Summary

Although major progress has been made in the treatment and prevention of cardiovascular disease (CVD), CVD-related mortality is still a major cause of death in Western societies. Retention of LDL-cholesterol in the vessel wall is an early step in atherosclerosis development. In contrast to native LDL particles, which are not taken up by macrophages, oxidatively-modified LDL particles can be taken up in excess by macrophages, resulting in their transformation into foam cells. Accumulation of foam cells in the vessel wall leads to formation of fatty streaks, which ultimately may evolve to atherosclerotic plaques. Acute cardiovascular events are initiated by rupture of unstable atherosclerotic plaques. Over the past decades evidence has accumulated that atherosclerosis is precipitated by dyslipidemia and hyperglycemia. In addition, inflammation, oxidative stress, and impaired nitric oxide production are also involved in all stages of atherosclerosis, from early endothelial dysfunction to late stage plaque rupture. To gain more insight in how oxidative stress, inflammation and nitric oxide formation relate to atherosclerosis, we investigated a selection of biomarkers reflecting these processes. All studies were performed in a population-based cohort of elderly subjects stratified for glucose tolerance (The Hoorn Study).

In chapter 2 we investigated the determinants of plasma oxidized low-density lipoprotein (oxLDL) and the association of this oxidative stress marker with flow-mediated dilation (FMD) of the brachial artery, which reflects endothelial function and is an early marker of atherosclerosis. It is likely that the concentration of oxLDL depends not only on the degree of oxidative stress, but also on the amount of substrate available for oxidation, i.e. the number of LDL particles. In line with this notion, we observed that oxLDL was strongly correlated with the plasma concentration of LDL-cholesterol and apolipoprotein B-100 (apoB100). The strong correlation between these variables makes it difficult to disentangle their independent contributions to CVD risk and prompted us to test the hypothesis that the oxLDL / LDL-cholesterol ratio and the oxLDL / apoB100 ratio are more informative than the separate variables. In support of this hypothesis, brachial FMD was not significantly related to plasma levels of oxLDL, LDL-cholesterol, or apoB100; whereas a negative association between FMD and the oxLDL / LDL-cholesterol and LDL / apoB100 ratios was observed. The latter association withstood adjustment for age, sex, Framingham risk score, renal function and obesity. We concluded from this study that correction of
oxLDL for LDL particle number may improve the clinical usefulness of oxLDL measurement.

In chapter 3 the use of myeloperoxidase (MPO) as biomarker for cardiovascular risk stratification is reviewed. To this end, results of published cross-sectional studies, case-control studies, and prospective cohort studies investigating the relation between MPO and CVD were discussed. Most of the studies showed a significant positive association between high plasma concentrations of MPO and CVD-related morbidity and/or mortality. Of note, the strongest associations were observed in populations with acute cardiovascular symptoms. In several studies measurement of MPO was associated with improved risk stratification above and beyond risk stratification obtained with markers used in routine clinical practice, such as troponin-T and CK-MB. This chapter also summarizes the mechanisms by which MPO may contribute to CVD. MPO plays a beneficial role in host defense by producing hypochlorous acid and other highly reactive compounds that kill pathogenic microorganisms. In the vascular system, however, these MPO-derived reactive substances may interfere with endothelial function and lead to structural damage. In addition, MPO reduces the bioavailability of nitric oxide, is involved in oxidation of LDL and impairment of HDL function, and contributes to plaque thinning and rupture by activating metalloproteinases. Overall, both in vitro experiments and pathophysiological observations support a causal role of MPO in initiating CVD and precipitating acute cardiovascular events. Additionally, attention is paid to pitfalls in the laboratory analysis of MPO. This issue is further expanded on in chapter 4, where matched serum, heparin-plasma, and EDTA-plasma of healthy volunteers were compared for MPO concentrations. In heparin-plasma MPO concentrations were twice as high as in EDTA-plasma and in serum even four times higher MPO concentrations were found. More importantly, MPO concentrations in EDTA-plasma were not significantly correlated with MPO concentrations in heparin-plasma or serum. We argued that ex vivo release of MPO from leukocytes induced by heparin or due to coagulation is most likely responsible for these differences in MPO concentrations. Accordingly, we recommended to use EDTA-plasma for the measurement of MPO, because these values may most accurately reflect the concentration of MPO in the circulation.

In chapter 5 the correlates of the EDTA-plasma MPO concentration and the association of MPO concentration with FMD of the brachial artery were investigated.
In a multivariable linear regression model, daily vitamin C intake and plasma levels of C-reactive protein (CRP) were significant independent determinants of plasma MPO concentration, together explaining approximately 9% of its variation. We hypothesized that the association between MPO and FMD would be stronger in subjects with elevated glucose concentrations, because hyperglycemia is associated with increased levels of hydrogen peroxide, an essential cosubstrate of MPO. Indeed, a significant inverse association between MPO and FMD was found in subjects with abnormal glucose metabolism (impaired glucose metabolism and type 2 diabetes mellitus combined), but not in subjects with normoglycemia. This negative association was not attenuated after adjustment for established CVD risk factors or other potential confounders. The results of this study suggest that hyperglycemia-induced vascular oxidative stress may enhance MPO activity, thereby strengthening the negative impact of MPO on endothelium-dependent vasodilation.

In chapter 6A the association between MPO and blood pressure was investigated. MPO may influence blood pressure in at least two ways. First, MPO activity may scavenge the endogenous vasodilator nitric oxide. Second, MPO-derived reactive substances may damage the arterial wall, thereby reducing its elasticity. Indeed, our data demonstrated that a high plasma level of MPO was associated with a clinically relevant increase in both systolic and diastolic blood pressure. This association was strongest in subjects with (hyperglycemia-induced) oxidative stress, consistent with enhancement of MPO activity in the vasculature by increased local production of reactive oxygen species. These observations, together with emerging evidence that MPO-derived oxidants contribute to the initiation and propagation of CVD, identify MPO as a promising target for drug development. In chapter 6B we propose that, in addition to other mechanisms, direct inhibition of MPO and scavenging of MPO-derived reactive species are plausible mechanisms of how melatonin may protect the vasculature and contribute to lowering of blood pressure.

Production of nitric oxide by the endothelium is important for vascular function and is generally considered to counteract the process of atherogenesis. Arginine is the substrate for nitric oxide synthase (NOS), whereas asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NOS. Therefore, a low arginine to ADMA ratio may be associated with reduced nitric oxide production, leading to endothelial dysfunction and accelerated atherogenesis. In chapter 7 we investigated whether inflammation, which has been linked to both endothelial dysfunction and
increased CVD risk, is associated with altered concentrations of arginine and ADMA. For this purpose we evaluated two inflammatory markers, CRP and MPO, the latter being also directly related to oxidative stress. Increased levels of both MPO and CRP were associated with a lower arginine to ADMA ratio. In the case of MPO this was mainly due to an elevation of ADMA concentrations, whereas elevated CRP was associated with reduced arginine levels. With increasing plasma levels of oxLDL the positive association between MPO and ADMA was strengthened, compatible with amplification of oxidative stress by MPO. Notably, dimethylarginine dimethylaminohydrolase (DDAH), the enzyme responsible for degradation of ADMA, is highly sensitive to oxidative stress. Therefore, inhibition of DDAH by MPO-induced oxidative stress is a plausible mechanism by which MPO may cause increased ADMA levels. The negative relation between CRP and arginine was independent of oxLDL levels, suggesting involvement of inflammation rather than oxidative stress. A likely mechanism is increased consumption of arginine by the inducible form of arginase that is upregulated during inflammation. Taken together, the results described in this chapter support the notion that both MPO and CRP, by different mechanisms, are associated with reduced vascular nitric oxide production, possibly leading to endothelial dysfunction.

In chapter 8 the relation between plasma levels of homoarginine and blood pressure was examined. Homoarginine has a strong structural resemblance to arginine. It has been shown that homoarginine can serve as substrate for NOS, but with less efficiency than arginine. In addition, homoarginine may compete with arginine for cell entry by the cationic amino acid transporters, leading to a reduced cellular uptake of arginine. Hence, we hypothesized that high homoarginine concentrations may result in reduced nitric oxide production by endothelial cells, possibly leading to increased blood pressure. In support of this hypothesis, we observed a positive, robust, and clinically relevant association between plasma levels of homoarginine and both systolic and diastolic blood pressure. In contrast, plasma levels of arginine were inversely associated with diastolic blood pressure, but only after adjusting for homoarginine concentrations. These observations lead to the conclusion that homoarginine and arginine may potentially act as antagonists in blood pressure regulation.
Conclusions

The first objective of this thesis was to study the relationships of novel markers of inflammation, oxidative stress, and nitric oxide signaling with vascular function and blood pressure.

![Diagram of overlapping circles showing relationships between inflammation, oxidative stress, nitric oxide synthesis, hypertension, and vascular dysfunction.]

**Figure** Oxidative stress, inflammation, and a reduced nitric oxide synthesis (filled circles), alone and in conjunction, contribute to cardiovascular disease. We studied associations between markers of these processes and their associations with hypertension and vascular dysfunction (open circles). The numbers refer to the chapters in this thesis.

As graphically represented in the Figure, we observed associations between markers of inflammation (MPO and CRP) and key players of nitric oxide synthesis (the NOS substrate arginine and inhibitor ADMA). We also found both oxLDL and MPO to be associated with vascular dysfunction. Furthermore, MPO was associated with hypertension. Finally, a positive association between homoarginine and blood pressure was observed, that was antagonized by arginine.

Our second objective was to investigate whether these relationships are influenced by hyperglycemia and/or oxidative stress. This we could confirm, because oxidative stress and hyperglycemia strengthened the associations of MPO with blood pressure and vascular function. Also the positive association between MPO and ADMA was found to be enhanced by oxidative stress. Overall, the results of these studies confirm that relationships between biomarkers and measures of outcome
may be conditional, e.g. dependent on the level of oxidative stress or hyperglycemia. Acknowledgement of this fact may be crucial for interpreting differences between studies and study populations.

**Study limitations and strengths**

Our study population consisted of approximately equal-sized groups with normal glucose metabolism, impaired glucose metabolism, and type 2 diabetes mellitus. Therefore, the high prevalence of hyperglycemia may have caused a selection bias by favoring inclusion of individuals with a high cardiovascular risk. On the other hand this design made it possible to investigate hyperglycemia-related effect modification of the relation between biomarkers and measures of outcome. Since atherosclerosis is a disease that progresses with age, the observations within this elderly population may differ from those in younger subjects. Another limitation is that our epidemiological studies had a cross-sectional design and can therefore only reveal associations, which may or may not reflect causality.

The fact that the same cohort was used in our studies was very useful for reasons of comparability. Other strengths of the present study are the considerable number of participants included and the vast array of variables that were at our disposal to control for potential confounding.

**Future perspectives**

Plasma concentrations of oxLDL have been shown to be associated with CVD, but a comparison of the predictive power of oxLDL versus oxLDL/LDL-cholesterol or oxLDL/ApoB100 for CVD morbidity and mortality has never been performed. This would be interesting, since we observed that FMD was more strongly related with these ratios than with unadjusted oxLDL concentrations.

In several studies MPO has been found to be a significant predictor of CVD events. With increasing oxidative stress and glucose concentrations, we found stronger associations between MPO and blood pressure and vascular dysfunction. It would be worthwhile to investigate whether hyperglycemia or oxidative stress also strengthen the predictive power of MPO for (non)fatal CVD events. In addition, it may be interesting to compare the predictive values of serum, plasma, and leukocyte MPO concentrations as well as MPO activity for CVD events.
MPO contributes to hypertension and vascular dysfunction, but is also an important player in the innate immune system. Direct inhibition of MPO may therefore have positive cardiovascular effects, but on the downside lead to impairment of host defense. Reduction of oxidative stress, preferably locally in the vasculature, may be a better, more selective approach. Nitroxides, with Tempol as one of its best characterized members, form a promising class of compounds suitable to achieve this goal. Nitroxides are stable free radicals of low toxicity that exert a range of antioxidant effects, including catalytic dismutation of superoxide and breakdown of hydrogen peroxide. By reducing the levels of its cosubstrate hydrogen peroxide, MPO will be “starved” and no longer be able to produce hypochlorous acid and other reactive substances. In animal studies nitroxides have been shown to increase the bioavailability of nitric oxide and reduce blood pressure, and inhibition of the formation of hypochlorous acid by MPO has also been observed. Therefore, next to other beneficial effects, nitroxides hold potential to alleviate the adverse effects of MPO on blood pressure and vascular function.

We observed that homoarginine and arginine were antagonistically related to blood pressure, but the positive association between homoarginine and blood pressure was more pronounced than the negative relation between arginine and blood pressure. This is remarkable because circulatory concentrations of homoarginine are one to two orders of magnitude lower than concentrations of arginine. This novel finding warrants further investigation of the role of homoarginine in nitric oxide signaling and possibly as novel CVD risk marker or even risk factor.