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Prostaglandins and Isoprostanes in Relation to Risk Factors for Atherosclerosis

Role of Inflammation and Oxidative Stress

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2005

ISSN 1651-6206
ISBN 91-554-6156-5
urn:nbn:se:uu:diva-4803

Dissertation presented at Uppsala University to be publicly examined in Auditorium Minus, Museum Gustavianum, Uppsala, Friday, April 1, 2005 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Helmersson, J. 2005. Prostaglandins and Isoprostanes in Relation to Risk Factors for Atherosclerosis. Role of Inflammation and Oxidative Stress. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 9. 82 pp. Uppsala. ISBN 91-554-6156-5

Inflammation and oxidative stress may be involved in atherogenesis. This thesis describes clinical studies of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), an inflammatory mediator, and the isoprostane 8-iso- $PGF_{2\alpha}$, a reliable indicator of oxidative stress, and cytokine-related inflammatory mediators and indicators in healthy subjects and in a population-based cohort of Swedish men.

$PGF_{2\alpha}$ and 8-iso- $PGF_{2\alpha}$ formation in healthy subjects varied considerably between days with a mean intra-individual coefficient of variation of 41 % and 42 %, respectively. A morning urine sample reflected the basal level of 8-iso- $PGF_{2\alpha}$ formation as accurately as a 24-hour urine collection, and represents a more practical alternative to the 24-hour urine collection in clinical studies. $PGF_{2\alpha}$ formation (as measured by urinary 15-keto-dihydro- $PGF_{2\alpha}$) was increased in patients with type 2 diabetes and in smokers independent of other cardiovascular risk factors. These results indicated an on-going cyclooxygenase (COX)-mediated inflammatory reaction related to these conditions. Further, an increased formation of isoprostanes (as measured by urinary 8-iso- $PGF_{2\alpha}$) was found in patients with type 2 diabetes and in smokers, indicating a high level of oxidative stress in these men. The smokers had also increased levels of the cytokine interleukin-6, indicating an on-going cytokine-related inflammatory reaction. The inflammatory indicators C-reactive protein and serum amyloid A were related to overweight but not independently associated to type 2 diabetes. High levels of serum selenium in middle-aged men predicted reduced formation of $PGF_{2\alpha}$ and 8-iso- $PGF_{2\alpha}$ 27 years later.

In summary, low-grade, chronic COX-mediated and possibly cytokine-related inflammation, and oxidative stress, seem to be joint features of type 2 diabetes and smoking, two major risk factors of atherosclerosis, in elderly men. Inflammation and oxidative stress may represent a possible common pathogenetic link between established risk factors for atherosclerosis and atherogenesis.

Keywords: prostaglandin $F_{2\alpha}$, F_2 -isoprostane, interleukin-6, C-reactive protein, serum amyloid A, tocopherols, cardiovascular risk factors, variation, inflammation, oxidative stress, human

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ISSN 1651-6206

ISBN 91-554-6156-5

urn:nbn:se:uu:diva-4803 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4803>)

LIST OF PAPERS

This thesis is based on the papers listed below, which will be referred to in the text by their Roman numerals.

- I Helmersson J., Basu S. F₂-isoprostane excretion rate and diurnal variation in human urine. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 1999, 61: 203–5
- II Helmersson J., Basu S. F₂-isoprostane and prostaglandin F_{2α} metabolite excretion rate and day to day variation in healthy humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 2001, 65: 99–102
- III Helmersson J., Vessby B., Larsson A., Basu S. Association of type 2 diabetes with cyclooxygenase-mediated inflammation and oxidative stress in an elderly population. *Circulation*, 2004, 109: 1729–34
- IV Helmersson J., Larsson A., Vessby B., Basu S. Active smoking and a history of smoking are associated with enhanced prostaglandin F_{2α}, interleukin-6 and F₂-isoprostane formation in elderly men. *Atherosclerosis*, 2005, *In press*
- V Helmersson J., Ärnlov J., Vessby B., Larsson A., Alftan G., Basu S. Serum selenium predicts formation of F₂-isoprostanes and prostaglandin F_{2α} in a 27 year follow-up study of Swedish men. Submitted for publication

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Related papers (not included in the thesis):

Helmersson J., Vessby B., Larsson A., Basu S. Cyclooxygenase-mediated prostaglandin $F_{2\alpha}$ is decreased in elderly population treated with low-dose aspirin. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 2005, *In press*

Basu S., Helmersson J. Factors regulating isoprostane formation in vivo. *Antioxidants & Redox Signaling*, 2005, 7: 221–235

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ABBREVIATIONS

ANOVA	analysis of variance
b	regression coefficient
BMI	body mass index
CE	cholesterol ester
CLA	conjugated linoleic acid
COX	cyclooxygenase
HsCRP	high sensitivity C-reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
DHA	docosahexaenoic acid, 22:6 n-3
EPA	eicosapentaenoic acid, 20:5 n-3
GC-MS	gas chromatography-mass spectrometry
GSHPx	glutathione peroxidase
HbA _{1c}	hemoglobin A _{1c}
HDL	high density lipoprotein
5-HPETE	5-hydroperoxyeicosatetraenoic acid
ICD	International statistical classification of diseases and related health problems
IL-6	interleukin-6
8-Iso-PGF _{2α}	8-iso-prostaglandin F _{2α}
LDL	low density lipoprotein
5-LO	5-lipoxygenase
LTA ₄	leukotriene A ₄
LXA ₄	lipoxin A ₄
n	number
NFκB	nuclear factor κB
NSAID	non-steroidal anti-inflammatory drugs
P	probability
15-PGDH	15-hydroxy prostaglandin dehydrogenase
PGF _{2α}	prostaglandin F _{2α}
PGI ₂	prostacyclin
PLA ₂	phospholipase A ₂
r	correlation coefficient
SAA	serum amyloid A protein
s	serum
TNF-α	tumour necrosis factor α

INTRODUCTION

Inflammation

Inflammation is a complex process, involving white blood cells, plasma, endothelium and tissue, and is associated with various diseases. In the acute phase of the inflammatory reaction, platelets, macrophages, mast cells and the complementary system are activated, due to tissue injury. These cells communicate and interact with endothelial cells using different mediators such as serotonin, prostaglandins, thromboxanes, leukotrienes and histamine. This interaction results in changes in the vascular tonus (vasoconstriction and vasodilatation), increased vasopermeability and subsequent formation of tissue oedema. Circulating leukocytes adhere and migrate through the endothelium into the tissue due to adhesion molecules and chemotactic mediators that are formed at sites of tissue injury and from the activated leukocytes. Leukocytes (macrophages) in the tissue will gather more leukocytes from the circulation by formation of chemotactic factors and cytokines (interleukin-1 [IL-1], IL-8, tumour necrosis factor α [TNF- α]). The cytokines stimulate the formation of cyclooxygenase (COX)-mediated products in various celltypes¹ mainly by inducing COX-2 expression^{2,3} in damaged tissue. The cytokine interleukin-6 (IL-6), formed by recruited white blood cells, fibroblasts and endothelial cells, starts a systemic response to a local inflammatory lesion.⁴ This results in formation of acute phase proteins (C-reactive protein [CRP], serum amyloid A [SAA] and other) in the hepatocytes which are subsequently excreted into the circulation. Chronic inflammation occurs when the elimination process for different reasons is disturbed, or if the tissue injury is of a chronic nature. Predominantly macrophages, plasma cells and lymphocytes are recruited from the circulation and gather in the tissue. The inflammatory mediators in the chronic stage are substantially the same as in the acute phase.

Quantification of inflammatory response

Inflammation *in vivo* can be quantified by various indicators reflecting different segments of the inflammatory reaction. In this thesis COX-mediated

prostaglandins, a cytokine and cytokine-mediated acute-phase proteins, are studied.

Eicosanoids derived by the COX and lipoxygenase pathway

Eicosanoids are oxygenated metabolites of the 20-carbon unsaturated fatty acid arachidonic acid. The eicosanoids derived enzymatically include prostaglandins,⁵ thromboxanes,⁶ leukotrienes⁷ and lipoxins.⁸ They are formed from free arachidonic acid which has been de-esterified from the phospholipids by phospholipase A₂ (PLA₂), and other hydrolyzing enzymes.

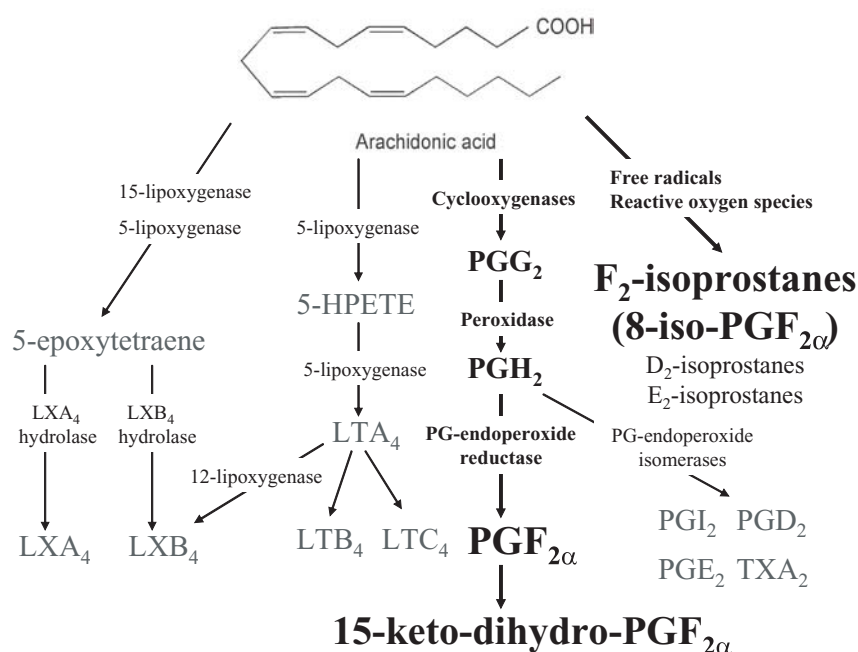


Figure 1. Cascade of eicosanoids formed from arachidonic acid.

Prostaglandin F_{2α} (PGF_{2α}), PGE₂, PGI₂ (prostacyclin), PGD₂, PGA₂ and thromboxane A₂ (TXA₂) are formed through the unstable intermediates PGG₂ and PGH₂ by activation of COX (see Figure 1). {Samuelsson, 1979-80 #647} The pattern of the PG endoperoxide reductase and isomerases expressed, respectively, may influence the profile of prostanoids produced in the particular cell. The prostanoids are potent mediators and modulators of inflammation¹⁰⁻¹² and coagulation, regulators of vascular tonus and are also involved in reproduction.¹³ COX-1 is constitutively expressed in a number of cells and tissues, and as stated above catalyses the formation of primary

prostaglandins that are involved in various physiological functions.¹⁴ COX-2 is upregulated at sites of inflammation and the eicosanoids produced are presumed to be regulators in the inflammatory reaction.^{2,3} It is now believed that both COX-1 and COX-2 might be of importance for both physiological regulation and in pathophysiological states of inflammation.¹⁵ Very recently a third type of COX has been presented. “COX-3” appears to be a splice variant of COX-1 and is speculated to be inhibited by paracetamol.¹⁶

Leukotriene A₄ (LTA₄) is formed through the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by 5-lipoxygenase (5-LO) and can be further converted to LTB₄ and LTC₄. {Samuelsson, 1979-80 #408} Leukotrienes are bronchoconstrictive, vasoconstrictive and mediators in the inflammatory process. Leukotriene antagonists are used as medication for asthma bronchiale.¹⁸

Lipoxin A₄ (LXA₄) and LXB₄ are formed either through 5-epoxytetraene by 15-LO, 5-LO and LXA₄ or LXB₄ hydrolase, respectively, or by 5-LO and 12-LO interactions (see Figure 1). Recently, a third pathway of lipoxin formation, namely aspirin-triggered lipoxins (15-epi-LXA₄, 15-epi-LXB₄), has been discovered.¹⁹ 15-epi-LXA₄ and 15-epi-LXB₄ can be formed by 5-LO in the resolution phase of inflammation through aspirin induction, by involving COX-2. Lipoxins and aspirin-triggered lipoxins are thought to possess anti-inflammatory effects.

Prostaglandin F_{2α}

PGF_{2α} is a major prostaglandin formed *in vivo* in physiological and pathophysiological situations and has mainly potent vasoconstrictive and pro-inflammatory properties (see Figure 2). The major research on PGF_{2α} has been done in the fields of reproduction, renal function and regulation of intraocular pressure. A PGF_{2α}-analogue, latanoprost, is today frequently used to decrease the intraocular pressure in glaucoma patients.²⁰

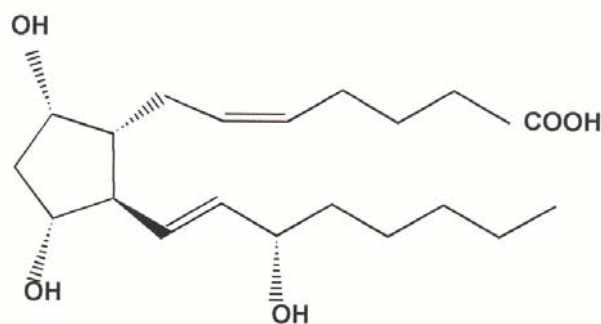


Figure 2. Molecular structure of PGF_{2α}. Figure by S. Basu.

PGF_{2α} metabolizes via 15-hydroxy prostaglandin dehydrogenase (15-PGDH) and Δ^13 -reductase to form 15-keto-dihydro-PGF_{2α} (see figure 3).^{21,22}

PGF_{2α} has a very short half-life in plasma (<1 min)²¹ and can easily be formed as an artefact during collection and handling of samples.²³ Due to these reasons PGF_{2α} quantification in plasma and urine is not reliable. The major metabolite in plasma, 15-keto-dihydro-PGF_{2α}, has a longer half-life, is not formed as an artefact and should preferably be used as an indicator of PGF_{2α}.^{14,22,23}

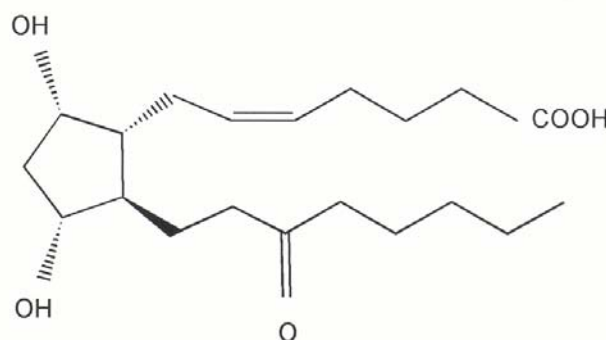


Figure 3. Molecular structure of 15-keto-dihydro-PGF_{2α}. Figure by S. Basu.

The knowledge about PGF_{2α} *in vivo* is limited due to mainly methodological difficulties of quantification. A specific and validated radioimmunoassay for the detection of 15-keto-dihydro-PGF_{2α} developed by Basu is used in this thesis.²⁴ With this assay the PGF_{2α} metabolite can be reliably quantified in different compartments, including plasma and urine. The analysis method is particularly suitable for large cohort studies. The original suggestion of PGF_{2α} being a pro-inflammatory mediator *in vivo* has recently been verified in experimental studies by the use of this radioimmunoassay. Increased PGF_{2α} formation in plasma, urine and myocardium after induction of inflammation with endotoxin infusion have been shown in several animal studies.²⁵⁻²⁷ Further, PGF_{2α} formation was increased in plasma and joint fluid in patients with severe rheumatic arthritis.²⁸

The role of PGF_{2α} in chronic subclinical inflammation, for example in atherosclerosis, is not known. The role of PGF_{2α} in diseases associated with cardiovascular complications and atherosclerosis, including type 2 diabetes, smoking, hypertension, hyperlipidemia and obesity has not been studied. The use of PGF_{2α} as an indicator of COX-mediated inflammation *in vivo* in different clinical conditions and experimental settings in humans requires knowledge about the extent of the biological variation of this prostaglandin.

To increase the comprehension of this thesis the name of the primary prostaglandin PGF_{2α} is frequently used. When referring to *in vivo* studies this

always means that quantification of $\text{PGF}_{2\alpha}$ has been made by quantification of 15-keto-dihydro- $\text{PGF}_{2\alpha}$ as earlier discussed. Similarly, TXA_2 formation *in vivo* means that quantification have been made by measurement of its stable metabolites 11-dehydro- TXB_2 or 2,3-dinor- TXB_2 . PGI_2 formation *in vivo* means quantification of 2,3-dinor-6-keto- $\text{PGF}_{1\alpha}$ and never 6-keto- $\text{PGF}_{1\alpha}$.

Cytokine-related substances

The cytokines include a myriad of polypeptides or glycoproteins that mediate pro-inflammatory and anti-inflammatory effects. Cytokines can mediate effects through cell-surface receptors in a variety of cell types, locally or systemically. Most cytokine activity results in an altered gene expression in the target cell. A large number of cohort studies have been undertaken to study cytokines and acute phase proteins in relation to cardiovascular disease and cardiovascular risk factors.

IL-6

IL-6 has received particular interest in the field of atherosclerosis. IL-6 has a variety of biological activities including stimulation and activation of B-cells, T-cells and macrophages and induction of acute-phase proteins in hepatocytes. IL-6 might also possess anti-inflammatory properties. Circulating high levels of IL-6 has been associated with an unfavourable clinical outcome in patients hospitalized for unstable angina pectoris²⁹ and shown to predict myocardial infarction in apparently healthy men.³⁰

CRP

CRP is an acute phase protein mainly formed in the hepatocytes under the control of IL-6, and maybe also IL-1 and $\text{TNF-}\alpha$. During acute infection or inflammation CRP can rise up to 100-fold.³¹ The basic biological function originally described for CRP was the ability to detect and bind to phosphocholine in damaged and apoptotic cells and activate the complement pathway. Recent data suggest a more complex role for CRP in the inflammatory process which includes both pro-inflammatory and anti-inflammatory involvement.

Highly sensitive assays for CRP (hsCRP) have been developed to allow a more detailed description of the protein in the low concentration range (<10 mg/L). Thus, slightly increased CRP levels (>3 mg/L) has been interpreted as an indicator of chronic low grade inflammation, a condition which will remain unnoticed with the conventional detection assays of CRP. Several reports have focused on the possible role for hsCRP in atherogenesis.^{32,33} HsCRP has been shown to predict different cardiovascular events, stroke and sudden death.³⁴

SAA

Like CRP, SAA is an acute phase protein primarily formed in the liver by control of IL-1, IL-6 and TNF- α and is possibly dependent on activation of the transcription factor nuclear factor κ B (NF κ B). SAA can increase up to 1000-fold in plasma in response to an acute inflammation.^{35,36} SAA has the features of an apolipoprotein and is incorporated into the high density lipoprotein (HDL) particle during inflammation. In analogy with CRP, highly sensitive assays for SAA detection have been developed to detect chronic low-grade inflammation. Slightly increased levels of SAA in apparently healthy people predict coronary heart disease.³⁷

Oxidative stress

Oxidative stress is a condition of excess formation of free radicals either by physiological and pathophysiological processes, such as diseases and states of insufficient antioxidative defence. A free radical is a molecule that exists independently and contains one or more unpaired electrons (marked with \bullet). Examples of free radicals are OH \bullet (hydroxyl radical), O $_2^{\bullet-}$ (superoxide radical), RO $_2^{\bullet}$ (peroxyl radical). Because of the unpaired electrons, free radicals are extremely reactive and react instantly with different biological molecules including lipids, DNA and protein. When free radicals start to attack long-chain polyunsaturated fatty acids this may result in several rapid chain reactions. Henceforth, the term oxidative stress in this thesis refers to oxidative modification of lipids.

Quantification of oxidative stress

A large number of assays are available to quantify oxidative stress and oxidative damage to lipids including conjugated dienes, thiobarbituric acid-reactive substances (TBARS), malondialdehyde (MDA) and hydroperoxides. Most of them are suitable for quantification of oxidative stress *in vitro*.

Isoprostanes

In 1990, Morrow and Roberts discovered the isoprostanes.³⁸ These compounds later became the golden standard indicators of free radical mediated lipid peroxidation *in vivo*.³⁹⁻⁴¹ Isoprostanes belong to a family of prostaglandin derivatives that are mainly formed by peroxidation of arachidonic acid catalysed by free radicals (see Figure 1). Unlike primary prostaglandins, isoprostanes do not require COX for their biosynthesis. Another difference from the biosynthesis of primary prostaglandins is that isoprostanes are

formed *in situ*, esterified to phospholipids, and released in the free form by mainly phospholipases.⁴² When free radicals attack arachidonic acid four PGG₂-like endoperoxide intermediates are formed which are further reduced to four different chemically stable F₂-isoprostane regioisomers. These are named 5-, 12-, 8- or 15-series F₂-isoprostanes depending on the location of the hydroxyl-group.^{43,44} Each regioisomer includes eight diastereomers, and thus 64 different F₂-isoprostanes can be generated. Depending on the structure of the cyclopentane ring other isoprostanes of type D₂, E₂, A₂, J₂ or isothromboxanes can be formed.

8-Iso-PGF_{2α}

8-Iso-PGF_{2α} (also called 15-F_{2t}-IsoP⁴³, iPF_{2α}-III⁴⁵) is one major 15-series F₂-isoprostane (see Figure 4). This isomer possesses biological effects *in vivo*, including vasoconstriction and bronchoconstriction. 8-Iso-PGF_{2α} may further cause platelet aggregation, induce DNA synthesis in endothelial cells, and enhance granulocyte activity and adhesion to endothelial cells.⁴⁶ These effects may possibly be mediated by interaction with the TXA₂ receptor.⁴⁷ A unique isoprostane receptor has been proposed⁴⁸ but needs to be confirmed in further studies.

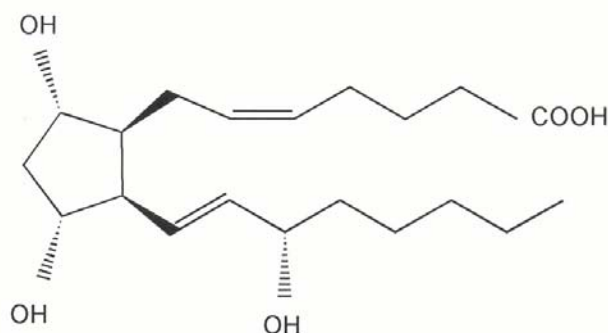


Figure 4. Molecular structure of 8-iso-PGF_{2α}. Figure by S. Basu.

8-Iso-PGF_{2α} in humans is metabolized by 15-PGDH to 15-keto-8-iso-PGF_{2α} and further by Δ^13 -reductase to 15-keto-dihydro-8-iso-PGF_{2α}. 15-keto-dihydro-8-iso-PGF_{2α} is finally metabolized by β -oxidation to 2,3-dinor-5,6-dihydro-8-iso-PGF_{2α} or 2,3-dinor-8-iso-PGF_{2α} with a half-life of 16 min.⁴⁹⁻⁵¹ The dinor-metabolites are excreted to the urine and the parent compound 8-iso-PGF_{2α} is excreted to the urine when formed in excess.

Free or total (free and esterified) 8-iso-PGF_{2α} can be quantified with different analysis techniques including gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC)-MS, enzymeimmunoassays and radioimmunoassays.⁵² Immunoassays are particularly suitable for large cohort

studies. A specific and validated radioimmunoassay developed by Basu has been used in this thesis.^{53,54}

8-Iso-PGF_{2α} formation has been shown to be increased in pathophysiological states of oxidative stress and inflammation in animals and humans, including ischemia-reperfusion injury, risk factors related to atherosclerosis, and experimental settings of oxidative stress and inflammation as recently reviewed.^{39,52,55-57} The use of F₂-isoprostanes as indicators of oxidative stress *in vivo* in different clinical conditions and experimental settings in humans requires knowledge as to how levels of this indicator vary during the day and between days in healthy humans. Studies that deal with these issues are surprisingly rare compared to the number of studies that use F₂-isoprostanes as an indicator of oxidative stress.

Collection of samples for quantification

8-Iso-PGF_{2α} can be quantified in several body fluids and tissues. Both plasma and urine measurement are believed to accurately show the systemic level of 8-iso-PGF_{2α}.^{44,52,55} Due to the short half-life and rapid excretion of 8-iso-PGF_{2α} it is important to imply accuracy in sampling schedule in the planning of a study.⁴⁹ To detect acute changes in 8-iso-PGF_{2α} formation, as in an acute oxidative injury during an experimental or intervention study, a frequent continuous sampling of preferably plasma is required due to the rapid formation and excretion of the compound. In situations where a more stable basal increase or decrease is expected e.g. after dietary supplementation, or in patients with chronic diseases, a urine sample mirrors a longer period of time and is therefore more reliable. Further, urine collection is non-invasive, and the storage of samples is easier since there is no evidence that isoprostanes are formed as artefacts during handling and storage in urine samples as in plasma samples.⁵⁸ However, a 24-hour urinary collection requires good compliance of the subject or patient studied and a single urine sample is easier to collect for practical reasons. Whether a single urine sample reflects the daily excretion of 8-iso-PGF_{2α} accurately is not known.

Antioxidants

Vitamin E

Vitamin E is a lipid-soluble antioxidant present in plasma. The compound scavenge peroxyl radicals and is probably the most important inhibitor of free-radical induced chain reactions of lipid peroxidation. There are eight known substances with vitamin E activity, α-, β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol. α-Tocopherol is the most abundant form of vitamin E in human tissues and plasma and has the greatest biological activity, followed by γ-tocopherol. When vitamin E acts as an antioxidant it donates a hydro-

gen atom to the fatty acid chain radical and thereby becomes a free radical itself. Thus, vitamin E might have a pro-oxidant effect. Vitamin C has been proposed to recycle the vitamin E radical to its reduced state.⁵⁹ The activity of vitamin E may also include other features not related to the chain-breaking antioxidative effect. γ -Tocopherol has been proposed to have anti-inflammatory properties *in vitro* and *in vivo* independent of the antioxidant activity.^{60,61}

Prospective observational studies suggest that a high intake of vitamin E (hence α -tocopherol) by diet or supplements may reduce the risk for myocardial infarction or stroke.⁶² However, clinical trials with α -tocopherol supplementation have not shown any reduction in cardiovascular incidence. γ -Tocopherol in serum has been shown to be reduced in patients with coronary heart disease.⁶³

Selenium

The essential trace element selenium is present in the soil of the earth in varying doses depending on geography. As a consequence, serum (s)-selenium levels vary largely within populations.⁶⁴ The major sources for dietary selenium in Sweden are meat and poultry (24%), fish and shellfish (23%), dairy products (14%) and egg (10%).⁶⁵ Most dietary selenium is incorporated into non-specific selenium-binding proteins or specific selenoproteins including selenoprotein P, selenoprotein W and different glutathione peroxidases (GSHPxs). GSHPxs are antioxidative enzymes that remove non-radicals and free radicals, such as hydrogen peroxide and lipid hydroperoxides, by catalyzation of the oxidation of reduced glutathione (GSH). In addition, it has been proposed that GSHPxs might be involved in the arachidonic acid metabolism by reducing the hydroperoxide PGG₂ to PGH₂ and thereby influence the formation of prostaglandins and thromboxane, hence GSHPxs may regulate inflammation.⁶⁶ The proposed antioxidative and anti-inflammatory capacity of Se and GSHPxs remains to be confirmed *in vivo*.

Atherosclerosis

Atherogenesis

Atherosclerosis is a complex and on-going process, eventually leading to the development of cardiovascular diseases (CVD). CVD is the major cause of death in Europe, United States and Asia.⁶⁷ Atherogenesis is mainly accumulation of lipids in the arteries accompanied by an on-going chronic inflammatory reaction.^{68,69} LDL is transported in a dose-dependent fashion into the artery wall and undergoes oxidation.⁷⁰ The subsequent endothelial dysfunction is a consequence of the injury inflicted by oxidized LDL or other inju-

ries elicited by excess free radicals e.g. due to disease or cigarette smoking. Macrophages and T-lymphocytes migrate into the intima due to inflammatory mediators in the endothelium. Macrophages accumulate oxidized LDL, form foam cells, and subsequently fatty streaks, the first visible sign of an atherosclerotic lesion.⁷¹ Smooth muscle cells migrate into the intima and form intermediate lesions⁷¹ and platelets adhere and aggregate on the endothelial surface. The advanced, complicated atherosclerotic lesion consists of macrophages and lipids in a necrotic core covered by a fibrous cap visible in the artery lumen. All these steps in the atherosclerotic process are mediated by a number of mediators produced by the endothelium and the leukocytes at the site of progression, including eicosanoids,⁷² adhesion molecules,^{73,74} oxidized LDL,⁷⁰ and cytokines.⁴

Risk factors for atherosclerosis

Several clinical risk factors for development of atherosclerosis have been described, including smoking, hyperlipidemia, hypertension, diabetes mellitus, obesity and the metabolic syndrome (insulin resistance syndrome). The processes involved in the accelerated development of atherosclerosis, and subsequent CVD due to these risk factors are not fully understood, but might include chronic inflammation and oxidative stress.

Type 2 diabetes

Type 2 diabetes mellitus is a disease accompanied by hyperglycemia. The disease is rapidly increasing throughout the world together with overnutrition, obesity and low physical activity level. The main underlying causes are thought to be loss of insulin sensitivity and progressive pancreatic β -cell failure. A majority of the patients with type 2 diabetes develop macro- and microvascular complications in conjunction with low-grade inflammation.

Smoking

Smoking is still common worldwide despite the established association between cigarette smoking and development of coronary heart disease and stroke. The risk of disease due to smoking is dose-related and there is a reduced risk of coronary heart disease among former smoker compared to active smokers.⁷⁵

Selenium

Low levels of selenium might be a risk factor for development of CVD in some populations (Finland, Denmark)^{76,77} but not in other (Netherlands, US).^{78,79} Previously published data from the ULSAM cohort indicated that low levels of s-selenium may predict all cause mortality in univariate analysis.⁸⁰

AIMS

The two main aims of this thesis were:

- to study intra-individual variation of prostaglandin and isoprostane formation in healthy subjects, and

- to study prostaglandin and isoprostane formation as indicators of COX-mediated inflammation and oxidative stress, respectively, in a cohort of Swedish men in relation to risk factors for atherosclerosis.

More specific aims of the studies were:

- to study day-to-day intra-individual variation of a prostaglandin ($\text{PGF}_{2\alpha}$) and an F_2 -isoprostane (8-iso- $\text{PGF}_{2\alpha}$) in urine in healthy humans (Paper II),

- to study whether spot urine samples, in the morning or during the day, reflect the diurnal 8-iso- $\text{PGF}_{2\alpha}$ excretion in healthy humans (Paper I),

- to study $\text{PGF}_{2\alpha}$ and 8-iso- $\text{PGF}_{2\alpha}$ formation and cytokine-mediated inflammatory indicators, respectively, in relation to type 2 diabetes in elderly Swedish men (Paper III),

- to study the $\text{PGF}_{2\alpha}$ and 8-iso- $\text{PGF}_{2\alpha}$ formation and cytokine-mediated inflammatory mediators, respectively, in elderly Swedish active and former smoking men (Paper IV), and

- to study the effect of s-selenium on $\text{PGF}_{2\alpha}$ and 8-iso- $\text{PGF}_{2\alpha}$ formation, and on cytokine-mediated inflammatory mediators, respectively, in a follow-up study of Swedish men (Paper V).

METHODS

Study participants and design

Paper I

Urinary samples were collected from healthy volunteers, five men and five women (age 28–59), during 24 hours with the use of a 24-h urine collecting cup.

Paper II

Morning urinary samples were collected from 10 healthy female and 3 healthy male volunteers (age 22–59 years) in 10 successive days.

Paper III-V

Uppsala Longitudinal Study of Adult Men (ULSAM)

Paper III-V are based on the reinvestigation of the population-based ULSAM-cohort, which was performed in 1997–2001, when the participants were approximately 77 years old. Information from the previous reinvestigations ULSAM-70 (1991–1995), ULSAM-60 (1980–1985) and the original investigation ULSAM-50 (1970–1973) is also included in the thesis. The ULSAM survey has been previously described in several reports^{81,82} and is described in detail on the ULSAM homepage (<http://www.pubcare.uu.se/ULSAM>). In brief, the cohort was originally started in 1970–73, when all men born 1920–1924 and living in Uppsala, were invited to participate in a health screening (at age 50). Out of 2841 invited men 2322 participated (82%). At age 60, 2130 eligible men were invited and 1860 agreed to participate. At the following reinvestigation at age 70, 1681 eligible men were invited and 1221 agreed to participate. Finally, in the last reinvestigation at age 77, 1398 eligible men were invited and 839 agreed to participate. The Ethics Committee at Uppsala University approved the study and all participants gave informed consent.

Study participants

Paper III

This paper describes a cross-sectional study based on the participants in the reinvestigation of ULSAM at age 77. Out of the 839 men who agreed to participate in the reinvestigation, 765 men had a plasma glucose value and a completed medical questionnaire, thus making it possible to classify the subject as a patient with diabetes or a control (see Figure 5). Urinary samples for analysis of prostaglandins and isoprostanes were collected in 101 patients with diabetes and 585 controls. Men with insulin therapy alone ($n = 3$) were excluded to avoid the risk of including men with type 1 diabetes. The men with type 2 diabetes were divided into two groups: newly diagnosed (<7 years since diagnosis), or diabetes diagnosis ≥ 7 years ago (see Figure 5). Urine was available in 40 of the newly diagnosed men and in 54 with a diabetes diagnosis at least since the age of 70. A subgroup of men without a clinical history of myocardial infarction, angina pectoris or ischemic stroke, and without intake of low-dose aspirin or non-steroidal anti-inflammatory drugs (NSAID) was additionally analyzed. Out of these, urine samples were available in 50 patients with diabetes and 349 controls.

Paper IV

This paper is based on participants from the reinvestigation at age 77. Out of 839 men, 763 had completed the questionnaire and serum samples were available. Men with diabetes and unknown diabetes status were excluded (see Figure 5). The remaining men in the study population were classified as current smoker, former smoker and non-smoker. Urine samples for analysis of prostaglandins and isoprostanes were available in 44 current smokers, 359 former smokers and 178 non-smokers. The former smokers were classified into 6 different groups according to their reported time since smoking cessation. Urine samples were available in 23 subjects who quit smoking within the last year, 54 who quit smoking 1–5 years ago, 14 who quit smoking 6–7 years ago, 48 who quit smoking 8–17 years ago, 89 who quit smoking 18–27 years ago and 131 who quit smoking more than 28 years ago.

Paper V

This paper describes a longitudinal study with participants from the reinvestigation at age 77 who also attended the original investigation at age 50. Out of the 839 men at the reinvestigation at age 77, 706 men had urine samples for analysis of prostaglandins and isoprostanes. Out of these, 615 attended the original investigation at age 50 and had a serum sample analyzed for selenium, thus constituting the present study population. Baseline s-selenium data were related to fatty acid composition at age 50, dietary data and fatty acid composition at age 70 and fatty acid composition at age 77 (see Figure 6 for number of men in respective analysis).

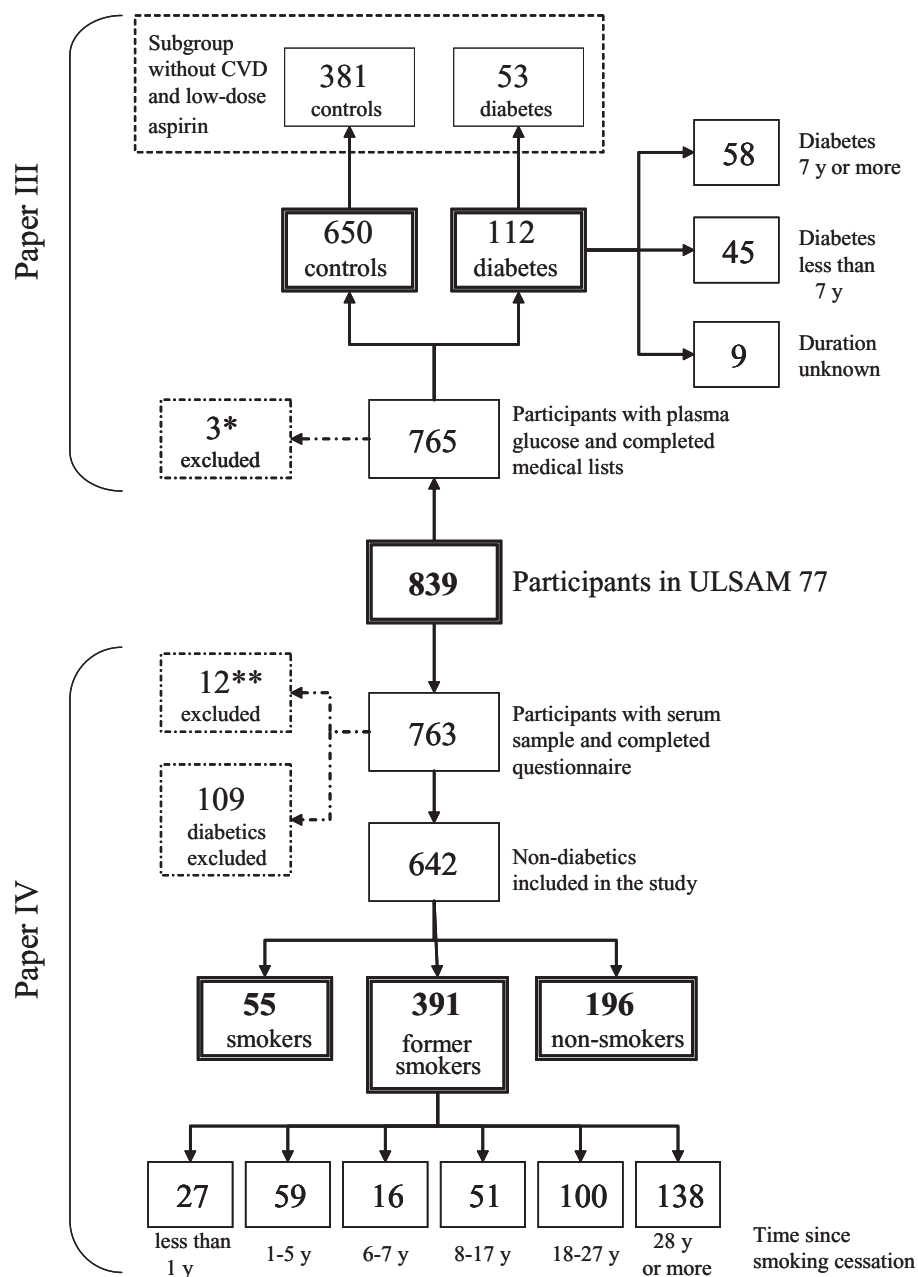


Figure 5. Outline of study participants in Paper III and IV.

* Excluded because of insulin treatment only.

** Excluded because of unknown diabetes status.

CVD, previous history of myocardial infarction, angina pectoris and ischemic stroke.

Paper III-V

At age 77 (Paper III-V)

The 44 first men (of the 839) invited to the ULSAM reinvestigation at 77 years were not asked to give blood or urine samples. Of the other invited men, 706 participants collected 24-hour urine samples. The samples were stored frozen at -70°C until analysis. Blood samples were drawn from the antecubital vein after an overnight fast (12 h) in 792 participants. Plasma was separated and stored at -70°C .

Body weight was measured to the nearest 0.1 kg and height to the nearest cm. BMI (body mass index) was calculated as the ratio of the weight (in kg) to the height (in m) squared. Waist circumference was measured midway between the lowest rib and the iliac crest to the nearest cm. Blood pressure was measured to the nearest even mmHg with the subject in the supine position after 10 min of rest. The blood pressure values reported are the mean value of two measurements.

Information about smoking habits was obtained by a self-administered questionnaire.⁸³ Information concerning pharmacological treatment was recorded with a questionnaire. Information about hospitalization because of myocardial infarction, angina pectoris, ischemic stroke and heart failure was obtained from the Swedish Hospital Discharge Registry.

At age 70 (Paper III-V)

Blood samples were drawn after an overnight fast and stored in -70°C until analysis.

Current smoking status was obtained during an interview by a nurse or a technician and previous smoking habits by a questionnaire. Pharmacological treatment was recorded with a questionnaire. A 7-day dietary assessment was performed.

At age 60 (Paper IV)

Participants were asked to fill in a questionnaire about their smoking habits.

At age 50 (Paper IV and V)

Blood samples were drawn after an overnight fast and stored in liquid nitrogen before analysis.

Body weight was measured to the nearest kg and height to the nearest cm and BMI was calculated. Blood pressure was measured with the subject in the supine position after 10 min rest and was read to the nearest 5 mmHg.

Current smoking habits were obtained during interviews by the same physician in all participants and previous smoking habits by a questionnaire. Pharmacological treatment was recorded with a questionnaire.

Biochemical analyses

PGF_{2α} (paper II-V)

The urinary samples (50 µl) were analysed without any prior extraction or purification for 15-keto-dihydro-PGF_{2α} by a radioimmunoassay developed by Basu described in detail elsewhere.²⁴ In brief, an antibody was raised in rabbits by immunisation with 15-keto-13,14-dihydro-PGF_{2α} coupled to bovine serum albumin (BSA) at the carboxylic acid by 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibody with PGF_{2α}, 15-keto-PGF_{2α}, PGE₂, 15-keto-13,14-dihydro-PGE₂, 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 11β-PGF_{2α}, 9β-PGF_{2α}, TXB₂, 8-iso-PGF_{3α} was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001 and 0.01% respectively. The detection limit was approximately 45 pmol/l. The intra-assay coefficient of variation (CV) was 12.2% at low concentrations and 14.0% at high concentrations. Levels of 15-keto-dihydro-PGF_{2α} were corrected for urinary creatinine.

F₂-isoprostanes (Paper I-V)

The urinary samples (50 µl) were analysed without any prior extraction or purification for free 8-iso-PGF_{2α} by a radioimmunoassay developed by Basu and described in detail elsewhere.⁵³ In brief, an antibody was raised in rabbits by immunisation with 8-iso-PGF_{2α} coupled to BSA at the carboxylic acid by 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibodies with closely related eicosanoids and eicosanoid metabolites including 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 8-iso-PGF_{2β}, PGF_{2α}, 15-keto-PGF_{2α}, 15-keto-13,14-dihydro-PGF_{2α}, TXB₂, 11β-PGF_{2α}, 9β-PGF_{2α} and 8-iso-PGF_{3α} was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6% respectively. The detection limit of the assay was approximately 23 pmol/l. The intra-assay CV was 14.5% at low concentrations and 12.2% at high concentrations. Levels of 8-iso-PGF_{2α} were corrected for urinary creatinine.

Creatinine (Paper I-V)

Creatinine concentration was determined in each urine sample by a colorimetric method using IL Test creatinine 181672-00 in a Monarch 2000 centrifugal analyser (Instrumentation Laboratories, Lexington, MA, USA).

CRP and SAA (Paper III-V)

High sensitivity CRP and SAA measurements were performed by latex enhanced reagent (Dade Behring, Deerfield, IL, USA) with the use of a Behring BN ProSpec analyzer (Dade Behring). The intra-assay CV of the

hsCRP method was 1.4% at both 1.23 mg/L and 5.49 mg/L and the intra-assay CV of the SAA method was 5.9% at 12.8 mg/L and 3.2% at 81.7 mg/L. One hsCRP outlier (116 mg/L) and two SAA outliers (573, 736 mg/L) were excluded from the statistical analysis.

IL-6 (Paper IV-V)

IL-6 was analyzed by an ELISA kit (IL-6 HS, R&D Systems, Minneapolis, MN). Samples and standards were pipetted in a microtiter plate coated with monoclonal antibody against IL-6. After incubation and washing enzyme substrate solution was pipetted followed by anti-IL-6 antibody. The colour reaction was proportional to the bound IL-6. The total CV of the method was 7% and inter-assay CV was 5%.

Antioxidants (Paper III-V)

Tocopherols and β -carotene

At age 77

Serum α - and γ -tocopherol were analysed with high performance liquid chromatography with a Hitachi pump and LiChrospher 100 NH₂, 250 x 4 mm column.⁸⁴ The fluorescence detector had an excitation wavelength of 395 nm and an emission wavelength of 327 nm. Tocopherol levels were corrected for the sum of total cholesterol and triglyceride concentrations.⁸⁵ Intra-assay CV for the method is 4.5% for α -tocopherol and 7.2% for γ -tocopherol.

At age 50

α -Tocopherol and β -carotene were simultaneously determined by high-performance liquid chromatography in serum samples that had been stored in liquid nitrogen for about 15 years. A diode-array detector at a wavelength of 292 nm and 460 nm for α -tocopherol and β -carotene, respectively, was used to measure the light absorption of the compounds. The inter-assay CV was 6.9% for α -tocopherol and 6.5% for β -carotene.⁸⁶ Tocopherol levels were corrected for the sum of total cholesterol and triglyceride concentrations.⁸⁵

Selenium

Se was determined in serum samples at age 50 that had been stored in liquid nitrogen for about 15 years, with the use of the graphite-furnace atomic absorption spectrometric method as described by Alfthan and Kumpulainen.⁸⁷ In brief, samples were diluted (1+9) with a solution containing nickel nitrate and nitric oxide and measured by a standard additions method. CV of the method was 4.4% at 87 μ g/L.

Other biochemical analyses and dietary assessment (Paper III-V)

At age 77

Serum cholesterol and triglycerides were analyzed by enzymatic techniques with the use of IL Test Cholesterol Trinder's Method 181618-10 and IL Test Triglyceride Enzymatic-colorimetric Method 181610-60 in a Monarch apparatus (Instrumentation Laboratories, Lexington, USA). HDL particles were separated by precipitation with magnesium chloride and phosphotungstate.⁸⁸ LDL cholesterol was calculated with Friedewald's formula: $LDL = \text{serum cholesterol} - HDL - (0.42 \times \text{serum triglycerides})$.

Plasma glucose concentrations were analyzed by the glucose dehydrogenase method (Gluc-DH, Merck, Darmstadt, Germany). Intra-individual CV was 3.2%. HbA_{1c} was analyzed with fast performance liquid chromatography (Bio-Rad). Plasma insulin was assayed with an enzymatic immunological assay (Mercodia) in a Coda Automated EIA Analyzer (Bio-Rad Laboratories, CA, USA).

Fatty acid composition in serum cholesterol esters was analysed in a subsample of the men with diabetes and healthy controls with the use of thin layer chromatography and gas-liquid chromatography (Hewlett Packard GLC system). Extraction, methylation and separation of the serum lipids has previously been described in detail by Boberg et al.⁸⁹ The amounts of eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), are given in percentage of the total sum of the fatty acids analyzed.

At age 70

Plasma glucose was analyzed as described above. Fatty acid composition in serum cholesterol esters were analyzed in a subsample of the men as described above.

A dietary assessment was performed at age 70 with the use of a 7-day dietary record which was optically readable. The precoded menu-book has previously been used⁹⁰ and validated by Becker et al.⁹¹ The daily intake of selenium, EPA and DHA were calculated by use of information from a database from the National Food Administration (SLV database, 1990).

At age 50

Blood glucose was analyzed with the glucose oxidase method with the use of spectrophotometry. Serum cholesterol and triglyceride concentrations were measured in samples stored on liquid nitrogen for about 10 years with the use of a Technicon dual-channel system (N-70 and N24a), respectively.⁸¹ The values were multiplied with a conversion factor (1.06 for serum cholesterol and 0.9 for serum triglycerides) to enable comparisons with the method used at age 77.

Fatty acid composition in serum cholesterol esters were measured on serum samples that had been stored in liquid nitrogen for about eight years.

The analyses were performed with thin-layer chromatography and gas-liquid chromatography as previously described.⁹² The amounts of EPA and DHA are given in percentage of the total sum of the fatty acids analyzed.

Definitions (Paper III-V)

Type 2 diabetes diagnosis

Type 2 diabetes mellitus at age 77 and 70 was diagnosed according to the World Health Organization (WHO) definition from 1999.⁹³ fasting plasma glucose ≥ 7.0 mmol/L, or intake of oral anti-diabetic drugs alone, or in combination with insulin treatment. Control men had a plasma glucose < 7.0 mmol/L and no anti-diabetic drugs. The men with type 2 diabetes at age 77 were divided into newly diagnosed (< 7 years since diagnosis, hence not diabetes at age 70), and established diabetes (≥ 7 years since diagnosis, diabetes already diagnosed at age 70). Diabetes at age 50 was diagnosed if fasting blood glucose ≥ 6.1 mmol/L or intake of anti-diabetic medication.

Smoking status

Active smoking at age 77 was defined if the subject answered yes to the question “Do you smoke? Är du rökare? (Sv.)” in the self-administered questionnaire. The duration of smoking among the current smokers was estimated with the answers from questions concerning smoking-duration in the questionnaires from age 77, 70, 60 and 50.

Former smoker (at age 77) was defined if the participant did not smoke at age 77 but had been smoking previously according to either:

1. Interview (concerning current smoking status) or questionnaire (concerning previous smoking) at age 70
2. Questionnaire (concerning current and previous smoking status) at age 60
3. Interview (concerning current and previous smoking status) at age 50

The time since smoking cessation was estimated with information collected with the questionnaires at age 77 and 60 and from the interviews at age 70 and 50.

A non-smoker (at age 77) was a person who had never been a smoker according to questionnaires at age 77 and 60 and interviews at age 70 and 50, hence a non-smoker at every investigation and reinvestigation.

Low-dose aspirin, hypertension and hyperlipidemia definition

Low-dose aspirin was defined as daily intake of 75–160 mg acetylsalicylic acid.⁹⁴ Hypertension was defined as an office blood pressure $> 140/90$ mmHg or antihypertensive medication. Hyperlipidemia was defined as a serum cho-

lesterol >6.5 mmol/l, triglycerides >2.3 mmol/L or when the patient was taking lipid-lowering medication.

Data from Swedish Hospital Discharge Registry

Registry data from 1970–2001 were obtained. Classifications 410, 412 (ICD [International Statistical Classification of Diseases and Related Health problems]-9) or I21–I22, I24–I25 (ICD-10) were coded as previous myocardial infarction. The classifications 413 (ICD-9) or I20 (ICD-10) were coded as angina pectoris and 433–436 (ICD-9) or I63–I66 (ICD-10) as ischemic stroke. The classifications 428 (ICD-9) or I50 (ICD-10) were coded as heart failure.

Statistics

Variables with skewed distribution according to Shapiro-Wilks test ($W < 0.95$) were log-transformed to reach normal distribution. Calculations were performed with the statistical software package JMP 3.2 (SAS Institute, Cary, NC) and Stata 6.0 and 8.2 (Stata Corporation, College Station, TX). Probability values < 0.05 were regarded as statistically significant.

Paper I and II

Overall difference between 8-iso-PGF_{2α} excretion in the morning urine samples, spot urine samples from varying hours (mean of 4–5 spot urine samples) and 24-hour urine samples in the 10 participants were tested by repeated measurement analysis of variance (ANOVA). Associations between 8-iso-PGF_{2α} in urine samples collected in the morning and in spot urine samples collected at varying hours, respectively, and in 24-hour urine samples were analysed with Pearson's correlation analysis. The between-day within-subject variation (biological and analytical) was obtained by ANOVA. CV was calculated as the square root of the within-subject variance estimate divided by the overall mean. The association between the mean values of 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} were tested by Spearman's rank correlation analysis.

Paper III-V

Differences between groups were tested with analysis of variance (ANOVA), unpaired t-tests, Kruskal-Wallis, Mann-Whitney, Fisher's or Chi-square tests in univariate analyses. Linear regression models, analysis of covariance and partial correlation were used to adjust the univariate associations for potential confounding factors. Associations between selenium (con-

tinuous variable and quartiles) and eicosanoids and cytokines, respectively, were tested in linear regression models. Pearson's or Spearman's rank correlation analyses were used to test associations between continuous variables.

Smoking (only Paper III), diabetes (only Paper IV), BMI, hypertension, treatment with low-dose aspirin (only Paper IV), serum cholesterol and triglycerides, clinical history of heart failure, myocardial infarction, angina pectoris or ischemic stroke, waist circumference and fasting insulin (only Paper III) at age 77 years were covariates in the multivariate analyses in Paper III and IV. BMI, serum α -tocopherol, β -carotene, CE-EPA and CE-DHA, diabetes, hyperlipidemia, hypertension, and smoking at baseline (age 50) and development of diabetes, myocardial infarction or ischemic stroke during the 27 years of follow-up were considered as potential confounders in the multivariate analysis in Paper V.

RESULTS

Concentration and variation of F₂-isoprostanes and PGF_{2α} in healthy humans (Paper I and II)

PGF_{2α}

Mean excretion rate of 15-keto-dihydro-PGF_{2α} during 10 days was 464 ± 188 pmol/mmol creatinine (mean \pm SD, n=13). The intra-subject CV in 15-keto-dihydro-PGF_{2α} in morning urine during 10 consecutive days varied from 23–54% with a CV of 41% for all 13 healthy subjects. Mean values (10 days) of urinary 15-keto-dihydro-PGF_{2α} correlated positively with mean values (10 days) of urinary 8-iso-PGF_{2α} in healthy humans, see Figure 7.

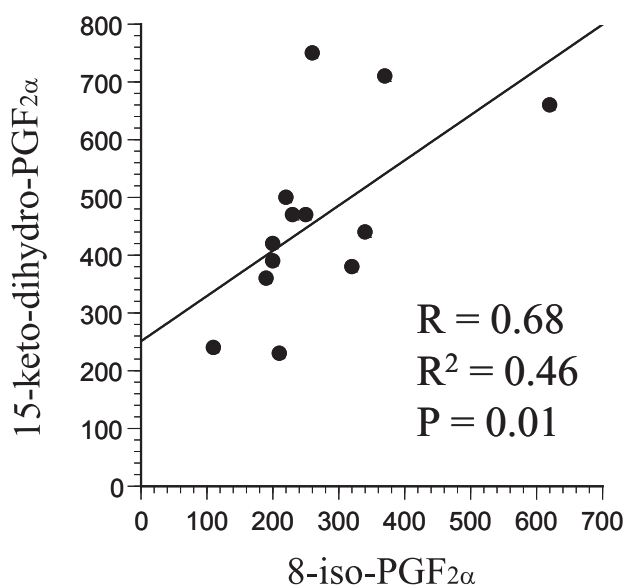


Figure 7. Linear correlation between mean of 8-iso-PGF_{2α} and mean of 15-keto-dihydro-PGF_{2α} in pmol/mmol during 10 days in 13 healthy subjects.

F₂-isoprostanes

Variation within the day

Urinary levels of 8-iso-PGF_{2α} for varying times, morning sample and 24-h urine sample are presented in Figure 8. Mean 8-iso-PGF_{2α} levels in spot urine samples collected at various times during the day and 8-iso-PGF_{2α} levels in morning urine, respectively, were well correlated with the levels of 8-iso-PGF_{2α} in 24-hour urine (see Figure 9). 8-Iso-PGF_{2α} in a single spot urine sample during the day did not correlate with 8-iso-PGF_{2α} in 24-hour urine in the 10 subjects ($R = 0.34$, $P = 0.33$). Further, there was no significant difference between the mean values of 8-iso-PGF_{2α} in urine collected at different times during the day (441 ± 326 pmol/mmol creatinine, mean \pm SD), in urine collected in the morning (541 ± 487 pmol/mmol), or in the 24-h urine sample (466 ± 221 pmol/mmol), $P = 0.86$ (overall ANOVA).

Variation between days

The intra-subject CV in 8-iso-PGF_{2α} in morning urine during 10 consecutive days varied from 18–104% with a CV of 42% for all 13 healthy subjects. Mean excretion rate of 8-iso-PGF_{2α} in the morning urine in the 13 subjects for 10 successive days was 272 ± 114 pmol/mmol creatinine.

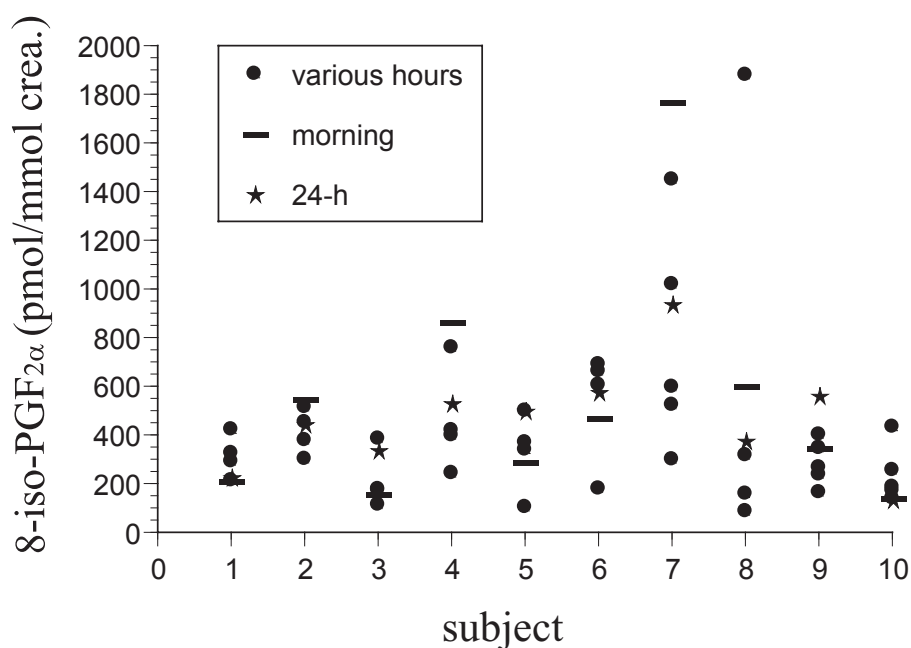


Figure 8. 8-Iso-PGF_{2α} in urine samples collected in spot urine samples at various hours, in the morning and during 24 hours in 10 healthy subjects. Printed with permission from Basu and Helmersson⁵⁶

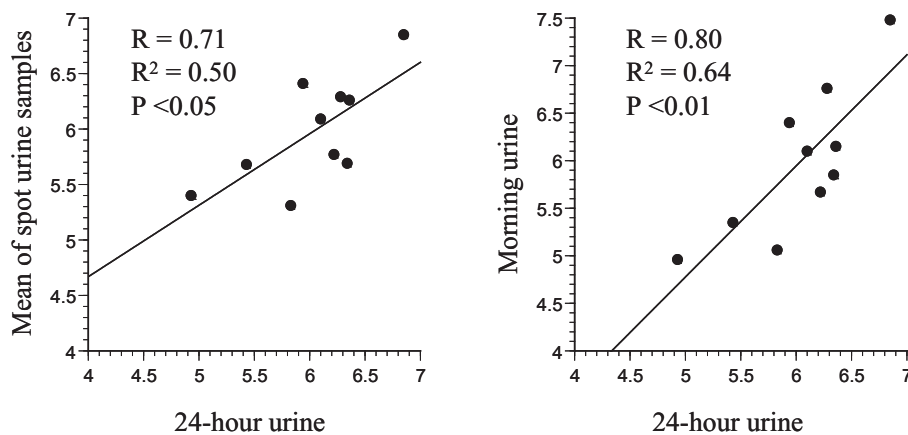


Figure 9. Linear correlations of 8-iso-PGF_{2α} in spot urine samples during a day (mean) and in morning urine samples, respectively, and 24-h urine samples in 10 healthy subjects. 8-Iso-PGF_{2α} (pmol/mmol) has been log transformed. Printed with permission from Basu and Helmersson.⁵⁶

Prostaglandin F_{2α}, F₂-isoprostanes, and other inflammatory indicators in 77-year old men

Characteristics of the study participants

Type 2 diabetes

As expected, the men with type 2 diabetes had a higher BMI and larger waist circumference in addition to higher fasting plasma glucose, insulin, triglycerides and HbA_{1c} than the controls. Serum cholesterol, including HDL and LDL cholesterol were lower in men with diabetes compared to the controls. CVD (heart failure, myocardial infarction, angina pectoris, hypertension, ischemic stroke) and low-dose aspirin treatment were more common among the men with diabetes than the controls. Fasting plasma glucose and HbA_{1c} were higher in the subgroup of men who have had diabetes seven years or more compared to the newly diagnosed diabetics, but cholesterol, triglycerides, fasting insulin, BMI, medical history of CVD, smoking, medication with statins or low-dose aspirin did not differ between the two subgroups of diabetic patients.

Smokers

Metabolic variables, medical history and medication did not differ among current smokers, former smokers and non-smoking controls except BMI which was significantly lower among the active smokers compared to the others.

PGF_{2α} (COX-mediated inflammation)

Type 2 diabetes

Urinary 15-keto-dihydro-PGF_{2α} was elevated in patients with type 2 diabetes compared to controls and this association did not change when adjusted for potential confounders (see Table 1). The same associations were found between patients with diabetes and controls in the subgroup without myocardial infarction, angina pectoris, and ischemic stroke and without treatment with low-dose aspirin or NSAID.

Men with newly diagnosed (<7 years ago) as well as manifest type 2 diabetes (≥ 7 years ago) had elevated levels of 15-keto-dihydro-PGF_{2α} compared to controls (Figure 10A), even when the association was adjusted for glucose and other mentioned potentially confounding factors.

Smoking

Urinary 15-keto-dihydro-PGF_{2α} was increased in current smokers and former smokers compared to non-smokers (see Table 2). 15-Keto-dihydro-PGF_{2α} did not significantly correlate to the estimated number of smoking years (P = 0.70), or the time since smoking cessation (P = 0.52).

Table 1. PGF_{2α}, F₂-isoprostanes, acute phase proteins and tocopherols in controls and patients with diabetes

	Controls	Type 2 diabetes	P	P (Adj.)	P (H)	P (BMI)
PGF _{2α} [⊗] pmol/mmol	312 ± 171 n = 585	385 ± 230 n = 101	<0.001	<0.001	<0.001	<0.001
HsCRP mg/L	3.59 ± 5.30 n = 648	4.73 ± 6.99 n = 110	<0.05	<0.05	0.08	0.28
SAA mg/L	8.40 ± 19.7 n = 645	14.0 ± 37.9 n = 111	<0.05	<0.05	0.10	0.25
8-Iso PGF _{2α} pmol/mmol	197 ± 91 n = 585	226 ± 125 n = 101	<0.01	<0.01	<0.01	<0.01
α-Tocopherol mg/mmol	1.57 ± 0.27 n = 646	1.50 ± 0.26 n = 110	<0.01	<0.01	<0.01	<0.05
γ-Tocopherol mg/mmol	0.090 ± 0.033 n = 646	0.095 ± 0.033 n = 110	0.11	0.31	0.34	0.33

[⊗] as measured by 15-keto-dihydro-PGF_{2α}. Values are given in mean ± SD.

P (Adj.) means smoking, low-dose aspirin treatment, cholesterol as covariates in the multiple regression model. P (H) means additionally adjusted for hypertension and heart failure. P (BMI) means additionally adjusted for BMI.

F₂-isoprostanes (oxidative stress)

Type 2 diabetes

Urinary 8-iso-PGF_{2α} was elevated in men with diabetes in the whole cohort (Table 1) and in the subgroup without myocardial infarction, angina pectoris, and ischemic stroke and without treatment with low-dose aspirin or NSAID. The division of the diabetes patients into a newly diagnosed and a manifest group depending on the estimated duration of the disease, revealed that only the men with manifest diabetes (duration ≥ 7 years) had significant increased formation of 8-iso-PGF_{2α} (see Figure 10B).

Smoking

Urinary 8-iso-PGF_{2α} was increased among current smokers and former smokers compared to non-smokers (see Table 2). 8-Iso-PGF_{2α} did not significantly correlate to the estimated number of smoking years ($P = 0.60$), or the time since smoking cessation ($P = 0.28$).

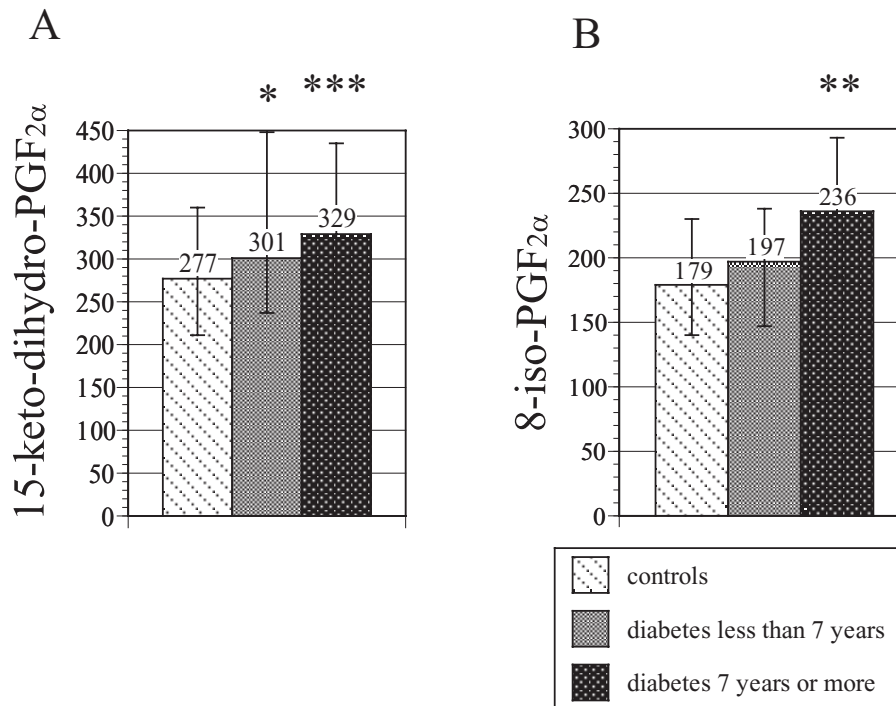


Figure 10. 15-keto-dihydro-PGF_{2α} (A) and 8-iso-PGF_{2α} (B) in controls, men with newly diagnosed diabetes (<7 years ago, $n = 40$), and men with diabetes diagnosed ≥ 7 years ago ($n = 54$). Values are given in pmol/mmol creatinine in median (inter-quartile range). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. controls.

Table 2. $PGF_{2\alpha}$, F_2 -isoprostanes, cytokine-related mediators and tocopherols in non-, former and never smokers.

	Non-smokers	Former smokers	Current smokers	P	P (Adj.)
$PGF_{2\alpha}$ [⊙] pmol/mmol	249 (197–341) n = 178	282 (213–365)‡ n = 359	321 (258–425)***† n = 44	<0.01	<0.01
IL-6 ng/L	2.47 (1.85–4.28) n = 195	2.87 (2.07–4.54)* n = 392	3.57 (1.86–5.29)* n = 55	0.01	<0.01
HsCRP mg/L	1.65 (0.98–3.23) n = 196	1.8 (0.85–4.23) n = 391	2.31 (1.16–5.98) n = 55	0.14	0.10
SAA mg/L	3.91 (2.40–6.42) n = 195	4.08 (2.41–6.76) n = 389	4.44 (2.13–5.72) n = 55	0.82	0.75
8-Iso- $PGF_{2\alpha}$ pmol/mmol	167 (134–214) n = 178	184 (141–239)* n = 359	191 (164–278)** n = 44	0.01	<0.01
α -Tocopherol mg/mmol	1.63 (0.32) n = 195	1.56 (0.25)** n = 391	1.49 (0.24)***• n = 54	<0.001	<0.001
γ -Tocopherol mg/mmol	0.089 (0.033) n = 196	0.090 (0.033) n = 391	0.091 (0.030) n = 54	0.90	0.87

⊙ as measured by 15-keto-dihydro- $PGF_{2\alpha}$

Values are given in median (interquartile range) or mean (SD)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. non smokers,

‡ $P = 0.07$ vs. non-smokers ($P < 0.05$ if adjusted),

• $P = 0.05$ vs. non-smokers ($P < 0.05$ if adjusted)

† $P < 0.05$ vs. former smokers, P (Adj.) means adjusted for low-dose aspirin medication, serum cholesterol, triglycerides, BMI, hypertension, clinical history of heart failure, myocardial infarction, angina pectoris or ischemic stroke.

IL-6, CRP and SAA (cytokine-mediated inflammation)

Type 2 diabetes

Serum hsCRP and SAA was slightly higher in men with diabetes than controls (see Table 1). Adjustment with either BMI or waist circumference, however, removed the differences.

Smoking

IL-6, but not hsCRP or SAA, was increased in current and former smokers compared to the non-smoking controls (Table 2). In former smokers IL-6, hsCRP and SAA were highest in the men who quit smoking recently and decreased linearly with increased time since smoking cessation ($b = -0.04$, $P < 0.05$, $b = -0.08$, $P < 0.05$, $b = -0.07$, $P < 0.05$, respectively).

Vitamin E

Type 2 diabetes

Serum α -tocopherol, but not γ -tocopherol, was decreased in men with diabetes compared to controls (see Table 1). Only the men with newly diagnosed diabetes, but not manifest diabetes, had low levels of α -tocopherol.

Smoking

Lipid adjusted α -tocopherol, but not γ -tocopherol, was decreased in current and former smokers compared to the non-smoking controls (Table 2). α -Tocopherol did not significantly correlate to the estimated number of smoking years in current smokers. In former smokers α -tocopherol was lowest in the men who quit smoking within the last year and increased linearly with time since smoking cessation ($b = 0.01$, $P < 0.05$). γ -Tocopherol was highest in the men who quit smoking in the last year and decreased linearly with time since smoking cessation ($b = -0.004$, $P < 0.05$).

Relations between inflammation, oxidative stress and metabolic measurements

PGF_{2 α} , 8-iso-PGF_{2 α} and γ -tocopherol, respectively, correlated positively to each other but not to any other metabolic variable. IL-6, hsCRP and SAA correlated positively and α -tocopherol negatively to BMI, waist circumference, HbA_{1c} or glucose, and insulin (see Table 3).

Table 3. Significant correlation coefficients between inflammatory and oxidative stress indicators and metabolic variables in all 77-year old men

	PGF _{2α}	F ₂ -isoprostane	hsCRP	SAA	IL-6	α -tocopherol	γ -tocopherol
PGF _{2α}	-	0.36***	-	-	0.12**	-	0.09*
F ₂ -isoprostane	0.36***	-	-	-	-	-0.08*	0.08*
BMI	-	-	0.16***	0.19***	0.10**	-0.09*	-
Waist circumference	-	-	0.21***	0.23***	0.15***	-0.13***	-
Glucose	0.07†	-	-	0.07†	0.09*	-0.10**	-
HbA _{1c}	-	-	0.12***	0.17***	0.16***	-	-
Insulin	-	-	0.11**	0.15***	0.17***	-0.18***	-

† $P = 0.06$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Serum selenium in relation to $\text{PGF}_{2\alpha}$, F_2 -isoprostanes, and other inflammatory indicators in a 27 year follow-up study (Paper V)

Characteristics of the study population at baseline (50 years)

S-selenium correlated positively with α -tocopherol, β -carotene and cholesterol in serum and diastolic blood pressure at baseline. Men with diabetes (4%), hypertension (21%), hyperlipidemia (63%) and current smokers (40%) did not have altered levels of selenium at baseline.

$\text{PGF}_{2\alpha}$

S-selenium at age 50 was inversely associated with urinary 15-keto-dihydro- $\text{PGF}_{2\alpha}$ at follow-up ($b = -0.054$, $P < 0.01$) in the linear regression model. This association was still significant when adjusted for BMI, diabetes, hyperlipidemia, hypertension, smoking, s- β -carotene, s- α -tocopherol and s-CE EPA and DHA at baseline, and interim diabetes, ischemic stroke, and myocardial infarction during follow-up ($P < 0.01$). Men in the highest quartile of selenium at age 50 had decreased levels of 15-keto-dihydro- $\text{PGF}_{2\alpha}$ at follow-up (see Table 4). These associations did not change when adjusted for the above mentioned potentially confounding variables.

F_2 -isoprostanes

S-selenium at age 50 was inversely associated with urinary 8-iso- $\text{PGF}_{2\alpha}$ at follow-up ($b = -0.048$, $P < 0.01$) in the linear regression model. This association was still significant when adjusted for BMI, diabetes, hyperlipidemia, hypertension, smoking at baseline, and development of diabetes, ischemic stroke, and myocardial infarction during follow-up ($b = -0.043$, $P = 0.01$), and borderline significant when s- β -carotene, s- α -tocopherol and s-CE EPA and DHA at baseline were adjusted for ($b = -0.037$, $P = 0.05$). Men in the highest quartile of selenium at age 50 had decreased levels of 8-iso- $\text{PGF}_{2\alpha}$ at follow-up (see Table 4). Adjustment with all of the above mentioned potentially confounding variables reduced the P values to some extent but the associations were still significant.

IL-6, hsCRP and SAA

S-selenium at age 50 was not linearly associated with IL-6, hsCRP or SAA at 27 years of follow-up. Quartiles of s-selenium were not associated to IL-6, hsCRP or SAA in univariate (see Table 4), or multivariate regression models.

Table 4. *Inflammatory and oxidative stress indicators at follow up in quartiles of s-selenium at age 50*

	Quartiles of selenium				P
	1	2	3	4	
	n = 168 30–68 µg/L	n = 144 69–76 µg/L	n = 159 77–85 µg/L	n = 144 86–131 µg/L	
PGF _{2α} [⊙] (pmol/mmol)	342 ± 183***	333 ± 220*	333 ± 184**	279 ± 148	<0.01
8-iso-PGF _{2α} (pmol/mmol)	209 ± 104**	206 ± 102*	203 ± 86*	177 ± 70	0.01
HsCRP (mg/L)	3.80 ± 6.20	3.73 ± 5.54	4.15 ± 5.47	3.87 ± 5.42	0.42
SAA (mg/L)	10.6 ± 32.0	8.69 ± 16.2	9.66 ± 22.0	9.14 ± 19.5	0.46
IL-6 (ng/L)	4.16 ± 2.90	3.85 ± 2.64	4.13 ± 2.80	3.75 ± 2.78	0.60
α-Tocopherol (µmol/mmol)	3.57 ± 0.63	3.58 ± 0.53	3.58 ± 0.60	3.71 ± 0.75	0.20
γ-Tocopherol (µmol/mmol)	0.209 ± 0.073	0.218 ± 0.080	0.220 ± 0.067	0.221 ± 0.084	0.47

Range of s-selenium at age 50 is given in each quartile. [⊙]PGF_{2α} is quantified by urinary 15-keto-dihydro-PGF_{2α}. Values are presented in mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. quartile 4.

Tocopherols

Selenium was positively associated with levels of s-α-tocopherol at follow-up in the univariate linear regression model (b = 0.016, P < 0.05). The association disappeared when the model was further adjusted for baseline s-α-tocopherol, s-β-carotene, s-CE EPA and DHA (b = 0.004, P = 0.57). Selenium was not linearly associated with γ-tocopherol at follow-up. Men in the highest selenium quartile at age 50 had increased levels of γ-tocopherol at follow-up compared to the lowest selenium quartile (see Table 4), but this was, however, only significantly different (P < 0.05), when adjusting for all the above mentioned confounders including α-tocopherol, β-carotene, s-CE EPA and DHA at baseline. α-Tocopherol at age 50 was correlated with α-tocopherol at age 70 (R = 0.20, P < 0.001) and age 77 (R = 0.24, P < 0.001).

Fatty acids and dietary assessment

Selenium at age 50 was correlated with reported dietary intake of selenium (mean dietary intake of Se 26 ± 8 µg/day) at 20 years of follow-up (R = 0.08, P = 0.01, n = 1001) but this was not significant in the subgroup of men who were followed up for all the 27 years (R = 0.07, P = 0.09, n = 554). Selenium at age 50 correlated positively with s-CE-EPA at baseline, at 20 years of

follow-up and at 27 years of follow-up (Table 5). Selenium was also positively correlated with s-CE-DHA at baseline and at 20 years of follow-up.

Further, s-CE EPA at baseline correlated well with s-CE EPA at 20 years of follow-up ($R = 0.41$, $P < 0.001$), and with s-CE EPA at 27 years of follow-up ($R = 0.27$, $P < 0.001$), respectively. S-CE DHA at baseline correlated well with s-CE DHA at 20 years of follow-up ($R = 0.42$, $P < 0.001$), and s-CE DHA at 27 years of follow-up ($R = 0.20$, $P < 0.01$), respectively.

Table 5. *Correlation of S-selenium at age 50 (baseline) with the long chain fatty acids EPA and DHA in the study population in paper V*

	N	R	P
S-CE EPA (20:5 n-3) at age 50 (baseline)	598	0.34	<0.001
S-CE DHA (22:6 n-3) at age 50 (baseline)	598	0.32	<0.001
S-CE EPA (20:5 n-3) at age 70 (20 year of follow-up)	307	0.22	<0.001
S-CE DHA (22:6 n-3) at age 70 (20 years of follow-up)	294	0.14	<0.05
Dietary EPA (20:5 n-3) at age 70 (20 year of follow-up)	554	0.08	0.08
Dietary DHA (22:6 n-3) at age 70 (20 year of follow-up)	554	0.13	<0.01
S-CE EPA (20:5 n-3) at age 77 (27 years of follow-up)	216	0.15	<0.05
S-CE DHA (22:6 n-3) at age 77 (27 years of follow-up)	217	0.08	0.22

DISCUSSION

PGF_{2α}

Individual variation in healthy humans

Variation between days and within the day

The intra-subject CV of 15-keto-dihydro-PGF_{2α} during 10 days varied from 23–54% with a mean value of 41% for all 13 subjects. This variation includes both a biological variation and an analytical variation of the assayed samples. Previously published results show that the analytical CV for 15-keto-dihydro-PGF_{2α} is 12–14%.²⁴ Although the analytical variation was not calculated in this study the observed individual variation is probably mainly related to biological variation. The observed variation of urinary 15-keto-dihydro-PGF_{2α} is of the same magnitude as the variation of the closely related 8-iso-PGF_{2α}. Although not studied in this thesis, the intra-individual variation in plasma levels of 15-keto-dihydro-PGF_{2α} would probably be higher because of the formation and release kinetics in forms of intermittent peaks which has been seen in animal studies in the field of reproduction.^{14,95,96}

The variation of urinary 15-keto-dihydro-PGF_{2α} within the day was not studied in this thesis. A study aiming at evaluating the circadian variation of plasma 15-keto-dihydro-PGF_{2α} during 24 hours indicated that the plasma values in late afternoon was lower than in early morning.⁹⁷ Excretion of other vasoconstricting prostanoids, TXA₂ and LTE₄, have shown similar patterns of circadian variation during the day with higher levels at 3–6 o'clock in the morning than at 3–6 o'clock in the afternoon.⁹⁸ Another study showed no significant diurnal variation in formation of TXA₂ or PGI₂ although the CVs were not reported.⁹⁹

Individual variation is a factor to take into account when planning a clinical study. The day-to-day variation described in this thesis only applies to healthy individuals, pathological conditions may alter the variation. If not a pronounced increase or decrease in levels of PGF_{2α} are expected in the study an under-dimensioned study population clearly increase the risk of a type 2 error when analysing the data.

Factors influencing the intra-individual biological variation in healthy humans

PGF_{2α} is primarily formed from free arachidonic acid which has been hydrolysed by mainly PLA₂ from tissue phospholipids. Theoretically, several regulatory stages in formation, metabolism and excretion of PGF_{2α} and 15-keto-dihydro-PGF_{2α} may be altered, and explain the observed variation in urinary 15-keto-dihydro-PGF_{2α}. In the de-esterification process, hydrolyzing enzymes, mainly phospholipases A₂, hydrolyses esterified arachidonic acid to the free form. The access of PLA₂ is essential and might, at least theoretically, be a rate-limiting factor. For example hydrogen peroxide has been shown to activate PLA₂.^{100,101} Arachidonic acid is present at a fairly constant rate in the phospholipids and might not be a major regulatory factor. Free arachidonic acid is oxygenated by cyclooxygenases and peroxidases to form the intermediate PGH₂ and further converted to PGF_{2α} by PGF synthase and variations in each step may theoretically influence levels of PGF_{2α}. PGF_{2α} is further inactivated through metabolism by 15-PGDH and Δ¹³-reductase to form the measured compound 15-keto-dihydro-PGF_{2α} which then is degraded by β-oxidation systems. Alterations in any of these enzymes could theoretically account for the observed variation in 15-keto-dihydro-PGF_{2α}. Finally, the excretion step of 15-keto-dihydro-PGF_{2α} could potentially be a source of variation. In paper II the biological and analytical variation in urinary creatinine could also account for some of the variation seen in urinary 15-keto-dihydro-PGF_{2α}.

There is limited knowledge about everyday factors which might affect individual levels of 15-keto-dihydro-PGF_{2α} from day-to-day in healthy humans. Long endurance exercise did not increase plasma 15-keto-dihydro-PGF_{2α}.⁹⁷ But again, a single plasma 15-keto-dihydro-PGF_{2α} value only mirrors a very short moment of time and increases or decreases in 15-keto-dihydro-PGF_{2α} might easily be overlooked unless a frequent sampling regime is undertaken. An attack of migraine headache did not alter the urinary levels of 15-keto-dihydro-PGF_{2α} in otherwise healthy subjects.¹⁰² No significant peak variations in plasma 15-keto-dihydro-PGF_{2α} levels have been shown in healthy women during menstruation cycle,^{103,104} although this needs to be confirmed in studies with a more frequent sampling schedule of plasma, or urine sampling in larger settings. Presumably, individual variations of 15-keto-dihydro-PGF_{2α} might also be affected by oxidative stress.

Paper II-V show a variation of 15-keto-dihydro-PGF_{2α} between individuals. Until proven otherwise, it is generally assumed that differences in 15-keto-dihydro-PGF_{2α} levels between individuals are related to variation in the formation of PGF_{2α} rather than to variation in metabolism and excretion. No variation in formation of PGF_{2α} has been seen related to sex or age.^{97,102} Premenopausal women had slightly lower levels of PGF_{2α} formation than postmenopausal women, but this was not statistically significant, $P = 0.08$.¹⁰²

Normal pregnancy is associated with increased formation of $\text{PGF}_{2\alpha}$.¹⁰⁵ Finally, dietary supplementation with conjugated linoleic acid (CLA), 18:2, *cis*-9, *trans*-11 and 18:2, *trans*-10, *cis*-12 has been shown to increase $\text{PGF}_{2\alpha}$ formation.¹⁰⁶⁻¹⁰⁹ Daily low-dose aspirin medication seemed to be related to reduced levels of $\text{PGF}_{2\alpha}$ formation in elderly men.⁹⁴

Pathological conditions and diseases related to inflammation or oxidative stress might alter $\text{PGF}_{2\alpha}$ formation. So far, this has been shown for different rheumatic diseases²⁸ and in acute myocardial infarction during percutaneous coronary intervention.¹¹⁰

$\text{PGF}_{2\alpha}$ formation in type 2 diabetes

Paper III is the first study to show an increased formation of $\text{PGF}_{2\alpha}$ in patients with type 2 diabetes. The association was independent of a clinical history of previous various CVD and cardiovascular risk factors including smoking, BMI, hypertension and serum cholesterol. $\text{PGF}_{2\alpha}$ formation was elevated both in newly diagnosed patients and in patients with disease duration of 7 years or more. $\text{PGF}_{2\alpha}$ was weakly positively correlated to plasma glucose and insulin but not to HbA_{1c} , BMI or waist circumference. This suggests that COX-mediated inflammation is enhanced in elderly men with type 2 diabetes.

The result is further supported by studies evaluating the formation of closely related COX-derived vasoactive eicosanoids TXA_2 and PGI_2 in type 2 diabetes. Enhanced formation of TXA_2 has been shown in patients with type 2 diabetes.¹¹¹⁻¹¹³ Furthermore, the formation of PGI_2 in patients with diabetes has been shown to be decreased.¹¹⁴

Collectively, the studies suggest that COX-mediated inflammatory eicosanoids are altered in patients with diabetes towards an inflammatory, vasoconstricting and platelet aggregatory state. These changes might be related to the pathogenesis and complications of the diabetic disease.

General aspects of inflammation and type 2 diabetes

Cytokine-mediated acute phase proteins

Other studies with cytokine-related mediators have found evidence in favour of an on-going subclinical inflammatory activity in type 2 diabetes. Enhanced IL-6 levels is cross-sectionally related to diabetes with and without macrovascular diseases¹¹⁵ and elevated levels of SAA to diabetes with and without syndrome X.¹¹⁶ Elevated levels of CRP, and possibly IL-6, is shown to predict diabetes development.¹¹⁷⁻¹¹⁹ However, CRP has not convincingly been associated to diabetes cross-sectionally. In several studies relating high CRP levels to diabetes disease the possible confounding effect of cardiovascular diseases is not studied.^{120,121} This confounder is important since CRP

predicts cardiovascular diseases. CRP levels are elevated in patients with diabetes and macrovascular disease¹¹⁵ and syndrome X,¹¹⁶ but not in diabetes without these complications. This is in line with the results regarding CRP in this study. The results showed higher CRP levels among the patients with diabetes, apparently independent of CVD and stroke, but this association was abolished when adjusted for either BMI, waist circumference or insulin. A positive correlation between CRP and BMI was found in our study and has previously been shown.¹²² Further, CRP was in our study shown to be positively correlated with waist circumference and insulin, respectively. SAA levels and diabetes is not extensively studied by others. In this study SAA followed the same pattern as CRP.

Summary

Type 2 diabetes seems to be associated with low-grade inflammation. The chronic inflammation may be related to the accelerating atherosclerosis often seen in patients with type 2 diabetes. Results from this thesis with elderly Swedish men suggest however a more convincing relationship between the COX-mediated inflammatory indicator $\text{PGF}_{2\alpha}$ and diabetes than between cytokine-related inflammatory indicators and diabetes. The cytokine-mediated inflammatory process seen in these elderly men with diabetes seems to be closer related to bodyweight and possibly insulin resistance.

$\text{PGF}_{2\alpha}$ in smokers

Paper IV is the first study to show an increased formation of $\text{PGF}_{2\alpha}$ in active and former smokers compared to non-smoking controls independent of other cardiovascular risk factors and clinical history of CVD including ischemic stroke. This suggests that smoking is associated with subclinical COX-mediated inflammation.

Several papers have shown that formation of the closely related eicosanoid TXA_2 is increased among smokers.¹²³⁻¹³¹ Studies on PGI_2 formation in smokers have shown conflicting results, some have reported unaltered formation^{123,127,129} while other have reported increased formation.^{124-126,128,131}

Togetherly, smoking is likely to be associated with an alteration in COX-mediated inflammatory eicosanoids towards an inflammatory, vasoconstricting and platelet aggregatory state. These changes might be related to the cardiovascular complications seen in smokers i.e. perifer vasoconstriction and CVD.

General aspects of inflammation and smoking

IL-6 and cytokine-mediated acute phase proteins

Paper IV showed that IL-6 was increased in active and former smokers compared to non-smokers. These results confirm other studies of IL-6 in current smokers and non-smokers.¹³²⁻¹³⁴ No associations were found with smoking status and hsCRP and SAA in Paper IV. However, increased levels of CRP¹³⁵⁻¹³⁹ and SAA¹³⁹ have been found in larger studies in active smokers compared to non-smokers. The discrepancy in results might be related to low power in Paper IV where 55 active smokers were analysed compared to 4187, 96, 929, 64, 415 smokers in the other cited studies, respectively. The results in Paper IV which showed that IL-6, hsCRP and SAA formation were inversely correlated to time since smoking cessation in the larger group of former smokers (n = 391), supports this theory. Smoking seems to be associated with cytokine-mediated inflammation as measured by IL-6, hsCRP and SAA, and this subclinical inflammatory process appears to be present even after smoking cessation.

Antioxidants and PGF_{2α} formation

Effects of selenium

Paper V is the first human population-based study to indicate that high levels of s-selenium are associated with reduced levels of PGF_{2α} formation at follow-up. This would indicate that selenium status might be of importance for the COX-mediated inflammatory eicosanoid formation. It has been shown that selenium deficiency in rats can lead to increased formation of PGF_{2α} in their liver cells by induction of glutathione S transferase activity. Based on *in vitro* studies it has been proposed that selenium deficiency is associated with increased levels of TXA₂ formation and decreased levels of PGI₂ formation as earlier reviewed.^{64,140,141} These results fit well with our clinical cohort data.

Effects of vitamin E

α-Tocopherol

S-α-tocopherol did not correlate with PGF_{2α} formation but correlated positively with the cytokine-mediated inflammatory indicators IL-6, hsCRP and SAA in 77 year old men (data not shown). On the other hand, α-tocopherol correlated negatively with other metabolic variables that usually are positively correlated to cytokines and cytokine-mediated inflammatory indicators in ULSAM and other settings.¹⁴² α-Tocopherol presents an unpredictable pattern in relation to inflammation in elderly men. The reasons for this are not clear.

α-Tocopherol supplementation did not change the PGF_{2α} formation in healthy humans.^{109,143} However, patients with type 2 diabetes,¹¹² hypercho-

lesterolemia¹⁴⁴ or hyperhomocysteinemia¹⁴⁵ who were supplemented with α -tocopherol decreased their levels of TXA₂. α -Tocopherol supplementation in healthy and patients with type 2 diabetes did also decrease CRP and IL-6 formation.¹¹⁵

γ -Tocopherol

γ -Tocopherol have been shown to correlate positively with PGF_{2 α} formation in ULSAM and in other studies.¹⁴² γ -Tocopherol also correlated positively with the cytokine-mediated inflammatory indicators IL-6, hsCRP and SAA, respectively, in ULSAM (data not shown). From these results it could be speculated that γ -tocopherol might be upregulated in relation to the degree of subclinical inflammation. The increase in 15-keto-dihydro-PGF_{2 α} during supplementation with CLA was significantly correlated with the increase in γ -tocopherol.¹⁴² γ -Tocopherol has been proposed to have anti-inflammatory effects *in vitro* and *in vivo*. Rats injected with γ -tocopherol showed a reduced level of PGE₂, leukotriene B₄ and TNF- α in an inflammatory model.⁶¹

F₂-isoprostanes

Individual variation in healthy humans

Variation within the day

Results from Paper I (Figure 8) show the individual variation in isoprostane levels during 24-hours. However, there was no significant difference at the group level when comparing the mean levels of 8-iso-PGF_{2 α} measured in spot urine samples during the day, in urine samples collected in the morning, or in the 24-hour urine samples. This finding was confirmed in a larger study with healthy individuals where the mean level of 8-iso-PGF_{2 α} in the morning urine in the whole group did not differ from the mean level in the 24-h urine collection.¹⁰⁶ Further, no diurnal variation of 8-iso-PGF_{2 α} at the group level could be observed when mean values from five different time periods during a day were compared.¹⁴⁶ No statistical circadian variation could be observed on the group level between mean values of 8-iso-PGF_{2 α} in urine collected during three 8-h collection periods during 24 hours.¹⁴⁷ The individual values were not presented in the referred study. Taken together, there is clearly a diurnal variation in levels of urinary 8-iso-PGF_{2 α} during the day in each individual subject. On the other hand, when 8-iso-PGF_{2 α} is evaluated on a group level, as in most clinical studies, the existing evidence agrees on a non-existing circadian variation.

Collection of urinary samples for analysis of 8-iso-PGF_{2 α}

Good correlations between 8-iso-PGF_{2 α} values in morning urine samples or in several spot urine samples collected during the day (mean), respectively,

and the 24h-urine samples were found (see Figure 9). Similar results have been found in other studies where a good correlation between urinary 8-iso-PGF_{2α} in samples collected in the morning and collected during 24 hours were reported.^{146,148} 8-iso-PGF_{2α} in a single spot urine sample taken any time during the day did however not correlate with 8-iso-PGF_{2α} in the 24-h urine collection, indicating that if only a single urine sample are to be used the morning urine sample is superior to reflect the diurnal excretion of 8-iso-PGF_{2α}. It appears that urine collected in the morning or in several urine spot samples, adequately represents the daily F₂-isoprostane excretion in a healthy person.

Variation between days

The intra-subject CV during 10 days varied from 18–104% with a mean value of 42% for all 13 subjects. This variation includes both a biological variation and an analytical variation of the assayed samples. The analytical variation was not calculated in this study but previously published results show that CV for the analysis method is 12–15%.⁵³ Thus, the observed individual variation is probably mainly related to biological variation. This result is in line with the observed within-subject biological variation of 25% in plasma 8-iso-PGF_{2α} when 20 healthy subjects were sampled monthly for four months.¹⁴⁹ The intra-subject variation during three different days varied from 2.1–10.5% in non-smokers and 4.5–24.3% in smokers.¹⁵⁰ However, the observed variation of 8-iso-PGF_{2α} in urine in Paper II is of the same magnitude as the observed within-subject biological variation in other hormones and peptides.¹⁵¹ Individual variation between days is a factor to be taken into account when planning a clinical study and the variation may vary depending on the setting. If a moderate increase or decrease in 8-iso-PGF_{2α} is expected in the study the size of the study population needs to be carefully considered to avoid the risk of a type 2 error when analysing the data.

Factors influencing the intra-individual biological variation in healthy humans

Formation of 8-iso-PGF_{2α} requires arachidonic acid, oxygen and free radicals. This compound is primarily formed from esterified arachidonic acid *in situ* present in the tissue phospholipids.⁴² Theoretically, there are several regulatory stages of formation, metabolism and excretion of 8-iso-PGF_{2α} which may be altered and account for the observed variation in levels of urinary 8-iso-PGF_{2α}.⁵⁶ In initial formation of isoprostanes from arachidonic acid the availability of oxygen and free radicals seems to be a rate-limiting step rather than access to arachidonic acid. In the de-esterification process, isoprostanes are enzymatically hydrolysed to form free bioactive isoprostanes and the availability of hydrolyzing enzymes such as PLA₂ might theoretically be an important rate-limiting step.¹⁰¹ It has been shown in rabbits that free isoprostanes are further metabolised by 15-PGDH, Δ¹³-

reductase and finally β -oxidation systems.⁴⁹ Although not studied, a relative deficiency in any of these enzymes could theoretically account for variations in the levels of isoprostanes. Finally, the excretion step could potentially be a source of variation. Generally, urinary creatinine, although a potential pitfall, is used to estimate the glomerular filtration rate. In this study the biological and analytical variation in urinary creatinine could account for some variation seen in the urinary isoprostanes.

Lifestyle factors could possibly be related to intra-individual variations of isoprostanes in healthy humans. A high-fat Big Mac meal (Big Mac[®] hamburger, fried potatoes and soft drink without sugar) contains measurable amounts of 8-iso-PGF_{2 α} but this did not affect the postprandial levels of plasma 8-iso-PGF_{2 α} .^{152,153} A 2-day low-fat diet (only 5 energy% as fat) did not alter the levels of 8-iso-PGF_{2 α} .¹⁴⁶ Thus, the lipid content *per se* does not appear to have an acute effect on isoprostanes. However, a 24-hour fasting has been shown to elevate isoprostane levels by 40%.¹⁴⁶ Alcohol intake causes a dose-dependent acute increase of isoprostanes in healthy humans¹⁵⁴ and might be a source of individual day-to-day variation. Smoking cessation and restarting has been shown to result in significant decrease and increase, respectively, within a few days.¹⁵⁵ Exhaled F₂-isoprostanes in breath condensate increased 15 min after cigarette smoking¹⁵⁶ but in another study no increase in isoprostanes could be seen within 30 min of cigarette smoking.¹⁵⁷ Smoking appears to be a factor which can contribute to intra-individual variation, however, no smokers were included in paper I or II. Prolonged endurance exercise has been shown to temporarily increase the levels of isoprostanes.¹⁵⁸⁻¹⁶¹ Eccentric exercise, leading to muscle damage and inflammation, can cause an increase in isoprostanes within a few days.^{162,163} Acute infection and inflammation caused by infusion of endotoxin in humans caused an acute elevation of isoprostanes.¹⁶⁴ Thus, a variety of different factors associated with daily life can cause intra-individual alteration in isoprostane levels between days.

The results in Paper I–V show that 8-iso-PGF_{2 α} levels vary between individuals. Variations between individuals could generally be related to a number of factors, both physiological (including age, gender, ethnicity, pregnancy, diet, dietary supplementation), and pathophysiological (different diseases) as recently reviewed in Basu and Helmersson.⁵⁶ Until proven otherwise, it is generally assumed that differences in isoprostane levels between individuals are related to variation in the formation of the compound rather than variation in metabolism and excretion.

F₂-isoprostanes in type 2 diabetes

The results from Paper III show that 8-iso-PGF_{2 α} formation was increased in patients with type 2 diabetes compared to controls. This study confirms the results of other studies that have shown increased levels of urinary and

plasma isoprostanes related to type 2 diabetes,^{112,148,165-167} but it is the first study to suggest that this association is independent of other cardiovascular risk factors.

The increase in 8-iso-PGF_{2α} formation seen in these elderly men with diabetes was 15%, not as pronounced as seen in other studies. Our diabetes patients had a median value of HbA1c of 5.8% which indicates a higher proportion of well regulated diabetic patients compared to the other studies presented. It has been proposed that good glycemic control in general is related to lower levels of 8-iso-PGF_{2α} compared to bad glycemic control.¹¹² Some reports show a linear correlation between 8-iso-PGF_{2α} and glucose^{112,166} while other studies do not.¹⁶⁵ Another possible explanation for the difference in 8-iso-PGF_{2α} increment is the age difference. Our diabetic patients are 77 years old, and it is possible that elderly persons may respond differently to oxidative stress¹⁶⁸ than younger persons.

Only the patients with established diabetes (diagnosis since 7 years or more), had increased levels of 8-iso-PGF_{2α} in the present study and the results seemed to be independent of the level of glycemic control. Another study in Indian Mauritians has shown that impaired fasting glucose and newly diagnosed diabetes is associated with increased 8-iso-PGF_{2α} formation.¹⁶⁶ The reason for the inconsistent results is not clear.

Oxidative stress hypothesis and type 2 diabetes

Increased 8-iso-PGF_{2α} in type 2 diabetic patients suggests a higher level of free radical induced lipid peroxidation in diabetic patients. A higher level of DNA oxidation,¹⁶⁹ glycoxydated proteins¹⁷⁰ and nitrotyrosine formation¹⁷¹ has also been suggested. Thus, oxidative stress seems to be an important feature in diabetes and might be one of the possible pathophysiological factors that may contribute to the macro- and microvascular complications associated with diabetes. Sources of oxidative stress have been suggested to be auto-oxidative glycosylation, where glucose reacts with proteins and free radicals are formed (which later also contribute to the formation of advanced glycosylation end products),¹⁷² induction of sorbitol and fructose by the polyol pathway in insulin-independent cells¹⁷³ and reduced antioxidant capacity.¹⁷²

Since most studies performed have been cross-sectional observation studies it is unclear whether diabetes is a consequence of oxidative stress or *vice versa*. However, it has been suggested that oxidative stress due to overnutrition and impaired physical activity is the mechanism underlying insulin resistance, beta-cell and endothelial dysfunction which eventually could lead to postprandial hyperglycemia with subsequent impaired fasting glucose and diabetes.¹⁷⁴ Oxidative stress may also induce inflammation or be induced by inflammatory mediators.

F₂-isoprostanes in smokers

8-Iso-PGF_{2α} formation was increased in current smokers compared to non-smokers which confirms other studies where elevated levels of F₂-isoprostanes in plasma, urine and breath condensate have been found in smokers.^{130,150,156,157,175,176} The association seen in this study appeared to be independent of other cardiovascular risk factors and low-dose aspirin treatment.

Paper IV also showed that even former smokers had increased levels of 8-iso-PGF_{2α}. This was an unexpected finding since other studies have shown that smoking cessation significantly decreases^{130,157} or normalizes^{155,176} the levels of isoprostanes within two weeks. However, the increase in 8-iso-PGF_{2α} in former smokers was only 10% compared to the non-smokers and considering the biological variation of the measured compound the number of study participants must be high in order to prove a statistically significant difference. The former smokers in paper IV were 359 and the non-smokers were 178, many times more than in the other referred studies. However it cannot be ruled out that some of the former smokers live in a smoking environment. Passive smoking has been shown to enhance the formation of isoprostanes in children of the household.¹⁷⁷

8-Iso-PGF_{2α} was not linearly correlated to time since smoking cessation in former smokers or number of smoking years in active smokers. The number of cigarettes smoked was unfortunately unknown in Paper IV. Other studies evaluating the dose-response effect of cigarette smoking on isoprostane formation have shown inconsistent results.^{130,157}

The active smokers in Paper IV show only a minor increment (14%), in isoprostane formation compared to the non-smokers. Other published studies have shown greater differences in isoprostane formation between smokers and non-smokers. This discrepancy might be related to the observation pointed out by Morrow et al that there is heterogeneity of isoprostane formation in smokers.¹⁵⁷ Some smokers appear to be more resistant to the toxic effects of smoking than others. The smokers in Paper IV represent a highly selected study population of relatively healthy 77-year old men who might be more resistant to toxic effects of smoking than the average person because of genetics, environment or other factors.

Oxidative stress and smoking

Collectively, studies indicate that smokers have a higher level of oxidative stress than non-smokers and it can be speculated that these might be a mechanistic link between smoking and atherogenesis. Cigarette smoke contains free radicals and other toxic substances that could easily contribute to the induction of free radicals.¹⁷⁸ F₂-isoprostanes have been shown to possess

vaso- and bronchoconstrictive properties⁴⁶ that might contribute to the complications of cigarette smoking.

Antioxidants and F₂-isoprostanes

Effects of selenium

The results of Paper V indicate that a high level (highest quartile), selenium in serum was associated with reduced levels of 8-iso-PGF_{2α} (thus, a reduced level of oxidative stress), 27 years later. The inverse relation between selenium status and isoprostanes is supported by cross-sectional studies which also have indicated an inverse association between s-selenium and 8-iso-PGF_{2α}.^{179,180} The fact that we found an association between selenium and isoprostanes despite the long follow-up time would indicate that selenium status might be an important factor in the formation of the basal levels of 8-iso-PGF_{2α}. Further, there is a well established antioxidative role of selenium as a scavenger of reactive oxygen species in the selenoprotein glutathione peroxidase system.^{140,141}

Effects of vitamin E and C

α-Tocopherol

Serum α -tocopherol was weakly inversely correlated with urinary isoprostanes in 77-year old men in the cross-sectional analysis. Some studies confirm this inverse correlation between α -tocopherol and isoprostanes¹⁴⁵ while others have shown no correlation.^{181,182} The cross-sectional picture of the relation between α -tocopherol and oxidative stress levels may point towards a consumption of α -tocopherol as a response to free radicals. Acute induction of oxidative stress by septic shock may also lead to a consumption of α -tocopherol.¹⁸³

Several intervention studies have been undertaken to establish the effect of vitamin E (α -tocopherol), alone or in combination with vitamin C, on isoprostane formation, as recently reviewed by Basu and Helmersson.⁵⁶ In brief, isoprostane formation in healthy men and women is not affected by α -tocopherol or vitamin C supplementation.^{109,184,185} Vitamin E and C supplementation have also been tested in patients where isoprostane formation was increased at baseline. A decrease in elevated 8-iso-PGF_{2α} levels has been shown after vitamin E supplementation in patients with type 2 diabetes, hyperhomocysteinemia and hypercholesterolemia,^{112,144,145,186-188} whereas vitamin C supplementation alone had no effect.^{186,187,189} Supplementation with vitamin E alone in smokers did however not alter the 8-iso-PGF_{2α} formation^{130,181} whereas vitamin C supplementation alone was shown to decrease the formation.^{130,190} Vitamin E and vitamin C theoretically potentiate each other's antioxidant effect, but placebo-controlled combination trials with

vitamins E and C have revealed no such effect on isoprostane formation.^{186,187,191,192}

γ-Tocopherol

γ-Tocopherol was positively correlated to isoprostanes in 77-year old men which might point toward an upregulation of γ-tocopherol in steady-state situations of oxidative stress.

Interventions with γ-tocopherol are sparse, but γ-tocopherol injected to rats has shown to reduce isoprostane formation in an inflammatory model.⁶¹

Connections between inflammation and oxidative stress

Formation of PGF_{2α} and 8-iso-PGF_{2α} is shown to be positively well correlated in healthy men and women and in the ULSAM cohort of elderly men. Further, isomers of CLA given to humans increased the levels of PGF_{2α} and 8-iso-PGF_{2α}^{106,107,109} and even CRP¹⁰⁸ simultaneously, and the increase of CRP and 8-iso-PGF_{2α} were correlated.¹⁰⁸

Experimental studies of rats have shown that carbon tetrachloride, a classic inducer of free radicals, induces 8-iso-PGF_{2α} and subsequently PGF_{2α} formation.¹⁹³ Likewise, infusion of 8-iso-PGF_{2α} in the rabbit caused an induction of PGF_{2α} formation.¹⁹⁴ These results indicate that COX-mediated eicosanoid formation can be activated by free radicals or by 8-iso-PGF_{2α}. Endotoxins, known to induce acute inflammation, given to pigs increased PGF_{2α} formation rapidly, and subsequently induced 8-iso-PGF_{2α} formation.²⁵ There seems to be a link between free radical generation and the inflammatory process and more specific connections between formation of 8-iso-PGF_{2α} and PGF_{2α}, but the exact mechanisms for these connections are not known.

Possible molecular mechanisms

PLA₂, which hydrolyses phospholipids in the membrane allowing arachidonic acid to form, can be activated by hydrogen peroxide (reactive oxygen species).^{100,101} Reactive oxygen species might also upregulate PLA₂ by the IKK/NF-κB pathway.¹⁹⁵ NF-κB is a nuclear transcription factor regulating cytokines, cell-adhesion molecules, acute-phase proteins, PLA₂ and COX-2 in complex cascades. There might be other regulating pathways than the NF-κB pathway by which free radicals induce inflammatory mediators. 8-Iso-PGF_{2α} has been shown to induce IL-8 in macrophages by activation of mitogen-activated protein kinases rather than the NF-κB pathway.¹⁹⁶ Further, oxygen radicals (OH•), can be formed during the prostaglandin synthesis (PGG₂–PGH₂), for the self-deactivation of COX.^{197,198}

Inflammation and oxidative stress in atherogenesis

Relevance of PGF_{2α} and other prostanoids

Out of the arachidonic acid-derived metabolites TXA₂ and PGI₂ have yet been most studied and have been related to atherothrombosis.¹⁹⁹ TXA₂ produced by platelets is mainly a mediator of acute occlusive events and PGI₂ has antiplatelet, antiadhesive and antiproliferative properties and might retard atherogenesis. PGI₂ inhibits cholesteryl ester accumulation and reduces LDL-receptor activity in macrophages and antagonizes the proliferative effects of growth factors in the smooth muscle cell. When endothelial cells are injured or activated, as in the proatherogenic state, cytokines and growth factors are released and PGI₂ formation is subsequently decreased which may cause a net effect of cell proliferation and cholesteryl ester deposition.⁷² The role of PGF_{2α} in this process has not been studied earlier, mainly because of methodological problems with quantification of this compound. COX-2 is upregulated in atherosclerotic lesions as part of the inflammatory process²⁰⁰ (cytokines could activate COX-2 expression through NF-κB). As a result of the upregulation of COX, it can be speculated that a cascade of different prostaglandins could be formed (depending on substrate and synthetase activity in the cells), as mediators and modulators of the inflammatory process. Exploring the formation of PGF_{2α} *in vivo* in relation to diseases, which could lead to atherosclerosis, is one first approach to better understand the role of PGF_{2α} in atherogenesis. The development of a radioimmunoassay specific for non-invasive quantification of a metabolite of PGF_{2α} offers a unique opportunity to study the role of this compound in diseases related to atherosclerosis and atherogenesis. So far we have shown that type 2 diabetes, smoking and intima media thickness in the carotid artery (Wohlin et al, unpublished observation), in elderly men is related to enhanced levels of PGF_{2α}. On the other hand, obesity and elevated fasting insulin, which also are risk factors for atherosclerosis, do not seem to be related to this prostaglandin in this setting.

The biological effects of PGF_{2α} through the PGF_{2α} receptor have mainly been focused on the reproductive organs and the eye. Known biological effects of PGF_{2α} that can be applied to the cardiovascular field are vasoconstriction and increase in heart ventricular weight in rats *in vivo* and proliferation of myocytes *in vitro*.²⁰¹ The known role of PGF_{2α} as an important mediator in the inflammatory process leads to the speculation that PGF_{2α} may be involved in atherogenesis.

Relevance of isoprostanes

An enhanced formation of isoprostanes has been reported in association with several risk factors for atherogenesis, including type 2 diabetes and smoking (as shown in paper III, IV and by others), hypercholesterolemia, hyperhomo-

cysteinemia, obesity and hypertension with renovascular disease.⁵⁶ It has also recently been suggested that isoprostane formation is increased in patients with coronary heart disease independent of other risk factors.²⁰² Thus, it is possible that isoprostanes carry prognostic information in addition to known risk factors. Moreover, it has been suggested that isoprostane formation may be increased by acute myocardial infarction.¹¹⁰ Isoprostanes are considered relevant indicators of oxidative stress in conditions related to cardiovascular diseases, but have isoprostanes themselves any role in atherogenesis?

Isoprostanes have been found in atherosclerotic plaques.²⁰³⁻²⁰⁵ The area of atherosclerotic lesions has been shown to correlate to urinary 8-iso-PGF_{2α} in rats fed an atherogenic diet.²⁰⁶ Thus far known *in vivo* effect of 8-iso-PGF_{2α} is mainly vasoconstriction. *In vitro* studies have shown a number of biological activities for 8-iso-PGF_{2α} that can be related to atherogenesis, including vasoconstriction, aggregation of platelets and enhanced granulocyte adhesion to endothelial cells as earlier reviewed.⁴⁶ A specific isoprostane receptor has not been identified but it is believed that at least some of the biological activity of 8-iso-PGF_{2α} is mediated by the thromboxane receptor.^{47,207} Further, isoprostane formation in monocytes or macrophages may modify their function and isoprostanes may accumulate in vascular smooth muscle cells in atherosclerotic plaques. Formation of isoprostanes in oxidised LDL may lead to uptake by monocytes or macrophages resulting in the formation of foam cells.¹⁷³ However, the *in vivo* role of 8-iso-PGF_{2α} in physiological concentrations is not known. Further, no study of the F₂-isoprostane as a prognostic marker and a predictor of CVD has yet been carried out. From what we know today the evidence is not convincing enough to implicate F₂-isoprostanes in cardiovascular pathology.

Relevance of IL-6, CRP and SAA

Slightly increased levels of circulating IL-6, hsCRP and SAA have been shown to predict CVD.^{29,30,34,37} The molecular basis for the role of IL-6, CRP and SAA in atherosclerosis is not as clear as the epidemiological studies. Most of the biological effects have only been established *in vitro*. IL-6 is not only produced in immune cells but also in cells directly involved in atherosclerosis like endothelial cells, vascular smooth muscle cells and myocytes as reviewed in Kanda et al.²⁰⁸ IL-6 activates B-cells, T-cells and macrophages.

Several reports have focused on the possible role for CRP in atherogenesis.^{32,33} CRP may contribute to endothelial dysfunction by induction of cytokines (IL-6, IL-8), and adhesion molecules (ICAM, VCAM, E-selectin), and inhibition of endothelial nitric oxide synthetase and PGI₂. Further, CRP may stimulate the adhesion of monocytes to the endothelial cells and stimulate uptake of oxidised LDL, cytokine-production and chemotaxis. CRP may also

induce production of matrix metalloproteinase-1 in monocytes and macrophages and induce proliferation of smooth muscle cells as recently reviewed.³³

The physiological and pathophysiological functions of SAA are largely unknown. It has been proposed that SAA may play a role in atherogenesis and its pathophysiological action may be linked to cholesterol transport.³⁶ The cholesterol carrying capacity of HDL in the reverse cholesterol transport might be reduced in the presence of SAA.

Vitamin E

Type 2 diabetes and smoking

α -Tocopherol in serum was reduced in patients with type 2 diabetes and in smokers, two conditions associated with oxidative stress and inflammation. Some studies in type 2 diabetes show the same association^{209,210} while other studies did not show any altered α -tocopherol levels in patients with diabetes or smokers, respectively, and controls.^{166,211,212} Other antioxidants (carotenes and vitamin C) were reduced in serum of smokers.^{157,211} Smokers have been shown to have an increased disappearance rate of α -tocopherol which is in line with the results of Paper IV. The reasons for this discrepancy in results might be related to the age of the study population or in the case of diabetes related to disease duration. In conclusion, the role of tocopherols in relation to oxidative stress and inflammation is not clear. In Paper IV it was observed that the time since smoking cessation was inversely related to α -tocopherol and positively related to γ -tocopherol and it might be possible that α -tocopherol is consumed and γ -tocopherol upregulated in situations of chronic oxidative stress and inflammation.

A further example of the complexity of the relationship between antioxidants and diabetes is the recent ULSAM-study which showed that both low serum β -carotene and low dietary intake of β -carotene independently predicted diabetes development in men during 27 years of follow-up.²¹³ However, a randomized trial with β -carotene supplementation did not result in reduced incidence of diabetes.²¹⁴

Cardiovascular diseases

In parallel with the antioxidant issue discussed above it has been suggested that patients with coronary artery disease have reduced levels of α -tocopherol.²¹⁰ Other studies have suggested that γ -tocopherol rather than α -tocopherol is reduced in patients with coronary heart disease.⁶³ Several cohort studies have shown that those who take vitamin E supplements or have a high intake of vitamin E in the diet have a slightly lower risk of coronary

heart events and all-cause mortality.^{62,215} On the other hand, a large number of randomized, controlled, primary or secondary prevention trials with vitamin E supplementation have shown no reduced risk of CVD or mortality.²¹⁵⁻²¹⁷ A recent meta-analysis including prevention trials evaluating high doses of vitamin E concluded that high dose supplementation might even increase all-cause mortality.²¹⁸ This meta-analysis also included smaller studies performed in patients with chronic diseases and might not be generalized to the general population as pointed out by the authors. Still, there is a clear discrepancy in the results of observational cohort studies and randomized intervention studies. No trial reported has estimated the level of oxidative stress in the study participants. It would be scientifically more appropriate to estimate the levels of oxidative stress at baseline and after vitamin E supplementation in order to determine if the vitamin E supplements had any effect on oxidative injury. Isoprostane determinations may be one reliable tool in this evaluation.⁵⁷ It has been suggested that α -tocopherol may have a pro-oxidative effect which may be related to the conflicting results seen in the cohort studies and in the trials. Many questions remain to be discussed including the lengths of trials, baseline levels of vitamin E, natural or synthetic supplements and when the supplementation should start. It is also difficult to evaluate to what extent cohort studies are influenced by immeasurable confounding factors. The complexity in the field of vitamin E and CVD should be recognized.

Discussion of methods

Quantification of PGF_{2 α} and isoprostanes

Radioimmunoassay

In this thesis the formation of free 8-iso-PGF_{2 α} and formation of 15-keto-dihydro-PGF_{2 α} were quantified by radioimmunoassays developed by Basu. Several other assay techniques for quantification of isoprostanes are used, including GC-MS, LC-MS and immunoassays.⁵² Mass spectrometry based methods usually have high precision but relatively low sample analysing capacity. Immunoassays generally have lower precision than mass spectrometry methods but are more applicable in large epidemiological studies. The radioimmunoassay for 8-iso-PGF_{2 α} used in this thesis requires no sample preparation before analysis and the antibodies have a very low cross-reactivity to related eicosanoids.⁵³ Most other analysis techniques require purification of samples and derivatisation procedures, which may lead to accuracy problems. The radioimmunoassay by Basu has recently been validated with GC-MS methods in a classic oxidative stress model in an international network-based study.⁵⁴

Urine or plasma measurements

15-Keto-dihydro-PGF_{2α} and 8-iso-PGF_{2α} can be detected in several body fluids, including plasma and urine. In this thesis, urinary levels were used to assess the integrated picture of lipid peroxidation over the past 24 hours. Although there are practical advantages with urinary collection and storage, it has been debated whether urinary measurements of eicosanoids reflect the renal rather than the systemic formation. However, it is not likely that the major part of isoprostanes found in the urine is formed in the kidneys. When 8-iso-PGF_{2α} was injected to a rabbit it was rapidly excreted and markedly increased levels of urinary 8-iso-PGF_{2α} could be quantified within 10 min.⁴⁹ Further, experimental studies in healthy humans and animals have shown that alterations in urinary levels of 8-iso-PGF_{2α} caused by intervention are well correlated to alterations in plasma levels.^{26,106,109,193} Cross-sectional human observational studies have shown that plasma and urinary 8-iso-PGF_{2α} are correlated.¹⁰⁵

Likewise, it is not likely that urinary levels of 15-keto-dihydro-PGF_{2α} mainly reflect PGF_{2α} formation in the kidneys. PGF_{2α} injected into a cow or horse was followed by a rapid excretion of the metabolite 15-keto-dihydro-PGF_{2α} in urine.^{14,95} Further, plasma and urinary 15-keto-dihydro-PGF_{2α} has been shown to be correlated in experimental animal studies and in human observational studies.^{105,219} Thus, it is reasonable to believe that urinary 8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} reflect the total systemic formation of each compound. However, in situations of kidney damage urinary excretion might be affected and plasma and urinary levels might not reflect the systemic formation.

Confounding factors

Cardiovascular risk factors

Covariates in the multiple regression models in Paper III-V were chosen mainly based on results from other studies. The same covariates were usually used in all models regardless of the dependent variable being an inflammatory or oxidative stress indicator. Hypercholesterolemia has previously been shown to be related to increased formation of isoprostanes.^{144,182,220} This could not be confirmed in the ULSAM cohort (data not shown), but hyperlipidemia was still considered a possible confounding factor and thus adjusted for. Increased formation of isoprostanes have been found in urine and pericardial fluid in patients with heart failure,^{221,222} and IL-6, CRP and TNF-α, respectively, have been shown to predict heart failure.²²³ Hypertension is not associated with increased formation of isoprostanes^{224,225} but might be related to a state of inflammation²²⁶ and was adjusted for. Acute, or a history of, myocardial infarction is possibly related to increased formation of isoprostanes.^{110,202} Stroke,²²⁷ stable and unstable

angina pectoris^{202,228,229} have been associated with enhanced formation of isoprostanes, thus treated as potential confounding factors. Although no correlation between eicosanoids and BMI could be found in this setting, obesity has been reported to be associated with enhanced formation of isoprostanes, thromboxanes and CRP,²³⁰ thus BMI was considered a possible confounding factor.

Low-dose aspirin treatment

Low-dose aspirin (75–160 mg acetylsalicylic acid), was frequently used among the study participants. Aspirin inhibits the generation of prostaglandins and thromboxanes by irreversibly blocking the active site of COX.²³¹ The subjects in ULSAM with daily treatment with low-dose aspirin have decreased formation of PGF_{2α} and possibly slightly lower levels of 8-iso-PGF_{2α}.⁹⁴ Low-dose aspirin has not been shown to affect levels of acute-phase proteins but a study showed that elevated CRP levels could be reduced by daily intake of 300 mg aspirin.²²⁸ Thus, low-dose aspirin and other NSAID medication were considered as potential confounding factors.

Strengths and limitations

The large number of participants in the cohort and the population-based setting are major advantages when exploring low-grade inflammation and oxidative stress. The fact that participants are of the same age, sex and ethnic group may be related to both advantages and limitations. The oxidative stress indicator 8-iso-PGF_{2α} formation may vary in relation to these above mentioned factors and studies exploring the formation of 8-iso-PGF_{2α} should preferably be age-, sex- and ethnicity-matched.⁵⁶ The disadvantage with this setting is that results from the papers based on ULSAM in this thesis cannot be extrapolated to the general population.

The ULSAM reinvestigation at age 77 was not originally designed for the hypothesis presented in this thesis, therefore some important aspects of smoking could not be taken into consideration i.e. cigarette-years and passive smoking. Further, data on vitamin supplementation could not be evaluated. However, detailed information of daily medication was available from almost all participants.

Paper V

The ULSAM population is well-characterized and has been followed up for more than 27 years. The associations observed in Paper V despite the long follow-up time may indicate that selenium is an important factor in the regulation of prostaglandins and maybe even in isoprostane formation. On the other hand, a long follow-up time may also increase the risk for introduction of confounding factors. However it could be speculated that the participants

did not change their dietary habits during the study in a way that would bias the results. This speculation is based on the finding that the men in the highest quartile of selenium at age 50 did not report a significantly higher intake of vitamins in the diet at age 70 (data not shown).

The design of the study does not exclude a cross-sectional association between selenium and eicosanoid formation. Further, in parallel with other studies of antioxidants and disease, there is a possibility that selenium is merely an indicator of another variable that in reality is of importance for eicosanoid formation. Quantification of GSHPx activity at baseline or follow-up could have strengthened the hypothesis presented in Paper V.

It should be noted that the part of the subgroup of the population studied in Paper V represents those who were well enough to participate in the reinvestigation at age 77, thus presumably the healthiest men in the cohort. This resulted in a relative loss of power to detect associations and possibly lead to the result that selenium status in this subgroup of the cohort did not correlate to cardiovascular risk. An alternative study design, however not possible in this thesis, would have been selenium measurements at age 50, evaluation of inflammation and oxidative stress at age 60 or 70, and subsequently evaluation of cardiovascular morbidity and mortality.

CONCLUSIONS

The mean intra-individual variation of $\text{PGF}_{2\alpha}$ and 8-iso- $\text{PGF}_{2\alpha}$ formation between days in healthy individuals is 41% and 42%, respectively. The biological variation is a factor to be taken into account when planning a clinical study.

A single morning urine sample, or a mean of several spot urine samples during the day, seems to reflect the diurnal 8-iso- $\text{PGF}_{2\alpha}$ excretion. A morning urine sample can thus be used to estimate the basal level of 8-iso- $\text{PGF}_{2\alpha}$ formation and represents a more practical alternative to the 24-hour urine collection in clinical studies.

$\text{PGF}_{2\alpha}$ formation was increased in active and former smokers, and in patients with type 2 diabetes independent of other cardiovascular risk factors. This suggests that a chronic low-grade COX-mediated inflammatory process is present in association with two major risk factors for atherosclerosis. These studies are the first to describe $\text{PGF}_{2\alpha}$ formation in a large cohort and in relation to the cardiovascular field.

8-Iso- $\text{PGF}_{2\alpha}$ formation was increased in active and former smokers, and patients with type 2 diabetes independent of other cardiovascular risk factors, indicating a higher level of oxidative stress associated with these conditions.

Further, active and former smokers had increased levels of cytokine IL-6, which could indicate the presence of low-grade inflammation mediated by cytokines in smokers. CRP and SAA seemed more closely associated to overweight than type 2 diabetes in this setting of elderly men.

High levels of s-selenium at age 50 predicted reduced formation of $\text{PGF}_{2\alpha}$ and 8-iso- $\text{PGF}_{2\alpha}$, but not cytokine-related inflammatory mediators, in the 27-year follow-up. Selenium seemed to be related to an inhibitory effect on COX-mediated inflammation and oxidative stress, respectively.

FUTURE PERSPECTIVES

Speculatively, a low-grade, chronic COX-mediated inflammation, oxidative stress, and possibly cytokine-mediated inflammation may be included in the underlying factors which contribute to the accelerated atherosclerosis reported in association with type 2 diabetes, smoking and low selenium status in some populations. Further work is needed to confirm these speculations. $\text{PGF}_{2\alpha}$ formation in relation to other risk factors for atherosclerosis also needs to be explored. Another approach is to study if $\text{PGF}_{2\alpha}$ or 8-iso- $\text{PGF}_{2\alpha}$, respectively, predicts clinical cardiovascular diseases in prospective studies. The outcome variable may also be estimation of atherosclerosis such as intima media thickness.

Cause and effect cannot be estimated from association studies. Prospective studies of $\text{PGF}_{2\alpha}$ and 8-iso- $\text{PGF}_{2\alpha}$, respectively, and diabetes development are needed. The existing ULSAM cohort might not be optimal for these studies because the baseline at 77 years of age could be a limitation in the evaluation of the study.

$\text{PGF}_{2\alpha}$ formation in relation to disease has so far only been studied in men, the relations need to be studied in women as well.

To explore if selenium has any effect on prostaglandin and isoprostane formation intervention studies are needed.

Variation in prostaglandins and isoprostane formation due to genetic variations in the COX-1 and COX-2 gene, and genes important for the cytokines, are unknown and need to be explored. Further, the role of genetic variation in the COX-genes in development of diabetes and cardiovascular diseases has yet not been addressed.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to everyone who contributed to this thesis. I am particularly grateful to:

Samar Basu, my principal supervisor and inspiring mentor, for enthusiastic and patient introduction to and guidance in science in general and in the field of prostaglandins and isoprostanes in particular, for always being available for discussions and support, for enjoyable company during scientific meetings in France, United States and the Caribbean, for introducing me to key scientists in the field of inflammation and oxidative stress during scientific meetings, for believing in me, and for great friendship.

Bengt Vessby, my second supervisor and head of the Section for Clinical Nutrition Research, for patiently introducing me to the scientific way of thinking, for giving me the opportunity to be a PhD-student in your group, for generous opportunities to take part in international scientific meetings and courses, for clinical and scientific guidance in the field of metabolic diseases and lipids, and for leading the way in the field of fatty acids with enthusiasm.

Hans Lithell, former head of the Section for Geriatrics, for providing a generous and creative research environment, and for your excellent work and devotion to the ULSAM cohort, *Lars Lannfelt*, present head of the Section for Geriatrics and head of Geriatriska kliniken, Akademiska sjukhuset, for your commitment for the maintenance of the ULSAM cohort and for introducing the dimension and knowledge of genetics into ULSAM research.

Eva Sejby, *Barbro Simu*, and *Siv Tengblad*, expert technicians in the lab, for patient guidance and support during my confused days in the lab, for joyful coffee breaks, for help with sample collection and logistics in all my studies, for daily work with the ULSAM cohort in various ways including sample preparation, organization and storage, and for being excellent organizers of several get-together lunches and dinners at the department. A special thanks to Eva for guide and support in the intricate world of pipettes, scintillation fluid, beta-counters, and standard curves during my work with the radioimmunoassays.

Rawya Mohsen for taking excellent care of the ULSAM database, and for helping out with internet, computers and software necessary for the scientific work, and *Mattias Pålsson* for taking good care of the network and computers, and for being available for consultation on technical issues with very short notice.

All study participants at the department and all men participating in the ULSAM studies over the years. Your contributions are greatly appreciated.

Anders Larsson, co-author, for valuable contribution of cytokine-related inflammatory indicators to this thesis and for fruitful discussions in the field of inflammation.

Johan Ärnlov, co-author, for constructive collaboration and inspiration to Paper V, for guidance in the field of epidemiology and longitudinal studies, for introducing me to the clinical work at Överviktsenheten, and for joyful company during lunches and coffee-breaks.

Annika Smedman for happy team work, for introducing me to software necessary for scientific research, for sharing my frustration of being dependent on SJ, for the support and help with the summary of the thesis, and for being a loyal friend.

Liisa Byberg for advice in the statistical jungle, for guidance in the design of webpages, and for being a loyal friend, and *Björn Zethelius* for sharing expertise in statistics and in the field of diabetes, and for being my private car driver

All good friends and colleagues at the Sections for Clinical Nutrition Research and Geriatrics with special reference to *Brita Karlström*, head of the dieticians at Geriatriska kliniken, for dedicated work with dietary issues, and for your warm and caring attitude towards everyone, *Margareta Öhrvall*, head of Överviktsenheten, for wise advice in clinical situations, *Merike Boberg* for advice in science, education and career, *Eva Warensjö* for merry lunch and coffee breaks, and for dedicated work with the ICTUS collaboration, *Agneta Andersson*, for being a cheerful and caring roommate, *Ulf Risérus* for fruitful discussions particularly in the field of inflammation and oxidative stress, and for good laughs at inconvenient places, *Ann-Cristin Åberg* for joyful lunch and coffee breaks, and for critical reading of my manuscripts, *Eva Södergren* for guidance in the field of oxidative stress, and for reading my early manuscripts, *Arvo Hänni* for collaboration and expertise in the field of metals (magnesium and cars), *Cecilia Nälsén* for organizing nutrition seminars, and for sharing knowledge of antioxidants, *Johan Sundström* for sharing statistical epidemiological knowledge and for future

selenium team work, *Martin Wohlin* for future collaboration in the field of atherosclerosis, *Klara Halvarsson-Edlund* for warm and friendly support and team work at Överviktsenheten, *Kristina Björklund-Bodegård* for cheerful lunch and coffee breaks, and for friendly advice concerning the life as a PhD-student and young clinician, *Karin Modin* for taking care of practical issues at the department, *Erika Olsson* for being supportive in the fight against migraine, *Lena Kilander* for hard work with the reinvestigation of ULSAM 77, *Anette Järvi* for sharing the knowledge about the mystery of carbohydrates, *Kristina Dunder*, *Hans-Erik Johansson*, *Kristin Franzon*, *Achraf Daryani*, *Agneta Nilsson* for patient work at Överviktsenheten, *Anders Sjödin*, *Elisabet Rytter*, *Bernice Wiberg*, *Erik Ingelsson*, *Kristina Ström-Möller*, *Marie von Post Skagegård*, and *Anu Hedman* for friendly and generous attitude.

Staff and dieticians at Överviktsenheten for taking good care of the participants of ULSAM

Kristin Elf for being a loyal friend, for giving good advice, for joyful lunch breaks, and for sharing the frustration of being a medical student, a green assistant physician, and writing a summary of a thesis.

My father *Seved* for inspiring me to get into the field of research, and for supporting me in various ways, my mother *Britta* for believing in me, and for supporting me in various ways, my younger brother *Erik* and younger sister *Martina* for helping me with computer programmes, and for being who they are.

Last but not least, *Mattias*, my companion for life, for endless love and support, for proofreading of manuscripts, and for giving me the opportunity to do this my own way. I love you and you are the best!

This work was financially supported by grants from the Swedish Society of Medicine, Swedish Heart-Lung foundation, Thuréus Foundation for Geriatric Research, the Faculty of Medicine at Uppsala University, Swedish Nutrition Foundation, and Geriatrics Research Foundation.

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