Introduction
1. Novel Oxidative Stress Markers in Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death in Western Society\(^1\). Although the last decades have been accompanied by great advancements in the insight of the treatment of atherosclerosis, the incidence of CVD is still rising\(^2\). Therefore, to provide early detection and better targeted therapies for CVD, a more complete understanding of the pathophysiologic mechanisms is essential and biomarkers are needed that allow clinical application and study of the role of oxidative and inflammatory stress in the development of atherosclerotic disease. In this chapter we will introduce the concept of oxidative stress in CVD and review currently available biomarkers for inflammation and for oxidative stress. In the final section we provide evidence that an alternative group of compounds, Advanced Glycation Endproduct (AGEs), may also serve as a novel marker and risk factor for atherosclerosis.

**Oxidative Stress**

Atherosclerosis is a chronic inflammatory disease, which is thought to be a response to an initial injury in the vascular wall\(^3\). Although several other theories have been postulated, Goldstein and Brown were the first to demonstrate that oxidative modification of structures contained in Low Density Lipoprotein (LDL)-particles renders them susceptible to phagocytosis by resident macrophages\(^4\), laying the basis for the oxidative stress in atherosclerosis hypothesis\(^5\).

At moderate concentrations, Reactive Oxygen Species (ROS) have many physiological functions, such as regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production, and signal transduction from membrane receptors in various physiological processes. However, an excess in ROS results in oxidative stress which is hallmarked by an imbalance between free (oxygen) radical production on the one hand and depleted (enzymatic) antioxidant defence mechanisms on the other hand, leading to pathological conditions\(^6\). Antioxidants are divided into three major categories of molecules: antioxidants that bind metal ions, reducing the production of hydroxyl radical, intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and extracellular antioxidant substances, such as vitamins\(^7;8\). On the other side of the balance, several sources of ROS generation have been identified. Firstly, endogenous production of ROS is mainly driven by intracellular processes. For atherosclerosis, the two main production sites are the mitochondrial respiration chain\(^9\) and cellular membrane oxidases such as NAD(P)H oxidase, nitric oxide synthetase and myeloperoxidase, originating mainly from endothelial cells, smooth muscle cells (SMCs), and macrophages\(^10\). Additionally, two major exogenous sources of ROS implicated in atherosclerosis are tobacco smoke and diets containing high saturated fatty acid\(^6\). When the “recycling” mechanisms fail to counterbalance ROS generation, intracellular as well as extracellular oxidative modification of macromolecules may occur, resulting in the formation of more advanced by-products of oxidative stress. Major substrates for oxidative modification are (intracellular) proteins, lipids and DNA, which impairs their physiological function\(^6\) and accumulate as biological garbage during ageing\(^11;12\). In addition to this rather random biochemical damage of organic substrates, several stress signalling pathways and/
or intracellular mediators have been identified being activated in response to oxidant injury, resulting in downstream induction of inflammation and apoptosis. Cumulative effects of these mechanisms have been implicated in many age-related diseases; the focus of this chapter will be on atherosclerosis and ensuing events.

**Oxidative stress and Atherosclerosis**

As mentioned above, the initial injury to the vascular wall is thought to result from oxidation of LDL, producing oxidized LDL (oxLDL). Oxidation of LDL promotes increased phagocytosis by resident macrophages in the vascular wall, which have been shown to express specific scavenger receptors for oxLDL. These receptors do not recognize native LDL. Uptake of oxLDL ultimately transforms them into foam cells. This is considered the initial step of atherosclerotic plaque formation. Furthermore, ROS may react with endothelium-derived nitric oxygen (NO), which is an important vasodilator, to inactivate its vasodilatory effect, promoting endothelial dysfunction. By secretion of cytokines, growth factors and adhesion molecules, more monocytes and other leukocytes are recruited to the vascular lesion site and promote fatty streak formation. Additionally, production of growth factors by foam cells triggers the proliferation and migration of smooth muscle cells (SMC) to the intima, initiating the formation of a fibrous cap. The latter, combined with the continuous influx of inflammatory cells converts the fatty streak to a more advanced lesion. Since inflammatory cells, endothelial cells and SMC all generate ROS, the oxidative stress driven plaque formation may be regarded as a vicious circle. Moreover, oxidative stress not only facilitates stable plaque formation, but also creates an environment that makes the plaque more vulnerable to rupture. This may ultimately result in an Acute Coronary Syndrome (ACS), including myocardial infarction as well as unstable angina. For example, ROS activate matrix metalloproteins (MMPs) in cultured vascular cells, negatively influencing plaque stability by the breakdown of interstitial collagen of the fibrous cap. Additionally, ROS may promote acute coronary artery luminal occlusion by modulating haemostatic factors. Thus, based on preclinical studies it can be concluded that oxidative stress plays a crucial role in the development of atherosclerosis and ensuing atherothrombotic events.

**Clinical application of the oxidative stress**

Although a causative role of traditional risk factors in the development of atherosclerosis is evident, their predictive value is poor. Even when applying current NCEP/ATPIII guidelines and integrating risk factors into global risk scores (Framingham or SCORE risk score), a substantial proportion of subjects at risk are not detected. Age, by far the most potent risk factor, is closely associated with several conventional risk factors, which are only moderately more prevalent in persons with documented cardiac or peripheral vascular disease. Several major risk factors like dyslipidaemia and hypertension are only present in approximately 50% of all patients with manifest atherosclerosis. Moreover, even the degree of atherosclerosis, such as the number of significantly stenotic vessels is not a very strong predictor of long-term events. It has, therefore, been postulated that it is not the extent of atherosclerosis, but inflammatory activity and associated morphologic instability that defines its propensity to cause an acute coronary syndrome, threatened limb, or stroke. Since the role of inflammation and oxidative stress is beyond reasonable doubt crucial in
the development of “vulnerable” atherosclerotic plaques, the search for clinically applicable biomarkers to identify subjects with high sub clinical “inflammatory activity” and oxidative stress is continuing\textsuperscript{23}. In the following sections, emerging and established biomarkers will be reviewed, focusing on their incremental power to predict future cardiovascular events.

**Clinical usefulness of a marker**

Before going into more detail, the following section will outline what the reader/clinician should look for when appraising new markers of oxidative stress. A biomarker of oxidative stress is classically defined as a biological molecule whose chemical structure has been modified by ROS\textsuperscript{24}. An ideal biomarker should fulfill several conditions. First of all, this compound should be stable and not susceptible to artificial generation \textit{ex vivo} and to loss during processing and storage. From an analytical point of view the method for biomarker measurement should be sensitive, specific, and reproducible. The biomarker should be valid from a biological and clinical point of view. In this specific case, this means that different levels of the biomarker should reflect not only different degrees of atherosclerosis progression but also reflect different severity stages of the clinical condition of the patient. Thus, changes in its levels should closely correspond to changes in the patient’s clinical status and prognosis. A valid marker should also be of prognostic value in the general population, identifying subjects that are at increased risk of developing the disease by its higher levels. Additionally, it should preserve its prognostic power in patients having the disease, and it should predict an adverse outcome\textsuperscript{25}. Furthermore, from a statistical point of view, it should add independent and incremental information to standard risk algorithms\textsuperscript{26}. In vascular disease this means NCEP/ATPIII-based risk stratification, including currently available risk scores, such as the Framingham Risk Score (FRS; based on a North American population) and the SCORE risk score (SCORE; based on a European population). The predictive power of a marker can be assessed by calculating the relative risk or odds ratio. These approaches compare the incidence of disease among persons who have a given risk factor with the risk among those who do not have that risk factor. Significant risk estimates, fully adjusted for other known confounders, indicate that the marker adds independent information and thus has potential clinical importance (26). Potentially useful markers should produce an odds ratio of at least 2 to 3 to be of sufficient significance. Also, most markers are measured using a continuous scale. Therefore, cut-off values should be calculated with high sensitivity and specificity in predicting the disease. A well accepted method of calculating cut-off values is by using receiver operating characteristics (ROC) curves. It can be calculated whether adding new markers to standard risk algorithms produces significantly better ROC curves, hence resulting in better risk estimation. It has been shown that very large odd ratios are needed in order to add incremental and statistically significant information to other risk markers\textsuperscript{27}. Using this strategy a potentially useful marker should at least have an odds ratio of 4 and an area under the ROC curve of 0.8 (expert opinion, ACC scientific sessions 2006). Finally, when applying a risk marker to clinical practice, it should preferably be therapeutically modifiable. Changes achieved by any therapeutic intervention should translate into a significant clinical improvement and the minimally clinically important difference should be determined\textsuperscript{28}. A biomarker might be employed in the drug development process for early evaluation of efficacy of new drugs.
INFLAMMATORY BIOMARKERS

Literature on the role of inflammatory biomarkers in CVD is extensive. This section will give a short overview of the prognostic value of the most promising or widely available biomarkers. Focus will mainly be on C-reactive protein (CRP), as currently the most robust biomarker available. For more detail, the reader is referred to some excellent recent reviews13;29-34.

CRP and cardiovascular disease

The most extensively studied, clinically applicable marker for inflammation in cardiovascular disease is CRP. This is a pentraxin acute-phase protein which is predominantly produced by hepatocytes upon stimulation by several inflammatory stimuli35. In addition to its wide use in assessing acute systemic inflammation in infectious disease, it has emerged to detect low grade inflammation in coronary and possibly other arterial walls36. Several pro-inflammatory cytokines stimulate CRP production, including interleukin (IL) 1 and 6, and tumor necrosis factor alpha (TNF-α), which are produced by cells involved in the inflammatory foci in the subendothelial space of atherosclerotic arteries37.

Because of the ease of the assay and its wide availability, it has been possible to measure CRP on a large scale in various clinical studies38. In primary prevention studies, CRP has been shown to predict future cardiovascular events beyond traditional risk stratification and to provide incremental information on top of ATP/NCEP, Framingham Risk Score based risk stratification39;40;40-48. Based on these data, current clinical guidelines advice using CRP as a supplemental stratifier in subjects at intermediate risk according to Framingham Risk Score, i.e. with a 10-20% ten year risk for coronary artery disease (CAD)49. Furthermore, CRP is markedly elevated in (most) patients presenting with ACS, even if cardiac enzymes (troponine, creatinine kinase) are negative32;50. Therefore, strengthened by evidence from the studies in primary prevention, the current hypothesis is that CRP is involved in inflammatory/oxidative process preceding acute plaque rupture/erosion. In full blown ST-elevation as well as in non-ST-elevation myocardial infarction and unstable angina, CRP appears to be an independent risk marker for short term (<1 months)51;52, and longer term survival50;53. Additionally, it has been recently demonstrated that lowering CRP levels below 2 mg/dL with statin therapy initiated after ACS, reduces the risk for recurrent events independent of achieved cholesterol levels54.

Although data on the clinical usefulness of CRP are very convincing, there is growing evidence that its applicability is limited, for several reasons. In the first place, the causative role of CRP in atherosclerosis has been disputed. There is growing evidence that CRP may be etiologically involved in the development of atherosclerotic plaques55. Proposed mechanisms include induction of endothelial dysfunction, promotion of foam cell formation, inhibition of endothelial progenitor cell survival and differentiation, and activation of complement in the intima of atherosclerotic plaques and ischemic myocardium56. However, most in-vitro studies that used CRP to study its effect on cells involved in atherosclerotic plaque formation (macrophages, endothelial cells, SMCs) may have been confounded by contaminating compounds such as endotoxins57. Additionally, in recent studies the additive value of CRP seems limited, after appropriate correction for other risk factors58;59. Therefore, a continuing need remains for novel biomarkers that are as easily applicable as CRP, are reproducible and...
stable, and provide incremental information to standard risk stratification strategies. In the following text we will reflect on several emerging markers.

Other Markers of Inflammation
Inflammatory markers in atherosclerotic disease are generally divided into several subcategories, including acute phase reactants (CRP, serum amyloid A (SAA), and fibrinogen), cytokines (Interleukin-6, 18, and 10, monocyte chemoattractant protein-1(MCP-1), tumor necrosis factor alpha (TNF-α)), endothelial cell activation biomarkers (sICAM, sVCAM, sE-selectin, and von Willebrand Factor (vWF)), and matrix metalloproteinases (MMP 1,2,3, and 9). In contrast to CRP a pathophysiological role in the atherosclerotic process has been suggested for all of these markers. Most of these markers are elevated in ACS and to a lesser extent in stable atherosclerotic disease, and are associated with the severity of the disease. Most evidence comes from studies in ACS. In addition to CRP, another acute phase protein, SAA, seems to predict short (14-days) and long term (1 and 2 year) outcome of ACS. From the endothelial cell activation biomarkers, vWF provides the most convincing evidence. Elevated levels and its rise after admission are predictive of a short term (<30 days) adverse outcome in ACS. Also leukocyte adhesion molecules seem to be promising, but prognostic data in ACS are scarce and somewhat conflicting. Furthermore, some cytokines appear to be of clinical value in ACS. Of these, data on IL-6 is the most convincing, demonstrating in two cohorts that IL-6 predicts the incidence of events. This molecule is of special interest since it directly stimulates the production of CRP in the liver. Prognostic data are also available for MCP-1, TNF-α, and IL18, as well as for IL10 (protective). Our group has shown that a relatively unknown biomarker, neopterin, which is a marker for monocyte activation, also predicts future cardiovascular events in ACS. These data have recently been confirmed by others. Prognostic data on matrix metalloproteinases are lacking. In patients with unidentified or documented stable coronary artery disease, several inflammatory markers also may also be useful in identifying high risk subjects. The acute phase protein fibrinogen appears to be the most promising one, but several other markers have prognostic potential, including Il-6, Il-1β, soluble adhesion molecules, neopterin, and soluble CD40 ligand. In conclusion, with the exception of fibrinogen, evidence that these alternative inflammatory biomarkers are effective in population-based screening is currently modest. Nonetheless, there is considerable evidence that many have usefulness in the setting of ACS. However, since inflammation is secondary to the initial injury of presumably oxidized LDL, novel markers for oxidative stress may be more pathophysiologically relevant in assessing disease risk.

OXIDATIVE STRESS BIOMARKERS

Monitoring Oxidative Stress
Since ROS are highly unstable, their measurement in human serum or plasma samples does not necessarily reflect their *in vivo* concentration. Therefore, only secondary (end) products of oxidative stress have emerged for application in clinical studies. Numerous tests have been examined in clinical studies. In general terms, these can be divided into markers of oxidative damage (to lipids, protein, and DNA) and of antioxidant status (either individual
Introduction

The large number of proposed biomarkers of oxidative stress and a lack of comparative studies presents a substantial diagnostic challenge. No universally set of standards for monitoring oxidative stress had been determined. A technological concern is that biomarkers of oxidation are often at risk for auto-oxidation themselves and their measurement cannot easily be made on stored samples. In other cases (e.g., lipid hydroperoxides, glutathione), the analyand being measured is short-lived and unstable and therefore requires immediate assay or dilution in a preservation buffer. This represents a challenge when assays cannot be performed on site within hours or days. The following text will present an overview of the most studied biomarkers for oxidative stress, their application in clinical studies, and potential prognostic value (see also figure 1).

Early and late Lipid peroxidation products

Peroxidation of polyunsaturated fatty acids, mostly in membrane phospholipids, is a chain reaction that can continue until substrate is completely consumed or termination occurs due to antioxidants. Lipid peroxidation leads to structural and functional damage to membranes as well as to several secondary products. Secondary products of lipid peroxidation are fragments of the original molecules or, when arachidonic acid is involved, isoprostanes. Initially lipid peroxidation leads to the production of conjugated dienic hydroperoxides. These unstable substances decompose either into various aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), and dienals or into alkanes as pentane and ethane. Lipid peroxidation products are relatively well accepted group of oxidative stress indices.

Malondialdehyde (MDA)

In clinical studies MDA levels have been measured both by means of a spectrocolorimetric assay and by sensitive high-performance liquid chromatography (HPLC) with fluorescence detection. These methods measure MDA directly or as a chromogen produced by the reaction of thiobarbituric acid reactive substances (TBARS) with MDA. This assay is extremely easy to use, but is not very specific for MDA and may also measure substances arising from pathways other than lipid peroxidation and free radical damage. MDA itself can also come from the degradation of endoperoxides and from in vitro oxidation if preservatives are not added. Therefore, this assay usually gives an overestimation of free radical damage. Even if the specificity of the measurement is improved by HPLC to separate the MDA-TBA adduct from interfering chromogens, there is still the possibility that part of the MDA present in the sample does not derive from oxidative damage of fatty acids. More recently, several gas chromatography/mass spectroscopy (GC/MS) methods have been developed that overcome the limitations of previous assays. TBARS levels have been shown to correlate with severity of disease as determined by angiography, independently of other known risk factors in different studies. Others found MDA levels to be significantly higher in smokers than non-smokers and to inversely relate to paraoxanase-1 (PON1) and GSH-PX activities. TBARS levels are found to be significantly higher amongst patients with unstable as compared with stable angina. It was also shown that although TBARS were not elevated in patients with an acute MI, MDA generation was stimulated after successful thrombolysis. Interestingly a recent study, conducted in a large cohort of patients with stable CAD,
Figure 1. Different pathways leading to the formation of labile reactive oxygen species (ROS) and ensuing modifications of organic molecules. Oxidative stress is defined as the balance between ROS generation and antioxidant defence mechanisms. Some of the antioxidant enzymes may be measured as indices for oxidative stress. ROS may be derived from exogenous sources or form spontaneously in vivo. Important endogenous pathways are nicotinamide adenine dinucleotide (NADPH) oxidase and myeloperoxidase (MPO), and A2-phospholipases (Type II secretory PLA2 (Lp-PLA2)) in phagocytic cells and lipoprotein-associated phospholipase A2 (sPLA2)) in low density lipoprotein (LDL) particles. MPO and Lp-PLA2/ sPLA2 (activity) are useful as markers for oxidative stress. ROS generation may rapidly modify several organic structures. Modification of (phospho)lipids in cells membranes or LDL particles leads to formation of lipid peroxidation products and oxidized LDL. Reaction of ROS with DNA or amino acids results in modified tyrosines and oxidized DNA. All of these oxidatively modified organic molecules can be measured in vivo, using different approaches described in the text. G6PD indicates glucose 6 phosphate dehydrogenase; NO2-Tyr, nitrotyrosines; Cl-Tyr, chlorotyrosines; 8OHiG, 8-Hydroxy-2-deoxyguanosine.
indicated that TBARS level may represent a strong and independent prognostic predictor of cardiovascular events\textsuperscript{106}.

**4-Hydroxynonenal (HNE)**

HNE is an aldehydic end-product of oxidative breakdown of membrane n-6 polyunsaturated fatty acids and has been shown to be a strongly involved in atherosclerosis\textsuperscript{107}. These aldehydes may be measured by highly specific and sensitive gas chromatography/mass spectrometry (GC/MS) methods\textsuperscript{108} but only in specialized laboratories. Studies in patients with CVD have not been published\textsuperscript{109}.

**F\textsubscript{2}-isoprostanes**

Isoprostanes are stable end-products of lipid peroxidation derived from arachidonic acid. A large number of end-products can theoretically be generated, but interest has focused on the F\textsubscript{2}-isoprostanes and, in particular, on 8-isoprostaglandin F\textsubscript{2} alpha (8-iso-PGF\textsubscript{2α}). In the human circulation, isoprostanes are present mainly in their ester forms, whereas only hydrolyzed isoprostanes are excreted in the urine.

Various approaches are available for the measurement of F\textsubscript{2}-isoprostanes, including gas chromatography/mass spectrometry (GC-MS), GC–tandem MS, liquid chromatography/tandem MS, and immunoassays\textsuperscript{110}. Immunoassay results for 8-iso-PGF\textsubscript{2α} correlate with GC-MS measurement in urine, little information is available to ascertain their precision and accuracy, and they are limited by reduced specificity in the presence of biological fluids, such as plasma\textsuperscript{30}. At present, there is no widespread consensus as to the best methodology for measurement, but chromatographic methods should be viewed as superior to immunoassays.

Increased F\textsubscript{2}-isoprostanes are associated with the presence of cardiovascular risk factors. Urinary and plasma isoprostanes are increased in patients with hypercholesterolemia\textsuperscript{111}. A relatively weak positive association also exists between urinary excretion of isoprostanes and hypertension\textsuperscript{112}. Plasma and urinary isoprostanes are increased in both type 1 and type 2 diabetes\textsuperscript{111;113} and are reduced by an improvement in glycemic control. Given the documented increase of isoprostanes in type 2 diabetes, it is not surprising that there is a graded relationship with body mass index\textsuperscript{113}. Cigarette smoking represents a strong oxidant stressor, and studies have shown that both urinary and plasma isoprostanes are increased in smokers and that they return to baseline within several weeks of stopping smoking\textsuperscript{114-117}.

In addition to their association with cardiovascular risk factors, isoprostanes are elevated in unstable angina and in the coronary sinus and urine of patients undergoing reperfusion therapy during acute myocardial infarction\textsuperscript{118;119}. Although these data are promising, no prognostic data have yet been published. The assays used to measure isoprostanes allow very precise measurement of specific compounds, but require highly sophisticated techniques that are expensive and do not allow routine measuring\textsuperscript{30}. Furthermore, isoprostanes may be artificially generated \textit{ex vivo} and samples should be measured immediately after blood collection or stored at -70 degrees Celsius. These demands limit their widespread use in clinical studies and practice\textsuperscript{120}.\noindent
Table 1. Overview of Oxidative Stress Biomarkers that have been prospectively tested

<table>
<thead>
<tr>
<th>Marker</th>
<th>First author, year</th>
<th>Essay</th>
<th>Subjects</th>
<th>N</th>
<th>Time</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde</td>
<td>Walter, 2004 (1)</td>
<td>HPLC (TBARS)</td>
<td>sCAD</td>
<td>634</td>
<td>3 yr</td>
<td>MI, MI death, stroke</td>
</tr>
<tr>
<td>Hydroperoxide</td>
<td>Vassalle, 2005 (2)</td>
<td>d-ROMs test</td>
<td>CVD</td>
<td>157</td>
<td>20 m</td>
<td>cardiac death</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Shimada, 2004 (3)</td>
<td>ELISA (DLH3 ab)</td>
<td>sCAD</td>
<td>238</td>
<td>52 m</td>
<td>MI, CHD death, revasc</td>
</tr>
<tr>
<td></td>
<td>Holvoet, 2004 (4)</td>
<td>ELISA (4E6 ab)</td>
<td>Eldery</td>
<td>3,033</td>
<td>± 5yr</td>
<td>MI</td>
</tr>
<tr>
<td>MPO</td>
<td>Brennan, 2003 (5)</td>
<td>ELISA</td>
<td>ACS</td>
<td>604</td>
<td>1m/6m</td>
<td>MI, revasc, or death</td>
</tr>
<tr>
<td></td>
<td>Baldus, 2003 (6)</td>
<td>ELISA</td>
<td>ACS</td>
<td>1090</td>
<td>6 m</td>
<td>death</td>
</tr>
<tr>
<td>Lp-PLA2</td>
<td>Packard, 2000 (7)</td>
<td>ELISA</td>
<td>HC</td>
<td>1740</td>
<td>± 5yr</td>
<td>MI, CHD death, revasc</td>
</tr>
<tr>
<td>sPLA2</td>
<td>Kugiyama, 1999 (8)</td>
<td>ELISA</td>
<td>sCAD</td>
<td>142</td>
<td>2 yr</td>
<td>MI, CHD death, revasc</td>
</tr>
<tr>
<td></td>
<td>Kugiyama, 2000 (9)</td>
<td>ELISA</td>
<td>ACS</td>
<td>52</td>
<td>2 yr</td>
<td>MI, CHD death, revasc</td>
</tr>
<tr>
<td></td>
<td>Mallat, 2005 (10)</td>
<td>fluorimetric (activity)</td>
<td>ACS</td>
<td>446</td>
<td>6.5 m</td>
<td>MI, death</td>
</tr>
<tr>
<td></td>
<td>O’Donoghue, 2006 (11)</td>
<td>radiometric(activity)ACS*</td>
<td>3648</td>
<td>2 yr</td>
<td>MI, MI death, stroke</td>
<td></td>
</tr>
<tr>
<td>GPX1</td>
<td>Blankenberg, 2003 (12)</td>
<td>Ery GPX1 activity</td>
<td>sCAD</td>
<td>636</td>
<td>4.7yr</td>
<td>MI, CVD death, death</td>
</tr>
</tbody>
</table>

MPO indicates myeloperoxidase; oxLDL, oxidized low density lipoprotein, Lp PLA2, lipoprotein-associated phospholipase A2 (PLA2); sPLA2, Type II secretory PLA2; GPX1, Glutathione-peroxidase-1, ELISA, enzyme linked immuno sorbent assay; ab, antibody; ery, erythrocyte; MI, myocardial infarction, CHD, coronary heart disease; revasc, rescularisation procedure; CVD, cardiovascular disease; sCAD, stable coronary artery disease; ACS, acute coronary syndrome; HC, hypercholesterolemia; Eldery, health well functioning elderly; yr, year; m, month(s). All biomarkers well at least age and sex independently and significantly associated with the outcome.
**D-Roms test**

Recently, a commercially available assay of organic peroxides (i.e. hydroxyperoxide) known as the Free Oxygen Radical Test (FORT) or d-ROMs test has become available\(^\text{121}\). This assay is relatively inexpensive and can be performed in minutes and is based on the ability of transition metals to catalyse in the presence of peroxides with formation of free radicals which are trapped by an alchilamine. In a recent publication, d-ROMs test was for the first time found to be an independent predictor for cardiac death in patients with cardiovascular disease monitored over 20 months\(^\text{122}\).

**Conjugated dienes (CD)**

The peroxidation of unsaturated fatty acids is accompanied by the formation of conjugated dienes that absorb ultraviolet light at 230–235 nm\(^\text{123}\). Their measurement is useful for ex vivo studies, but application of this methodology to lipid extracts from human samples in body fluids may be readily confounded by contamination. A further limitation of this methodology is that generation of dienes continues to occur ex vivo following sampling\(^\text{109}\). Clinical studies are, therefore, limited. CD were found to be elevated in both stable and unstable angina\(^\text{105}\), and higher in unstable compared with stable angina\(^\text{103}\). Cigarette smoking does not appear to affect CD plasma levels\(^\text{124}\). However, no prognostic data are available.

**Oxidized LDL**

Oxidized LDL is not one homogeneous entity, but represents multiple chemical and immunogenic modifications of the lipids, phospholipids, and apoB-100 on the surface of LDL particles. The term “oxLDL” has been used in a generic sense to describe many different types of oxLDL.

**Direct antigenic measurement of oxLDL**

Currently, three major oxLDL plasma ELISAs have been developed based on murine monoclonal antibodies DLH3 (Kohno, Japan\(^\text{125}\)), 4E6 (Holvoet, Belgium\(^\text{126}\)), and E06 (Tsimikas, USA\(^\text{127}\)) that recognize various epitopes of oxLDL in vitro. Circulating oxLDL is associated with subclinical atherosclerosis in adults\(^\text{128}\);\(^\text{129}\) angiographically determined CAD\(^\text{130}\), symptomatic CVD, ACS and vulnerable plaques\(^\text{127}\);\(^\text{128}\);\(^\text{131}-\text{134}\). Furthermore, oxLDL appears to be of prognostic value. In a large cohort study (Health, Aging, and Body Composition study) oxLDL was measured in plasma from 3,033 well-functioning elderly people. Although oxLDL was not an independent predictor of total CHD risk, those with high oxLDL (upper 5\(^\text{th}\) quintile only) showed a greater disposition to myocardial infarction\(^\text{128}\). However, major overlap existed in oxLDL levels between those without and with prevalent CVD. In a nested-case control study, the oxidized LDL/plasma cholesterol ratio was higher amongst cases that had developed myocardial infarction than in controls and hypercholesterolaemic controls\(^\text{135}\). In presumably healthy males oxLDL levels predicted the progression of carotid intima-media thickness, independent of other cardiovascular risk factors\(^\text{136}\). Recently, it was demonstrated that higher levels of oxLDL measured with the DLH3 antibody were independent predictors of future cardiac events in patients with documented CAD in 52 months\(^\text{137}\). Interestingly, some studies have studied oxLDL (using E06 antibody) as levels relative to apoB levels,
since the E06 antibody binds to apoB. These levels appear to increase after statin therapy in patients with ACS\textsuperscript{138} and in children with familial hypercholesterolaemia\textsuperscript{139}, in parallel with increasing Lp(a) levels. It was hypothesized that this indicates an enhanced clearance of oxLDL facilitated by statins\textsuperscript{30}. In summary, oxLDL may serve as a biomarker, because there are several ELISA kits available to measure oxLDL in large scale studies. However, before incorporation into routine clinical practice, additional work is needed to compare the different assays in their clinical utility, and more insights are needed into their ability to provide independent prognostic information, particularly in relation to other risk factors such as LDL-C and CRP. Additionally, more prospective studies are needed with serial measurements to assess changes in these markers with various interventions\textsuperscript{30}.

**Antibodies against oxidized LDL**

The oxidation of LDL predominantly takes place in the arterial wall, generating several epitopes (for example oxidized phospholipids, MDA-LDL, or glycated LDL) to which polyclonal autoantibodies are generated by the adaptive immune system, but probably also innate natural antibodies\textsuperscript{140}. The concentration of oxLDL in plasma is low and antibodies to oxLDL are, therefore, thought be directed to oxLDL present in subendothelial space\textsuperscript{141}. T-cell clones responsive to oxLDL have indeed been isolated from human vascular plaques\textsuperscript{142}. Antibodies against oxidized LDL are detectable in human atherosclerotic lesions as well as in plasma\textsuperscript{143;144}. However, it is currently unclear whether antibodies to oxLDL are pro-atherogenic or protective against atherosclerosis\textsuperscript{140;145}. For the measurement of the immune response, two systems of generating oxLDL \textit{in vitro} are widely used, one generating oxLDL with MDA and one by oxidation with copper\textsuperscript{146}. Additionally, some methods measure only IgG, others IgM or both. Results from clinical studies are conflicting and puzzling. The majority of studies shows a differential relation of IgG and IgM antibodies with atherosclerosis. Most cross-sectional studies reveal a positive association of IgG antibodies with stable atherosclerosis, acute coronary syndromes, and sub-clinical atherosclerosis, as measured by IMT\textsuperscript{147-155}, but some studies find no association or even an inverse relation\textsuperscript{156-163}. Interestingly, IgM antibodies seem inversely related to atherosclerosis in most studies\textsuperscript{154;156;162;164;165}. However contradictory data have also been published\textsuperscript{152;166}. Prospective studies are limited. Salonen and colleagues were the first to demonstrate that high IgG titres were predictive of carotid atherosclerosis progression\textsuperscript{152}. These data were later confirmed by others\textsuperscript{167}. However, as was the case in the cross-sectional studies, contradictory data have also been published\textsuperscript{168}. Furthermore, it has also been shown that circulating LDL immune complexes are inversely related to antibodies to oxLDL and interfere with the measurement of oxLDL antibodies. These immune complexes on the other hand have been shown to positively predict the development of CAD\textsuperscript{169;170}. These discrepancies may be explained by the fact that several methods and definitions of oxLDL have been used in these studies. An alternative explanation may be that antibodies to oxLDL may have different functions during the various stages of atherosclerotic plaque progression, and that they may also have a different impact in different populations\textsuperscript{146;171}. An additional complicating factor is that immunization with \textit{Streptococcus Pneumoniae} may induce IgM antibody formation\textsuperscript{171}. In conclusion, although measuring antibodies to oxLDL is very interesting for clarification of the pathophysiology of atherosclerosis, the inconsistent results from clinical studies,
combined with varying definitions and preparation methods of oxLDL, do not make them useful markers for oxidative stress at this point in time.

**Susceptibility of LDL to in vitro oxidation**

Susceptibility of LDL to in vitro induced oxidative stress has been suggested as an indirect measure for oxidative stress induced damage. The in vivo peroxidation process is mimicked by incubating LDL with the strong redox oxidant copper, measuring LDL oxidation using its absorbance at 234 nm\(^{172}\). In one study a higher susceptibility of LDL to oxidation was observed in CAD patients who had shown progression in stenosis\(^{173}\). In another study an inverse association was described between resistance of LDL to oxidation and severity of coronary stenosis in patients\(^{174}\). However, these findings were not confirmed in other studies\(^{175};^{176}\). It was also reported that susceptibility of LDL is not associated with IMT in a case-control study\(^{163}\). Recently, it was reported that this marker was positively related to the extent of CAD\(^{100}\). Unfortunately, no prospective study has so far evaluated the clinical usefulness of this marker.

**Enzymes promoting Oxidative Stress**

**Myeloperoxidase**

Myeloperoxidase (MPO) is an abundantly available heme peroxidase enzyme that is produced upon degranulation of activated neutrophils and monocytes at sites of inflammation, including the coronary circulation\(^{177};^{178}\). MPO uses hydrogen peroxide as substrate to generate highly reactive species, such as hypochlorous acid, tyrosyl radical, and nitrogen dioxide, which can oxidatively modify protein and lipid targets in the vascular wall\(^{179}\). Furthermore, MPO, by generating these substrate radicals, catalytically consumes nitric oxide, promoting endothelial dysfunction\(^{180}\). Moreover, MPO has been shown to activate metalloproteinases, inducing plaque instability\(^{181}\). There is substantial evidence that MPO plays a role in atherosclerosis. Biochemical analyses localize the enzyme and its oxidation products within human atherosclerotic lesions\(^{182}\).

Measurement of MPO in clinical studies is performed using ELISA assays, which are commercially available\(^{30}\). Elevated plasma levels of MPO have been demonstrated to predict the presence of angiographic CAD. Patients with MPO levels in the fourth quartile are 15- to 20-fold more likely to demonstrate abnormal coronary angiograms compared with subjects in the lowest quartile, even after adjustments for Framingham risk score and C-reactive protein\(^{183}\). Additionally, serum levels of MPO were recently noted to independently predict brachial artery flow-mediated dilation in 298 subjects with CAD or multiple risk factors for CAD\(^{184}\). Two recent studies have revealed that MPO is a powerful predictor of adverse outcome in patients with ACS\(^{185};^{186}\). In individuals with low troponin levels, MPO identified patients at increased risk for early cardiovascular events that occur within days after the onset of symptoms\(^{186}\), making it a promising new marker for oxidative stress in ACS\(^{187}\). In contrast, individuals with total or subtotal MPO deficiency, appear markedly protected from developing CVD\(^{188}\). Almost all clinical data for MPO have been collected in the setting of ACS or established CAD, so the ability of MPO to provide information beyond traditional risk factors used in the Framingham Risk Score is unknown. Therefore studies are needed to
confirm its diagnostic and prognostic ability in asymptomatic individuals.

**A2 phospholipases (PLA₂)**

PLA₂ are enzymes that cleave sn-2 side chains of oxidized phospholipids in LDL to form free fatty acids and lysophospholipids, which are metabolised to form several inflammatory mediators. Two major subtypes have been extensively studied: Type II secretory PLA₂ (sPLA₂) and lipoprotein-associated phospholipase A₂ (Lp-PLA₂), which can be quite easily measured using immunoassays measuring absolute levels or activity. Both of these markers have been shown to be elevated and have prognostic power independently of classic risk factors in stable CAD, but also in ACS. In these studies, the association of PLA₂ was independent of established risk factors and CRP. Interestingly, a recent study demonstrated that intensive lipid lowering after ACS reduces Lp-PLA₂ activity by approximately 20%, whereas conventional therapy lowers it by only 4%. Moreover, although Lp-PLA₂ activity was not associated with a higher risk of recurrent CV events shortly after the event, it appeared to be of weak, but independent prognostic significance when measured 30 days after the event. Furthermore, in a prospective, case control study Lp-PLA₂ was independently associated with CAD in apparently healthy middle-aged men and women. Conflicting data comes from Japanese populations, in which an inherited deficiency of Lp-PLA₂ appears to confer increased risk of myocardial infarction, stroke, and peripheral arterial disease. The current data support continued research evaluating Lp-PLA₂ as a potential predictor of cardiovascular disease. In particular, further evidence is needed to quantify the extent to which Lp-PLA₂ measures are independent of traditional risk factors, particularly LDL-C. Additional data are also needed in more diverse populations, as most of the data are derived from middle-aged males.

**Other oxidative stress markers**

**Modified Tyrosines**

A potentially useful oxidative stress marker is the plasma level of oxidatively modified tyrosines, including nitrotyrosines (NO₂-Tyr), and stable halogenated Tyr residues: chlorotyrosines (Cl-Tyr), and 3-bromotyrosine. These are formed as a result of hydrogen peroxide interactions with amino acids. One pathway for generating nitric oxide–derived oxidants involves interaction with superoxide anion, leading to formation of peroxynitrite. Peroxynitrite is a potent oxidant that promotes nitration of protein tyrosine residues producing a distinctive "molecular fingerprint" for nitric oxide–derived oxidants, nitrotyrosine. An alternative mechanism for generating nitric oxide–derived oxidants involves myeloperoxidase (see previous paragraph). Measuring modified tyrosines in a clinical setting is troublesome. At present, only MS/MS-based methods, both GC-MS/MS and LC-MS/MS are reliable methods, however these techniques are expensive and require specialized laboratories. As is the case with several other oxidative stress markers tyrosines can also be formed as an artefact during sample preparation and analysis. Therefore clinical studies are limited. One JAMA paper reported that plasma levels of nitrotyrosine correlate with the severity of coronary artery disease and that their levels are modulated by statin therapy.
**Oxidative damage to DNA**

Data indicate that oxidative DNA damage and repair are markedly increased in human atherosclerotic plaques\(^{201}\). Oxidative damage to DNA occurs mainly at the C8 of the purine ring, which leads to G to T substitution and production of 8-Hydroxy-2-deoxyguanosine (8OHdG)\(^{202-204}\). This oxidation product has been suggested as a marker for oxidative stress and although it can be measured in plasma of serum is of most value when measured in urine\(^{204}\). Is was shown that levels of oxidative DNA damage in patients with angiographically documented coronary artery disease (CAD) were elevated\(^{205}\). 8OHdG can be measured with HPLC or ELISA. However, there are concerns that these do not have a high specificity for 8OHdG.

**Antioxidant defense markers**

**Antioxidant vitamins**

Nonenzymatic detoxification of ROS is provided by lipid- and water-soluble antioxidants. Lipid-soluble antioxidants present in LDL include vitamin E and carotenoids, and water-soluble antioxidants present in the extracellular fluid of the arterial wall include vitamin C. Many vitamins have been ascribed to have anti-oxidant properties *in vivo*\(^{206}\). Most extensively studied have been vitamin C (ascorbic acid) and E (α-tocopherol), and β-carotene. Circulating levels of these anti-oxidants have been shown to be inversely related to and protective of cardiovascular disease\(^{207-219}\). However, nowadays, there is much debate about the potential anti-oxidant capacity of these vitamins *in vivo*\(^{220}\). Concerns have arisen because contrary to the small early trials, large anti-oxidant supplementation trials did not demonstrate beneficial effects of vitamins on long term clinical end-points\(^{221}\). Moreover, overadministration may even have adverse effects\(^{221}\). Because oxidative stress is defined as the imbalance between oxidants and antioxidants in favour of the oxidants\(^{222}\), antioxidants may serve as indirect markers of oxidative stress. However, vitamins are highly non-specific for oxidative stress since they have other functions as well, which may result in their depletion\(^{220}\). Furthermore, some vitamins may even induce cell death\(^{223}\). Therefore, before using them as oxidative stress markers, the aforementioned issues need to be resolved.

**Glutathione-peroxidase-1**

Glutathione-peroxidase-1 (GPX1) is a selenocysteine containing an anti-oxidant enzyme that catalyses hydrogen peroxide to water and lipid peroxide to alcohol\(^{224}\). Studies of GPX1 knockout mice demonstrate that GPX1 functions as the primary protection against acute oxidative stress, particularly in neuropathological situations such as stroke and cold-induced head trauma, when high levels of ROS occur during reperfusion or in response to injury\(^{225}\). In humans GPX1 can be assessed in circulating erythrocytes by measuring total activity of GPX1 by the coupled enzyme procedure\(^{226}\). In an interesting study of 636 patients referred for coronary angiography, the baseline levels of GPX1 activity were inversely associated with increased risk of cardiovascular events during a 5-year follow-up. Although related to gender and smoking status, levels of GPX1 remained significantly associated with risk after adjustment for major vascular risk factors\(^{226}\). In a genetic study it was suggested that functional variants in the GPX1 gene are associated with increased IMT of carotid arteries.
and risk of cardiovascular and peripheral vascular diseases in type 2 diabetic patients. Furthermore it has been shown that GPX1 activity is positively associated with the intake of dietary supplements and negatively correlates with tobacco consumption.

**Anti-oxidant enzyme systems**

Antioxidants may inhibit atherogenesis and improve vascular function by different mechanisms. Enzymatic protection against ROS and the breakdown products of peroxidized lipids and oxidized protein and DNA are provided by many enzyme systems such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glucose 6 phosphate dehydrogenase (G6PD). SOD catalyzes dismutation of the superoxide anion into \( H_2O_2 \), which is then deactivated to \( H_2O \) by CAT or GPx. GPx also reduces organic peroxides into their corresponding alcohols. GPx uses GSH as a hydrogen donor whereby GSH is oxidized. The regeneration of GSH is catalyzed by GR. Apart from these important enzymatic antioxidants, paraoxonase (PON1) appears to have antioxidative properties as well. PON1 is a high-density lipoprotein (HDL)-associated antioxidant enzyme capable of hydrolyzing lipid peroxides in oxidized lipoproteins. Genetic studies have demonstrated that genetic variation resulting in diminished activity of these enzymes is associated with increased risk for atherosclerosis due to enhanced susceptibility to oxidative damage. Also expression, and plasma concentration or activity of these enzymes, mainly SOD, have been shown to be associated with stable CAD and AMI. Interestingly, recent reports suggest that a common gene variant of extracellular SOD (ecSOD) is actually associated with increased risk of ischemic heart disease. In conclusion, the balance between the individual antioxidants and their concentration relative to oxidants makes it difficult to interpret the individual levels. Therefore, these are not very suitable as markers for oxidative stress when measured alone.

**Thiols**

Plasma measurement of thiols, such as glutathione may provide an interesting measure of *in vivo* oxidative stress. Intracellularly, glutathione (GSH) is a major antioxidant that helps eliminate peroxides and other oxidants. The oxidized disulfide form of GSH, GSSG, is formed during the reaction of glutathione peroxidase with hydrogen peroxide, or by a direct reaction of GSH with peroxynitrite and other oxidants. The steady-state balance of GSH and GSSG can be expressed as the redox state of the GSH/GSSG couple. A recent study demonstrated that GSH/GSSG independently predicts the presence of early atherosclerosis in an otherwise healthy population, even after adjusting for the presence of traditional risk factors and inflammation measured as hs-CRP level.
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2. Advanced Glycation Endproducts

A novel group of compounds that may potentially serve as markers for inflammatory and oxidative stress are a heterogenous group of moieties referred to as Advanced Glycation Endproducts (AGEs). These are the end products of advanced modifications of proteins and accumulate with diabetes, renal disease and aging, including atherosclerosis. AGEs were originally characterized by their yellow-brown fluorescent colour and their ability to form cross-links to and between amino groups of proteins. Their classical formation pathway is the so called Maillard reaction, discovered by a French food scientist, which is a slowly occurring non-enzymatic glycation of proteins. This reaction starts with non-enzymatic glycation, occurring through covalent binding of aldehyde or ketone groups of reducing sugars to free amino groups of proteins, forming a labile Schiff’s base. This may undergo rearrangements to a much more stable ketoamine, called Amadori’s product. Dehydration and condensation reactions finally result in the production of protein adducts and cross-links, traditionally called AGEs. This pathway has been classically associated with the development of long-term complications in diabetes and renal disease. However, later insights have pointed out that AGEs may actually form from various other important pathways. These much faster occurring alternative routes are highly oxidative stress driven, and have been implicated in the pathogenesis of atherosclerotic disease (Figure 1).

**AGEs and the role of oxidative stress**

It is now established that an important proportion of the accumulation of AGE-like structures occurs under influence of oxidative stress. Crucial intermediates of these pathways are the so-called reactive carbonyl compounds (RCC), which may form rapidly under oxidative stress by auto-oxidation of sugars (and derivatives), glycolytic intermediates, but also from peroxidation of fatty acids and oxidative modification of nucleic and amino acids. Carbohydrates react with amino groups in proteins via their carbonyl groups, and then undergo rearrangement to more reactive dicarbonyl compounds such as 1- and 3-deoxyglucosone (DGs), which may react further to yield glyoxal (GO) or methylglyoxal (MG). These RCC react with lysine and arginine residues in protein to form a wide variety of AGEs and cross-link structures. For these carbohydrate derived RCC, oxidative stress is not strictly required, however oxidation reactions accelerate the chemical modification of proteins. Most AGEs require oxygen for their formation, and therefore they may also be referred to as glycoxidation products.

More importantly, chemical modification of protein may also be brought about by lipid peroxides. As mentioned in the previous section, oxidative modification of lipids begins with the formation of conjugated dienes, initiating chain reactions that produce various RCC, including malondialdehyde (MDA) and 4-hydroxyxenal (HNE). These may react with amino residues of protein generating characteristic Advanced Lipoxidation Endproducts (ALEs). These ALEs may in turn also carry on to form protein cross-links and fluorescent products, analogous to AGEs. A third relevant class of compounds comprises protein adducts that are formed from either carbohydrates or lipids. These include N-(carboxymethyl)lysine (CML), N-(carboxyethyl)lysine (CEL), glyoxal-lysine dimer (GOLD), and methylglyoxal-
lysine dimer (MOLD)\textsuperscript{1} and are quantitatively the most prominent biomarkers of nonenzymatic modification and cross-linking of protein in aging and disease\textsuperscript{11,16-18}. In this thesis, all aforementioned compounds, including carbohydrate derived (AGEs), lipid derived (ALEs), and both carbohydrate and lipid derived (such as CML) structures, will be referred to as AGEs, because of their uniform characteristic of forming AGE-like adducts to proteins, and from a historically most point of view.

**AGEs, cross-linking, cellular processes, and oxidative stress**

AGEs have classically been characterized by their ability to form cross-links in and between...
long-lived matrix molecules such as collagen, resulting in insolubilization, yellowing, and stiffening, interrupting their function and integrity\textsuperscript{19}. These are all typical features of the aging process and decrease the elasticity of various organ systems, such as heart, cornea, and lens\textsuperscript{19}. Since the half life of collagen is estimated to be 15 years, these cross-links persist for a long time\textsuperscript{20}. Moreover, cross-linking is thought to impair the enzymatic digestibility of the extracellular matrix, thickening basement membranes in capillaries, glomeruli, lens, and lungs\textsuperscript{21}. The observation that these cross-links may also be formed by lipid peroxides, such as MDA and HNE, argues that this is not merely a feature of glycation in diabetes, but appears to be a common product of oxidative stress-related aging\textsuperscript{22}. A natural defence against glycation \textit{in vivo} is provided by the enzyme glyoxalase I . Glyoxalase I detoxifies RCC, thereby removing deleterious species. Depletion of glutathione, as observed in settings of oxidative stress, suppresses activity of glyoxalase I\textsuperscript{23}. This is a potential mechanism by which oxidative stress could lead to enhanced accumulation of RCC-derived AGEs. Conversely, AGEs may themselves promote oxidative and inflammatory stress by activating their major receptor (RAGE) via activation of NADPH oxidase and mitochondrial pathways in inflammatory cells\textsuperscript{24}. RAGE is a multiligand member of the immonuglobulin superfamily of cell surface molecules\textsuperscript{25}. Ligand binding takes place at the extracellular domain of the receptor, consisting of one V-type immunoglobulin domain followed by two C-type immunoglobulin domains\textsuperscript{25}. In addition to AGEs, RAGE interacts with a diverse class of ligands, including S100/calgranulins, amphoterin, amyloid-\beta peptide, and the class of \beta\text{-sheet fibrils\textsuperscript{26}}. RAGE can be found on many cells, including macrophages, endothelial cells, and SMCs\textsuperscript{27}. When RAGE is engaged by one of its ligands, multiple signalling pathways are activated as well as downstream consequences such as the expression of NF-kB regulated genes\textsuperscript{26}. This initiates an inflammatory stress response, accompanied by abundant formation of inflammatory mediators, such as TNF-\alpha or IL-6, and ROS\textsuperscript{28}. Since these substances create a milieu of abundant oxidative and inflammatory stress, AGEs favour their own production and create a vicious circle. Activation of RAGE by AGEs and the ensuing events are commonly referred to as the "AGE-RAGE axis\textsuperscript{8}". In addition to RAGE, other receptors found on several cells recognize AGE-proteins, including AGE-R1, -R2\textsuperscript{29}, -R3\textsuperscript{30}, ScR-II\textsuperscript{31}, and CD36\textsuperscript{32}. Interestingly, AGE-R1 has recently been shown to have anti-inflammatory effects, since it exerts a protective function against RAGE promoted cell activation\textsuperscript{33}. Additionally, macrophage scavenger receptors (MSR-A) also recognize AGEs, promoting macrophage mediated damage\textsuperscript{34}. All of these AGE-related events form an integrative mechanism by which oxidative stress may lead to age-related disease\textsuperscript{11}. In the following section, we will review evidence of the role of AGEs in the development of one of these diseases, namely atherosclerosis.

The role of AGEs in the development of atherosclerosis

Classically, AGEs have been implicated as playing an important role in the pathogenesis of micro- but also macrovascular complications, diabetes and renal disease\textsuperscript{7}. However, taking the aforementioned oxidative stress-related mechanisms into account, it is unnecessary to argue that AGE formation may also be of interest in atherosclerosis. A fundamental observation linking AGEs to atherosclerosis is the identification of AGE structures in atherosclerotic plaques of patients with diabetes or renal disease\textsuperscript{35-39}, but also in non-
diabetic patients with CAD\textsuperscript{40}. Moreover, these structures co-localize with macrophages that overexpress RAGE\textsuperscript{34}, indicating the pathophysiolocal importance of AGEs in atherosclerosis. In the following sections we will provide several lines of \textit{in vitro}, animal, and human evidence for the role of AGE in atherosclerosis.

\textit{In vitro evidence}

In experimental models, AGEs have been demonstrated to interact with their receptors on endothelial cells, smooth muscle cells and macrophages\textsuperscript{41} and induce (by means of transcription factor NF-kB activation) focal adhesion molecule, cytokine expression and atheromateous plaque formation\textsuperscript{42}. Additionally, cross-linking also occurs in proteins in the vascular wall\textsuperscript{43}, and there are many sites in which AGE-modified structures can be found, such as type IV collagen, laminin and fibronectin\textsuperscript{44}. The most common structure is collagen, which is the main constituent of the extra-cellular matrix\textsuperscript{45}. The cross-links formed between collagen molecules result in stiffening of the vascular wall as well as reduction of myocardial compliance\textsuperscript{43}. Hence, hypertension and vessel leaking may ensue\textsuperscript{46}. Furthermore, excessive deposition of AGEs might attract monocytes to bind to the luminal surface, to transmigrate the vessel wall and to release mediators that potentially contribute to the development of vascular lesions\textsuperscript{41}. Evidence suggests that AGEs quench nitric oxide (NO) in vitro\textsuperscript{47} resulting in NO depletion and increased smooth muscle cell proliferation\textsuperscript{48}. NO synthetase gene expression is reduced in the presence of AGEs\textsuperscript{49}. These actions may result in endothelial dysfunction, demonstrated by the association between urinary excretion of pentosidine, an AGE product, and markers of endothelial dysfunction, such as plasma concentrations of von Willebrand factor and s-VCAM-1 in patients with type 1 diabetes\textsuperscript{50}, and the impairment of endothelium-dependent vasodilation, another marker of endothelial dysfunction, in type 2 diabetics\textsuperscript{51}.

Another important mechanism by which AGEs may promote atherosclerosis is by modification of lipoproteins\textsuperscript{52;53}. Glycoxidation of LDL makes it less recognizable by the native LDL receptor, resulting in delayed clearance from the circulation, with increasing LDL-C levels. Moreover, it also promotes LDL uptake by scavenger receptors on macrophage and smooth muscle cells, which may eventually lead to foam cell formation\textsuperscript{54}. It has been shown that glycated LDL stimulates the production of pro-inflammatory and pro-thrombotic mediators\textsuperscript{55;56}. Furthermore, AGEs may also modify HDL, altering its atheroprotective effects\textsuperscript{57}. For example, glycated HDL loses its ability to prevent monocyte adhesion to the endothelium\textsuperscript{58} and has a decreased activity of enzymes associated with its surface such as paraoxonase\textsuperscript{57}, CETP and LCAT\textsuperscript{59}. A lower ability of glycated HDL to mediate cholesterol efflux and a decreased ability to protect LDL from oxidative damage have been observed\textsuperscript{58-60}. Interestingly, in one study by the group of Vlassara, diabetic subjects were either randomised to a diet high in AGEs or one low in AGEs for 6 weeks. Isolated pooled LDL from patients with a high AGE diet tended to be more glycated and oxidized than the low AGE diet group. When incubated with umbilical cord endothelial cells the glycated LDL resulted in redox-sensitive mitogen-activated protein kinase activation, including enhanced production of soluble vascular cell adhesion molecule-1\textsuperscript{58}. In conclusion, extensive experimental evidence exists that AGEs may function at many different levels as initiators and promoters of atherosclerosis\textsuperscript{61}.
Animal studies

The role of AGEs in atherosclerosis is strengthened by the results from experimental animal studies. It has been shown that direct intravenous infusion of glycated albumin in euglycemic rabbits results in enhanced expression of adhesion molecules and thickening of the intima. In another study, local application of AGE-BSA to the vessel wall resulted in enhancement of intimal hyperplasia of the carotid artery. These observations are supported by animal intervention studies demonstrating that the atherosclerotic process can be attenuated by diminishing the AGE burden using the soluble receptor for AGEs (sRAGE) or an AGE inhibitor or cross-link breaker (i.e. aminoguanidine and ALT-711). Forbes and colleagues randomized streptozotocin-induced diabetic apolipoprotein E-deficient mice that were allocated to receive no treatment, the AGE cross-link breaker ALT-711, or the inhibitor of AGE formation aminoguanidine (AG) for 20 weeks. Accumulation of AGEs in aortas and plasma, collagen associated CML and CEL, and decreases in skin collagen solubility were ameliorated by both treatments. Moreover, this was accompanied by a 30-40% decrease in plaque area in both treatment arms. This study should be regarded as proof of the principle that AGEs play a crucial pathophysiological role in atherosclerotic plaque formation. Recently, the key role of RAGE in the generation of oxidative stress and subsequent cellular damage was pointed out in an animal model of ischemia/reperfusion injury after myocardial infarction. It was demonstrated that CML is generated by ischemia/reperfusion and subsequently activates RAGE, augmenting vascular and inflammatory cell activation. Animals lacking cellular expression of RAGE were more likely to be protected from RAGE-mediated damage.

Additional evidence of the role of AGEs in atherosclerosis comes from research on the influence of exogenous AGEs. Lin et al randomised apolipoprotein E deficient mice (apo E -/-) to a diet low in AGE content (L-AGE) or a diet with a 10-fold higher AGE (H-AGE) content. After one week a femoral artery injury was inflict and their diet was maintained for another 4 weeks. At the end of the study the mice on L-AGE had a less pronounced neointima formation as compared to the mice on H-AGE. Additionally macrophage infiltration as well as smooth muscle cell formation was obviously reduced in the vascular wall of the mice on a low AGE diet. Moreover, the reduction in neointimal formation correlated with an approximately 40% decrease in circulating AGE levels and there was a significant reduction in AGE deposition in the vascular wall. A later study on this subject has demonstrated the beneficial effect of a low AGE diet in streptozotocin-induced Apo E deficient diabetic mice on the development of aortic atherosclerotic lesions. After 2 months mice on a low AGE diet developed 50% smaller lesions at the aortic root as compared with mice on a high AGE diet. Immunohistochemical comparison showed markedly suppressed tissue AGE, AGE-receptor-1, 2 and RAGE expression, a reduced number of inflammatory cells, tissue factor, vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 in the low AGE diabetic group.

Human studies

In humans there are only limited data on the association of AGEs with (non-diabetic) atherosclerosis. One study revealed that patients with type 2 diabetes and coronary heart disease presented with higher AGE levels than patients with diabetes alone. Additionally, in another study elevated levels of AGEs correlated with the number of stenotic vessels in non-
diabetic patients with coronary artery disease. Furthermore, strong association between tissue accumulation of AGE collagen fluorescence in skin, myocardium, and aortic plaques, and carotid to femoral pulse wave velocity, and the severity of atherosclerotic lesions was described in diabetic patients admitted for coronary artery bypass surgery. These findings also appear to be true for non-diabetic subjects, as reviewed by others. Interestingly, in a study with type 2 diabetes subjects, serum AGEs were independent determinants of endothelium-dependent vasodilation of the brachial artery, a well accepted measure for endothelial dysfunction.

In humans, the vascular effects of food AGEs have been studied by the group of Vlassara. They found that in 4 weeks, a low AGE diet had marked effects on plasma levels of AGEs, carbonyl products in non-diabetic patients with nephropathy. Additionally, restriction of dietary AGEs in patients with nephropathy reduced serum CML bound LDL and apoB, an initial suggestion of a possibly beneficial influence on the development of atherosclerosis. In type 2 diabetic patients a study has been conducted by the same group to assess the influence of dietary AGEs on markers of the early atherosclerotic process. After two and also 6 weeks serum AGE’s increased in the high AGE diet group and decreased in the low AGE group. The differences in serum AGE’s were accompanied by marked differences in several vascular inflammation markers, including CRP, vascular adhesion molecule-1, but also by a difference in AGE-LDL. Thus, pre-clinical as well clinical evidence points out that AGE may play a crucial role in the development of cardiovascular disease in disease classically associated with enhanced accumulation of these compounds, but probably also in atherosclerosis in general.

Although several biochemical analytical methods have been applied to measure AGE levels in clinical studies, there is no reliable golden standard (for more information the reader is referred to Chapter 2). We have recently introduced an instrument to non-invasively assess tissue AGE accumulation in vivo, which utilises the characteristic fluorescence pattern of AGEs. This instrument has been validated to collagen linked fluorescence (CLF) and specific AGEs, including the exclusively glucose derived pentosin and partially lipid derived CML and CEL, in human skin samples of healthy subjects and in patients with diabetes or renal failure. Skin AF is elevated in diabetes and renal disease and to a larger extent if these diseases are accompanied by cardiovascular disease. Furthermore skin AF appears to be a strong prognostic marker for future cardiovascular disease in renal failure and diabetes.

A detailed description of this method and its clinical evaluation is given in Chapter 2.

**Conclusion**

Experimental studies have demonstrated that oxidative stress plays a crucial, pathophysiological role in the development of atherosclerotic plaques. Most convincingly it has been shown that oxidative modification of LDL in the vascular wall initiates atherosclerosis, because it enhances the uptake of LDL by phagocytic cells. Furthermore, oxidative stress may also promote several later steps of vascular lesion formation, mainly by the quenching of NO and the triggering and propagating of inflammatory reactions.

Since currently available risk assessment, using traditional risk factor based algorithms or clinical parameters identifies only some patients at risk, measuring markers of oxidative stress and inflammation in clinical practice may provide additional information to identify...
the vulnerable patient. Among inflammatory biomarkers, CRP is by far the most robust, with much prognostic data available. However, several other markers, such as fibrinogen, soluble CD40 ligand, neopterin, and several cytokines and soluble adhesion molecule, may also be of value. Only indirect measurement for oxidative stress is possible because free radicals are too short lived and aggressive to be measured *in vivo*. Among currently available markers for oxidative stress, direct antigen detection oxLDL and serum levels of the enzymes A₂ phospholipases₂ (PLA₂ and Lp-PLA₂) and myeloperoxidase seem the most promising. More specific and accurate measurement of, for example, isoprostanes is currently impractical for clinical studies and routine practice.

A new group of compounds that may potentially serve as markers of inflammation and oxidative stress are AGEs. These have been shown to be pathophysiologically involved in atherosclerosis, beyond diabetes and renal disease, and can be easily and non-invasively measured by using skin AF as marker for AGE deposition in the skin. In contrast to all other currently available biomarkers for oxidative stress, this tool might ultimately be suitable as a bedside instrument for quick assessment of AGES as an indirect measure for oxidative stress in a clinical setting. However, there are currently no data available on its use in non-diabetic patients or in non-diabetic patients with normal renal function. In the following chapters, data obtained from patients with CAD and sub clinical atherosclerosis and one rare related disease model will be presented and the association of skin AF with cardiovascular disease in general will be presented.
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Introduction


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3. Aims of the thesis

In **Chapter 1** we have given an overview of currently available markers for inflammation and oxidative stress, and put their usefulness in clinical practice into perspective. We presented clinical data on several well studied and defined biomarkers, including CRP and other more specific inflammatory markers. Additionally, we have discussed the relatively novel field of biomarkers for oxidative stress, their important pathophysiological role in atherosclerotic disease and their limitations, concerning reproducibility, low prognostic power, and more importantly availability for clinical practice. In the final section of the chapter we introduced a relatively new group of compounds, the Advanced Glycation End products (AGEs) and their potential role as markers for inflammation and oxidative stress, especially in atherosclerotic disease. In the current thesis, we aim to investigate the clinical usefulness of several well accepted biomarkers and present a completely new approach to measure disease risk, skin Autofluorescence (skin AF).

**Chapter 2** is a randomised controlled trial in patients with a history of cardiovascular disease that do not reach their low density lipoprotein (LDL-C) goal on standard care statin therapy (simvastatin 40 mg). The potency of current available statins compared with placebo has been well documented. Additionally, aggressive statin therapy compared with standard therapy has been shown to be beneficial in lowering the risk in future cardiovascular morbidity and mortality in patients with stable atherosclerotic manifestations, but also in patients with acute coronary syndromes. However, it is yet unclear whether the additional beneficial effect results from lowering LDL-C to a greater extent only, or whether there is a contribution of the so called “pleiotropic effect” designated to statins. This may include their ability to reduce inflammation and oxidative stress beyond lipid lowering. In this chapter we aim to investigate whether intensified therapy with statins reduces levels of circulating, well accepted biomarkers for inflammation and oxidative stress.

We have outlined that several biomarkers for oxidative stress exist, but the inherent limitations discussed in Chapter 1 limit their clinical usefulness and availability. Therefore, there is a continuing need to investigate new markers, and a potential new group of compounds may be AGEs. In **Chapter 3** we review current methods to measure AGEs in plasma and tissue (i.e. skin samples) with immuno(histo)chemistry and other sophisticated biochemical assays and introduce the concept of measuring tissue AGE accumulation, using a simple and non-invasive method, skin AF. Since an important concern with non-invasive photology of the human skin is the interference of skin colour, and more specifically skin pigmentation, with the measurement, we investigate the effect of different skin photo types on skin reflectance and autofluorescence.

In **Chapters 4 – 7** we present clinical studies with a prototype of the current AGE-Reader, which is the instrument to measure skin AF. It has been previously demonstrated that skin AF is a strong predictor of future cardiovascular events in patients with diabetes or renal failure. However, it is not known whether skin AF may also be of value in atherosclerosis in general.

Skin AF may be most valuable in a primary prevention setting, to easily identify high risk patients, without performing costly invasive procedures. In **Chapter 4** we therefore investigate whether skin AF is related to intima media thickness in asymptomatic subjects.
with multiple risk factors for atherosclerosis, and whether skin AF adds incremental information to commonly used risk scores (Framingham risk score and SCORE risk score) in identifying those subjects with the highest (>95th percentile) IMT values, who are at high risk for developing cardiovascular disease. In Chapter 5 we investigate whether skin AF is elevated in patients with stable CAD, who visited our hospital for elective coronary angiography. Additionally, we aim to validate skin AF with levels of three circulating markers for inflammation (C-reactive protein), monocyte/macrophage activation (neopterin), and the soluble receptor for AGEs (sRAGE). In Chapter 6 we address the question of whether skin AF is acutely and temporally elevated in patients with an acute ST-elevation myocardial infarction (STEMI) compared with patients with stable coronary artery disease and whether this is related to clinically available markers for inflammation (C-reactive protein) and glycation (glycated haemoglobin A1c) in these patients. Most importantly, we investigate whether elevated levels of skin AF in STEMI patients are of prognostic relevance in predicting the one year incidence of major cardiac events, including all cause death, hospitalisation for non-fatal myocardial infarction and heart failure.

Finally, in Chapter 7 we present a study of patients with the very rare Glycogen Storage Disease 1a (GSD1a). These patients lack the liver enzyme glucose-6-phosphatase, which catalyses the gluconeogenesis, resulting in extremely low blood glucose levels, but also very adverse lipid profiles and microalbuminuria. In the past, these patients died prematurely from hypoglycemia, however since the introduction of better dietary therapies these patients live much longer. Because of their adverse cardiovascular risk profile, it is plausible that these patients would develop atherosclerosis at an early age. However, it has been shown that the contrary is true and evidence exists that they may be protected against oxidative stress. In this chapter we try to find an explanation for this discrepancy by comparing the levels of AGEs in GSD1a patients - measured using skin AF and measured from skin biopsy homogenates - with the levels found in age matched healthy controls subjects. Additionally, since this is a low age group (18 – 35), there will be a unique opportunity to validate skin AF with the golden standard (i.e. skin biopsies) in this age group, without the interference of intercurrent diseases.

Finally, we summarize all the results presented in this thesis and discuss them to further define future studies in solving missing links and new fields of interest.