breach of the physical barrier of skin and muscle. All such factors could be responsible for the observed difference. Moreover, the difference in observed effects between the orthopedic surgery and the LPS studies, could also be explained by the different mouse models used. Although both the ApoE3*Leiden mice and the ApoE -/- mice are established models in the atherosclerotic research field, there are some differences between the two. Apolipoprotein E (ApoE) is involved in the efficient uptake of lipoprotein particles and as a result mediates the clearance of cholesterol remnants. Normally, these remnant particles are rapidly removed from circulation. However, mutations in the gene encoding for ApoE (i.e. ApoE3*Leiden) or the double knock-out of the gene (ApoE -/- ) cause hyperlipidemia, which can be greatly enhanced with a high-fat diet, leading to an increased susceptibility for atherosclerosis.
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It is known that in the ApoE3*Leiden model plaques grow in a slower, more human-like way than the ApoE -/- model. Furthermore ApoE is important for hepatic clearance of LPS and the complete absence of this apolipoprotein enhances the immune response to LPS. This could have had effect on the positive outcome of the study in Chapter 3 were we used the ApoE -/- model instead of the ApoE3Leiden model.

Although our data in Chapter 3 are compatible with a causal role for surgery-induced inflammation, it would be interesting to address these findings to more in-depth studies that are designed to clarify inflammatory pathways. In addition, other non- or indirect inflammatory effects of surgery should be further explored, such as hemodynamic disturbances, sympathetic nerve system activation or enhanced platelet activity as these are potential contributors to atherosclerotic lesion development and increased cardiovascular risk. Furthermore it would be interesting to test our findings in advanced mouse models to study actual plaque complications rather than atherosclerotic plaque development alone.

As discussed before in Chapter 3 and 4, we used mouse models to evaluate our hypothesis. Mice models are crucial for the proof-of-principle of most basic research questions and have shown to be invaluable for the progress of biomedical science. However, caution is warranted when translating these studies directly to the human situation. Hereby overcoming for example the fact that in mice, plaques seldom rupture and therefore are considered less adequate to use for the evaluation of human-like plaque complications. We therefore translated our findings from the aforementioned murine studies into clinical studies of which we report in Chapter 5, 6, 7, 8.

In Chapter 5 we studied the effect of systemic inflammation on atherosclerotic coronary plaques of post-AMI patients with or without infection at time of death. We found that AMI was accompanied by advanced inflammation of human coronary atherosclerotic plaques in patients that died 6 hrs-14 days after onset of AMI (Fig. B1). The presence of instable plaques (thin fibrous caps and/or multiple inflammatory cells reaching up to the luminal endothelial layer of the plaque) was also increased in these groups. A particularly interesting finding was that the presence of infection (e.g. sepsis, pneumonia, pancreatitis, peritonitis) at time of death enhanced the mast cell density in the intima and media of coronary arteries, but this phenomenon was not accompanied by increased plaque instability nor an increase in other cell densities (Fig. B2). It thus seems that acute systemic inflammation, both cardiac and infectious, influenced atherosclerotic lesions in a different way.

In Chapter 6 we studied the presence of mast cells (MCs) in the intima and media of unstable and stable coronary lesions at different time points after AMI. We found MCs in the intima and media of both stable and unstable atherosclerotic coronary lesions after AMI, especially increasing in the media of unstable plaques in the patients that died 5-14 days after AMI (Fig B1). These results suggest that MCs present in coronary lesions

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might contribute to the onset of MI through their plaque-destabilizing or spasm-inducing properties. MI, in turn, might trigger intra-plaque infiltration of MCs, thereby inducing the transition of stable plaques into unstable plaques resulting in an increased risk of re-infarction.

Taken together, Chapters 5 and 6 thus indicate that MCs play a role in destabilizing plaques and contribute to the onset of AMI. This presumes a role for MC stabilizers, such as cromolyn, in the clinical management of patients with AMI and/or patients at risk for AMI. MC stabilizers prevent degranulation and the subsequent release of histamine, tryptase and chymase. Indeed, in several atherosclerotic murine studies inhibition of MCs reduced plaque instability, whereas activation of MCs increased plaque instability. It has to be noticed that the conclusions of our studies are based on a tryptase staining. Tryptase is stored in the MCs’ granules and is released when activated. It would be interesting to include in future projects not only the MC density, but also the extent of the released MC mediators, since it is known that the mechanism by which MCs are activated affects the profile of excreted MC mediators, which in turn could influence atherogenicity. For example, there is a difference in the mediators when activated by TLR4 or components of the complement system when compared to activation by IgE. Under ischemic conditions, MCs are known to release more growth factors like VEGF instead of tryptase.

In Chapter 4 and 6 we evaluated plaque stability in order to identify the so called vulnerable plaques which are prone to rupture. These thin-capped, macrophage rich plaques with large necrotic cores and signs of hemorrhage have been an integral part of our understanding on the pathophysiology of the acute coronary syndromes (ACS) for decades. The vulnerable plaque is still of great value as a readout parameter for the severity of atherosclerotic disease, it is in this context however interesting to realize that plaque rupture still plays an important causal role in acute coronary syndromes, but that superficial erosion appears on the rise. This is most likely explained by the changing risk profile of the studied western Caucasian population and the success of statin treatment which “stabilizes” plaques. This is for instance reflected by the increase of non-ST segment elevation myocardial infarction. In future research it therefore would be valuable to include quantification of plaque erosion. Further it seems that the consequences of a plaque disruption depend not only on the state of the atheromatous lesion itself, indicated by the high amount of ruptured and healed plaques found in the arteries of already young patients, but also on the fluid phase of the blood, for example the concentrations of fibrinogen, tissue factor, endogenous inhibitors of fibrinolysis, and pro-coagulant microparticles, which is known to be potentially altered by inflammation itself. Future studies must include the potential role of these factors in the development of AMI.
Next, we designed a study to replicate our finding of pro-atherogenic effects of major orthopedic surgery, in humans. In order to do so, we set out to compare large artery \(^{18}\text{F}-\text{FDG}\) uptake in patients scheduled for total knee or total hip replacement. Patients underwent \(^{18}\text{F}-\text{FDG}\)-PET/CT scanning one day prior to surgery and two to three days after surgery. \(^{18}\text{F}\)-2-fluoro-2-deoxy-D-glucose (FDG) imaging using positron-emission tomography (PET)/computed tomography (CT) provides a noninvasive assessment of inflammation and, as such, could be a valuable imaging biomarker in atherosclerosis. Major surgery may reduce \(^{18}\text{F}-\text{FDG}\) uptake in atherosclerotic plaques due to competitive uptake of the surgery area and change in pharmacokinetics of \(^{18}\text{F}-\text{FDG}\). The aim of the study presented in Chapter 7 was to validate the use of simplified quantitative \(^{18}\text{F}-\text{FDG}\) uptake parameters for evaluation of inflammation in atherosclerotic plaques after surgery. We found that tissue to background ratio (TBR) calibrated using venous blood samples could be used as surrogate (simplified) measures to assess changes in \(^{18}\text{F}-\text{FDG}\) uptake in atherosclerotic plaques of patients recovering from major orthopaedic surgery. This led to further evaluation of one of our major research questions: does major orthopaedic surgery increase intra-plaque inflammation in humans? Patients are currently being enrolled for this study in the Alrijne hospital. Results are expected in 2018 and will be published separately from this thesis. The preliminary results in \(n=4\) patients are presented in Chapter 8 and do not suggest an increase of \(^{18}\text{F}-\text{FDG}\) uptake in the major arteries of patients recovering from major orthopaedic surgery (Fig A3).

The process of intracoronary thrombus formation and naturally occurring resolution is poorly understood. We do know that inflammatory cells play a role in the formation and resolution of venous thrombi, but their role in coronary thrombosis is less clear. In Chapter 9, we analyzed inflammatory cells in thrombi derived from patients with STEMI in relation to histologically classified thrombus age. The VU University Medical Center thrombus aspirate collection provided us the unique opportunity to study patient thrombus material. We found that fresh thrombi (76.1%) were the most abundant as compared to lytic (16.8%) and organized (7.1%) thrombi. Neutrophils were significantly less present in organized compared to fresh and lytic thrombi, respectively. Monocytes/macrophages were significantly more present in lytic than in fresh thrombi (Fig. C1). We additionally found that thrombi from patients aged <50 years as compared to >50 years old contained significantly more neutrophils and monocytes/macrophages irrespective of thrombus age (Fig. C2). Furthermore platelet area was smaller in patients on aspirin, again irrespective of thrombus age. To summarize, the composition of inflammatory cells differs with thrombus age in thrombosuction material of STEMI patients that in part depends on patient age and medication.

Angiographically successful primary percutaneous coronary intervention (p-PCI) not always restores cardiac microvascular blood flow. This phenomenon, called MicroVascular Injury (MVI), worsens prognosis and is poorly understood. We hypothesized that
inflammatory cell density of acute coronary thrombi would be increased in patients with MVI as presented in Chapter 10. In line with our expectations, we found a higher mean symptom-to-balloon time, larger enzymatic infarct size, lower left ventricular ejection fraction and a higher incidence of IMH in the MVI patients. We however could not establish a difference in thrombus content of platelets, erythrocytes, macrophages/monocytes, lymphocytes, neutrophils and neutrophil extracellular traps (NETs) between patients with MVI and patients without MVI (Fig. C3). This study thus argues against inflammatory cell alterations in the coronary thrombus as a possible influence on the occurrence of MVI.

In this Chapter, as well as in Chapters 2, 3, 4, 5, 6, 9, we used inflammatory cell densities in order to make suggestions about inflammatory status of atherosclerotic plaques and coronary thrombus. Research and diagnostics in the field of pathology has its very origin in the observation and quantification of the presence of inflammatory cells in tissues and proved to be very closely related to clinical observations. However, additional analysis of the activation status of inflammatory cells, would also be of interest when studying the pathophysiology of acute inflammation and its role in plaque destabilization and thrombus formation.

**CONCLUSION**

In the previous sections we gave an overview of the main findings of this thesis and what we add to the existing knowledge. We also tried to carefully address several methodological issues regarding our research. Some of these issues are not specific for our research, but are experienced by most scientists in the field of atherosclerotic research. Others give important clues for future projects, of which we make note in these sections. This thesis provides evidence for a role of systemic inflammation in the pathophysiology of acute myocardial infarction. We found, at least in part, supportive mechanistic evidence for the observational studies reporting increased incidence of cardiovascular events during or after clinical conditions that are accompanied by a systemic inflammatory response, e.g. orthopedic surgery, infection/sepsis and AMI itself.

We describe age-induced inflammatory changes in the microvasculature of the heart that can be postponed with a fish oil constituent. Furthermore, we report evidence concerning orthopedic surgery induced atherosclerotic plaque growth and increased necrotic core area, indicative of more vulnerable plaques. Next we show that mast cells (MCs) are increased in the vessel wall surrounding atherosclerotic plaques in patients that have signs of infection and that MCs are associated with more unstable plaques. Our work also shows that MCs are elevated in the vessel wall that surrounds plaques in patients that died of acute myocardial infarction. Again this was associated with a concomitant increase of unstable plaques. We hypothesize a bi-directional role of MCs as
they are not only a factor in the origin of AMI, but we believe that they are also involved in the acceleration of atherogenesis once an AMI has occurred. Besides increased coronary artery densities of MCs, we also found an increased number of macrophages, neutrophilic granulocytes and lymphocytes in atherosclerotic lesions and the surrounding vessels wall of patients that died more than 6 hours-14 days after myocardial infarction as opposed to patients that died immediately after a coronary event. This also highlights that myocardial infarction induced inflammation favors the atherosclerotic process and could explain the high amount of re-infarctions. Lastly, we found a relationship between immune cell composition of the coronary thrombus and patient characteristics and thrombus age, indicative for a role of inflammatory cells in atherothrombosis development. We did not find a clear relationship between inflammatory thrombus composition and the occurrence of the microvascular injury phenomenon. This finding argues against inflammatory cell involvement in the coronary thrombus as a possible influence on the occurrence of MVI.

Given that 17.5 million people die each year as a result of cardiovascular disease and the incidence is increased with socio-economic status, researchers should obtain a deeper insight in the pathophysiology of these diseases. Paramount in this task is increasing knowledge about triggers of acute destabilization of these mostly gradually developing conditions and subsequently the development of preventative strategies to postpone or even avoid acute myocardial infarction. This thesis provides a framework for the role of acute inflammation in causes and consequences of AMI. Based on our work we believe that one day, accumulating knowledge on the specific link between inflammation and cardiovascular events, will shift the foundation of treatment from damage control or tertiary prevention to primary prevention.

REFERENCES