Isoprostanes and Phospholipases, 
Markers and Mediators of Oxidative Stress

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"Le savoir est l'unique fortune que l'on peut entièrement donner sans en rien la diminuer."

Knowledge is the only asset that can be wholly donated without depriving the benefactor whatsoever.

Amadou Hampâté Bâ
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Introduction

1 Introduction

1.1 Oxidative stress: introduction

Oxygen is the molecule essential for aerobic respiration and thus, for animal life. Paradoxically, it is also involved in many diseases and degenerative conditions. Indeed, atmospheric oxygen in its ground-state is a biradical (O$_2$) whose reduction leads to superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), perhydroxyl radical (HO$_2^*$) and hydroxyl radical (OH$^*$). These chemical entities are known as reactive oxygen species (ROS). ROS are present in all aerobic organisms. In human, ROS are continuously produced by the polymorphonuclear leukocytes or neutrophils of the immune system to kill bacteria (Forman and Thomas 1986). However, they are unstable, therefore extremely reactive and can damage the molecules they react with. Although all cells contain antioxidants that aim at reducing or preventing this damage, the situation where ROS prevail over antioxidants can occurs and is known as oxidative stress. Oxidative stress contributes to tissue injury following irradiation and hyperoxia and is thought to be a cause of neurodegenerative diseases such as Alzheimer's disease (Gilbert 2000) Since oxidation of low-density lipoprotein (LDL) in the endothelium is a precursor to plaque formation, oxidative stress is also thought to be linked to cardiovascular diseases.

ROS can react with lipids, nucleic acids or proteins. Oxidative damage to lipids has been most frequently investigated and is addressed in detail below. In DNA, both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage and cross-linking protein, and yielding products such as 8-hydroxyguanine (Imlay and Linn 1986). Oxidative attack on proteins results in amino-acid modifications, fragmentation of the peptide chain, altered electrical charge and increased susceptibility to proteolysis. However, oxidative damage to proteins is not pertinent for the present work.

1.2 Phospholipid oxidation

1.2.1 History

The reaction of oxygen free radicals with lipids has been noticed since antiquity because of the development of undesirable odours and flavours in food and because of its involvement in rancidity, for example in the storage of oils. This phenomenon was taken advantage of by ancient
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civilizations such as the Greek, Roman and Egyptian who created the first oil paint recipe by using a mixture of bee wax, pigments such as iron and copper, and vegetable oils like walnut oil. This recipe was rendered quick-drying and the resulting colours more brilliant and more intense by the replacement of walnut oil with linseed oil by the Flemish Painter Jan Van Eyck in the 15\textsuperscript{th} century. It further evolved in the hand of Italian painters such as Leonardo Da Vinci, who avoided too dark colours by cooking this oil mixture at low temperature. This and other modifications of this recipe were kept secret in Italian ateliers, securing their uniqueness and radiance throughout Europe over almost three centuries. Van Eyck’s recipe was also the basis for the recipe of the ink used by Johann Gutenberg in 1454 (Mills and White 1999). Today, lipid oxidation is still crucial in the fabrication of chemical and industrial products such as paints, inks, resins or lacquers but also of food products such as margarine.

Owing to its critical character in the fabrication of products used for human consumption, the oxidation of lipids has been extensively researched. As soon as 1800, the Swiss chemist Nicolas-Théodore de Saussure observed that linseed oil could absorb more than twelve times its own volume of oxygen over a period of four months. Indeed, the drying process of air- and hence, oxygen-exposed oil paint, for example, mainly includes the oxidative degradation of unsaturated fatty acids leading to the formation of aldehyde groups later transformed into carboxylic groups, yielding dicarboxylic acids (Surowiec et al. 2004). The knowledge about lipid peroxidation evolved over the years and led, through the first demonstration of free radical oxidation of membrane phospholipids by Porter et al. in 1980, to a new era with a constant stream of works devoted to biochemistry, biology and medicine.

1.2.2 Enzymatic oxidation

Arachidonic acid, whether diet-supplied or derived from endogenous linolenic acid, is the center molecule for enzymatic oxidation and can be oxidized through three pathways (Figure 1). Through the cyclooxygenase (COX) enzymes, which catalyze the addition of molecular oxygen to various polyunsaturated acids, arachidonic acid is converted to prostaglandin (PG) G\textsubscript{2}, which is subsequently reduced to PGH\textsubscript{2}. Under the action of cell-specific isomerases and thromboxane (TXA\textsubscript{2}) synthase, other PGs and TXA\textsubscript{2} are produced.

The lipoxygenase enzymes catalyze reactions between O\textsubscript{2} and methylene-containing polyunsaturated fatty acids. Among others, arachidonic acid is converted by these enzymes to hydroperoxyicosatetraenoic acids (HPETEs), which are important metabolic intermediates.
The CYP450 epoxygenase pathway forms epoxyicosatrienoic acid (EET) as well as dihydroxy acids.

Figure 1: Simplified scheme of the arachidonid acid cascade

1.2.3 Auto-oxidation
The peroxidation of lipids or auto-oxidation involves three steps: initiation, propagation and termination. (Figure 2) (Halliwell and Chirico 1993)
The initiation refers to the abstraction of an H atom from the methylvinyl group of the fatty acid by a ROS, leaving a carbon-centered radical that forms a resonance structure sharing the unpaired electron. The propagation stage is characterized by a reaction between this resonance structure and oxygen in its ground state, yielding a peroxyl radical. This peroxyl radical can then abstracts an H atom from a second fatty acid, forming a lipid hydroperoxide and leaving another carbon-centered free radical that can take part in a second H-abstraction. Therefore, a single H-abstraction by a hydroxyl radical creates a chain reaction involving the most abundant form of oxygen in the cell, namely oxygen in its ground state. The alternative fate of peroxyl radicals, especially from polyunsaturated fatty acids such as arachidonic acid, is to be transformed in cyclic peroxides or cyclic endoperoxides. Termination is achieved when radicals cross-link or react with chain-breaking antioxidants such as \( \alpha \)-tocopherol (vitamin E) to form conjugated products that are not radicals.
In 1990, Morrow et al. (1990) reported for the first time the formation by free-radical catalyzed lipid peroxidation of prostaglandin F₂ isomers, named F₂-isoprostanes. Depending on the position of the radical after free radical attack (C8, C9, C11 or C12), four F₂-isoprostanes could be formed (8-F₂-IsoP, 5-F₂-IsoP, 15-F₂-IsoP, 12-F₂-IsoP respectively) (Figure 3), each of which can comprise a mixture of eight racemic diastereoisomers. Thus, there is a possibility of 64 F₂-isoprostanes and the same applies for the E₂ and D₂ families (Morrow et al. 1994).
Because its structure solely differs from that of prostaglandin F\(_2\alpha\) in the orientation of the bound at C8, 15-F\(_{2\text{trans}}\)-IsoP is also named 8-iso-prostaglandin F\(_2\alpha\) (8-iso-PGF\(_2\alpha\)) (Figure 4A). The same applies for isoprostanes of the other families, such as 8-iso-prostaglandin E\(_2\) (8-iso-PGE\(_2\)) (Figure 4B). This nomenclature will be used throughout the present work.

1.2.4 Photo-oxidation

In the presence of light and sensitizers such as bilirubin, myoglobin or riboflavin, oxygen can react rapidly with unsaturated lipids to form hydroperoxides (Figure 5).

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**Figure 3: The F\(_2\) isoprostanes**

**Figure 4: Structure of the isoprostanes**

**Figure 5: Photo-oxidation of an unsaturated fatty acid**
1.3 Isoprostanes

1.3.1 Fate of the isoprostanes

Consistent with the known preference of biological systems to esterify arachidonic acid at the sn2 position of phospholipids, isoprostanes compounds appear primarily esterified at the same position (Kayganich-Harrison et al. 1993). The enzymes cleaving the sn2 bound of phospholipids are the phospholipases A2. Therefore, isoprostanes are presumably released in the plasma by a phospholipase A2 (Figure 6) following their formation in situ.

Figure 6: Cleavage of an isoprostane from a phospholipid

Subsequently, isoprostanes are filtered by the kidneys and excreted in urine (Morrow et al. 1992). Thus, they are found free and esterified to phospholipids in plasma, and free in urine.

8-iso-PGF$_{2\alpha}$ is chemically stable, whereas 8-iso-PGE$_2$ can spontaneously further degrade to 8-iso-PGA$_2$. Metabolites of 8-iso-PGF$_{2\alpha}$ have also been identified in human urine, resulting from $\beta$-oxidation with or without subsequent reduction of the $\Delta^5$ double bond, resulting in the formation of 2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ (Roberts et al. 1996) and 2,3-dinor-8-iso-PGF$_{2\alpha}$ respectively (Chiabrando et al. 1999) (Figure 7).

Figure 7: Chemical structures of 8-iso-PGF$_{2\alpha}$ and its metabolites 2,3-dinor-8-iso-PGF$_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$

In human, 2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ appears to be the major metabolite, accounting for 20% of the total excreted products following infusion of 8-iso-PGF$_{2\alpha}$ (Roberts et al. 1996).
1.3.2 Analysis

1.3.2.1 Preliminary considerations

Although no standard reference values exist, plasma concentrations of 8-iso-PGF$_{2\alpha}$ in the range of 0.01 to 0.1 nM have been reported in healthy individuals, with urine concentrations being up to 100 times higher (Basu 1998b, Morrow 1990) but highly correlated (Basu 1998b, Oguogho et al. 1999) to plasma levels. Since, in contrast to plasma, urine contains virtually no lipids such as arachidonic acid, thereby bearing no risk of ex vivo auto-oxidation and isoprostanes formation, and because it can be noninvasively collected, urine is the preferred medium for isoprostanes quantification. Nevertheless, as a precaution, samples should be supplemented with antioxidants such as ethylenediaminetetraacetic acid (EDTA) or 4-hydroxy-2,2,6,6,-tetramethylpiperidine 1-yl (4-hydroxy-TEMPO), and stored at least at –20 ºC. Initially, a 24-hour collection of urine was performed in order to adjust for renal function and eventual intra-day variations in isoprostanes’ urinary excretion. However, it was shown that intra-day variations were negligible (Helmersson and Basu 1999, Wang et al. 1995, Richelle et al. 1999), and that quantification of isoprostanes in a single time point-urine sample normalized for its creatinine content to adjust for renal function was an equivalent procedure. Considering that 64 F$_2$-isoprostanes possibly exist, the quantification of a single isoprostane appeared advantageous to ease the comparison of methods and results from different laboratories. Most investigators focused on 8-iso-PGF$_{2\alpha}$.

Thus, ideally, 8-iso-PGF$_{2\alpha}$ will be quantified in urine and expressed as pg/mg creatinine or as pmol/mmol creatinine, or as ng/h in case of a 24-hour collection.

1.3.2.2 Analytical methods

Techniques such as gas chromatography-mass spectrometry (GC-MS), radioimmunoassay (RIA) and enzyme immunoassay (EIA) have been used to quantify isoprostanes. Mass spectrometry was originally applied to characterize the structure of isoprostanes (Morrow et al. 1990, Waugh et al. 1997, Roberts et al. 1998, Reich et al. 2000) and became the reference analytical method for their quantification. Despite its lower selectivity, GC-MS in the negative ion chemical ionization (NICI) mode reliably detects 8-iso-PGF$_{2\alpha}$ with a detection limit of approximately 5 pg/mL (Schwedhelm and Böger 2003) if the appropriate prior purification procedures are undertaken, that is one solid phase extraction (SPE) and two thin layer chromatography (TLC) steps or one SPE, one TLC and one high-performance liquid chromatography (HPLC) step successively. GC-MS-MS reduced sample preparation to one SPE followed with one TLC step. However,
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 extraction of 8-iso-PGF$_{2\alpha}$ by means of immunoaffinity chromatography (IAC) efficiently replaces the time-consuming SPE, TLC and HPLC steps. No other procedure as those specified above leads to resolution of 8-iso-PGF$_{2\alpha}$ from other isomers by NICI. The actual quantification of 8-iso-PGF$_{2\alpha}$ by mass spectrometry is performed by addition of an internal standard, most of the time a deuterated analogue of 8-iso-PGF$_{2\alpha}$.

Mass spectrometry, however, is a considerably expensive analytical method. For this reason, immunoassays have been developed. One RIA has been quantitatively validated by GC-MS analysis of 8-iso-PGF$_{2\alpha}$ and presented a negligible cross-reactivity with other F$_2$-isoprostanes isomers (Wang et al. 1995). Although the commercially available EIA for the quantification of 8-iso-PGF$_{2\alpha}$ (Cayman Chemical) has the advantage not to require radioactive tracers, its comparability with GC-MS remains uncertain, with correlation coefficients ranging from $r = 0.63$ (Proudfoot et al. 1999) to $r = 0.80$ (Devaraj et al. 2001) and $r = 0.88$ (Bessard et al. 2001). Moreover, none of the immunoassays has been tested for cross-reactivity with F$_2$-isoprostanes metabolites.

1.3.3 Significance

1.3.3.1 Markers of oxidative stress

As product of free-radical induced lipid peroxidation, isoprostanes are reliable markers of oxidative stress. The quantification of isoprostanes altogether and of 8-iso-PGF$_{2\alpha}$ in particular is considered to be the best method for measurement of lipid peroxidation in vivo (Basu 2004) and has been used as such in many studies investigating a role of oxidative stress in various diseases. Consequently, increased isoprostane formation and urinary excretion is reportedly involved in numerous pathophysiological states (Table 1). Among others, F$_2$-isoprostanes are elevated in chronic renal insufficiency (Ikizler et al. 2002), pulmonary hypertension (Cracowski et al. 2001a), arthritis (Basu et al. 2001b), chronic obstructive pulmonary diseases (Montuschi et al. 2000a, Pratico et al. 1998a), asthma (Montuschi et al. 1999, Wood et al. 2003), cystic fibrosis (Ciabattoni et al. 2000, Wood et al. 2001) and in Parkinson disease or schizophrenia (Pratico et al. 1998c).

At the latest since the report of Schwedhelm et al. (2004) who showed that 8-iso-PGF$_{2\alpha}$ is correlated with the number of cardiovascular risk factors, 8-iso-PGF$_{2\alpha}$ is more specifically recognized as risk marker for coronary heart diseases. Indeed, conditions such as diabetes
mellitus (Devaraj et al. 2001), hypercholesterolemia (Davi et al. 1997), obesity (Keaney et al. 2003) and hypertension (Wang et al. 1995) have been associated with an increase in isoprostanes. Likewise, chronic healthy smokers have higher free and esterified F₂-isoprostane plasma concentrations and urinary excretion of F₂-isoprostane and F₂-isoprostane metabolites compared with healthy nonsmokers (Montuschi et al. 2000a, Bachi et al. 1996, Pilz et al. 2000, Reilly et al. 1996). This hint to a pro-oxidant effect of smoking in vivo is further validated by the fact that isoprostanes levels return to baseline values two weeks after smoking cessation (Morrow et al. 1995). Since LDL oxidation may lead to atherosclerosis, these findings may provide a causative link between smoking and the development of atherosclerosis (Morrow et al. 1995 and 1997). In line with this hypothesis, F₂-isoprostanes have been found in atherosclerotic lesions (Waddington et al. 2003) and 8-iso-PGF₂α in coronary arteries from coronary heart disease patients (Mehrabi et al. 1999).

1.3.3.2 Mediators of oxidative stress
Isoprostanes levels are not mere indicators of oxidative stress, they are also a marker of disease severity. For instance, pericardial F₂-isoprostane concentrations increase with the functional severity of heart failure and are associated with ventricular dilatation, suggesting a role for oxidative stress on ventricular remodeling and the progression to heart failure in vivo (Mallat et al. 1998). In patients with lung diseases, 8-iso-PGF₂α concentrations in exhaled breath condensate reflect the degree of airway inflammation (Montuschi et al. 1999, 2000a and 2000b). Likewise, urinary isoprostane concentrations correlate with disease severity in scleroderma (Stein et al. 1996). In the Alzheimer’s disease, F₂-isoprostane concentrations in cerebrospinal fluid are elevated early in the course of dementia (Montine et al. 1999a), and correlate with disease severity (Pratico et al. 2000, Montine et al. 1999c) and progression. Increase in F₂-isoprostane levels is an early event in asthma (Montuschi et al. 1999, Dworski et al. 1999), hepatic cirrhosis (Pratico et al. 1998b), Alzheimer’s disease (Montine et al. 2002, Pratico et al. 2002) and scleroderma (Cracowski et al. 2001b), suggesting a role for oxidative stress or at the least a prognostic value of isoprostanes in these pathological states.
Table 1: Conditions associated with increased oxidative stress according to assessment of F$_2$-isoprostanes

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<thead>
<tr>
<th>Disease</th>
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<tr>
<td><strong>Cardiovascular diseases</strong></td>
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<tr>
<td>Atherosclerosis</td>
<td>Waddington et al. 2003</td>
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<td>Pratico et al. 1997</td>
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<td>Ischemia/reperfusion injury</td>
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<td>Coronary artery disease</td>
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<td>Heart failure</td>
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<td>Renovascular disease</td>
<td>Minuz et al. 2002</td>
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<td><strong>Risk factors for cardiovascular diseases</strong></td>
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<td>Smoking</td>
<td>Reilly et al. 1996</td>
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<td>Hypercholesterolemia</td>
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<td>Reilly et al. 1998</td>
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<td>Diabetes</td>
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<td>Davi et al. 1999</td>
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<td>Hyperhomocysteinemia</td>
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<td>Male gender</td>
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<td>Obesity</td>
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<td>Hypertension</td>
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<td><strong>Neurological diseases</strong></td>
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<td>Alzheimer’s disease</td>
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<td>Chronic obstructive pulmonary disease</td>
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Although the association between increased oxidative stress and disease does not necessarily imply a causative link, the fact is that isoprostanes are not only biomarkers of oxidative stress but have numerous biological effects, suggesting they may function as pathophysiologic mediators of oxidant injury. 8-iso-PGF$_2$\(\alpha\) in particular has established itself as biologically active molecule. Indeed, it exerts vasoconstriction in a concentration-dependent manner in several vascular beds (Montuschi et al. 2004; also see Table 2). 8-iso-PGF$_2$\(\alpha\) affects the integrity and fluidity of cell membranes in tissues, a mechanism also involved in oxidative stress (Basu 2004). It also stimulates monocyte adhesion to endothelial cells (Leitinger et al. 2001) that synthesize and release various factors regulating angiogenesis, inflammatory responses, hemostasis as well as vascular tone and permeability. Therefore, the early increase in F$_2$-isoprostane levels in diseases such as scleroderma (Cracowski et al. 2001b) is suggestive of an association between isoprostanes and endothelial and/or vascular dysfunction. In compliance with this hypothesis, isoprostanes correlate with scleroderma activity (Stein et al. 1996). Furthermore, 8-iso-PGF$_2$\(\alpha\) formation is increased during LDL-oxidation \textit{in vitro} (Lynch et al. 1994) and exerts activities that

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<td>Scleroderma</td>
<td></td>
<td></td>
<td></td>
<td>Cracowski et al. 2001b</td>
<td>Stein et al. 1996</td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td></td>
<td></td>
<td></td>
<td>Cracowski et al. 2002</td>
<td>Basu et al. 2001a</td>
<td></td>
</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
could be relevant to the pathophysiology of atherosclerosis: it activates platelets (Patrono and FitzGerald 1997), induces mitogenesis in vascular smooth muscle cells in rat (Takahashi et al. 1992) and favors minimally oxidatively modified LDL-induced adhesive effect on neutrophiles (Fontana et al. 2002).

Table 2: Vasoconstrictive effects of 8-iso-PGF$_{2\alpha}$ according to vascular bed and species

<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary artery</td>
<td>rat</td>
<td>Morrow et al. 1990</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>pig, cattle</td>
<td>Kromer et al. 1996</td>
</tr>
<tr>
<td>Cerebral arterioles</td>
<td>pig</td>
<td>Hou et al. 2000</td>
</tr>
<tr>
<td>Retinal vessels</td>
<td>piglet</td>
<td>Lahaie et al. 2000</td>
</tr>
<tr>
<td>Portal vein</td>
<td>rat</td>
<td>Marley et al. 1997</td>
</tr>
<tr>
<td>Mammary artery</td>
<td>human</td>
<td>Cracowski et al. 2000</td>
</tr>
</tbody>
</table>

Hence, isoprostanes are markers of oxidative stress with significant biological effects. However, their action mechanism to these effects is still unclear. Although the early hypothesis concerning the existence of a unique isoprostane receptor remains (Longmire et al. 1994, Fukunaga et al. 1993), increasing and more recent evidence point to an interaction with the thromboxane receptor (TXAR) (Kinsella et al. 1997, Janssen et al. 2002, Tang et al. 2005, Tazzeo et al. 2003), a G-protein-coupled receptor which plays a key role in homeostasis. Indeed, its activation by its physiological agonist TXA$_2$ causes platelet aggregation and vasoconstriction, stimulates the release of prostacyclin from endothelial cells (Hunt et al. 1992) and can mediate mitogenic response in vascular smooth muscle (Dorn et al. 1992, Ali et al. 1993). In human, there are two TXAR subtypes, termed TXAR-α and TXAR-β (Hirata et al. 1991, Nusing et al. 1993, Raychowdhury et al. 1994). The two receptor subtypes are encoded by a single gene on chromosome 19p13.3 (Nusing et al. 1993) and are identical with regard to their 328 N-terminal amino acid residues, but differ in their C-terminal cytoplasmic domains, so that TXAR-α have 15 amino acids residues in its C-tail sequence, whereas the TXAR-β’s C-tail have 79 amino acid residues (Figure 8). Whereas TXAR-α expression levels appear similar in most human cell and tissue types, extensive differences in the expression levels of the TXAR-β were observed (Miggin and Kinsella 1998). Despite the presence of mRNA for both isoforms in human platelets, TXAR...
isoform-specific antibodies have revealed that they solely express the TXAR-α subtype (Habib et al. 1999). Relative expression levels of both subtypes in other cell types or tissues remains largely unknown.

Figure 8: Structural organization of the TXAR-α and –β isoforms

1.3.4 Substances influencing the isoprostanes

1.3.4.1 Metals

Some of the reactions producing free radicals in aerobic organisms involve metal ions. Metals such as iron, copper, chromium, vanadium and cobalt can accept or donate a single electron in redox reactions, thereby catalyzing the production of ROS in reactions like Fenton's, first described by in 1894:

\[
ROOH + Fe^{2+} \rightarrow OH^- + RO \cdot + Fe^{3+}
\]
\[
ROOH + Fe^{3+} \rightarrow R^+ + \cdot OOH + Fe^{2+}
\]
These radicals can then initiate and/or propagate lipid peroxidation. Thus, the presence of metals in biological systems in a free form does not only propagate the chain reactions but also amplifies them, thereby significantly increasing the level of oxidative stress.

More specifically, a detrimental role of iron was hypothesized by J.L. Sullivan (1981) to explain the sex difference in cardiovascular diseases. It was later proven that iron overload associated with conditions such as hereditary hemochromatosis could lead to serious complications such as arrhythmia (Niederau et al. 1985.) or congestive heart failure (Niederau et al. 1996, Bathum et al. 2001). Hereditary hemochromatosis is most frequent in Caucasians and originates in 90 to 94% of the cases from a cystine to tyrosine mutation at position 282 (C282Y) on the autosomal hemochromatosis (HFE) gene, the gene which regulates the amount of iron absorbed from food (Feder et al. 1996). Through a not yet fully understood mechanism, HFE patients absorb excessive amounts of iron from food. This leads, most of the time after decades, to iron overload. The prevalence of HFE C282Y homozygotes in Caucasians is about 0.5%.

1.3.4.2 Antioxidants (vitamins, glutathione)

An antioxidant is a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell 1990). Antioxidants can be of endogenous or exogenous (dietary) origin, lipid- or water-soluble, according to what compartment they act in. They can be divided into three main groups according to their antioxidative mechanism, namely (Young and Woodside 2001):

- chain breaking antioxidants: small molecules such as vitamin C, vitamin E and presumably vitamin A that can donate or receive an electron in the termination step of the lipid peroxidation, or carotenoids, which can effectively scavenge free radicals (Fukuzawa et al. 1998) and thereby inhibit the initiation step of the lipid peroxidation. Besides, coenzyme Q10 (coQ10), which is ubiquitous in the organism and whose lipo- and aquaphilicity allows its insertion into the membrane phospholipid bilayer, reacts with ROS or with lipid radicals to yield the ubisemiquinone radical, which is converted back to coQ10 in the mitochondria or through quinone reductases. Moreover, coQ10 can regenerate the vitamin E from its tocopheroxyl radical issued from the termination of lipid peroxidation (Genova et al. 2003).

- antioxidant enzymes such as superoxide dismutase (SOD), glutathione (GSH) peroxidase and catalase, which catalyse the breakdown of ROS;
- metal binding proteins like iron-binding proteins transferrin and lactoferrin, and the copper-binding protein ceruloplasmin: by binding metal ions, they prevent them from reacting to form radicals.

1.3.4.3 Pharmacological substances influencing phospholipases

Phospholipases A₂ (PLA₂s) are a large family of enzymes that specifically release fatty acids from the second carbon atom (sn2, thus PLA₂) of the triglyceride backbone of phospholipids, producing a free fatty acid and a lyso-phospholipid. PLA₂s are ubiquitous enzymes, although the individual enzyme expression patterns differ. Initially, PLA₂s were named based on location of activity (e.g. pancreatic and synovial) or mode of activity (Ca²⁺-dependent and Ca²⁺-independent). A much more structured and accurate system has been developed based on the catalytic site (His/Asp, Ser/Asp or Ser/His/Asp hydrolase) as well as on functional and structural features, yielding the cytosolic (cPLA₂), Ca²⁺-independent intracellular (iPLA₂), lysosomal (IPLA₂) PLA₂s (Table 3), which also hydrolyze the sn1 bond of phospholipids, and the secretory (sPLA₂) PLA₂s and the platelet activating factor-acetylhydrolase (PAF-AH) families, which both selectively cleave the sn2 bond of phospholipids and which we will focus on (Dennis 1994, Schaloske and Dennis 2006).

Table 3: human cPLA₂, iPLA₂ and lPLA₂ enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alternate names</th>
<th>Size (kDa)</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPLA₂ (Ca²⁺-dependent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVA</td>
<td>cPLA₂α</td>
<td>85</td>
<td>Ubiquitous, except in mature T and B lymphocytes</td>
</tr>
<tr>
<td>IVB</td>
<td>cPLA₂β</td>
<td>114</td>
<td>Pancreas, brain, heart, liver</td>
</tr>
<tr>
<td>IVC</td>
<td>cPLA₂γ</td>
<td>61</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>IVD</td>
<td>cPLA₂δ</td>
<td>92-93</td>
<td>Keratinocytes</td>
</tr>
<tr>
<td>iPLA₂ (Ca²⁺-independent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIA-1</td>
<td>iPLA₂</td>
<td>84-85</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>VIA-2</td>
<td>iPLA₂β</td>
<td>88-90</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>VIB</td>
<td>iPLA₂γ</td>
<td>88-91</td>
<td>Ubiquitous</td>
</tr>
</tbody>
</table>
Introduction

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Size (kDa)</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB</td>
<td>13-15</td>
<td>Pancreas</td>
</tr>
<tr>
<td>IIA</td>
<td>13-15</td>
<td>Synovial liquid, spleen, thymus, tonsil, bone marrow, intestine, liver</td>
</tr>
<tr>
<td>IID</td>
<td>14-15</td>
<td>Pancreas, spleen</td>
</tr>
<tr>
<td>IIE</td>
<td>14-15</td>
<td>Brain, heart, uterus</td>
</tr>
<tr>
<td>IIF</td>
<td>16-17</td>
<td>Testis, embryo</td>
</tr>
<tr>
<td>III</td>
<td>55</td>
<td>Kidney, heart, liver, skeletal muscle</td>
</tr>
<tr>
<td>V</td>
<td>14</td>
<td>Heart, lung, macrophage</td>
</tr>
<tr>
<td>X</td>
<td>14</td>
<td>Spleen, thymus, leukocyte</td>
</tr>
<tr>
<td>XII</td>
<td>19</td>
<td>Heart, skeletal muscle, kidney, pancreas</td>
</tr>
</tbody>
</table>
One of their functions is the release of arachidonic acid from phospholipids. However, additionally, group IB sPLA₂ appears involved in the digestion of dietary phospholipids as well (Kudo and Murakami 2002). Group IIA sPLA₂ exerts physiologically significant antibacterial properties, especially against Gram-positive bacteria (Beers et al. 2002). Through a region distinct from its catalytic site and similar to a region of the coagulation factor Va, group IIA, IID and V sPLA₂s can bind to the coagulation factor Xa, thereby bringing about an anti-coagulant effect (Mounier et al. 2000).

sPLA₂s in general seem involved in inflammatory diseases such as adult respiratory stress syndrome, inflammatory bowel disease and pancreatitis (Nevalainen et al. 2000). More specifically, group IIA sPLA₂’s concentration in serum and tissue correlates with disease severity in inflammatory states such as rheumatoid arthritis (Seilhamer et al. 1989), Crohn’s disease (Minami et al. 1994), adult respiratory distress syndrome (Touqui et al. 1999) and asthma (Bowton et al. 1997). Group IIA and V PLA₂s have been found to act jointly in inflammatory states (Gilroy et al. 2004). Furthermore, group IIA sPLA₂ is also associated with collagen fibers in the extracellular matrix of human atherosclerotic plaques (Sartipy et al. 2000) and group V PLA₂ promotes atherosclerotic lesions by modifying LDL particles (Wooton-Kee et al. 2004). Group IIA, IID and V sPLA₂s are highly cationic and bind tightly to cell surfaces that are rich in the anionic heparin sulphate proteoglycans. Thus, besides their secreted form, significant portions of these enzymes are membrane-bound in mammalian cells (Kudo and Murakami 2002).

Many different classes of compounds have been found to inhibit the sPLA₂ family. Glucocorticoids, for instance, may inhibit sPLA₂s by inducing dephosphorylation of the active form of the enzyme (Bailey 1991). The non-steroidal anti-inflammatory drug indomethacin and antimalarial agents such as chloroquine and mepacrine, also known as quinacrine, non-competitively inhibit PLA₂ activity by interfering with the substrate-enzyme interface (Chang et al. 1987, Jain and Jahagirdard 1985) or with Ca²⁺ (Volpi et al. 1981). In one clinical study, a 6-week daily intake of atorvastatin 40 mg or of simvastatin 40 mg led to a reduction in the group IIA PLA₂ protein levels (Wiklund et al. 2002). The influence on enzyme activity was not investigated.

Structure-based designed inhibitors have recently been described, among which the propane sulfonic anid LY311727 (Schevitz et al. 1995) and the indole analogue Me-Indoxam (Smart et al. 2004). LY311727 is potent in the low nanomolar range and preferentially binds to group IIA
sPLA₂ over group IB PLA₂ (Balsinde et al. 1999). However, it has been shown to bind to group V sPLA₂ as well (Chen and Dennis 1998). Hence, no isoenzyme-specific sPLA₂ is available yet.

1.3.4.3.2 The PAF-AH family

The platelet activating factor (PAF) is a potent phospholipid that binds the PAF-receptor, causing increased vascular permeability and activating platelets and leukocytes, thereby mediating platelet aggregation, inflammation and anaphylaxis (Kudo and Murakami 2002). PAF-AHs are Ca²⁺-independent PLA₂s with a Ser/His/Asp hydrolase catalytic site that can hydrolyze the acetyl group from the sn2 position of PAF, liberating acetate and lyso-PAF (Table 5, Figure 9). Whereas group VIIIA and VIIIB sPLA₂s selectively hydrolyze PAF, the intracellular group VIIB sPLA₂ also hydrolyzes sn2 acyl chains containing as long as five carbons of the phosphatidylcholine backbone (Hattori et al. 1995). Its extracellular counterpart, group VIIA sPLA₂, which we will focus on, hydrolyzes phospholipids with oxidized fatty acyl groups of up to nine carbons in length from the sn2 position of phosphatidylcholine and phosphatidylethanolamine (Kudo and Murakami 2002). Unlike the other PLA₂s, group VIIA sPLA₂ can hydrolyze its substrates in monomer form as well as in vesicles (Soubeyrand et al. 1998).

Table 5: mammalian PAF-AH enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alternate names</th>
<th>Sike (kDa)</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIIA</td>
<td>Lipoprotein associated PLA₂ (lp-PLA₂), plasma PAF-AH</td>
<td>45</td>
<td>Plasma</td>
</tr>
<tr>
<td>VIIB</td>
<td>PAF-AH II</td>
<td>40</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>VIIIA</td>
<td>PAF-AH Ib (α1 subunit)</td>
<td>26</td>
<td>Brain</td>
</tr>
<tr>
<td>VIIIB</td>
<td>PAF-AH Ib (α2 subunit)</td>
<td>26</td>
<td>Brain</td>
</tr>
</tbody>
</table>
Figure 9: Catabolism of PAF by the PAF-AHs


In plasma, 70% of PAF-AH circulates with LDL, in which it exerts a longer half-life than in high-density lipoprotein (HDL) (Kudo and Murakami 2002, Stafforini et al. 1989). Accordingly, some studies have demonstrated concurrent decreases in PAF-AH protein levels (Eisaf and Tselepis 2003, Blake et al. 2001, Koenig et al. 2006) and activity (Eisaf and Tselepis 2003) in plasma and LDL cholesterol in response to different lipid-lowering drugs. A reversible group VIIA sPLA$_2$ inhibitor, SB-480848, is currently being investigated in clinical trials (Blackie et al. 2003).
1.4 Objectives

1) The comparability of IAC-GC-MS to assess 8-iso-PGF$_{2\alpha}$ with other analytical methods such as an EIA is still debated. One objective will be to provide a more definite answer to this question.

2) Also, the value of 8-iso-PGF$_{2\alpha}$ as marker of oxidative stress in various clinical setting involving various diseases will be investigated. The goal will be to consider the value of 8-iso-PGF$_{2\alpha}$ in assessing a) oxidative stress in various pathological states and b) the effect of pharmacological and non-pharmacological treatments on oxidative stress. These clinical studies will involve measurement of 8-iso-PGF$_{2\alpha}$ in human urine with IAC-GC-MS.

3) Besides their value as markers of oxidative stress, further biological effects of the isoprostanes will be investigated, namely on platelet aggregation and angiogenesis. If applicable, the action mechanisms will be elucidated.

4) That isoprostanes are cleaved from phospholipids by PLA$_{2\alpha}$s has yet to be confirmed. A goal will be to find out, in vitro as well as in vivo in a clinical setting, whether an increase in PLA$_{2\alpha}$s parallels or leads to a rise in 8-iso-PGF$_{2\alpha}$ and how strong that association is. For methodological reasons, group IIA and V PLA$_{2\alpha}$s and plasma PAF-AH will be focused on.
2 Methods

2.1 8-iso-PGF$_2$α extraction and quantification

2.1.1 Immunoaffinity chromatography

Urine or cell supernatant samples were collected in polypropylene bottles containing 1 mM each of 4-hydroxy-TEMPO and EDTA, immediately 5 mL-aliquoted and stored at –20 °C until analysis.

For extraction of the 8-iso-PGF$_2$α by immunoaffinity, 20 µL of 0.25 ng/µL internal standard $[^2]$H$_4$-15(S)-8-iso-PGF$_2$α were added to 5 mL of the sample, to a final concentration of 1 ng/mL. Samples were then centrifuged for 5 min at 2,000 rpm and directly applied to the immunoaffinity columns (Cayman Chemical, Ann Arbor, USA). Columns were subsequently washed with 10 mL of column buffer, then with 10 mL of ultra-pure water. Samples were eluted with 3 mL of elution solution. Columns were regenerated by washing with 10 mL of ultra-pure water followed by 10 mL of column buffer, and stored back at 4 °C containing 10 mL of column buffer.

2.1.2 8-iso-PGF$_2$α derivatizations

The eluted samples were evaporated down to 0.3-0.5 mL under nitrogen and at 40 ºC, then transferred into silanised vials and desiccated, under nitrogen and at room temperature, until crystallization. For the first derivatization (Figure 10), the following reagents were added in each sample:

- 10 µL methanol, stored on molecular sieve beads
- 100 µL acetonitrile, stored on molecular sieve beads
- 10 µL Hünig’s base
- 10 µL PFB-bromide 33% v/v in acetonitrile

Samples were then incubated at 30 ºC for 1 hour and desiccated under nitrogen at room temperature until they crystallised.

For the second derivatization (Figure 10), 100 µL of BSTFA was added in each sample and they were incubated at 60 ºC for 1 hour. They were subsequently stored at 4 ºC until quantification by GC-MS.
2.1.3 GC-MS

The GC-MS analysis of 8-iso-PGF$_{2\alpha}$ was performed by means of quadrupole mass spectrometer 1200 (Varian, Walnut Creek, USA) connected with a gas spectrograph CP-3800 (Varian). The gaseous separation occurred by means of a 30 m x 0.25 mm (length x diameter) FactorFour$^\text{TM}$-5MS capillary column (Varian), with a film thickness of 0.25 µm. The capillary column was heated according to the following temperature sequence: 70 ºC for 2 min, heating to 280 ºC at a rate of 25 ºC/min, heating at 325 ºC at a rate of 5 ºC/min. The carrier gas was helium, with a constant flow of 1 mL/min. The temperature of the injector, 150 ºC at the injection, was increased immediately thereafter to 300 ºC at a rate of 200 ºC/min. Injection volume was 2.0 µL in the
Methods

split/splitless mode. The transfer line and the ion source were heated at a constant temperature of 300 °C and 170 °C respectively. Under the chosen NICI conditions, the ionization energy was 70 eV and the electron current 150 µA. Methane was used for chemical ionization in the ion source. For the detection of the ions, the electron-multiplier was set to a tension of 1.4 kV. 8-iso-PGF$_{2\alpha}$ was detected at a mass-to-charge (m/z) ratio of 569.4 and the internal standard [${^3}$H]$_4$-8-iso-PGF$_{2\alpha}$ at a m/z ratio of 573.4 in the SIM (single ion monitoring) mode.

2.2 Cell Culture

Human coronary artery endothelial cells (HCAECs, PromoCell) were delivered as an aliquot of cells frozen after the third passage. Upon delivery, the cells were thawed, subcultured for one further passage, aliquoted and stored in liquid nitrogen. An aliquot of HCAECs in their fourth passage was thawed anew for each performed experiment, so as to ensure that no properties characteristic of the endothelial cells would be lost.

HCAECs were cultured in a medium formulated for microvascular cells (Endothelial Cell Growth Medium MV [EGM], PromoCell) containing vitamin C, hydrocortisone, gentamicine, amphotericine B, fetal bovine serum (FBS) and other growth factors. For the experiments, these supplements were replaced with 0.1% bovine serum albumin (BSA) and 10% FBS.

FBS is an animal serum commonly used to supplement culture media due its high nutritional content. It is low in protein and nevertheless effective in promoting and sustaining growth of vertebrate mammalian cells. Inactivation is usually performed through a heat treatment, which destroys the complement, thereby ensuring that cells are not lysed due to antibody binding and complement activation.

Cells were cultured in 25- or 75-cm$^2$ flasks or in 6-well plates. Medium was changed every other day and the cells were sub-cultured when a confluence of 75% was reached. Cells were handled under a sterile laminar hood with vertical airflow. All incubations occurred under 5% CO$_2$ and 37°C.

2.2.1 Expression of phospholipases in HCAECs

HCAECs underwent a 9-hour incubation first in vehicle (EGM-2 basal medium supplemented with 0.1 % BSA and 10 % FBS), then with following test substances: vehicle (control), 5 U/L bee venom group III PLA$_2$ as positive control, 15 µM mepacrine and 5 U/L group III PLA$_2$ + 15 µM mepacrine. 8-iso-PGF$_{2\alpha}$ was subsequently quantified in the supernatant according to section 2.1.
Protein were harvested as described in section 2.4.1 in order to be quantified (section 2.4.2) to normalize the 8-iso-PGF$_{2\alpha}$ content in the supernatant of each well with its protein content and to be used for immunoblotting (section 2.4.3).

2.2.2 Tube formation

HCAECs were let to incubate for 1 hour on a 48-well plate previously thin-coated with Matrigel™ according to manufacturer’s instructions, at 37 ºC and 5 % CO$_2$. Cell number was approximately 30,000 cells/well. They were suspended in 300 µL of basal medium enriched with 5 % FBS. After one hour, medium was removed and replaced with basal medium enriched with 5 % FBS in which the vascular endothelial growth factor (VEGF) was diluted, with or without the test substances. The stock solutions of the test substances were in ethanol, so that the final concentration of ethanol in the wells was 0.1%. This concentration was tested as vehicle. The cells were then let to incubate for 24 hours, at 37 ºC and 5 % CO$_2$. A photo of each well was taken at a 2.5x magnification with an AxioCam PRc 5 camera, and analyzed with Zeiss LSM Image Browser v. 3.2.0. Tubes were measured in a randomly chosen 25 cm$^2$-area of the wells (Figure 11A) and only node-to-node continuous structures were counted as tubes (Figure 11B).

Matrigel™ is a solubilized preparation of extracellular matrix extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. It mainly contains laminin and collagen whose interactions provide a foundation for the assembly of other basement membrane components.

VEGF is a potent growth and angiogenic cytokine that causes the differentiation of mesodermal cells into endothelial cells as well as their proliferation, thereby promoting angiogenesis and vascular permeability. VEGF is expressed in vascularized tissues and plays a prominent role in normal and pathological angiogenesis.
Figure 11: Tube formation experiment: A) a tube network of HCAECs, B) close-up on completed and uncompleted tube structures

The vehicle (0.1% v/v ethanol) showed a positive effect on tube formation. However, VEGF was able to significantly further improve the tube formation (Figure 12).

Figure 12: Effect of vehicle and of VEGF on the basal tube formation (n=7)

2.3 Reverse transcriptase-polymerization chain reaction (RT-PCR)
RT-PCR is a variation of the standard PCR technique in which complementary DNA (cDNA) is synthesized from a mature mRNA template via reverse transcription and subsequently amplified using standard PCR protocols (Figure 13).
Figure 13: Distinction between standard and the RT-PCR principles

2.3.1 mRNA extraction

For extraction of the mRNA, cells in 25-cm² flasks or in 6-well plate were first washed with phosphate buffer saline (PBS) and 800 µL of RNAzol per well/flask was added. The content of each well/flask was transferred in an Eppi containing 200 µL of ice-cold chloroform. Each well/flask was rinsed with 500 µL of RNAzol, which were then pipetted in the corresponding Eppi as well. Eppis were vortexed for 30 sec and let 15 min on ice, then centrifuged 15 min at 4 ºC and 12,000 g. The aqueous supernatant was transferred in new Eppis containing 1 mL of 2-propanol. These were vortexed and stored at –20ºC for 24 hrs. They were centrifuged anew at 12,000 g and 4 ºC for 30 min. The supernatant was discarded, the pellet washed with 200 µL of ice-cold ethanol 70% v/v and centrifuged once more at 4 ºC and 12,000 g for 10 min. Ethanol was pipetted out and the RNA pellet air-dried for 1 to 2 min and resuspended in 12 µL of 0.1% v/v diethyl pyrocarbonate (DEPC).
2.3.2 cDNA extraction
RNA content was determined by photometry and the mRNA accordingly diluted to 0.1 µg/µL. 1 µg of mRNA served for extraction to cDNA using the Omniscript Reverse Transcriptase Kit according to manufacturer’s instructions. Briefly, 10 µL (1µg) mRNA was diluted in 10 µL Master mix (10 U Rnase inhibitor, 4 U reverse transcriptase, 1 µM oligo-dT primer, 5 mM dNTP mix, 1x reverse transcriptase buffer) and incubated at 37°C for 1 hour.

2.3.3 Primers
Customs primers were from Höttner und Hüttner AG (Tübingen, Germany). The forward primer was the following nucleic sequence: 5’-GTGTTGGCTGCCCTTTCTG-3’ which belongs to the exon 2 common to both receptors subtypes. The first reverse primer, 5’-GCGCTCTGTCCACTTCCTAC-3’, was designed to anneal in the exon 3 of the mRNA coding for the alpha-subtype receptor, whereas a second reverse primer was aimed at the exon 4 of the mRNA encoding the beta-subtype: 5’-CAAATTCAGGGTCAAAGAGCA-3’ (Figure 13). The expected final cDNAs had a weight of 281 and 386 base pairs (bp) for the subtypes alpha and beta respectively.

2.3.4 Polymerase Chain Reaction (PCR)
For each receptor subtype, the following solution was prepared:
cDNA from section 2.3.2 2.00 µL
(or aqua ad injectabilia, in blank)
dNTPs (2.5 mM) 2.50 µL
Forward primer (0.5 pmol/µL) 1.25 µL
Reverse primer (0.5 pmol/µL) 1.25 µL
Buffer Y 2.50 µL
Thermus aquaticus (Taq)-Polymerase 0.25 µL
aqua ad injectabilia q.s. 25.0 µL
After an initial 5 min at 94 ºC, 20 cycles were run as follows (GeneAmp, Applied Biosystems):
94ºC for 30 seconds,
62ºC for 30 seconds at the first cycle and 0.2ºC decrement at each subsequent cycle,
72ºC for 30 seconds.
Subsequently, 15 cycles were run as follows:
94°C for 30 seconds,
58°C for 30 seconds,
72°C for 30 seconds,
followed by a final step of 72°C for 7 minutes, and cooling to 4°C.
The amplified cDNA was run (100 V, 45 min) on a horizontal 1.5% w/v agarose gel for DNA >
1,000 bp submerged in 1x TBE buffer and a picture under UV was subsequently shot
(ChemiGenius² Bio-imaging System, Syngene).

2.4 Western Blot

2.4.1 Protein extraction
Cells were rinsed with PBS and lysed with 80 µL of lysis buffer per 25 cm². They were then
mechanically harvested by means of a cell scraper. The so obtained suspension was transferred in
an Eppi set on ice and centrifuged at 4 °C and 12,000 g for 5 min. The supernatant was 25 µL-
aliquoted and frozen at –80 °C until further use.

2.4.2 Protein quantification
Proteins extracted from cells were quantified according to the method described by Bradford
(Bradford, 1976). For that purpose, a standard curve was established in the concentrations 3.45,
6.90, 10.35 and 13.8 µg/mL corresponding respectively to 10, 20, 30 and 40 µL of standard
protein solution in 790, 780, 770 and 760 µL of aqua ad injectabilia. Similarly, 10 µL of the
diluted or undiluted cell extracts were pipetted in 790 µL of aqua ad injectabilia. 200 µL of
Bradford reagent was added in all cuvettes and after stirring and a 15-min incubation, the
absorbance was assessed at a wavelength of 595 nm. Each measurement was performed in
duplicate.

2.4.3 Immunoblotting
50 µg of protein were mixed with 7.5 µL of 3x Laemmli buffer and the volume adjusted to 50 µL
with aqua ad injectabilia. SDS-polyacrylamide gel electrophoresis was performed at 150 V for
1.5 hr in 1x running buffer, using 10% v/v acrylamide for the collecting gel and 25% v/v
acrylamide for the separating gel. Proteins were electrotransferred onto nitrocellulose membrane
(or on a PVDF membrane for proteins < 15 kDa) at 250 A for 1 hr, in 1x transferring buffer.
Methods

Blots were saturated for 1.5 hr in TBS-T containing 5% w/v of milk powder as a source of albumin. Membranes were further incubated with the corresponding antibody (1/1,000) in TBS-T containing 1% w/v milk powder, overnight and at 4 °C. Membranes were washed three times for 5 min in TBS-T and incubated 1.5 hr with a 1/2,000 dilution of a secondary antibody i.e. a donkey anti-rabbit antibody or a goat anti-mouse antibody, according to the origin of the primary antibody. Excess antibody was washed and positive bands were revealed by addition of an ECL mixture after a 10-min exposure time.

2.5 Platelet aggregation

Venous blood samples were collected from healthy adult volunteers who had not been taking any drugs during the previous 2 weeks; a citrate mixture-containing monovette (0.106 M trisodium citrate, 0.1% v/v citrate solution) was used as anticoagulant. Platelet-rich plasma (PRP) was then prepared by centrifugating the blood at 200 g for 15 min at room temperature and platelet-poor plasma (PPP) by centrifugating PRP at 2,000 g for 10 min at room temperature. PPP was used as blank to set full light transmission on the aggregometer. Aggregation was measured after 3 minutes starting at addition of agonist, as percent maximum light transmission, according to Born (Born 1962). Each measurement was performed in duplicate. All substances were solved in 10% v/v ethanol, so that the final concentration of ethanol in plasma was 0.5%. For each agonist, a sub-threshold concentration, the highest concentration inducing less than a 10% increase in light transmission, and a threshold concentration, the lowest concentration inducing irreversible aggregation, that is an increase in light transmission above 65%, were defined (definitions by Pratico et al. 1992) (Figure 14).
Methods

Figure 14: Induction of A) reversible and B) irreversible platelet aggregation by the addition of sub-threshold (0.5 μM) and threshold (1 μM) concentrations of the thromboxane receptor agonist U46619

2.6 Phospholipase activity assay

PAF-AH and group II and V phospholipases activities were assayed by use of commercially available assay kits (Cayman Chemical), following sample concentration with Amicon Ultra-4 Centrifugation Filter Devices with a cut-off weight of 10 kDa. A 2:1 concentration ratio was achieved by centrifugating the samples for 5 min at 400 g.

2.7 Organ bath

2.7.1 Preparation of rat aortic rings

All experiments were conducted in accordance with institutional guidelines and approved by the local committee on animal experiments. Since the use of anaesthetics such as nembutal could have confounded the results through vasorelaxating effects (Akata 2007), the test animals were sedated with ether and subsequently exsanguinated. The thoracic aorta was excised and placed in ice-cold Krebs-Henseleit solution. The vessel was pinned in a Petri dish filled with chilled Krebs solution (Figure 15A), cleaned of fat and connective tissue (Figure 15B) and cut into segments of approximately 0.5 cm in length. Aortic rings were horizontally mounted in 30-mL myograph chambers (Figure 15C and D) containing a Krebs solution at 37°C (Heat Circulator C20CS,
Methods

Lauda) and continuously bubbled with a mixture of 95% O₂ and 5% CO₂. The rings were adjusted over a period of 45 min approximately to maintain a passive force of 30 mN. After equilibration, the aortic rings were challenged with 80 mM KCl as training contraction. Changes in isometric force were recorded using IBJ Amon 2.61.

![Figure 15](image)

Figure 15: Picture of a rat thoracic aorta A) freshly excised, B) exempt of fat and connective tissue, C) mounted on a transducer apparatus and D) bubbled in Krebs-Henseleit solution in a myograph chamber

2.7.2 Vasorelaxation studies

The endothelium-dependent vasodilatation was determined by measuring the vasorelaxation to increasing concentrations of acetylcholine (ACh, 10⁻⁹-10⁻⁴ M) after phenylephrine-induced (PE, 0.5 µM) contraction of the vessels. Rings were then washed twice to restore baseline tension. Next, the endothelium-independent vasodilatation was determined by measuring the vasorelaxation to increasing concentrations of nitroprusside (NTP, 10⁻⁹-10⁻⁴ M) after PE-induced (0.5 µM) contraction of the vessels (Figure 16).
Methods

Figure 16: Vasorelaxation pattern of a thoracic rat aorta

2.7.3 Data analysis

Experimental values of relaxation were calculated as the changes from the PE-induced contraction produced. Data are shown as the percentage of contraction of \( n \) experiments, expressed as the mean ± SEM. IBJ Bmon 2.61 was used for data analysis and Mann-Whitney or Student T-test was used to assess significance of non-parametric or parametric data, respectively. A P-value <0.05 was considered to indicate significance.
3 Results

3.1 The analytics of the isoprostanes

After purification by SPE, it was possible to detect both 5- and 15-F$_2$-IsoP. These substances, whether deuterated or not, were all detected not only at the same m/z ratios (Figure 17), but also at the same retention time (Figure 18).

![Image of reconstructed ion chromatograms](Image)

**Figure 17**: reconstructed ion chromatogram (RIC) of synthetic A) 15-F$_2$-IsoP; B) [²H]$_4$-15-F$_2$-IsoP; C) 5- F$_2$-IsoP; D) [²H]$_4$-5- F$_2$-IsoP
When selectively extracted by means of IAC and subsequently detected by GC-MS, physiological and deuterated 8-iso-PGF$_{2\alpha}$ were also detected at the m/z ratio 569.3 and 573.3 respectively with a retention time of about 14.9 min (Figure 19).

Figure 18: Spectra of A) 15-F$_2$-IsoP; B) [H$_4$]-15-F$_2$-IsoP; C) 5- F$_2$-IsoP; D) [H$_4$]-5- F$_2$-IsoP
Results

Physiological 8-iso-PGF$_{2\alpha}$

Internal standard $[^2\text{H}_4]$-8-iso-PGF$_{2\alpha}$

Figure 19: Chromatogram of a sample spiked with 1 ng/mL of internal standard $[^2\text{H}_4]$-8-iso-PGF$_{2\alpha}$ and analyzed with GC-MS in SIM mode

Three samples, as is and spiked with 100, 500 and 1000 pg/mL of 8-iso-PGF$_{2\alpha}$, were aliquoted and sent to laboratories quantifying isoprostanes with following methods: IAC-GC-tandem MS according to Tsikas et al. (2003), SPE-HPLC-GC-MS according to Proudfoot et al (1999) or EIA performed by manufacturer, Cayman Chemical (Ann Harbor, USA), in order to assess the comparability of results reported. Additionally, we subjected the same samples to the method described in section 2.1, i.e. IAC-GC-MS. The resulting data are illustrated on Figure 20:
Results

Figure 20: Comparison of 8-iso-PGF$_{2\alpha}$ quantification methods
Although analyzed in separate laboratories, data yielded by the methods IAC-GC-MS and IAC-GC-tandem MS were closely correlated (Figure 21A). Despite being both specific for 8-iso-PGF$_2$α, the correlation between IAC-GC-MS and EIA was rather poor (Figure 21B) compared with that with the unspecific method SPE-HPLC-GC-MS (Figure 21C). Furthermore, the values yielded by the latter were significantly higher.

Figure 21: Correlation between IAC-GC-MS and A) IAC-GC-MS-MS, B) EIA, C) SPE-GC-MS

The data of the specific methods were reported on Bland-Altman plots (Bland and Altman 1986) displaying, for each sample, the percent difference between the values yielded by two methods A and B ($\frac{100 \times (A - B)}{A + B}$) on the y axis, versus their average ($\frac{A + B}{2}$) on the x axis. The mean difference for IAC-GC-MS-MS compared with IAC-GC-MS was −37.1 pg-mL and the agreement limits were −99.9 pg/mL and +25.6 pg/mL (Figure 22).
Results

Thus, 95% of samples measured with IAC-GC-MS will yield a value 99.9 pg/mL below or 25.6 pg/mL above the value yielded by the same sample measured with IAC-GC-MS-MS. When IAC-GC-MS was compared with EIA, the mean difference was –18.0 pg/mL and the agreement limits were –118 pg/mL and +82.3 pg/mL (Figure 23).

The selective quantification of the isoprostane 8-iso-PGF$_{2\alpha}$ as index of oxidative stress was used in subsequent studies.
Results

3.2 8-iso-PGF$_{2\alpha}$ as marker of oxidative stress

3.2.1 The haemochromatosis clinical study

This study was conducted in collaboration with the Department of Biochemistry and Molecular Biology II of the University Medical Center Hamburg-Eppendorf, Germany. The objective was to show that iron overload in HFE-related hemochromatosis is associated with increased oxidative stress assessed through 8-iso-PGF$_{2\alpha}$ urinary excretion, and that oxidative stress is impacted by iron-removal treatment (phlebotomy).

3.2.1.1 Study description

Study participants

All participants were between 18 and 65 years of age. The groups were matched according to age and gender. Individuals with renal insufficiency (serum creatinine >1.5 mg/dL) and infectious diseases (body temperature > 37 °C) were excluded from the study. Approval from the local ethics' committee for studies in humans was obtained before the beginning of the study, and all participants gave their written informed consent to the study.

Subjects with a history of increased values of serum ferritin and/or serum iron and suspected of having iron overload were referred by physicians from around Germany to our outpatient unit for the diagnosis or exclusion of hereditary hemochromatosis. For each patient, the HFE C282Y genotype was determined, the serum iron parameters (serum iron, transferrin saturation, serum ferritin), and liver iron concentration (LIC) were measured. Diagnosis of hemochromatosis was established by at least three of the following criteria: (a) transferrin saturation > 62%; (b) serum ferritin > 300 µg/L for men, 200 µg/L for women; (c) LIC > 1,000 µg/g wet weight; (d) hepatic iron index (LIC/age) > 30. In addition, all subjects were homozygote for the C282Y polymorphism in the HFE gene and naïve to hemochromatosis treatment. Patients were recruited over a period of 18 months, from December 2001 to June 2003. Two patients declined taking part in the follow-up.

Controls were recruited from the general population via local newspaper advertisement. They were exempt of the HFE C282Y mutation and presented a normal phenotype regarding iron parameters (serum iron, transferrin saturation, serum ferritin). Three of the 24 individuals screened for the control group presented a HFE C282Y-heterozygous genotype, which matches the estimated prevalence of 10% for this genotype (Feder et al. 1996). Although they had normal serum iron, serum ferritin, and transferrin saturation, they were excluded...
Results

from the study.

All subjects were being treated and stable for concomitant disorders, e.g., antihypertensive medication, stable asthma, or thyroxin supplementation. There was no anaemia in the participants.

Study protocol

Samples and data regarding the participants were labelled in a way that enabled data confidentiality. Blood and urine samples were collected in fasting state.

Study parameters were defined as 8-iso-PGF$_{2\alpha}$ excretion in urine, vitamins A and E, serum iron, serum ferritin, transferrin and transferrin saturation in serum. LIC and non-transferrin-bound iron (NTBI) were quantified in patients only.

LIC was measured prior to starting phlebotomy in the patient group by superconducting quantum interference device (SQUID) device and calculated from the specific magnetic susceptibility of $1.6 \times 10^{-3}$ SI units (g of iron/g tissue) for ferritin iron. Organ volume was determined by sonography.

Plasma samples were 2 mL-aliquoted and frozen at –80 °C. Testing for the HFE C282Y mutation and the determination of LIC, serum iron, transferrin saturation, serum ferritin, NTBI and vitamins were performed in the Department of Biochemistry and Molecular Biology II of the University Medical Center Hamburg-Eppendorf, Germany.

Urine samples were 5 mL-aliquoted and frozen at –20 °C in 1 mM EDTA and 1 mM 4-hydroxy-TEMPO until analyzed. 8-iso-PGF$_{2\alpha}$ was quantified by GC-MS after purification by immunoaffinity chromatography according to section 2.1. 8-iso-PGF$_{2\alpha}$ was detected at a m/z ratio of 569.4 and the internal standard [²H₄]-8-iso-PGF$_{2\alpha}$ at a m/z ratio of 573.4.

All other biochemical analyses (e.g. creatinine, cholesterol) were performed using certified assays in the local clinical laboratory.

Patients were treated with weekly removal of 400–500 mL of blood until a mild anemia was induced (Hb < 11.5 g/dL) and serum ferritin was below 30 µg/L. This accounted for 13–84 phlebotomies according to the individual iron load in the patients. After the initial therapy, a maintenance therapy was initiated, which consisted in 400–500-mL phlebotomies, 2–5 times a year. The follow-up serum and urine samples were collected immediately before one of the routine blood removals of the maintenance therapy.

Statistical analysis

Statistical analysis was performed with SPSS 10.0. Parameters were found not to be normally
Results

distributed according to the Shapiro-Wilk test. For that reason, groups are presented as nonparametric data, i.e. median and interquartile range (IQR). Comparisons between patients and controls were performed with the two-sided Mann-Whitney U test. Comparisons of patients before and after treatment were performed with the Wilcoxon test. A P < 0.05 was accepted for statistical significance.

3.2.1.2 Study results

Baseline data

Results are given in Table 6 and Table 7. There was no significant difference between cases and controls in age and body mass index (BMI). Baseline total and low-density lipoprotein (LDL) cholesterol were significantly lower in cases than in controls. There was no difference in other lipid parameters. Transferrin was significantly lower in patients than in healthy participants while serum iron, ferritin, and transferrin saturation were significantly higher. NTBI, first identified by Hershko et al. (1998) as a marker of iron overload, was present in patients. 8-iso-PGF$_{2\alpha}$ urinary excretion was 192% higher in patients, as illustrated in Figure 24. When excluding smokers, median 8-iso-PGF$_{2\alpha}$ urinary excretion in controls was 117 pg/mg creatinine [interquartile range 105–190] compared with 228 pg/mg creatinine [157–298] P = 0.002, in patients at baseline. Serum vitamin A was decreased in patients while vitamin E levels were similar to those of control. Frequencies of cardiovascular risk factors were similar in cases and controls, except for diabetes mellitus (Table 7).

Serum iron and ferritin values were significantly lower in female patients than in males (median 31.9 µM interquartile range [28.8–36.7] vs. 39.6 µM [31.9–42.4] for iron, P = 0.03 and 419 µg/L [230–828] vs. 850 µg/L [583–2762] for ferritin, = 0.04, females vs. males, respectively). In female controls, only ferritin was significantly decreased compared with males (28.1 µg/L [19.1–61.9] vs. 152.8 µg/L [91.7–186.9], P < 0.001). However, in neither of the groups was there a sex difference in 8-iso-PGF$_{2\alpha}$ urinary excretion.
Results

### Table 6: Groups' biochemical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 21)</th>
<th>Patients at baseline (n = 21(^b))</th>
<th>Treated patients (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
</tr>
<tr>
<td>Cholesterol parameters</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>227</td>
<td>204–235</td>
<td>193</td>
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<tr>
<td>HDL (mg/dL)</td>
<td>57</td>
<td>48–75</td>
<td>55</td>
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<tr>
<td>LDL (mg/dL)</td>
<td>138</td>
<td>118–159</td>
<td>120</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>125</td>
<td>84–149</td>
<td>144</td>
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<tr>
<td>LDL/HDL ratio</td>
<td>2.30</td>
<td>1.78–3.05</td>
<td>1.99</td>
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<tr>
<td>8-iso-PGF(_{2\alpha}) (pg/mg creat)</td>
<td>128</td>
<td>106–191</td>
<td>245</td>
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<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.90</td>
<td>0.75–0.95</td>
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<td>Iron parameters</td>
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<td>Serum iron (µM)</td>
<td>10.8</td>
<td>7.5–15.2</td>
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<tr>
<td>Transferrin (g/L)</td>
<td>2.5</td>
<td>2.4–2.7</td>
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<td>Transferrin saturation (%)</td>
<td>17.0</td>
<td>11.5–24.5</td>
<td>95.0</td>
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<tr>
<td>Ferritin (µg/L)</td>
<td>83.9</td>
<td>25.8–164.4</td>
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<td>LIC (µg of iron/g of liver)(a, b)</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>NTBI (µM)</td>
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<td>n.a.</td>
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<tr>
<td>Vitamin E (µg/mL)</td>
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<td>13.1–19.2</td>
<td>14.7</td>
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<tr>
<td>Vitamin A (µg/mL)</td>
<td>3.00</td>
<td>2.11–3.39</td>
<td>0.34</td>
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</table>

NTBI, non-transferrin-bound iron; LIC, liver iron concentration; n.a.: not available.
Significance was defined as P < 0.05.
IQR: interquartile range.
\(a\) As reference point, values of LIC in healthy individuals are defined by Nielsen et al. (1995) as 100–500 µg/g liver.
\(b\) For LIC, n = 20: one female participant could not undergo this examination due to overweight.
\(c\) Statistics were computed using the Mann-Whitney two-sided test.
\(d\) Statistics were computed using the Wilcoxon test.
Table 7: Groups' other characteristics

<table>
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<td>Myocardial infarction</td>
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<td>Other diseases&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Age</td>
<td>49.0</td>
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<tr>
<td>BMI</td>
<td>23.8</td>
<td>19.5-25.6</td>
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<sup>a</sup> Hypercholesterolemia was defined as: LDL cholesterol 160 mg/dL, total cholesterol 240 mg/dL or lipid-lowering medication.

<sup>b</sup> Previous breast cancer (n = 1), varicose veins (n = 5), hypothyroidism (n = 2), anxiety disorder (n = 1), gastrointestinal disorder (n = 1), stable asthma (n = 1).

Follow-up data

A phlebotomy therapy was initiated in all patients. The median follow-up period was 25 [17.5–32.5] months. All iron parameters were significantly decreased compared with baseline. All iron parameters were normalized, except the transferrin, which, at follow-up, remained significantly lower than in controls. There was a significant increase in serum vitamin A levels, albeit not up to control levels. There was a decrease in vitamin E in cases that, although nonsignificant compared with baseline, caused cases to have significantly lower vitamin E levels after phlebotomy treatment than controls. As shown on Figure 24, 8-iso-PGF<sub>2α</sub> urinary excretion was significantly decreased after treatment, to levels similar to controls' levels.
Results

Figure 24: Median creatinine indexed of 8-iso-PGF$_{2\alpha}$ urinary excretion in controls and patients before and after phlebotomy treatment

Figure 25 illustrates the individual change in 8-iso-PGF$_{2\alpha}$ urinary excretion in the patients. When individuals who smoked were excluded from the analysis, levels of 8-iso-PGF$_{2\alpha}$ urinary excretion in treated patients was 141 pg/mg creatinine [111–198] with $P = 0.43$ compared with controls and $P < 0.001$ compared with baseline. Although only one of the patients started a lipid-lowering treatment in the course of the study, total cholesterol and LDL cholesterol were significantly decreased in the whole group after phlebotomy treatment in comparison to baseline, leading to a significant lowering of the LDL/HDL (high-density lipoprotein) ratio, an indicator of cardiovascular risk. Triglycerides and HDL cholesterol levels were not significantly modified.

Figure 25: Individual effect of blood letting on oxidative stress measured through 8-iso-PGF$_{2\alpha}$ urinary excretion in cases.
3.2.2 The VASSc study

This was an open case-control study conducted in collaboration with the Grenoble University Hospital in France. The goal was to test whether there is a link between micro- and/or macrovascular dysfunction and oxidative stress damage in patients with systemic sclerosis. The endpoints used to test this hypothesis in the correlation analysis were the peak postocclusive hyperemia and urinary 8-iso-PGF$_{2\alpha}$ levels.

3.2.2.1 Study description

Study participants

This was a descriptive monocentric controlled study performed using methodology previously described (Boignard et al. 2005). The study was approved by the Institutional Review Board of Grenoble University Hospital, France, and all subjects gave informed written consent. 68 subjects were enrolled: 43 patients suffering from systemic sclerosis and 25 healthy volunteers. Patients suffering from systemic sclerosis (SSc) were recruited from the Vascular Medicine Department. Healthy volunteers were recruited through local newspaper advertisements. The inclusion criteria in the SSc group was the diagnosis of systemic sclerosis according to the criteria of LeRoy and Medsger (2001). All subjects were 18 years of age or older. Exclusion criteria were cigarette smoking, diabetes mellitus, hypercholesterolemia, or any associated severe disease (cancer, cardiac and pulmonary failure, myocardial infarction, angina pectoris). Furthermore, patients taking statins, nitrates, and/or nonsteroidal anti-inflammatory drugs were excluded. All patients were asked to discontinue any vasodilator therapy given for Raynaud’s phenomenon (RP) from 1 week before inclusion until the end of the study. Patients unable to discontinue vasodilator therapies during the study period were not included.

The onset of the disease was defined as the first occurrence of symptoms of systemic sclerosis apart from the RP. Digital pitting scars, esophageal dysfunction, and RP were diagnosed clinically. Skin thickness was quantified using the modified Rodnan skin score (Furst et al. 1998). The diagnosis of pulmonary fibrosis was suspected on the basis of clinical data and systematic radiographs and confirmed in all cases by computed tomography scans.
Study protocol
Subjects arrived at the Clinical Research Center between 8 and 9 am in a fasting state. They were placed in the supine position in a quiet room with a stable ambient temperature. Blood samples were taken for plasma hsCRP, interleukin-1α, and interleukin-6 quantification and urinary samples for quantification of 8-iso-PGF₂α. After clinical examination, subjects were placed in the supine position, with both forearms resting at heart level. Blood pressure and heart rate were recorded, followed by baseline laser Doppler measurements at the left middle finger pad. A 5-min postocclusive hyperemia was then performed, followed by nitroglycerin challenge after a 20-min resting period.

Laser Doppler measurements
Cutaneous blood flow was measured using a laser Doppler flowmeter (PeriFlux System 5000; Perimed, Järfälla, Sweden). Laser probes (PR457) were attached to the distal pad of the third left finger. They were left in place during the whole laser Doppler measurement. The laser Doppler flowmeter was interfaced to a personal computer through a converter using Perisoft (Perimed) data acquisition software.

Laser Doppler blood flow was recorded in millivolts (mV), which are directly related to blood flow in the microcirculation of the surface tissue. Blood flow was divided by the mean arterial pressure in order to take into consideration potential variations in blood pressure to yield values of cutaneous vascular conductance (mV/mm Hg). The hyperemia was studied in the following sequence: 30 min of rest, postocclusive hyperemia with a 30-min recovery period, followed by the nitroglycerin challenge. The recovery periods were determined in a previous experimentation (Boignard et al. 2005), to ensure that cutaneous vascular conductance had returned to baseline values between protocols.

After 30 min of rest, the baseline cutaneous conductance being measured in the last 10 min, digital blood flow was occluded for 5 min by inflating a cuff placed on the left arm to 50 mm Hg above the systolic blood pressure. The cuff was then released and the flow responses were recorded. Endothelium-independent vasodilation was tested after another 30-min resting period, the baseline cutaneous conductance, blood pressure and heart rate being measured in the last 10 min. A single high dose of sublingual nitroglycerin (0.4 mg) was administered. Then, digital blood flow was occluded for 5 min by inflating a cuff placed on the left arm to 50 mm Hg above the systolic blood pressure. The cuff was then released and the flow responses recorded.
Results

The amplitude of the response was determined by recording the peak cutaneous vascular conductance, expressed in mV/mm Hg. The values of the biological zero were systematically subtracted from the data for each individual patient. The kinetics of the response were determined by calculating the time to peak hyperemia, expressed in seconds. The day-to-day reproducibility was assessed in a previous study (Boignard et al. 2005). Briefly, each examination was repeated 1 day after the end of the first series on the same subject. The median absolute difference for the peak hyperemic conductance was 2 mV/mm Hg [10th-90th percentile 0.5–9]. The median absolute difference for the time to peak hyperemia was 20 sec [5–40]. The coefficient of correlation for peak hyperemic conductance and the time to peak hyperemia was 0.94 and 0.56, respectively. Since correlation coefficients are poor indicators of reproducibility, Bland and Altman plots were constructed to measure the agreement between both measures. For the two measures, more than 95% of the differences were less than 2 standard deviations, and neither proportional error nor systematic errors were detected.

Biology

Spontaneous morning micturition samples (20 mL) were collected in polyethylene tubes, immediately aliquoted, and stored at -20°C. At the end of the inclusion period, all samples were transferred from Grenoble, France, to Hamburg, Germany, in dry ice via an express courier. Urinary concentrations of 8-iso-PGF$_{2\alpha}$ were determined by GC–MS after purification by immunoaffinity chromatography according to section 2.1. Final results were expressed as picograms of 8-iso-PGF$_{2\alpha}$ per milligram of creatinine. Observers were blinded to the source of samples for technical analysis.

Blood samples were collected in EDTA tubes and immediately centrifuged at 4°C. Plasma was aliquoted and stored at -80°C. Plasma concentration of hsCRP was measured by a high-sensitivity assay (N Latex Mono test) on a Behring BN II nephelometer with polystyrene microbeads coated with mono-clonal mouse antibodies. The detection limit of the assay was 0.2 mg/L. Plasma concentrations of interleukin-1α and interleukin-6 were determined with a commercially available immunoassay (Beckman, Villepinte, France). Observers were blinded to the source of samples for technical analysis.
Results

Data analysis
Quantitative data were expressed as the means ± standard deviation (SD) or median, with 10th and 90th percentiles according to the normality of the values. Qualitative data are expressed as number and percentage. Normality and variance homogeneity analyses were tested before quantitative data analysis (ANOVA and Student t-test for between-group comparisons and correlation tests for the relationship between quantitative variables). When data did not follow a normal distribution, nonparametric statistical methods were performed: Mann–Whitney test for comparisons between groups, and Spearman rank correlation test for the relationship between quantitative variables. P values < 0.05 were considered significant.

3.2.2.2 Study results
Clinical and biological characteristics
The demographic, clinical, and biological characteristics of the subjects are listed in Table 8. Among the 43 patients with systemic sclerosis, 1 patient was treated with methotrexate, 2 with cyclophosphamide, 2 with hydroxychloroquine, one with iloprost, and 2 with azathioprine. 16 patients in the SSc group were taking calcium channel blockers and 5 were taking buflomedil, a vasodilator. Both calcium channel blockers and buflomedil were stopped 7 days before enrollment in the study.
### Results

Table 8: Demographic, clinical, and biological characteristics of systemic sclerosis patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n = 25)</th>
<th>Systemic sclerosis (n= 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years</td>
<td>51 (10)</td>
<td>52 (11)</td>
</tr>
<tr>
<td>Female</td>
<td>22 (88%)</td>
<td>39 (90%)</td>
</tr>
<tr>
<td>Raynaud's phenomenon</td>
<td>0 (0%)</td>
<td>43 (100%)</td>
</tr>
<tr>
<td>Median number of fingers involved</td>
<td>n.a.</td>
<td>10 (8–10)</td>
</tr>
<tr>
<td>Thumb involved</td>
<td>n.a.</td>
<td>33 (77%)</td>
</tr>
<tr>
<td>Feet involved</td>
<td>n.a.</td>
<td>33 (77%)</td>
</tr>
<tr>
<td>Median SSc disease duration, years</td>
<td>n.a.</td>
<td>5 (1–16)</td>
</tr>
<tr>
<td>Digital pitting scars</td>
<td>0 (0%)</td>
<td>23 (53%)</td>
</tr>
<tr>
<td>Sclerodactyly</td>
<td>0 (0%)</td>
<td>34 (79%)</td>
</tr>
<tr>
<td>Median Rodnan modified skin score</td>
<td>n.a.</td>
<td>6 (0–23)</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>0 (0%)</td>
<td>11 (26%)</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension</td>
<td>0 (0%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Esophageal dysmotility</td>
<td>0 (0%)</td>
<td>23 (53%)</td>
</tr>
<tr>
<td>Mean creatinine clearance (mL/min)</td>
<td>88 (22)</td>
<td>89 (25)</td>
</tr>
<tr>
<td>Mean microalbuminuria (mg/L)</td>
<td>15 (11)</td>
<td>16 (17)</td>
</tr>
<tr>
<td>Mean cardiac rate (beat/min)</td>
<td>62 (10)</td>
<td>70 (13)</td>
</tr>
<tr>
<td>Mean systolic/diastolic blood pressure (mm Hg)</td>
<td>114 (13)/ 67(8)</td>
<td>118 (19)/ 68(11)</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-centromere</td>
<td>0 (0%)</td>
<td>18 (42%)</td>
</tr>
<tr>
<td>Anti-topoisomerase I</td>
<td>0 (0%)</td>
<td>11 (26%)</td>
</tr>
<tr>
<td>Mean plasma LDL cholesterol (mg/dL)</td>
<td>112 (30)</td>
<td>112 (30)</td>
</tr>
<tr>
<td>Mean plasma glycemia (mmol/L)</td>
<td>4.6 (0.6)</td>
<td>4.6 (0.5)</td>
</tr>
</tbody>
</table>

Data for normality and variance homogeneity are expressed using the mean (SD). In other cases, they are expressed as the median (10th–90th percentiles). NA: not applicable.
Results

Comparison of the postocclusive hyperemia, lipid peroxidation, and inflammatory parameters

There was a nonsignificant trend toward a higher baseline cutaneous vascular conductance in healthy volunteers (15 mV/mm Hg, 2–46) compared with patients with systemic sclerosis (8.2 mV/mm Hg, 1–26). The postocclusive hyperemia was altered in subjects with systemic sclerosis compared with controls (Table 9). When data were expressed as an increase over baseline, the median increase was lower in the systemic sclerosis group (Table 9). Whereas 8-iso-PGF$_{2\alpha}$ levels were increased in the systemic sclerosis group compared with healthy controls, no significant increase was found for the systemic parameters of inflammation (Table 9).

Table 9: Microvascular function, lipid peroxidation, and inflammatory biomarkers in patients with systemic sclerosis and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n = 25)</th>
<th>Systemic sclerosis (n = 43)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median digital pad temperature (°C)</td>
<td>33 (24–35)</td>
<td>28 (24–33)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Postocclusive response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median peak cutaneous vascular conductance (mV/mm Hg)</td>
<td>39.9 (13–63)</td>
<td>28 (7–48)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Median increase vs. baseline (mV/mm Hg)</td>
<td>16.3 (5–36)</td>
<td>10 (5–31)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Median time to peak (s)</td>
<td>32 (16–79)</td>
<td>77 (19–208)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Response to 0.4 mg sublingual nitroglycerin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median cutaneous vascular conductance increase over baseline (mV/mm Hg)</td>
<td>2 (0.1–7.7)*</td>
<td>2.3 (–0.6–12)*</td>
<td>n.s.</td>
</tr>
<tr>
<td>Median 8-iso-PGF$_{2\alpha}$ urinary excretion (pg/mg creatinine)</td>
<td>207 (109–291)</td>
<td>230 (155–387)</td>
<td>0.048</td>
</tr>
<tr>
<td>Median hsCRP (mg/L)</td>
<td>1.1 (0.2–13)</td>
<td>1.1 (0.5–12)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Median interleukin-1α (ng/L)</td>
<td>10 (5–19.5)</td>
<td>13.5 (10–34)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Median interleukin-6 (ng/L)</td>
<td>17 (10–31)</td>
<td>20 (10–45)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Laser Doppler probes were placed on the left middle finger pad. Data are expressed using the median (10th–90th percentiles).

n.s.: not significant

* Sublingual nitroglycerin induced a moderate but significant increase in baseline cutaneous vascular conductance in all groups, Wilcoxon rank tests: p < 0.001 in healthy controls and systemic sclerosis.

There was a significant inverse correlation between isoprostanes and postocclusive hyperemia in systemic sclerosis patients expressed as raw data (p = 0.007) or as an increase over baseline (p = 0.04, Figure 26). Conversely, no correlation was found with the nitroglycerin response or the basal flux. No correlation was observed between the inflammatory biomarkers and the postocclusive response. In healthy controls, 8-iso-PGF$_{2\alpha}$ levels did not correlate with the
Results

postocclusive hyperemia. Furthermore, 8-iso-PGF$_2$α levels did not correlate with any biomarker of inflammation in both groups.

Figure 26: Correlation of 8-iso-PGF$_2$α urinary levels with (A) peak postocclusive hyperemia vascular conductance, (B) postocclusive response (peak postocclusive hyperemia minus baseline), and (C) response to 0.4 mg sublingual nitroglycerin (cutaneous vascular conductance 4 min after the nitroglycerin challenge minus baseline) in patients with systemic sclerosis.

Laser Doppler probes were placed on the left middle finger pad.

3.2.3 The VIVALDI study

This study was performed in collaboration with Boehringer-Ingelheim. It was designed as a prospective, multicenter, randomized, double-blind, double-dummy, parallel group trial to investigate the efficacy of telmisartan 80 mg versus valsartan 160 mg in hypertensive type 2 diabetic patients with overt nephropathy. The objective of the study was to compare the effect of
Results

telmisartan with that of valsartan on 24-hour proteinuria, blood pressure and oxidative stress after one year’s treatment in hypertensive patients with type 2 diabetes and overt nephropathy.

3.2.3.1 Study description

Study population
Inclusion criteria were age between 30 and 80 years, a clinical history of type 2 diabetes (glycosylated hemoglobin [HbA1c] > 10%), nephropathy (serum creatinine < 265 μmol/L or 3.0 mg/dL and proteinuria > 900mg/24h), hypertension (mean cuff systolic/diastolic blood pressure (SBP/DBP) > 130/80 mmHg or antihypertensive treatment). Premenopausal women who were not surgically sterile or using contraception, pregnant and nursing women were not eligible. Other exclusion criteria were a recent acute cardiovascular event, congestive heart failure, metformin treatment in patients with elevated serum creatinine levels, >30% increase in serum creatinine during run-in, secondary hypertension, hepatic dysfunction, biliary obstructive disorders, renal arterial stenosis, chronic immunosuppressive therapy, a history of drug or alcohol dependency, and SBP > 180 mmHg and/or DBP > 110 mm Hg on two consecutive visits during run-in. All patients provided written informed consent.

Study design
Randomization was preceded with a 2-week screening period and a further 2-week placebo run-in period to wash out any previous treatment with angiotensin converting-enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs). Participants were thereafter randomized to telmisartan 80 mg or valsartan 160 mg titrated after two weeks from 40 and 80 mg respectively. Patients were requested to take the study medication with water in the morning at approximately the same time every day. If the goal blood pressure of 130/80 mm Hg could not be reached with the study medication, additional antihypertensive therapy could be initiated at any time during the study, starting two weeks after randomization.

Statistical analysis
Primary analysis was based on log-transformed urinary protein excretion rate data and treatment effects were compared using ANOVA including terms for treatment and center as main effects, and baseline urinary protein excretion rate as a covariate, to establish non-inferiority. Using an estimate for the standard deviation of the change from baseline in urinary protein excretion rate
being as much as 2 g/24 h (Lewis et al. 2001), a sample size of 340 patients per treatment group would have a power of 0.9 at the 5% (2-sided) level of significance to demonstrate non-inferiority of telmisartan compared with valsartan.

3.2.3.2 Results

Participants characteristics

Participants were recruited from 128 centers in 11 countries in Europe, 3 in Asia and in South Africa. A total of 1372 patients were enrolled, 885 of which were randomized. The two treatment groups were similar (Table 10).

Table 10: Characteristics of the study participants at baseline

<table>
<thead>
<tr>
<th></th>
<th>Temisartan (n=443)</th>
<th>Valsartan (n=442)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, %</td>
<td>63.0</td>
<td>65.2</td>
</tr>
<tr>
<td>Age, years</td>
<td>60.9 ± 9.2</td>
<td>61.4 ± 9.1</td>
</tr>
<tr>
<td>Ethnic origin, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>20.1</td>
<td>18.1</td>
</tr>
<tr>
<td>Black</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>White</td>
<td>77.9</td>
<td>80.3</td>
</tr>
<tr>
<td>Glycosylated hemoglobin, %</td>
<td>7.8 ±1.4</td>
<td>7.7 ± 1.3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>30.0 ± 5.2</td>
<td>30.4 ± 5.6</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>18.1</td>
<td>18.3</td>
</tr>
<tr>
<td>Duration hypertension, years</td>
<td>11.1 ± 9.5</td>
<td>11.6 ± 9.7</td>
</tr>
<tr>
<td>Duration type 2 diabetes, years</td>
<td>13.9 ± 8.1</td>
<td>14.4 ± 8.4</td>
</tr>
<tr>
<td>Duration diabetic nephropathy, years</td>
<td>2.7 ± 3.4</td>
<td>2.8 ± 3.5</td>
</tr>
<tr>
<td>Concomitant antihypertensive medication, %</td>
<td>59.6</td>
<td>63.1</td>
</tr>
<tr>
<td>Diuretic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretic + beta-blocking agent</td>
<td>3.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Beta-blocking agent</td>
<td>36.8</td>
<td>37.6</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>71.1</td>
<td>71.0</td>
</tr>
<tr>
<td>Calcium channel blocker + beta-blocking agent</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Other antihypertensive</td>
<td>35.2</td>
<td>39.1</td>
</tr>
<tr>
<td>Other concomitant medication, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>45.1</td>
<td>44.6</td>
</tr>
<tr>
<td>Other lipid-lowering agents</td>
<td>8.4</td>
<td>10.9</td>
</tr>
<tr>
<td>Oral anti-diabetic agents</td>
<td>58.2</td>
<td>57.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>58.7</td>
<td>56.8</td>
</tr>
<tr>
<td>Other drugs</td>
<td>81.3</td>
<td>84.8</td>
</tr>
</tbody>
</table>

Unless otherwise specified, data are mean ± SD
Results

Endpoints

24-h urinary protein excretion rate (95% CI) was reduced by a mean of 33% (27%-39%) of baseline with telmisartan and 33% (27%-38%) with valsartan, demonstrating non-inferiority of telmisartan (P=0.849). Changes from baseline in secondary end points are summarized in Table 11. Reduction in urinary albumin excretion was similar to that of urinary protein and corresponded to reductions of 39% with telmisartan and 36% with valsartan. No difference between treatments was noted for changes from baseline in serum creatinine. However, a significant difference in creatinine clearance was noted in favor of valsartan (P=0.001). There was no significant difference between treatments in changes in glomerular filtrate rates (GFR). Urinary excretion of 8-iso-PGF$_{2\alpha}$ decreased by 14% (P<0.01) with telmisartan and by 7% (P<0.01) with valsartan (P=0.04 between treatments). During treatment, SBP and DBP were reduced in both treatment groups with no significant differences between telmisartan and valsartan. Despite the use of additional antihypertensive therapy during the study, 55.6% of telmisartan-treated patients and 57.4% of those receiving valsartan had a SBP $\geq$140 mm Hg and/or a DBP $\geq$90 mm Hg at the end of the study. Optimal blood pressure (<120/80 mm Hg) was achieved in 5.2% of telmisartan-treated patients and 6.4% of valsartan-treated patients.

Table 11: Biochemical parameters at endpoint compared with baseline

<table>
<thead>
<tr>
<th></th>
<th>Telmisartan</th>
<th>Valgsartan</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Endpoint</td>
<td>Baseline</td>
</tr>
<tr>
<td>Urinary protein excretion (g/24h)</td>
<td>3.6 (3.28)</td>
<td>2.89 (3.05)</td>
<td>3.56 (2.86)</td>
</tr>
<tr>
<td>Urinary albumin excretion (g/24h)</td>
<td>1.65 (2.17)</td>
<td>0.95 (1.94)</td>
<td>1.76 (2.06)</td>
</tr>
<tr>
<td>GFR (mL/min/1.73m$^2$)</td>
<td>56.7 (26.3)</td>
<td>50.88 (30.0)</td>
<td>56.5 (25.4)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.36 (42.5)</td>
<td>1.50</td>
<td>1.37 (42.3)</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min/1.73m$^2$)</td>
<td>57.2 (36.0)</td>
<td>45.8 (33.5)</td>
<td>58.5 (35.7)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>148 (16)</td>
<td>142 (18)</td>
<td>149 (15)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>82 (10)</td>
<td>79 (10)</td>
<td>82 (10)</td>
</tr>
<tr>
<td>8-iso-PGF$_{2\alpha}$ urinary excretion (ng/h)</td>
<td>11.3 (7.9)</td>
<td>9.9 (7.0)</td>
<td>12.6 (9.7)</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>6.28 (10.23)</td>
<td>7.03 (11.02)</td>
<td>6.15 (9.10)</td>
</tr>
<tr>
<td>Glycosylated hemoglobin (%)</td>
<td>7.8 (1.4)</td>
<td>7.8 (1.5)</td>
<td>7.7 (1.3)</td>
</tr>
</tbody>
</table>

Data are mean (SD)

* difference between treatments
3.3 The isoprostanes as mediators of the oxidative stress

3.3.1 Haemochomatosis (HFE) rat model

This study was conducted in collaboration with the Department of Biochemistry and Molecular Biology II of the University Medical Center Hamburg-Eppendorf, Germany. The goal of this study was to investigate if iron overload-induced oxidative stress is associated with altered endothelial function, using an established HFE animal model (Nielsen and Heinrich 1993, Nielsen et al.1993). In this model, rats are fed with a diet enriched with 3,5,5-trimethylhexanoyl ferrocene (TMH-ferrocene), a chemically stable, nonionic, lipophilic iron donor that readily diffuses into hepatocytes, where a cytochrome P450-dependent reaction metabolizes the molecule, releasing iron into the hepatocyte (Cable and Isom 1999). TMH-ferrocene does not catalyze oxidation reactions (Bilello et al. 2003).

3.3.1.1 Study description

Female Wistar rats were fed a control or iron-enriched diet for 10 weeks (n=8 each group). At the end of this period, urine samples were collected. Prior to sacrifice, rats were fasted overnight. On sacrifice day, they were weighed and anaesthetized with ether. The abdomen and thorax were cut open, heparin was injected in the cardiac apex, blood samples were collected and the animals were exsanguinated. The thoracic aorta was excised and immediately cleaned and used for organ bath according to 2.7. The liver was removed and frozen at –20 degrees until analysis. Endothelium-dependent and -independent relaxation of isolated aortic rings was measured in organ bath experiments. Urine samples were collected in 4-hydroxy-TEMPO- or EDTA-containing recipients and stored at –80 degrees until analysis. 8-iso-PGF$_2\alpha$ urine excretion was quantified according to 2.1. The determination of liver iron content (LIC) was performed in the Department of Biochemistry and Molecular Biology II of the University Medical Center Hamburg-Eppendorf, Germany.

3.3.1.2 Study results

The groups were significantly different with regard to weight, animals under normal diet being heavier (mean 308 g, SD 19 vs. 239±15 g, P<0.05, Figure 27A). Conversely, animals under iron-enriched diet exhibited liver hypertrophy (liver weight 20.7±2.1 g vs. 9.1±1.6 g, P<0.001, Figure
Results

27B), which translated in a significantly higher liver to total body weight ratio (8.7%±0.4 vs. 3.0%±0.4, P<0.001, Figure 27C).

![Figure 27: Difference in A) body weight, B) liver weight and C) liver to body weight ratio under control diet and iron-enriched diet](image)

Moreover, feeding a diet enriched with TMH-ferrocene for 10 weeks resulted in a very severe liver siderosis in the HFE-group compared with controls (0.05±0.02 mg iron/g body weight vs. 8.88±0.81 mg iron/g body weight, P<0.01, Figure 28A). Furthermore, 8-iso-PGF$_{2\alpha}$ urinary excretion was significantly increased in HFE in comparison with control (median 2755 pg/mg creatinine, IQR [1605-5384] vs. 698 pg/mg creatinine, [344-938], P<0.01, Figure 28B).
Results

Figure 28: A) Liver iron content and B) urinary excretion of 8-iso-PGF$_{2\alpha}$ under control and iron-enriched diet

The endothelium-dependent and -independent relaxation responses were substantially altered in HFE vs. control group. This effect was concentration-dependent. The HFE group exhibited a submaximal relaxation to ACh representing 55.4% of that of the control group, which was evoked by a lift of the curve (Figure 29A). Although the maximal NTP-induced relaxation was unaffected in the HFE group, the response curve to NTP was shifted to the right (Figure 29B) and the EC$_{50}$ of NTP increased from 0.022 µM in the control group to 0.073 µM in the HFE group.

Figure 29: Endothelium-dependent (A) and -independent (B) relaxation to contraction induced with 0.5 µM PE
3.3.2 Platelet aggregation

Isoprostanes did not induce irreversible aggregation in concentrations up to 50 µM (Figure 30).

Figure 30: Effect of increasing concentrations of U46619, 8-iso-PGF$_2α$ and 8-iso-PGE$_2$ on platelets (n=6)

Isoprostanes inhibited the aggregation induced by the thromboxane receptor agonist U46619 in a concentration-dependent manner (Figure 31). The inhibition was significant at concentrations 10 µM and higher (P < 0.001).
Figure 31: Effect of pre-incubation with 8-iso-PGE\(_2\) and 8-iso-PGF\(_{2\alpha}\) on the aggregation induced by a threshold concentration of U46619 (1µM) (n=6)

3.3.3 Tube formation assay
The thromboxane receptor agonist U46619 significantly inhibits the tube formation, so did the isoprostanes 8-iso-PGF\(_{2\alpha}\) and 8-iso-PGE\(_2\), at a concentration of 30 µM. The tube formation was completely restored when U46619 and 8-iso-PGE\(_2\) were incubated with equimolar concentrations of the thromboxane receptor antagonist SQ29548. SQ29548 could only partially reverse the 8-iso-PGF\(_{2\alpha}\)-induced tube formation inhibition (Figure 32).
Results

Figure 32: Effect of the TXAR agonist U46619 and of the isoprostanes 8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$ on VEGF-induced tube formation in HCAECs (n=8)

A 10-fold lower concentration of 8-iso-PGF$_{2\alpha}$ induced an equally lower inhibition of the tube formation (Figure 33).

Figure 33: Effect of various concentrations of 8-iso-PGF$_{2\alpha}$ on tube formation by HCAECs
Results

3.3.4 Identification of the TXAR subtype(s) present in HCAECs
PCR was performed as described in section 2.3 to investigate the presence of the mRNA the TXAR isoforms in different cell lines. mRNA of both TXAR isoforms were present in HCAECs (Figure 34).

![Expression of A) the TXAR-α and the B) TXAR-β mRNA in HCAEC (lane 1), negative control (aqua ad injectabilia) (lane 2)](image)

Figure 34: Expression of A) the TXAR-α and the B) TXAR-β mRNA in HCAEC (lane 1), negative control (aqua ad injectabilia) (lane 2)

3.4 The role of phospholipases A₂ in the release of the isoprostanes

3.4.1 *In vitro* data
When investigating the role of sPLA₂s in the release of 8-iso-PGF₂α in HCAECs *in vitro* as described in section 2.2.1, the mean production rate of 8-iso-PGF₂α in vehicle was $7.54 \pm 1.14$ pg 8-iso-PGF₂α/mg protein/hour. The production of 8-iso-PGF₂α in HCAECs was significantly increased in the presence of the bee venom group III sPLA₂ and conversely inhibited under incubation with the unspecific sPLA₂ inhibitor mepacrine (Figure 35).
Results

![Graph showing 8-iso-PGF$_{2\alpha}$ production rate](image)

Figure 35: Effect of the PLA$_2$ inhibitor mepacrine on the release of 8-iso-PGF$_{2\alpha}$ in HCAECs (n=12)

Western blotting according to 2.4 revealed that HCAECs express both group IIA and group V sPLA$_2$s (Figure 36).

![Western blot images](image)

Figure 36: Expression of A) group IIA and B) group V sPLA$_2$s in HCAECs

3.4.2 The atorvastatin (ATV) study

We investigated if a statin treatment (atorvastatin 40 mg/day for 6 weeks) in hypercholesterolemic patients naïve to lipid-lowering therapy would lead to a lowering in the
Results

PAF-AH and/or PLA2s activity and to 8-iso-PGF2α, thereby hinting to which enzyme(s) is/are involved in the release of the latter.

3.4.2.1 Study description

Participants

24 participants aged between 35 and 60 years old were included in this study. Hypercholesterolemia was defined as LDL cholesterol levels ≥ 160 mg/dL (4.2 mmol/L). All hypercholesterolemic participants were naïve to statins or other lipid-lowering medications. Exclusion criteria were: history of alcoholism or drug abuse; pregnancy or breastfeeding status; liver disease or liver insufficiency (serum AST or ALT >1.5-fold above the upper limit of the normal range, 10-35 U/L for women, 10-50 U/L for men); advanced kidney disease (creatinine clearance < 30 mL/min), nephrotic syndrome or dysproteinemia; diabetes mellitus. We assessed eligibility and obtained written informed consent as stipulated in the study protocol approved by the local Review Board for Studies in Humans.

All participants were invited to the study centre in the morning of day one and a 24 hour-urine sample collection was started. Urine was collected in a container prepared with 4-hydroxy-TEMPO and EDTA as antioxidants. 24 hours later, patients returned to the study centre in the morning. A fasting blood sample was drawn, blood samples were centrifuged (2000g, 20 min, 4°C) immediately, and plasma was divided into aliquots and stored at -20°C until analysis. Collected urine was retreated and urine samples were divided into aliquots and kept frozen at -20°C until analysis. Participants were given the study medication or the placebo in a neutral packaging, instructed about the intake scheme and dismissed. Two weeks later, they returned to the study centre to have their biochemical parameters controlled in order to detect any intolerance reaction. On day 42 of the study, participants returned to the study centre and underwent an investigation identical to the one on the first day.

Biochemical Analyses

Urinary concentration of 8-iso-PGF2α was determined by GC-MS as described in section 2.1. Activities of plasma group IIA and V PLA2s and PAF-AH were assessed using commercially
available assay kits (Cayman Chemicals), following sample concentration with Amicon Ultra Centrifugation Filter Devices. hsCRP was measured on a Dade Behring BN II nephelometer with polystyrene microbeads coated with monoclonal mouse antibodies (Ledue et al. 1998). Plasma total cholesterol, LDL and HDL levels as well as plasma and urinary creatinine concentrations were determined by standard laboratory methods using certified assays in the local clinical laboratory.

Calculations and Statistical Methods

All data were tested for normal distribution with the Shapiro-Wilk test. The distribution of 8-iso-PGF$_{2\alpha}$ and hsCRP was skewed, as reported previously (Schwedhelm et al. 2004, Keaney et al. 2003, Koenig et al. 1999). Differences between groups are given as mean (standard deviation) except for not normally distributed parameters (median and interquartile range). Comparisons between study end and baseline involving not normally distributed parameters were performed with the Wilcoxon test. All other comparisons were performed by Student t-test. Correlation coefficients are Pearson’s. A p < 0.05 was accepted for statistical significance. For statistical analyses, SPSS version 13.0 was used.

3.4.2.2 Study results

Participants’ characteristics at baseline are presented in Table 12. There were two smokers in the placebo group and none in the treatment group.

At the end of the study, none of the biochemical parameters (total, HDL- and LDL- cholesterol plasma levels (Figure 37A), 8-iso-PGF$_{2\alpha}$ urinary excretion (Figure 37D), PAF-AH (Figure 37B) and PLA$_{2\alpha}$’ activity (Figure 37C), hsCRP) was modified in the placebo group (Table 12). The mean change in 8-iso-PGF$_{2\alpha}$ urinary excretion was +0.69 ng/h (95% confidence interval –0.52 to +1.90 ng/h).
### Results

Table 12: Participants’ characteristics at baseline and study end

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th></th>
<th>P</th>
<th>ATV</th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline</td>
<td>study end</td>
<td>vs. baseline</td>
<td>baseline</td>
<td>study end</td>
<td>vs. baseline</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td>-</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td>-</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>58.3 (5.0)</td>
<td>58.3 (5.0)</td>
<td>-</td>
<td>58.9 (7.3)</td>
<td>58.9 (7.3)</td>
<td>-</td>
</tr>
<tr>
<td>BMI kg/m² (SD)</td>
<td>25.3 (1.8)</td>
<td>25.3 (1.8)</td>
<td>-</td>
<td>24.2 (4.1)</td>
<td>24.2 (4.1)</td>
<td>-</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>284 (30)</td>
<td>289 (35)</td>
<td>0.844</td>
<td>320 (61)</td>
<td>182 (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mg/dL (SD)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>LDL</td>
<td>202 (21)</td>
<td>206 (34)</td>
<td>0.469</td>
<td>231 (54)</td>
<td>103 (31)</td>
<td>&lt;0.001</td>
</tr>
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<td>mg/dL (SD)</td>
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<td></td>
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</tr>
<tr>
<td>HDL</td>
<td>50.6 (13.1)</td>
<td>51.4 (11.5)</td>
<td>0.625</td>
<td>59.1 (11.6)</td>
<td>62.3 (11.8)</td>
<td>0.101</td>
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<td>mg/dL (SD)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-iso-PGF₂α</td>
<td>8.0 (6.4-11.1)</td>
<td>8.9 (7.4-12.3)</td>
<td>0.721</td>
<td>9.8 (6.6-12.5)</td>
<td>8.4 (6.9-13.3)</td>
<td>0.875</td>
</tr>
<tr>
<td>urinary excretion ng/h (IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLA₂S’ activity</td>
<td>4.35 (0.61)</td>
<td>5.25 (2.06)</td>
<td>0.172</td>
<td>4.12 (1.14)</td>
<td>4.45 (0.83)</td>
<td>0.247</td>
</tr>
<tr>
<td>nmol/min/mL (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAF-AH activity</td>
<td>16.7 (2.0)</td>
<td>17.3 (1.5)</td>
<td>0.196</td>
<td>16.1 (2.8)</td>
<td>10.9 (2.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>nmol/min/mL (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP mg/L (IQR)</td>
<td>1.50 (0.70-2.37)</td>
<td>1.60 (0.70-2.00)</td>
<td>0.507</td>
<td>1.35 (0.83-1.80)</td>
<td>0.85 (0.70-2.17)</td>
<td>0.553</td>
</tr>
</tbody>
</table>

P: placebo vs. ATV at baseline
Results

Figure 37: Change in A) LDL-cholesterol plasma levels, B), PAF-AH and C) PLA2s’ activity, and D) 8-iso-PGF2α urinary excretion in the placebo and atorvastatin groups

In the atorvastatin group, a non-significant reduction in 8-iso-PGF2α urinary excretion was observed, whereas the PLA2s’ activity remained virtually unaffected. Total and LDL-cholesterol were significantly lowered, which was paralleled by a decrease of PAF-AH activity in all patients with active treatment (Figure 38).
Results

ATV: atorvastatin
n.s.: not significant

Figure 38: Individual change in 1A) LDL-cholesterol plasma levels and 1B) PAF-AH activity in the ATV-treated group

The change in PAF-AH activity was correlated with the change in LDL-cholesterol levels ($r=0.574$, $P=0.03$) (Figure 39A) and with that in total cholesterol ($r=0.562$, $P=0.03$) but not with the change in 8-iso-PGF$_{2\alpha}$ urinary excretion (Figure 39B).
Results

Figure 39: Correlation between the change in PAF-AH activity and the change in A) LDL-cholesterol levels and B) 8-iso-PGF$_{2\alpha}$ urinary excretion, in participants treated with atorvastatin.

The mean change in 8-iso-PGF$_{2\alpha}$ urinary excretion was +0.21 ng/h (95% CI –0.92 to +1.35 ng/h). No intolerance complaint was recorded in this group, although the liver enzyme ALT was slightly increased (43.5 U/L, SD [15.6] vs. 32.6 U/L, SD [12.6], P=0.004). The creatine kinase (155.7 U/L [97.5] vs. 175.2 U/L [185.3] at baseline, P=0.659) and the AST (33.8 U/L, SD [9.6] vs. 30.1 U/L, SD [9.6] at baseline, P=0.147) were not modified, nor was the inflammatory marker hsCRP (0.85 mg/L, IQR [0.70-2.17] vs. 1.35 mg/L, IQR [0.83-1.80] at baseline, P=0.553).
4 Discussion

4.1 Review of the analytics of 8-iso-PGF$_{2\alpha}$

After purification by SPE, it was possible to detect both 5- and 15-F$_2$-IsoP. However, these substances, whether deuterated or not, were detected not only at the same m/z ratios (Figure 17), but also at the same retention time (Figure 18). Hence, purification of a single given sample by SPE would not allow efficient resolution of 15-F$_2$-IsoP from 5-F$_2$-IsoP and possibly from other 8- and 12-F$_2$-isoprostanes by GC-MS in that sample. Much more, 5- and 15-F$_2$-IsoP would appear as a single peak. This is in accordance with the methods published by Lee et al. (2004) and Proudfoot et al. (1999) reporting total amounts of F$_2$-isoprostanes after purification by SPE. In at least one of these methods (Proudfoot et al. 1999), a column with similar polarity was used for chromatographical separation, namely an Agilent DB-5MS. Moreover, depending on the extraction method, e.g. with different SPE columns, it is not certain that the denomination "total F$_2$-isoprostanes" refers to identical mixtures of stereoisomers. Selective extraction of 8-iso-PGF$_{2\alpha}$ by means of IAC followed by quantification by GC-MS also allows its detection at the already mentioned m/z ratios and retention time (Figure 19), but guarantees the identity of the quantified product. Thus, the selective extraction of 8-iso-PGF$_{2\alpha}$ appears to be the most appropriate method to allow inter-laboratory comparison.

In practice, values yielded by IAC-GC-MS were slightly but consistently higher than those yielded by GC-MS-MS. Thus, GC-MS and GC-tandem MS following IAC purification are interchangeable, with a reference range higher for GC-MS than for GC-tandem MS. In contrast, the selective extraction of 8-iso-PGF$_{2\alpha}$ by EIA rather poorly correlates with IAC-GC-MS and yields the most spread apart agreement limits. The agreement limits were calculated from a modified Bland-Altman plot using a percent y scale, as recommended in cases when the standard deviation increases with concentration and/or with a proportional difference (Pollock et al. 1992). Moreover, EIA appears to overestimate samples in the lower and medium range, supposedly due to cross-reactivity with other products such as other isoprostanes or even prostaglandins, and underestimates those in the higher scale, maybe because of a not large enough range of detection. This finding is in line with a previous report from Proudfoot et al. (1999). In a clinical setting, such bias would lead to a high rate of false positive and negative results, yielding a poor predictive value. Thus, this method is not advisable. Expectedly, the non-specific SPE-HPLC-
Discussion

GC-MS method yielded much higher results than the specific ones. However, since it does not assess the same parameter as the latter, it is neither comparable nor interchangeable with IAC-GC-MS or IAC-GC-tandem MS. However, its good correlation with IAC-GC-MS is a further indication that 8-iso-PGF$_2\alpha$ alone is a reliable and representative index of the total amount of isoprostanes and thus, of oxidative stress.

In summary, the method used for assessment of oxidative stress should be taken into account when reviewing the literature. Based on our data, specific quantification of 8-iso-PGF$_2\alpha$ by chromatographic methods appears the most advisable. Nevertheless, the establishment of this method as routine means of assessment of oxidative stress in the medical practice not only demands a consensus in the scientific community, but also wide access to the necessary equipment and the determination of reference values.

4.2 Assessment of 8-iso-PGF$_2\alpha$ as marker of oxidative stress

4.2.1 8-iso-PGF$_2\alpha$ as marker of increased oxidative stress in a pathological state: the VASSc study

Results indicate that there is an inverse correlation between 8-iso-PGF$_2\alpha$ levels in urine and postocclusive hyperemia in patients suffering from systemic sclerosis. In contrast, there was no link between 8-iso-PGF$_2\alpha$ and the endothelial-independent nitroglycerin response. This suggests that microvascular dysfunction is linked to the oxidative stress generation in patients with systemic sclerosis.

Since 8-iso-PGF$_2\alpha$ is an independent risk marker for coronary heart disease while also correlating to classical cardiovascular risk factors (Schwedhelm et al. 2004), subjects with a history of coronary heart disease and those with potential confounding factors such as diabetes, smoking, and/or hypercholesterolemia were excluded from the present study. 8-iso-PGF$_2\alpha$ urinary levels were elevated in the systemic sclerosis population compared with healthy controls. The repeatability of the results in different patient groups using different methods further supports the fact that oxidative stress biomarkers are increased in patients with systemic sclerosis, irrespective of their clinical classification. Indeed, quantification of 8-iso-PGF$_2\alpha$ with GC-MS with electronic impact (Bessard et al. 2001) in previous studies (Cracowski et al. 2001b, Cracowski et al. 2002, Cracowski et al. 2005), a more specific but less sensitive method, revealed exactly the same
pattern. No correlation was found between 8-iso-PGF$_{2\alpha}$ urinary levels and inflammatory biomarkers, unlike what was previously described in Crohn disease (Cracowski et al. 2002). However, plasma CRP was elevated in patients with Crohn disease compared with controls, whereas this was not the case in the SSc group. Therefore, 8-iso-PGF$_{2\alpha}$ urinary levels seem to be a more sensitive biomarker than hsCRP in patients with SSc.

The correlation between 8-iso-PGF$_{2\alpha}$ levels and postocclusive hyperemia is driven by the four patients with the highest isoprostane levels. However, these four patients had a normal response to nitroglycerin. Together with the fact that our whole systemic sclerosis population had a normal response to nitroglycerin, this strongly suggests that the impairment of the postocclusive hyperemia is specific and related neither to a decreased capillary density nor to a nonspecific alteration of vascular smooth muscle cells. However, data reported here show a correlation between oxidative stress status and postocclusive hyperemia in systemic sclerosis, they did not provide evidence for a causal link.

Systemic sclerosis remains a complex disease, in which the interactions between vascular dysfunction, oxidative stress generation, leukocyte activation and matrix remodeling are poorly understood. Patients with SSc exhibit both vascular structure and functional abnormalities that are interdependent (Herrick 2000). There is strong evidence of a defect in the microvascular endothelial-dependent vasodilation in SSc (Herrick 2000). Laser Doppler flowmetry is a tool that allows the investigation of microvascular dysfunction in systemic sclerosis (Kahaleh et al. 2003). Although it has no proven interest in the clinical management of individual patients, postocclusive hyperemia has been shown to differ in patients with systemic sclerosis compared with healthy controls, which reflects an abnormal microvascular structure and function (Wigley et al. 1990). Postocclusive hyperemia is characterized by a sudden rise in blood flow after cuff release, which can be characterized by measuring the amplitude of the rise. The reproducibility of the time to peak hyperemia was not satisfactorily comparable to the peak hyperemic conductance. Therefore, the peak hyperemic conductance was used in the correlation analysis. This peak can then be corrected for baseline, but since baseline conductance is highly variable, this method of quantification is debatable (Bircher et al. 1994). This limitation is the reason both raw and corrected amplitude were reported. Laser Doppler flowmetry remains a semiquantitative approach to investigating skin blood flow (Carpentier 1999). Expression of data in terms of perfusion units does not take into consideration potential variations in blood pressure, which could alter microcirculatory conductance, with a specific relevance for the nitroglycerin challenge.
to account for potential variations in blood pressure that could alter microcirculatory conductance, data were expressed in terms of cutaneous vascular conductance rather than flux. This gives as a better physiological index to study vasodilation. Based on recent observations that local hyperemia induced by 44°C local heating of the skin was impaired in patients with systemic sclerosis (Boignard et al. 2005), data were preferentially expressed as a percentage of maximal vasodilation. As a consequence, the endothelium-independent relaxation induced by 0.4 mg sublingual nitroglycerin was used as a control. This minimizes the individual variability and provides support for endothelial versus structural (endothelium-independent) vasodilation.

4.2.2 8-iso-PGF$_2$α as evaluation tool of the effect of a non-pharmacological therapy on oxidative stress: the HFE study

There are as many studies that conclude to a deleterious role of iron (Tuomainen et al. 1999, Jiang et al. 2004, Piperno et al. 2002) as there are that show its innocuousness (Bozzini et al. 2002, Sempos et al. 1994, Liao et al. 1994). However, none of these studies was conducted exclusively in C282Y homozygote patients. Data reported here show for the first time significantly elevated urinary excretion of 8-iso-PGF$_2$α, in untreated patients with HFE-related hemochromatosis in comparison with controls with normal iron stores. Secondly, these data confirm that hemochromatosis in C282Y homozygotes is associated with lower levels of serum vitamin A.

This report shows that hemochromatosis is associated with oxidative stress, based on elevated 8-iso-PGF$_2$α urinary excretion measured by GC-MS. This elevated urinary excretion of 8-iso-PGF$_2$α could be reversed after normalization of the iron parameters through blood letting. 8-iso-PGF$_2$α urinary excretion is considered a highly reliable marker of oxidative stress (Vassalle et al. 2003, Schwedhelm et al. 2004) and GC-MS, one of the best methods to quantify it (Schwedhelm and Böger 2003, section 4.1). Other studies showed an association between iron overload and thiobarbituric acid-reactive substances, another marker of lipid peroxidation (Young et al. 1994, Gaenzer et al. 2002), and others found a link between 8-iso-PGF$_2$α and iron overload in other populations (Pulliam et al. 2003, Salahudeen et al. 2001, Yeoh-Ellerton and Stacey 2003). Oxidative stress might be responsible for the impaired endothelial function and increased intima thickness that have been associated with iron overload (Pulliam et al. 2003). Indeed, when deferoxamine, an iron chelator, was infused in patients with coronary artery disease without
regard to their iron status, results proved iron to have a deleterious action on endothelial function, possibly through inactivation of endothelium-derived NO (Duffy et al. 2001). Furthermore, iron has been hypothesized to catalyze a cascade of intracellular biochemical reactions that produces reactive oxidative species (Ong and Halliwell 2004). Accordingly, high iron availability is associated in vitro with increased intracellular oxidant species as well as with enhanced hydrogen peroxide-induced intracellular production of oxidant species (Breuer et al. 1997). Altogether, our data and these observations strengthen an association between iron and oxidative stress.

Moreover, the F$_2$-isoprostanes, which includes 8-iso-PGF$_2\alpha$, are increasingly pointed at not only as marker, but also as mediator of oxidative stress. Indeed, F$_2$-isoprostanes induce platelet activation (Patrono and FitzGerald 1997, section 4.3.1.1), which could lead to thrombotic disorders. They are vasoconstrictor in pulmonary artery, coronary arteries, cerebral arterioles, retinal vessel, and portal vein (Montuschi et al. 2004). 8-iso-PGF$_2\alpha$ promotes monocyte adhesion, a process that contributes to atherosclerosis (Leitinger et al. 2001). This implies that C282Y homozygote hemochromatosis patients are at risk of further diseases caused by 8-iso-PGF$_2\alpha$ and/or other isoprostanes.

Hypovitaminemia A is secondary to hemochromatosis. Although low levels of serum vitamin A have already been observed in hemochromatosis patients (Young et al. 1994, Brissot et al. 1978), the mechanism remains unknown. Vitamin A being involved in free radicals scavenging (Fang et al. 2002), its low levels in hemochromatosis patients could result from increased consumption secondary to increased oxidative stress, as reported here. Another hypothesis uttered in liver diseases is that of impaired vitamin A absorption due to reduced bile acid (Urayama et al. 1998). Another possibility yet is that of faulty mobilization of vitamin A from the liver (Nyberg et al. 1988). All these hypotheses are compatible with the restoration of serum vitamin A levels after treatment. Thus, the data confirm the association of hypovitaminemia A with iron overload, but does not point to a definite mechanism leading to this deficiency.

Hemochromatosis did not lead to lowered serum levels of vitamin E. Data regarding vitamin E levels in hemochromatosis is not unanimous: some have found lowered levels of vitamin E in all hemochromatosis patients (Young et al. 1994) whereas for others, a specific phenotype and biochemical presentation of the disease (high serum iron, low free iron binding capacity, high ferritin levels) rather than the hemochromatosis genotype alone seem to be the determining factors in the lowering in vitamin E (von Herbay et al. 1994). Regardless, the lack of a significant difference in vitamin E between the groups excludes a confounding role of the antioxidant status.
in the association we establish between increased lipid peroxidation and hemochromatosis. Phlebotomy treatment led to impaired vitamin E levels, possibly through a “leak” mechanism similar to that observed for cholesterol (Kumar 1994, Facchini and Saylor 2002). These and previous data (Kumar 1994, Facchini and Saylor 2002) account for a decrease in total and LDL cholesterol and in the LDL/ HDL ratio, an indicator of cardiovascular risk, secondary to blood-letting therapy. However, the present study was neither aimed nor designed to investigate the effect of hemochromatosis on lipid metabolism. In addition, this change in the patients' lipid profile could be due to other factors than hemochromatosis, such as lifestyle changes.

The main limitation of this study is the number of subjects. Because of the low prevalence of the studied genotype, larger cohort studies aiming at confirming the current results or looking to determine the predictive value of 8-iso-PGF$_{2\alpha}$ urinary excretion will have to be multicentered. It is worth noting that given the prevalence of the studied genotype, such case group represents a selection among a population of 4000 individuals. This is the largest study in HFE C282Y homozygote patients. Another limitation is that since the hemochromatosis genotype may remain unexpressed for years before biochemical parameters are modified and clinical symptoms appear (Adams 2000), it is impossible to know if the increase in 8-iso-PGF$_{2\alpha}$ urinary excretion is proportional not only to the magnitude but also to the duration of the iron overload. Indeed, the prognosis in hemochromatosis depends on both the amount and duration of iron excess (Wojcik et al. 2002). A further limitation is the inclusion of smokers, since smoking status is associated with oxidative stress (Morrow et al. 1995). However, given the prevalence of the studied genotype, it was not affordable to exclude these. The statistical analysis showed that the inclusion of smokers did not have a significant influence on the results.

The observed enhanced oxidative stress measured through 8-iso-PGF$_{2\alpha}$ urinary excretion and hypovitaminemia A in C282Y homozygote patients imply that these individuals are at risk of further diseases caused by 8-iso-PGF$_{2\alpha}$, as the isoprostanes are increasingly pointed at not only as markers but also as mediators of oxidative stress. Altogether, the normalization of 8-iso-PGF$_{2\alpha}$ urinary excretion described here and other studies showing a normalized survival rate in early treated hemochromatosis patients (Niederau et al. 1996, Milman et al. 2001, Strohmeyer et al. 1988) stress the need for awareness in physicians.
4.2.3 8-iso-PGF$_{2\alpha}$ as evaluation tool of the effect of pharmacological treatment on oxidative stress: the VIVALDI Study

Telmisartan and valsartan belong to the angiotensin II (Ang II) type 1 (AT$_1$) receptor blockers (ARBs), a class of medication indicated for the treatment of hypertension. Indeed, blockade of the AT$_1$ receptor allows the Ang II to bind to the AT$_2$ receptor, which mediates blood pressure lowering effects such as vasodilatation (Hernández-Hernández et al. 2002). In this study, telmisartan and valsartan are equally efficient in reducing blood pressure and nephropathy in type 2 diabetes patients. In contrast, although both medications significantly lowered oxidative stress assessed by 8-iso-PGF$_{2\alpha}$ urinary excretion, telmisartan was superior to valsartan in this regard.

Because of the significant difference in creatinine clearance and since 24-h urine was performed, the urinary excretion in 8-iso-PGF$_{2\alpha}$ was expressed in ng/h rather than in pg 8-iso-PGF$_{2\alpha}$/mg creatinine. The effect of telmisartan and valsartan on oxidative stress is presumably mediated by inhibition of ROS production. Indeed, through the AT$_1$, Ang II potently stimulates NAD(P)H oxidases (NOX) in various cell types, among others in cardiomyocytes, vascular smooth muscle cells (VSMC) and in endothelial cells (Ushio-Fukai and Alexander 2004). ROS produced following Ang II-mediated stimulation of NOX enzymes act as second messengers through signaling pathways such as mitogen-activated protein kinases, tyrosine kinases and transcription factors, and lead to events such as inflammation, hypertrophy, remodeling and angiogenesis (Cai et al. 2003). The physiological function of the NOX family is the generation of ROS. This family consists of 7 isoenzymes that share a common structure consisting of 6 transmembrane domains which include two heme-binding regions, and a cytoplasmic C-terminus which contains the NAD(P)H-binding regions (Krause 2004). The main studied NOX isoform is the phagocytic NOX2 which is composed of several subunits, namely the two catalytic units gp91phox, p22phox, and the cytosolic units p47phox, p67phox, the G protein Rac and p40phox (Li and Frei 2006). NOX2 is microbicidal, but it is also involved in several pathological processes, including the development of cardiovascular diseases (Ushio-Fukai and Alexander 2004). For example, studies in p47phox- and gp91phox-deficient mice show that ROS produced by this oxidase contribute to cardiovascular diseases including atherosclerosis and hypertension (Cai et al. 2003). Oscillatory shear stress-induced O$_2^\cdot$ production is inhibited in endothelial cells isolated from p47phox-deficient mice (Hwang et al. 2003). Similarly, administration of the experimental AT$_1$ receptor antagonist BAY 10-6734 in a rabbit hypercholesterolemic model of early atherosclerosis
Discussion

normalized $O_2^-$ production and endothelial function and reduced early atherosclerotic lesion formation (Warnholtz et al. 1999). Thus, inhibition of NOX could explain the effect of the ARBs telmisartan and valsartan on oxidative stress.

Considering that telmisartan and valsartan exert their effects through the same action mechanism, the superiority of telmisartan in lowering oxidative stress is presumably due to its pharmacokinetic and pharmacodynamic properties. Indeed, of all ARBs, valsartan exerts the lowest affinity to the AT$_1$ receptor and telmisartan the highest one, characterized as pseudo-irreversible because higher than that of the physiological agonist Ang II (Kakuta et al. 2005, Maillard et al. 2002). Besides, telmisartan has a half-life of 24 hours, compared with 6 hours for valsartan (Hernández-Hernández et al. 2002). Finally, telmisartan is highly lipophilic, a property that, although without consequences on its effect on blood pressure, could contribute to higher efficiency in reducing the expression of p22phox (Takai et al. 2005). These differences presumably justify the significantly higher reduction in oxidative stress, i.e. in 8-iso-PGF$_{2\alpha}$, observed under telmisartan.

4.3 Assessment of the isoprostane 8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$ as mediator of oxidative stress

4.3.1 Biological effects of the isoprostanes 8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$

4.3.1.1 Platelet aggregation

Platelet activation can be triggered by several stimuli, e.g. thrombin, ADP, adrenaline, collagen, PAF, TXA$_2$ or vasopressin. These stimuli induce the liberation of TXA$_2$ by platelet, leading to platelet aggregation. Platelet aggregation is an essential step of the coagulation process and thereby, of hemostasis (Gawaz 2001). Conditions such as hypercholesterolemia, hypertension, diabetes mellitus are associated with hyperaggregability of circulating blood platelets, favoring atherosclerosis and the onset of acute coronary syndrome or myocardial infarction (Chesebro and Fuster 1992, Fuster et al. 1992).

The isoprostanes 8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$ were not able to induce irreversible aggregation. At concentrations $\approx 10^{-5}$ M, they induce a slight increase in light transmission, corresponding to activation and reversible aggregation (Figure 30). In this regard, 8-iso-PGE$_2$ was more potent than 8-iso-PGF$_{2\alpha}$. These data are in line with those of Longmire et al. (1994) in PRP and Cranshaw et al. (2001) in whole blood. The aggregation induced by the TXAR agonist U46619 ($EC_{50}$ 82 nM in human platelets, (Tymkevycz et al. 1991)) was significantly inhibited by both isoprostanes (Figure 31), which is in accordance with the literature as well. Indeed, Longmire et
al. (1994) reported an inhibition by 8-iso-PGE\(_2\) of the irreversible aggregation induced by both TXAR agonists U46619 (1 \(\mu\)M) and IBOP (0.33 \(\mu\)M), at EC\(_{50}\) 0.5 \(\mu\)M and 5 \(\mu\)M respectively. Altogether, these and the present data discount the only report describing the ability of 8-iso-PGF\(_{2\alpha}\) to induce platelet activation and reversible aggregation rather than an irreversible aggregation indeed, but also an additive pro-aggregatory effect in the presence of U46619 (Pratico et al. 1996). Furthermore, they seem to hint to a role of 8-iso-PGF\(_{2\alpha}\) and 8-iso-PGE\(_2\) as TXAR partial agonists in platelets, an observation that was initially considered contradictory to the previously reported strong TXAR agonist effect in human (Crankshaw 1995) and rat vascular smooth muscle cells (Takahashi et al 1992), leading to the hypothesis of the existence of an isoprostane receptor (Longmire et al. 1994) exhibiting a structure similar to that of the TXAR. This hypothesis was invalidated by the identification of two TXAR subtypes named \(\alpha\) und \(\beta\) (Raychowdhury et al. 1994) and the evidence that these two subtypes are present in human and rat vascular smooth muscle cells (Krauss et al., 1996) whereas platelets solely express the TXAR-\(\alpha\) subtype (Habib et al. 1999). Furthermore, transgenic mice overexpressing the TXAR-\(\beta\) in the vasculature but not in platelets exhibited an increased pressor response to 8-iso-PGF\(_{2\alpha}\) whereas the effect on platelets was the same as in wild type mice, i.e. as previously described. Moreover, the pressor response and the effect on platelet aggregation were abolished in TXAR-knockout mice. (Audoly et al. 2000). Thus, 8-iso-PGF\(_{2\alpha}\) and 8-iso-PGE\(_2\) appear to bind to both TXAR subtypes but with distinct affinity and/or effects of different nature, i.e. of agonistic and antagonistic nature.

### 4.3.1.2 Angiogenesis

Angiogenesis, i.e. the formation of new capillaries from pre-existing vessels, is primordial in the development of collateral circulation. When dysregulated, angiogenesis contributes to numerous disorders: insufficient vessel growth is involved in Alzheimer disease, atherosclerosis, hypertension, diabetes mellitus, Crohn disease, pulmonary fibrosis, nephropathy, osteoporosis and several other diseases while on the other hand, excessive or abnormal angiogenesis can favour or lead to cancer, primary pulmonary hypertension, ascites and a number of other pathologies (Carmeliet 2003). Extracellular matrix (ECM) plays an important role in endothelial cell adhesion, differentiation and proliferation (Rakusan 1995) and therefore, in angiogenesis (Figure 40). When cultured on an ECM-like substance such as Matrigel\textsuperscript{TM}, endothelial cells build
up tube-like structures (Grant et al. 1990, Grant et al. 1991), similar to the capillary vessels formed in vivo (Rakusan 1995, Folkman and Haudenschild 1980). The in vitro formation of endothelial cells tubes can be enhanced by exogenously added growth factors such as VEGF. Binding of VEGF to its receptor, a member of the tyrosine kinase family, induces vascular permeability (Ferrara et al. 1992) and endothelial cell growth and migration (Leung et al. 1989) through relatively well described pathways (Figure 41). VEGF is elevated in the serum of patients with acute ischemia from day 3 to 28 postinfarction (Tamura et al. 1999) and stimulates revascularization of the myocardium and the development of collateral vessels (Ferrara and Bunting 1996). In vitro, VEGF exerts its effects in a concentration dependent manner with maximal effects at concentrations $\geq 20$ ng/mL (Ashton and Ware 2004).

![Figure 40: Simplified scheme of the angiogenesis process](image)

8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$ inhibited the VEGF-induced tube formation in HCAECs, an effect that mimicked that of the TXAR agonist U46619 (Figure 32). Such inhibition has also been observed in human umbilical vein endothelial cells (HUVECs) with the TXAR agonist IBOP (Ashton et al. 1999). 8-iso-PGF$_{2\alpha}$ could inhibit HCAECs’ VEGF-induced tube formation at a concentration of 30 $\mu$M as well as at a 10-fold lower concentration (Figure 33). This effect was
reversed by simultaneous incubation with an equimolar concentrations of the TXAR antagonist SQ29548 (K_i 4.1 nM (Abramovitz et al. 2000)) (Figure 32), suggesting that the effect of the isoprostanes are mediated through the TXAR, an observation that seems in opposition with the effects of these isoprostanes on platelet aggregation. The hypothesis of an isoprostane receptor distinct from but analogous to the TXAR was born from similar apparently paradoxical observations. However, as mentioned before, platelets only express the TXAR-α subtype (Habib et al. 1999), whereas TXAR-β, but not TXAR-α expression is required for the inhibition of VEGF-induced angiogenesis (Ashton and Ware 2004). Indeed, IBOP did not alter VEGF-induced tube formation by HUVECs solely expressing TXAR-α, while tube formation by HUVECs expressing only TXAR-β was reduced to control levels. Ashton and Ware found out that TXAR-β stimulation abrogated the activation of PDK1 and c-Src. We have shown that the mRNA for both TXAR isoforms is present in HCAECs (Figure 34). Although this observation could not be confirmed at the protein levels because of the lack of commercially available isoform-specific antibodies, one could hypothesize that 8-iso-PGF$_{2α}$ and 8-iso-PGE$_2$ are partial agonists on the TXAR-α, an assumption supported by both the present data and by at least one radioligand binding study (Yin et al. 1994), and full agonists on the TXAR-β. In line with this theory, nanomolar amounts of 8-iso-PGF$_{2α}$ have been shown to reduce the release of NO by endothelial cells (Minuz et al. 1998), an observation that could also result from c-Src and PDK1 inactivation by isoprostanes through activation of the TXAR-β.
A limit of these results is the concentration used in the tube formation assay which, indeed, are much higher than 8-iso-PGF$_{2\alpha}$ physiological concentrations in vivo, that range between 0.01 to 0.1 nM in plasma in healthy individuals, with urine concentrations being up to 100 times higher (Basu 1998b, Morrow 1990). However, there are possibly 64 F$_2$-isoprostanes in vivo, and several other isoprostanes family. Furthermore, owing to the fact that pathological concentrations are higher than in healthy individuals, concentrations in situ being possibly even higher than systemic concentrations, the concentrations used in our experiment could be relevant in vivo. Macrophages, for instance, can liberate between 0.06 and 0.10 ng/mg protein of free F$_2$-isoprostanes during the oxidative modification of LDL, with total levels (free and esterified F$_2$-isoprostanes) ranging from 0.027 to 0.057 ng/mg protein (Gopaul et al.1994).
4.3.1.3 Endothelial dysfunction

In accordance with the results obtained in the clinical study, the rats fed with the iron-enriched diet exhibited significantly increased 8-iso-PGF$_2\alpha$ urinary excretion. Moreover, the endothelium-dependent as well as –independent relaxation was significantly altered in this animal model. Endothelial dysfunction is associated with numerous physiological and pathological processes, such as atherosclerosis, hypertension, heart and renal failure, obesity, type I and II diabetes, hypercholesterolemia, rheumatic arthritis or smoking (Félétou and Vanhoutte 2006). Data reported here account for an association between iron overload and endothelial dysfunction.

In line with clinical observations (section 3.2.1), the increased urinary excretion in 8-iso-PGF$_2\alpha$ reflects increased oxidative stress, i.e. increased formation of ROS, presumably through the Fenton reaction (section 1.3.4.1). It is known that under stimulation with acetylcholine, endothelial cells release nitric oxide (NO), the main determinant of endothelium-dependent relaxation. NO activates guanylate cyclase, increasing cGMP that in turn decreases intracellular Ca$_{2+}$, resulting in direct relaxation of vascular smooth muscle and hence, in vasodilation (Moncada and Higgs 2006, Furchgott and Vanhoutte 1989). When produced in closed vicinity, NO and the hydroxyl radical O$_2^- \cdot$ react to form peroxynitrite, ONOO$^- \cdot$, thereby reducing the bioavailability of NO (Félétou and Vanhoutte 2006). Moreover, peroxynitrite also uncouples the nitric oxide synthase by oxidizing complexes within the enzyme as well as its essential cofactor, tetrahydrobiopterin. Furthermore, it directly inhibits NO's main target, guanylate cyclase (Münzel et al. 2005). All these mechanisms can lead to an underproduction of NO by endothelial cells, and account for the significantly submaximal endothelium-dependent relaxation in the animals having been fed the iron-enriched diet.

In solution, sodium nitroprusside spontaneously decomposes to NO, which shorts cuts endothelial cells, allowing assessment of endothelium-independent relaxation by VSMC. In this regard, it has been shown that ROS promote the contraction of VSMC by facilitating the mobilization of calcium and increasing the sensibility of the contractile proteins to calcium ions (Jin et al. 1991, Suzuki and Ford 1992). Such observations (decreased NO bioavailability and increase in intracellular calcium) have also been directly generated in platelets after incubation with isoprostane 8-iso-PGF$_2\alpha$ (Minuz et al. 1998), suggesting that isoprostanes are not mere markers of oxidative stress in this case: they could antagonize vasorelaxation by activation of the TXAR on VSMC (Yang et al. 2004) and endothelial cells (Davidge 2001), leading to increased formation of the vasoconstrictive prostanoid TXA$_2$. Accordingly, induction of oxidative stress by acute
inhibition of glutathione synthesis in rats not only led to higher plasma total isoprostanes levels, but also to decreased plasma NO levels, impaired vasopressor response to sodium nitroprusside in vivo and higher release of TXA2 from the aorta (Ganafa et al. 2002). Finally, externally added 8-iso-PGF2α has been shown to induce vasoconstriction of rat thoracic aorta in vitro, an effect that was enhanced in endothelium-denuded aorta rings compared with intact ones, and inhibited by the TXAR antagonist GR32191 (Cracowski et al. 2002). Altogether, these data indicate that impaired endothelium-independent relaxation could be secondary to the action of ROS on intracellular calcium or/and to that of isoprostanes on the TXAR. In any case, this impairment could be abolished by sufficiently high concentrations of sodium nitroprusside as source of NO. Besides, although both diseases involve different physiopathological mechanisms, the lack of impairment of the endothelium-independent that we report in scleroderma (section 3.2.2.2) indicates that the clinical relevance of this observation in hemochromatosis is uncertain and should be investigated.

Hence, endothelial dysfunction in this hemochromatosis model could be secondary to the actions on ROS or to that of isoprostanes acting as TXAR agonists.

4.3.2 Significance regarding the isoprostanes 8-iso-PGF2α and 8-iso-PGE2 as pharmacological targets

4.3.2.1 In vitro experiment
HCAECs liberated 8-iso-PGF2α and this liberation was enhanced by incubation with bee venom group III PLA2. The general PLA2 inhibitor mepacrine inhibited the liberation of 8-iso-PGF2α in HCAECs, whether intrinsic to the cells or extrinsic induced by group III PLA2 from bee venom. Immunoblotting of full cell lysate revealed that HCAECs express both group IIA and V PLA2s, indicating that one or both of these enzymes is or are involved in the liberation of 8-iso-PGF2α in HCAECs. Secretory porcine and snake PLA2s have been shown to have a strong tendency to form dimers (Reynolds et al. 1995, Myatt et al. 1991, Romero et al. 1987), and this property appears to apply to the human group IIA and V PLA2s as well. The necessity of dimer formation for catalytic activity has not been demonstrated and the physiological relevance of dimer formation is not understood.

Bee venom group III PLA2 was chosen as positive control because it aggressively and with little specificity attacks the phospholipids in membrane of intact cells. Mepacrine interacts with the
enzyme's binding sites, thereby disrupting the substrate-enzyme interface (Jain and Jahagirdar 1984), and possibly antagonizes calmodulin, a calcium-binding protein that binds to Ca\(^{2+}\)-activated or -inhibited proteins such as kinases or phosphatases (Volpi et al. 1981). It inhibits PLA\(_2\) from porcine pancreas with a K\(_i\) of 59 µM (Jain and Jahagirdar 1984). Up to 100 µM, it has little or no effect on the activity of other phospholipases such as phospholipase C (Walenga et al. 1981) or phospholipase D (Kiel and Feinmark 1996). The effect of mepacrine on phospholipase B is not documented. Moreover, incubation of muscle homogenates with mepacrine has been described to inhibit lipid peroxidation (Jackson et al. 1984). Thus, the observed lowering in 8-iso-PGF\(_{2\alpha}\) liberation under mepacrine incubation is in accordance with this previous report and seems attributable to PLA\(_2\)s inhibition.

However, this experiment is limited by the number of PLA\(_2\)s potentially present in endothelial cells, which makes it impossible to point at a precise phospholipase A\(_2\). Unfortunately, due to the nonavailability of isoenzyme-specific PLA\(_2\) inhibitors, the design of this experiment could not lead to more precise conclusions. Furthermore, mepacrine is hydrophobic, which allows its passage through cell membranes. Thus, the in vitro observations we report could be led back to a secretory PLA\(_2\) as well as to another PLA\(_2\) subfamily, such as intracellular PLA\(_2\)s. Thus, we investigated the involvement of secretory PLA\(_2\)s in vivo in a clinical setting.

4.3.2.2 In a clinical setting

In this study, a reduced PAF-AH activity secondary to cholesterol lowering after a 6-week treatment with atorvastatin 40 mg was observed. The treatment did not induce any change in other secretory phospholipases activity, nor in the 8-iso-PGF\(_{2\alpha}\) urinary excretion.

The biological functions of PAF-AH appear paradoxical and their pro- or anti-inflammatory and -atherogenic effects are still a matter of debate. PAF-AH catabolizes the pro-inflammatory PAF (Kudo and Murakami 2002) and, in vitro, its activity in HDL parallels HDL’s ability to prevent LDL oxidation (Van Lenten et al. 1995). A genetic deficiency in PAF-AH studied in Japanese subjects was significantly associated with asthma, stroke, myocardial infarction, brain hemorrhage and nonfamilial cardiomyopathy (Tjoelker and Stafforini 2000, Hiramoto et al. 1997). On the other hand, PAF-AH can transform lyso-PAF back into the biologically active PAF and its analogues. This reaction may occur in the atherogenic small dense LDL particles and may confer them higher pro-inflammatory potential in atherosclerosis-prone areas (Macphee et al. 1996). The lysophospholipids and oxidized fatty acids generated by PAF-AH from highly
oxidized LDL are pro-inflammatory, upregulating adhesion molecules and cytokine production, thereby having a deleterious effect on the arterial wall (Macphee et al. 1999, Macphee 2001, Macphee and Suckling 2002, Macphee et al. 1996). PAF-AH has been postulated as independent risk factor for cardiovascular diseases in healthy middle-aged men (Montuschi et al. 2004) and women (Ballantyne et al. 2004) as well as in men with a history of coronary event (Packard et al. 2000). One other possible mechanism of the pro-inflammatory effect of PAF-AH could have been an involvement in the liberation of isoprostanes in the blood stream. Indeed, isoprostanes are not mere markers, but also mediators of oxidative stress: they are vasoconstrictors in several vascular beds (Montuschi et al. 2004), activate platelets (section 4.3.1), stimulate monocyte adhesion to endothelial cells (Leitinger et al. 2001), inhibit angiogenesis (section 4.3.1) and are involved in endothelial dysfunction in pathological states such as hemochromatosis (section 4.3.1). However, the results of the present study, especially the correlation between the change in PAF-AH activity and that in LDL-cholesterol levels in such small group, contest an independent role of PAF-AH in cardiovascular disease and, in accordance with recent reports (Kardys et al. 2006, Albert et al. 2005), rather suggest that these observations were actually closely connected to LDL-cholesterol levels. Moreover, contrary to recent observations that plasma samples from PAF-AH-deficient subjects do not release F2-isoprostanes from esterified precursors ex vivo and that PAF-AH transgenic mice have a higher capacity to release F2-isoprostanes compared with nontransgenic littermates (Stafforini et al. 2006), the unchanged urinary excretion of 8-iso-PGF2α despite the marked atorvastatin-induced lowering in PAF-AH activity rather shows that the involvement of plasma PAF-AH in the release of 8-iso-PGF2α in vivo in hypercholesterolemic patients is marginal to nil. This is in turn compatible with the observation from Stafforini et al. (2006) that the catabolism rate of PAF-AH was much slower for isoprostanes than for its other substrates, a hint that this enzyme is not the main one responsible for isoprostane liberation.

The intake of atorvastatin 40 mg over 6 weeks significantly lowered PAF-AH activity but as the exact role of PAF-AH remains controversial, the potential benefits of this reduction are uncertain as well. According to the literature, overly increased PAF-AH expression and/or activity are associated with pathological states (Macphee et al. 1999, Macphee 2001, Macphee and Suckling 2002, Macphee et al. 1996). Thus, although the decrease in the inflammatory marker hsCRP was not significant (Table 12), we hypothesize that in such pathological state as hypercholesterolemia, a lowering in PAF-AH activity to normal physiological levels lowers atherogenesis and inflammatory potential and is beneficial. However, since overly decreased PAF-AH expression...
and/or activity are associated with pathological states as well (Tselepis and Chapman 2002, Tjoelker and Stafforini 2000, Hiramoto et al. 1997), these should not be excessively lowered either. Indeed, PAF-AH is secreted in response to inflammatory stimuli (Castro Faria Neto et al. 2005), yet it is unclear if this happens in response to or as part of an inflammatory cascade. If proven to be a primarily anti-inflammatory and –atherogenic enzyme, PAF-AH’s reported pro-inflammatory and –atherogenic properties could be a mere imbalance or reversal in its functions brought about by unknown pathophysiological conditions.

The reason why a significant decrease in 8-iso-PGF$_{2\alpha}$ was not observed is not clear. The choice of the statin used does not explain this constancy. Indeed, a few studies have reported a decrease in 8-iso-PGF$_{2\alpha}$ after atorvastatin treatment (Sinzinger and Oguogho 2003, Sugiyama et al. 2005). Sugiyama et al. (2005) observed a significant decrease in 8-iso-PGF$_{2\alpha}$ as soon as 4 weeks of a 10 mg daily intake of atorvastatin. An insufficient lowering of the cholesterol levels, which has been associated in some studies (De Caterina et al. 2002) with that in 8-iso-PGF$_{2\alpha}$ urinary excretion, is not the explanation either. Indeed, in a therapy scheme intended to produce a 20% reduction of total cholesterol levels after 60 days, simvastatin induced after as soon as one month a significant reduction of 8-iso-PGF$_{2\alpha}$ urinary excretion levels (De Caterina et al. 2002). Despite a higher than 40% lowering in total cholesterol, this result could not be reproduced for atorvastatin in the present setting. Since liver and muscle enzymes were not significantly increased, with the exception of ALT, which, however, did not reach 1.5 fold of the upper limit of the normal range, the hypothesis of oxidation injury in the muscles or liver to explain the lack of change in 8-iso-PGF$_{2\alpha}$ (Sinzinger et al. 2001) seems irrelevant as well. Of course, the explanation could be that 8-iso-PGF$_{2\alpha}$ is in fact liberated by PLA$_2$s (group IIA and V), which activity remained unaltered. Indeed, at least one study suggested a link between 15-F$_2$-isoprostanes and group IIA and V PLA$_2$s (Staff et al. 2003).

Wiklund et al. (2002) reported a significant reduction in group IIA PLA$_2$ plasma protein levels after a 6-week daily intake of atorvastatin 40 mg. Thus, a likely argument for the lack of change in PLA$_2$s’ activity in our study could be the fact that we measured enzyme activity where others measured plasma protein levels. Indeed, protein quantification does not give information about the catalytic activity of PLA$_2$ present in the sample, and reaction rates can remain unchanged or even rise with comparable protein levels (Staff et al. 2003). Since PLA$_2$s are involved in inflammatory processes, a reason yet for the lack of change in their activity could be that
atorvastatin, as reflected by the hsCRP (Table 12), did not significantly affect the inflammatory status of treated participants, although Sugiyama et al. (Sugiyama et al. 2005) reported a significant decrease in hsCRP after a 4-week 10 mg/day intake of atorvastatin. Taken together, these data and ours demand that the possible association between PLA$_2$s and 8-iso-PGF$_{2\alpha}$ be further investigated.

A limit of this study is that the biochemical parameters were not measured in the same biological compartment: all parameters were quantified in plasma, except 8-iso-PGF$_{2\alpha}$. Nevertheless, there is an excellent correlation between plasma and urine 8-iso-PGF$_{2\alpha}$ (Oguogho et al. 1999). Since artefactual isoprostane formation through lipid autooxidation is less of an issue in urine samples than in plasma (Schwedhelm and Böger 2003), 8-iso-PGF$_{2\alpha}$ quantitation in urine is more reliable and was favoured. The possibility that a larger number of participants would have allowed drawing more definite conclusions regarding the parameters possibly linked to inflammation, e.g. hsCRP, PLA$_2$s and 8-iso-PGF$_{2\alpha}$, cannot be ruled out. However, in a previous study including 12 individuals with similar 8-iso-PGF$_{2\alpha}$ baseline levels (Troost et al. 2000), we were able to detect a 24% significant decrease in 8-iso-PGF$_{2\alpha}$ urinary excretion, i.e. $\approx$ 2 ng/h. Finally, it is undeniable that PAF-AH plasma protein levels instead of activity could have lead to different conclusions, because similar to PLA$_2$s, there is no direct correlation between protein levels and activity (O’Donoghue et al. 2006), suggesting that a fraction of the protein can become inactive, and because activity seems more closely related to LDL cholesterol than protein levels (Iribarren et al. 2006). However, since the involvement of in PAF-AH in the enzymatic liberation process of isoprostanes was the main interest of this study, its activity was more relevant than its protein levels to us.

Thus, although an association between group IIA and V phospholipase and 8-iso-PGF$_{2\alpha}$ must be further looked into, our data strongly suggest that the previously reported increased cardiovascular risk associated with PAF-AH was connected with LDL cholesterol. Furthermore, we eliminate a key role of the enzyme PAF-AH in the release of 8-iso-PGF$_{2\alpha}$ in our patients.
5 Summary

Isoprostanes are by-products of the formation of the prostaglandin from arachidonic acid, catalysed by the prostaglandin H$_2$ synthase. They differ from the prostaglandins in their regio- and stereochemistry: there are potentially four regioisomers with 64 corresponding stereoisomers each. Especially the isoprostanes 8-iso-Prostaglandin F$_{2\alpha}$ und 8-iso-Prostaglandin E$_2$ exert biological activity via the thromboxane A$_2$ receptor. They can, as reported in the present work, modulate platelet aggregation, inhibit angiogenesis and affect endothelial function.

Since the formation of isoprostanes is catalysed by free radicals, the influence of iron on the formation of 8-iso-Prostaglandin F$_{2\alpha}$, e.g. through the Fenton reaction, was investigated in vitro and in vivo. For that purpose, several quantification methods of 8-iso-Prostaglandin F$_{2\alpha}$ were compared, showing that specific quantification of 8-iso-Prostaglandin F$_{2\alpha}$ by chromatographic methods appeared the most reliable. This method was used in all investigations thereafter, yielding to the observation that iron overload is associated with increased formation of 8-iso-Prostaglandin F$_{2\alpha}$, a decline in potentially antioxidative mechanisms and an impairment of the endothelial function.

A further difference between prostaglandins and isoprostanes is that the latter are synthesized esterified to phospholipids, from which they subsequently must be cleaved. In vitro experiments aiming at the identification of the enzyme(s) responsible for this cleavage hinted to an involvement of the group VII phospholipase A$_2$ (PAF-AH). However, this observation could not be confirmed in vivo. Thus, several enzymes still come in question in the liberation of isoprostanes from phospholipids. A pharmacotherapy targeting this or these enzyme(s) could lead to a reduction in the formation of free radicals and in the liberation of isoprostanes. In this matter, blockers of the angiotensin receptor and statins are plausible pharmacological candidates, through mechanisms that remain to be elucidated.
Zusammenfassung

Isoprostane entstehen aus Arachidonsäure in Analogie zu der durch die Prostaglandin H$_2$-Synthase katalysierten Bildung der Prostaglandine. Im Unterschied zu den Prostaglandinen unterscheiden sich die Isoprostane durch ihre Regio- und Stereochemie. Es sind vier Regioisomere mit jeweils 64 Stereoisomeren möglich. Unter den Isoprostanen nehmen 8-iso-Prostaglandin F$_{2\alpha}$ und 8-iso-Prostaglandin E$_2$ eine Sonderstellung ein, da sie eine über den Thromboxan-Rezeptor vermittelte biologische Aktivität aufweisen. Im Rahmen dieser Arbeit konnte gezeigt werden, dass 8-iso-Prostaglandin F$_{2\alpha}$ und 8-iso-Prostaglandin E$_2$ die Plättchenaggregation regulieren, die Angiogenese hemmen und die Endothelfunktion beeinflussen.

Die Bildung der Isoprostane wird durch freie Radikale katalysiert. Es wurde daher untersucht, welchen Einfluss freies Eisen auf die Bildung von 8-iso-Prostaglandin F$_{2\alpha}$ in vitro und in vivo, z.B. über die Fenton-Reaktion, hat. Hierzu wurden verschiedene analytische Methoden zur Quantifizierung von 8-iso-Prostaglandin F$_{2\alpha}$ miteinander verglichen. Eine auf Basis der Gaschromatographie-(Tandem-)Massenspektrometrie entwickelte Methode war am geeignetsten und wurde für alle weiteren Analysen verwendet. So konnte gezeigt werden, dass eine Eisenüberladung sowohl zu einer gesteigerten Bildung von 8-iso-Prostaglandin F$_{2\alpha}$, zu einer Abnahme antioxidativ wirksamer Schutzmechanismen und einer Verschlechterung der Endothelfunktion führt.

Ein weiterer Unterschied zwischen Prostaglandinen und Isoprostanen besteht darin, dass Isoprostane aus in Phospholipiden veresterter Arachidonsäure gebildet werden. Sie müssen also nachfolgend freigesetzt werden. Versuche, welche Enzyme für die Freisetzung von 8-iso-Prostaglandin F$_{2\alpha}$ verantwortlich sein können, zeigten in vitro eine Beteiligung der Phospholipase A$_2$ VII (PAF-AH). In vivo konnte diese Beobachtung jedoch nicht bestätigt werden. Somit kommen für die Freisetzung der Isoprostane möglicherweise noch andere Enzyme in Frage. Eine Pharmakotherapie könnte somit zweierlei bewirken, eine Reduktion der Radikalbildung als auch der Isoprostanfreisetzung. Eigene Untersuchungen zeigen, dass sowohl Hemmer des Angiotensinrezeptors Inhibitoren als auch Lipidsenker (Statine) hier vielversprechende Ansätze
sein können. In zukünftige Arbeiten sollen die dahinterstehenden Mechanismen weiter aufgeklärt werden.
6 References


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7 Materials

7.1 Chemicals

<table>
<thead>
<tr>
<th>Substance Name</th>
<th>Risks and safety statements, when applicable</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-F$_2$-IsoP</td>
<td></td>
<td>Jügesheim, Germany</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
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<td>Cayman Chemical</td>
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<tr>
<td>2-propanol</td>
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<tr>
<td></td>
<td>S: 7-16-24/25-26</td>
<td>Jügesheim, Germany</td>
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<tr>
<td></td>
<td>R: 45-46-23/24/25-48</td>
<td>Merck KgaA</td>
</tr>
<tr>
<td>40% bis-Acrylamide solution</td>
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<td>Bio-Rad Laboratories</td>
</tr>
<tr>
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<tr>
<td>8-iso-PGE$_2$</td>
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<td>Bio-Rad Laboratories</td>
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<tr>
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<td>Cayman Chemical</td>
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<td>Acetic acid C$_2$H$_4$O$_2$</td>
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<td>Ann Arbor, USA</td>
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<tr>
<td></td>
<td>R: 11-20/21/22-36</td>
<td>Darmstadt, Germany</td>
</tr>
<tr>
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<td>Agarose</td>
<td>R: 8-22-36/37/38-42/43</td>
<td>Bio-Rad Laboratories</td>
</tr>
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<td>Bee venom PLA$_2$</td>
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<td>Bio-Rad Precision Plus Protein Standards</td>
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<td>KH₂PO₄</td>
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<td>WAK Chemie, Steinbach, Germany</td>
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<td>RNAzol</td>
<td>Merck-Schuchardt</td>
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<td>Sodium azide NaN₃</td>
<td>J.T. Baker</td>
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<td>Sodium chloride</td>
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<td>Steinheim, Germany</td>
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<tr>
<td>U46619</td>
<td>Cayman Chemical</td>
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Materials

VEGF

17, 17, 18, 18-[\(^2\)H\(_4\)]-5-F\(_2\)-IsoP

17, 17, 18, 18-[\(^2\)H\(_4\)]-15-F\(_2\)-IsoP

17,18,19,20-[\(^2\)H\(_4\)]-8-iso-PGF\(_{2\alpha}\)

Ann Arbor, USA
Peprotech
London, England
Cayman Chemical
Ann Arbor, USA
Cayman Chemical
Ann Arbor, USA
Cayman Chemical
Ann Arbor, USA

7.2 Cells, Cell Culture Media and Consumable

EBM-2

EGM-2 MV

Fetal bovine serum (FBS), heat inactivated

HCAEC

Cambrex, Walkersville, USA
Cambrex, Walkersville, USA
Invitrogen
PromoCell, Heidelberg, Germany

7.3 Consumables Supplies

1,5- and 2-mL Eppis

10-, 100-, 1000- and 5000-µL pipettes

10-, 100- and 1000-µL pipette tips

15- and 50-mL Falcon tubes

25- and 75-cm\(^2\) cell culture flasks

5000-µL pipette tips

6- and 48-well plates

8-iso-PGF\(_{2\alpha}\) immunoaffinity columns

Blotting paper

Cell Scraper

Eppendorf AG, Hamburg, Germany
Eppendorf AG, Hamburg, Germany
Sarstedt AG & Co, Nümbrecht, Germany
Sarstedt AG & Co, Nümbrecht, Germany
Sarstedt AG & Co, Nümbrecht, Germany
Eppendorf AG, Hamburg, Germany
Nunc, Roskilde, Denmark
Cayman Chemical, Ann Arbor, USA
Whatman, Dassel, Germany
Sarstedt AG & Co, Nümbrecht, Germany
### Materials

<table>
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<tr>
<th>Item</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Curix X-ray film cassettes</td>
<td>Agfa, Köln, Germany</td>
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<tr>
<td>Cuvettes for photometry</td>
<td>Sarstedt AG &amp; Co, Nümbrecht, Germany</td>
</tr>
<tr>
<td>Factor-Four-5MS column</td>
<td>Varian, Palo Alto, USA</td>
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<tr>
<td>Glassware</td>
<td>Schott Duran, Mainz, Germany</td>
</tr>
<tr>
<td>High performance chemiluminescence film</td>
<td>Amersham Biosciences, Little Chalfont, England</td>
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<tr>
<td>Latex Gloves</td>
<td>Kimberly-Clark, Zaventem, Belgium</td>
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<tr>
<td>Microcuvettes for aggregometry, 1.0 x 4.0</td>
<td>Rolf Greiner Biochemics, Flacht, Germany</td>
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<tr>
<td>Molecular sieve beads</td>
<td>Merck KGaA, Darmstadt, Germany</td>
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<tr>
<td>Monovettes</td>
<td>Sarstedt AG &amp; Co, Nümbrecht, Germany</td>
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<td>Nitrile gloves</td>
<td>Ansell, Brussels, Belgium</td>
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<tr>
<td>Nitrocellulose transfer membrane</td>
<td>Schleicher &amp; Schuell BioScience GmbH, Dassel, Germany</td>
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<td>Omniscrypt Reverse Transcriptase Kit</td>
<td>QIAGEN GmbH, Hilden, Germany</td>
</tr>
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<td>PAF-AH activity Assay Kit</td>
<td>Cayman Chemical, Ann Arbor, USA</td>
</tr>
<tr>
<td>Pasteur Pipettes</td>
<td>Brand, Wertheim, Germany</td>
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<tr>
<td>Polypropylene tubes</td>
<td>Greiner Bio-one, Frickenhausen, Germany</td>
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<tr>
<td>Primers</td>
<td>Höttner und Hüttner AG, Tübingen, Germany</td>
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<td>Serological pipettes</td>
<td>Sarstedt AG &amp; Co, Nümbrecht, Germany</td>
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<tr>
<td>sPLA&lt;sub&gt;2&lt;/sub&gt; activity assay kit</td>
<td>Cayman Chemical, Ann Arbor, USA</td>
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<tr>
<td>Sterile filter (0.22, 0.45 µm)</td>
<td>Qualilab, Bruchsal, Germany</td>
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### Equipment

-20 °C Freezer                                             | Liebherr, Rostock, Germany                         |
-80 °C Freezer                                             | Kryotec, Hamburg, Germany                          |
96-well plate reader, Sunrise                              | Tecan, Crailsheim, Germany                         |
Accu-jet pipetting aid                                     | Eppendorf AG, Hamburg, Germany                     |
Analytical balance CP225 D                                 | Sartorius, Göttingen, Germany                      |
AxioCam PRc 5 camera                                        | Zeiss, Göttingen, Germany                          |
Benchtop centrifuge Rotina 35 R
ChemiGenius 2 Bio-imaging System, agarose gel viewing system
Eppendorf tubes shaker Thermomixer Compact
Evaporator, TurboVap LV
GC-MS
GeneAmp PCR System 9700
Heat Circulator C20CS
Heating blocks
Homogenisator, Polytron
Hood with laminar vertical airflow, LaminAir HB 2448
Lambda 2S Photometer, for protein quantification
Magnetic stirrer MR 3002, heating
Micro centrifuge 5415 R
Microscope, Axiovert 25
Optical aggregometer, Apact 2
Orbital shaker DuoMax 1030
Organ bath myograph chambers
PCR Sprint Thermal Cycler
pH-meter, digital
Precision balance BP3100 S
Pump for Rotational-Vacuum-Concentrator
Refrigerators
Rotational-Vacuum-Concentrator 2-25
SmartSpec 3000 Photometer, for RNA/DNA quantification
Thermometer, digital
Ultra-Pure Water System Milli-Q Plus
Vacuum pump Mini-Vac E1
Vacuum manifold

Hettich, Tuttlingen, Germany
Syngene, Cambridge, UK
Eppendorf AG, Hamburg, Germany
Zymark, Hopkinton, USA
Varian, Palo Alto, USA
Applied Biosystems, Foster city, USA
Lauda, Lauda-Königshofen, Germany
Fisher Bioblock Scientific, Tournai, Belgium
Kinematica AG, Littau-Lucerne, Switzerland
Heraeus, Hanau, Switzerland
PerkinElmer, Jügesheim, Germany
Heidolph, Schwabach, Germany
Eppendorf AG, Hamburg, Germany
Zeiss, Oberkochen, Germany
LABor, Hamburg, Germany
Heidolph, Schwabach, Germany
Otto Jahn OHG, Oberhausen, Germany
ThermoHybaid, Waltham, USA
Knick, Berlin, Germany
Sartorius, Goettingen, Germany
Vacuubrand, Wertheim, Germany
Liebherr, Rostock, Germany
Martin Christ, Osterode am Harz, Germany
Bio-Rad Laboratories, Hercules, USA
Eutech Instruments, Nijkerk, Holland
Millipore, Schwabach, Germany
PeqLab, Erlangen, Germany
Macherey-Nagel GmbH & Co. KG, Düren,
Materials

Germany

Vertical and Horizontal Systems for Electrophoresis, with Power Supply Unit
Vortexer, Reax top
CO₂ Incubator HERACell

Bio-Rad Laboratories, Hercules, USA
Heidolph, Schwabach, Germany
Thermo Electron Corporation, Langenselbold, Germany

7.5 Gases

Carbogen, Carbon dioxide, Argon, Helium, Methane were provided by Linde (Hannover).

7.6 Softwares

LSM Image Browser v. 3.2.0, Zeiss
SPSS 10.0.5
GeneSnap 6.02, Syngene
AxioVision 4.3.0, Zeiss
IBJ Amon 2.61

7.7 Buffer and Solution Recipes

Column buffer:
Potassium phosphate dibasic trihydrate K₂HPO₄·3H₂O 17.40 g
Potassium phosphate monobasic KH₂PO₄ 3.22 g
Sodium chloride NaCl 29.20 g
Sodium azide NaN₃ 0.5 g
Ultra-pure water 1,000 mL

Elution solution :
Absolute ethanol 95% v/v
Ultra-pure water 5% v/v

Lysis buffer:
Nonidet P-40 Substitute 10 mL
**Materials**

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<th>Material</th>
<th>Volume</th>
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<td>Triton X-100</td>
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<td>Tris base</td>
<td>1.2 g</td>
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<td>Potassium chloride KCl</td>
<td>186 mg</td>
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<tr>
<td>Sodium chloride NaCl</td>
<td>8.7 g</td>
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<tr>
<td>Glycerol phosphate</td>
<td>6.48 g</td>
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<td>Sodium fluoride NaF</td>
<td>2.1 g</td>
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<tr>
<td>Sodium orthovanadate Na$_3$VO$_4$</td>
<td>183.9 g</td>
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<tr>
<td>Protease Inhibitor Cocktail</td>
<td>0.1%</td>
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<tr>
<td><em>Aqua ad injectabilia</em></td>
<td>q.s. 1,000 mL</td>
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**Basal medium**

0.1% BSA in EBM-2 medium

1 x *Tris-Borate-EDTA (TBE) buffer*:

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<thead>
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<th>Volume</th>
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<tbody>
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<td>Tris base</td>
<td>10.8 g</td>
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<tr>
<td>Boric acid</td>
<td>5.5 g</td>
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<tr>
<td>0.5M EDTA, pH 8.0</td>
<td>4 mL</td>
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<td><em>Aqua ad injectabilia</em></td>
<td>q.s. 1,000 mL</td>
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1x *Phosphate buffered saline (PBS)*:

according to manufacturer’s instructions

**Separating gel**:

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<td>bis-Acrylamide 40% solution</td>
<td>2.53 mL</td>
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<td><em>Aqua ad injectabilia</em></td>
<td>5.48 mL</td>
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<tr>
<td>Tris 2 M pH 8.8</td>
<td>2.0 mL</td>
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<tr>
<td>SDS 20%</td>
<td>50 µL</td>
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<tr>
<td>TEMED</td>
<td>5 µL</td>
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<tr>
<td>APS 10% m/v solution</td>
<td>50 µL</td>
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**Collecting gel**:

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<tr>
<td>bis-Acrylamide 40% solution</td>
<td>0.6 mL</td>
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</table>
### Materials

- *Aqua ad injectabilia*: 3.8 mL
- Tris 0.5 M pH 8.8: 1.6 mL
- SDS 20%: 30 µL
- TEMED: 6 µL
- APS 10% m/v solution: 30 µL

#### 3x Laemmli solution:
- 1 M Tris: 18.75 mL
- Glycerol: 30 mL
- 20% SDS: 30 mL
- 2-Mercaptoethanol: 15 mL
- Bromphenol blue: 2 mg

#### 5x Running buffer:
- Tris base: 15 g
- Glycin: 72 g
- SDS 20%: 25 mL
- Ultra-pure water: q.s. 1 L

#### 10x Transfer buffer:
- Tris base: 30.2 g
- Glycin: 144.2 g
- SDS 20%: 10 mL
- Ultra-pure water: q.s. 1 L

#### 1x Transfer buffer:
- 10x Transferring buffer: 100 mL
- Methanol: 100 mL
- Ultra-pure water: q.s. 1 L

#### 10x Tris-buffered saline (TBS) buffer:
- Tris base: 24.2 g
**Materials**

NaCl 80.0 g  
Ultra-Pure water q.s. 1 L  
Adjust pH to 7.6 with 1N hydrochloric acid HCl

*1x TBS-Tween (TBS-T) buffer:*

- 10x TBS buffer 100 mL  
- Tween 20 1 mL  
- Ultra-pure water q.s. 1 L

*Krebs-Henseleit solution:*

- Sodium chloride NaCl 13.79 g  
- Potassium chloride KCl 0.715 g  
- Calcium chloride CaCl₂ 0.2739 g  
- Magnesium sulphate heptahydrate MgSO₄·7H₂O 0.5915 g  
- Potassium dihydrogen Phosphate KH₂PO₄ 0.3265 g  
- Sodium bicarbonate NaHCO₃ 1.05 g  
- Glucose 2.1775 g  
- Indomethacin 3.578 mg  
- Ultra-pure water q.s. 1 L
8 Abbreviations

4-hydroxy-TEMPO 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-yl
8-iso-PGF$_{2\alpha}$, 8-iso-PGE$_{2}$, 8-iso-PGA$_{2}$ 8-iso-Prostaglandin F$_{2\alpha}$, 8-iso-Prostaglandin E$_{2}$, 8-iso-Prostaglandin A$_{2}$

95% CI 96% confidence interval
A ampere
ACE angiotensin converting-enzyme
ACh Acetylcholine
ADP Adenosine diphosphate
ALT alanine aminotransferase
Ang II angiotensin II
APS Ammonium persulfate
ARB angiotensin type 1 receptor blocker
AST aspartate aminotransferase
AT$_{1}$ (or AT$_{2}$) angiotensin type 1 (or 2)
ATV atorvastatin
BMI body mass index
Bp base pairs
BSA bovine serum albumin
BSTFA N,O-Bis(Trimethylsilyl)trifluoroacetamide
cm, mm, µm centimeter, millimetre, micrometre
CoQ10 coenzyme Q10
COX cyclooxygenase
DBP diastolic blood pressure
DEPC diethyl pyrocarbonate
DNA, cDNA deoxyribonucleic acid, complementary DNA
dNTP deoxyribonucleotide triphosphate
ECL enhanced chemoluminescent
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
<table>
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<th>Abbreviation</th>
<th>Meaning</th>
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<td>EGM</td>
<td>endothelial Cell Growth Medium</td>
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<tr>
<td>EIA</td>
<td>enzyme immunooassay</td>
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<td>EMB</td>
<td>endothelial cell basal medium</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>g, mg, µg, ng, pg</td>
<td>gram, milligram, microgram, nanogram, picogram</td>
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<td>gas chromatography-mass spectrometry</td>
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<td>hemoglobin</td>
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<td>hemochromatosis</td>
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<td>HPETE</td>
<td>hydroperoxyicosatetraenoic acid</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>hour</td>
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<tr>
<td>hsCRP</td>
<td>high-sensitive C-reactive protein</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein cells</td>
</tr>
<tr>
<td>IAC</td>
<td>immunoaffinity chromatography</td>
</tr>
<tr>
<td>IBOP</td>
<td>[15-(1a,2b(5Z),3a-(1E,3S),4a)]-7-[3-hydroxy-4-(p-iodophenoxo)-1-butetyl-7-oxabicycloheptenoic acid</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L, mL, µL</td>
<td>liter, milliliter, microliter</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LIC</td>
<td>liver iron concentration</td>
</tr>
<tr>
<td>m</td>
<td>mass</td>
</tr>
<tr>
<td>M, mM, µM, nM, pM</td>
<td>molar, millimolar, micromolar, nanomolar, picomolar</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge</td>
</tr>
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</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>minute</td>
</tr>
<tr>
<td>mm Hg</td>
<td>millimetre of mercury</td>
</tr>
<tr>
<td>N, mN</td>
<td>Newton, millinewton</td>
</tr>
<tr>
<td>NICI</td>
<td>negative ion chemical ionization</td>
</tr>
<tr>
<td>Nox</td>
<td>NAD(P)H oxidase</td>
</tr>
<tr>
<td>NTBI</td>
<td>non-transferrin-bound iron</td>
</tr>
<tr>
<td>NTP</td>
<td>nitroprusside</td>
</tr>
<tr>
<td>ºC</td>
<td>grad Celsius</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAF-AH</td>
<td>platelet activating factor-acetylhydrolase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR, RT-PCR</td>
<td>polymerization chain reaction, reverse transcriptase-PCR</td>
</tr>
<tr>
<td>PE</td>
<td>phenylephrine</td>
</tr>
<tr>
<td>PFB-bromide</td>
<td>2,3,4,5,6-pentafluorobenzyl bromide</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PLA₂, sPLA₂, cPLA₂, iPLA₂, lPLA₂</td>
<td>phospholipase A₂, secretory PLA₂, cytosolic PLA₂, Ca²⁺-independent intracellular PLA₂, lysosomal PLA₂</td>
</tr>
<tr>
<td>PPP</td>
<td>platelet-poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluorid</td>
</tr>
<tr>
<td>q.s.</td>
<td>up to</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RNA, mRNA</td>
<td>ribonucleic acid, messenger RNA</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Raynaud’s phenomenon</td>
</tr>
<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium lauryl sulfate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SIM</td>
<td>single ion monitoring</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>SQUID</td>
<td>superconducting quantum interference device</td>
</tr>
<tr>
<td>SSc, lSSc, lcSSc, dcSSc</td>
<td>systemic sclerosis, limited SSc, limited cutaneous SSc, diffuse cutaneous SSc</td>
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<td>SQ29548</td>
<td>[1S-[1α,2α(Z),3α,4α]]-7-[3-[(Phenylamino)carbonyl]hydrazino)methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TBS, TBS-T</td>
<td>Tris-buffered saline, TBS-Tween</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>TXAR</td>
<td>thromboxane receptor</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>U46619</td>
<td>9,11-dideoxy-9α,11α-methanoepoxy Prostaglandin F₂α</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>V, mV</td>
<td>volt, milliV</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cells</td>
</tr>
</tbody>
</table>
9 Curriculum Vitae

Ghainsom Djomkaem Kom, born December 10, 1980 in Sherbrooke, Canada

Diplomas and Certifications

Institute of Experimental and Clinical Pharmacology, Hamburg, Germany
University Hospital Hamburg-Eppendorf

RPEBC (Registered at the Pharmacy Examining Board of Canada) May 2003
Montréal, Canada

B.Pharm. - International profile (refer to Other Experiences) Sept. 1999 - May 2003
Université Laval Québec city, Canada

Working Experience

June-August 2001, June-August 2000
Worcester, USA

June-July 2003 Intern at Pharmacie Brunet <www.brunet.ca>
Montréal, Canada

July-August 2002 Intern for Hexal AG <www.hexal.com>
Holzkirchen, Germany

Spring 2002 Intern for Anapharm Inc. <www.anapharm.com>
Québec city, Canada

Spring 2001 Intern at CLSC-Haute-Ville-des-Rivières, a Public Hospice
Québec city, Canada
Curriculum Vitae

June-August 2000    Volunteering Technical Assistant at the *Laboratory of HIV Vaccine*, Department of Infectious Diseases, Umass Medical Center <www.umass.edu>

Worcester, USA

Other Experiences

Spring 2003    Clinical Semester: Rotations in the Surgery/Palliative Care, Geriatrics and Cardiology Departments, Saint-Sacrement Hospital
Québec city, Canada

Fall 2002    Exchange Semester: Galenics and analytical pharmacy project: Manufacturing of a Solution of Ascorbic Acid Intended for Parenteral Administration
Rouen, France

Languages

French, English, German

Prizes and Scholarships

April 2007-September 2007: scholarship for foreign students with excellent performances from the *Department of Research and International affairs* of the University of Hamburg
April 2005-March 2007: doctoral scholarship from the *Department of Research and International affairs* of the University of Hamburg
October 2004: 75€ from Cayman Chemicals, 3rd Poster Prize at the joint 2nd International Conference on Phospholipase A2 and 8th International Congress on Platelet Activating Factor
May 2000: 125$CAN from the Pharmacies Brunet, reward for the best information article of the month on hemorrhoids
1999-2000: 1000$CAN from the Desjardins Foundation, reward for excellence
1995-1996: ~500$CAN Scholarship, First prize of the «Opération zéro faute», the national French exam sponsored by the Cameroonian Union of Breweries
Publications and congress participations

10 Publications and congress participations

Articles


Congress posters and presentations


Kom GD, Schwedhelm E, Böger RH. The Phospholipase A\textsubscript{2} Inhibitor Mepacrine Prevents the \textit{in vitro} Liberation of 8-iso-Prostaglandin F\textsubscript{2α} by Human Coronary Artery Endothelial Cells. Naunyn Schmiedeberg’s Archives of pharmacology 2006; 372 (S1): 45.
47\textsuperscript{th} Spring Meeting of the German society for experimental and clinical pharmacology and toxicology, Mainz, April 4-6, 2006.

Kom GD, Schwedhelm E, Maas R, Schneider L, Benndorf RA, Böger RH. Platelet activating factor acetylhydrolase is not involved in the liberation of 8-iso-Prostaglandin F\textsubscript{2α}: a case-control study, Journal of Vascular Research, 2005; 42 (S2): II/121.
3\textsuperscript{rd} European Meeting of Vascular Biology and Medicine, Hamburg, September 28-30, 2005.

3\textsuperscript{rd} European Meeting of Vascular Biology and Medicine, Hamburg, September 28-30, 2005.

71\textsuperscript{st} Spring Meeting of the German Society for Cardiology and Cardiovascular Research, Mannheim, March 31-April 02, 2005

Gnann A, Benndorf RA, Schwedhelm E, Kom GD, Ergün S, Böger RH. Isoprostanes inhibit migration and \textit{in vitro} tube formation of endothelial cells \textit{via} the thromboxane A\textsubscript{2} receptor. Naunyn Schmiedeberg’s Archives of pharmacology 2005; 371 (S1): R24.
46\textsuperscript{th} Spring Meeting of the German society for experimental and clinical pharmacology and toxicology, Mainz, March 15-17, 2005.

Kom GD, Schwedhelm E, Tsikas D, Gutzki FM, Böger RH. Platelet Activating Factor Acetylhydrolase, Secretory Phospholipase A\textsubscript{2} and 8-iso-Prostaglandin F\textsubscript{2α} in Coronary Heart Disease: a Case-control Study.
11 Annexes

Statement

I herewith declare on oath that the work reported in the thesis submitted at the University of Hamburg and entitled

Isoprostanes and Phospholipases – Markers and Mediators of Oxidative Stress

was realized in person in the Clinical Pharmacology of the Institute of Experimental and Clinical Pharmacology and Toxicology under the supervision of Prof. Rainer H. Böger and that no other resources than those therein listed were used in the writing process of the mentioned thesis.

I furthermore certify that neither the present nor another thesis was submitted as doctoral thesis in another national or in a foreign university.

Hamburg, May 23, 2007
Acknowledgement

I thank Prof. Böger for having taken a chance and the necessary steps to give me the chance to work in his team. I thank him for a mentoring that steadily promotes scientific discussion and innovation, in internal and external meetings. I thank him for his constant ear and support in all matters.

I also thank Prof. Korth for having accepted to stand in for the present work vis-à-vis the Department of Chemistry of the University of Hamburg.

Thanks to Edzard Schwedhelm, Renke Maas, Friedrich Schulze, Anna Steepaß, Mariola Kastner, Cornelia Woermann, Lisa Silberhorn, my fellow students and all the staff at the Institute for the great working atmosphere and the permanent willingness to exchange ideas and practical tips. I particularly thank Edzard Schwedhelm for his knowledgeability and his availability.

This work was supported in part by scholarships of the Department of Research and International affairs of the University of Hamburg.

Last but not least, I thank my family, my parents Ambroise and Dorothée, my brother Nouepeyiô, my sister Messà, and Patrice, for their unconditional and constant support. I thank Makéba for having been the best incentive of all.