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Biomarkers of Arachidonic Acid Metabolism as Predictors for Presence of Cardiovascular Disease

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Content

1 Summary

Eicosanoids are important lipid mediators primarily generated from arachidonic acid (AA) which is liberated out of membrane phospholipids by phospholipase A_2 (PLA_2). AA is metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) pathways to a number of different metabolites such as thromboxanes, prostaglandins, hydroxyeicosatetraenoic acids (HETE) or leukotrienes. Several lines of evidence implicate an important role of eicosanoids in central processes of inflammation and due to their widespread synthesis in blood and vascular cells these metabolites may play a central role in atherogenesis. On account of this, the analysis of the AA metabolism in whole blood might provide a new marker of atherosclerotic burden and is predestinated as biomarker for diagnosis and prediction of coronary artery disease (CAD).

In previous work, we developed an *in vitro* assay where whole blood from patients was stimulated with 1 µg/mL lipopolysaccharide (LPS) for 24 hours. Samples were centrifuged and AA metabolites such as AA, 5-HETE, 11-HETE, 12-HETE, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) were analyzed out of supernatant by liquid chromatography tandem mass spectrometry. Furthermore, messenger ribonucleic acid for quantitative gene expression analysis of *COX-2* and *prostaglandin E synthase* (*PGES*) was isolated out of cellular components.

In the current thesis, we improved the *in vitro* whole blood activation model and established additional quantitative real time polymerase chain reaction assays for *PLA_2*, *COX-1*, *thromboxane synthase* (*TXAS*), *prostaglandin F synthase* (*PGFS*), *5-LOX*, *5-LOX activating protein* (*FLAP*) and *12-LOX* gene expression analysis to cover major routes of AA metabolism and investigate the discriminatory potential of these eicosanoid pathways for inflammatory diseases.

Furthermore, different stimuli (LPS, tumor necrosis factor alpha and oxidized low-density-lipoprotein) for whole blood activation have been evaluated. Our data revealed most significant effects for LPS which were time- but not dose-dependent.

Since we observed a rapid eicosanoid response upon stimulation we asked whether eicosanoid response on gene expression and metabolite level is preformed or underlies *de novo* synthesis. To this end, transcriptional and translational inhibition experiments were performed to characterize these regulatory mechanisms. Data suggested that the production of metabolites underlies *de novo* synthesis upon stimulation, which is controlled both, at the level of transcription and translation.

We then applied the new *in vitro* whole blood assay to test the variability of the LPS-induced AA metabolism in healthy subjects. We showed major inter-individual differences for all investigated

target genes as well as metabolites, suggesting that varying eicosanoid response might be predisposing towards different susceptibility to inflammatory diseases.

The predictive potential of the individual eicosanoid response for the presence of atherosclerosis was examined in 92 patients with or without CAD using the newly developed whole-blood assay. We found that the eicosanoid response on gene expression (*COX-1*, *COX-2*, *PGES* and *FLAP*) and metabolite level (AA, 5-, 11- and 12-HETE) was significantly ($P<0.05$) different in patients with or without CAD. These data allowed developing a score consisting of three biomarkers of AA metabolism with an area under the curve (AUC) of 83.6, which is superior to currently available scores of blood markers of CAD.

Taken together, this work established an *in vitro* activation assay for the metabolites of AA metabolism, characterized its regulating mechanism and showed its potential for diagnostic testing of patients to the presence of CAD.

2 Zusammenfassung

Eicosanoide sind bedeutende Lipidmediatoren, welche aus Arachidonsäure (AA) nach deren Freisetzung aus Membranphospholipiden durch die Phospholipase A_2 (PLA_2) gebildet werden können. AA wird anschließend über den Cyclooxygenase (COX-) und Lipoxygenase (LOX-) Stoffwechselweg metabolisiert, wobei eine Reihe von verschiedenen Metaboliten wie Thromboxane, Prostaglandine, Hydroxyeicosatetraensäuren (HETE) sowie Leukotriene entstehen. Eicosanoide haben Einfluss auf die zentralen Prozesse von Entzündungsreaktionen und aufgrund ihrer weit verbreiteten Bildung in Zellen des Blutes sowie des Gefäßsystems spielen diese Metabolite ebenfalls eine Rolle in der Entstehung der Atherosklerose. Die Untersuchung des AA Metabolismus in Vollblut könnte einen neuen Parameter für atherosklerotische Veränderungen darstellen und ist somit prädestiniert als Biomarker für die Diagnostik und Prädiktion einer Koronaren Herzerkrankung (KHK).

In Vorarbeiten unserer Gruppe entwickelten wir einen *in vitro* Assay, bei dem Vollblut von Patienten mit 1 µg/ml Lipopolysaccharid (LPS) für 24 Stunden stimuliert wurde. Die Proben wurden anschließend zentrifugiert und die Metabolite des AA Metabolismus (AA, 5-HETE, 11-HETE, 12-HETE, Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), Prostaglandin E_2 (PGE_2) und Thromboxan B_2 (TxB_2)) in den Überständen mittels Flüssigchromatografie und Tandem-Massenspektrometrie analysiert sowie quantifiziert. Des Weiteren wurde mRNA (messenger RNA) für quantitative Genexpressionsanalysen der *COX-2* sowie *Prostaglandin E-Synthase* (*PGES*) aus den zellulären Bestandteilen des Ansatzes isoliert.

Im Rahmen der vorliegenden Promotionsarbeit entwickelten wir das *in vitro* Vollblutmodel weiter und etablierten zusätzliche Assays für *PLA_2*, *COX-1*, *Prostaglandin F-Synthase* (*PGFS*), *Thromboxansynthase* (*TXAS*), *5-LOX, 5-LOX aktivierendes Protein* (*FLAP*) und *12-LOX* Genexpressionsanalysen, um so alle Hauptwege des AA Metabolismus abzudecken und das diskriminierende Potential dieser Stoffwechselwege für entzündliche Erkrankungen zu untersuchen.

Des Weiteren wurden verschiedene Stimulanzien (LPS, Tumornekrosefaktor Alpha und oxidiertes Low-Density-Lipoprotein) für die Vollblutaktivierung ausgetestet. Unsere Ergebnisse zeigten, dass die stärksten Effekte LPS-vermittelt waren und eine ausgeprägte Zeit- jedoch keine Dosisabhängigkeit aufwiesen.

Da wir eine sehr schnelle Eicosanoidantwort auf den inflammatorischen Stimulus beobachten konnten, fragten wir uns, ob die Eicosanoidantwort auf Genexpressions- und Metabolitenebene bereits vorhanden ist oder einer *de novo* Synthese unterliegt. Um die regulatorischen Mechanismen besser zu charakterisieren, führten wir Experimente zur Transkriptions- und Translationshemmung durch. Die Daten suggerierten, dass die Produktion der Metabolite einer

de novo Synthese nach Stimulation unterliegen, welche sowohl auf der Ebene der Transkription als auch Translation kontrolliert wird.

Wir verwendeten nun das neue *in vitro* Vollblutmodel an, um zu testen, wie hoch die Variabilität des LPS-abhängigen AA Metabolismus in gesunden Individuen ist. Wir konnten große interindividuelle Unterschiede für alle von uns untersuchten Zielgene sowie -metaboliten aufzeigen und nahmen an, dass diese variierende Eicosanoidantwort prädisponierend für die Suszeptibilität für entzündliche Erkrankungen sein könnte.

Das prädiktive Potential der individuellen Eicosanoidantwort für das Vorhandensein von Atherosklerose wurde in 92 Probanden mit und ohne KHK mittels des neu entwickelten Vollblutassays geprüft. Wir fanden signifikante Unterschiede ($P<0.05$) in der Eicosanoidanwort sowohl auf Genexpressions- (*COX-1*, *COX-2*, *PGES* und *FLAP*) als auch auf Metabolitenebene (AA, 5-, 11- and 12-HETE) zwischen Patienten mit und ohne KHK. Diese Daten erlaubten die Entwicklung eine Scores, welcher aus 3 Biomarkern des AA Metabolismus besteht, eine AUC von 83.6 aufweist und bereits publizierten Scores mit Blutbiomarkern für die Prädiktion einer KHK überlegen ist.

Zusammenfassend wurde in dieser Arbeit ein *in vitro* Aktivierungsassay für die Metabolite des Arachidonsäuremetabolismus entwickelt, es wurden die zugrundeliegenden regulatorischen Mechanismen charakterisiert sowie das Potential dieses Stoffwechselweges zur diagnostischen Testung von Patienten hinsichtlich dem Vorliegen einer KHK aufgezeigt.

3 Abbreviations

AA	Arachidonic Acid
ActD	Actinomycin D
AST	Aspartate Aminotransferase
AUC	Area Under the Curve
BLT	Leukotriene Receptor
CAD	Coronary Artery Disease
CCL	Chemokin Ligand
CCS	Coronary Calcium Score
cDNA	complementary Deoxyribonucleic Acid
CHOD-PAP	Cholesterol Oxidase-Phenol-Aminophenazone
CHX	Cycloheximide
CLIA	Chemiluminescence Immunoassay
CO_2	Carbon Dioxid
COX	Cyclooxygenase
CMR	Cardiac Magnetic Resonance
CT	Computed Tomography
$CuCl_2$	Copper (II) Chloride
CYP	Cytochrome
DEPC	Diethyl Pyrocarbonate
DMSO	Dimethyl Sulfoxide
DNase	Desoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
ECG	Echocardiography
EcoRI	Restriction Enzyme of an *E. coli* strain that carries the cloned EcoRI gene from *E. coli RY13*
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate
EET	Epoxyeicosatrienoic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EP	PGE_2 Receptor
ESI	Electrospray Ionization
FAM	Fluorescein Amidite
FLAP	5-Lipoxygenase Activating Protein
FSB	First Strand Buffer
H_2O	Dihydrogenmonoxid (Water)

HDL	High-Density-Lipoprotein
HETE	Hydroxyeicosatetraenoic Acid
HindIII	Restriction Enzyme of an *E. coli* strain that carries the HindIII gene from *Haemophilus influenzae*
HpETE	Hydroxyperoxyeicosatetraenoic Acid
HPLC	High Performance Liquid Chromatography
hsCRP	high sensitive C-reactive Protein
ICA	Invasive Coronary Angiography
IDL	Intermediate-Density-Lipoprotein
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IQR	Interquartile Range
IS	Internal Standard
LB	Luria-Bertani
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LDL	Low-Density-Lipoprotein
LOX	Lipoxygenase
LMP	Low Melt Point
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxines
MeOH	Methanol
$MgCl_2$	Magnesium Chloride
MMP	Matrix Metalloproteinase
MRM	Multiple Reactions Monitoring
mRNA	messenger Ribonucleic Acid
NaCl	Sodium Choride
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B-cells
NIT	Non-Invasive Testing
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PGD_2	Prostaglandin D_2
PGDS	Prostaglandin D Synthase
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E Synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFS	Prostaglandin F Synthase

PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGIS	Prostacyclin Synthase
PLA_2	Phospholipase A_2
oxLDL	oxidized Low-Density-Lipoprotein
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RIA	Radioimmunoassay
RNase	Ribonuclease
RT	Reverse Transcription
SEM	Standard Error of the Mean
SMC	Smooth Muscle Cells
SPE	Solid Phase Extraction
SPECT	Single Photon Emission Computed Tomography
TAE	TRIS-Acetate-EDTA
TAMRA	Carboxytetramethylrhodamine
TE	TRIS-EDTA
TNFα	Tumor Necrosis Factor Alpha
TRIS	Tris-(hydroxymethyl)-aminomethan
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
TXAS	Thromboxane Synthase
VLDL	Very-Low-Density-Lipoproteins
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactoside
$ZnSO_4$	Zinc Sulfate

4 Remarks on Gene Nomenclature

All gene symbols are assigned and typeset following the guidelines established by the HUGO Gene Nomenclature Committee. According to these guidelines, the following specifications will be used:

Human gene and mRNA symbols are written in capital letters and italic font (e.g. *PGES)*

Protein symbols are written in capital letters and normal font (e.g. PGES)

Database links

HUGO Gene Nomenclature Committee: http://www.genenames.org/

5 Introduction

5.1 Arachidonic Acid Metabolism

Phospholipids are an integral part of the lipid bilayer of the cell membranes, where arachidonic acid (AA) as an unsaturated fatty acid can be liberated by Phospholipase A_2 (PLA_2).[1] Free AA is then processed enzymatically by cyclooxygenase (COX), lipoxygenases (LOX) and cytochrome P450 pathways or non-enzymatically by peroxidation to a number of different eicosanoids (e.g. prostanoids, hydroxyeicosatetraenoic acids (HETE), leukotrienes (LT), lipoxines (LX) or epoxides (epoxyeicosatrienoic acids) (Figure 1). Almost all cell types participate in eicosanoid synthesis, though metabolite level depends on local production and distribution of specific precursors as well as enzymes that are differentially regulated in variable cells.[2,3]

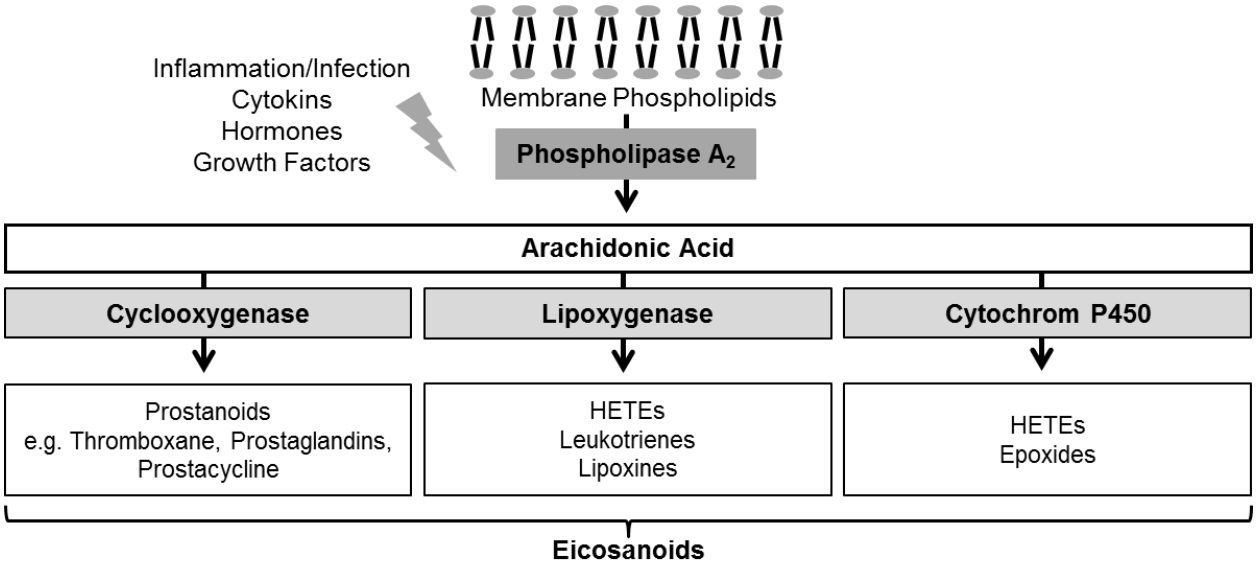


Figure 1. Schematic Representation of Enzymatic Arachidonic Acid Metabolism. Enzymes (grayed boxes) and corresponding metabolites of arachidonic acid (AA) metabolism: Phospholipase A_2 liberates AA out of membrane bound phospholipids, whiles subsequent metabolisation by cyclooxygenase, lipoxygenase or cytochrom P450 pathways reveals a broad spectrum of eicosanoids such as prostanoids, hydroxyeicosatetraenoic acids (HETE), leukotrienes, lipoxines and expoxides.[2]

COX is the rate limiting enzyme for the biosynthesis of prostanoids by converting AA to Prostaglandin H_2 (PGH_2) followed by subsequent metabolism by thromboxane synthase (TXAS), prostaglandin (PG) synthases (PGFS, PGES and PGDS) or prostacyclin synthase (PGIS). Conversion leads to the formation of different metabolites such as thromboxane A_2 (TxA_2) and the inactive metabolite TxB_2, $PGF_{2\alpha}$, PGE_2, PGD_2 as well as prostacyclin (PGI_2) (Figure 2).[4,5] Macrophages are the main source of the mentioned prostanoids, however, other

cell types also synthesize specific COX-products: platelets TxA_2, endothelial cells PGI_2 and epithelial cells, fibroblasts as well as smooth muscle cells PGE_2.[6]

There are two main isoforms of the cyclooxygenase and most cells are able to express both isoenzymes. COX-1 is expressed in tissues serving for the basic metabolic rate of AA metabolism and inducible COX-2 can be activated by inflammatory stimuli, cytokines, hormones or growth factors and is mainly involved in the regulation of inflammation.[6]

COX- derived eicosanoids play a central role in physiological and pathophysiological processes and exert their effects by binding to membrane receptors that trigger changes in cytosolic second messenger generation, activation of protein kinase or alteration of membrane potential.[2] They are regulators of vascular and respiratory smooth muscle tone (TxA_2, PGE_2 and $PGF_{2\alpha}$), platelet aggregation (TxA_2 and PGI_2) or vascular permeability (PGE_2).[7-9] Prostanoids are also modifiers of pain, fever (PGE_2), mast cell maturation and eosinophil recruitment in allergy (PGD_2). In inflammatory processes, TxA_2 is considered to be a proinflammatory factor, while PGI_2 and PGE_2 are predominantly anti-inflammatory mediators.[10,11] In consideration of their extraordinary effects, eicosanoids are also investigated as targets in renal function, cancer, gastrointestinal integrity, diseases of the brain and parturition.[12]

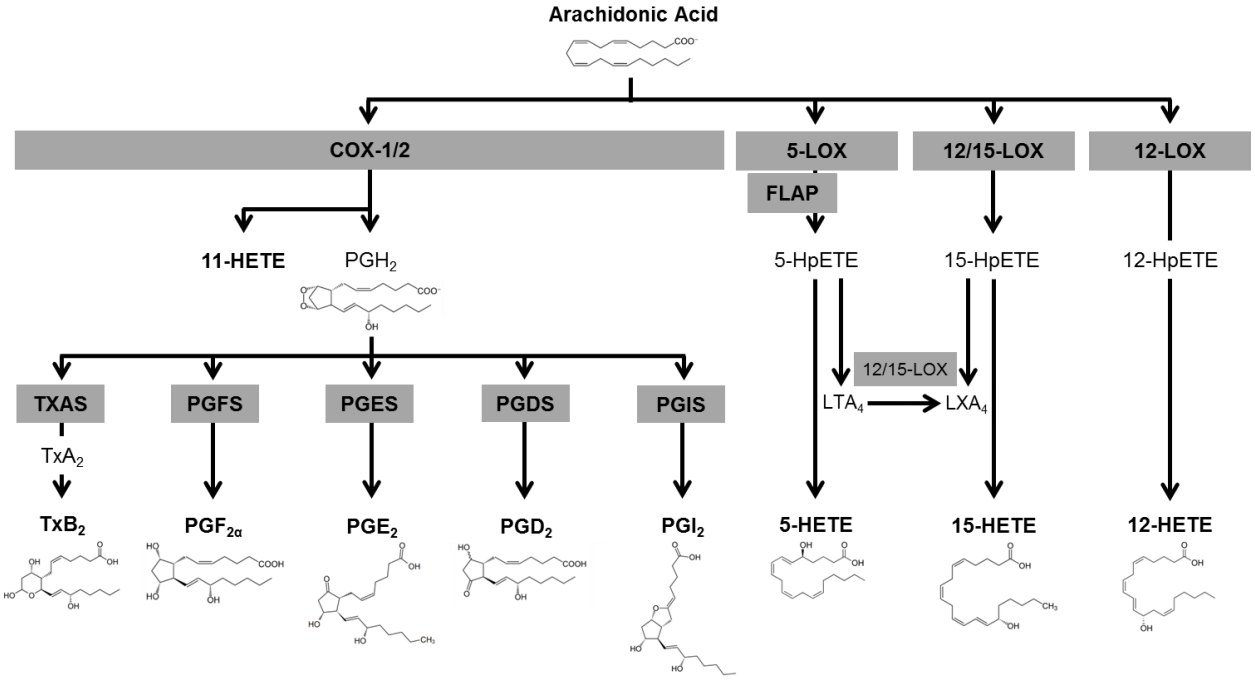


Figure 2. Cyclooxygenase and Lipoxygenase Dependent Arachidonic Acid Metabolism. Enzymes (grayed boxes) and corresponding metabolites of cyclooxygenase (COX) and lipoxygenase (LOX) pathways: COX converts arachidonic acid to prostaglandin H_2 (PGH_2) followed by subsequent metabolisation by thromboxane synthase (TXAS), prostaglandin (PG) synthases (PGFS, PGES and PGDS) or prostacyclin synthase (PGIS) to thromboxane A_2 (TxA_2) and the inactive metabolite TxB_2, $PGF_{2\alpha}$, PGE_2, PGD_2 as well as prostacyclin (PGI_2). 5-LOX catalyzes in conjunction with its cofactor five lipoxygenase activating protein (FLAP) the metabolisation of AA to 5-hydroxyperoxyeicosatetraenoic acid (HpETE) and subsequently to leukotriene A_4 (LTA_4) and 5-hydroxyeicosatetraenoic acid (5-HETE). 12-LOX mediates the conversion of AA to 12-HETE.[13]

In addition to COX-derived prostaglandins, leukotrienes and related hydroxyeicosatetraenoic acids (HETEs) are formed by 5-, 12-, as well as 15-LOX pathways.[14] The main metabolic pathway is represented by 5-LOX that catalyses in conjunction with its cofactor 5- lipoxygenase activating protein (FLAP) the metabolism of AA to 5- hydroxyperoxyeicosatetraenoic acid (HpETE) and subsequently to leukotriene A_4 (LTA_4) and 5-HETE.[15,16] Another LOX-pathway is represented by 12-LOX that mediates the conversion of AA to 12-HETE. Lipoxygenized AA metabolites exert their biological effects by G protein-coupled receptors and reveal chemotactic properties, mediate vascular permeability as well as contractility and are proinflammatory lipid mediators primarily produced by inflammatory cells like leukocytes and macrophages.[13,17-19] Leukotrienes are also considered to be central modulators of allergic rhinitis and bronchoconstriction in asthma where therapeutic treatment includes 5-LOX inhibitors and leukotriene receptor antagonists.[20,21] 12-HETE as LOX-metabolite seems to be relevant in several pathological disorders like atherosclerosis, hypertension, hyperglycaemia and prostate cancer, but findings are discussed controversial.[22]

In summary, metabolites of AA metabolism reveal central physiological and pathophysiological effects. Due to their superordinate function on almost all processes in the human body, further investigation and analysis of eicosanoids in health and disease might be of great interest.

5.2 LPS *in vitro* Whole Blood Model

Different phenotypes of AA metabolism and its diagnostic potential have been poorly investigated so far because of the chemical and biological complexity as well as the analytical difficulties of these metabolites.

Eicosanoids are known as local mediators that act near their site of synthesis and have a short half-life due to metabolite instability and rapid metabolism.[16,23] Exemplarily, the intermediate TxA_2 revealed a half-life of approximately 32 seconds when prostaglandin G_2 is converted into TxB_2 in platelets, while the metabolism of PGE_2 occurred within 15 seconds and half-life of the corresponding stable metabolite is about 8 minutes.[24,25] On account of this, plasma concentration of eicosanoids beside inflammatory processes are reported to be very low.[26,27]

Therefore, immunoassays such as radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs) or chemiluminescence immunoassays (CLIAs) have been developed for quantifying eicosanoid concentration with lower limits of quantification in the picogram range.[28,29] However, limitations of these methods like low sample throughput and cross reactions of antibodies due to isomeric similarity between eicosanoids with the same molecular weight but different structural configuration have been reported.[30,31]

To overcome those limitations, our group has developed a high performance liquid chromatography (HPLC) method combined with tandem mass spectrometry (MS/MS) for increased sensitivity and specificity as well as facilitated high throughput of samples. Using a multiparametric approach, the method allowed the simultaneous identification and quantification of AA metabolites.[32] Furthermore, we have developed an *in vitro* whole blood activation model using lipopolysaccharide (LPS) that was already described as potent activator of COX- and LOX dependent AA metabolism (Figure 3).[6,33] Due to this standardized inflammatory stimulus, the release of eicosanoids was excited and consequently the concentration of AA metabolites was increased. For the *in vitro* model, whole blood from study subjects was mixed with cell culture medium containing 1 µg/mL LPS and incubated at 37 °C and 5 % CO_2 for 24 hours. Then, the samples were centrifuged and metabolites were analyzed out of supernatant where LC-MS/MS method allowed quantification of AA, 5-HETE, 11-HETE, 12-HETE, $PGF_{2\alpha}$, PGE_2 and TxB_2. Beside the analysis of eicosanoid profiles on mediator level, the investigation of corresponding gene expression level of AA-metabolizing enzymes might provide further insights on the activity of these pathways. Therefore we improved the whole blood activation assay and isolated messenger ribonucleic acid (mRNA) for gene expression analyses by quantitative real time polymerase chain reaction (qRT-PCR) out of cellular components and established RT-PCR assays for analysis of *COX-2* and *PGES* expression.[34]

In a first clinical application using the whole blood activation model, it was found that the determination of the eicosanoid response on mediator and gene expression level could be used as diagnostic as well as prognostic marker for inflammatory diseases. Here septic patients revealed significant reduced concentrations of AA, 11-HETE, PGE_2 and TxB_2 after 24 hours LPS activation compared to healthy controls and this suppression of the eicosanoid response was associated with severity of sepsis and worse clinical outcome. To further elucidate the sepsis-associated effects on release of COX-dependent metabolites, comparative analyses of corresponding target genes were performed in a second independent study cohort, where we found a reduced inducibility of *COX-2* and an upregulated *PGES* on gene expression level of septic patients compared to healthy controls.[34]

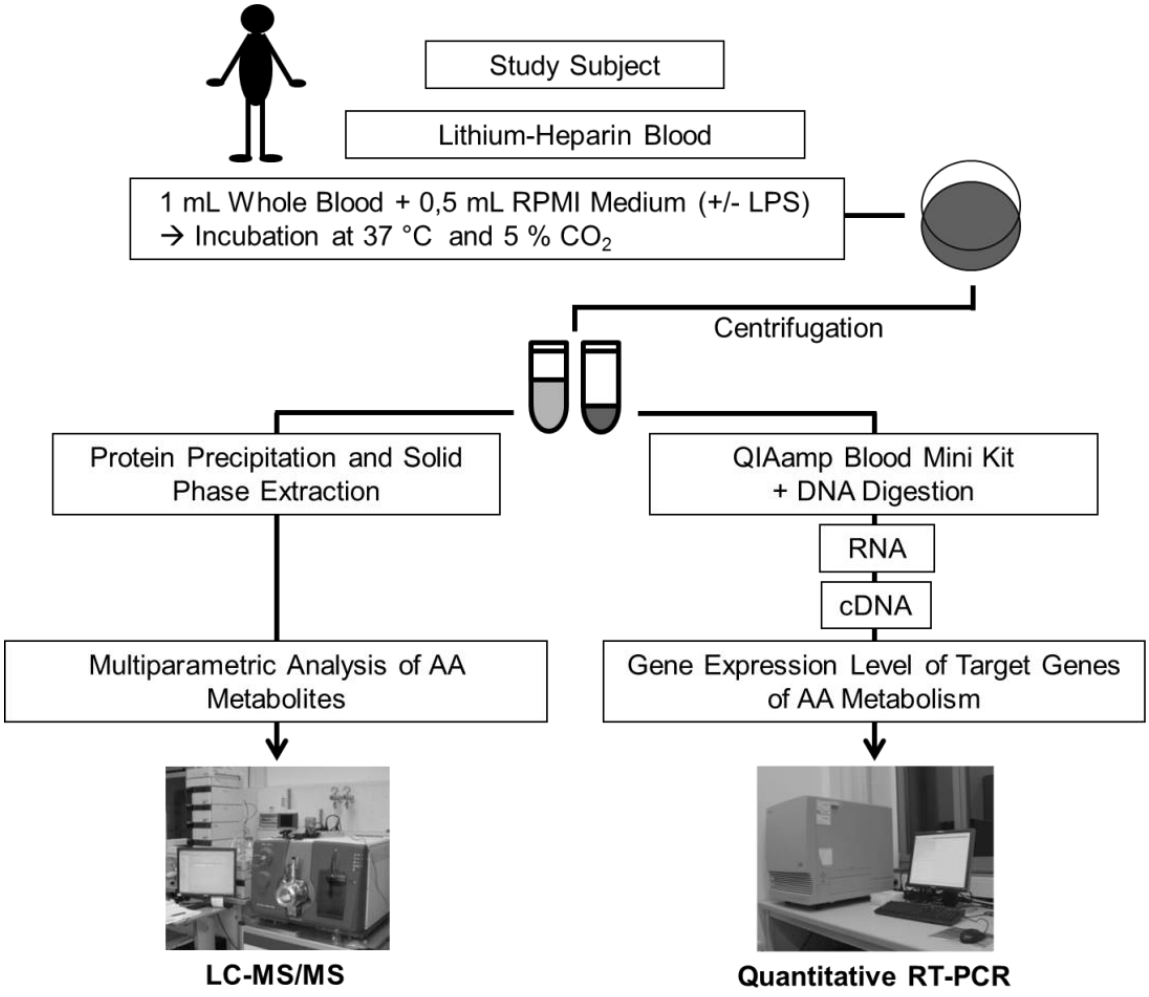


Figure 3. *In vitro* Whole Blood Activation Assay. Human whole blood (lithium heparin) was mixed with cell culture medium containing 1 µg/mL lipopolysaccharide (LPS) and incubated at 37 °C and 5 % CO_2 for 24 hours. Samples were centrifuged, metabolites were analyzed out of supernatant by liquid chromatography tandem mass spectrometry (LC-MS/MS) and messenger ribonucleic acid (mRNA) for quantitative gene expression analysis by reverse transcription polymerase chain reaction (RT-PCR) was isolated out of cellular components.

5.3 Arachidonic Acid Metabolism and Atherosclerosis

5.3.1 Pathogenesis of Atherosclerosis

Atherosclerosis is a chronic inflammatory disease that is regulated by diverse molecular mechanisms. It is initiated by endothelial dysfunction that causes increased vascular permeability. Chronic exposure to cardiovascular risk factors (e.g. hypertension, smoking, hyperlipidemia and hyperglycemia) promotes the development of endothelial cell changes and the progression of atherosclerosis due to lipid accumulation in the vessel wall.[35] Low-density-lipoprotein (LDL) particles infiltrate the subendothelial space and become oxidized, thereby provoking an inflammatory response that promotes adhesion as well as migration of leukocytes. Migrated monocytes convert into activated macrophages that take up modified lipoprotein particles through their scavenger receptors and transform into foam cells. Accumulation of foam cells as well as subsequent foam cell death lead to the deposit of extracellular lipids and the formation of fatty streaks. Furthermore, activated macrophages release proinflammatory mediators, creating an inflammatory environment that consequently promotes T-lymphocyte activation and migration of smooth muscle cells (SMCs). Thus, a developing atherosclerotic plaque is characterized by a lipid rich necrotic core and a fibrous cap out of SMC surrounded by collagen and elastic fibers (Figure 4).[36,37]

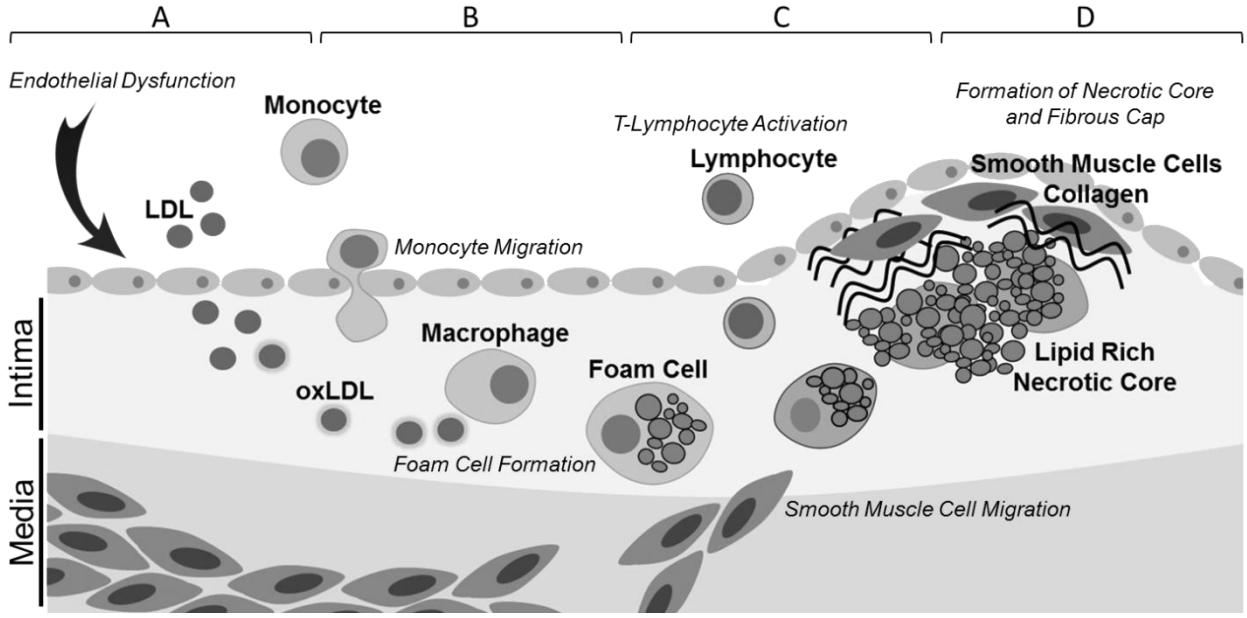


Figure 4. Pathogenesis of Atherosclerosis. The different stages of atherogenesis are shown. A Endothelial dysfunction causes lipid accumulation in the intima, where inflammatory response induces monocyte migration. B Monocytes convert into activated macrophages that take up oxidized low-density-lipoprotein and transform into foam cells. C Proinflammatory mediators promote T-lymphocyte activation and smooth muscle cell migration. D Atherosclerotic plaque is characterized by a lipid rich necrotic core and a fibrous cap out of smooth muscle cells, collagen and elastic fibers.

Apoptosis of vascular smooth muscle cells as well as increased activity of matrix-degrading enzymes (e.g. Matrix Metallopeptidase 9 (MMP-9)) may ultimately provoke rupture or erosion of the atherosclerotic plaque followed by platelet aggregation and thrombosis. While protrusion of the atheroma into the blood vessel lumen leads to stenosis, plaque rupture might determine specific endpoints of this disease such as myocardial infarction or stroke.[38]

5.3.2 Arachidonic Acid Metabolism as Modulator of Atherogenesis

Inflammatory processes influence all phases of atherogenesis and get modulated by several cellular components including monocytes, B- and T-lymphocytes, platelets as well as various pro- and anti-inflammatory mediators (e.g. tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6, chemokine ligand 2 (CCL-2)).[39-41] Eicosanoids as metabolites of AA metabolism are known as modulators of inflammatory diseases and due to their widespread synthesis in blood and vascular cells, they can be considered as central factors in the complex interplay between blood components and cells of the arterial wall (Figure 5).[42]

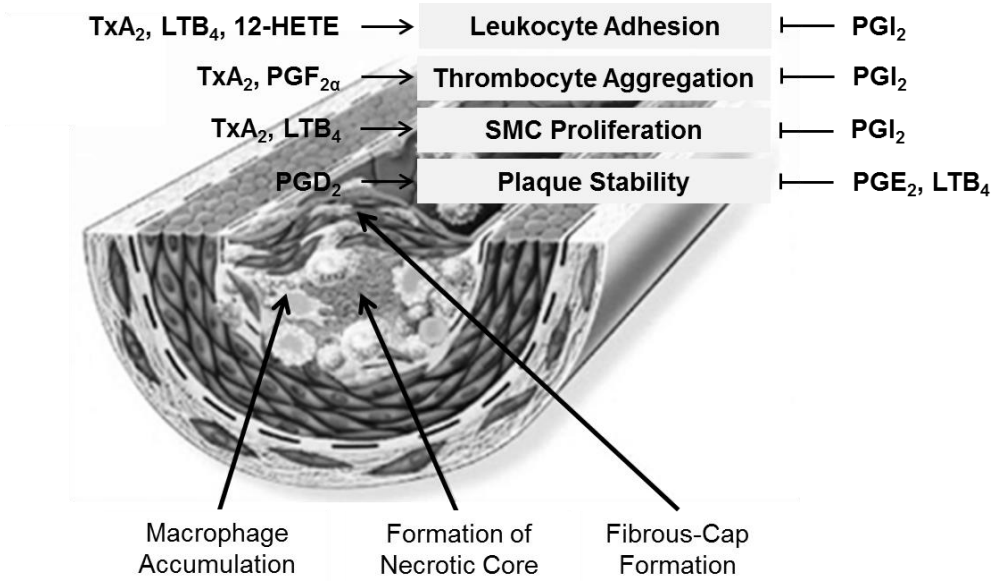


Figure 5. Arachidonic Acid Metabolites as Modulators of Pathophysiological Proceses of Atherogenesis. Image is modified from Ross R**.** Atherosclerosis—an inflammatory disease. N Engl J Med. 1999; 340:115-126.[43]

COX-derived eicosanoids such as TxA_2, $PGF_{2\alpha}$ and PGI_2 were identified as chemo-attractants, platelet aggregation factors during thrombus formation and modifiers of SMC proliferation that represent central pathophysiologic processes in atherosclerosis.[13,44-47] Furthermore, previous studies showed that symptomatic plaques revealed higher expression of *COX-2*, where functionally coupled COX-2/mPGES-1 promoted PGE_2 dependent MMP-9 production by

macrophages that led to increased plaque instability.[48] In accordance with this, *TXAS* mRNA levels and functional TxA_2 synthesis were increased in atherosclerotic lesions. It was suggested that enhanced intraplaque TxA_2 generation contributed to atherogenesis and thrombus formation in symptomatic patients.[49] In contrast, asymptomatic plaques revealed increased expression of *PGDS* and subsequent inhibition of MMP-9 biosynthesis by PGD_2. Results indicated that selective expression of eicosanoid pathways promoted either pro- or anti-atherogenic molecular pathways.[50] In addition to COX-dependent prostaglandins, eicosanoids generated by the LOX-pathways seem to be relevant in atherosclerosis. Lipoxygenized AA metabolites revealed chemotactic properties and Bäck and colleagues showed that human vascular smooth muscle cells expressed BLT_1 receptors and found that LTB_4 induced chemotaxis and proliferation of those cells.[13,51] Furthermore, present studies suggested that metabolites of the 12-LOX pathway induced the interaction of monocytes and endothelial cells followed by migration and differentiation into macrophages.[52,53] Investigation of atherosclerotic plaques in different vascular beds revealed expression of 5-LOX pathway mainly by macrophages, dendritic cells, foam cells, mast cells as well as neutrophilic granulocytes. Furthermore, the presence of 5-lipoxygenase expressing cells increased the progression of the disease.[54] Qiu and colleagues also showed significantly increased *5-LOX* expression in macrophages of human atherosclerotic plaques, whereas tissue incubated with arachidonic acid generated significant amounts of LTB_4.[55] Moreover, increased *5-LOX* expression and LTB_4 generation was associated with enhanced MMP-2 and MMP-9 expression in symptomatic plaques proposing that this pathway contributed to plaque rupture.[56]

Beside several studies that investigated the AA metabolism in plaques, further data indicated also an activation of those pathways in blood cells. Gómez-Hernández and colleagues showed that patients with carotid atherosclerosis revealed an overexpression of *COX-2*, *mPGES-1* and *EPs* (PGE_2 receptors) in plaques as well as in peripheral blood mononuclear cells (PBMC). They thus suggested that nuclear factor kappa B (NF-κB) inhibitors or EP antagonists may represent a promising therapeutic approach to treat plaque instability and rupture. Furthermore, study subjects revealed increased PGE_2 plasma levels that corresponded to findings of Jouve and colleagues that showed increased TxB_2 and PGE_2 levels in patients with atherosclerosis obliterans.[57,58] In line with this, Beloqui et al found that PGE_2 release of LPS stimulated monocytes was significant associated with carotid intima media thickness and correlated with cardiovascular risk factors in subjects free of clinically overt cardiovascular disease.[33]

In summary, these data suggest that analysis of the AA metabolism in whole blood on metabolite and gene expression level might provide a new marker of atherosclerotic burden and is predestinated as biomarkers for diagnosis and prediction of coronary artery disease**.**

5.4 Coronary Artery Disease and Prediction Strategies

Atherosclerosis is the most common cause of coronary artery disease (CAD), leading to progressive narrowing of the coronary arteries and chest pain as typical clinical sign.[59,60] Depending on clinical presentation, primarily an acute coronary syndrome with myocardial ischemia and/or myocardial infarction should be excluded. Rule-out strategies include clinical assessment, 12-lead electrocardiogram (ECG) and cardiac troponin as biomarker for myocardial cellular damage.[61,62] In remaining patients, presence of coronary heart disease will be subsequently suspended as the cause of clinical symptoms.[63] Following the guidelines, determination of pre-test probability that classified patients into subgroups of low, intermediate and high risk likelihood for CAD is recommended. Patients with low pre-test probability are assumed to have no obstructive CAD, in which other causes of chest pain should be excluded. Patients with intermediate likelihood should undergo non-invasive testing (NIT, e.g. stress echocardiography (ECG), single photon emission computed tomographic (SPECT) myocardial perfusion imaging, cardiac magnetic resonance (CMR) or coronary computed tomography (CT) angiography), while transfer to invasive coronary angiography (ICA) for patients with high pre-test probability or high-risk findings on NIT is acclaimed (Figure 6).[64]

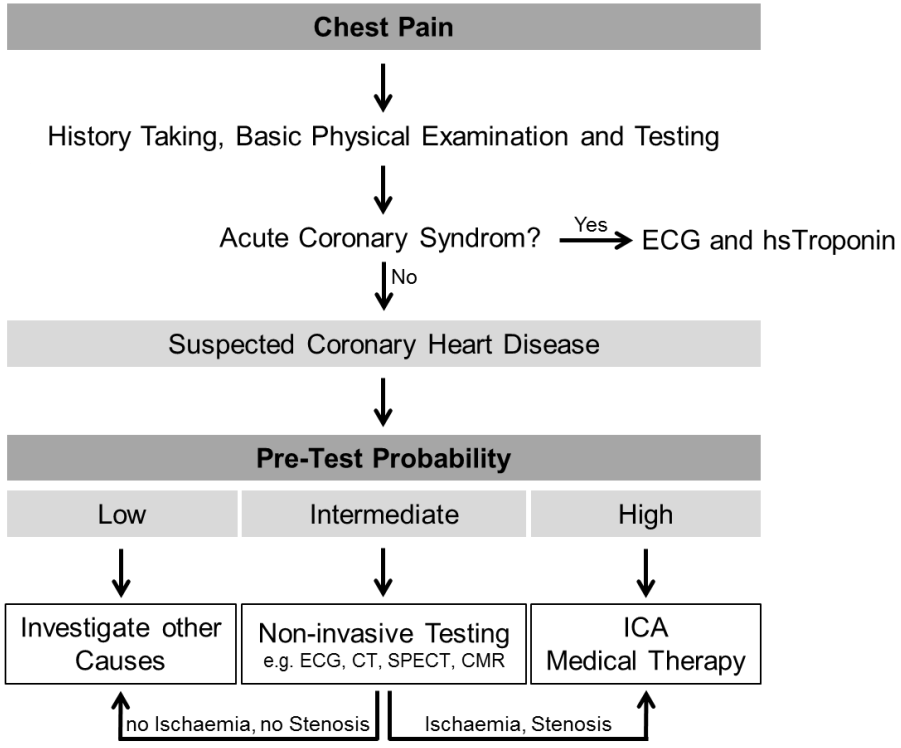


Figure 6. Diagnostic Management of Patients with Suspected Coronary Heart Disease. Electrocardiogram (ECG); high sensitivity Troponin (hsTroponin); Coronary Computed Tomography Angiography (CT); Single Photon Emission Computed Tomographic Myocardial Perfusion Imaging (SPECT); Cardiac Magnetic Resonance (CMR); Invasive Coronary Angiography (ICA). Image is modified from the 2013 ESC guidelines on the management of stable coronary artery disease: the Task Force on the management of stable coronary artery disease of the European Society of Cardiology.[64]

ICA is the gold standard to establish the diagnosis and guide treatment of CAD,[65] however previous studies have shown, that only 41 to 43 % of patients that underwent ICA due to suspected CAD had wall irregularities that corresponded to their clinical symptoms.[66] On the contrary, patients with a positive non-invasive pre-test revealed higher rate of obstructive CAD than patient without any non-invasive testing before angiography.[67]

The Diamond and Forrester score is one of the currently recommended pre-tests and considers age, sex and symptoms for the prediction of significant CAD. However, the score does not account for known cardiovascular risk factors associated with the presence of atherosclerosis. Therefore, Pryor and colleagues developed the Duke Clinical Score that additionally consider history of myocardial infarction, diabetes, dyslipidemia, smoking and electrocardiographically findings (Table 1).[68] A study with 633 patients, that underwent ICA due to suspected CAD, revealed an area under the curve of 0.642 for the Diamond and Forrester Score, in which the improved Duke Clinical Score showed an significantly increased AUC of 0.718 (*P< 0.001*).[69]

	Diamond and Forrester Score	**Duke Clinical Score**
Age	✓	✓
Sex	✓	✓
Chest Pain	✓	✓
History of Myocardial Infarction		✓
Diabetes		✓
Dyslipidemia		✓
Smoking		✓
Electrocardiography Findings		✓
C Statistic	**0.64**	**0.72**

Table 1. The Models of Diamond and Forrester Score and Duke Clinical Score.

Nevertheless, Genders and colleagues showed that the Duke Clinical Score overestimated the probability of coronary artery disease (C statistic 0.78). Thus, the Consortium [CAD Prediction] Score was developed with a basic, clinical and extended model (Table 2). The basic model uses information on age, gender and chest pain, the clinical score considers information on diabetes, dyslipidemia, smoking as well as hypertension. Compared with the basic score, the clinical model improved the c statistic from 0.77 to 0.79. The addition of the coronary calcium score as non-invasive diagnostic imaging procedure (extended model) increased the c-statistic up to 0.88.[70]

	Basic Model	Clinical Model	Clinical Model + CCS
Age	✓	✓	✓
Sex	✓	✓	✓
Chest Pain	✓	✓	✓
Diabetes		✓	✓
Dyslipidemia		✓	✓
Smoking		✓	✓
Hypertension		✓	✓
Coronary Calcium Score (CCS)			✓
C Statistic	**0.77**	**0.79**	**0.88**

Table 2. Consortium [CAD Prediction] Score and C-Statistics for the Basic, Clinical and Extended Model by Genders et al. [70]

However, availability and costs for cardiac CT for calcium scoring are hampering the widespread application[71] and alternatively, blood biomarkers have been evaluated regarding their impact on pre-test probability for CAD. The European Evaluation of Integrated Cardiac Imaging (EVINCI) study with 527 patients approved just a low accuracy for predicting functionally significant CAD (AUC 0.58) when using Gender´s Clinical Score. Though, the addition of High-Density-Lipoprotein (HDL) cholesterol, aspartate aminotransferase (AST) and high sensitive C-reactive Protein (hsCRP) improved the prediction of presence of CAD (EVINCI Score; AUC 0.70) (Table 3).[72]

	Clinical Score	EVINCI Score
Age	✓	✓
Sex	✓	✓
Chest Pain	✓	✓
Diabetes	✓	✓
Dyslipidemia	✓	✓
Smoking	✓	✓
Hypertension	✓	✓
HDL-Cholesterol		✓
AST		✓
hs-CRP		✓
C statistic	**0.58**	**0.70**

Table 3. EVINCI Score. The addition of HDL cholesterol, aspartate aminotransferase (AST) and high sensitive C-reactive Protein (hsCRP) improved the prediction of Gender´s Clinical Score. C-statistics for Clinical and EVINCI Score are shown.[72]

The data of the EVINCI study are in line with other studies that confirmed that lipid and inflammatory markers could be used for CAD prediction. For instance, Bogavac-Stanojevic et al. showed that a score combining non-HDL cholesterol, hsCRP, HDL cholesterol and lipoprotein a revealed an AUC of 0.80 while Haidari and colleagues showed that CRP, diabetes, total cholesterol, HDL cholesterol and total cholesterol/ HDL cholesterol ratio led to an accuracy of 78 % for the classification of suspected individuals.[73,74]

Nevertheless, further biomarkers detecting patients at risk are lacking and improvement of strategies to predict pre-test probability under inclusion of biochemical blood biomarkers would be of great interest.

5.5 Aims and Work Program

Eicosanoids are known as modulator of inflammatory diseases and due to their widespread synthesis in blood and vascular cells these metabolites play a central role in atherogenesis. It was thus the aim of the current thesis to investigate whether the analysis of the AA metabolism in whole blood might provide a new marker of atherosclerotic burden and could be used as biomarker for diagnosis and prediction of coronary artery disease.

In previous work, we developed an analytical test suited for the application in a clinical setting that enabled the simultaneous and highly standardized analysis of COX- and LOX-dependent eicosanoid response both on metabolite and gene expression level. LC-MS/MS method allowed quantification of AA, 5-HETE, 11-HETE, 12-HETE, $PGF_{2\alpha}$, PGE_2 and TxB_2, while RT-PCR assays for analysis of *COX-2* and *PGES* expression have been established.[34]

Aim 1 - Improvement of the Established Whole-Blood Activation Assay

For a complete investigation of the major pathways of AA metabolism additional gene expression assays will be established. Furthermore, different stimuli (LPS, TNFα and oxLDL) for whole blood activation and the time- and dose-dependency of eicosanoid response will be evaluated. In addition, functional studies will be performed to better characterize the molecular mechanism of eicosanoid release in whole blood.

Aim 2 - Determination of Eicosanoid Response of Healthy Subjects

The established assay will then be used to test the variability of the LPS-induced AA metabolism in healthy individuals, with the aim to characterize general eicosanoid profiles.

Aim 3 - Investigation of the Eicosanoid Response in Patients with or without Coronary Artery Disease (Leipzig Heart Study)

Suggesting that the individual regulated eicosanoid response might determine the risk for atherosclerosis, we aimed to investigate target genes and metabolites of AA metabolism as novel biomarkers for atherosclerotic burden. The predictive role of eicosanoid response will be investigated in patients with or without coronary artery disease using the standardized and improved whole-blood assay.

6 Materials

6.1 Laboratory Equipment

Roche Modular Analysis System	Roche Diagnostics
Sysmex XE-2100 Hematology Analyzer	Sysmex
Optima LE-80K Ultracentrifuge	Beckman Coulter
Spectrafluor Microplate Reader	Tecan
QTRAP 4000 LC-MS/MS System	Sciex
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific
GeneAmp PCR System 9700	Applied Biosystems
Gel System & Power Supply	peqlab (VWR)
Gel Documentation Instrument	peqlab (VWR)
Thermomixer comfort	Eppendorf
ViiA 7 Real-Time PCR-System	Thermo Fisher Scientific
Vortex Genie 2	Bender & Hobein AG
Centrifuges	Eppendorf
Pipettes	Eppendorf

6.2 Chemicals and Consumables

Specimen Collection

Safety-Multifly, 21G, tube 200mm	Sarstedt
Lithium Heparin Tubes, 9 mL	Sarstedt

***In vitro* Whole-Blood Model**

Cell Culture Dish 35 mm x 10 mm	Sigma-Aldrich (Merck)
RPMI 1640 Liquid Medium (FG 1383)	Biochrom (Merck)
Penicillin-Streptomycin (10.000 U/mL)	Gibco by Life Technologies (Thermo Fisher Scientific)
Lipopolysaccharides (LPS) from *Escherichia coli* O55:B5	Sigma-Aldrich (Merck)
Recombinant Human TNF-alpha Protein	R&D Systems
15 mL Tube, PP (polypropylene)	Sarstedt
1.5 mL Micro Tubes, PP (polypropylene)	Sarstedt

Preparation and Oxidation of LDL

Sodium Chloride (NaCl)	Sigma-Aldrich (Merck)
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2)	Sigma-Aldrich (Merck)
OptiSeal Tube, Polypropylene, 32.4 mL, 26 x 77 mm	Beckman
Water LiChrosolv	Merck
Servapor Dialysis Tubing, 16 mm	Serva
Tris-(hydroxymethyl)-aminomethan (TRIS)	Roth
Minisart NML Syringe Filter, 0.45 µm	Sartorius
Econo-Pac Chromatography Columns	Bio-Rad
Phosphate Buffered Saline (PBS) Dulbecco	Merck
Cholesterol CHOD-PAP	Roche
Calibrator for automated systems	Roche
HAM´s F-10 Nutrient Mix	Thermo Fisher Scientific
Copper(II) chloride ($CuCl_2$)	Sigma-Aldrich (Merck)
Folin & Ciocalteu's Phenol Reagent	Sigma-Aldrich (Merck)

Transcriptional and Translational Inhibitors

Actinomycin D	Sigma-Aldrich (Merck)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (Merck)
Cycloheximide	Sigma-Aldrich (Merck)
Ethanol	Merck

LC-MS/MS Analysis

Thromboxane B_2-d4	Cayman Chemical
Prostaglandin $F_{2\alpha}$-d4	Cayman Chemical
Prostaglandin E_2-d4	Cayman Chemical
5(S)-HETE-d8	Cayman Chemical
Arachidonic Acid (AA)-d8	Cayman Chemical
Methanol	Biosolve
Zinc sulfate ($ZnSO_4$)	Sigma-Aldrich (Merck)
Water	Thermo Fisher Scientific
Acetic acid (glacial)	Merck
Strata-x 33 µm polymeric sorbent 60 mg/3 mL	Phenomenex
Acetonitrile	J.T.Baker (Thermo Fisher Scientific)

Formic Acid	Biosolve
Autosampler (Glass) Vials	Waters

RNA Isolation

QIAamp RNA Blood Mini Kit	Qiagen
Contents:	
QIAamp Spin Columns	
QIAshredder Spin Columns	
Collection Tubes	
Buffer EL	
Buffer RLT	
Buffer RW1	
Buffer RPE	
RNase-free Water	
2-Mercaptoethanol	Sigma-Aldrich (Merck)
Ribonuclease (RNase)-Free Desoxyribonuclease (DNase)	Qiagen
Contents:	
RNase-Free DNase I (1500 kU)	
Buffer RDD	
RNase-Free Water	

Reverse Transcription

SuperScript II Reverse Transcriptase	Invitrogen (Thermo Fisher
Contents:	Scientific)
SuperScript II Reverse Transcriptase (200 U/µL)	
5X First Strand Buffer (FSB)	
DTT (100 mM)	
Primer Random $p(dN)_6$	Roche
Deoxynucleotide Triphosphate (dNTP) Mix	Promega
Recombinant RNasin Ribonuclease Inhibitor	Promega
TRIS-EDTA (TE) Buffer [10X]	G-Biosciences

Quantitative RT-PCR

Primer	Eurofins Genomics
Probes	Eurofins Genomics

AmpliTaq Gold DNA Polymerase with Buffer II and $MgCl_2$	Applied Biosystems
Contents:	
AmpliTaq Gold DNA Polymerase (5 U/µL)	
$MgCl_2$ Solution 25 mM	
10X PCR Buffer II	
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich (Merck)

Gel Electrophoresis

peqGold Low Melt Point (LMP) Agarose	peqlab (VWR)
TAE Buffer (Tris-acetate-EDTA)	Thermo Fisher Scientific
Molecular-Weight Size Marker X	Roche
Ethidium bromide aqueous solution 1 % w/v	Serva

Plasmid Standard Preparation

TOPO TA Cloning Kit Dual Promoter	Invitrogen (Thermo Fisher
Contents:	Scientific)
pCR II-TOPO vector	
Salt Solution	
S.O.C. Medium	
TOP10F´ competent cells	
Cell Culture Dish, 100 mm x 20 mm	Corning
Ampicillin	Thermo Fisher Scientific
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich (Merck)
5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)	Sigma-Aldrich (Merck)
Bacto Tryptone	BD Biosciences
Bacto Yeast Extract	BD Biosciences
Bacto Agar	BD Biosciences
FastPlasmid Mini-Prep Kit	5Prime
Contents:	
Lysis Solution	
RNase Solution	
Lysozyme	
Spin Column Assembly	
Wash Buffer	
Elution Buffer	
2-Propanol	Merck

EcoRI *Contents:* *EcoRI (20.000 U/mL)* *NEBuffer EcoRI*	New England Biolabs
BigDye Direct Cycle Sequencing Kit *Contents:* *BigDye Direct PCR Master Mix* *BigDye Direct M13 Fwd Primer* *BigDye Direct M13 Rev Primer*	Applied Biosystems by Life Technology (Thermo Fisher Scientific)
Sodium Acetate	Merck
HindIII *Contents:* *HIND III (20.000 U/mL)* *NEBuffer 2.1*	New England Biolabs

7 Methods

7.1 Study Population and Specimen Collection

Whole Blood for LDL-cholesterol preparation and the evaluation of the whole-blood activation assay was obtained from blood donors of the Institut für Transfusionsmedizin of the University Hospital Leipzig. Samples from 10 healthy blood donors (5 male, 5 female) were collected for investigation of differential eicosanoid response on gene expression and mediator level. Investigations were approved by the Ethics Committee of the Medical Faculty of the University Leipzig, Germany (Reg. No 178-2009).

Blood from 92 patients of the Leipzig Heart Study was collected to study eicosanoid response on gene expression and mediator level in patients with varying CAD severity. The study was approved by the Ethics Committee of the Medical Faculty of the University Leipzig, Germany (Reg. No 276-2005) and is registered with ClinicalTrials.gov (NCT00497887).[66]

Principally, whole blood was collected by peripheral venous blood puncture into 9 mL lithium heparin tubes. After blood collection, samples were stored at 4-7 °C for a maximum of 4 hours until activation experiments. A Sysmex XE-2100 hematology analyzer was used for analysis of blood count and an automated Roche Modular analysis system was used for analysis of clinical chemistry.

7.2 *In vitro* Whole Blood Model

7.2.1 Inflammatory Stimuli

Different stimuli were used for whole blood activation. LPS and TNFα were provide as lyophilized powders and were diluted into H_2O to final stock solution of 100 µg/mL. Preparation and oxidation of LDL-Cholesterol was performed as described below.

7.2.1.1 Preparation of LDL-Cholesterol from Plasma

Lipoproteins were isolated by ultracentrifugation from 250 mL plasma of healthy blood donors at densities (d) = 1.060 g/mL for separation of HDL-Cholesterol and (d) = 1.019 g/mL for separation of LDL-Cholesterol. Plasma was mixed with NaCl for a final density of 1.060 g/mL as well as 250 mg EDTA and transferred into OptiSeal Centrifuge Tubes, followed by a centrifugation step for 24 hours and 150.000 *x g* at 10 °C.

Amount of NaCl was calculated as follows:

$$Amount\ (g)NaCl\ per\ 1\ mL\ plasma = \frac{1.060\ \frac{g}{mL} - [density\ of\ plasma]}{(1 - 0.3175)}$$

The supernatant contained Very-Low-Density-Lipoproteins (VLDL) as well as LDL and was separated from the lower sample phase that contained HDL. Supernatant was mixed with H_2O to yield a final density of 1.019 g/mL and centrifuged for 24 hours at 150.000 *x g* at 10 °C. Upper Phase and lower phase were separated again, in which the lower phase contained LDL-Cholesterol and upper phase with VLDL and Intermediate-Density-Lipoproteins (IDL) was discarded.

Lower phase was mixed with NaCl to yield a final density of 1.060 g/mL and was centrifuged for 24 hours at 150.000 *x g* at 10 °C. Upper phase was separated, filled into a Serva Dialysis Tubing and dialyzed for 20 hours at 4 °C into 5 L 1X TRIS Buffer (Table 4). Then, obtained LDL was filtrated (Minisart NML Syringe Filter, 0.45 µm) and stored at 4 °C until the oxidation step.

Table 4. TRIS Buffer (10X)

60.5 g	Trishydroxymethylaminomethan (50 mM)
900 g	NaCl (0,9 %)
9.3 g	EDTA
ad 10 L	H_2O
10 L	(adjust to pH 7,4)

7.2.1.2 Oxidation of LDL-Cholesterol

For removal of excessed salt, a column chromatography (Econo-Pac Chromatography Columns) was performed. The column was primed with 4 mL PBS three times; LDL solution was applied and rinsed thoroughly with 2 mL PBS.

Cholesterol concentrations of LDL was determined with an enzymatic colorimetric assay (cholesterol oxidase-phenol-aminophenazone: CHOD-PAP).[75] To this end 10 µL of samples or standard solution (100, 200, 400 mg/dL) were mixed with 200 µL cholesterol reagent and incubated for 10 minutes at room temperature on an orbital shaker. Measurements were performed with a fluorescence and absorbance reader (Tecan) at 500 nm and LDL cholesterol concentrations were determined using the standard curve. Then, LDL fractions were diluted with HAMs F10 to yield a final concentration of 100 mg/dL. For oxidation of LDL, the diluted solution

was incubated with $CuCl_2$ in a final concentration of 5 µM for 24 hours at 37 °C and 5 % CO_2 (Table 5).

Table 5. $CuCl_2$ Stock Solution (0,5 M)

170 mg	$CuCl_2$
ad 2 mL	HAMs F10
2 mL	

Oxidation was stopped by addition of Na_2EDTA in a final concentration of 2 mg/mL and oxLDL was immediately transferred on ice. Thereafter, a second column chromatography was performed as described before, protein concentrations were determined[76] and oxLDL was used as stimuli for whole blood activation in a final concentration of 100 µg/mL.

7.2.2 Transcriptional and Translational Inhibitors

Actinomyin D (ActD) as an inhibitor of transcription and cycloheximide (CHX) as a translational inhibitor were used to identify if eicosanoid response underlay preformed release or *de novo* synthesis. ActD and CHX were provided as lyophilized powders. ActD was diluted into DMSO to a final stock solution of 1000 µg/mL. CHX was diluted into ethanol to final stock solution of 100 mg/mL.

7.2.3 Experimental Procedure

Whole blood activation experiments were performed according to the protocol by Bruegel et al.[34] 1 mL of whole blood was mixed with 500 µL medium spiked with or without different stimuli. Mixtures were incubated for 1, 4 and 24 hours at 37 °C and 5 % CO_2. Sample aliquots containing whole blood medium mix without stimuli were immediately processed without further incubation, serving as baseline. Samples were centrifuged for 10 min at 3220 *x g*; supernatants were transferred into 1.5 mL microtubes, covered with nitrogen and stored at -80 °C until LC-MS/MS Analysis of eicosanoid metabolites. Cellular components were used for RNA isolation and gene expression analysis.

Investigation of regulatory mechanism of AA metabolism was performed by transcriptional and translational inhibition experiments with ActD and CHX. Samples were preincubated for 1 hour with ActD or CHX and then activated with LPS for 1, 4 and 24 hours. Furthermore, samples with ActD or CHX without LPS activation were saved as baseline controls.

7.2.4 LC-MS/MS Analysis for Eicosanoid Metabolites

On mediator level, AA as general precursor of eicosanoids, 11-HETE, TxB_2, PGE_2 and $PGF_{2\alpha}$ as major representatives of COX pathway and 12-HETE and 5-HETE as representatives of *12-LOX* and *5-LOX* pathway, were determined in supernatants according to the protocol from Bruegel et al.[34] A protein precipitation and solid phase extraction (SPE) was used for extraction and concentration of eicosanoids from supernatants of the whole blood activation model. Thereafter quantification of metabolites was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using corresponding internal standards (IS).

7.2.5 Protein Precipitation and Solid Phase Extraction

The protein precipitation was performed after the addition of internal standards for metabolite quantification to each sample. Internal standard solution consists out of deuterium labeled eicosanoids TxB_2-d4, $PGF_{2\alpha}$-d4, PGE_2-d4, 5-(S)-HETE-d8 (20 ng/mL each) and AA-d8 (200 ng/mL) that has been diluted in methanol (MeOH). MeOH/$ZnSO_4$ precipitation solution was compounded out of 89 g/L $ZnSO_4$ in H_2O and MeOH 4/1 (v/v). 100 µL supernatant was mixed with 50 µL internal standard and 200 µL MeOH/$ZnSO_4$ precipitation solution and was centrifuged for 10 minutes at 10.000 *x g* at room temperature. 300 µL supernatant was transferred into a glass vial and diluted by addition of 800 µL H_2O and 100 µL glacial acetic acid. SPE was performed using Strata-x 33 µm polymeric sorbent 60 mg/3 mL tubes. SPE Tubes were conditioned with 2 mL MeOH and centrifugation for 1 minute at 160 *x g* at room temperature, followed by equilibration with 2 mL H_2O and centrifugation for 1 minute at 160 *x g* at room temperature. Diluted samples were loaded and SPE cartridges were centrifuged for 1 minute at 270 *x g* at room temperature followed by a wash step with 2 mL MeOH 10/90 (v/v) and centrifugation for 2 minutes at 270 *x g* and 5 minutes at 3320 *x g* at room temperature. Samples were eluted in 1 mL MeOH by centrifugation for 1 minute at 270 *x g* and 5 minutes at 3320 *x g* at room temperature. After protein precipitation and SPE samples were dried and concentrated by vacuum centrifugation at 37 °C. Then, samples were covered with nitrogen and stored at -80 °C until analysis.

7.2.6 Liquid Chromatography and Tandem Mass Spectrometry

For analysis by LC-MS/MS stored samples were solved in 100 µL acetonitrile/water 50/50 (v/v) + 0.02 % formic acid, centrifuged for 10 minutes at 15.000 rpm and 80 µL supernatant was transferred into an autosampler vial. A triple quadrupole mass spectrometer with an electrospray ionization (ESI) source was used in negative ion mode. Eicosanoids were analyzed

by multiple reactions monitoring (MRM; Figure 7) of defined mass transitions and retention times given in Table 6.

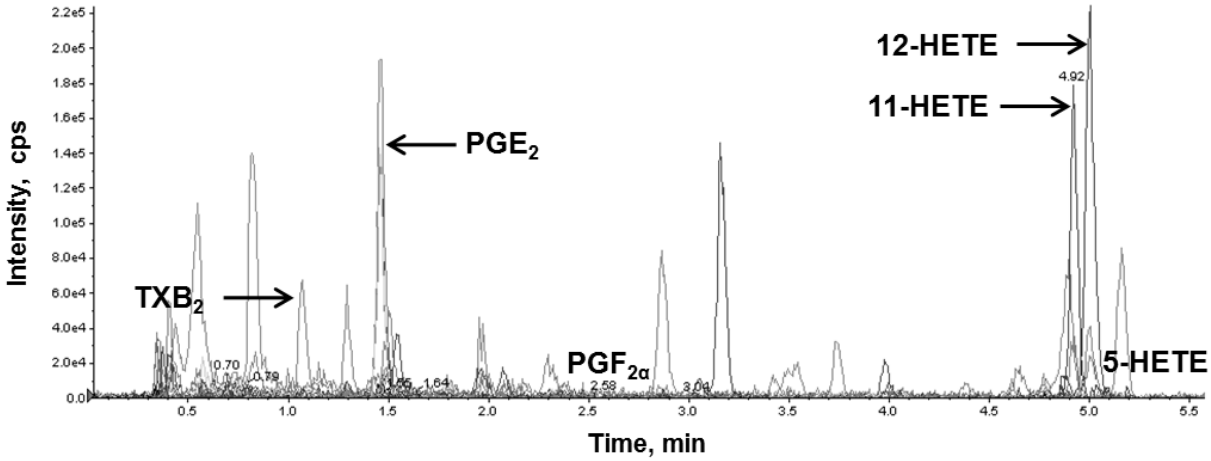


Figure 7. Chromatogram of Multiparametric LC-MS/MS Analysis.

Table 6. Mass Transitions and Chromatographic Retention Times of AA Metabolites

Parameter	MRM (*m/z*)	Retention time (min)
TxB_2	369/ 169	1.07
TxB_2-d4	373/ 173	1.07
PGE_2	351/ 271	1.46
PGE_2-d4	355/ 275	1.46
$PGF_{2\alpha}$	353/ 193	2.51
$PGF_{2\alpha}$-d4	357/ 197	2.51
11-HETE	319/ 167	4.81
12-HETE	319/ 179	4.89
5-HETE	319/ 115	5.09
5-HETE-d8	327/ 116	5.09
AA	303/ 259	6.57
AA-d8	311/ 267	6.57

Metabolite quantification was performed via corresponding internal deuterated standards using the Analyst 1.5 software and following calculation:

$$Metabolite\ Concentration = \frac{Analyte\ Peak\ Area\ x\ IS\ Concentration}{IS\ Peak\ Area\ x\ 2}$$

7.3 Quantitative PCR Analysis of Eicosanoid Metabolizing Enzymes

Gene expression of AA metabolizing enzymes was determined by quantitative fluorogenic RT-PCR using cDNA plasmid standard curves for quantification of absolute mRNA copies. Assays for *PLA_2* as superordinate target gene, *COX-1, COX-2, TXAS, PGES and PGFS* as corresponding target genes of COX pathway and *12-LOX*, *5-LOX* and *FLAP* as corresponding target genes of LOX pathway were established using gene-specific primers and probes spanning 2 exons in order to avoid amplification of genomic DNA (Table 13).

7.3.1 RNA Isolation

RNA was isolated using the QIAamp RNA Blood Mini Kit according to the manufacturer´s instructions including additional DNA digestion with Ribonuclease (RNase)-Free Desoxyribonuclease (DNase). To this end, cellular components of whole blood activation assay were transferred into a 15 mL Tube, mixed with 5.6 mL Lysis Buffer and incubated on ice for 10 minutes. During incubation, samples were mixed two times and centrifuged at 400 *x g* for 10 minutes at 4 °C. Supernatants were completely removed and discarded. Then, 2 mL Lysis Buffer was added and samples were centrifuged for 10 minutes at 400 *x g* and 4 °C. Supernatants were discarded and 600 µL RLT Buffer was added to pelleted leukocytes. Lysates were pipetted directly into a QIAshredder spin column and centrifuged for 2 minutes at maximum speed. 600 µL of 70 % ethanol was added to the homogenized lysates and samples were transferred to a QIAamp spin column and centrifuged for 15 seconds at 8000 *x g* at room temperature. QIAamp spin columns were transferred into a new 2 mL collection tube and 350 µL Buffer RW1 was applied and approach was centrifuged for 15 sec at 8000 *x g* for washing, followed by DNA digestion with RNase-free DNase. Therefore, 80 µL of RNase-free DNase Mix (Table 7) was added to the columns and incubated for 15 minutes at room temperature.

Table 7. Mastermix for DNA Digestion using RNase-free DNase

10 µL	RNase-free DNase (1000 U/mL)
70 µL	Buffer RDD
80 µL	

Then, a second wash step with 350 µL RW1 Buffer was performed. QIAamp spin columns were placed in a new 2 mL collection tube and 500 µL Buffer RPE was added and centrifuged for 15 seconds at 8000 *x g* at room temperature. Another 500 µL Buffer RPE was added and

columns were centrifuged at full speed for 3 minutes. Then, the approach was centrifuged for 1 min at full speed for drying and RNA was eluted into 30 µL RNase free water.

Finally, purity and concentration of nucleic acids were determined at 260 and 280 nm with the NanoDrop 2000c spectrophotometer and samples were stored at -80 °C until reverse transcription into cDNA.

7.3.2 Complementary DNA Synthesis by Reverse Transcription

RNA was reversed transcribed into cDNA with Super Script-II enzyme. 10 µL RNA was mixed with 1 µL random hexamer primer and incubated for 10 minutes at 68 °C. Then, 9 µL master mix (Table 8) was added, followed by incubation for 1 hour at 42 °C. Samples with RNA concentrations > 100 ng/µL were diluted with diethyl pyrocarbonate (DEPC) H_2O (Table 9) to yield a final concentration of 100 ng/µL.

Table 8. Mastermix fo Reverse Transcription

1 µL	RNAsin (40 U/µL)
1 µL	dNTP (10 mM)
2 µL	DTT (0.1 M)
4 µL	5 x First Strand Buffer
1 µL	Superscript II (200 U/µL)
9 µL	

The samples were diluted 1:7.5 with 1X TE buffer (Table 10) and stored at -20 °C until quantitative RT-PCR.

Table 9. DEPC Water

1 mL	DEPC	
999 mL	H_2O	autoclaved
1 L		

Table 10. 1X TE Buffer

5 mL	10X TE Buffer (100 mM Tris, 10 mM EDTA, pH 8.0)
45 mL	H_2O
50 mL	

7.3.3 Generation of Plasmid Standards

Gene expression analyses were performed by qRT-PCR and absolute mRNA copies were determined using cDNA plasmid standard curves for each gene. Generation of plasmid standards was accomplished as described below.

7.3.3.1 Polymerase Chain Reaction

Amplification of specific cDNA sequences of target genes was performed with polymerase chain reaction (Table 11; Table 12).

Table 11. Mastermix for PCR

5 µL	cDNA
2.5 µL	10 x PCR Buffer
5 µL	$MgCl_2$ (25 mM)
2 µL	dNTP (125 µM)
0.5 µL	Primer forward
0.5 µL	Primer reverse
0.25 µL	AmpliTaq Gold
9.25 µL	DEPC H_2O
25 µL	

Table 12. Cycler Parameter

95 °C	10 min	
95 °C	15 sec	40 Cycles
60 °C	1 min	
72 °C	7 min	

Specific primers and probes were selected to span two exons in order to avoid amplification of genomic DNA and have been obtained from MWG Biotech (Table 13).

Table 13. Primers and Probes used for Quantitative RT-PCR

Gene NM Number	**Primer and Probes**	
PLA2G4A	5'- primer	5'-CTGGATTGTGCTACCTACGTTG-3'
(PLA_2)	3'- primer	5'-TTCTCTGGAAAATCAGGGTGA-3'
NM_024420.2	flourogenic probe	5'*-CTGGTCTTTCTGGCTCCACCTGGTATATGTC-#3'
PTGS1	5'- primer	5'-CACCCATGGGAACCAAAG-3'
(COX-1)	3'- primer	5'-TGGGGGTCAGGTATGAACTT-3'
NM_000962.2	flourogenic probe	5'*-AGTTGCCAGATGCCCAGCTCCTGGC-#3'

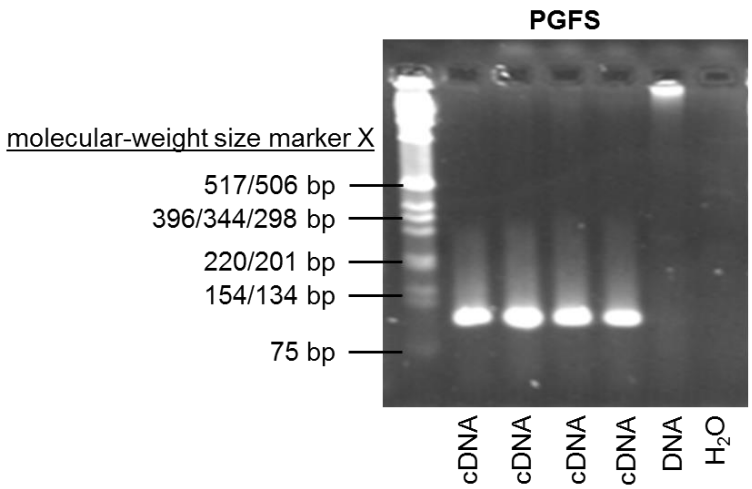
PTGS2	5'- primer	5'-CTTCACGCATCAGTTTTTCAAG-3'
(COX-2)	3'- primer	5'-TCACCGTAAATATGATTTAAGTCCAC-3'
NM_000963.2	flourogenic probe	5'*-ATAAGCGAGGGCCAGCTTTCACCAACG-#3'
TBXAS1	5'- primer	5'-GGAGACCTTCAACCCTGAAA-3'
(TXAS)	3'- primer	5'-AAGGGCAGGTACGTGAAGG-3'
NM_001061.2	flourogenic probe	5'*-CTGAGGCCCGGCAGCAGCACC-#3'
PTGES	5'- primer	5'-CTGGGATGACAGGCATGAAT-3'
(PGES)	3'- primer	5'-GACTCACATGGGAGCCTTTT-3'
NM_004878.4	flourogenic probe	5'*-CACTGTGCTCAGCCACCATCTGGAGTT-#3'
AKR1C3	5'- primer	5'-CATTGGGGTGTCAAACTTCA-3'
(PGFS)	3'- primer	5'-CCGGTTGAAATACGGATGAC-3'
NM_3003739	flourogenic probe	5'*-CCGCAGGCAGCTGGAGATGATCCTC-#3'
ALOX12	5'- primer	5'-GCATCGAGAGAAGGAACTGAAAGAC-3'
(12-LOX)	3'- primer	5'-CGATGGTCAGGGGTAACCCTTC-3'
NM_000697	flourogenic probe	5'*-ACAGCAGATCTACTGCTGGGCCACCTGG-#3'
ALOX5	5'- primer	5'-TCTGGTGTCTGAGGTTTTTGG-3'
(5-LOX)	3'- primer	5'-TCTCACGTGTGCCACCAG-3'
NM_000698.2	flourogenic probe	5'*-ATTGCAATGTACCGCCAGCTGCCTGC-#3'
ALOX5AP	5'- primer	5'-CATCAGCGTGGTCCAGAAT-3'
(FLAP)	3'- primer	5'-CAAGTGTTCCGGTCCTCTG-3'
NM_001629.2	flourogenic probe	5'*-CCATAAAGTGGAGCACGAAAGCAGGACCC-#3'

* 6-FAM

TAMRA

7.3.3.2 Agarose Gel Electrophoresis

An agarose gel electrophoresis was performed for product check of PCR reactions. Expected fragment size was analyzed using the molecular-weight size marker X. DNA fragments were separated using 3 % LMP agarose gels and TAE-buffer containing 2 M TRIS acetate and 5 mM Na_2ETDA (pH 8). Electrophoresis ran for 45 minutes at 120 volt and gel was stained with ethidium bromide (1 ng/mL). Photography of gels was performed using an UV transluminator (Figure 8).



Gene	Fragment Size (bp)
PLA_2	86
COX-1	94
COX-2	96
TXAS	72
PGFS	112
PGES	69
12-LOX	80
5-LOX	88
FLAP	95

Figure 8. Gel Electrophoresis of *AKR1C3*. The expected fragment size is 112 bp and no PCR product for DNA and negative controls (H_2O).

7.3.3.3 Subcloning of PCR Fragments into PCR II-TOPO Vector

The cloning reaction was performed for the insertion of PCR products into a 3973 bp pCR II-TOPO plasmid vector and following transformation into *Escherichia coli* cells using the TOPO TA Cloning Kit according to the manufacturer´s instructions (Figure 9).

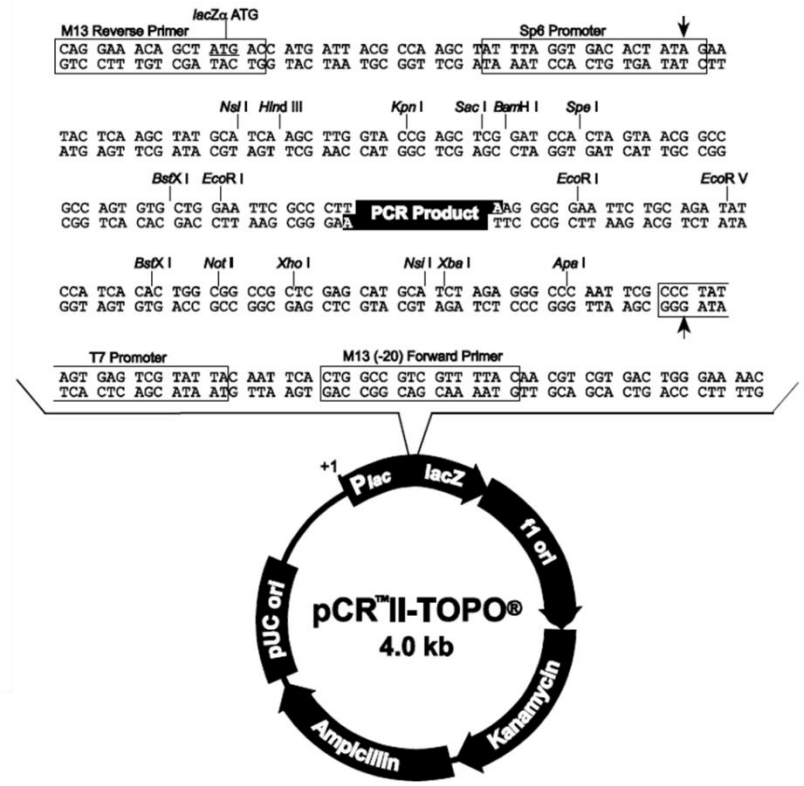


Figure 9. pCR II-TOPO Plasmid Vector

PCR products were mixed with 1 µL salt solution and water was added to a final volume of 5 µL. 0.5 µL TOPO cloning vector was given to the reaction and approach was incubated for 5 minutes at room temperature. During incubation *Escherichia coli* were thawed on ice. 2 µL of TOPO cloning reaction was added and samples were incubated for 15 minutes on ice. Then, cells were heat-shocked for 30 seconds at 42 °C, immediately transferred on ice and 250 µL S.O.C. medium was added. Reaction was incubated for 1 hour at 37 °C and spread on a prewarmed LB (Luria-Bertani) medium agar plates (Table 14) containing Ampicillin, IPTG (Isopropyl β-D-1-thiogalactopyranoside) and x-Gal (5-Brom-4-Chlor-3-indolyl-ß-D-galactosid). Successful transformation of PCR product leads to ampicillin resistance and loss of ß-galactosidase enzyme of *Escherichia coli*. Present x-Gal cannot be reduced to 5-Brom-4-Chlor-indogo and transformed colonies appear white. Therefore, white colonies were selected and cultured in 2 mL LB medium (Table 15) over night.

Table 14. LB (Luria-Bertani) Agar Plates (Ø 10 cm)

5 g	Tryptone	
2.5 g	Yeast Extract	
5 g	NaCl	
7,5 g	Bacto Agar	
ad 500 mL	H_2O	autoclaved
+ 1 mL	Ampicillin (25 mg/mL)	
+ 1 mL	IPTG (100 mM)	
+ 1 mL	X-Gal (2 % in dimethylformamide)	

Table 15. LB (Luria-Bertani) Medium

5 g	Tryptone	
2.5 g	Yeast Extract	
5 g	NaCl	
ad 500 mL	H_2O	autoclaved
+ 1 mL	Ampicillin (25 mg/mL)	

7.3.3.4 Preparation of Plasmid DNA

Preparation of plasmid DNA was performed using the Fast Plasmid Mini Kit according to the manufacturer´s instructions. 1.5 mL fresh *Escherichia coli* medium mixture was transferred into an Eppendorf tube and centrifuged for 1 minute at full speed. Supernatant was completely removed and 400 µL ice cold Complete Lysis Solution was added to the cells, mixed and

incubated at room temperature for 3 minutes. Lysates were given into a spin column assembly and centrifuged for 1 minute at full speed. The Columns were washed with 400 µL Diluted Wash Buffer (centrifugation 1 minute, full speed), waste was discarded and spin columns were centrifuged for another minute at maximum speed. Plasmid DNA was eluted into 50 µL Elution Buffer and EcoRI restriction analysis was performed. Reactions were prepared as described below (Table 16) and incubated for 1 hour at 37 °C. Fragment analysis was done by gel electrophoresis.

Table 16. Restriction Analysis with EcoRI Enzyme

4 µL	Plasmid DNA preparation
1 µL	NEBuffer EcoRI
1 µL	EcoRI (20.000 U/mL)
4 µL	H_2O
10 µL	

7.3.3.5 DNA Sequencing

DNA-Sequencing was done for confirming the accuracy of the PCR fragment that was cloned into the pCR II-TOPO vector. Components were prepared and reaction was ran as described below (Table 17; Table 18).

Table 17. DNA Sequencing Reaction

1 µL	Plasmid DNA
1 µL	M13 Primer (forward or reverse)
2 µL	BigDye Direct PCR Master Mix
6 µL	H_2O
10 µL	

Table 18. Cycling Conditions

96 °C	1 min	
96 °C	10 sec	25 cycles
50 °C	5 sec	
60 °C	2 min	

Sequencing products were purified by ethanolic precipitation. Therefore, 10 µL H_2O, 2 µL 3 M sodium acetate and 55 µL 100 % ethanol were added to the sequencing reaction and centrifuged for 20 minutes at full speed. Supernatant was discarded and 180 µL 70 % ethanol was added, following by centrifugation for another 20 minutes at full speed. Supernatant was removed, pellet was dried at 68 °C for 10 minutes and subsequent Sanger sequencing was performed by MWG Eurofins. Received sequences were compared to the expected fragments using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

7.3.3.6 Preparation of Standard Curves

Plasmid DNA was linearized using HindIII as restriction endonuclease that cuts the plasmid vector at a single restriction site avoiding cutting sites at the inserted PCR fragment. 34 µL Plasmid solution, 4 µL NEB-2 Buffer and 2 µL HindIII (20.000 U/mL) was mixed briefly and incubated at 37 °C over night. Additionally, restriction enzyme was inactivated for 20 minutes at 65 °C. Thereafter, concentration of samples was determined at the NanoDrop 2000c spectrophotometer and fragment analysis was performed by gel electrophoresis (Figure 10).

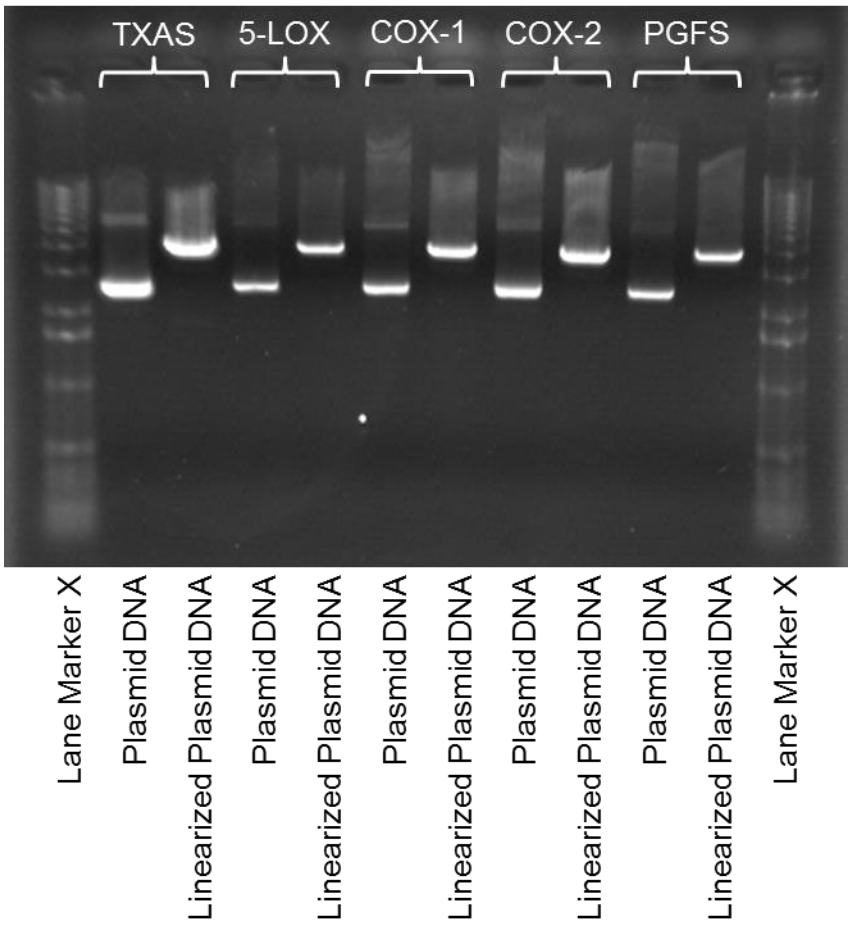


Figure 10. Gel Electrophoresis of Plasmid DNA and Linearized Plasmid DNA. Linearization leads to a mobility shift that can be used for discrimination and verification of effective HINDIII digestion.

Standard solution was prepared by diluting plasmid DNA to 10^9 single-strand molecules/ µL with 1 x TE Buffer. cDNA plasmid standard curves for each gene ranging from 10^2 to 10^6 copies were prepared using 10^9 standard and following dilution steps with 1 X TE containing 0.06 % 5 x First Strand Buffer (FSB) (Table 19).

Table 19. Dilution of Plasmid Standard Curves

Volume	Stock Solution	Volume (µL) 1 x TE + FSB	Single-Strand Molecules/ µL	2,5 µL/ Reaction
5 µL	10^9	245	2×10^7	
40 µL	10^7	160	4×10^6	
20 µL	10^6	180	4×10^5	10^6
20 µL	10^5	180	4×10^4	10^5
20 µL	10^4	180	4×10^3	10^4
20 µL	10^3	180	4×10^2	10^3
20 µL	10^2	180	4×10^1	10^2

7.3.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative Real-Time Polymerase-Chain-Reaction was performed for determination of expression level of target genes of AA metabolism. Specific primers and probes for qRT-PCR are given in Table 13. The TaqMan probes were labeled with a fluorescent reporter dye at the 5' end that is suppressed by the quencher dye at the 3' end of the probe as long as the probe is intact. During PCR the probe anneals between the forward and reverse primer sides. The 5' nuclease activity of the AmpliTaq Gold DNA Polymerase cleaves the probe, which Increased the distance of reporter and quencher dye and leads to an increase of reporter´s fluorescence that is proportional to originated PCR-products (Figure 11).

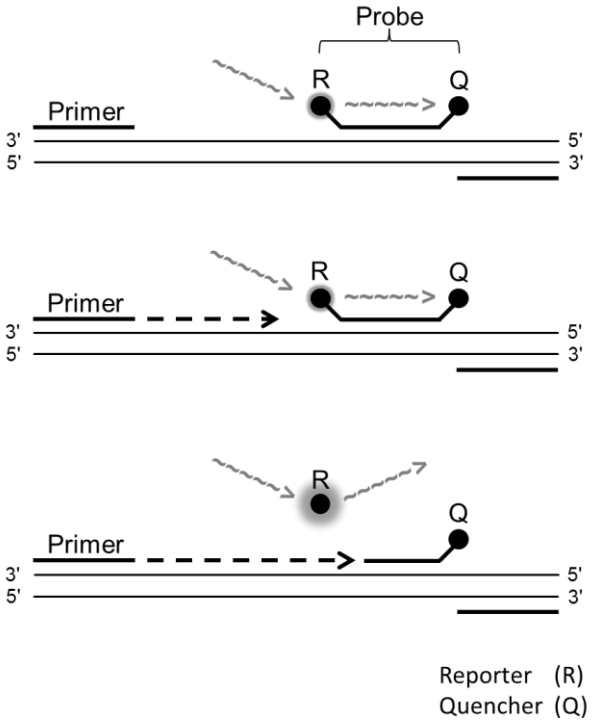


Figure 11. Quantification of PCR-products using Fluorogenic Probes.

Quantification of expression of target genes of AA metabolism was performed on a ViiA 7 Real-Time PCR-System. Absolute mRNA copies were determined using cDNA plasmid standard curves for each gene, ranging from 10^2 to 10^6 copies (Figure 12). Gene expression analysis was performed in quadruplicates; results were normalized to µg RNA.

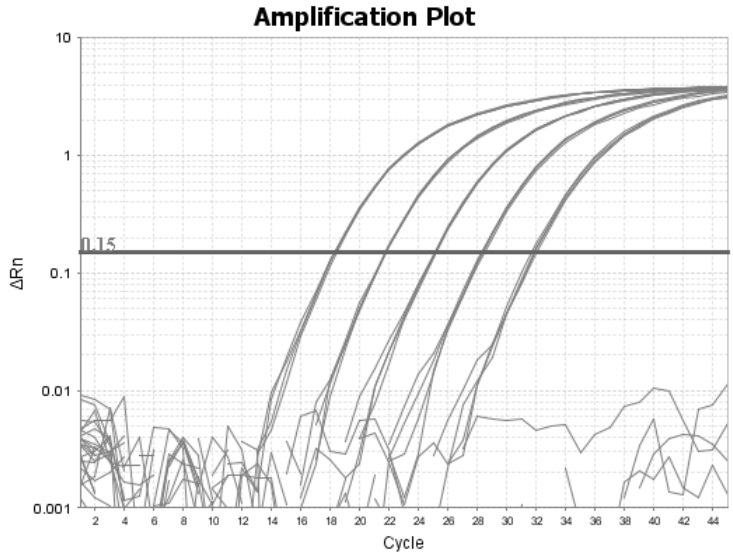


Figure 12. cDNA Plasmid Standard Curve of *PGES* ranging from 10^2 to 10^6 Copies.

The following protocol (Table 20; Table 21) was used for all qRT PCRs:

Table 20. Mastermix and qRT-PCR Reaction

4.5625 µL	H_2O
2.5 µL	$MgCl_2$ (25 mM Stock)
1.25 µL	10 x PCR Buffer
1.0 µL	dNTP
0.1875 µL	Primer forward 300 nM
0.1875 µL	Primer reverse 300 nM
0.25 µL	Probe (10 µM Stock)
0.0625 µL	AmpliTaq Gold
10 µL	

Reaction Volume

2.5 µL	cDNA/Standard
10 µL	Master Mix
12.5 µL	

Table 21. Cycling Conditions

50 °C	2 min	
95 °C	10 min	
95 °C	15 sec	45 cycles
60 °C	1 min	

7.4 Statistics

Eicosanoid metabolites and corresponding gene expression levels were quantified at baseline, after 1, 4 and 24 hours with and without inflammatory activation, respectively. Gene expression was normalized to µg RNA and both gene expression and metabolite release was given relative to medium control at 1, 4 and 24 hours to evaluate the kinetics of reactions. Data are presented as median, interquartile range (IQR) as well as range, analyses performed as duplicates are shown as mean. Significance of activation with different stimuli and differential eicosanoid response on gene expression and metabolite level of healthy subjects was calculated with the Wilcoxon-Mann-Whitney test.

Descriptive statistics was used for the investigation of dose-dependency of LPS activation and study of regulatory mechanism of AA metabolism since analyses were performed as duplicates.

Significance of relative changes against control values of patients with or without CAD was determined with the signed rank test. To evaluate associations with CAD status, first, patients without CAD were compared to all patients with CAD. If significantly different ($P < 0.05$), separate P-values were calculated for patients without CAD versus patients with CAD < 50 % stenosis, and patients with CAD ≥ 50 % stenosis. The χ^2-test was used for categorized variables and the Wilcoxon-Mann-Whitney test in case of continuous variables. Age and sex adjusted p-values were derived from logistic regression analysis. Absolute values entered logistic regression as base 2 logarithms, relative values entered without transformation, and age was categorized as ≥ 65 versus < 65 years. In addition, a logistic regression model for the combined influence of eicosanoid values on CAD status was developed. Since data were highly correlated, a bootstrap variable selection method was used. All variables with age and sex adjusted p-values < 0.1 entered logistic regression analyses in 500 bootstrap samples using stepwise variable selection. Among those variables with at least 150 selections the most important ones where chosen using a strategy proposed by Sauerbrei and Schumacher.[77] A score to estimate the probability of CAD was calculated from the final model and illustrated by Receiver-Operator Characteristics (ROC) curves and boxplots in the three CAD groups. All analyses were performed using SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 6 (Version 6.07, GraphPad Software, La Jolla, CA, USA).

8 Results

8.1 Differential Eicosanoid Response in Whole Blood upon Activation with LPS, oxLDL and TNFα

The previously established whole-blood activation model required 1 mL lithium heparin whole blood which was incubated with 1 µg/mL lipopolysaccharide (LPS) and incubated at 37 °C and 5 % CO_2 for 24 hours. Particularly in regard to the investigation of patients with atherosclerosis, we aimed to further evaluate stimuli that have been described in the context with this chronic inflammatory disease. Furthermore, we aimed to study different time points of whole blood activation to characterize the time response of gene expression and metabolite release of AA metabolism in greater detail. Therefore, whole blood of 3 healthy blood donors was activated with LPS (1 µg/mL), oxidized low-density-lipoprotein (oxLDL; 100 µg/mL) or tumor necrosis factor alpha (TNFα; 10 ng/mL) and incubated for 1, 4 and 24 hours. Cell culture studies were performed in duplicates, experiments without stimuli serving as baselines were performed as quadruplicate (Figure 13). Gene expression analyses were performed in quadruplicates.

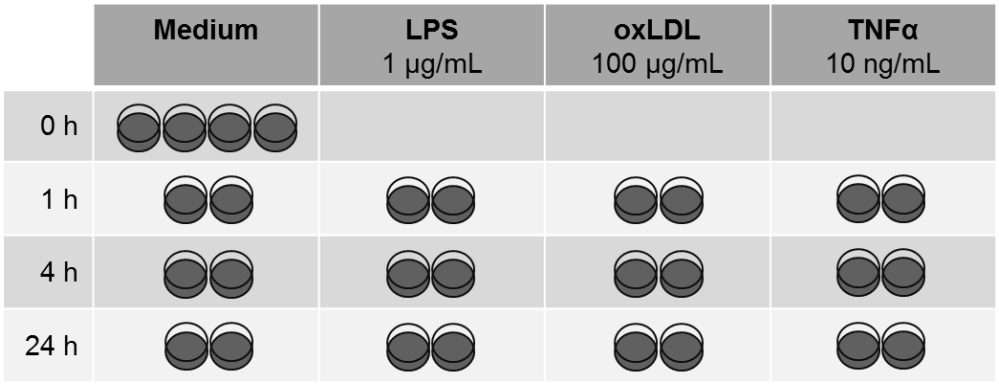


Figure 13. Investigation of the Eicosanoid Response due to Different Inflammatory Stimuli (n=3). 1 mL whole blood was mixed with 500 µL medium spiked with or without LPS, oxLDL and TNFα. Mixtures were incubated for 1, 4 and 24 hours at 37 °C and 5 % CO_2. Cell culture studies were performed in duplicates, experiments without stimuli serving as baselines were performed as quadruplicates.

We determined the gene expression of *COX-1, COX-2, PGFS* and *TXAS* as representatives of COX pathway and gene expression of *5-LOX* from the LOX pathway. Gene expression was normalized to µg RNA and relative to medium control at 1, 4 and 24 hours to evaluate the kinetics of gene expression. Corresponding metabolites 11-HETE, $PGF_{2\alpha}$, TxB_2 and 5-HETE were analysed in supernatants. Data are given as absolute values and relative to medium control at 1, 4, and 24 hours.

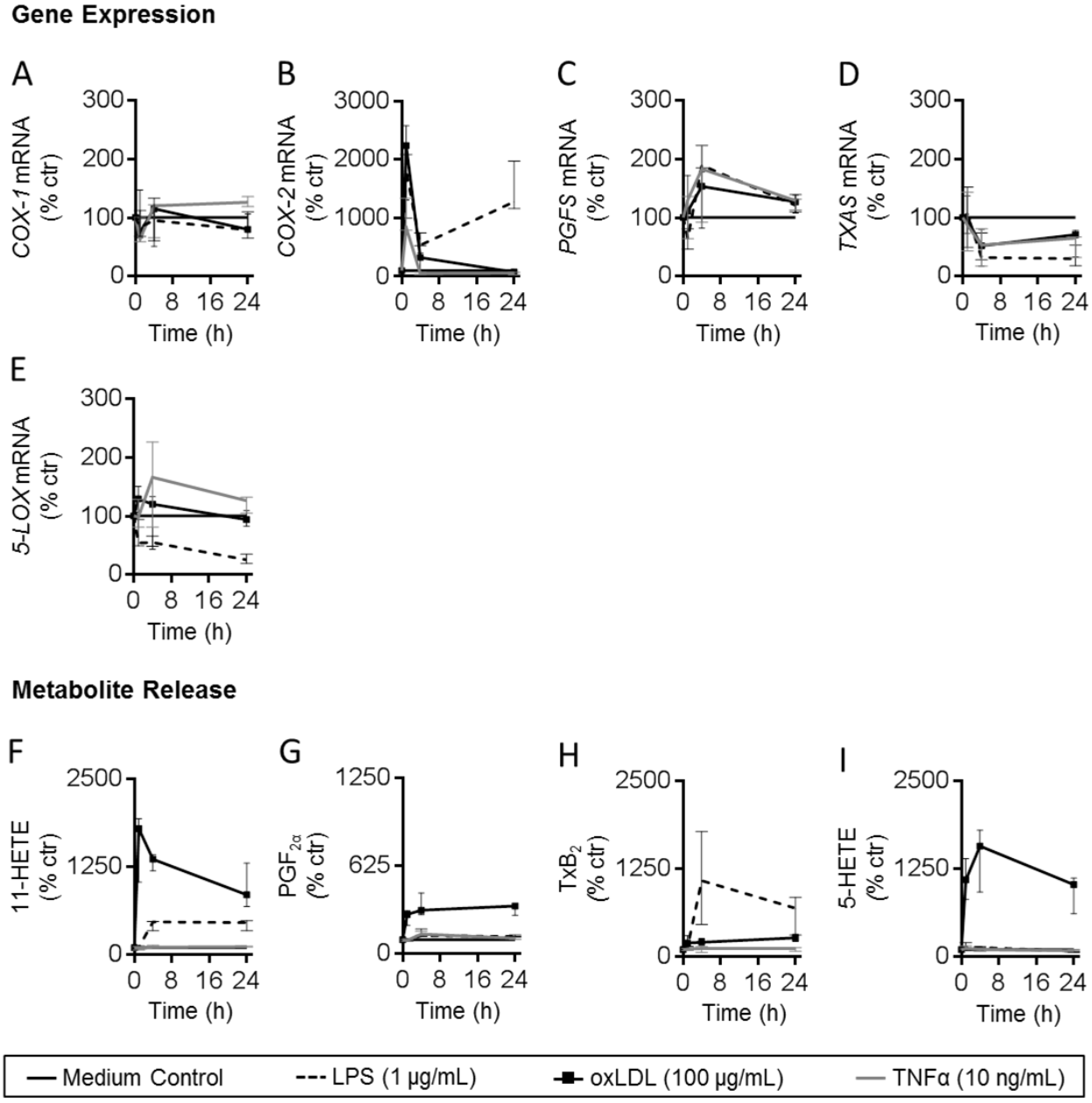


Figure 14. Eicosanoid Response after Activation with LPS, oxLDL or TNFα. Whole blood from study subjects (n=3) was activated with LPS (1 µg/mL), oxLDL (100 µg/mL) or TNFα (10 ng/mL) for 1, 4 and 24 hours. Cell culture studies were performed in duplicates, experiments without stimuli serving as baselines were performed as quadruplicates. Gene expression analyses were performed in quadruplicates. mRNA expression of *COX-1* (A), *COX-2* (B), *PGFS* (C), *TXAS* (D) and *5-LOX* (E) and corresponding metabolites 11-HETE (F), $PGF_{2\alpha}$ (G), TxB_2 (H) and 5-HETE (I) are shown. Data are given relative to medium control at 1, 4 and 24 hours (Median ± Range). Significances are given in Supplemental Table I.

COX-1 expression was not affected by stimulation with LPS and oxLDL, while incubation of whole blood with TNFα revealed a significant increase after 24 hours when normalized to medium control (*P<0.05*) (Figure 14 A; Supplemental Table I). Absolute *COX-1* expression levels showed no consistent trend after incubation with or without different stimuli for 1 or

4 hours, but significantly decreased after 24 hours incubation (*Medium and TNFα: P<0.05; LPS: P<0.001; oxLDL: P<0.01*) (Supplemental Table II). *COX-2* expression was significant upregulated (*P<0.05*) in cellular blood components after activation with LPS, oxLDL and TNFα for 1 hour, while only LPS revealed a significant increase at 4 as well as at 24 hours both for absolute as well as relative values (*P<0.05*). In contrast, incubation with oxLDL or TNFα tended to result in a downregulation of this pathway after 4 (TNFα) and 24 hours (oxLDL and TNFα) when normalized to medium control. Data were in line with absolute *COX-2* expression levels that significantly increased after 1 hour (*P<0.05*) and decreased after 24 hours (*P<0.01*) incubation with this stimuli (Figure 14 B; Supplemental Table I; Supplemental Table II).

TXAS and *5-LOX* expression was downregulated after 4 and 24 hours LPS activation (*P<0.05*; *P<0.01*), however oxLDL and TNFα showed no significant effects when data were normalized to medium control (Figure 14 D-E; Supplemental Table I). Absolute *TXAS* expression levels were downregulated with significant effects for 4 hours (*P<0.01*) and 24 hours (*P<0.05*) LPS incubation and 1 hour stimulation with oxLDL (*P<0.05*). *5-LOX* expression was downregulated after 1 hour for all investigated stimuli (*LPS: P<0.01; oxLDL and TNFα: P<0.05*), while LPS and oxLDL showed also significant effects after 24 hours (*LPS: P<0.001; oxLDL: P<0.05*) (Supplemental Table II).

PGFS expression revealed no significant effects for oxLDL and TNFα both for relative and absolute data. After activation of this pathway with LPS no consistent trend of time response could be observed, in which incubation showed a significant increase after 4 hours relative to medium control (*P<0.05*). (Figure 14 C; Supplemental Table I) Absolute *PGFS* expression levels tended to be downregulated with significant effects for LPS after 1 hour (*P<0.01*) and 24 hours (*P<0.05*) (Supplemental Table II).

On metabolite level, absolute and relative values of 11-HETE were significant upregulated after incubation with oxLDL for all investigated time points and LPS after 4 as well as 24 hours (*P<0.05*). Activation with TNFα revealed no consistent trend, in which a significant induction could be observed after 24 hours both for absolute and relative data (*P<0.05*) (Figure 14 F; Supplemental Table III; Supplemental Table IV). $PGF_{2\alpha}$ was significantly upregulated after treatment with oxLDL for all time points (*1 and 4 hours: P<0.05; 24 hours: P<0.01*) and TNFα as well as LPS revealed some opposing trends with significant increases after 4 (TNFα) and 24 hours (LPS) when normalized to medium control (*P<0.05*) (Figure 14 G; Supplemental Table III). Absolute $PGF_{2\alpha}$ release was upregulated after oxLDL stimulation for all investigated time points (*1 hour: P<0.05; 4 and 24 hours: P<0.01*), while activation with LPS and TNFα showed an initial decrease (*P<0.05*) followed by an increase after 4 hours (*P<0.05*) and 24 hours (*P<0.01*) (Supplemental Table IV). TxB_2 was upregulated for all investigated stimuli with significant effects for oxLDL after 4 and 24 hours when normalized to medium control (*P<0.05*).

Absolute TxB_2 release tended to be upregulated with significant effect after 1 hour (*P<0.05*) and 4 hours (*P<0.01*) oxLDL incubation (Figure 14 H; Supplemental Table III; Supplemental Table IV). LOX-dependent 5-HETE showed no consistent trend after activation with LPS with a significant increase after 4 hours and a decreased metabolite release after 24 hours. Activation of whole blood with oxLDL revealed significant increased 5-HETE levels for all investigated time points and no significant effect for stimulation with TNFα (Figure 14 I; Supplemental Table III). Absolute 5-HETE levels seemed to be increased with significant effects for 1 and 24 hours for all stimuli, while oxLDL also revealed significant effects for 4 hours incubation (*P<0.05*) (Supplemental Table IV).

In summary, whole blood activation with different stimuli revealed variable effects on gene expression and mediator level with a distinctive time-dependency of those reactions. In principal, *COX-2* and *PGFS* expression was upregulated, *TXAS* and *5-LOX* expression was downregulated while gene expression levels of *COX-1* were not altered. Furthermore, corresponding metabolites for both COX- and LOX-pathways showed mostly an upregulation, while stimulation with LPS revealed a significant downregulation of 5-HETE after 24 hours.

Together, we observed a minor response of whole blood arachidonic acid metabolism followed by activation with TNFα. Although oxLDL revealed a quantitative significant stimulation, the preparation and oxidation of LDL from plasma is not possible to allow its application in clinical routine settings due to high variability between the preparations and prolonged preparation time (estimated preparation time: 120 hours). Therefore, further experiments were performed with LPS as stimulant at three different time points (1, 4 and 24 hours).

8.2 Dose-Dependency of Eicosanoid Response on Gene Expression and Mediator Level

For the investigation of dose-dependency of LPS mediated eicosanoid response, whole blood was activated with different concentrations of LPS (100 ng/mL, 300 ng/mL or 1 µg/mL). The rational for these conditions was based on LPS concentrations that have been used for the stimulation of cytokine release in human whole blood.[78,79] The samples (n=3; pool) were incubated for 1, 4 and 24 hours. Cell culture studies were performed in duplicates, experiments without LPS serving as baselines were performed as quadruplicates (Figure 15). Gene expression analyses were performed in quadruplicates.

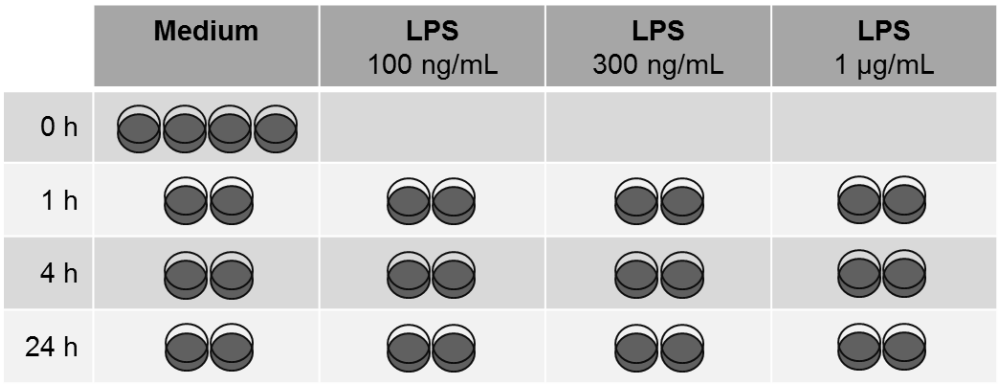


Figure 15. Investigation of Dose-Dependency of Eicosanoid Response. 1 mL whole blood (n=3, pool) was mixed with 500 µL medium spiked with or without LPS (100 ng/mL, 300 ng/mL or 1 µg/mL). Mixtures were incubated for 1, 4 and 24 hours at 37 °C and 5 % CO_2. Cell culture studies were performed in duplicates, experiments without LPS serving as baselines were performed as quadruplicates.

Eicosanoid response on gene expression (*COX-1, COX-2, PGFS* and *TXAS)* as well as metabolite (11-HETE, $PGF_{2\alpha}$, TxB_2 and 5-HETE) level was analysed. Gene expression was normalized to µg RNA and both, gene expression and metabolite release, are given relative to medium control at 1, 4 and 24 hours.

COX-1 expression revealed a minor 1.26 to 1.63 fold upregulation after 1 hour LPS activation, while expression level was not affected or tended to be downregulated after 4 and 24 hours when normalized to medium control. Absolute *COX-1* expression levels showed also no consistent trend after incubation with LPS, whereas expression seemed to be upregulated after 1 hour followed by a downregulation after 4 and 24 hours (Figure 16 A; Supplemental Table V). *COX-2* expression showed a 3.27 to 24.44 fold upregulation in cellular blood components after activation with LPS, while also absolute values were highly upregulated with gene expression levels between 8633.56 and 29424.23 $x10^3$ copies/ µg RNA (Figure 16 B; Supplemental Table

V). Furthermore, relative as well as absolute *PGFS* expression revealed no LPS mediated effects (Figure 16 C; Supplemental Table V). *TXAS* and *5-LOX* expression was downregulated after 4 and 24 hours (Figure 16 D-E; Supplemental Table V). With respect to the different concentration of LPS no differences for all investigated target genes as well as for all studied time points were observed.

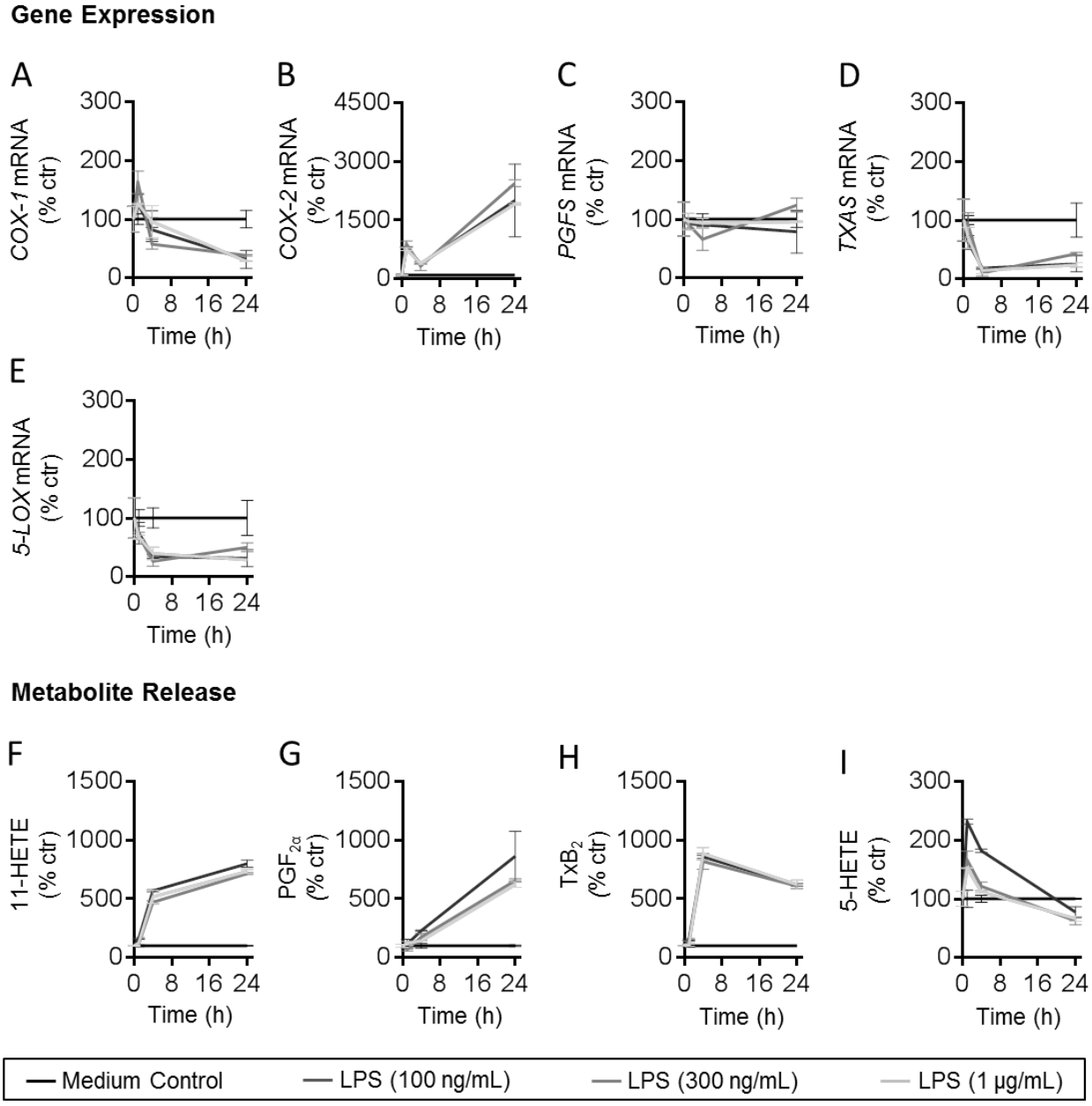


Figure 16. Eicosanoid Response after Incubation with Different Concentrations of LPS. Whole blood (n=3, pool) was activated with different concentrations of LPS (100 ng/mL, 300 ng/mL or 1 µg/mL). Cell culture studies were performed in duplicates, experiments without stimuli serving as baselines were performed as quadruplicates. Gene expression analyses were performed in quadruplicates. mRNA expression of *COX-1* (A), *COX-2* (B), *PGFS* (C), *TXAS* (D) and *5-LOX* (E) and corresponding metabolites 11-HETE (F), $PGF_{2\alpha}$ (G), TxB_2 (H) and 5-HETE (I) are shown. Data are given relative to medium control at 1, 4 and 24 hours (Mean ± SEM).

Analysis of corresponding metabolites revealed an upregulation of 11-HETE, $PGF_{2\alpha}$ and TxB_2 while 5-HETE showed a downregulation after 24 hours LPS incubation (Figure 16 F-I; Supplemental Table VI). Eicosanoid response on metabolite level also indicated no differences for several LPS concentrations.

Summing, the time-dependent eicosanoid response on gene expression and metabolite level was confirmed and no dose-dependency for the investigated time-points was detected. Thus, further experiments were performed with a final concentration of 100 ng/mL LPS.

8.3 Investigation of Regulatory Mechanism of AA Metabolism

Since we observed a rapid response of AA metabolism on mRNA expression and metabolite level, functional studies were performed to better characterize the regulation of AA metabolism on the transcriptional and translational level. To this end, actinomyin D (ActD) as an inhibitor of transcription and cycloheximide (CHX) as a translational inhibitor were used to investigate, if the eicosanoid response was controlled through transcriptional or translational changes.
Whole blood was activated with LPS (100 ng/mL) for 1, 4 and 24 hours after pre-incubation with 5 µg/mL ActD or 10 µg/mL CHX for 1 hour. Cell culture studies were performed in duplicates, experiments without LPS, ActD or CHX serving as baselines were performed as triplicates (Figure 17; Figure 18). Gene expression analyses were performed in quadruplicates.

Eicosanoid response on gene expression (*COX-1, COX-2, PGFS* and *TXAS)* as well as metabolite (11-HETE, $PGF_{2\alpha}$, TxB_2 and 5-HETE) level was analysed. Gene expression was normalized to µg RNA and results are given relative to medium control, while results after treatment with ActD are given relative to the ActD control at 1, 4 and 24 hours.

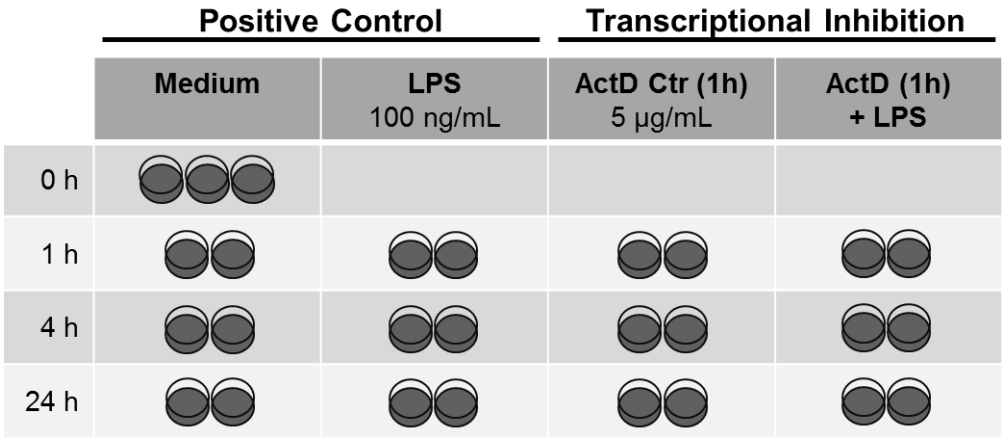


Figure 17. Transcriptional Inhibition Experiments with Actinomycin D (ActD). 1 mL whole blood (n=3, pool) was mixed with 500 µL medium spiked with or without LPS (100 ng/mL). For transcriptional inhibition, whole blood was pre-incubated with ActD for 1 hour. Mixtures were incubated for 1, 4 and 24 hours at 37 °C and 5 % CO_2. Cell culture studies were performed in duplicates, experiments without LPS or ActD serving as baselines were performed as triplicates.

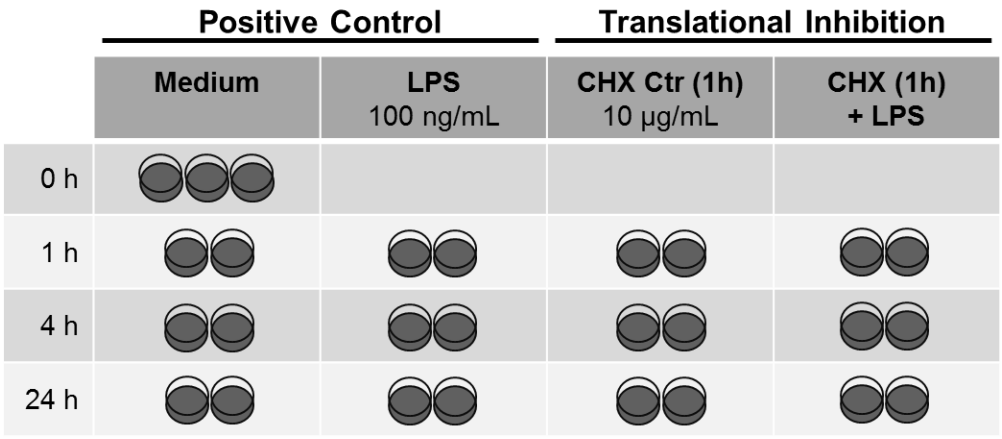


Figure 18. Translational Inhibition Experiments with Cycloheximide (CHX). 1 mL whole blood (n=3, pool) was mixed with 500 µL medium spiked with or without LPS (100 ng/mL). For translational inhibition, whole blood was pre-incubated with CHX for 1 hour. Mixtures were incubated for 1, 4 and 24 hours at 37 °C and 5 % CO_2. Cell culture studies were performed in duplicates, experiments without LPS or CHX serving as baselines were performed as triplicates.

Activation of whole blood with LPS revealed similar effects on gene expression and mediator level as described in previously performed experiments. We found a transcriptional inhibition of *COX-1*, *COX-2*, *PGFS* and consolidated downregulation of *TXAS* as well as *5-LOX* gene expression when results were normalized to medium control. Absolute gene expression levels showed a transcriptional inhibition of *COX-2*, *PGFS*, *TXAS* and *5-LOX* after 24 hours, while solely *COX-1* expression was not affected suggesting a prolonged half life time of mRNA. On metabolite level 11-HETE and TxB_2 release was inhibited after incubation with ActD, in which $PGF_{2\alpha}$ and 5-HETE revealed a slight increase after 1 hour (Figure 19; Table 22; Supplemental Table VII; Supplemental Table VIII). Translational inhibition with CHX induced a general inhibition of LPS induced 11-HETE, $PGF_{2\alpha}$, TxB_2 and 5-HETE release (Figure 20; Table 22; Supplemental Table IX) with minor increases of $PGF_{2\alpha}$ after 1 hour, 5-HETE after 4 hours as well as TxB_2 after 24 hours incubation.

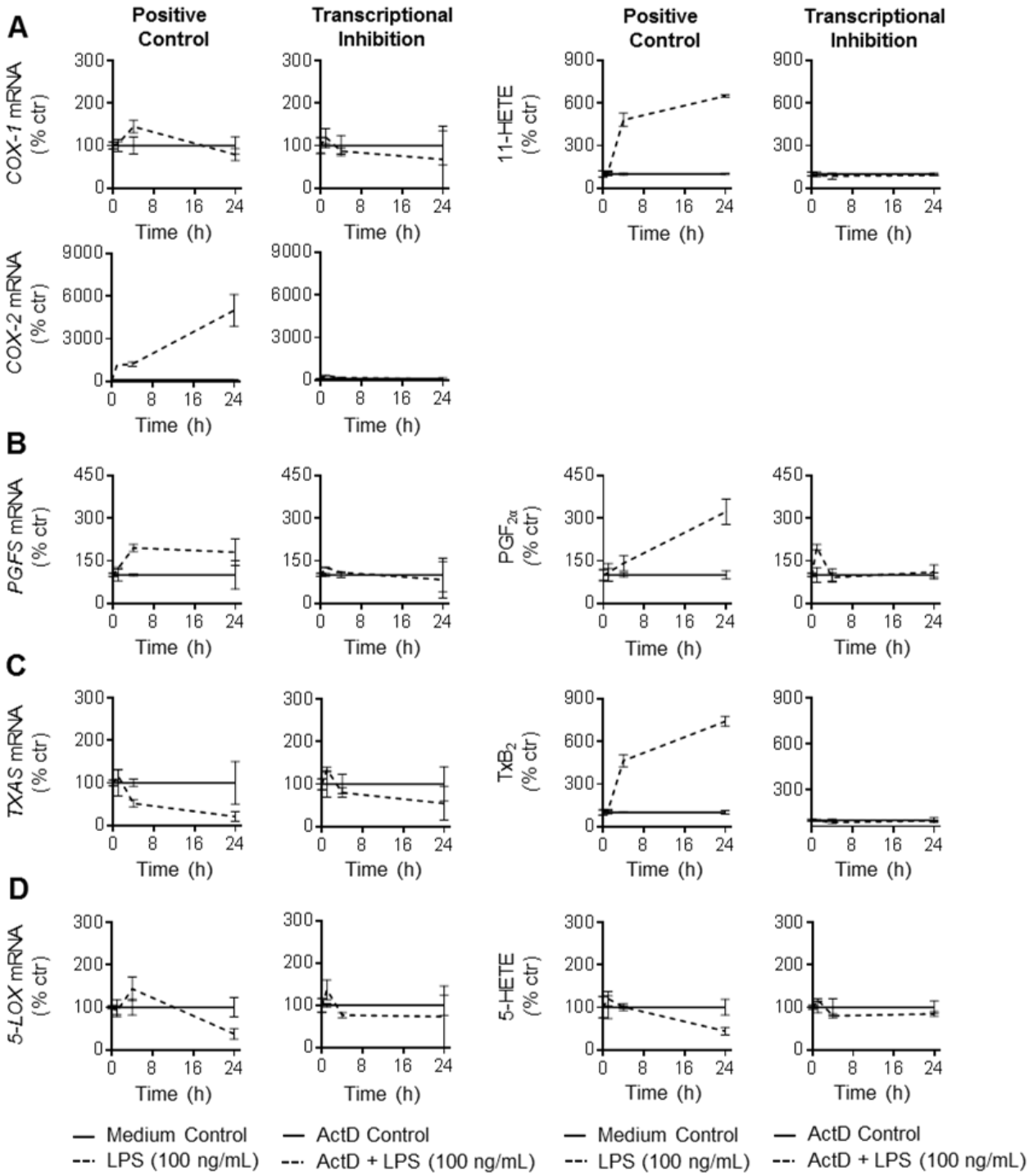


Figure 19. Transcriptional Inhibition of the Arachidonic Acid Metabolism with Actinomycin D (ActD). COX- and LOX- dependent eicosanoid response on gene expression and metabolite level after incubation with LPS (positive control) and transcriptional inhibition with actinomycin D (ActD) are shown. Cell culture studies were performed in duplicates, experiments without stimuli serving as baselines were performed as triplicates. Gene expression analyses were performed in quadruplicates. Data are given relative to medium or ActD control at 1, 4 and 24 hours (Mean ± SEM).

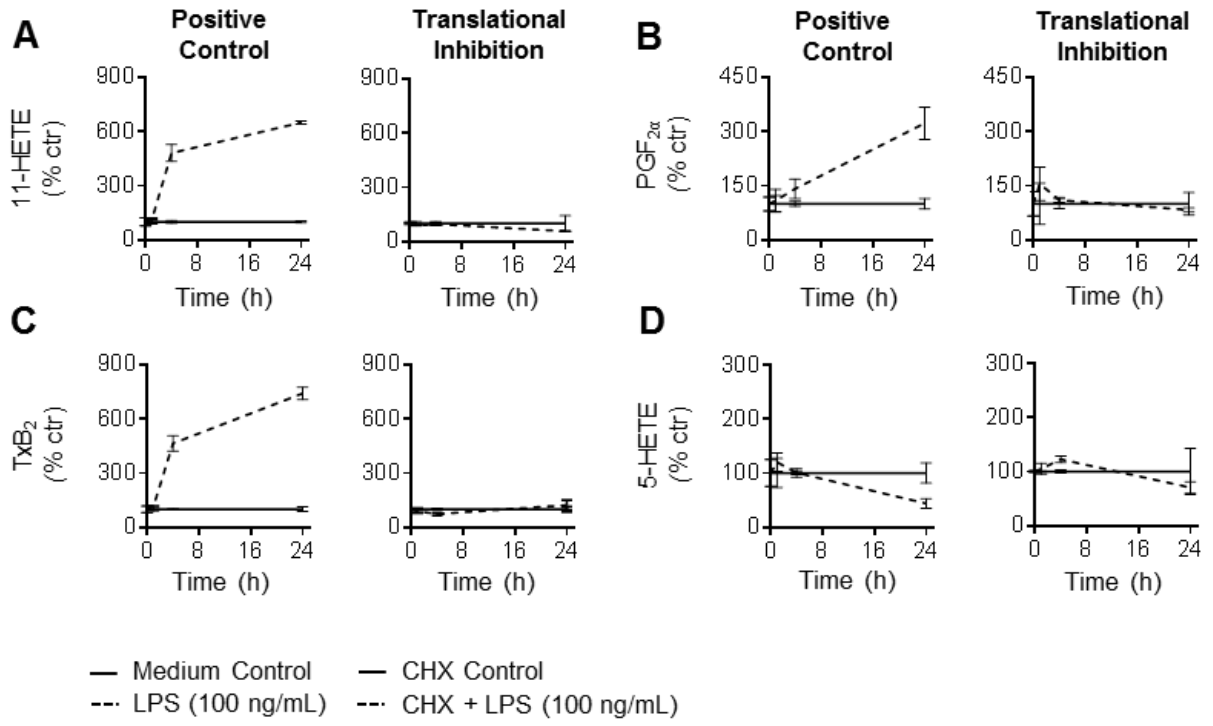


Figure 20. Translational Inhibition of the Arachidonic Acid Metabolism with Cycloheximide (CHX). Metabolite release of 11-HETE (A), $PGF_{2\alpha}$ (B), TxB_2 (C) and 5-HETE (D) after incubation with LPS (Positive Control) and translational inhibition with cycloheximide (CHX) are shown. Cell culture studies were performed in duplicates, experiments without stimuli serving as baselines were performed as triplicates. Gene expression analyses were performed in quadruplicates. Data are given relative to medium control or CHX at 1, 4 and 24 hours (Mean ± SEM).

Table 22. LPS induced Eicosanoid Response after Transcriptional and Translational Inhibition.

Target Gene/ Metabolite	Time (h)	Positive Control	Transcriptional Inhibition	Translational Inhibition
COX-1	1	=	(↑)	
	4	(↑)	↓	
	24	↓	↓	
COX-2	1	↑	(↑)	
	4	↑	(↑)	
	24	↑	↓	
11-HETE	1	=	=	=
	4	↑	=	=
	24	↑	=	=
PGFS	1	(↑)	(↑)	
	4	↑	↓	
	24	↑	↓	
$PGF_{2\alpha}$	1	(↑)	↑	↑
	4	↑	=	=
	24	↑	=	=

TXAS	1	(↑)	(↑)	
	4	↓	↓	
	24	↓	↓	
TxB_2	1	(↑)	=	=
	4	↑	=	=
	24	↑	=	(↑)
5-LOX	1	=	↑	
	4	↑	(↓)	
	24	↓	(↓)	
5-HETE	1	↑	↑	=
	4	=	(↓)	↑
	24	↓	(↓)	(↓)

Data suggested that production of 11-HETE and $PGF_{2\alpha}$ due to the inflammatory stimuli LPS underlie *de novo* synthesis and seemed to be coupled to induced *COX-2* and *PGFS* mRNA synthesis. In contrast, the strong involvement of the translational level was assumed since we found increased TxB_2 and 5-HETE metabolite release while *TXAS* and *5-LOX* gene expression decreased

In summary, eicosanoid response seemed to be regulated on transcriptional as well as translational levels, thus further experiments should consider both, data of gene expression and mediator release, equally.

8.4 Differential Eicosanoid Response of Healthy Subjects

In previous work, we developed an analytical test for the application in a clinical setting that enabled the simultaneous and standardized analysis of COX- and LOX-dependent eicosanoid response both on metabolite and gene expression level. LC-MS/MS method allowed quantification of AA, 5-HETE, 11-HETE, 12-HETE, $PGF_{2\alpha}$, PGE_2 and TxB_2, while RT-PCR assays for analysis of *COX-2* and *PGES* expression have been established.

For a more comprehensive profiling of the main metabolizing pathways of AA metabolism, further gene expression assays were needed. Therefore, additional RT-PCRs for *PLA_2*, *COX-1*, *TXAS*, *PGFS*, *5-LOX*, *FLAP* and *12-LOX* have been established using gene-specific primers and probes (Figure 21).

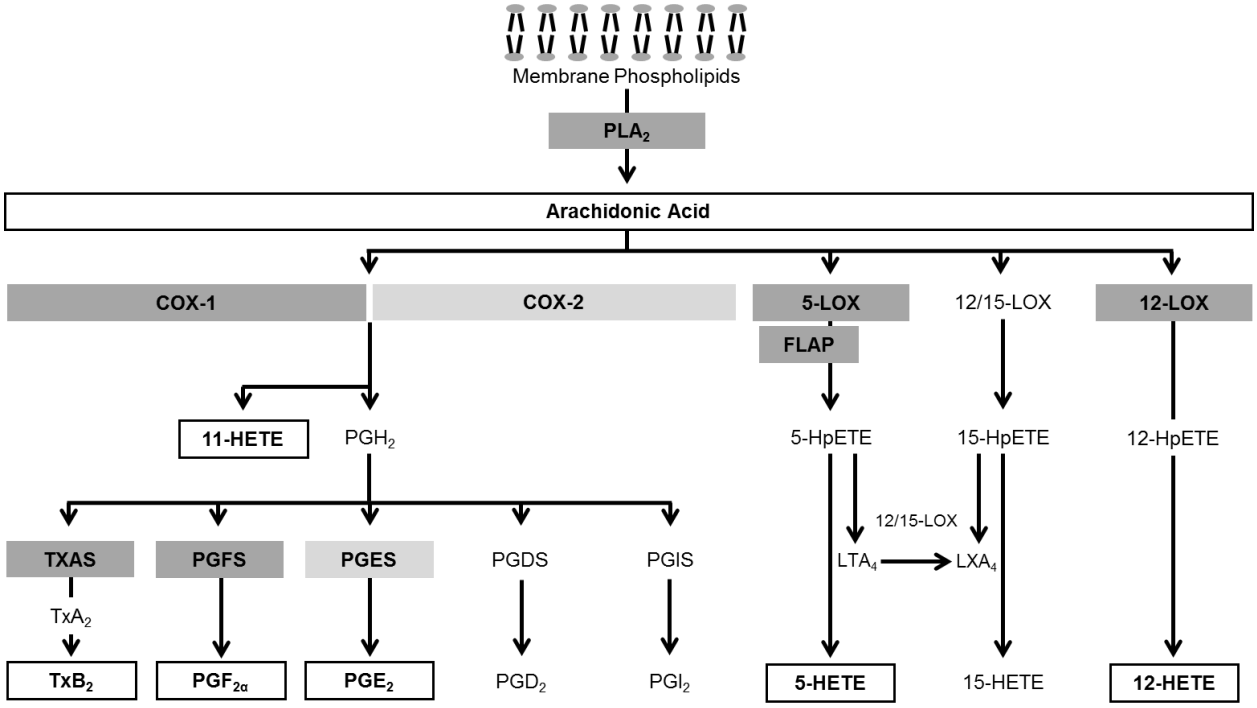


Figure 21. Improvement of the Analysis of Arachidonic Acid (AA) Metabolism. AA metabolism was analyzed on gene expression and metabolite level. Target genes (boxes colored light gray) and metabolites (framed boxes) of the previously developed *in vitro* whole-blood assay are shown. Additional established gene expression assays (*PLA_2*, *COX-1*, *TXAS*, *PGFS*, *5-LOX*, *FLAP*, *12-LOX*) are colored dark gray.

Using the newly developed *in vitro* whole blood assay, AA metabolism of 10 healthy subjects was investigated for the characterization of general eicosanoid profiles and a better understanding of the inter-individual variability of the eicosanoid response.

Whole blood of 5 men and 5 women was incubated with LPS (100 ng/mL) for 1, 4 and 24 hours. Analysis was performed in triplicates (Figure 22). Eicosanoid response on gene expression

(*PLA_2, COX-1, COX-2, PGES, PGFS, TXAS, 5-LOX*, *FLAP*, *12-LOX)* and metabolite (AA, 11-HETE, PGE_2, $PGF_{2\alpha}$, TxB_2, 5-HETE and 12-HETE) level was analysed. Gene expression was normalized to µg RNA and both, gene expression and metabolite release, are given relative to medium control at 1, 4 and 24 hours.

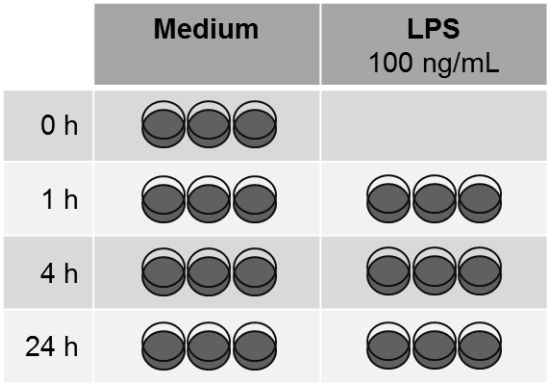


Figure 22. Investigation of the Eicosanoid Response of Healthy Subjects (n=10). 1 mL whole blood was mixed with 500 µL medium spiked with or without LPS (100 ng/mL). Mixtures were incubated for 1, 4 and 24 hours at 37 °C and 5 % CO_2. Cell culture studies were performed in triplicates.

Gene expression analysis over all healthy subjects revealed an upregulation for four investigated target genes (*COX-2, PGES, PGFS, FLAP*), a downregulation for two investigated pathways (*TXAS, 5-LOX*) and no consistent trend for *PLA_2*, *COX-1* and *12-LOX*.
In detail, *COX-2* (*P<0.01*), *PGES* (*P<0.01*) and *PGFS* (*P<0.05*) showed significant effects for 1, 4 and 24 hours, while *FLAP* was merely upregulated for 1 and 4 hours when normalized to medium control (*P<0.01*). Absolute gene expression levels for *COX-2*, *PGES* and *FLAP* showed an increase after incubation with LPS for 1, 4 and 24 hours, whereas *PGES* at 4 and 24 hours as well as *FLAP* at 24 hours showed also upregulated expression levels without LPS stimulation. Absolute mRNA level of *PGFS* mainly decreased. Normalized gene expression of *TXAS* and *5-LOX* showed significant downregulation for 1 and 24 hours (*P<0.01*), in which *TXAS* revealed also a significant effect for 4 hours (*P<0.01*). Absolute levels where in line with relative data and showed a decrease after 4 and 24 hours LPS incubation for both target genes and after 1 hour for *5-LOX* gene expression. *PLA_2, COX-1* and *12-LOX* were significant (*P<0.01*) upregulated after 1 hour and declined after 4 or 24 hours LPS activation when normalized to medium control. Absolute gene expression level of *PLA_2* significantly increased after 4 hours LPS incubation while *COX-1* and *12-LOX* expression decreased over time (Figure 23; Supplemental Table X).

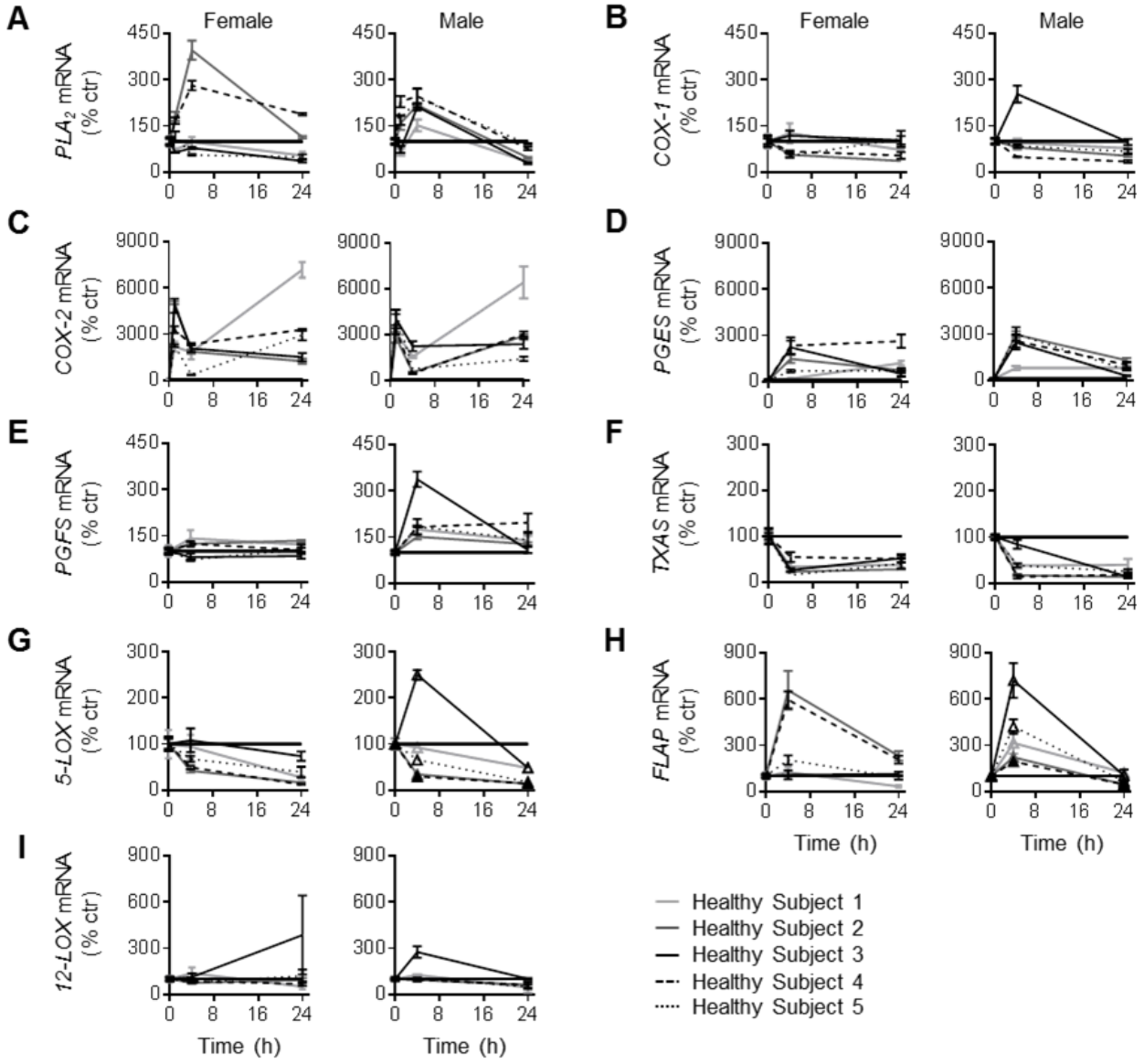


Figure 23. Differential Eicosanoid Response on Gene Expression Level in Healthy Subjects (n=10). Whole blood from female (n=5, left) and male (n=5, right) study subjects was activated with or without LPS (100 ng/mL) for 1, 4 and 24 hours. mRNA expression of *PLA₂* (A), *COX-1* (B), *COX-2* (C), *PGES* (D), *PGFS* (E), *TXAS* (F), *5-LOX* (G), *FLAP* (H) and *12-LOX* (I) are shown. Statistical significances are given in Supplemental Table X and Supplemental Table XI. Cell culture studies were performed in triplicates.

Differential gene expression levels between males and females could be found for *PGFS* after 4 hours for absolute and relative values and *TXAS* after 24 hours LPS activation when normalized to medium control (*P<0.05*). The other investigated pathways revealed no significant gender effects (Supplemental Table XI). In regard to the analysis of every single subject major inter-individual differences could be found for all investigated target genes both for COX- and LOX dependent pathways. For instance, activation of whole blood with LPS results in a 10 to 80-fold induction of *COX-2* gene expression, while downregulation of *5-LOX* ranged from 12 to

74 percent relative to medium control after 24 hours (Supplemental Table XI). The coefficients of variation (CV) ranged between 15.06 % for *PGFS* up to 100.04 % for *PGES* expression (Supplemental Table XII). Furthermore, some observed subjects revealed oppositional effects compared to the whole group, which could be shown for *PLA_2, COX-1, TXAS, PGFS, 12-LOX, 5-LOX* and *FLAP* for several time points after LPS stimulation (Figure 23; Supplemental Table X; Supplemental Table XI).

On metabolite level AA was significant downregulated after 1 and 24 hours LPS activation when normalized to medium control (*P<0.01*). In contrast, absolute AA release significantly increased over time (*P<0.01*). The major COX metabolites 11-HETE, TxB_2, PGE_2 and $PGF_{2\alpha}$ were significantly upregulated for all investigated time points (*P<0.01*), solely $PGF_{2\alpha}$ revealed a significant downregulation after 1 hour LPS incubation normalized to medium control (*P<0.01*). Absolute metabolite release revealed an increase for these metabolites after 4 and 24 hours, whereas 11-HETE, TxB_2 and PGE_2 release was also increased after 1 hour LPS stimulation (*P<0.05*). LOX dependent metabolites showed no consistent trend after whole blood activation normalized to medium control, in which 5-HETE was significant upregulated after 1 and 4 hours followed by a significant downregulation after 24 hours (*P<0.01*). 12-HETE revealed an initially significant downregulation continued by a significant upregulation after 24 hours (*P<0.01*). Absolute 5-HETE and 12-HETE levels were significant upregulated for all investigated time points (*P<0.05*) (Supplemental Table XIII).

Analyzing potential gender effects on metabolite level, we found significant differences of TxB_2 release after 24 hours between male and female subjects (*P<0.05*). No differential eicosanoid response could be shown for the further investigated AA metabolites when results were normalized to medium control. Absolute data revealed higher levels of 5-HETE (baseline and 4 hours without LPS), 11-HETE (1 hour with and 4 hours without LPS) and 12-HETE (baseline and 1 hour as well as 4 hours incubation with or without LPS) for female subjects when compared to males. Contrary, TxB_2 release was significantly lower in females compared to males for all investigated conditions except of 24 hours LPS activation (Supplemental Table XIV).

In line with the results of the analysis of target genes, inter-individual differences of metabolite release could be found with a coefficient of variation from 8.23 % (AA) up to 110.63 % (TxB_2) (Supplemental Table XV). 24 hours LPS activation of whole blood lead to major variabilities of COX-dependent metabolite release, whereat 11-HETE showed an 4- to 13-fold, PGE_2 an 12- to 81-fold, $PGF_{2\alpha}$ an 3- to 8-fold and TxB_2 an 2- to 16-fold increase. Also LOX pathways showed differences for every single subject, in which exemplarily 5-HETE ranged between a 1- to 3-fold

induction after 4 hours LPS activation. Similarly to investigated target genes, analysis of metabolite level revealed single subjects that showed oppositional effects compared to the whole group. Diverging eicosanoid release could be found for AA, 11-HETE, TxB_2, PGE_2, $PGF_{2\alpha}$ and 12-HETE (Figure 24; Supplemental Table XIII; Supplemental Table XIV).

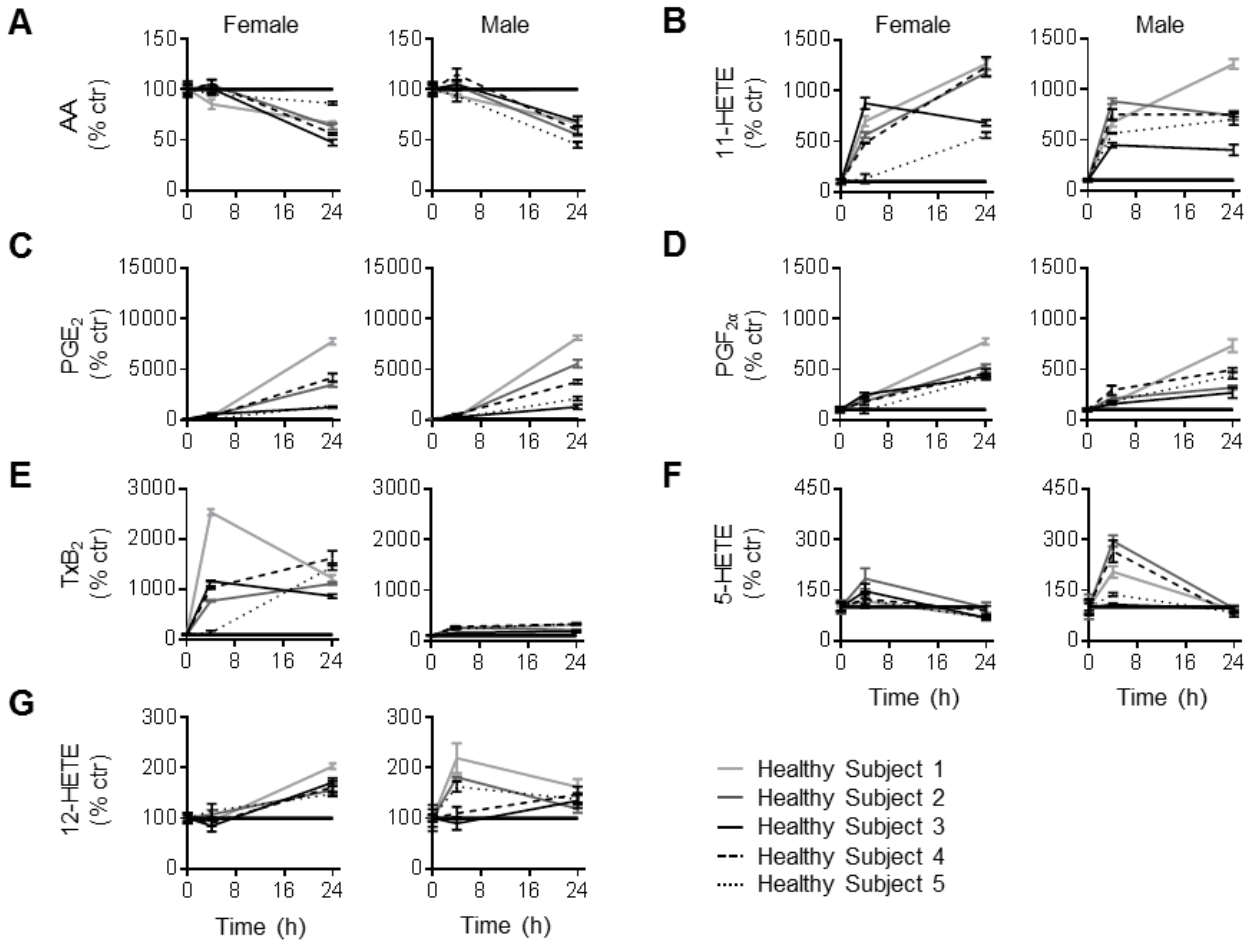


Figure 24. Differential Eicosanoid Response on Mediator Level in Healthy Subjects (n=10). Whole blood from female (n=5, left) and male (n=5, right) study subjects was activated with or without LPS (100 ng/mL) for 1, 4 and 24 hours. Metabolite release of AA (A), 11-HETE (B), PGE_2 (C), $PGF_{2\alpha}$ (D), TxB_2(E), 5-HETE (F) and 12-HETE (G) are shown. Statistical significances are given in Supplemental Table XIII and Supplemental Table XIV. Cell culture studies were performed in triplicates.

Summing up, investigation of 10 healthy subjects revealed characteristic eicosanoid profiles of COX and LOX-dependent target genes and corresponding metabolites due to an inflammatory stimulus. In general gene expression analysis over all healthy subjects revealed an upregulation for *COX-2, PGES, PGFS, FLAP*, a downregulation for *TXAS as well as 5-LOX* and no consistent trend for *PLA_2*, *COX-1* and *12-LOX*. On metabolite level AA seemed to be

downregulated, COX-dependent metabolites were upregulated and 5-HETE as well as 12-HETE revealed no consistent trend (Figure 25).

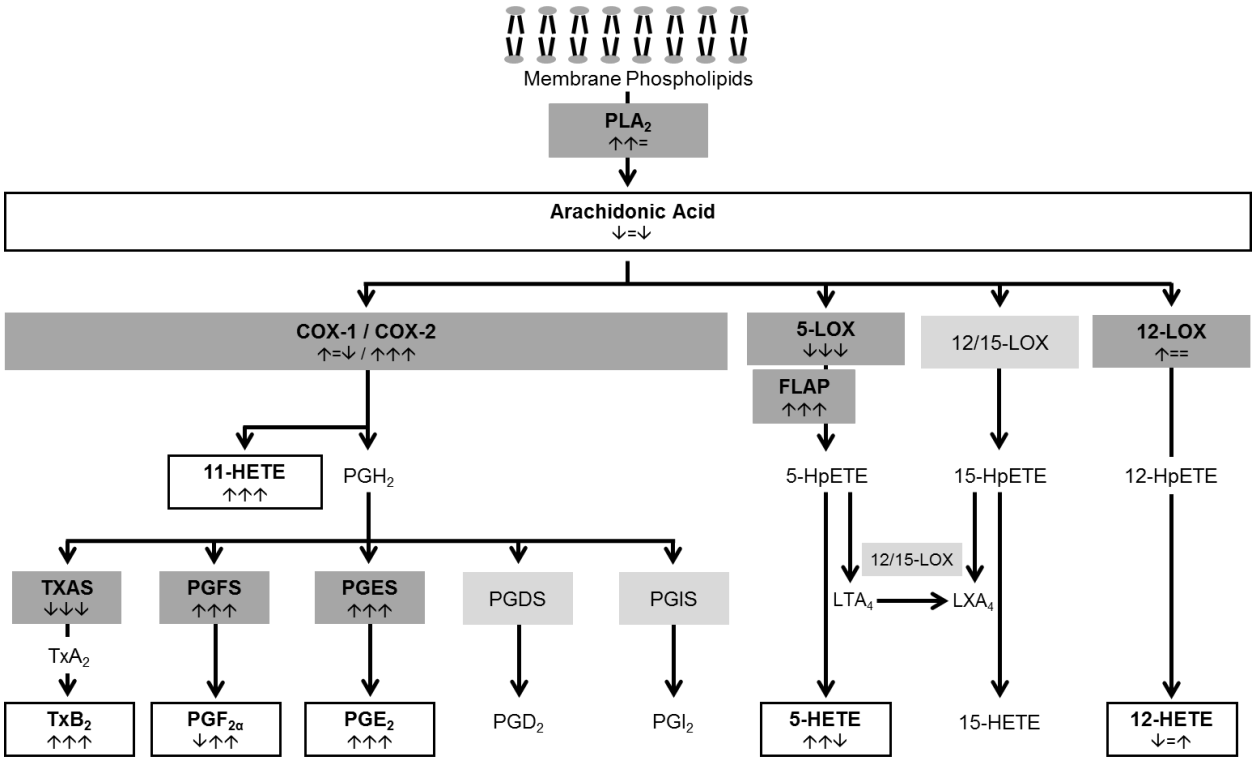


Figure 25. Eicosanoid Response on Gene Expression and Metabolite Level followed by LPS Incubation for 1, 4 and 24 Hours. Investigated enzymes on gene expression level (dark gray) and metabolites (framed) revealed significant upregulation (↑), downregulation (↓) or no LPS mediated effect (=).

Furthermore, we confirmed the previously described time dependency for all investigated pathways both on gene expression and mediator level. Since eicosanoid profiling after 1 hour LPS activation required a strict time management further clinical studies might consider solely LPS incubation for 4 and 24 hours.

In addition, strong inter-individual variabilities of target gene expression and mediator release could be found. Data suggested that those differences might also affect the susceptibility to inflammatory diseases. Therefore further studies will illuminate the eicosanoid response of patients with or without atherosclerosis as a chronic inflammatory disease.

8.5 Differential Eicosanoid Response in Patients with or without Coronary Artery Disease

Suggesting that individual regulated eicosanoid profiles determine the risk for atherosclerosis, we aimed to investigate target genes and metabolites of AA metabolism as novel biomarkers for atherosclerotic burden. The predictive role of eicosanoid response was investigated in patients with or without CAD. Standardized *in vitro* activation assays were performed using whole blood from 92 patients from the Leipzig LIFE Heart Study, which is a cross-sectional study of patients undergoing first diagnostic coronary angiography for suspected CAD.[66] In brief, whole blood was activated with LPS (100 ng/mL) and incubated for 4 and 24 hours. Analysis was performed in triplicates (Figure 26). Using quantitative PCRs, we investigated the expression of *PLA$_2$*, *COX-1*, *COX-2*, *TXAS*, *PGES*, *PGFS, 12-LOX*, *5-LOX* and *FLAP*. Analyses were performed in quadruplicates and data were normalized to µg RNA as well as relative to medium control at 4 and 24 hours to evaluate the kinetics of gene expression. We further investigated the release of AA, 4 metabolites of the COX-pathway (11-HETE, TxB_2, PGE_2, PGF_2) and LOX-derived 12-HETE and 5-HETE using LC-MS/MS. We evaluated absolute concentrations (ng/mL) and relative changes normalized to medium control.

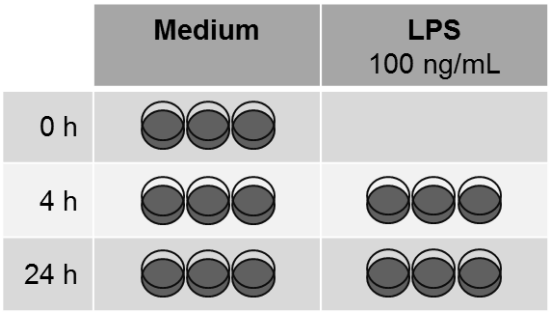


Figure 26. Investigation of Eicosanoid Response in Patients with or without CAD (n=92). 1 mL whole blood was mixed with 500 µL medium spiked with or without LPS (100 ng/mL). Mixtures were incubated for 4 and 24 hours at 37 °C and 5 % CO_2. Cell culture studies were performed in triplicates.

8.5.1 Characteristics of Study Patients

Standardized *in vitro* activation assays were performed using whole blood from 92 patients. Out of these, 40 patients did not reveal CAD, 20 patients showed wall irregularities with a luminal reduction < 50 % and 32 patients revealed angiographic confirmed stenosis ≥ 50 % in at least one major coronary artery. Patients without CAD were significantly younger than patients with CAD (*P<0.05*), showed a significantly lower intima media thickness (*P<0.01*) and higher HDL-cholesterol levels (*P<0.05*). No significant differences for gender, smoking, body mass index, waist hip ratio, for further lipid parameters such as cholesterol, LDL-cholesterol, Lipoprotein (a),

for C-reactive protein (CRP) and Troponin T were detected between the different study groups (Table 23).

Table 23. Characteristics of Study Population

Parameter	**no CAD***	**with CAD**	*with CAD*	
			CAD < 50 %†	CAD ≥ 50 %‡
Subjects, n	40	52	20	32
Age (years)§	61.5 (40.0 - 80.0)	68.0‖ (46.0 - 80.0)	68.0	67.5
Men, n (%)	25 (62.5)	40 (76.9)	15	25
Smoking, n (%)	3 (7.5)	10 (19.2)	4 (20.0)	6 (18.8)
Diabetes, n (%)	8 (20.0)	17 (32.7)	9 (45.0)	8 (25.0)
Body mass index (kg/m2)§	29.3 (21.3 - 46.9)	29.5 (23.3 - 49.4)	30.3	28.6
Waste hip ratio§	0.9 (0.7 - 1.1)	1.0 (0.8 - 1.1)	1.0	1.0
Intima media thickness§	0.7 (0.6 - 1.2)	0.8# (0.6 - 1.3)	0.8‖	0.9‖
Lp(a) (mmol/L)§	0.2 (0.1 - 1.4)	0.2 (0.1 - 1.9)	0.1	0.2
Cholesterol (mmol/L)§	5.9 (3.8 - 8.0)	5.7 (3.6 - 10.2)	5.4	5.9
LDL Cholesterol (mmol/L)§	3.6 (1.7 - 5.3)	3.4 (1.5 - 7.7)	3.3	3.5
HDL Cholesterol (mmol/L)§	1.4 (0.5 - 2.3)	1.2‖ (0.6 - 2.2)	1.2	1.2
Triglycerides (mmol/L)§	1.7 (0.8 - 12.3)	1.7 (0.7 - 8.9)	1.6	1.8
hsC-reactive Protein (mg/L)§	2.1 (0.2 - 21.7)	2.0 (0.2 - 41.2)	2.4	1.9
hsTroponin T (pg/mL)§	5.0 (5.0 - 36.7)	6.3 (5.0 - 70.9)	5.0	7.6‖

* Subjects with catheter based exclusion of coronary artery disease (CAD)

† Subjects with angiographic coronary wall irregularities < 50 % luminal reduction

‡ Subjects with angiographic stenosis ≥ 50 % luminal reduction in at least one major coronary artery

§ Data are given as median (range)

‖ *P<0.05* when compared with subjects w/o CAD

\# *P<0.01* when compared with subjects w/o CAD

8.5.2 Regulatory Genes of AA Metabolism are Differentially Expressed in Whole Blood from Patients with or without Coronary Artery Disease

We found that 3 genes (*COX-1* (Figure 27 A), *TXAS*, *5-LOX*) were downregulated and 3 genes (*COX-2*, *PGES*, *FLAP* (Figure 27 B-D)) were upregulated in cellular blood components after 4 and 24 hours activation with LPS when normalized to medium control. For PLA_2, *PGFS* and *12-LOX*, no consistent trend after LPS activation was observed (Supplemental Table XVI).

Comparing patients with or without CAD, we did not detect expression differences at baseline (Supplemental Table XVII). On the contrary, significantly 0.78- up to 0.80-fold lower mRNA expression levels were observed for *COX-2* and *PGES* after 24 hours in patients with CAD (*P<0.05*) (Figure 27 B-C; Supplemental Table XVII). For *COX-2*, this finding was also statistically significant after 4 hours with LPS activation (*P<0.05*) (Figure 27 B-C; Supplemental Table XVII), indicating a reduced inducibility of these eicosanoid pathways in patients with CAD. For *COX-1* and *FLAP*, we observed significant upregulated relative expression levels after 24 hours of LPS activation (*P<0.05*), in contrast absolute expression levels tended to be lower at 24 hours in patients with CAD (Figure 27 A; Figure 27 D; Supplemental Table XVII).

Comparing patients with different extent of stenosis (< or ≥ 50 %) separately to controls, a differential gene expression of *COX-1* could be shown for patients with < 50 % as well as with ≥ 50 % stenosis. Only patients with a < 50 % stenosis showed a significantly lower gene expression of *COX-2* and *PGES* in relation to controls, whereas *FLAP* gene expression was significantly higher in patients with ≥ 50 % stenosis.

Together, this indicated that AA-metabolizing regulatory genes, both of the COX- and LOX-pathway, were expressed at lower levels upon activation of whole blood from patients with CAD.

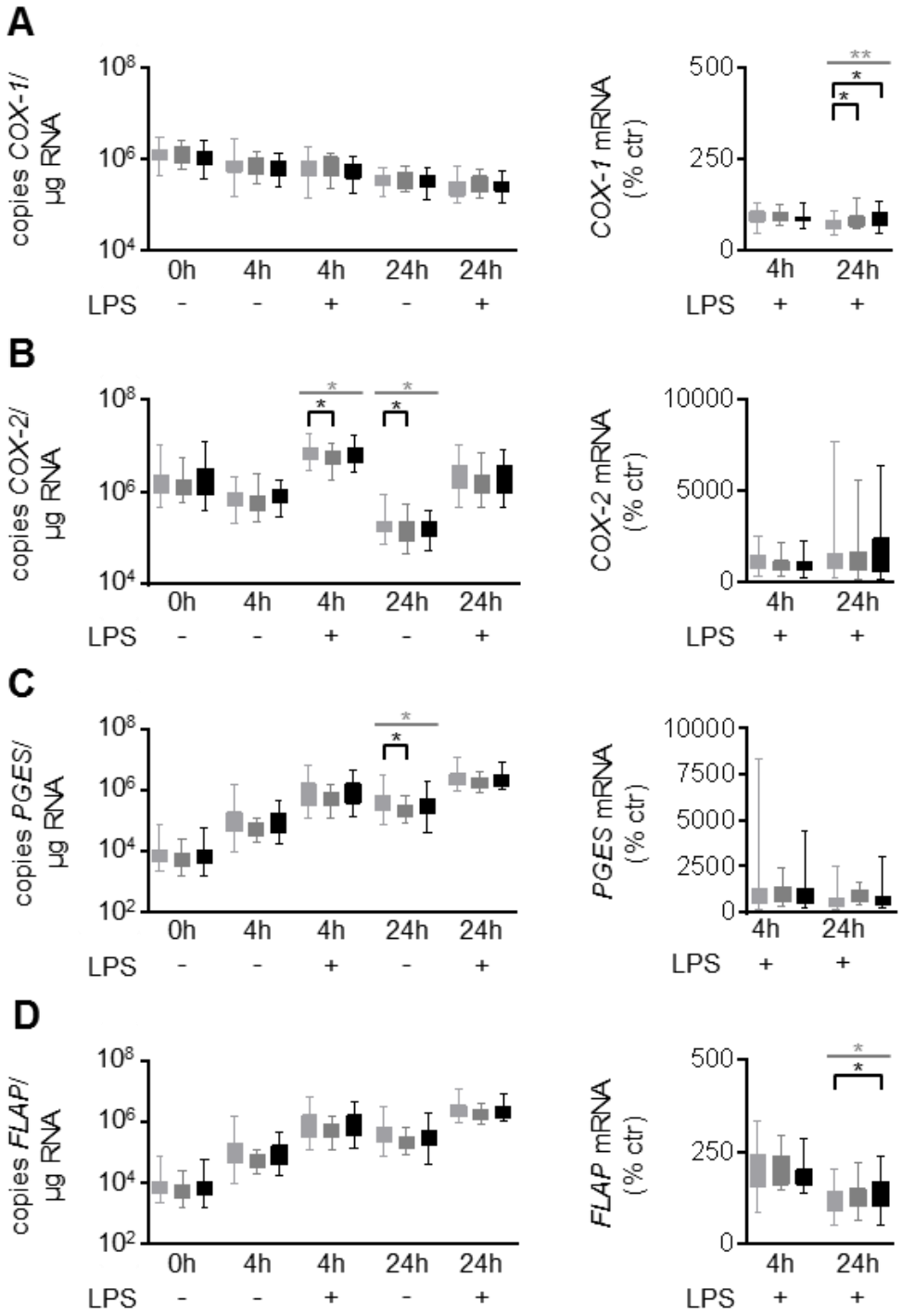


Figure 27. Differential Eicosanoid Response on Gene Expression Level in Patients with or without CAD. Whole blood from patients without CAD (n=40, light grey), with CAD < 50 % stenosis (n=20, dark grey) and with CAD ≥ 50 % stenosis (n=32, black) was activated with or without LPS (100 ng/mL) for 4 and 24 hours. mRNA expression of *COX-1* (A), *COX-2* (B), *PGES* (C) and *FLAP* (D) as differentially regulated target genes are shown. Data are shown as absolute values normalized to µg RNA (left) and as relative values normalized to medium control (right). Statistical significance for patients without CAD compared to patients with CAD is shown with grayed lines and subsequent analysis of all groups is shown with black brackets (* *P<0.05*, ** *P<0.01*). Cell culture studies were performed in triplicates, gene expression analyses were performed in quadruplicates.

8.5.3 Differential Release of AA and AA-derived Metabolites in Whole Blood from Patients with or without Coronary Artery Disease

We next investigated the release of corresponding metabolites of AA metabolism (AA, 11-HETE, TxB_2, PGE_2, PGF_2, 12-HETE, 5-HETE) using LC-MS/MS, which were recently shown to be differentially secreted in inflammatory disease.[34]

As a major finding, we detected that AA concentrations were significantly 0.74- up to 0.78-fold lower already at baseline in patients with CAD (*P<0.05*) (Figure 28 A). For all other investigated metabolites, no significant difference was observed between patients with or without CAD at this time point (Supplemental Table XIX). LPS activation led to a principal induction of AA and AA-metabolite release (Supplemental Table XVIII), but significantly lower levels of AA, 11-HETE, 12-HETE and 5-HETE were detected in whole blood supernatants of patients with CAD compared to controls (Figure 28 A-D; Supplemental Table XIX). For 5-HETE, this effect was more pronounced when evaluating the relative changes at 24 hours (Figure 28 D; Supplemental Table XIX). Comparing metabolite release of patients with < or ≥ 50 % stenosis separately to controls, the reduced release of AA was pronounced especially in patients with ≥ 50 % stenosis (Figure 28 A).

Together, this indicated a reduced release of AA as central precursor of eicosanoids and implied a reduced release of COX- and LOX-derived metabolites in patients with CAD, which was in accordance with results from gene expression analyses.

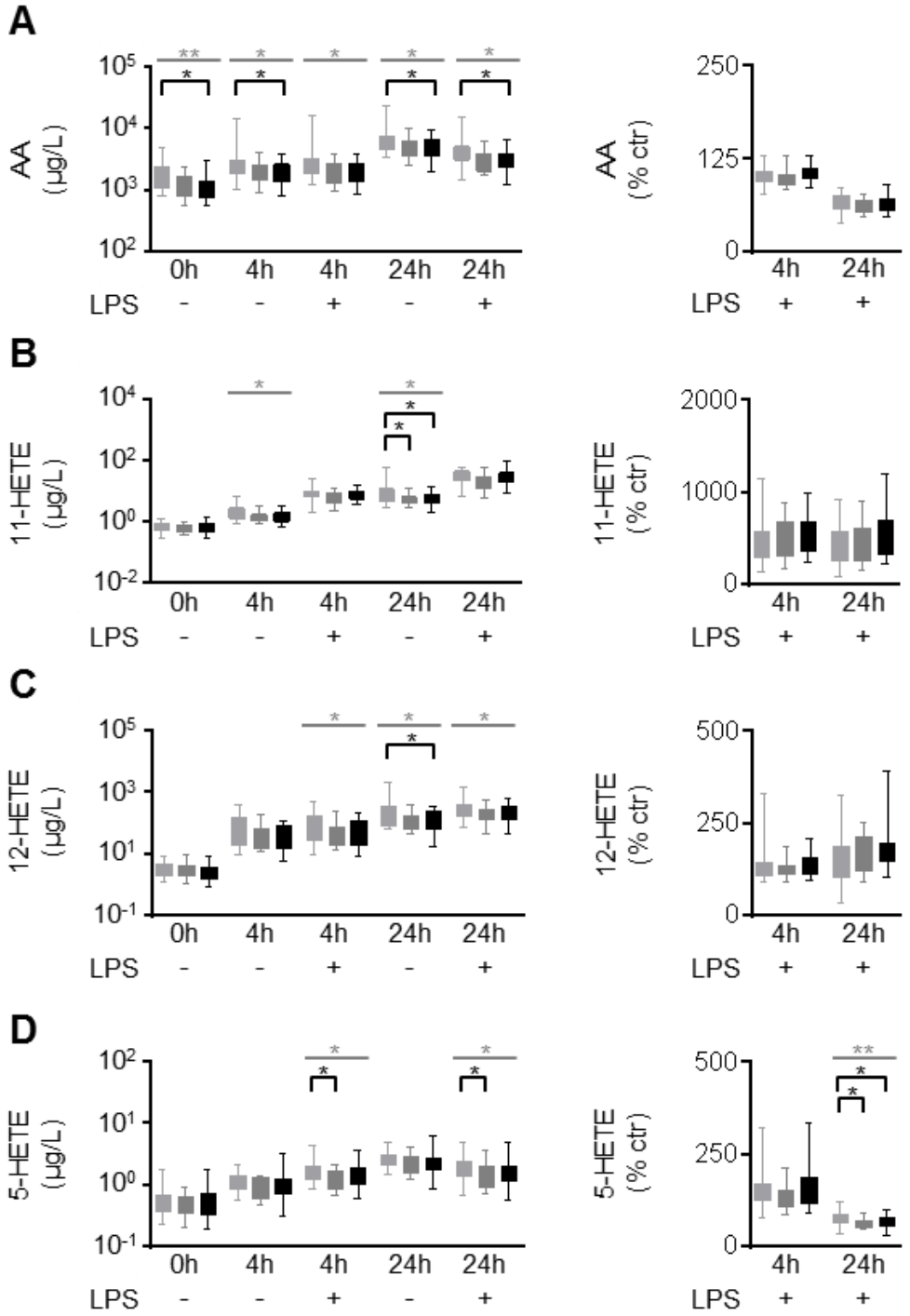


Figure 28. Differential Eicosanoid Response on Mediator Level in Patients with or without CAD. Whole blood from patients without CAD (n=40, light grey), with CAD < 50 % stenosis (n=20, dark grey) and with CAD ≥ 50 % stenosis (n=32, black) was activated with or without LPS (100 ng/mL) for 4 and 24 hours. Eicosanoid release of AA (A), 11-HETE (B), 12-HETE (C) and 5-HETE (D) as differentially regulated eicosanoids are shown. Data are shown as absolute values in µg/L (left) and as relative values normalized to medium control (right). Statistical significance for patients without CAD compared to patients with CAD is shown with grayed lines and subsequent analysis of all groups is shown with black brackets (*P<0.05, **<0.01). Cell culture studies were performed in triplicates.

8.5.4 Eicosanoids as Blood Biomarkers as Predictors of Coronary Artery Disease

We first evaluated the power of classical CAD risk factors, such as age and sex, and of IMT as non-invasive diagnostic imaging procedure in the discrimination of patients with or without CAD. In accordance with recent studies, AUCs of 67.5 for age combined with sex (Table 24) and 67.7 for IMT could be shown. As the CAD consortium scores were recently recommended by the European Society of Cardiology for evaluating pre-test probability of obstructive CAD,[80] we further investigated the discriminatory power of the CAD2 score, based on age, sex, type of chest pain, diabetes, hypertension, dyslipidaemia, and smoking, between patients with or without CAD, resulting in an AUC of 71.1 (Figure 30 B).

We subsequently investigated, if laboratory parameters, which are associated with mechanisms underlying CAD, including vascular inflammation or aberrant lipid regulation, discriminated between patients with and without CAD. As summarized in Figure 29 serum cholesterol, LDL-cholesterol, and triglyceride levels, as well as hsCRP or high sensitive Troponin T (TnT-hs) revealed limited significance in the prediction of CAD, with AUCs in the range of 50.0 to 61.0. For HDL-cholesterol, which was significantly different between cases and controls (Table 23), an AUC of 62.6 was calculated.

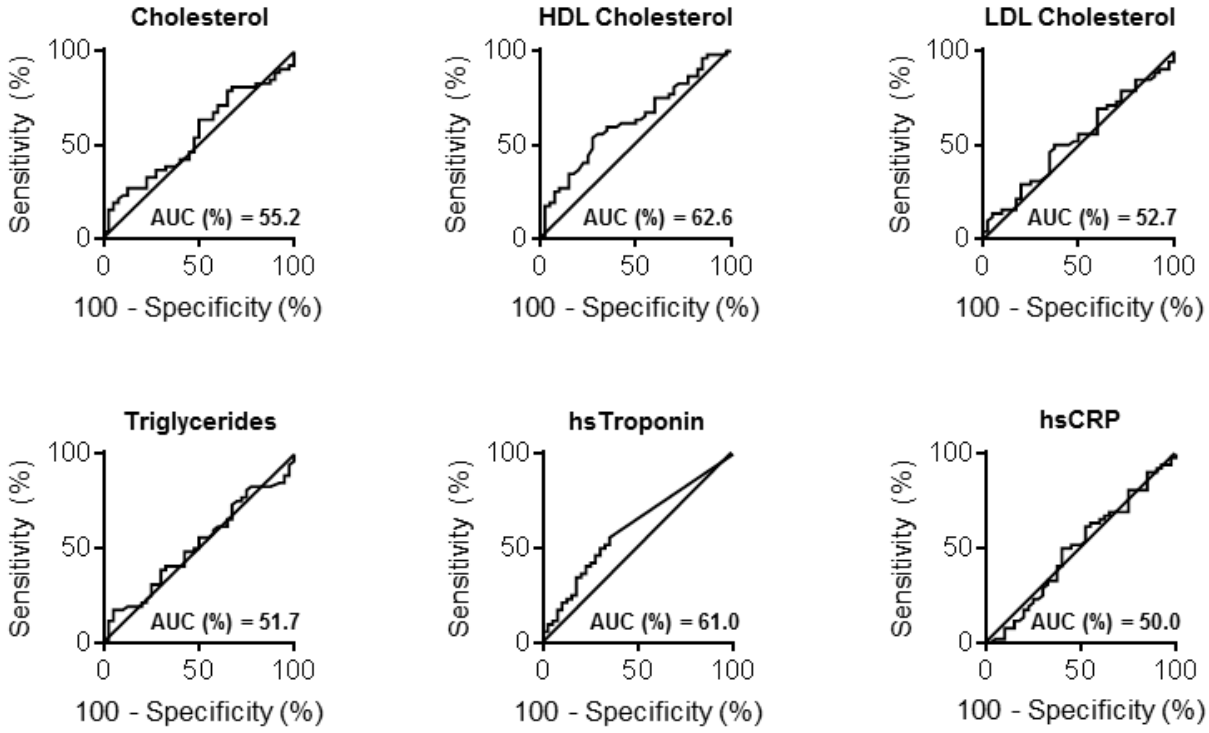


Figure 29. ROC-Analysis of Laboratory Parameters. ROC-analysis and AUCs of Cholesterol, HDL-Cholesterol, LDL-Cholesterol, Triglycerides, hs-Troponin and hs-CRP are shown.

Since mRNA expression of regulatory AA genes (*COX-1*, *COX-2*, *PGES* and *FLAP*) and AA metabolite release (AA, 5-, 11- and 12-HETE) was significantly different between patients with or without CAD, we tested their potential as biomarkers to predict the presence of CAD. Using a bootstrap analysis, we identified relative *COX-1* mRNA expression at 24 hours, absolute *COX-2* mRNA expression levels at 4 hours LPS activation and AA release at baseline (0 hours) as most relevant phenotypes of AA metabolism to differentiate between patients with or without CAD. These blood phenotypes were included into a logistic regression analysis and revealed an AUC of 79.8 (Table 24 Model 2). Addition of age and gender further improved the AUC to 83.6 (Table 24 Model 3; Figure 30 A). Combining AA blood phenotypes with the CAD2 score resulted in an AUC of 81.9 (Figure 30 C). No major differences between the calculated score for patients with < 50 % stenosis (AUC 83.7) and ≥ 50 % stenosis (AUC 83.5) compared to controls could be shown.

Table 24. Logistic Regression Models

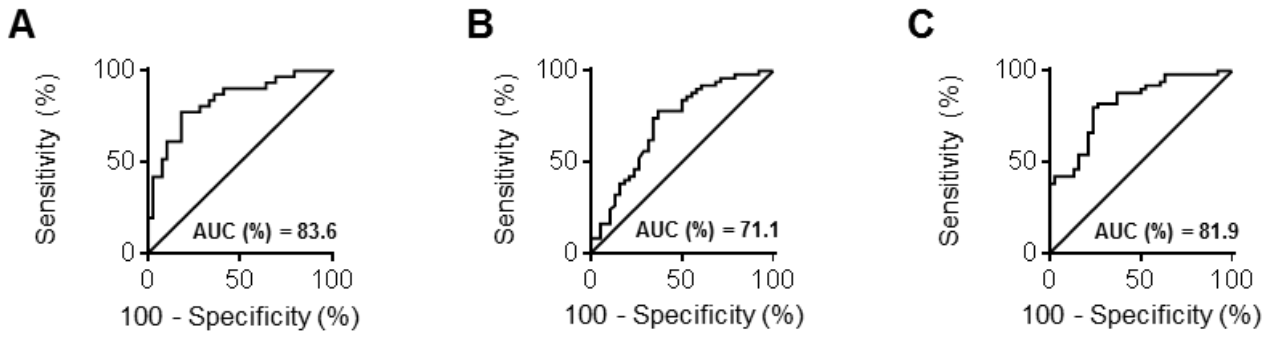
Variable	Odds Ratio*	95 % CI	*P-Value*	AUC
Model 1†				**67.5**
Age (≥ 65 vs. < 65 years)	3.45	1.4 - 8.51	*0.007*	
Sex (male vs. female)	2.61	0.98 - 7.00	*0.056*	
Model 2‡				**79.8**
AA (0h log2)	0.27	0.12 - 0.58	*0.001*	
COX-1 (24 h % ctr.)	1.04	1.01 - 1.07	*0.005*	
COX-2 (4 h % ctr.)	0.99	0.99 - 1.00	*0.055*	
Model 3§				**83.6**
Age (≥ 65 vs. < 65 years)	4.62	1.52 - 14.07	*0.007*	
Sex (male vs. female)	2.41	0.72 - 8.09	*0.156*	
AA (0h log2)	0.32	0.15 - 0.72	*0.006*	
COX-1 (24h % ctr.)	1.05	1.02 - 1.09	*0.002*	
COX-2 (4h % ctr.)	0.99	0.99 - 1.00	*0.048*	

* Odds ratios are presented per 1-unit difference

† Logistic Regression Model calculated with age and sex only, variable-specific AUC

‡ Logistic Regression Model calculated without age and sex, variable-specific AUC

§ Logistic Regression Model combining Logistic Regression Model 1 and 2



$$100 * exp\frac{(score)}{1 + exp(score)}$$

$$score = 7.8297 + (1.5309 * Age_{\geq 65}) + (0.8779 * SexMale) + (0.0514 * COX1\ 24_{h\,(\%)}) - (1.1270 * AA_{0h\,(log2)}) - (0.00111 * COX2\ 4_{h\,(\%)})$$

Figure 30. Receiver-Operator Characteristic (ROC) Curves for Patients without CAD Compared to all Patients with Angiographically Confirmed CAD. (A) Score calculated by logistic regression model. (B) Score calculated by CADII (C) ROC curve analysis for combined logistic regression model and CADII score.

Together, combining 3 whole blood biomarkers of AA metabolism with age and sex into our developed probability score allowed to predict presence of CAD with a specificity of 71.8 % and a sensitivity of 80.4 %.

9 Discussion

In the current thesis, we improved an *in vitro* whole blood activation model for the investigation of the eicosanoid response on gene expression and metabolite level using an inflammatory stimulus. Comparison of LPS, TNFα and oxLDL as activators of whole blood AA metabolism revealed most significant effects for LPS which was time- but not dose-dependent. Transcriptional and translational inhibition experiments were performed to illuminate the central molecular mechanisms of the eicosanoid pathways. Data suggested that the production of metabolites underlies *de novo* synthesis upon stimulation, which is controlled both, at the level of transcription and translation. Investigation of the eicosanoid response in healthy subjects revealed major inter-individual differences for all investigated target genes and metabolites. Results let us hypothesize that varying eicosanoid response might be predisposing towards a different susceptibility to inflammatory diseases. The predictive role of the individual eicosanoid response for the presence of atherosclerosis was examined in patients with or without coronary artery disease using the novel developed whole-blood assay. Overall, we found that the eicosanoid response on gene expression (*COX-1*, *COX-2*, *PGES* and *FLAP*) and metabolite level (AA, 5-, 11- and 12-HETE) was significantly different in patients with or without CAD. This allowed the development of a score consisting of three biomarkers of AA metabolism with an AUC of 83.6, which is superior to currently available scores of blood markers of CAD.

Eicosanoids are signaling molecules that exhibit major physiological as well as pathophysiological effects and play a central role in acute and chronic inflammatory diseases.[81,82] Nevertheless, studies investigating the different phenotypes of AA metabolism and their diagnostic potential as biomarkers for atherosclerosis are rare due to analytical difficulties of these metabolites. Immunoassays offer an opportunity to quantify the eicosanoid concentration in several body fluids e.g. plasma, serum and urine but struggled with limitations such as low sample throughput, single parametric metabolite analysis and potential cross reactions of antibodies.[28,29,31,83,84] Furthermore, studies investigating eicosanoid profiles based on gene expression and metabolite level including both COX- and LOX-dependent pathways, are rare since most studies concern metabolites only or examine parts of the eicosanoid networks.[85-88]

To overcome these limitations we have established a standardized whole blood activation assay using LPS as inflammatory stimulus that increases concentration of AA metabolites and facilitate its measurement by LC-MS/MS.[34] A previously developed method from our laboratory allowed simultaneous quantification of AA, 5-HETE, 11-HETE, 12-HETE, $PGF_{2\alpha}$, PGE_2 and TxB_2 as central mediators of AA metabolism. Beside the analysis of eicosanoid profiles on the

mediator level, this assay allowed the determination of gene expression of corresponding AA metabolizing enzymes. To this end, mRNA was isolated out of blood cells and RT-PCRs for the quantification of *COX-2* and *PGES* gene expression levels were established. The assay which was developed in this thesis additionally comprised RT-PCRs for the analysis of *PLA_2*, *COX-1*, *TXAS*, *PGFS*, *5-LOX*, *FLAP* and *12-LOX* gene expression to cover all major routes of AA metabolism and investigate the discriminatory potential of these eicosanoid pathways for inflammatory diseases.

LPS is already known as a potent inflammatory stimulus of the AA metabolism and acts by a toll-like receptor 4 (TLR4) associated nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathway.[89] However, oxLDL and TNFα are well described in the pathophysiological mechanism of atherosclerosis and might be alternatively activators for the established whole blood assay.[90,91] Therefore we examined and compared the eicosanoid response in whole blood after activation with LPS, oxLDL and TNFα. The rational for used concentrations of the stimuli was based on concentrations that have been applied to previously published cell culture experiments.[78,79,92-95] Furthermore, clinical observations on neutropenic patients with suspected gram-negative sepsis revealed, that endotoxin plasma concentration of these patients ranged from 0 to 7.7 x 10^9 pg/mL.[96] Due to the previously reported time dependency of the TxB_2, PGE_1 and PGE_2 release from human monocytes treated with LPS, we also investigated the eicosanoid response at different time points after stimulation, e.g. 1, 4 and 24 hours.[97]

As a major result we found that whole blood activation with different stimuli revealed characteristic effects on gene expression and metabolite level with a distinctive time- but no dose-dependency. In principal, *COX-2* and *PGFS* expression was upregulated, *TXAS* and *5-LOX* expression was downregulated while gene expression levels of *COX-1* were not altered. Furthermore, corresponding metabolites for both COX- and LOX-pathways showed mostly an upregulation, except for 5-HETE release after 24 hours LPS activation. The obvious effects of LPS on gene expression and metabolite levels of AA metabolism were comparable with previous studies that showed an increase of PGE_2, TxB_2 and LTB_4 release as well as *COX-2* expression after incubation of whole blood with LPS.[11] In contrast, activation with TNFα revealed only a minor response of cellular AA metabolism where solely *COX-2* expression, the corresponding metabolite 11-HETE as well as $PGF_{2\alpha}$ were significantly increased. These results were in line with previous data showing that macrophages of the human term decidua activated with TNFα revealed increased $PGF_{2\alpha}$ without alteration of any other prostaglandins.[98] Furthermore, activation of human umbilical vein endothelial cells (HUVEC) with TNFα was not associated with PGE_2 and 15-HETE, but only PGI_2 release, which is also consistent with the

minor effects on AA metabolism observed in our study.[99] Whole blood activation with oxLDL revealed a significant stimulation especially of *COX-2* mRNA expression and metabolite release of 11-HETE, $PGF_{2\alpha}$, TxB_2 and 5-HETE. On gene expression level *COX-2* mRNA expression was upregulated after 1 hour followed by a drop down after 4 and 24 hours of incubation. Findings are similar to observations of others which described that macrophages incubated with oxLDL revealed an inhibition of COX-2 protein and mRNA when co-stimulated with LPS for 4 hours.[100] With respect to the LOX pathway, Fair and colleagues investigated the effects of oxLDL on U937 cells as a model for mononuclear cells. They demonstrated that oxLDL increased the production of 15-HETE and 5-HETE on the metabolite level and raised *FLAP* transcript levels 10-fold compared to controls. *5-LOX* mRNA was not affected which is comparable to our findings in the whole blood model.[101]

All together our data confirmed the practicability of the whole blood activation assay for the investigation of the eicosanoid response on gene expression and metabolite level, whereas intensity of the effects depended upon the stimuli used. Because of deficient applicability of the standardized protocol for oxLDL preparation in clinical routine settings (estimated preparation time: 120 hours) and high batch variability as well as low effects of TNFα, LPS was used for the following experiments. Furthermore, analyses revealed a strong time- but no dose-dependent eicosanoid response on gene expression and mediator level after LPS activation for all investigated pathways. Therefore further experiments were performed with a final concentration of 100 ng/mL LPS resembling more physiological conditions at three different time points.

The AA metabolism can be understood as a network of interacting pathways with distinct upstream and downstream enzymes that contribute to a broad spectrum of metabolites.[102] The established whole blood assay allows illuminating central mechanism of the cyclooxygenase and lipoxygenase pathways on gene expression and metabolite level. Since we observed a rapid response upon stimulation we queried whether eicosanoid response on gene expression and metabolite level underlie *de novo* synthesis. To this end, actinomyin D (ActD) as an inhibitor of transcription and cycloheximide (CHX) as a translational inhibitor were used to perform functional studies to better characterize the molecular mechanism.[103,104]

Data suggested that the eicosanoid response was regulated at the transcriptional as well as translational level. For instance, incubation of whole blood with ActD led to a transcriptional inhibition of *COX-2* and *PGFS* that was not markedly overcomed by stimulation with LPS. Inhibition could be also shown for corresponding metabolites (11-HETE and $PGF_{2\alpha}$), suggesting that the eicosanoid release is dependent on *de novo* mRNA transcription of the respective enzymes, which is lightly controlled by inflammatory stimuli. In line with this, translational inhibition also revealed downregulation of eicosanoid metabolite release, indicating a short half-life time of these enzymes. Data were in line with the results of Nüsing et al. that supposed

enhanced *de novo* synthesis of COX when human monocytes were incubated with LPS and calculated half-life of 3.2 h for COX-2 enzyme.[105]
In contrast, the strong involvement of the translational level was assumed since we found increased TxB_2 and 5-HETE metabolite release while *TXAS* and *5-LOX* gene expression decreased. Results were in accordance to the experiments of Nüsing and colleagues that found no LPS effect on the biosynthesis of TXAS in human monocytes but determined a half-life of 28 hours for cellular TXAS activity.[105] In summary, eicosanoid response seemed to be regulated on all levels, thus further experiments should consider both, data of gene expression and mediator release, equally.

Another finding of this study was the high inter-individual variability of eicosanoid response in healthy subjects, both on gene expression and metabolite level. In general, gene expression upon LPS activation for *COX-2*, *PGES*, *PGFS* and *FLAP* was upregulated, revealed a downregulation for *TXAS* as well as *5-LOX* and showed no consistent trend for *PLA_2, COX-1 and 12-LOX.* On metabolite level, AA release was significant downregulated, COX metabolites were upregulated and LOX metabolites revealed no consistent trend.
The coefficients of variation (CV) ranged between 15.06 % (*PGFS)* up to 100.04 % (*PGES)* for analyzed target genes. Differences of metabolite release could be found with a coefficient of variation from 8.23 % (AA) up to 110.63 % (TxB_2). Data are consistent with results that described reproducible inter-individual variations of PGE_2 release by endotoxin-stimulated human monocytes.[106] Also methodical publications of other LC-MS/MS methods for the quantification of AA and related metabolites reported a wide range of eicosanoid concentrations found in plasma of healthy subjects.[107,108] Yasumoto and colleagues postulated that variations of COX, LOX and CYP metabolites were caused by the platelet activation ability of each subject and inter-individual differences might be related to the stimulation of vascular endothelia cells and white blood cell *in vivo*.[109] When analyzing the CV separately for male and female subjects, we found decreased CVs for *COX-1*, *PGES* and *FLAP* as well as 11-HETE, TxB_2, $PGF_{2\alpha}$ and 12-HETE. In line with this, we found significant gender differences of *PGFS* and TX*AS* gene expression levels as well as TxB_2, 5-HETE, 11-HETE and 12-HETE release between male and female subjects. Therefore analysis of these pathways should consider gender as cofunding. The other investigated pathways revealed no significant gender effects. Published data support these findings, e.g. Hennam and colleagues also found no different $PGF_{2\alpha}$ plasma concentration between men and women, while Suzuki et al showed sex specific differences of urinary 12-HETE levels.[110,111] Further studies described increased serum TxB_2 production in healthy males compared to females which is congruent to our results.[112]
Based on the hypothesis that inter-individual regulated eicosanoid response on gene expression and mediator level may predispose to different susceptibility to inflammatory

diseases, we investigated, if the AA metabolism may be used for the prediction of stable CAD in patients of the Leipzig LIFE Heart Study.

Our study indicated that patients with CAD were characterized by significant differences in their humoral eicosanoid response of *COX-1*, *COX-2*, *PGES* and *FLAP* expression and AA, 11-HETE, 5-HETE and 12-HETE release when compared to patients without CAD. CAD patients showed a suppression of COX and LOX pathways both on gene expression and mediator level in our whole blood activation assay. Combining data from relative *COX-1* mRNA expression at 24 hours, relative *COX-2* mRNA expression levels at 4 hours LPS activation and AA release at baseline (0 hours) as well as classical CAD risk factors such as age and sex led to the development of a novel probability score with an AUC of 83.6.

Pretest probability models such as the Diamond and Forrester or the Duke Clinical Score were recently described and combined age, sex and type of chest pain (Diamond and Forrester Score, AUC 0.64) as well as electrocardiographic findings and common atherosclerotic risk factor such as smoking, cholesterol and diabetes (Duke Clinical Score, AUC 0.72) for the prediction of CAD.[68] Furthermore, Genders and colleagues developed the Consortium [CAD prediction] Scores with an extended model, where the inclusion of the coronary calcium score, that measures the amount of calcium in the walls of the arteries using a special computed tomography (CT) of the heart, increased the c statistic to 88.0.[70] With respect to laboratory markers studies showed, that lipid and inflammatory parameters alone could be used for CAD prediction with an AUC up to 0.80.[73] In line with this, the EVINCI study revealed that addition of high-density-lipoprotein (HDL) cholesterol, aminotransferase (AST) and high sensitive c-reactive protein (hsCRP) improved the prediction of Gender´s Clinical Score.[72]

Overall, we found that the novelly developed score consisting of three biomarkers of AA metabolism determined using an *in vitro* whole blood activation model revealed the best performance in the prediction of CAD compared to all other studies. Data of the current study were raised from a deeply phenotyped study cohort using a highly standardized procedure for analysis. However, diagnostic value of this pretest score needs to be evaluated in larger, independent study of patients with atherosclerosis in different vascular beds. Furthermore, the current protocol of our *in vitro* model is technically challenging and time-intense, which limits the application in large clinical trials. Thus, the whole blood activation model is currently under modification for prospective widespread clinical application.

In summary, this thesis contributes to a better understanding of the AA metabolism and its regulation on gene expression and metabolite level. Data indicated a high inter-individual eicosanoid response in healthy subjects and major differences in the humoral eicosanoid response of patients with or without CAD. Thus, the implementation of eicosanoids as

biomarkers for inflammatory diseases seems to be a promising approach for the prediction of CAD.

10 References

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11 Supplement

Supplemental Table I. Kinetic Expression of Target Genes after Activation of Whole Blood with LPS, oxLDL or TNFα (n=3)

***COX-1* Expression Relative to Medium Control (% Ctr)**				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		84.29	68.44 - 112.02	*0.454*
4h	LPS	94.69	61.21 - 133.35	*0.879*
24h		79.18	65.29 - 110.37	*0.376*
1h		75.29	65.92 - 147.49	*0.897*
4h	oxLDL	114.84	51.49 - 118.90	*0.842*
24h		79.73	75.00 - 105.66	*0.300*
1h		63.24	58.76 - 99.66	*0.181*
4h	TNFα	119.86	65.67 - 122.44	*0.899*
24h		126.18	119.31 - 135.82	*< 0.05*
***COX-2* Expression Relative to Medium Control (% Ctr)**				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		1811.50	996.40 - 2085.62	*< 0.05*
4h	LPS	537.67	526.47 - 744.88	*< 0.05*
24h		1278.70	1163.71 - 1972.87	*< 0.05*
1h		2239.30	1306.66 - 2579.98	*< 0.05*
4h	oxLDL	324.87	84.52 - 399.06	*0.216*
24h		75.69	62.37 - 83.93	*0.054*
1h		842.76	790.05 - 1337.82	*< 0.05*
4h	TNFα	49.17	31.52 - 73.15	*0.056*
24h		57.25	29.45 - 69.53	*0.056*
***PGFS* Expression Relative to Medium Control (% Ctr)**				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		64.12	46.47 - 81.08	*0.069*
4h	LPS	189.10	184.61 - 223.24	*< 0.05*
24h		121.40	109.67 - 139.21	*0.112*
1h		110.92	76.89 - 171.93	*0.548*
4h	oxLDL	152.71	82.35 - 177.72	*0.319*
24h		126.78	108.12 - 137.65	*0.107*
1h		130.19	63.50 - 135.93	*0.712*
4h	TNFα	183.25	91.53 - 223.39	*0.233*
24h		128.73	112.58 - 130.78	*0.053*
***TXAS* Expression Relative to Medium Control (% Ctr)**				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		106.31	48.59 - 152.19	*0.944*
4h	LPS	32.37	27.93 - 73.80	*0.063*
24h		29.60	17.82 - 53.38	*< 0.05*
1h		95.79	72.55 - 137.13	*0.932*
4h	oxLDL	51.70	17.24 - 79.68	*0.108*
24h		71.11	32.91 - 78.25	*0.108*

***TXAS* Expression Relative to Medium Control (% Ctr)**				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		87.47	42.90 - 142.57	*0.784*
4h	TNFα	52.52	16.66 - 81.37	*0.117*
24h		65.47	32.48 - 67.19	*0.058*
***5-LOX* Expression Relative to Medium Control (% Ctr)**				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		53.75	48.97 - 93.60	*0.135*
4h	LPS	55.49	48.35 - 66.07	*< 0.05*
24h		25.25	19.23 - 34.81	*< 0.01*
1h		129.19	106.44 - 150.39	*0.152*
4h	oxLDL	119.96	42.78 - 133.42	*0.968*
24h		94.08	83.33 - 109.16	*0.611*
1h		97.66	80.84 - 127.73	*0.894*
4h	TNFα	165.58	80.83 - 225.56	*0.305*
24h		125.75	105.38 - 132.21	*0.121*

Supplemental Table II.Quantitative Target Gene Analysis of Whole Blood Activation with LPS, oxLDL or TNFα (n=3)

COX-1 (x10³ copies/µg RNA)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	2374.84	1713.84 - 3167.31	-
1h		2915.24	2789.43 - 4741.62	*0.062*
4h		2383.94	1066.88 - 3799.73	*0.765*
24h		941.86	874.64 - 971.70	*< 0.05*
1h	LPS	3265.71	1908.94 - 3996.68	*0.080*
4h		2257.33	1422.71 - 2325.71	*0.123*
24h		769.37	571.06 - 1039.54	*< 0.001*
1h	oxLDL	3125.49	2100.25 - 4299.78	*0.298*
4h		1956.34	1225.19 - 2834.40	*0.468*
24h		728.75	697.33 - 995.14	*< 0.01*
1h	TNFα	2905.41	1639.11 - 2998.66	*0.687*
4h		2495.07	1278.74 - 2918.79	*0.660*
24h		1159.34	1103.64 - 1279.25	*< 0.05*

COX-2 (x10³ copies/µg RNA)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	1390.14	977.88 - 1972.70	-
1h		794.75	447.38 - 1353.48	*< 0.05*
4h		1864.53	1381.77 - 2319.60	*0.368*
24h		329.81	304.72 - 394.89	*< 0.01*
1h	LPS	9330.69	7918.91 - 24518.17	*0.052*
4h		10292.59	10025.10 - 12211.99	*< 0.05*
24h		3896.40	3838.06 - 7790.70	*< 0.05*
1h	oxLDL	11542.41	10384.74 - 30308.44	*< 0.05*
4h		4488.93	1960.56 - 7440.69	*0.290*
24h		249.63	246.28 - 255.76	*< 0.01*
1h	TNFα	6278.96	5985.18 - 11406.56	*< 0.05*
4h		916.71	731.13 - 1010.76	*0.134*
24h		211.87	97.13 - 226.08	*< 0.01*

PGFS (x10³ copies/µg RNA)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	564.44	266.44 - 999.92	-
1h		672.06	281.25 - 703.30	*0.918*
4h		220.25	183.21 - 510.77	*< 0.05*
24h		226.25	165.62 - 295.23	*< 0.05*
1h	LPS	312.30	180.35 - 570.26	*< 0.01*
4h		491.69	338.21 - 965.88	*0.796*
24h		274.67	181.64 - 410.99	*< 0.05*
1h	oxLDL	516.77	311.96 - 1209.17	*0.397*
4h		336.33	325.60 - 420.61	*0.406*
24h		244.63	227.99 - 374.29	*0.099*
1h	TNFα	426.77	382.30 - 915.65	*0.878*
4h		409.28	403.61 - 467.50	*0.799*
24h		295.90	213.20 - 332.37	*0.081*

TXAS (x10³ copies/µg RNA)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	685.32	221.19 - 925.47	-
1h		428.39	114.00 - 520.51	*< 0.05*
4h		318.52	308.65 - 787.44	*0.755*
24h		295.10	231.00 - 1061.22	*0.923*
1h	LPS	252.91	173.49 - 455.41	*0.067*
4h		219.91	99.90 - 235.06	*< 0.01*
24h		123.30	87.35 - 189.06	*< 0.05*
1h	oxLDL	377.65	156.33 - 410.35	*< 0.05*
4h		164.69	135.78 - 245.94	*0.245*
24h		209.85	180.75 - 349.20	*0.176*
1h	TNFα	223.27	162.53 - 374.73	*0.055*
4h		167.28	131.20 - 251.14	*0.259*
24h		193.21	155.21 - 344.65	*0.140*
5-LOX (x10³ copies/µg RNA)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	5596.75	3372.51 - 6352.43	-
1h		3208.77	1695.42 - 4224.71	*< 0.05*
4h		4336.56	3778.93 - 10122.48	*0.564*
24h		2864.37	2853.72 - 3544.31	*0.089*
1h	LPS	1724.74	1586.92 - 2069.01	*< 0.01*
4h		2496.65	2096.81 - 5617.01	*0.111*
24h		894.79	548.74 - 997.01	*< 0.001*
1h	oxLDL	4496.80	2190.35 - 4825.62	*< 0.05*
4h		4533.22	4330.46 - 5785.98	*0.851*
24h		3126.65	2378.04 - 3334.57	*< 0.05*
1h	TNFα	3133.70	2165.53 - 3415.12	*< 0.05*
4h		8181.71	6256.99 - 9781.36	*0.309*
24h		3601.92	3007.17 - 4685.91	*0.144*

Supplemental Table III. Kinetic Metabolite Release after Activation of Whole Blood with LPS, oxLDL or TNFα (n=3)

11-HETE Release Relative to Medium Control (% Ctr)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		105.68	86.60 - 125.96	*0.646*
4h	LPS	468.65	338.98 - 474.17	*< 0.05*
24h		459.05	339.34 - 483.54	*< 0.05*
1h		1791.11	1032.33 - 1932.22	*< 0.05*
4h	oxLDL	1358.62	1190.84 - 1420.22	*< 0.01*
24h		852.89	686.06 - 1301.03	*< 0.05*
1h		96.39	69.42 - 112.54	*0.624*
4h	TNFα	107.26	100.75 - 135.06	*0.306*
24h		114.44	111.40 - 121.26	*< 0.05*
$PGF_{2\alpha}$ Release Relative to Medium Control (% Ctr)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		95.90	90.67 - 99.77	*0.226*
4h	LPS	128.92	123.20 - 175.54	*0.124*
24h		122.52	122.29 - 133.69	*< 0.05*
1h		280.19	201.72 - 306.78	*< 0.05*
4h	oxLDL	309.50	278.08 - 431.11	*< 0.05*
24h		340.41	275.45 - 341.67	*< 0.01*
1h		96.43	89.50 - 103.01	*0.444*
4h	TNFα	140.18	131.12 - 160.23	*< 0.05*
24h		109.54	100.64 - 115.24	*0.184*
TxB_2 Release Relative to Medium Control (% Ctr)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		134.81	114.52 - 147.35	*0.078*
4h	LPS	1083.26	461.01 - 1781.35	*0.118*
24h		690.17	308.53 - 847.38	*0.084*
1h		194.69	142.75 - 297.99	*0.134*
4h	oxLDL	206.19	187.13 - 260.92	*< 0.05*
24h		270.23	217.08 - 318.95	*< 0.05*
1h		112.39	89.31 - 117.72	*0.535*
4h	TNFα	117.25	62.63 - 143.60	*0.774*
24h		118.87	79.86 - 130.31	*0.591*
5-HETE Release Relative to Medium Control (% Ctr)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
1h		134.92	120.99 - 200.00	*0.166*
4h	LPS	133.60	129.53 - 141.64	*< 0.05*
24h		79.16	63.30 - 79.36	*< 0.05*
1h		1091.95	818.33 - 1395.65	*< 0.05*
4h	oxLDL	1573.09	920.52 - 1799.79	*< 0.05*
24h		1024.90	614.44 - 1117.39	*< 0.05*
1h		119.46	110.51 - 153.45	*0.167*
4h	TNFα	108.16	81.16 - 121.99	*0.783*
24h		85.46	84.47 - 103.14	*0.277*

Supplemental Table IV. Quantitative Metabolite Analysis after Whole Blood Activation with LPS, oxLDL or TNFα (n=3)

11-HETE Release (ng/mL)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	0.67	0.55 - 0.70	-
1h		0.60	0.57 - 1.06	*0.552*
4h		1.07	0.93 - 1.16	*< 0.01*
24h		3.03	2.66 - 3.48	*< 0.05*
1h	LPS	0.75	0.60 - 0.92	*0.363*
4h		5.01	3.15 - 5.49	*< 0.05*
24h		12.85	10.27 - 15.98	*< 0.01*
1h	oxLDL	10.94	10.68 - 10.98	*< 0.01*
4h		13.78	12.63 - 15.18	*< 0.01*
24h		25.80	23.88 - 34.58	*< 0.05*
1h	TNFα	0.64	0.57 - 0.74	*0.758*
4h		1.24	1.08 - 1.26	*0.054*
24h		3.37	3.22 - 3.98	*< 0.05*
$PGF_{2\alpha}$ Release (ng/mL)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	2.02	1.96 - 2.03	-
1h		1.83	1.77 - 2.04	*0.219*
4h		1.78	1.44 - 1.96	*0.171*
24h		2.10	2.10 - 2.35	*0.131*
1h	LPS	1.83	1.70 - 1.85	*< 0.05*
4h		2.41	2.29 - 2.52	*< 0.05*
24h		2.81	2.57 - 2.88	*< 0.01*
1h	oxLDL	5.13	4.11 - 5.43	*< 0.05*
4h		5.50	5.44 - 6.20	*< 0.01*
24h		7.14	6.48 - 7.18	*< 0.01*
1h	TNFα	1.82	1.71 - 1.89	*< 0.05*
4h		2.49	2.30 - 2.57	*< 0.05*
24h		2.37	2.30 - 2.42	*< 0.01*
TxB_2 Release (ng/mL)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	0.79	0.59 - 1.23	-
1h		1.13	0.52 - 1.64	*0.320*
4h		1.18	0.72 - 1.74	*< 0.05*
24h		0.69	0.60 - 0.90	*0.648*
1h	LPS	1.67	0.60 - 2.21	*0.186*
4h		12.75	8.01 - 12.75	*0.089*
24h		4.16	2.14 - 7.59	*0.157*
1h	oxLDL	2.20	1.56 - 2.34	*< 0.05*
4h		2.20	1.87 - 3.58	*< 0.01*
24h		1.92	1.87 - 1.95	*0.106*
1h	TNFα	1.27	0.62 - 1.46	*0.234*
4h		1.38	0.45 - 2.50	*0.314*
24h		0.72	0.72 - 0.90	*0.769*

5-HETE Release (ng/mL)				
Time	**Stimuli**	**Median**	**Range**	***P-Value***
0h	Medium	0.29	0.24 - 0.43	-
1h		0.46	0.33 - 0.62	*0.148*
4h		0.64	0.47 - 0.99	*0.060*
24h		1.15	1.00 - 1.84	*< 0.05*
1h	LPS	0.67	0.62 - 0.75	*< 0.05*
4h		0.91	0.63 - 1.29	*0.051*
24h		0.80	0.73 - 1.45	*< 0.01*
1h	oxLDL	4.99	4.65 - 5.07	*< 0.05*
4h		9.15	8.45 - 10.12	*< 0.05*
24h		11.28	10.29 - 12.85	*< 0.05*
1h	TNFα	0.51	0.50 - 0.74	*< 0.05*
4h		0.70	0.57 - 0.81	*0.059*
24h		1.04	0.98 - 1.55	*< 0.01*

Supplemental Table V. Dose-Dependency of Eicosanoid Response on Gene Expression Level (n=3, pool)

COX-1 (x10³ copies/µg RNA)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	1317.94	-	-	-
1h	1560.65	1959.06	2541.74	1970.82
4h	1110.78	910.99	641.17	1067.68
24h	1315.50	413.01	506.12	377.77
Gene Expression Level normalized to Medium Control (%)				
1h	-	125.53	162.86	126.28
4h	-	82.01	57.72	96.12
24h	-	31.40	38.47	28.72
COX-2 (x10³ copies/µg RNA)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	11336.26	-	-	-
1h	4421.39	36214.51	39424.23	33717.51
4h	2637.93	10138.63	8633.56	10487.62
24h	622.00	12477.74	15206.19	11923.34
Gene Expression Level normalized to Medium Control (%)				
1h	-	819.07	891.67	762.60
4h	-	384.34	327.29	397.57
24h	-	2006.08	2444.73	1916.94
PGFS (x10³ copies/µg RNA)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	220.44	-	-	-
1h	387.03	356.00	353.66	370.68
4h	375.10	340.58	248.58	349.79
24h	261.82	205.46	324.02	250.05
Gene Expression Level normalized to Medium Control (%)				
1h	-	91.98	91.38	95.77
4h	-	90.80	66.27	93.25
24h	-	78.48	123.76	95.51
TXAS (x10³ copies/µg RNA)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	468.49	-	-	-
1h	837.74	522.05	765.31	583.94
4h	511.82	93.69	55.41	75.08
24h	316.05	79.46	135.21	74.81
Gene Expression Level normalized to Medium Control (%)				
1h	-	62.32	91.35	69.70
4h	-	18.30	10.83	14.67
24h	-	25.14	42.78	23.67

5-LOX (x10^3 copies/µg RNA)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	9075.87	-	-	-
1h	9147.79	6828.73	6364.68	6140.50
4h	8049.21	2752.87	2156.47	3205.26
24h	2881.42	902.45	1449.37	849.42
Gene Expression Level normalized to Medium Control (%)				
1h	-	74.65	69.58	67.13
4h	-	34.20	26.79	39.82
24h	-	31.32	50.30	29.48

* Data are given as Mean

Supplemental Table VI. Dose-Dependency of Eicosanoid Response on Metabolite Level (n=3, pool)

11-HETE (ng/mL)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	0.51	-	-	-
1h	0.75	1.22	0.86	0.94
4h	1.18	6.68	5.54	6.10
24h	4.29	34.30	30.70	31.50
Metabolite Release normalized to Medium Control (%)				
1h	-	162.56	114.59	125.45
4h	-	568.09	471.06	519.15
24h	-	799.53	715.62	734.27
$PGF_{2\alpha}$ (ng/mL)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	0.34	-	-	-
1h	0.38	0.45	0.26	0.47
4h	0.28	0.64	0.49	0.39
24h	0.26	2.26	1.72	1.63
Metabolite Release normalized to Medium Control (%)				
1h	-	116.41	68.88	122.27
4h	-	230.27	175.32	141.98
24h	-	862.60	656.49	622.14
TxB_2 (ng/mL)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	1.54	-	-	-
1h	1.72	2.63	2.21	1.80
4h	1.93	16.55	15.80	17.05
24h	1.29	7.99	7.85	8.01
Metabolite Release normalized to Medium Control (%)				
1h	-	153.35	128.86	104.96
4h	-	857.51	818.65	883.42
24h	-	618.99	608.53	620.93
5-HETE (ng/mL)				
Time	**Medium***	**LPS (100 ng/mL)***	**LPS (300 ng/mL)***	**LPS (1 µg/mL)***
0h	0.50	-	-	-
1h	0.58	1.34	0.96	0.88
4h	0.80	1.46	0.97	0.89
24h	1.45	1.12	0.91	0.96
Metabolite Release normalized to Medium Control (%)				
1h	-	231.37	166.55	152.43
4h	-	182.10	121.34	111.51
24h	-	77.54	62.77	66.33

* Data are given as Mean

Supplemental Table VII. Eicosanoid Response on Gene Expression Level after Transcriptional Inhibition (n=3, pool)

COX-1 (x10³ copies/µg RNA)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	2161.93	-	-	-
1h	2017.10	2115.81	1798.04	2160.77
4h	1286.41	1856.22	1033.60	898.21
24h	383.33	301.26	811.45	547.02
Gene Expression normalized to Medium or ActD Control (%)				
1h	-	104.89	-	120.17
4h	-	144.29	-	86.90
24h	-	78.59	-	67.41
COX-2 (x10³ copies/µg RNA)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	7246.62	-	-	-
1h	3182.22	36131.97	2512.14	7916.03
4h	375.94	4515.48	866.25	1427.98
24h	81.96	4099.00	5.74	3.65
Gene Expression normalized to Medium or ActD Control (%)				
1h	-	1135.43	-	315.11
4h	-	1201.10	-	164.85
24h	-	5001.14	-	63.55
PGFS (x10³ copies/µg RNA)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	549.18	-	-	-
1h	511.48	633.21	207.75	261.76
4h	316.34	618.91	55.24	60.14
24h	159.59	288.04	1.80	1.49
Gene Expression normalized to Medium or ActD Control (%)				
1h	-	123.80	-	126.00
4h	-	195.65	-	108.86
24h	-	180.48	-	82.97
TXAS (x10³ copies/µg RNA)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	509.48	-	-	-
1h	606.12	704.95	292.33	390.83
4h	240.59	124.57	53.46	42.87
24h	214.49	44.13	2.68	1.46
Gene Expression normalized to Medium or ActD Control (%)				
1h	-	116.31	-	133.69
4h	-	51.78	-	80.20
24h	-	20.57	-	54.64

5-LOX (x10^3 copies/µg RNA)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	13489.10	-	-	-
1h	9400.01	8558.72	7912.81	10804.10
4h	4455.34	6388.93	3022.26	2323.61
24h	3677.47	1372.12	107.99	79.40
Gene Expression normalized to Medium or ActD Control (%)				
1h	-	91.05	-	136.54
4h	-	143.40	-	76.88
24h	-	37.31	-	73.53

* Data are given as Mean

Supplemental Table VIII. Eicosanoid Response on Metabolite Level after Transcriptional Inhibition (n=3, pool)

11-HETE (ng/mL)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	0.29	-	-	-
1h	0.47	0.54	0.89	0.81
4h	0.75	3.62	1.35	1.14
24h	2.66	17.30	3.53	3.20
Metabolite Release normalized to Medium or ActD Control (%)				
1h	-	114.38	-	91.07
4h	-	481.04	-	84.63
24h	-	650.38	-	90.64
$PGF_{2\alpha}$ (ng/mL)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	0.31	-	-	-
1h	0.26	0.29	0.18	0.36
4h	0.36	0.51	0.37	0.34
24h	0.48	1.54	0.42	0.46
Metabolite Release normalized to Medium or ActD Control (%)				
1h	-	108.75	-	197.56
4h	-	141.34	-	91.20
24h	-	323.53	-	109.60
TxB_2 (ng/mL)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	0.58	-	-	-
1h	0.75	0.88	0.69	0.67
4h	0.99	4.61	0.80	0.68
24h	0.51	3.81	0.31	0.30
Metabolite Release normalized to Medium or ActD Control (%)				
1h	-	117.29	-	97.46
4h	-	464.02	-	85.00
24h	-	741.97	-	96.01
5-HETE (ng/mL)				
Time	**Medium***	**LPS***	**ActD***	**ActD + LPS***
0h	0.40	-	-	-
1h	0.41	0.50	0.95	1.11
4h	0.66	0.67	0.92	0.73
24h	0.79	0.35	1.21	1.01
Metabolite Release normalized to Medium or ActD Control (%)				
1h	-	120.00	-	116.90
4h	-	100.38	-	80.14
24h	-	44.18	-	84.11

* Data are given as Mean

Supplemental Table IX. Eicosanoid Response on Metabolite Level after Translational Inhibition (n=3, pool)

11-HETE (ng/mL)				
Time	**Medium***	**LPS***	**CHX***	**CHX + LPS***
0h	0.29	-	-	-
1h	0.47	0.54	0.58	0.51
4h	0.75	3.62	0.88	0.84
24h	2.66	17.30	4.07	2.27
Metabolite Release normalized to Medium Control (%)				
1h	-	114.38	-	87.92
4h	-	481.04	-	95.78
24h	-	650.38	-	55.77
$PGF_{2\alpha}$ (ng/mL)				
Time	**Medium***	**LPS***	**CHX***	**CHX + LPS***
0h	0.31	-	-	-
1h	0.26	0.29	0.24	0.37
4h	0.36	0.51	0.31	0.34
24h	0.48	1.54	0.51	0.42
Metabolite Release normalized to Medium Control (%)				
1h	-	108.75	-	155.51
4h	-	141.34	-	110.37
24h	-	323.53	-	82.87
TxB_2 (ng/mL)				
Time	**Medium***	**LPS***	**CHX***	**CHX + LPS***
0h	0.58	-	-	-
1h	0.75	0.88	0.80	0.72
4h	0.99	4.61	0.81	0.60
24h	0.51	3.81	0.28	0.34
Metabolite Release normalized to Medium Control (%)				
1h	-	117.29	-	89.81
4h	-	464.02	-	73.70
24h	-	741.97	-	122.64
5-HETE (ng/mL)				
Time	**Medium***	**LPS***	**CHX***	**CHX + LPS***
0h	0.40	-	-	-
1h	0.41	0.50	0.83	0.88
4h	0.66	0.67	0.66	0.81
24h	0.79	0.35	0.93	0.66
Metabolite Release normalized to Medium Control (%)				
1h	-	120.00	-	105.22
4h	-	100.38	-	122.49
24h	-	44.18	-	70.48

* Data are given as Mean

Supplemental Table X. Target Gene Analysis of Healthy Subjects (n=10)

PLA_2 ($x10^3$ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value*[*]	*P-Value*[†]
0h	-	29.88	22.05	11.75 - 47.67	-	-
1h	-	20.25	4.73	16.04 - 38.72	*1.000*	-
	+	29.21	21.72	13.03 - 44.22	*1.000*	-
4h	-	38.06	12.97	21.13 - 58.79	*0.160*	-
	+	65.79	37.14	21.52 - 107.33	*< 0.01*	-
24h	-	35.65	12.62	21.69 - 49.26	*0.193*	-
	+	23.31	12.39	9.42 - 40.92	*0.432*	-
1h	% Ctr	138.43	96.78	64.95 - 229.29	-	*< 0.01*
4h	% Ctr	214.93	148.62	56.00 - 396.72	-	*< 0.05*
24h	% Ctr	52.36	54.65	29.47 - 188.65	-	*0.131*

COX-1 ($x10^3$ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value*[*]	*P-Value*[†]
0h	-	698.63	607.53	265.27 - 1957.96	-	-
1h	-	769.55	426.30	305.84 - 1624.68	*0.695*	-
	+	908.49	705.20	209.33 - 1513.79	*0.695*	-
4h	-	435.73	163.98	225.49 - 1551.45	*< 0.01*	-
	+	441.74	291.82	109.57 - 1306.85	*< 0.01*	-
24h	-	189.64	80.79	143.03 - 625.08	*< 0.01*	-
	+	139.50	106.31	59.97 - 509.94	*< 0.01*	-
1h	% Ctr	114.64	51.23	66.08 - 132.74	-	*< 0.01*
4h	% Ctr	82.26	61.73	48.19 - 253.28	-	*0.375*
24h	% Ctr	69.55	44.79	33.89 - 107.10	-	*< 0.05*

COX- 2 ($x10^3$ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value*[*]	*P-Value*[†]
0h	-	619.04	419.38	270.38 - 1884.13	-	-
1h	-	505.94	329.01	221.62 - 799.76	*0.232*	-
	+	16968.71	4397.23	10451.22 - 25120.51	*< 0.01*	-
4h	-	491.55	421.14	197.21 - 1018.49	*0.275*	-
	+	5354.02	2166.59	2244.82 - 10253.36	*< 0.01*	-
24h	-	107.16	59.68	68.50 - 161.97	*< 0.01*	-
	+	2650.94	2197.48	1039.68 - 7966.64	*< 0.01*	-
1h	% Ctr	3311.28	1662.89	2200.63 - 5043.49	-	*< 0.01*
4h	% Ctr	1701.24	1519.28	378.55 - 2354.32	-	*< 0.01*
24h	% Ctr	2939.86	1780.75	1255.85 - 7183.24	-	*< 0.01*

TXAS ($x10^3$ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value*[*]	*P-Value*[†]
0h	-	92.47	46.87	49.94 - 129.62	-	-
1h	-	88.92	30.68	57.46 - 184.95	*0.131*	-
	+	98.46	28.24	40.46 - 156.11	*0.131*	-
4h	-	108.28	51.93	45.14 - 153.96	*0.106*	-
	+	25.71	22.03	12.76 - 58.92	*< 0.01*	-
24h	-	116.00	46.47	56.17 - 141.99	*< 0.01*	-
	+	29.90	23.42	8.80 - 58.15	*< 0.01*	-

TXAS (x10³ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value**	*P-Value*†
1h	% Ctr	95.09	41.62	53.55 - 158.79	-	*< 0.01*
4h	% Ctr	30.11	21.30	13.82 - 56.16	-	*< 0.01*
24h	% Ctr	32.87	22.06	13.43 - 52.02	-	*< 0.01*

PGES (x10³ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value**	*P-Value*†
0h	-	2.04	1.81	0.68 - 7.68	-	-
1h	-	2.56	2.13	1.22 - 4.78	*0.232*	-
	+	11.04	5.33	4.17 - 17.49	*< 0.01*	-
4h	-	5.61	3.92	2.44 - 31.36	*< 0.01*	-
	+	88.86	71.21	56.15 - 211.36	*< 0.01*	-
24h	-	43.77	60.90	23.13 - 140.26	*< 0.01*	-
	+	371.63	331.57	124.25 - 1394.64	*< 0.01*	-
1h	% Ctr	404.90	275.46	179.26 - 684.24	-	*< 0.01*
4h	% Ctr	2277.19	1755.90	179.02 - 3011.33	-	*< 0.01*
24h	% Ctr	762.31	530.07	282.52 - 2620.87	-	*< 0.01*

PGFS (x10³ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value**	*P-Value*†
0h	-	277.51	86.54	130.21 - 326.69	-	-
1h	-	250.72	120.13	151.35 - 365.26	*0.846*	-
	+	270.29	56.26	157.49 - 353.27	*0.432*	-
4h	-	165.77	59.61	82.60 - 219.71	*< 0.01*	-
	+	233.58	79.53	145.45 - 401.34	*0.846*	-
24h	-	145.98	27.24	88.33 - 190.03	*< 0.01*	-
	+	184.54	56.43	112.42 - 282.12	*< 0.01*	-
1h	% Ctr	108.86	21.60	81.29 - 129.18	-	*< 0.01*
4h	% Ctr	145.90	59.36	71.85 - 338.06	-	*< 0.05*
24h	% Ctr	124.03	32.73	84.10 - 196.61	-	*< 0.05*

12-LOX (x10³ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value**	*P-Value*†
0h	-	102.87	55.59	28.51 - 158.56	-	-
1h	-	90.87	56.62	43.67 - 139.78	*0.492*	-
	+	100.43	48.91	27.98 - 167.48	*< 0.05*	-
4h	-	44.40	36.47	20.44 - 81.81	*< 0.01*	-
	+	49.80	43.15	18.00 - 103.15	*< 0.01*	-
24h	-	0.42	0.65	0.14 - 2.20	*< 0.01*	-
	+	0.35	0.37	0.10 - 1.11	*< 0.01*	-
1h	% Ctr	106.55	40.81	63.34 - 144.87	-	*< 0.01*
4h	% Ctr	99.21	40.31	74.20 - 274.73	-	*0.695*
24h	% Ctr	65.38	50.33	44.59 - 385.89	-	*0.193*

5-LOX (x10³ copies/µg RNA)

Time	LPS	Median	IQR	Range	*P-Value**	*P-Value*†
0h	-	3067.86	813.21	859.99 - 5362.00	-	-
1h	-	2404.31	1086.09	1125.46 - 3995.10	*0.432*	-
	+	2154.24	1408.42	693.23 - 4149.90	*< 0.01*	-

5-LOX (x10³ copies/µg RNA)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
4h	-	2282.21	1472.13	678.89 - 3102.32	*0.106*	-
	+	1556.50	1189.55	486.730 - 2612.96	*< 0.01*	-
24h	-	1725.41	872.89	635.75 - 2346.03	*< 0.01*	-
	+	422.24	276.83	116.99 - 819.89	*< 0.01*	-
1h	% Ctr	89.36	69.43	42.61 - 142.58	-	*< 0.01*
4h	% Ctr	66.46	51.98	29.41 - 249.66	-	*0.160*
24h	% Ctr	22.55	33.84	11.98 - 73.78	-	*< 0.01*
FLAP (x10³ copies/µg RNA)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	517.44	476.48	153.14 - 1777.86	-	-
1h	-	540.94	519.41	314.19 - 1238.08	*0.4922*	-
	+	946.64	1232.54	423.79 - 2805.52	*< 0.01*	-
4h	-	743.19	965.61	311.47 - 1830.15	*0.160*	-
	+	2003.16	2095.80	831.32 - 6006.27	*< 0.01*	-
24h	-	1557.73	1710.55	734.98 - 4836.03	*< 0.01*	-
	+	1539.30	1402.09	415.43 - 4409.45	*< 0.01*	-
1h	% Ctr	176.67	141.31	77.37 - 439.39	-	*< 0.01*
4h	% Ctr	268.14	402.02	107.10 - 721.68	-	*< 0.01*
24h	% Ctr	97.61	77.16	32.12 - 231.53	-	*1.000*

* Absolute values related to baseline gene expression

† Relative values normalized to medium control

Supplemental Table XI. Differential Gene Expression Levels between Female (n=5) and Male Subjects (n=5)

PLA₂ (x10³ copies/µg RNA)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	24.49	13.16 - 38.75	35.27	11.75 - 47.67	*1.000*
1h	-	20.53	16.04 - 38.72	19.96	28.49 - 26.04	*1.000*
	+	25.15	15.00 - 36.62	36.72	60.54 - 44.22	*0.676*
4h	-	38.43	21.13 - 58.79	37.69	29.16 - 44.51	*1.000*
	+	46.68	21.52 - 83.82	69.87	9.42 - 107.33	*0.210*
24h	-	43.78	21.69 - 49.26	31.98	17.33 - 41.53	*0.296*
	+	24.69	16.08 - 40.92	18.43	13.03 - 28.47	*0.144*
1h	% Ctr	117.43	64.95 - 181.73	159.43	65.26 - 229.29	*0.676*
4h	% Ctr	98.46	56.00 - 396.72	217.35	152.43 - 247.08	*0.676*
24h	% Ctr	54.58	36.73 - 188.65	47.16	29.47 - 91.37	*0.296*
COX-1 (x10³ copies/µg RNA)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	945.10	462.13 - 1230.25	634.00	265.27 - 1957.96	*0.531*
1h	-	878.21	570.30 - 1052.88	602.14	225.49 - 1624.68	*0.676*
	+	1087.05	376.83 - 1244.32	729.92	109.57 - 1513.79	*1.000*
4h	-	438.46	361.82 - 976.23	433.00	143.03 - 1551.45	*0.835*
	+	461.35	243.26 - 552.33	422.13	59.97 - 1306.85	*0.835*
24h	-	197.32	148.93 - 476.13	181.96	305.84 - 625.08	*0.835*
	+	185.00	70.40 - 509.94	138.79	209.33 - 418.11	*0.676*
1h	% Ctr	118.18	66.08 - 132.74	111.09	68.44 - 130.76	*1.000*
4h	% Ctr	67.23	53.29 - 125.97	84.23	48.19 - 253.28	*0.835*
24h	% Ctr	72.21	35.68 - 107.10	66.89	33.89 - 97.03	*0.531*
COX-2 (x10³ copies/µg RNA)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	814.33	318.36 - 1884.13	584.85	270.38 - 689.26	*0.403*
1h	-	366.93	221.62 - 799.76	523.19	465.47 - 795.75	*0.531*
	+	15315.05	10451.22 - 18181.79	19712.28	15479.00 - 25120.51	*0.095*
4h	-	319.40	248.57 - 593.00	705.46	197.21 - 1018.49	*0.144*
	+	5852.14	2244.82 - 7153.21	4819.65	4184.28 - 10253.36	*0.676*
24h	-	90.36	68.50 - 139.13	123.96	71.22 - 161.97	*0.835*
	+	2980.47	1039.68 - 5543.64	2321.41	1901.25 - 7966.64	*0.835*
1h	% Ctr	3297.09	2200.63 - 5043.49	3325.48	2830.59 - 4493.47	*1.000*
4h	% Ctr	1873.11	378.55 - 2354.32	762.94	473.21 - 2258.89	*0.403*
24h	% Ctr	2945.99	1255.85 - 7183.24	2933.73	1433.22 - 6426.62	*1.000*
TXAS (x10³ copies/µg RNA)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	96.07	49.94 - 105.84	88.88	57.70 - 129.62	*0.835*
1h	-	86.68	66.02 - 113.22	98.84	45.14 - 184.95	*0.531*
	+	97.89	40.46 - 113.86	99.04	18.83 - 156.11	*0.531*
4h	-	99.65	56.05 - 128.98	123.26	64.60 - 153.96	*0.403*
	+	26.08	12.76 - 54.18	25.35	8.80 - 58.92	*0.835*
24h	-	111.80	56.17 - 132.49	122.63	57.43 - 141.99	*0.210*
	+	42.53	23.98 - 58.15	25.04	51.15 - 47.41	*0.210*

TXAS (x10³ copies/µg RNA)

Time	LPS	Median*	Range*	Median	Range	P-Value
1h	% Ctr	93.92	61.29 - 137.94	96.27	53.55 - 158.79	*1.000*
4h	% Ctr	26.38	15.10 - 54.37	38.27	13.82 - 56.16	*0.835*
24h	% Ctr	40.48	27.88 - 52.02	18.42	13.43 - 39.61	*<0.05*

PGES (x10³ copies/µg RNA)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	2.96	0.68 - 7.68	1.91	0.73 - 3.23	*0.531*
1h	-	2.62	2.40 - 4.63	1.76	1.22 - 4.78	*0.531*
	+	11.61	8.31 - 13.66	9.72	4.17 - 17.49	*1.000*
4h	-	6.04	4.36 - 31.36	4.49	2.44 - 8.28	*0.296*
	+	75.89	56.15 - 134.10	102.70	59.64 - 211.36	*0.531*
24h	-	43.56	23.13 - 92.04	43.98	25.29 - 140.26	*0.531*
	+	373.25	319.00 - 650.57	326.59	124.25 - 1394.64	*0.835*
1h	% Ctr	443.59	179.26 - 570.69	366.20	248.84 - 684.24	*0.835*
4h	% Ctr	1465.51	179.02 - 2335.93	2552.41	796.51 - 3011.33	*0.060*
24h	% Ctr	732.23	505.14 - 2620.87	792.39	282.52 - 1291.55	*0.835*

PGFS (x10³ copies/µg RNA)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	277.85	130.21 - 326.69	277.17	173.85 - 304.82	*1.000*
1h	-	248.44	151.35 - 312.03	253.01	181.00 - 365.26	*0.676*
	+	288.46	172.48 - 312.22	247.89	157.49 - 353.27	*0.676*
4h	-	195.16	148.42 - 215.33	142.82	82.60 - 219.71	*0.296*
	+	183.33	145.45 - 240.63	262.86	216.92 - 401.34	*<0.05*
24h	-	162.94	108.09 - 190.03	143.49	88.33 - 146.95	*0.095*
	+	182.09	112.42 - 217.30	186.98	122.57 - 282.12	*0.835*
1h	% Ctr	113.97	96.46 - 125.67	96.72	81.29 - 129.18	*0.296*
4h	% Ctr	123.30	71.85 - 141.21	182.66	150.60 - 338.06	*<0.05*
24h	% Ctr	106.04	84.10 - 133.36	138.77	108.75 - 196.61	*0.060*

12-LOX (x10³ copies/µg RNA)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	83.80	28.51 - 141.35	112.10	49.18 - 158.56	*0.403*
1h	-	85.70	44.17 - 115.61	109.22	43.67 - 139.78	*0.296*
	+	81.03	27.98 - 167.48	111.99	33.60 - 128.75	*0.531*
4h	-	36.72	20.99 - 59.74	59.47	20.44 - 81.81	*0.676*
	+	38.62	18.00 - 71.53	63.92	19.54 - 103.15	*0.210*
24h	-	0.29	0.14 - 0.87	0.43	0.22 - 2.20	*0.296*
	+	0.43	0.10 - 0.75	0.27	0.18 - 1.11	*1.000*
1h	% Ctr	114.31	63.34 - 144.87	106.52	68.05 - 117.74	*0.531*
4h	% Ctr	85.77	74.20 - 137.34	102.80	92.97 - 274.73	*0.296*
24h	% Ctr	93.82	50.07 - 385.89	60.61	44.59 - 100.69	*0.210*

5-LOX (x10³ copies/µg RNA)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	3278.60	859.99 - 5362.00	2581.71	1949.40 - 3880.98	*0.403*
1h	-	1909.71	1125.46 - 2935.06	2670.40	1849.00 - 3995.10	*0.296*
	+	1942.23	693.23 - 4149.90	2366.24	1208.71 - 2905.51	*0.835*

5-LOX (x10³ copies/µg RNA)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
4h	-	2143.33	987.07 - 3102.32	2299.96	678.89 - 2965.16	*1.000*
	+	1418.11	486.73 - 2612.96	1694.90	676.40 - 2085.55	*1.000*
24h	-	1953.94	635.75 - 2346.03	1706.68	1182.83 - 2055.72	*0.835*
	+	469.03	116.99 - 819.89	305.06	225.82 - 819.68	*0.835*
1h	% Ctr	115.56	42.61 - 141.39	69.92	44.64 - 142.58	*0.835*
4h	% Ctr	67.62	41.80 - 107.93	65.30	29.41 - 249.66	*0.676*
24h	% Ctr	27.61	11.98 - 73.78	17.49	13.42 - 48.07	*1.000*
FLAP (x10³ copies/µg RNA)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	663.29	153.14 - 1100.53	421.53	319.21 - 1777.86	*0.676*
1h	-	534.16	314.19 - 1238.08	547.72	342.63 - 1200.48	*1.000*
	+	1380.51	563.10 - 1857.13	583.60	423.80 - 2805.52	*0.296*
4h	-	755.84	458.44 - 1830.15	560.02	311.47 - 1424.05	*0.296*
	+	3017.96	906.96 - 4504.97	1758.51	831.32 - 6006.27	*0.835*
24h	-	1792.04	734.98 - 4836.03	1323.43	923.16 - 4093.53	*1.000*
	+	2053.87	575.62 - 4409.45	1376.81	415.43 - 2608.74	*0.210*
1h	% Ctr	201.63	82.69 - 439.39	150.32	77.37 - 233.70	*0.403*
4h	% Ctr	204.24	107.10 - 658.31	314.01	194.00 - 721.68	*0.531*
24h	% Ctr	112.46	32.12 - 231.53	63.73	44.57 - 122.16	*0.403*

* Female Subjects

Supplemental Table XII. Coefficient of Variation (%) of Gene Expression Data from Healthy Subjects (n=10)

Time	LPS	*PLA$_2$*	*COX-1*	*COX-2*	*TXAS*	*PGES*	*PGFS*	*12-LOX*	*5-LOX*	*FLAP*
0h	-	44.74	56.07	68.13	30.19	82.70	24.72	41.55	39.62	70.72
1h	-	29.32	45.56	37.34	38.62	43.25	27.77	36.52	35.45	53.90
	+	42.35	49.57	27.24	35.21	33.66	23.34	45.70	46.78	65.49
4h	-	29.06	72.71	52.10	33.83	100.04	26.21	49.01	40.04	59.62
	+	38.75	66.77	37.67	51.00	47.62	29.85	53.12	47.23	63.45
24h	-	23.31	60.64	31.88	27.47	64.89	20.39	98.82	33.20	67.35
	+	41.83	79.00	62.34	47.20	70.08	27.51	69.07	52.46	64.05
1h	% Ctr	45.25	25.47	28.60	32.58	38.24	15.06	25.60	43.87	59.06
4h	% Ctr	52.52	61.61	55.65	48.58	54.28	47.53	49.80	77.44	64.24
24h	% Ctr	67.86	38.01	62.36	44.80	66.51	24.23	98.51	66.80	64.13
	Mean	**41.50**	**55.54**	**46.33**	**38.95**	**60.13**	**26.66**	**56.77**	**48.29**	**63.20**
Male Subjects										
0h	-	38.07	69.68	31.75	49.54	48.69	33.96	55.77	77.42	40.88
1h	-	23.89	52.57	25.05	37.30	35.32	19.37	58.11	30.91	39.03
	+	47.21	24.83	21.33	63.75	57.48	33.17	41.00	17.72	35.68
4h	-	48.44	37.24	15.50	44.14	37.47	30.03	55.33	98.24	39.69
	+	35.81	35.27	20.20	57.59	50.31	55.87	53.55	34.27	47.35
24h	-	52.25	32.63	19.65	46.86	76.93	29.49	75.44	55.34	29.18
	+	86.56	58.90	24.88	54.99	58.75	36.25	61.54	32.90	35.47
1h	% Ctr	30.17	38.58	9.47	44.04	28.72	30.89	59.68	40.74	43.96
4h	% Ctr	41.63	45.13	27.93	39.31	27.60	49.05	79.13	68.46	81.95
24h	% Ctr	41.69	73.26	16.97	73.64	96.07	23.83	61.43	74.53	71.18
	Mean	**44.57**	**46.81**	**21.27**	**51.12**	**51.73**	**34.19**	**60.10**	**53.05**	**46.44**
Female Subjects										
0h	-	76.70	33.25	19.52	25.54	37.02	29.38	88.37	49.06	51.56
1h	-	64.03	23.37	31.28	31.50	35.73	44.89	55.61	58.89	15.92
	+	57.67	21.50	27.83	31.36	38.61	37.62	96.32	48.00	49.54
4h	-	93.09	44.65	34.65	40.68	55.72	36.77	62.87	49.64	17.49
	+	79.26	43.29	24.57	40.24	48.13	51.13	80.32	54.65	26.44
24h	-	72.52	33.61	18.75	18.72	95.58	26.24	66.19	72.19	15.60
	+	78.22	69.17	31.51	56.17	82.12	54.74	56.24	91.14	39.65
1h	% Ctr	23.54	19.03	18.79	47.89	22.52	37.95	46.90	39.18	49.49
4h	% Ctr	71.91	68.44	36.45	96.30	55.83	53.25	56.95	38.49	17.53
24h	% Ctr	36.69	58.18	23.04	64.26	34.32	49.46	46.66	46.61	48.78
	Mean	**65.36**	**41.45**	**26.64**	**45.27**	**50.56**	**42.14**	**65.64**	**54.78**	**33.20**

Supplemental Table XIII. Metabolite Release of Healthy Subjects (n=10)

AA (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	629.67	351.33	330.33 - 1176.67	-	-
1h	-	651.83	304.00	375.667 - 1286.67	*0.193*	-
	+	638.00	347.83	365.333 - 1113.33	*0.232*	-
4h	-	743.50	419.67	522.33 - 1580.00	*< 0.01*	-
	+	772.50	399.67	549.67 - 1350.00	*< 0.01*	-
24h	-	2206.67	1006.67	1610.00 - 4010.00	*< 0.01*	-
	+	1415.00	813.33	907.50 - 1996.67	*< 0.01*	-
1h	% Ctr	98.34	13.36	86.53 - 112.18	-	*< 0.01*
4h	% Ctr	102.23	11.73	85.44 - 114.38	-	*0.922*
24h	% Ctr	61.32	12.12	44.95 - 86.59	-	*< 0.01*
11-HETE (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.24	0.09	0.14 - 0.60	-	-
1h	-	0.379	0.102	0.165 - 0.82	*< 0.01*	-
	+	0.351	0.081	0.218 - 0.68	*< 0.01*	-
4h	-	0.58	0.30	0.36 - 1.00	*< 0.01*	-
	+	3.05	1.54	0.98 - 7.50	*< 0.01*	-
24h	-	1.91	0.80	1.67 - 3.76	*< 0.01*	-
	+	20.15	10.55	7.76 - 39.47	*< 0.01*	-
1h	% Ctr	108.29	44.131	76.136 - 136.32	-	*< 0.01*
4h	% Ctr	618.28	268.41	131.12 - 881.80	-	*< 0.01*
24h	% Ctr	745.95	556.45	398.63 - 1266.31	-	*< 0.01*
TxB_2 (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	1.73	3.05	0.15 - 3.91	-	-
1h	-	1.87	3.32	0.274 - 3.66	*< 0.05*	-
	+	2.00	3.33	0.241 - 3.79	*< 0.01*	-
4h	-	2.11	2.95	0.27 - 3.80	*< 0.05*	-
	+	7.02	4.52	1.30 - 9.84	*< 0.01*	-
24h	-	1.46	1.69	0.23 - 2.20	*0.921*	-
	+	5.47	2.88	3.29 - 7.53	*< 0.01*	-
1h	% Ctr	112.05	27.06	87.85 - 154.26	-	*< 0.01*
4h	% Ctr	269.67	802.84	135.39 - 2537.13	-	*< 0.01*
24h	% Ctr	604.30	913.90	178.40 - 1621.08	-	*< 0.01*
PGE_2 (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.10	0.03	0.08 - 0.14	-	-
1h	-	0.13	0.04	0.086 - 0.19	*< 0.05*	-
	+	0.13	0.06	0.058 - 0.20	*< 0.05*	-
4h	-	0.10	0.03	0.04 - 0.21	*0.557*	-
	+	0.31	0.12	0.17 - 0.64	*< 0.01*	-
24h	-	0.16	0.06	0.10 - 0.22	*< 0.01*	-
	+	4.99	3.72	1.55 - 12.03	*< 0.01*	-

PGE_2 (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
1h	% Ctr	115.44	47.69	41.04 - 187.00	-	*< 0.01*
4h	% Ctr	364.23	210.16	80.69 - 562.94	-	*< 0.01*
24h	% Ctr	3615.49	4131.27	1232.11 - 8125.00	-	*< 0.01*
$PGF_{2\alpha}$ (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.22	0.05	0.17 - 0.38	-	-
1h	-	0.23	0.05	0.157 - 0.39	*0.492*	-
	+	0.21	0.09	0.155 - 0.32	*0.625*	-
4h	-	0.22	0.09	0.17 - 0.30	*0.106*	-
	+	0.42	0.17	0.27 - 0.68	*< 0.01*	-
24h	-	0.29	0.09	0.20 - 0.39	*< 0.01*	-
	+	1.44	0.66	0.66 - 2.65	*< 0.01*	-
1h	% Ctr	99.61	33.54	52.10 - 145.42	-	*< 0.01*
4h	% Ctr	180.13	46.80	89.14 - 294.05	-	*< 0.01*
24h	% Ctr	450.72	108.81	267.56 - 775.39	-	*< 0.01*
12-HETE (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.70	1.20	0.26 - 2.47	-	-
1h	-	2.52	1.98	0.920 - 8.40	*< 0.01*	-
	+	1.64	2.27	0.658 - 7.44	*< 0.01*	-
4h	-	3.89	3.60	1.42 - 11.87	*< 0.01*	-
	+	4.22	2.62	2.14 - 11.12	*< 0.01*	-
24h	-	24.53	12.90	14.60 - 70.47	*< 0.01*	-
	+	34.77	18.00	19.63 - 120.67	*< 0.01*	-
1h	% Ctr	87.21	23.50	53.85 - 111.47	-	*< 0.01*
4h	% Ctr	108.56	72.08	84.30 - 219.14	-	*0.275*
24h	% Ctr	151.55	24.30	118.41 - 202.96	-	*< 0.01*
5-HETE (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.17	0.09	0.10 - 0.23	-	-
1h	-	0.24	0.12	0.108 - 0.43	*< 0.05*	-
	+	0.45	0.29	0.205 - 0.79	*< 0.01*	-
4h	-	0.34	0.14	0.24 - 0.48	*< 0.01*	-
	+	0.52	0.35	0.27 - 0.94	*< 0.01*	-
24h	-	0.79	0.18	0.64 - 1.26	*< 0.01*	-
	+	0.65	0.15	0.58 - 0.96	*< 0.01*	-
1h	% Ctr	204.61	81.24	128.66 - 372.22	-	*< 0.01*
4h	% Ctr	142.04	83.73	107.34 - 295.88	-	*< 0.01*
24h	% Ctr	86.78	25.63	67.71 - 98.26	-	*< 0.01*

* Absolute values related to baseline release

† Relative values normalized to medium control

Supplemental Table XIV. Differential Eicosanoid Release between Female (n=5) and Male Subjects (n=5)

AA (ng/mL)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	712.00	411.67 - 1176.67	547.33	330.33 - 771.00	*0.531*
1h	-	719.00	444.67 - 1286.67	520.00	375.67 - 871.00	*0.531*
	+	693.33	450.67 - 1113.33	582.67	365.33 - 798.50	*0.40*
4h	-	784.67	522.33 - 1580.00	702.33	590.67 - 1093.33	*1.000*
	+	817.67	549.67 - 1350.00	727.33	561.33 - 1166.67	*1.000*
24h	-	2390.00	1610.00 - 4010.00	1980.00	1703.33 - 3190.00	*0.403*
	+	1893.33	907.50 - 1996.67	1163.33	978.33 - 1896.67	*0.296*
1h	% Ctr	96.43	86.53 - 112.18	99.42	91.68 - 112.05	*0.531*
4h	% Ctr	100.89	85.44 - 105.23	103.56	93.48 - 114.38	*0.676*
24h	% Ctr	63.18	47.22 - 86.59	59.46	44.95 - 68.30	*0.676*
11-HETE (ng/mL)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	0.31	0.23 - 0.60	0.22	0.14 - 0.26	*0.060*
1h	-	0.38	0.31 - 0.82	0.32	0.17 - 0.44	*0.403*
	+	0.42	0.34 - 0.68	0.34	0.22 - 0.36	*0.022*
4h	-	0.74	0.53 - 1.00	0.45	0.36 - 0.58	*0.037*
	+	2.97	0.98 - 7.50	3.13	2.01 - 4.38	*0.835*
24h	-	1.88	1.78 - 3.76	1.95	1.67 - 2.58	*0.531*
	+	23.25	10.22 - 39.47	15.77	7.76 - 20.93	*0.095*
1h	% Ctr	102.13	83.09 - 136.32	114.44	76.14 - 131.92	*1.000*
4h	% Ctr	563.09	131.12 - 876.80	671.65	448.99 - 881.80	*0.531*
24h	% Ctr	1179.44	559.49 - 1266.31	741.25	398.63 - 1251.00	*0.676*
TxB_2 (ng/mL)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	0.20	0.15 - 0.32	3.25	3.15 - 3.91	*0.012*
1h	-	0.291	0.27 - 0.46	3.61	3.27 - 3.66	*0.012*
	+	0.435	0.24 - 0.55	3.76	3.46 - 3.79	*0.012*
4h	-	0.48	0.27 - 0.96	3.43	3.27 - 3.80	*0.012*
	+	3.66	1.30 - 7.21	8.19	6.01 - 9.84	*0.037*
24h	-	0.40	0.23 - 0.87	2.09	2.05 - 2.20	*0.012*
	+	4.42	3.29 - 7.53	6.34	3.72 - 7.16	*0.676*
1h	% Ctr	126.15	87.85 - 154.26	104.62	94.96 - 115.75	*0.144*
4h	% Ctr	1033.19	135.39 - 2537.13	246.83	158.16 - 277.87	*0.144*
24h	% Ctr	1223.67	865.85 - 1621.08	309.77	178.40 - 342.74	*0.012*
PGE_2 (ng/mL)						
Time	**LPS**	**Median***	**Range***	**Median**	**Range**	**P-Value**
0h	-	0.09	0.08 - 0.11	0.10	0.08 - 0.14	*0.210*
1h	-	0.10	0.09 - 0.16	0.14	0.11 - 0.19	*0.210*
	+	0.15	0.06 - 0.20	0.13	0.09 - 0.16	*0.676*
4h	-	0.09	0.04 - 0.21	0.11	0.10 - 0.17	*0.296*
	+	0.27	0.17 - 0.52	0.34	0.26 - 0.64	*0.403*
24h	-	0.16	0.12 - 0.22	0.12	0.10 - 0.18	*0.403*
	+	4.24	2.12 - 12.03	5.73	1.55 - 9.58	*1.000*

PGE_2 (ng/mL)

Time	LPS	Median*	Range*	Median	Range	P-Value
1h	% Ctr	124.04	41.04 - 187.00	83.33	61.03 - 119.48	*0.144*
4h	% Ctr	371.42	80.69 - 562.41	259.45	197.51 - 562.94	*0.676*
24h	% Ctr	3477.31	1232.11 - 7763.44	3753.68	1273.97 - 8125.00	*0.676*

$PGF_{2\alpha}$ (ng/mL)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	0.24	0.19 - 0.38	0.18	0.17 - 0.24	*0.095*
1h	-	0.24	0.21 - 0.29	0.20	0.16 - 0.39	*0.210*
	+	0.271	0.17 - 0.32	0.19	0.16 - 0.27	*0.144*
4h	-	0.27	0.21 - 0.30	0.22	0.17 - 0.29	*0.144*
	+	0.41	0.27 - 0.68	0.43	0.34 - 0.53	*0.835*
24h	-	0.34	0.23 - 0.39	0.27	0.20 - 0.30	*0.095*
	+	1.62	0.96 - 2.65	1.20	0.66 - 1.47	*0.060*
1h	% Ctr	106.97	80.90 - 115.62	92.24	52.10 - 145.42	*0.835*
4h	% Ctr	180.61	89.14 - 248.71	179.64	157.47 - 294.05	*1.000*
24h	% Ctr	462.42	422.40 - 775.39	439.02	267.56 - 733.33	*0.531*

12-HETE (ng/mL)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	1.59	0.88 - 2.47	0.38	0.26 - 0.52	*0.012*
1h	-	3.08	2.88 - 8.40	1.10	0.92 - 2.17	*0.012*
	+	3.43	2.05 - 7.44	1.17	0.66 - 1.22	*0.012*
4h	-	5.24	4.14 - 11.87	1.64	1.42 - 3.63	*0.012*
	+	5.59	4.45 - 11.12	2.97	2.14 - 3.98	*0.012*
24h	-	24.20	20.53 - 70.47	24.87	14.60 - 34.03	*0.676*
	+	39.10	31.97 - 120.67	34.20	19.63 - 49.97	*0.144*
1h	% Ctr	85.77	71.18 - 111.47	93.897	53.85 - 109.273	*1.000*
4h	% Ctr	93.71	84.30 - 114.48	162.68	89.43 - 219.14	*0.144*
24h	% Ctr	161.57	147.43 - 202.96	137.53	118.41 - 161.83	*0.060*

5-HETE (ng/mL)

Time	LPS	Median*	Range*	Median	Range	P-Value
0h	-	0.22	0.17 - 0.23	0.13	0.10 - 0.16	*0.012*
1h	-	0.27	0.15 - 0.43	0.17	0.11 - 0.28	*0.296*
	+	0.62	0.24 - 0.68	0.40	0.21 - 0.79	*0.835*
4h	-	0.41	0.33 - 0.48	0.26	0.24 - 0.35	*0.022*
	+	0.56	0.39 - 0.75	0.49	0.27 - 0.94	*1.000*
24h	-	0.85	0.70 - 1.21	0.71	0.64 - 1.26	*0.403*
	+	0.62	0.58 - 0.82	0.68	0.59 - 0.96	*0.676*
1h	% Ctr	177.05	157.15 - 240.40	229.72	128.66 - 372.22	*0.531*
4h	% Ctr	122.94	116.88 - 183.92	204.48	107.34 - 295.88	*0.296*
24h	% Ctr	69.92	67.71 - 98.26	91.56	76.09 - 95.67	*0.296*

* Female Subjects

Supplemental Table XV. Coefficient of Variation (%) of Metabolite Release Data from Healthy Subjects (n=10)

Time	LPS	AA	11-HETE	TxB_2	PGE_2	$PGF_{2\alpha}$	12-HETE	5-HETE
0h	-	39.33	45.72	93.70	20.31	27.40	75.95	28.65
1h	-	41.15	45.17	87.46	26.10	27.56	80.67	39.30
	+	35.26	32.59	83.76	33.82	25.16	84.91	40.21
4h	-	37.52	33.00	78.48	42.51	19.69	80.58	24.73
	+	32.37	55.86	43.30	41.83	29.17	60.75	37.69
24h	-	31.01	31.03	69.98	23.58	20.15	54.75	25.83
	+	29.44	46.19	29.10	59.76	38.77	67.77	17.60
1h	% Ctr	8.78	20.98	19.59	42.10	27.44	21.32	34.25
4h	% Ctr	8.23	36.92	110.63	45.06	28.78	36.83	38.85
24h	% Ctr	19.47	36.97	72.73	65.91	32.96	15.06	14.60
	Mean	**28.26**	**38.44**	**68.87**	**40.10**	**27.71**	**57.86**	**30.17**
Male Subjects								
0h	-	42.52	11.24	37.71	28.72	28.23	16.43	42.04
1h	-	44.94	37.08	53.60	23.71	12.05	28.49	42.54
	+	29.75	37.66	54.87	30.06	24.57	42.51	36.38
4h	-	25.29	13.86	51.16	52.79	16.13	61.42	45.27
	+	66.21	24.22	43.44	56.68	37.28	46.28	36.08
24h	-	36.85	22.19	57.51	55.59	19.21	20.63	34.23
	+	43.91	15.73	64.16	34.33	35.69	70.41	27.67
1h	% Ctr	20.64	20.57	16.55	21.61	15.12	44.15	10.35
4h	% Ctr	50.47	20.33	12.72	78.23	32.57	48.80	8.26
24h	% Ctr	34.23	18.09	12.81	23.42	27.94	73.13	22.88
	Mean	**39.48**	**22.10**	**40.45**	**40.52**	**24.88**	**45.22**	**30.57**
Female Subjects								
0h	**-**	20.03	24.73	23.88	10.96	14.60	20.83	34.07
1h	**-**	35.72	37.18	38.93	4.61	40.29	22.69	37.92
	+	17.86	46.88	24.34	3.82	20.88	21.82	33.33
4h	**-**	20.60	16.63	43.35	6.10	21.19	24.46	29.77
	+	27.38	50.74	25.74	17.07	19.26	39.84	32.01
24h	**-**	18.79	31.72	29.45	2.77	15.22	26.83	26.10
	+	34.59	20.86	32.39	26.42	29.39	55.06	28.12
1h	**% Ctr**	23.69	39.93	27.49	7.74	39.02	27.10	7.69
4h	**% Ctr**	25.12	39.97	34.68	19.74	27.59	45.28	8.65
24h	**% Ctr**	39.82	9.48	11.45	27.08	40.45	66.26	16.13
	Mean	**26.36**	**31.81**	**29.17**	**12.63**	**26.79**	**35.02**	**25.38**

Supplemental Table XVI. Target Gene Analysis of AA Metabolism in Patients of the Leipzig Heart Study

PLA2 **(x10³ copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	27.5	14.7	9.8 - 75.5	-	-
4h	-	31.5	20.7	8.5 - 101.8	*< 0.001*	-
	+	34.9	21.7	0.6 - 92.1	*< 0.001*	-
24h	-	40.9	25.5	15.3 - 118.8	*< 0.001*	-
	+	12.3	7.7	3.3 - 36.0	*< 0.001*	-
4h	% ctr	127.1	79.9	52.7 - 374.6	-	*< 0.001*
24h	% ctr	37.4	24.0	14.7 - 97.4	-	*< 0.001*
COX-1 **(x10³ copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	1205.2	661.3	367.2 - 3041.0	-	-
4h	-	695.0	449.0	154.4 - 2726.3	*< 0.001*	-
	+	643.4	458.2	137.7 - 1812.8	*< 0.001*	-
24h	-	347.6	175.7	130.8 - 701.3	*< 0.001*	-
	+	242.5	155.8	106.8 - 684.0	*< 0.001*	-
4h	% ctr	88.4	21.2	47.9 - 132.5	-	*< 0.001*
24h	% ctr	76.5	25.8	40.8 - 141.0	-	*< 0.001*
COX-2 **(x10³ copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	1287.4	1329.1	412.3 - 13122.7	-	-
4h	-	713.2	433.0	212.9 - 2598.5	*< 0.001*	-
	+	6699.7	4224.8	1839.5 - 19204.4	*< 0.001*	-
24h	-	159.8	97.8	47.3 - 901.0	*< 0.001*	-
	+	1650.3	2595.5	457.5 - 10729.8	*< 0.01*	-
4h	% ctr	881.8	595.9	220.4 - 2514.0	-	*< 0.001*
24h	% ctr	1079.8	1243.1	164.7 - 7674.7	-	*< 0.001*
TXAS **(x10³ copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	114.0	116.1	36.8 - 719.1	-	-
4h	-	91.2	84.8	27.1 - 444.1	*< 0.001*	-
	+	33.4	30.0	7.9 - 286.3	*< 0.001*	-
24h	-	142.4	108.6	25.1 - 770.8	*< 0.001*	-
	+	38.9	31.9	11.5 - 235.5	*< 0.001*	-
4h	% ctr	34.9	15.3	10.2 - 173.0	-	*< 0.001*
24h	% ctr	29.8	15.6	8.9 - 65.9	-	*< 0.001*
PGES **(x10³ copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	6.4	6.2	1.6 - 70.8	-	-
4h	-	80.6	108.0	9.1 - 1507.5	*< 0.001*	-
	+	614.7	1069.5	117.5 - 6743.9	*< 0.001*	-
24h	-	319.9	378.6	40.8 - 3083.0	*< 0.001*	-
	+	1899.3	1520.2	791.3 - 11996.3	*< 0.001*	-

***PGES* (x10^3 copies/µg RNA)**						
4h	% ctr	830.9	760.2	196.6 - 8319.9	-	*< 0.001*
24h	% ctr	621.6	552.4	146.5 - 3029.8	-	*< 0.001*
***PGFS* (x10^3 copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value*†**
0h	-	191.3	138.6	60.7 - 795.1	-	-
4h	-	194.2	145.8	30.5 - 810.7	*0.662*	-
	+	146.3	110.9	41.9 - 690.6	*< 0.001*	-
24h	-	144.5	144.5	25.4 - 489.2	*< 0.001*	-
	+	141.2	93.1	29.4 - 668.3	*< 0.001*	-
4h	% ctr	74.0	51.0	17.5 - 185.2	-	*< 0.001*
24h	% ctr	102.3	50.0	34.3 - 229.8	-	*0.553*
***12-LOX* (x10^3 copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value*†**
0h	-	177.7	131.6	48.4 - 480.8	-	-
4h	-	79.7	64.1	12.2 - 262.2	*< 0.001*	-
	+	76.4	63.3	14.4 - 281.4	*< 0.001*	-
24h	-	2.3	4.9	0.3 - 19.8	*< 0.001*	-
	+	2.3	2.9	0.3 - 19.0	*< 0.001*	-
4h	% ctr	95.7	19.9	50.6 - 148.4	-	*< 0.05*
24h	% ctr	93.1	46.2	40.2 - 351.0	-	*0.636*
***5-LOX* (x10^3 copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value*†**
0h	-	8638.6	4615.1	4638.8 - 23344.6	-	-
4h	-	7068.8	3488.4	3165.6 - 17808.2	*< 0.001*	-
	+	5459.1	3157.5	1593.3 - 14083.8	*< 0.001*	-
24h	-	6399.2	3395.3	2659.4 - 15684.1	*< 0.001*	-
	+	3003.9	2373.9	1065.7 - 11806.4	*< 0.001*	-
4h	% ctr	71.1	24.4	29.2 - 140.9	-	*< 0.001*
24h	% ctr	47.1	28.1	16.7 - 147.1	-	*< 0.001*
***FLAP* (x10^3 copies/µg RNA)**						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value*†**
0h	-	7958.8	4719.4	3382.5 - 27743.4	-	-
4h	-	14777.5	9366.5	5712.2 - 50606.2	*< 0.001*	-
	+	28059.2	18391.7	7917.4 - 92589.6	*< 0.001*	-
24h	-	23446.1	15620.8	9613.0 - 73713.3	*< 0.001*	-
	+	27396.4	18293.0	7506.7 - 96017.7	*< 0.001*	-
4h	% ctr	186.8	68.3	83.0 - 334.5	-	*< 0.001*
24h	% ctr	118.1	56.0	49.0 - 239.0	-	*< 0.001*

* Absolute values related to baseline release

† Relative values normalized to medium control

Supplemental Table XVII. Differential Eicosanoid Response on Gene Expression Level in Patients with or without CAD

PLA₂ (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	29.4	25.8	*0.379*	26.5	24.7
4h	-	31.2	31.8	*0.772*	35.3	31.1
	+	39.0	33.9	*0.570*	34.3	32.2
24h	-	43.6	38.8	*0.222*	39.4	38.8
	+	12.2	12.4	*0.998*	12.9	12.4
4h	% ctr	133.5	120.3	*0.251*	141.8	111.6
24h	% ctr	34.4	40.2	*0.329*	44.5	38.4
COX-1 (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	1278.7	1159.9	*0.396*	1473.7	1130.5
4h	-	712.6	622.7	*0.616*	801.3	584.4
	+	686.8	580.8	*0.701*	668.4	500.2
24h	-	369.9	334.9	*0.511*	347.3	325.7
	+	215.7	268.0	*0.183*	324.6	265.4
4h	% ctr	93.1	86.0	*0.906*	93.2	84.6
24h	% ctr	73.2	77.7	*< 0.01*	78.1//	77.7//
COX-2 (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	1296.0	1281.6	*0.771*	1198.3	1341.2
4h	-	680.6	726.6	*0.695*	614.7	784.3
	+	7638.9	5977.7	*< 0.05*	5385.0//	6723.0
24h	-	166.3	158.0	*< 0.05*	158.3//	158.0
	+	1657.0	1643.7	*0.187*	1608.7	1643.7
4h	% ctr	1063.7	837.6	*0.062*	973.3	806.0
24h	% ctr	1048.6	1129.2	*0.517*	1014.9	1258.6
TXAS (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	112.1	122.0	*0.143*	90.5	131.4
4h	-	109.7	89.3	*0.239*	64.5	105.1
	+	34.8	28.1	*0.149*	26.2	33.5
24h	-	155.8	128.6	*0.058*	127.9	133.1
	+	38.1	39.1	*0.447*	36.4	41.5
4h	% ctr	35.9	33.9	*0.280*	36.1	32.8
24h	% ctr	28.3	30.2	*0.145*	30.1	31.3
PGES (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	6.6	6.1	*0.199*	4.6	6.6
4h	-	88.8	74.7	*0.198*	55.6	93.7
	+	723.2	527.8	*0.307*	488.6	805.7
24h	-	350.3	282.2	*< 0.05*	225.2//	394.2
	+	1936.2	1864.8	*0.211*	1787.9	1982.6

PGES (x10³ copies/µg RNA)						
4h	% ctr	750.4	927.7	*0.554*	741.4	942.3
24h	% ctr	538.8	663.4	*0.223*	814.3	658.6
PGFS (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	197.9	191.3	*0.360*	230.2	171.1
4h	-	191.7	197.3	*0.638*	197.3	197.4
	+	144.8	147.1	*0.622*	187.7	128.9
24h	-	156.7	142.2	*0.486*	140.7	144.2
	+	141.2	132.7	*0.429*	158.7	125.1
4h	% ctr	73.3	75.9	*0.931*	84.3	72.8
24h	% ctr	105.1	99.5	*0.805*	104.3	92.5
12-LOX (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	188.0	164.8	*0.075*	220.1	142.4
4h	-	80.5	79.5	*0.213*	91.8	66.8
	+	77.5	74.7	*0.208*	92.3	64.9
24h	-	2.9	2.3	*0.143*	2.4	2.3
	+	2.3	2.3	*0.373*	2.7	1.9
4h	% ctr	97.8	94.2	*0.580*	95.9	92.8
24h	% ctr	88.4	93.5	*0.106*	93.1	94.7
5-LOX (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	8913.5	8604.4	*0.394*	8900.4	8336.8
4h	-	6943.5	7211.0	*0.738*	7053.5	7261.1
	+	5484.5	5459.1	*0.568*	5658.2	4925.5
24h	-	7039.5	6343.5	*0.166*	6399.2	6276.1
	+	3035.7	2897.7	*0.943*	2913.7	2897.7
4h	% ctr	70.8	72.1	*0.975*	78.1	69.7
24h	% ctr	41.9	47.6	*0.089*	45.3	51.4
FLAP (x10³ copies/µg RNA)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	7552.2	8096.2	*0.878*	6642.4	8382.4
4h	-	15316	14360	*0.613*	14108	14907
	+	28877	28059	*0.614*	27830	28980
24h	-	23968	22536	*0.277*	23921	22135
	+	27382	28532	*0.480*	28997	25892
4h	% ctr	202.0	185.5	*0.534*	206.6	179.2
24h	% ctr	106.1	129.7	*< 0.05*	117.9	136.0//

* Subjects with catheter based exclusion of coronary artery disease (CAD)

† *P-Value* subjects w/o CAD compared to subjects with CAD

‡ Subjects with angiographic coronary wall irregularities < 50 % luminal reduction

§ Subjects with angiographic stenosis ≥ 50 % luminal reduction in at least one major coronary artery

// *P < 0.05* when compared to subjects w/o CAD

Supplemental Table XVIII. Quantitative Metabolite Analysis of AA Metabolism in Patients of the Leipzig Heart Study

AA (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	1242.7	896.3	544.0 - 4973.3	-	-
4h	-	1968.3	1286.7	809.0 - 14400.0	*< 0.001*	-
	+	2135.0	1253.3	833.0 - 16333.3	*< 0.001*	-
24h	-	5158.3	3241.7	1956.7 - 23433.3	*< 0.001*	-
	+	3507.5	1678.3	1230.0 - 14900.0	*< 0.001*	-
4h	% ctr	101.1	12.8	76.5 - 130.3	-	*0.112*
24h	% ctr	65.4	15.4	38.3 - 90.3	-	*< 0.001*
11-HETE (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.6	0.3	0.3 - 1.3	-	-
4h	-	1.6	1.1	0.7 - 6.4	*< 0.001*	-
	+	7.5	4.0	1.9 - 26.3	*< 0.001*	-
24h	-	6.1	3.7	1.9 - 58.3	*< 0.001*	-
	+	28.2	19.5	5.6 - 100.8	*< 0.001*	-
4h	% ctr	439.5	319.3	146.3 - 1150.1	-	*< 0.001*
24h	% ctr	396.3	304.5	83.8 - 1206.3	-	*< 0.001*
TxB_2 (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.5	0.6	0.1 - 4.0	-	-
4h	-	1.6	2.4	0.1 - 14.7	*< 0.001*	-
	+	13.0	10.0	2.1 - 47.1	*< 0.001*	-
24h	-	1.5	2.5	0.2 - 14.9	*< 0.001*	-
	+	10.9	11.5	0.6 - 54.2	*< 0.001*	-
4h	% ctr	747.0	1015.9	163.9 - 17205.9	-	*< 0.001*
24h	% ctr	612.3	811.6	52.5 - 3662.8	-	*< 0.001*
PGE_2 (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.2	0.2	0.1 - 2.2	-	-
4h	-	0.3	0.2	0.1 - 2.3	*< 0.001*	-
	+	1.2	1.0	0.1 - 8.0	*< 0.001*	-
24h	-	0.4	0.3	0.1 - 2.5	*< 0.001*	-
	+	8.0	8.8	0.2 - 82.5	*< 0.001*	-
4h	% ctr	406.1	317.9	72.8 - 1444.5	-	*< 0.001*
24h	% ctr	2174.4	2627.8	103.4 - 29262.8	-	*< 0.001*
$PGF_{2\alpha}$ (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.2	0.1	0.1 - 0.4	-	-
4h	-	0.3	0.1	0.1 - 0.6	*< 0.01*	-
	+	0.7	0.3	0.1 - 1.7	*< 0.001*	-
24h	-	0.4	0.3	0.1 - 1.3	*< 0.001*	-
	+	1.9	1.2	0.2 - 13.1	*< 0.001*	-

$PGF_{2\alpha}$ (ng/mL)						
4h	% ctr	243.0	148.0	23.5 - 1036.0	-	*< 0.001*
24h	% ctr	520.9	395.7	117.3 - 3071.1	-	*< 0.001*
12-HETE (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	2.7	2.5	0.9 - 10.0	-	-
4h	-	44.3	78.7	6.2 - 405.3	*< 0.001*	-
	+	57.6	99.2	8.9 - 518.3	*< 0.001*	-
24h	-	157.5	172.4	17.4 - 2066.7	*< 0.001*	-
	+	260.8	249.7	45.7 - 1600.0	*< 0.001*	-
4h	% ctr	121.0	30.5	89.4 - 332.4	-	*< 0.001*
24h	% ctr	159.6	62.7	32.9 - 390.8	-	*< 0.001*
5-HETE (ng/mL)						
Time	**LPS**	**Median**	**IQR**	**Range**	***P-Value****	***P-Value**†*
0h	-	0.5	0.3	0.2 - 1.8	-	-
4h	-	1.0	0.6	0.3 - 3.3	*< 0.001*	-
	+	1.4	0.8	0.6 - 4.5	*< 0.001*	-
24h	-	2.2	1.1	0.9 - 6.4	*< 0.001*	-
	+	1.6	0.9	0.6 - 4.9	*< 0.001*	-
4h	% ctr	140.4	46.4	79.5 - 335.0	-	*< 0.001*
24h	% ctr	70.1	18.4	31.1 - 123.8	-	*< 0.001*

* Absolute values related to baseline release

† Relative values normalized to medium control

Supplemental Table XIX. Differential Eicosanoid Response on Mediator Level in Patients with or without CAD

AA (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	1391.7	1081.8	*< 0.01*	1119.5	1033.7//
4h	-	2358.3	1743.3	*< 0.05*	1725.0	1755.0//
	+	2386.7	1860.0	*< 0.05*	1681.7	1913.3
24h	-	5976.7	4470.0	*< 0.05*	4505.0	4470.0//
	+	3808.3	2886.7	*< 0.05*	2651.7	2963.3//
4h	% ctr	99.3	102.1	*0.733*	95.8	103.8
24h	% ctr	66.7	64.0	*0.311*	59.7	65.7
11-HETE (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	0.6	0.6	*0.927*	0.6	0.6
4h	-	1.9	1.4	*< 0.05*	1.3	1.6
	+	8.0	6.9	*0.234*	6.2	7.7
24h	-	6.8	5.6	*< 0.05*	5.0//	5.9//
	+	31.4	25.6	*0.121*	19.3	28.4
4h	% ctr	437.2	494.8	*0.486*	399.6	512.8
24h	% ctr	412.3	384.4	*0.482*	352.5	438.5
TxB_2 (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	0.5	0.4	*0.532*	0.4	0.6
4h	-	2.0	1.3	*0.324*	0.9	1.5
	+	13.2	12.3	*0.583*	10.3	13.7
24h	-	1.9	1.3	*0.439*	1.0	1.6
	+	10.9	10.6	*0.997*	8.6	11.3
4h	% ctr	584.8	850.6	*0.785*	803.6	903.1
24h	% ctr	465.9	711.9	*0.894*	745.0	644.9
PGE_2 (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	0.2	0.2	*0.880*	0.2	0.3
4h	-	0.3	0.3	*0.841*	0.2	0.3
	+	1.2	1.1	*0.935*	0.8	1.7
24h	-	0.4	0.3	*0.415*	0.2	0.4
	+	8.6	6.2	*0.870*	4.3	8.4
4h	% ctr	408.8	405.5	*0.796*	335.8	405.5
24h	% ctr	2706.4	1980.7	*0.523*	1860.6	2002.6
$PGF_{2\alpha}$ (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	0.2	0.2	*0.541*	0.2	0.3
4h	-	0.3	0.3	*0.530*	0.3	0.3
	+	0.7	0.7	*0.717*	0.6	0.7
24h	-	0.4	0.3	*0.266*	0.3	0.4
	+	2.1	1.9	*0.927*	1.4	2.1

$PGF_{2\alpha}$ (ng/mL)						
4h	% ctr	249.8	233.8	*0.666*	184.7	276.6
24h	% ctr	515.2	522.4	*0.319*	495.6	538.6
12-HETE (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	2.8	2.5	*0.260*	2.7	2.4
4h	-	54.4	41.0	*0.060*	36.5	45.9
	+	81.8	47.9	*< 0.05*	47.4	49.4
24h	-	196.7	133.0	*< 0.05*	124.5	161.3//
	+	311.0	211.8	*< 0.05*	167.5	262.5
4h	% ctr	120.0	124.3	*0.354*	118.8	129.0
24h	% ctr	147.9	167.2	*0.073*	160.5	175.3
5-HETE (ng/mL)						
Time	***LPS***	***w/o CAD****	***with CAD***	*P-Value†*	*CAD < 50%‡*	*CAD ≥ 50%§*
0h	-	0.5	0.5	*0.967*	0.5	0.5
4h	-	1.0	0.9	*0.385*	0.9	0.9
	+	1.5	1.2	*< 0.05*	1.1//	1.4
24h	-	2.5	2.2	*0.179*	2.0	2.2
	+	1.9	1.4	*< 0.05*	1.2//	1.4
4h	% ctr	138.2	141.3	*0.058*	134.9	147.5
24h	% ctr	76.9	66.8	*< 0.01*	63.3//	70.1//

* Subjects with catheter based exclusion of coronary artery disease (CAD)

† *P-Value* subjects w/o CAD compared to subjects with CAD

‡ Subjects with angiographic coronary wall irregularities < 50 % luminal reduction

§ Subjects with angiographic stenosis ≥ 50 % luminal reduction in at least one major coronary artery

// *P<0.05* when compared to subjects w/o CAD

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- Helen Keller -

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13 Curriculum Vitae

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Research

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10/2015	Poster Award, 12. Jahrestagung der Deutschen Vereinten Gesellschaft für Klinische Chemie und Laboratoriumsmedizin **Kleinhempel A**, Holdt LM, Nagel D, Ceglarek U, Beutner F, Kortz L, Thiery J, Teupser D, Bruegel M. Differential Eicosanoid Response on Gene Expression and Mediator Level in Patients with or without Coronary Artery Disease

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