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STUDIES ON ANTI-INFLAMMATORY AND VASOACTIVE EFFECTS OF MPGES-1 INHIBITION

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Studies on anti-inflammatory and vasoactive effects of mPGES-1 inhibition
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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POPULAR SCIENCE SUMMARY OF THE THESIS

Prostanoids are hormone-like molecules that are derived from polyunsaturated fatty acids and are involved in enabling and maintaining physiological processes in the body. Prostaglandin E_2 (PGE_2) is an active driver of inflammation and pain. PGE_2 is produced by two enzymes. The first one, which is called cyclooxygenase converts the polyunsaturated fatty acid arachidonic acid to PGH_2 (the precursor of PGE_2 and other prostanoids). The second enzyme, called microsomal prostaglandin E synthase 1 (mPGES-1) converts PGH_2 to PGE_2. To treat inflammation and pain drugs like Ibuprofen or Aspirin are commonly used. They block cyclooxygenases and thereby the formation of PGE_2 and other prostanoids, which successfully reduces inflammation symptoms. However, when these drugs are used regularly they can increase the risk for side effects on the gut and the cardiovascular system. It is believed that this is because inhibition of cyclooxygenases blocks not only PGE_2 but also other prostaglandins that are important for the gastrointestinal and cardiovascular system. Besides PGE_2 there are other primary prostaglandins including PGI_2 and PGD_2, which are suggested to be cardio-protective and anti-inflammatory. If these and especially PGI_2 are blocked, this can result in imbalances and severe complications including gastric ulcers and bleeding, elevated blood pressure, increased risk for heart attack or worsening of heart failure. To avoid or reduce side effects, selectively blocking PGE_2 is a promising treatment alternative. This could be achieved by drugs that block the mPGES-1 enzyme. For the development of safe and effective drugs, it is important to know how well blocking of the enzyme reduces PGE_2 and what effects this may have on specific diseases. This is investigated in pre-clinical studies with pharmacological probes inhibiting the enzyme. One aspect of pre-clinical studies involves testing the inhibitors in systems mimicking disease conditions, which is often done in human cell cultures and animal models and requires inhibitors that do work in both species.

This thesis aimed to characterize five new inhibitors of mPGES-1 regarding their efficacy to block PGE_2 and to study their effects in models of inflammation and cardiovascular disease. We found that the new inhibitors are well suited for pre-clinical studies being effective in inhibiting human and rat mPGES-1 and blocking PGE_2 production in animal models of inflammation. Moreover, we found that the inhibition of mPGES-1 maintained PGD_2 compared to inhibitors of cyclooxygenase in immune cells. We could show that PGD_2 is metabolized to metabolites that may promote the resolution of inflammation. Furthermore, we could show that inhibition of mPGES-1 reduced human artery tone and increased relaxation. In addition, in a mouse model of myocardial infarction (MI), inhibition of mPGES-1 was protective and promoted cardiac healing after MI compared to placebo treatment or treatment with an inhibitor of cyclooxygenase. In summary, results from this thesis contribute to a better understanding on how the pharmacological inhibition of mPGES-1 could affect inflammation and cardiovascular disease. The findings support the hypothesis of an overall beneficial cardiovascular profile of mPGES-1 inhibitors and highlight the complexity of the underlying mechanism.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Prostaglandine sind hormonähnliche Moleküle, die sich von mehrfach ungesättigten Fettsäuren ableiten und an der Ermöglichung und Aufrechterhaltung physiologischer Prozesse im Körper beteiligt sind. Prostaglandin E_2 (PGE_2) ist eines dieser Moleküle und ein aktiver Treiber von Entzündungen und Schmerzen. PGE_2 wird von zwei Enzymen produziert. Das erste, Cyclooxygenase genannt, wandelt die mehrfach ungesättigte Fettsäure Arachidonsäure in PGH_2 um (Vorstufe von PGE_2 und anderen Prostaglandinen). Das zweite Enzym, das als mikrosomale Prostaglandin-E-Synthase 1 (mPGES-1) bezeichnet wird, wandelt PGH_2 in PGE_2 um. Zur Behandlung von Entzündungen und Schmerzen werden häufig Medikamente wie Ibuprofen oder Aspirin eingesetzt. Diese Medikamente blockieren Cyclooxygenasen und damit die Bildung von PGE_2 und anderen Prostaglandinen. Die Blockierung von Cyclooxygenasen und der Produktion von PGE_2 reduziert Symptome wie Entzündungen und Schmerzen sehr erfolgreich. Wenn diese Medikamente jedoch über einen längeren Zeitraum angewendet werden, können sie das Risiko für Nebenwirkungen auf den Darm und das Herz-Kreislauf-System erhöhen. Es wird angenommen, dass dies daherkommt, dass die Blockierung von Cyclooxygenasen neben PGE_2 auch andere Prostaglandine blockiert, die für das gastrointestinale und kardiovaskuläre System wichtig sind. Neben PGE_2 gibt es vier primäre Prostaglandine, darunter PGI_2 und PGD_2, denen eine kardioprotektive und entzündungshemmende Wirkung zugeschrieben wird. Wenn diese und insbesondere PGI_2 blockiert werden, kann dies zu Ungleichgewichten und schweren Komplikationen bis hin zur Herzinsuffizienz führen. Um Nebenwirkungen zu vermeiden oder zu reduzieren, ist die Blockierung von nur PGE_2 eine vielversprechende Behandlungsalternative. Dies könnte durch Medikamente erreicht werden, die speziell das mPGES-1-Enzym blockieren und dabei die Bildung der anderen Prostaglandine nicht beeinträchtigt. Für die Entwicklung sicherer und wirksamer Medikamente ist es wichtig zu wissen, wie gut die Blockierung des Enzyms PGE_2 reduziert und welche Auswirkungen dies für den Verlauf bestimmter Erkrankungen haben könnte. Dies wird unter anderem in präklinischen Studien untersucht in welchen die Wirkstoffkandidaten (Inhibitoren) getestet werden. Ein Aspekt der vorklinischen Studien beinhaltet das Testen der Inhibitoren in Systemen, die Krankheitszustände nachahmen, was oft in Tiermodellen durchgeführt wird und Inhibitoren erfordert, die bei beiden Spezies funktionieren.

Das Ziel dieser Arbeit war es, fünf neue Inhibitoren von mPGES-1 hinsichtlich ihrer Wirksamkeit zur Blockierung von PGE_2 zu charakterisieren und ihre Wirkung in Modellen für Entzündungen und kardiovaskuläre Erkrankungen zu untersuchen. Wir haben festgestellt, dass die neuen Inhibitoren gut für vorklinische Studien geeignet sind, da sie bei der Hemmung von mPGES-1 vom Menschen und von der Ratte und bei der Blockierung der PGE_2-Produktion in Entzündungs-Tiermodellen wirksam sind. Darüber hinaus fanden wir heraus, dass die Hemmung von mPGES-1 PGD_2 im Vergleich zu Inhibitoren der Cyclooxygenase in

Immunzellen aufrechterhält. Wir konnten zeigen, dass PGD_2 zu Metaboliten verstoffwechselt wird, die die Auflösung von Entzündungen fördern können und womöglich zu den therapeutischen Effekten von mPGES-1 Inhibitoren beitragen. Außerdem konnten wir zeigen, dass die Hemmung von mPGES-1 den Tonus von menschlichen Arterien der Mikrozirkulation reduziert und deren Entspannung erhöht. Des Weiteren konnten wir feststellen, dass die Hemmung von mPGES-1 in einem Mausmodell für Myokardinfarkt schützend wirkt und die Herzheilung nach dem Infarkt im Vergleich zu keiner Behandlung oder einer Behandlung mit einem Inhibitor der Cyclooxygenase fördert.

Zusammenfassend tragen die Ergebnisse dieser Arbeit zu einem besseren Verständnis bei, wie die pharmakologische Hemmung von mPGES-1 Entzündungen und kardiovaskuläre Erkrankungen beeinflussen könnte. Die Ergebnisse unterstützen die Hypothese eines insgesamt vorteilhaften kardiovaskulären Profils von mPGES-1 Inhibitoren und unterstreichen die Komplexität der zugrunde liegenden Mechanismen.

ABSTRACT

Inflammation is the basis for various serious illnesses such as rheumatic diseases, cardiovascular diseases, and cancer. Prostaglandin E_2 (PGE_2) is a pro-inflammatory lipid mediator produced by cyclooxygenases (COX1/2) and the microsomal prostaglandin E synthase 1 (mPGES-1). Nonsteroidal anti-inflammatory drugs (NSAIDS) targeting COX successfully reduce pain and inflammation. However, gastrointestinal and cardiovascular side effects associated with blockade of all prostaglandins limit their use. Selective inhibition of mPGES-1 is an alternative therapeutic strategy to impede PGE_2 production while sparing or even upregulating other lipid mediators. When and where the shunting of PGH_2 to other prostanoids occurs and whether it interferes with or contributes to the therapeutic effects of mPGES-1 inhibition is not fully understood. The overall aim of this thesis was to study the anti-inflammatory and vasoactive effects of mPGES-1 inhibition in models of inflammation and cardiovascular disease and to investigate the possible shunting of PGH_2 to PGD_2 and PGI_2. The methodological approach was principally based on liquid chromatography-tandem mass spectrometry, biochemical assays as well as wire-myography.

In **Paper I** five new mPGES-1 inhibitors were characterized. The inhibitors selectively suppressed PGE_2 formation in *in-vitro* and *in-vivo* assays, reduced acute paw swelling in rats, and reduced adrenergic vasoconstriction. The results of this study serve as a basis for the application of these inhibitors in pre-clinical research. Depletion of mPGES-1 may lead to the re-direction of PGH_2 into the PGD_2/15-deoxy-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2) pathway, which has been described to be anti-inflammatory and pro-resolving. In **Paper II**, we studied the biosynthesis and metabolism of 15dPGJ_2 via conjugation to glutathione in immune cells and upon inhibition of mPGES-1. The results of this study demonstrate the formation of 15dPGJ_2-glutathione and 15dPGJ_2-cysteine conjugates in immune cells, the involvement of MGST3 in this pathway and the preservation of the PGD_2/15dPGJ_2 pathway upon inhibition of mPGES-1. Another important aspect of mPGES-1 inhibition is the redirection of excess PGH_2 into the PGI_2 pathway. A decrease in PGI_2 levels as a result of COX-2 inhibition has been associated with increased cardiovascular risk in patients treated with selective NSAIDs. In **Paper III** we aimed to study the effects of mPGES-1 inhibition on human resistance arteries. Inhibition of mPGES-1 significantly reduced adrenergic vasoconstriction and enhanced relaxation. Our results suggest that multiple pathways in addition to shunting to PGI_2 may be involved in the vasoactive effects of mPGES-1 inhibition in human microcirculation. In **Paper IV** we studied the effects of mPGES-1 inhibition in a mouse model of MI. The results of this study indicate that pharmacological inhibition of mPGES-1 could improve cardiac function after MI and increase the PGI_2/PGE_2 metabolite ratio in urine compared with controls.

The results from this thesis contribute to a better understanding of the mechanisms underlying the effects seen after inhibition of mPGES-1 in models of inflammation and cardiovascular disease.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to by their corresponding Roman numerals throughout this thesis. Reprints were made with permission from the publishers. *Equal author contribution.

I. **Biological characterization of new mPGES-1 inhibitors in pre-clinical models of inflammation and vascular tone**
Karin Larsson*, Julia Steinmetz*, Filip Bergqvist*, Samsul Arefin, Linda Spahiu, Johan Wannberg, Sven-Christian Pawelzik, Ralf Morgenstern, Patric Stenberg, Karolina Kublickiene, Marina Korotkova, Per-Johan Jakobsson. *British Journal of pharmacology, 2019; 176(24): 4625-4638*

II. **Biosynthesis of prostaglandin 15dPGJ$_2$-glutathione and -cysteine conjugates in macrophages and mast cells via MGST3**
Julia Steinmetz-Späh, Jianyang Liu, Rajkumar Singh, Maria Ekoff, Sanjaykumar Boddul, Xiao Tang, Filip Bergqvist, Helena Idborg, Pascal Heitel, Elin Rönnberg, Daniel Merk, Fredrik Wermeling, Jesper Z. Haeggström, Gunnar Nilsson, Dieter Steinhilber, Karin Larsson, Marina Korotkova, Per-Johan Jakobsson. *Accepted Manuscript, Journal of Lipid Research, 2022.*

III. **Effects of microsomal prostaglandin E synthase-1 (MPGES-1) inhibition on resistance artery tone in patients with end stage kidney disease**
Julia Steinmetz-Späh*, Samsul Arefin*, Karin Larsson, Jabin Jahan, Neja Mudrovcic, Lars Wennberg, Peter Stenvinkel, Marina Korotkova, Karolina Kublickiene*, Per-Johan Jakobsson*. *British Journal of Pharmacology, 2022;179(7):1433-1449*

IV. **Microsomal prostaglandin E synthase-1 inhibition protects cardiac remodeling after myocardial infarction in mice**
Yuze Zhang*, Julia Steinmetz-Späh*, Helena Idborg, Liyuan Zhu, Huihui Li, Haojie Rao, Zengrong Chen, Ziyi Guo, Lejia Hu, Chuansheng Xu, Hong Chen, Marina Korotkova, Per-Johan Jakobsson*, Miao Wang*. *Submitted Manuscript.*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

During the time as a doctoral student, the author has contributed to the following publications. These are however not the content of this thesis.

I. **Integration of magnetic resonance imaging and protein and metabolite CSF measurements to enable early diagnosis of secondary progressive multiple sclerosis**
Stephanie Herman, Payam Emami Khoonsari, Andreas Tolf, Julia Steinmetz, Henrik Zetterberg, Torbjörn Akerfeldt, Per-Johan Jakobsson, Anders Larsson, Ola Spjuth, Joachim Burman and Kim Kultima. *Theranostics, 2018;8(16):4477-4490*

II. **Descriptive proteome analysis to investigate context-dependent treatment responses to OXPHOS inhibition in colon carcinoma cells grown as monolayer and multicellular tumor spheroids**
Julia Steinmetz, Wojciech Senkowski, Johan Lengqvist, Jenny Rubin, Elena Ossipova, Stephanie Herman, Rolf Larsson, Per-Johan Jakobsson, Mårten Fryknäs, Kim Kultima. *ACS Omega, 2020;5(28):17242-17254*

III. **Bisbolane sesquiterpenes from the leaves of Lindera benzoin reduce prostaglandin E2 formation in A549 cells**
Astrid Henz Ryen, Thomas Göls, Julia Steinmetz, Ammar Tahir, Per-Johan Jakobsson, Anders Backlund, Ernst Urban, Sabine Glasl-Tazreiter. *Phytochemistry Letters, 2020, 38(4):6-11.*

IV. **Implications for Nrf2 in early vascular ageing: focus on calcification, senescence and therapeutic approaches**
Samsul Arefin, Sarah Buchanan, Sam Hobson, Julia Steinmetz, Shno Alsalhi, Paul Shiels, Karolina Kublickiene, Peter Stenvinkel. *Clinica Chimica Acta, 2020, 505:108-118*

V. **Establishment of an in vitro 3D model for neuroblastoma enables preclinical investigation of tumor-stroma drug targeting**
Anna Kock, Filip Bergqvist, Julia Steinmetz, Lotta Elfman, Marina Korotkova, John inge Johnsen, per-Johan jakobsson*, Per Kogner*, Karin larsson*. *FASEB, 2020, 34(8):11101-11114*

VI. **Microsomal prostaglandin E synthase-1 inhibition promotes shunting in arachidonic acid metabolism during inflammation *in-vitro***
Bing Peng*, Jianyang Liu*, Julia Steinmetz-Späh, Helena Idborg, Marina Korotkova, Per-Johan Jakobsson. M*anuscript.*

CONTENTS

1 Introduction 1
1.1 Lipid mediators as therapeutic targets for the treatment of inflammation 2
1.1.1 Eicosanoids 2
1.1.2 Prostanoids 2
1.1.3 Targeting COX-1/COX-2 8
1.1.4 Targeting mPGES-1 10
1.1.5 mPGES-1 inhibitor development 12
1.2 Effects of mPGES-1 inhibition on inflammation – focusing on the PGD_2 pathway 15
1.2.1 Anti-inflammatory and pro-resolving effects of PGD_2 and $15dPGJ_2$ 15
1.2.2 Characteristics of $15dPGJ_2$ 16
1.2.3 Effects of mPGES-1 depletion on the PGD_2 pathway 18
1.3 Effects of mPGES-1 inhibition on inflammation and vascular tone 19
1.3.1 The cardiovascular system 19
1.3.2 Prostanoids and the regulation of vascular tone 20
1.3.3 Effects of mPGES-1 depletion on vascular biology 23
2 Research aims 25
3 Methodological approach 27
3.1 Enzyme activity assays 27
3.2 Wire myography 29
3.3 Sample preparation for LC-MS/MS analysis 30
3.4 LC-MS/MS analysis 33
3.5 Statistical analysis 35
3.6 Ethical considerations 36
4 Results and discussion 39
4.1 Inhibitors of mPGES-1 for pre-clinical studies 39
4.2 Inhibition of mPGES-1 preserves the PGD_2/$15dPGJ_2$ pathway – characterization of the $15dPGJ_2$ metabolism in macrophages and mast cells 43
4.3 Inhibition of mPGES-1 has vasoactive effects 47
4.4 Inhibition of mPGES-1 improves cardiac function after MI 51
5 Summary and perspectives 57
6 Acknowledgements 61
7 References 63

LIST OF ABBREVIATIONS

5-HETE	5-hydroxyeicosatetraenoic acid
5-HpETE	5-hydroperoxyeicosatetraenoic acid
12-HHT	12-hydroxyheptadecatrienoic acid
15-PGDH	15-hydroxyprostaglandin-dehydrogenase
$15dPGJ_2$	15-deoxy-$\Delta^{12,14}$-PGJ_2
$15dPGJ_2$-Cys	15-deoxy-$\Delta^{12,14}$-PGJ_2-Cysteine
$15dPGJ_2$-GS	15-deoxy-$\Delta^{12,14}$-PGJ_2-Glutathione
AC	Adenylyl cyclase
ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
BK	Bradykinin
BMDM	Bone marrow derived macrophages
CA	Carrageenan
CAIA	Collagen antibody-induced arthritis
cAMP	Cyclic adenosine monophosphate
CBMC	Cord-blood derived mast cells
cGMP	Cyclic guanosine monophosphate
CIA	Collagen-induced arthritis
CKD	Chronic kidney disease
COX	Cyclooxygenase
Coxib	COX-2 selective inhibitor
cPGES	Cytosolic prostaglandin E synthase
CRTH2	Chemoattractant receptor-homologous molecule expressed on TH2 cells
DAG	Diacylglycerol
DC	Dendritic cell
ESKD	End-stage kidney disease
eNOS	Endothelial nitric oxide synthase
GPCR	G protein-coupled receptor
GSH	Glutathione
GST	Glutathione S-transferase
GTP	Guanosine triphosphate

HFrEF	Heart failure with reduced ejection fraction
H-PGDS	Hematopoietic PGD synthase
IL	Interleukin
IL-1β	Interleukin-1beta
IP3	Inositol triphosphate
IS	Internal standard
Keap1	Kelch-like ECH-associated protein 1
KO	Knock-out
LAD	Left anterior descending
LOX	Lipoxygenase
L-PGDS	lipocalin-type PGD synthase
LPS	Lipopolysaccharide
LT	Leukotriene
MAPEG	Membrane associated proteins in eicosanoid and GSH metabolism
MDA	Malondialdehyde
MeOH	Methanol
MGST1	Microsomal glutathione S-transferase 1
MGST2	Microsomal glutathione S-transferase 2
MGST3	Microsomal glutathione S-transferase 3
MI	Myocardial infarction
MI/IR	Myocardial infarction ischemia-reperfusion
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
mPGES-1	Microsomal prostaglandin E synthase 1
mPGES-2	Microsomal prostaglandin E synthase 2
MRP	Multidrug resistance protein
NF$_{\kappa}$B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
PG	Prostaglandin

PGIS	PGI_2 (prostacyclin) synthase
PIP2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
PMN	Polymorphonuclear cells
PPARγ	Peroxisome proliferator-activated receptor gamma
PSS	Physiological salt solution
RA	Rheumatoid arthritis
red-15dPGJ_2-GS	15-deoxy-$\Delta^{12,14}$-PGJ_2-Glutathione with reduced carbonyl on C11
RT	Retention time
SPE	Solid phase extraction
SR	Sarcoplasmic reticulum
TBA	2-thiobarbituric acid
Tgfb1	Gene for transforming growth factor beta
Timp1	Gene for tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TP	Thromboxane receptor
TX	Thromboxane
TXS	TXA_2 (thromboxane A_2) synthase
VSMC	Vascular Smooth muscle cells
WT	Wild type

1 INTRODUCTION

Inflammation is an essential function of the immune system that defends and repairs following exposure to harmful stimuli and involves immune cells, blood vessels and molecular mediators. The course of an inflammatory response begins with an initiating stimulus which can be an infection, burn or other traumatic injury that leads to the release of inflammatory mediators (e.g., cytokines, chemokines, and bioactive lipids) by resident immune cells. This results in an increase in blood flow, temperature and, vascular permeability, as well as recruitment of effector cells leading to the formation of exudate, pain and contingently loss of function [1]. Phagocytosis and intracellular killing of microorganisms by activated leukocytes eventually result in containment of the danger stimuli and homeostasis can be restored. The final phase of the inflammatory response is the resolution phase, an active self-limiting process that involves the counteraction of pro-inflammatory mediators, the decline of inflammatory cells and an increase in tissue repair through reactivation of resident immune cells [2]. At the molecular level, this includes a shift in the production of lipid mediators from pro-inflammatory lipid mediators like prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) to pro-resolving lipid mediators such as prostaglandin D_2 (PGD_2), lipoxin A_4 (LXA_4) and resolvin E_1/D_1 (RvE_1/RvD_1) [3]. Unlike acute inflammation which is a rapid and self-limiting reaction that neutralizes pathogens and repairs tissue damage, chronic inflammation is a persistent process that can be viewed to fail to resolve. This results in the constant activation of immune cells, and an overproduction of pro-inflammatory mediators causing tissue destruction and loss of function thereby contributing to several chronic diseases, such as rheumatoid arthritis (RA), chronic kidney disease, and cardiovascular disease [4, 5].

In this thesis, the inhibition of microsomal prostaglandin E synthase 1 (mPGES-1), the terminal synthase for inflammatory PGE_2 production, was studied in *in-vitro*, *ex-vivo,* and *in-vivo* models of inflammation and cardiovascular disease. This thesis aims to improve the understanding of the mechanisms underlying the effects of mPGES-1 inhibition in addition to the reduction of PGE_2. In particular, a possible shift towards anti-inflammatory and cardio-protective lipid mediators (PGD_2 and PGI_2) induced by blockage of mPGES-1 was investigated. Results obtained from this thesis extend the knowledge of the anti-inflammatory and vasoactive effects of mPGES-1 inhibition and how these might contribute to the beneficial therapeutic effects of mPGES-1 inhibition in inflammatory diseases.

1.1 LIPID MEDIATORS AS THERAPEUTIC TARGETS FOR THE TREATMENT OF INFLAMMATION

1.1.1 Eicosanoids

Endogenous lipids generated from ω-6 and ω-3 polyunsaturated fatty acids play an essential role in all phases of inflammation. Lipids are the major components of cell membranes and are the source of pathophysiological mediators with fundamental roles in inflammation and maintenance of tissue homeostasis [6, 7]. Essential fatty acids such as the ω-3-α-linoleic acid and the ω-6 linoleic acid, cannot be synthesized by mammals and therefore must be supplied in the diet in the form of vegetable oil, nuts, fish, eggs and meat. Eicosanoids are derived from 20-carbon polyunsaturated fatty acids and primarily from the ω-6-arachidonic acid (20:4) which is obtained directly from food or through the uptake of linoleic-acid rich food which is converted to arachidonic acid in animal cells [8]. Arachidonic acid can be metabolized to a wide range of biologically active and clinically important oxidized lipid metabolites (oxylipids) [9]. Arachidonic acid is involved in multiple oxidative metabolic pathways, namely cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase (cytochrome P450) pathways [10]. Arachidonic acid metabolism results in eicosanoids including prostaglandins (PGs), thromboxanes (TX), hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LT), lipoxins and epoxyeicosatrienoic acids (EETs).

1.1.2 Prostanoids

Since the discovery of prostanoids in the 1930s [11-13] and later the description of their biosynthesis from arachidonic acid [14, 15], a variety of physiological and pathophysiological activities have been revealed.

Prostanoids derive from arachidonic acid, which is found in the phospholipid bilayer of cell membranes. Phospholipases and primarily the cytosolic enzyme phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the sn-2 bond of phospholipids, generating free arachidonic acid and a lysophospholipid. The translocation of $cPLA_2$ to the cell membrane where it cleaves arachidonic acid from membrane phospholipids is induced by an increase in intracellular Ca^{2+} levels [9].

For the synthesis of prostaglandins and thromboxanes, arachidonic acid is then converted to PGH_2 by cyclooxygenases (COX-1 and COX-2). Cyclooxygenases are membrane-bound enzymes with two active sites that exert cyclooxygenase and peroxidase activities. At the cyclooxygenase site arachidonic acid is oxidized with two molecules of oxygen (O_2) forming the unstable endoperoxide intermediate PGG_2. At the peroxidase site, PGG_2 is subsequently reduced to PGH_2 [16]. COX-1 and COX-2 are isozymes that differ in their expression pattern and tissue localization. While COX-1 is considered to be mainly constitutively expressed, COX-2 is also inducible and contributes to the production of prostanoids during inflammation. PGH_2 serves as substrate for specific downstream synthases that produce PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and TXA_2 defined as prostanoids (Figure 1) [17].

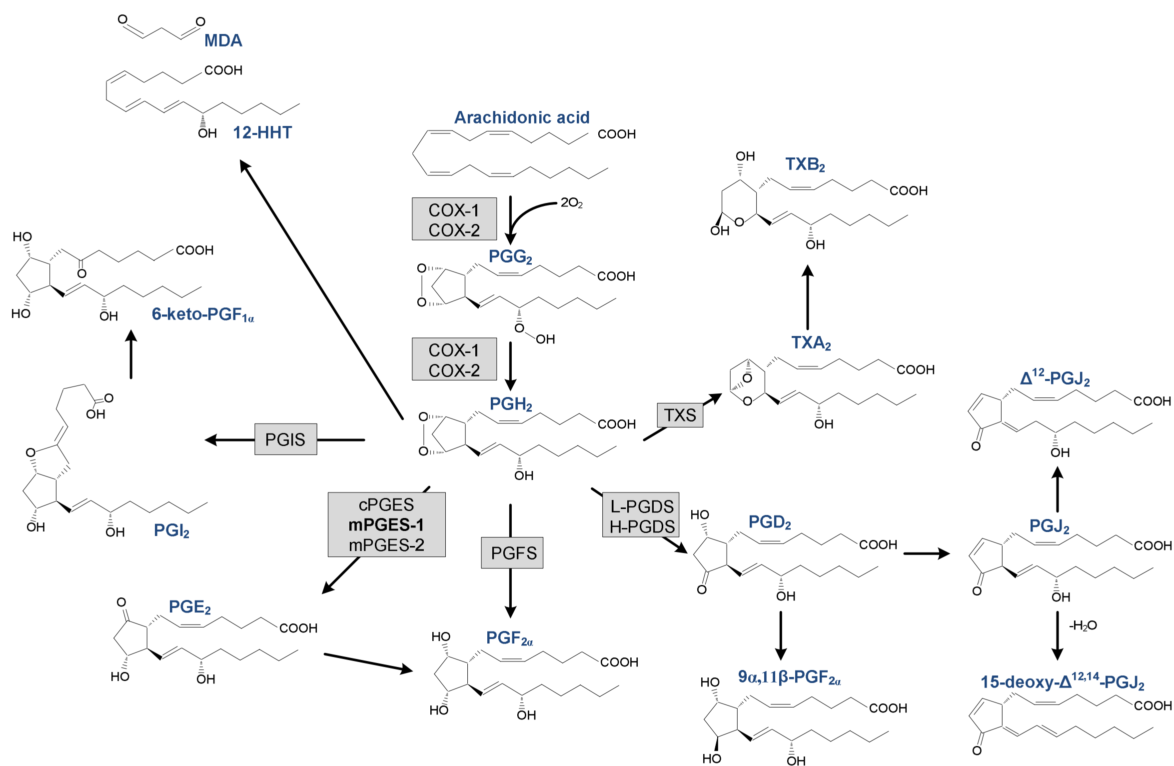


Figure1: Biosynthesis of the primary prostanoids (PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and TXA_2). TXA_2 and PGI_2 were primarily analyzed by their stable metabolites TXB_2 and 6-keto-$PGF_{1\alpha}$ respectively. PGH_2 can decompose to malondialdehyde (MDA) and 12-hydroxy-heptadecatrienoic acid (12-HHT). Prostanoid metabolism yields additional metabolites (including urinary metabolites) that are not covered here. Prostanoid metabolites are reviewed in detail in [18].

Prostanoids pass through the plasma membrane by passive diffusion and transport via the multidrug resistance protein MRP4 for efflux or prostaglandin transporters for influx into the cell [19, 20]. Leukotrienes and especially the glutathione S-conjugates (LTC4, LTD4, LTE4) can be transported by MRP1 and MRP2 [19]. Prostanoids act endocrine or paracrine via the activation of rhodopsin-like seven transmembrane spanning G protein coupled receptors (GPCRs). Nine primary prostanoid receptors have been identified, including the receptors for PGD_2 (DP1, DP2 (CRTH2)), PGE_2 (EP1-4), TXA_2 (TP), PGI_2 (IP) and $PGF_{2\alpha}$ (FP) receptors [21]. Each prostanoid receptor couples to specific G-proteins resulting in signaling pathways that eventually activate target gene transcription. Coupling to the Gq protein results in activation of phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-biphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 increases intracellular Ca^{2+} levels and DAG activates protein kinase C (PKC). Coupling to Gi protein inhibits adenylyl cyclase (AC), reducing cyclic adenosine monophosphate (cAMP) levels. Coupling to the Gs protein activates AC and stimulates cAMP levels and protein kinase A (PKA) [22]. To avoid undesired and continuous activity, termination of prostanoid signaling is critical and occurs by non-selective dehydration at the C15 position by the 15-hydroxyprostaglandin-dehydrogenase (15-PGDH), which forms an unstable 15-keto prostaglandin. Double-bond reduction at the C13 position carried out by the Δ^{13}-15-ketoprostaglandin-reductase (13-PGR) subsequently generates a 15-keto-13,14-dehydro-prostaglandin which can then be

completely inactivated by shortening of the carbon chain through β-oxidation [23]. The degradation of TXA_2 and PGI_2 is an exception to this pathway and occurs non-enzymatically.

PGE_2 is the most abundant prostaglandin in the human body and can be produced by three prostaglandin E synthases, microsomal prostaglandin E synthase 1 (mPGES-1), microsomal prostaglandin E synthase 2 (mPGES-2) and cytosolic prostaglandin E synthase (cPGES) [24]. The mPGES-1 enzyme is a membrane-bound inducible enzyme that belongs to the family of membrane-associated proteins in eicosanoid and glutathione (GSH) metabolism (MAPEG) and is the main contributor to PGE_2-related pathophysiology [25]. It has been shown that mPGES-1 preferentially couples functionally with the inducible COX-2 enzyme generating inflammatory PGE_2, whereas cPGES couples with COX-1 and mPGES-2 with COX-1 and COX-2 to maintain homeostasis via baseline PGE_2 production [26, 27]. PGE_2 exerts its biological effects through four receptors, namely the E-series of prostaglandin receptors (EP1-4) located in cell membranes. Each EP receptor is coupled to a different downstream signaling pathway. Activation of EP1 leads to an increase in intracellular Ca^{2+} via the Gq protein pathway [28]. The EP2 and EP4 receptors couple to the Gs protein, activating cAMP and PKA [22]. The EP4 receptor can additionally signal through a phosphatidylinositol 3- kinase (PI3K) dependent pathway [29]. The EP3 receptor exists in multiple isoforms that allow coupling to multiple G proteins. Primarily, EP3 inhibits cAMP levels via activation of Gi [30].

PGE_2 is involved in many physiological processes, such as regulation of blood flow, vascular permeability, smooth muscle cell function, renal filtration, gastrointestinal integrity, and female reproduction [31-35]. In addition to its function in maintaining homeostasis, PGE_2 is a key modulator of inflammation and pain and exerts its effects as a consequence of COX-dependent synthesis, EP receptor expression and degradation by 15-PGDH. PGE_2 is widely recognized as a main driver of acute inflammation. During the inflammatory response epithelial cells, fibroblasts and infiltrating immune cells are the main source of PGE_2 production [36]. PGE_2 initiates the inflammatory response, induces vasodilation, and mediates the recruitment of neutrophils, mast cells and macrophages [36-39]. Mast cells are primarily tissue-resident cells that are important for host defense and in allergic reactions. PGE_2 is involved in the local attraction, induction, and degranulation of mast cells via EP1 signaling [38, 40]. Moreover, PGE_2 has been shown to contribute to the induction of a migratory dendritic cell (DC) phenotype and to enhance the production of pro-inflammatory IFN-γ by DC [41]. In addition, PGE_2 promotes the activation of T_H17 cells, contributing to the production of pro-inflammatory IL-17, which facilitates the recruitment of monocytes and neutrophils in the initial phase of inflammation [42, 43]. Macrophages are one of the main sources of prostanoid production in inflammation. COX-2 derived PGE_2 production by macrophages following TLR4 activation by LPS leads to a positive feedback loop of autocrine and paracrine macrophage activation and recruitment [44]. However, PGE_2 is not only a mediator of acute inflammation, but also involved in the initiation and maintenance of chronic inflammation and mPGES-1 is overexpressed in several chronic inflammatory diseases [24, 45-47]. In macrophages for example, PGE_2 signaling through EP2 and via

activation of NF-$_k$B was shown to result in increased induction of COX-2 and the macrophage chemokine CCL2, establishing persisted inflammation in a mouse model of intracranial aneurism [48]. Moreover, PGE_2 has been shown to promote T_H1-cell differentiation and to enhance IL-22 production from T_H22 cells *in-vitro* via EP2 and EP4 thereby exacerbating chronic inflammation [49].

Although PGE_2 is widely known as one of the main drivers of inflammation and blocking PGE_2 production leads to reduction of inflammation and pain, PGE_2 also has immunosuppressive activity. PGE_2 has been shown to suppress neutrophil function and recruitment [50, 51], to support reprogramming of pro-inflammatory activated M1-type macrophages into alternatively activated M2-type macrophages [52], and to stimulate reprogramming of pro- to anti-inflammatory lipid mediators inducing the resolution of inflammation [53]. In addition, recent data suggest that PGE_2 contributes to the resolution of inflammation by promoting the clearance of activated myeloid cells from sites of inflammation [54] and that PGE_2 plays a role in maintaining immune tolerance during the post-resolution phase, thereby acting immuno-suppressive [55].

PGD_2 first considered as a minor and biologically inactive prostaglandin [56], was later discovered to be abundantly produced in the brain and important for several physiological and pathological functions [57, 58]. PGD_2 is produced by two PGD_2 synthases, the hematopoietic PGD synthase (H-PGDS) and the lipocalin-type PGD synthase (L-PGDS). H-PGDS belongs to the glutathione S-transferase (GST) family and isomerizes PGH_2 to PGD_2 [59]. H-PGDS is expressed in hematopoietic cells and especially in mast cells. The L-PGDS is GSH independent and initially considered to drive the production of PGD_2 in the brain [60]. Later L-PGDS was found in heart, kidney, and lung tissue for example [61-63]. PGD_2 exerts its biological effects via two receptors (DP1 and DP2 or CRTH2) that are expressed on a variety of inflammatory cells (e.g., dendritic cells and T_H1 cells, type 2 polarized lymphocytes, mast cells, monocytes and macrophages [64-66]). PGD_2 receptor 1 (DP1) couples to Gs proteins, raising intracellular cAMP levels. PGD_2 receptor 2, also called the chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2) couples to Gi proteins, decreasing intracellular cAMP levels and increasing intracellular Ca^{2+} [67, 68]. Physiological functions of PGD_2 include for example temperature regulation, hormone release and sleep regulation. Further, PGD_2 inhibits platelet aggregation and induces vasodilation via the DP1 receptor [69-71] but also acts as a broncho- and vasoconstrictor via activation of the thromboxane receptor (TP) [72]. PGD_2 is predominantly produced by mast cells and contributes to the pathogenesis of allergic diseases, primarily through the activation of DP2, stimulating eosinophil chemotaxis and degranulation as well as recruitment of immune cells to the airways [73-75]. PGD_2 synthesis and receptor signaling are therefore considered targets for anti-inflammatory treatment strategies in these diseases [76].

Moreover, PGD_2 is the source of several bioactive metabolites. PGD_2 can be metabolized to $9\alpha,11\beta$-$PGF_{2\alpha}$, which is a stereoisomer of $PGF_{2\alpha}$ and a marker of inflammation during anaphylaxis [77]. PGD_2 is also the precursor of the J series of cyclopentenone prostaglandins

including PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2). In contrast to Δ^{12}-PGJ_2, which is formed to large extend by the enzymatic action of albumin, 15dPGJ_2 is a non-enzymatic product formed from PGJ_2 by double bond rearrangement and dehydration [78]. 15dPGJ_2 has first been described in 1983 and is the most studied of the PGD_2 metabolites [79]. In addition to the pro-inflammatory effects described above, a growing body of research suggests that PGD_2 and its downstream product the 15dPGJ_2 play important roles in resolving the inflammatory response. The anti-inflammatory and pro-resolving effects of PGD_2 and 15dPGJ_2 are described in more detail in section 1.2.

$PGF_{2\alpha}$ was first isolated from human seminal fluid and found to stimulate smooth muscles [80]. Endogenously, $PGF_{2\alpha}$ is rapidly degraded to its stable metabolite 15-keto-dihydro-$PGF_{2\alpha}$ [81]. $PGF_{2\alpha}$ is formed by reduction of PGH_2 by PG endoperoxide synthase or reductase but it can also be formed from other prostaglandins such as PGE_2 or PGD_2 by 9-ketoreductase and 11-ketoreductase, respectively [82-84]. $PGF_{2\alpha}$ exerts its biological effects via binding to the prostaglandin F receptor (FP). FP couples to Gq proteins, leading to an increase in intracellular Ca^{2+} [85, 86]. FP has also been described to activate the small G protein Rho and to couple with Gi proteins [87, 88]. The FP receptor is the least sensitive of the prostaglandin receptors and responds to the binding of other prostaglandins, albeit to a lesser extent (PGD_2>PGE_2>PGI_2/TXA_2).

$PGF_{2\alpha}$ is abundantly produced in the female reproductive system and required for several physiological functions such as embryo development, labor induction and ovarian function [89-91]. $PGF_{2\alpha}$ acts as a vaso- and bronchoconstrictor via the receptors FP and TP and is involved in the stimulation of smooth-muscle cell hypertrophy [92, 93]. Moreover, $PGF_{2\alpha}$ is abundantly formed at sites of inflammation and found in synovial fluid of patients with chronic inflammatory diseases such as rheumatoid arthritis and osteoarthritis [94, 95]. In addition, $PGF_{2\alpha}$ has been suggested to play a role in the development and progression of cardiovascular diseases. $PGF_{2\alpha}$ was shown to promote cardiac fibrosis [96], to be released from endothelial cells [97], and to be involved in blood pressure control [98].

PGI_2 (prostacyclin) was first described by Moncada et al., in 1976 as "unstable substance (PGX) that inhibited human platelet aggregation" [99]. It has a short half-life of up to two minutes and breaks down at physiological pH to its stable inactive hydration product 6-keto-$PGF_{1\alpha}$. In urine, PGI_2 can be measured as 6-keto-$PGF_{1\alpha}$ and its major metabolite 2,3-dinor-6-keto-$PGF_{1\alpha}$ (also referred to as PGI_2-M), of which the latter is thought to reflect systemic PGI_2 production *in-vivo* [100]. PGI_2 is generated from PGH_2 by PGI_2 synthase (PGIS) which belongs to the cytochrome P450 enzyme family [101, 102]. PGIS has been shown to be expressed in various cell types including fibroblasts, dendritic cells, endothelial cells, and smooth muscle cells. PGI_2 signaling is mediated via the IP receptor, which couples to the Gs protein and increases cAMP levels which in turn stimulates the activation of PKA that subsequently regulates various signaling pathways [103].

In addition to activating the IP receptor, PGI_2 can also activate nuclear receptors such as PPARα, PPARβ and PPARγ, which have been shown to be present in human vascular smooth

muscle cells (VSMC) and endothelial cells *in-vitro* and are responsible for many pathophysiological events related to inflammation [104]. While PGI_2 is anti-inflammatory and protective in the vasculature, it is also associated with pathologies such as RA where elevated PGI_2 levels have been found in synovial fluid [105], and results from mouse models suggest that PGI_2-IP signaling increases inflammation and pain [106, 107]. During the onset of inflammation, PGI_2 together with PGE_2, plays an important role in increasing vascular permeability and dilatation, which contributes to an increased blood flow and edema formation [105]. Moreover, knock-out of the IP receptor in mice resulted in increased susceptibility to thrombosis and decreased inflammation and pain [108]. The role of PGI_2 in vascular biology is described in more detail in section 1.3.

TXA_2 (thromboxane A_2) is the major arachidonic acid metabolite produced by platelets and has potent and contrasting effects to PGI_2 on vascular tone and platelet function. TXA_2 is chemically unstable and is rapidly hydrolyzed to the stable inactive metabolite TXB_2 [109]. TXA_2 is generated from PGH_2 by TXA_2 synthase (TXS), a member of the cytochrome P450 enzyme family. In addition to platelets, TXA_2 synthase is also expressed in monocytes and macrophages [110-112]. Coupling of TXA_2 synthase and the constitutively expressed COX-1 contributes to the primary production of TXA_2 in humans whereby coupling to COX-2 enhances TXA_2 production during inflammation [113, 114]. TXA_2 exerts its biological functions via signaling through the TP receptor. In most tissues, there are two isoforms of the TP receptor (α and β), whereas in platelets only the TPα is expressed [115]. TP couples to the Gq protein, mobilizing intracellular Ca^{2+}. It has been shown that TP also couples to the G12,13 protein activating Rho-mediated signaling which results in platelet shape change and aggregation, among other effects [116].

TXA_2 is a potent constrictor that can cause platelet aggregation and vasoconstriction via activation of its TP receptor on platelets or VSMC in the vasculature, bronchi, intestine, and bladder [117-119]. Physiological roles of TXA_2 include platelet activation and aggregation as well as modulation of blood flow. However, an overproduction of TXA_2, contributes to several severe cardiovascular pathologies. It has been shown that TXA_2 contracts bronchial smooth muscle in asthma, is involved in diseases of the kidney and that TXA_2-mediated platelet aggregation and vasoconstriction can cause myocardial infarction (MI) [120-122]. Moreover, TXA_2-mediated platelet activation and interaction between leukocytes and endothelial cells contribute to the initiation and progression of atherosclerosis [123, 124]. For example, inhibition of TXA_2 synthesis for the preventive treatment of coronary heart disease by low-dose aspirin, which irreversibly inhibits COX-1 in platelets (they cannot reproduce COX-1 during their lifetime) is associated with a 30 % decreased risk for MI [125, 126].

1.1.3 Targeting COX-1/COX-2

The first description of an anti-pyretic and analgesic substance derived from the willow bark dates back to 1763 and was later identified as salicin [127]. In 1899, acetylsalicylic acid, also called “aspirin”, was introduced to the market, followed by other compounds such as indomethacin. Later in 1971, research by John Vane and colleagues demonstrated the inhibitory effect of aspirin on prostaglandins and further that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the enzymes responsible for prostaglandin synthesis [128-130]. It was discovered that cyclooxygenases (COX) are responsible for the conversion of arachidonic acid into prostaglandins and that they are targets of NSAIDs [131, 132].

Two COX isoforms COX-1 and COX-2 were discovered to catalyze the conversion of arachidonic acid to PGH_2. During inflammation it is COX-2 expression and COX-2 derived PGH_2 which are increased while COX-1 levels remain stable. It is generally believed that prostanoid production derived from COX-1 mainly enables homeostasis and prostanoids derived from COX-2 primarily play a role in inflammation and upon induction. The expression pattern of the COX-enzymes varies between tissues, and COX-inhibition may therefore have different effects in different tissues.

NSAIDs and COX-2 selective inhibitors (coxibs) have been used for many years as treatment to inhibit PGE_2 synthesis by inhibiting COX-1 and COX-2 to reduce inflammation and pain [133]. Except for aspirin, which covalently and irreversibly modifies COX-1 and COX-2 with increased selectivity for COX-1, other NSAIDs inhibit the activity of the enzymes by competing with arachidonic acid. Traditional NSAIDs such as aspirin, indomethacin, diclofenac, ibuprofen or naproxen inhibit both COX enzymes whereas COX-2 selective inhibitors (e.g., celecoxib, etoricoxib) have increased COX-2 selectivity [134]. Because of their anti-pyretic, anti-inflammatory, and anti-analgesic properties, NSAIDs are the first-choice treatment for many diseases. NSAIDs are commonly used in the general population for the treatment of minor aches and pains, and extensively for the treatment of chronic pain and inflammatory diseases such as osteoarthritis (OA), rheumatoid arthritis (RA), migraine, and musculoskeletal injuries [134]. Many chronic inflammatory diseases are age-related, and thus the use of NSAIDs also correlates with patient age [135, 136]. However, long-term treatment with dual COX inhibitors is associated with an increased risk of gastrointestinal ulceration. Inhibition of constitutive prostanoid production targeting COX-1 or COX-2 has been shown to result in an imbalance in prostanoid homeostasis and altered cell integrity, contributing to gastrointestinal side effects or reduced renal performance. COX-1 has been shown to be constitutively expressed in the gastrointestinal tract of humans, and PGE_2 as well as PGI_2 contribute to increased blood flow and reduction of gastric acid secretion [137]. Inhibition of COX-2 had been shown to be safer in terms of gastrointestinal side effects compared with traditional NSAIDs [138, 139], despite findings of constitutive expression of COX-2 in gastric mucosa from healthy human and rabbit [140]. In the kidney, prostanoids produced by both COX-enzymes play important roles in the maintenance of renal function [141]. COX-1 is constitutively expressed in the human vasculature and collecting ducts while COX-2 is constitutively expressed in epithelial cells and the kidney [142]. In animal studies

using COX-2 knock-out (KO) mice, severe nephropathy was observed, and in humans, treatment with NSAIDs resulted in sodium retention due to inhibition of COX-2 and a decrease in glomerular filtration rate due to COX-1 inhibition [143, 144].

Selective COX-2 inhibition was first assumed to be a safer alternative than COX-1 inhibition resulting in less gastrointestinal side effects due to inhibition of only the induced pro-inflammatory prostanoids. This led to the development of selective COX-2 inhibitors such as celecoxib (Celebrex®), rofecoxib (Vioxx®) and later valdecoxib (Bextra®), lumiracoxib (Prexige®) and etoricoxib (Arcoxia ®) [142, 145-148]. However, shortly after the launch of selective COX-2 inhibitors it became clear that blocking the inducible COX-2 in endothelial cells caused severe cardiovascular problems such as MI, pulmonary hypertension and heart failure [134, 149] and resulted in the withdrawal of rofecoxib and valdecoxib, and rejected FDA approval (US market) for lumiracoxib and etoricoxib [147, 148, 150, 151]. COX-2 inhibition causes an imbalance of pro-thrombotic TXA_2 (mainly produced by platelets via COX-1 in the vasculature) and anti-thrombotic PGI_2 (mainly produced by vascular cells via COX-2) [152, 153]. In contrast, aspirin used at low doses was shown to inhibit platelet COX-1 and TXA_2 production pre-systemically and due to first-pass effects spares PGI_2 resulting in anti-thrombotic properties [154, 155]. Deletion of the COX enzymes in mice demonstrated that vascular control by both enzymes is essential and today, COX-1 and COX-2 inhibitors except for low dose aspirin are associated with a negative class-effect on cardiovascular safety [156].

The gastric and cardiovascular side effects, associated particularly with conventional NSAIDs and coxibs, raise concerns about the use of these drugs, and alternatives are of interest and clinical need. Several strategies have been developed to overcome unwanted side effects such as dual COX and LOX inhibitors, nitric-oxide (NO)-NSAIDs, and mPGES-1 inhibitors. Arachidonic acid is converted to LTs when metabolized by 5-LOX. LTs are pro-inflammatory lipid mediators that can act as potent vasoconstrictors and have been shown to be involved in cardiovascular disease [157, 158]. Moreover, cysteinyl-leukotrienes (LTC_4, LTD_4, LTE_4) have been shown to be involved in gastric inflammation and bleeding [159]. The combination of COX-1/COX-2 and 5-LOX inhibition has been suggested to prevent the formation of TXA_2 in platelets, downregulate inflammatory prostanoids and counteract gastric damage by blocking LTs. Several dual COX/LOX inhibitors have been developed and licofelone for example was evaluated in clinical trials for the treatment of osteoarthritis and presented anti-inflammatory and, anti-analgesic and, fewer gastrointestinal side effects compared to conventional NSAIDS [160]. However concerns about renal and cardiovascular side effects comparable to COX-2 inhibitors may be the reasons that this drug has not progressed further since [161]. In order to overcome unwanted gastro-intestinal and cardiovascular side effects, NO-NSAIDs releasing NO and the NSAID became of interest. NO is a potent vasodilator and plays a role in gastrointestinal mucosa healing and defense. Pre-clinical studies indicate that NO-NSAIDs could be safer alternatives for the treatment of inflammation and hypertension [162, 163]. Although the compound naproxcinod (NO-naproxen) has entered clinical trial for the treatment of osteoarthritis, further studies

regarding long term cardiovascular and gastrointestinal safety as well as on the mechanisms of action are needed [164, 165]. Recently, inhibition of renal COX-2 was shown to induce cardiovascular dysfunction via upregulation of the endothelial nitric oxide synthase (eNOS) inhibitor asymmetrical dimethylarginine (ADMA) which is competing with eNOS for L-arginine [166]. The inhibition of the ADMA pathway suggests a cardioprotective role of COX-2 and supplementation of L-arginine may improve cardiovascular function after NSAID treatment. The effect of L-arginine supplementation is currently investigated in a clinical trial in healthy volunteers receiving celecoxib (ClinicalTrials.gov Identifier, NCT number: NCT04765644). Moreover, the selective inhibition of mPGES-1, the terminal synthase of PGE_2 downstream of COX, was suggested as alternative target. The hypothesized anti-inflammatory properties lie in the selective reduction of inflammatory PGE_2, without causing unwanted side effects by sparing the other prostaglandins. The role of mPGES-1 in inflammation and the effects of mPGES-1 inhibition are summarized in the following sections.

1.1.4 Targeting mPGES-1

Microsomal prostaglandin E synthase 1 (mPGES-1) is a microsomal, GSH-dependent and inducible enzyme identified by Jakobsson and colleagues in 1999 [25]. Initially mPGES-1 was referred to as MGST1-like 1 due to its homology (38% of amino acid sequence) with microsomal glutathione S-transferase 1 (MGST1). Both MGST1 and mPGES-1 are members of the MAPEG family [25]. The six mammalian members of this protein family are 5-lipoxygenase activating protein (FLAP), leukotriene C4-synthase (LTC4S), microsomal glutathione S-transferase 1 (MGST1), microsomal glutathione S-transferase 2 (MGST2), microsomal glutathione S-transferase 3 (MGST3), and mPGES-1. MAPEG proteins are integral membrane proteins that perform various functions such as glutathione transferase activity, glutathione dependent peroxidase activity or isomerase activity as well as substrate presentation in the case of FLAP [167, 168]. The enzyme mPGES-1 is a 16 kDa membrane protein with a homotrimeric confirmation where each monomer is organized with four transmembrane alpha-helices. Besides catalytic activity for PGE_2 production, mPGES-1 possess GST activity and peroxidase activity, although the latter are rather modest [169]. The tripeptide GSH is required as an essential co-factor for mPGES-1 activity and is thought to enter the active site through an access cavity from the cytosol [170]. Two amino acids (Arg-126 and Asp-49) close to the GSH molecule were found to be essential for the catalytic activity of mPGES-1 [171]. In addition, Thr-131, Leu-135 and Ala-138 were identified as key residues responsible for interspecies differences between the human and rat mPGES-1 enzyme [172]. The level of mPGES-1 is upregulated by inflammatory stimuli as shown early on in A549 cells treated with IL-1β [25]. Additional mechanisms of induction for mPGES-1 have been described, like LPS and TNFα [26, 173].

Role of mPGES-1 *in-vivo*. To understand the role of mPGES-1 in inflammation, mouse models with genetic deletion of mPGES-1 were developed. mPGES-1 knock-out mice (KO) do not differ from wild-type mice (WT) in terms of general physiological parameters

(body weight, behavior, tissue histology or appearance) [174]. However, macrophages from mPGES-1 KO mice stimulated with LPS were unable to produce PGE_2 above baseline levels without any effects on LPS-induced cytokine production (IL-6, IL-12, tumor necrosis factor α (TNF-α)) or cell viability [174, 175]. The mPGES-1 KO mouse model has been very useful for better understanding of the effects of mPGES-1 and mPGES-1 derived PGE_2 under physiological and pathological conditions.

Studies using mPGES-1 deficient mice demonstrated an important role of mPGES-1/PGE_2 in gastrointestinal and renal homeostasis. For example, it was shown that PGE_2 is needed for gastrointestinal homeostasis as EP4 activation ameliorated colitis symptoms and mucosal damage in a model of experimental colitis [176]. Moreover, basal renal functions (urinary osmolalities, blood pressure) are preserved in mPGES-1 KO compared to WT mice although PGE_2 excretion in urine was decreased by 50 % [177]. However, when fed a high salt diet mPGES-1 KO mice developed severe hypertension and impaired ability to excrete an acute salt load. Additionally high-salt induced expression of NO synthases was reduced in mPGES-1 KO mice, revealing a critical role for the mPGES-1/PGE_2/NO/cGMP pathway to adapt to salt loading [178]. In line, mPGES-1 KO mice showed delayed urinary PGE_2 excretion and reduced ability to excrete water after acute water load [179]. In a mouse model of chronic renal failure (based on surgical reduction of renal mass), deletion of mPGES-1 reduced renal and urinary PGE_2 excretion and improved renal failure while worsening anemia [180]. In a mouse model of renal fibrosis (based on unilateral ureteral obstruction causing tubular injury by an obstructed urine flow) the deletion of mPGES-1 led to an increase in inflammatory and fibrosis markers (such as interleukin-1β, transforming-growth factor (TGF)-β1, fibronectin and collagen III) [181], indicating a protective effect of mPGES-1 in renal inflammation and fibrosis.

The mPGES-1 enzyme has been proposed as an alternative target to COX, especially in conditions where continuous treatment with NSAIDs and coxibs would be beneficial. Understanding the role of mPGES-1 in inflammatory conditions with elevated PGE_2 levels such as rheumatic diseases has therefore been of major interest. In mice with collagen-induced arthritis (CIA) the deletion of mPGES-1 resulted in lower disease incidence and severity [174] and lower levels of anti-collagen II antibody [182]. In addition, edema formation was significantly reduced in mPGES-1 KO mice [174]. This was tested in a paw swelling model in mice injected locally with type II collagen. It was found that paw swelling was accompanied by leukocyte infiltration, which were attenuated in paws of mPGES-1 KO mice [174]. In mPGES-1 KO mice with collagen antibody-induced arthritis (CAIA), the disease severity was significantly reduced, accompanied with reduced PGE_2 levels in paws, without affecting the disease incidence compared to WT mice [183]. In the same study, only mild cartilage destruction and approximately 40 % less bone loss was observed in mPGES-1 KO mice with CAIA compared to WT controls. Further, mPGES-1 has been shown to be upregulated in atherogenesis and its deletion retarded the progress *in-vivo* [184]. In this study, mice lacking mPGES-1 and the low-density lipoprotein receptor showed a reduction in plaque burden and unaffected blood pressure was observed. Urinary PGE_2 metabolite was depressed, and PGI_2-

M was increased, while urinary TXB_2 metabolite levels were unaffected. Other studies focusing on the effects of PGE_2 and fever revealed that PGE_2 in the brain is a mediator of the cytokine induced febrile response following e.g., pathogen infection [185, 186]. LPS-treated mice lacking mPGES-1 showed similar PGE_2 formation as untreated control WT mice and did not develop a fever response [186]. Similar results were described for COX-2 deletion [185]. In addition, mPGES-1 was shown to regulate pro-inflammatory macrophage polarization and deletion of mPGES-1 upregulated anti-inflammatory gene expression in macrophages [187]. In contrast the deletion of COX-2 resulted in skewing of cultured macrophages towards the M1 type [188].

1.1.5 mPGES-1 inhibitor development

Interest in mPGES-1 as a pharmaceutical target has increased over the last decade, supported by increasing evidence from pre-clinical investigations. In recent years, several mPGES-1 inhibiting compounds have been developed, but most of these compounds had drawbacks such as interspecies differences, high lipophilicity, and poor selectivity and were therefore not suitable for pre-clinical and clinical investigations [189]. For mPGES-1 inhibitors to be suitable for use in pre-clinical studies and eventually clinical trials, certain criteria should ideally be met. The inhibitor needs to be highly potent against mPGES-1 and have increased selectivity over other enzymes in the prostanoid pathway. Another criterion is potency in a complex biological matrix such as whole blood. To enable pre-clinical *in-vivo* studies, sufficient bioavailability and inhibition of both the murine and human mPGES-1 would be ideal [190]. In order to meet the above mentioned criteria, pre-clinical evaluation of novel mPGES-1 inhibitors includes cell-free assays, cell-based assays and *in-vivo* animal models [191]. The cell free assays evaluate the inhibitory potential of the compounds towards a recombinant mPGES-1 enzyme, overexpressed in cells or bacteria. The enzyme-containing microsomal membrane fractions are isolated and utilized in enzyme activity assays. The enzyme activity assays provide information on the inhibitory potential of a compound of interest and allow simultaneous screening of a large number of compounds. A compound that gives adequate results (lead compound) will be further optimized and tested against other recombinant proteins in the prostaglandin cascade and from other species, and subsequently in cell-based assays and whole blood. To accomplish this, cell lines or primary cells are induced with inflammatory stimuli such as LPS, IL-1β or TNFα in the presence or absence of the compounds of interest and PGE_2 formation is monitored by LC-MS/MS or immunoassays. The whole blood assay allows determination of efficacy in a cell- and protein-rich environment that mimics an *in-vivo* like situation. Pre-clinical investigations based on animal experiments are critical for evaluating the pharmacokinetics and pharmacodynamics, efficacy and toxicity of lead compounds characterized in *in-vitro* experiments. Based on these criteria, several mPGES-1 inhibiting compounds have been reported and characterized. One of the first compounds described to inhibit mPGES-1 was the FLAP inhibitor MK-886. MK-886 inhibited recombinant rat and human mPGES-1 (IC_{50} = 3.2 µM and 1.6 µM, respectively) [192, 193] and was used as a reference in activity assays for later developed inhibitors. MK-886 showed only weak potency in cell-based assays and human whole blood, similar to

compounds developed on the basis of MK-886 (e.g. compound 23 and 30) making them inapplicable for *in-vivo* studies [192]. The first selective mPGES-1 inhibitor to show potency in cell-based assays and human whole blood was MF63. MF63 potently inhibited the activity of human recombinant mPGES-1 (IC_{50} = 1.3 nM), PGE_2 production in A549 cells (EC_{50} = 0.42 µM) with 50 % FBS present and, LPS induced PGE_2 production (EC_{50} = 1.3µM) in whole blood. In *in-vivo* studies with guinea pigs MF63 inhibited PGE_2 formation without affecting PGI_2 synthesis or causing gastrointestinal irritation. However, MF63 showed no activity towards rat or mouse mPGES-1 [194]. Since the publication of MF63 in 2005, several mPGES-1 inhibitors have been described; detailed descriptions of individual compounds can be found in the following articles [195-197]. Most mPGES-1 inhibiting compounds described in the literature lack cross-species inhibitory activity and are therefore not suited for *in-vivo* pre-clinical investigations. The first mPGES-1 inhibitors that inhibited both human and murine enzyme were quinazolinone 7 (FR20) and derivatives [198]. FR20 inhibited both recombinant human mPGES-1 (IC_{50} = 0.13 µM) as well as membrane fractions of murine RAW264.7 and NIH cells (8.4% and 10.7% inhibition at 1µM inhibitor concentration, respectively). Inhibition of PGE_2 in whole blood displayed medium potency with 51% residual production at 100 µM. Other examples of cross-species mPGES-1 inhibitors were: compound 8 and compound 26 by Eli Lilly [199], compound 28 [200], compound II (CII) [201], compound III (CIII) [202], compound 8n [203], compound A [204], and compound 4b [205]. CIII was characterized in 2013 as a cross-species mPGES-1 inhibitor that inhibited both human and rat recombinant enzyme (IC_{50} = 0.09 µM and 0.9 µM respectively) [202]. CIII showed no concentration dependent inhibition on other enzymes of the prostaglandin cascade (COX-1, COX-2, PGIS, H-PGDS and L-PGDS). In A549 cells CIII showed almost full inhibition of PGE_2 formation at 10 µM. In whole blood, CIII demonstrated dose-dependent inhibition of PGE_2 without affecting thromboxane synthesis. In mouse peritoneal macrophages CIII reduced PGE_2 production, whereas a shunting towards PGI_2 production was observed. *In-vivo* in the air-pouch model, CIII dose-dependently reduced carrageenan (CA)-induced cell migration into the pouch as well as PGE_2 synthesis in pouch exudates [202]. CIII has been studied in various models of inflammation and cancer (see below) and was also used in the studies presented in this thesis. Since most mPGES-1 inhibitors developed, lack cross-species inhibitory activity, characterization of mPGES-1 inhibition in pre-clinical models is limited, and only three compounds have reached clinical trials to date. The small number of pre-clinical and clinical investigations maintains the concerns of unwanted side effects similar to NSAIDs and leaves unresolved a clear indication which diseases might be the primary targets for an mPGES-1 inhibitor. One of the mPGES-1 inhibitors that reached phase I clinical trial in 2013 was LY3023703 developed by Eli Lilly [206]. LY3023703 inhibited LPS-induced PGE_2 production in blood of healthy volunteers *ex-vivo*. An increase in systemic PGI_2, as measured by the urinary metabolite PGI_2 metabolite was found. The program was however stopped due to compound specific liver toxicity observed in two study participants [207]. The second inhibitor to reach clinical trial in 2015 was GRC-27864 produced by Glenmark [208]. GRC-27864 was well tolerated by healthy volunteers up to a dose of 1000 mg. The compound inhibited LPS-induced PGE_2 production

ex-vivo in blood of healthy volunteers and urinary PGE_2 metabolite without affecting urinary PGI_2-M. Recently, the mPGES-1 inhibitor GS-248 developed by Gesynta Pharma AB reached clinical trial in 2019 and results from phase I investigations were reported [209]. GS-248 reduced LPS-induced PGE_2 synthesis in blood of healthy volunteers at subnanomolar concentrations. Urinary PGE_2 metabolite was significantly inhibited, and an increase of urinary PGI_2 metabolite was observed after treatment with GS-248. A phase II investigation to evaluate the safety and efficacy profile of GS-248 in Raynaud's syndrome in patients with systemic sclerosis has been completed (ClinicalTrials.gov Identifier, NCT number: NCT04744207). In the phase II study, GS-248 was well tolerated and potently inhibited mPGES-1 but no significant effects on symptoms of the patients were observed. A phase II investigation of GS-248 in endometriosis is currently prepared (https://www.gesynta.se/news-media).

Role of pharmacological inhibition of mPGES-1 *in-vivo*. In contrast to studies on mPGES-1 deletion, pre-clinical *in-vivo* studies on pharmacological inhibition of mPGES-1 are limited for the reasons mentioned above. A summary of recent advances in mPGES-1 inhibitor characterization and pre-clinical studies can be found in a review by Bergqvist et al., [197]. In the air-pouch model of inflammation (tested in rat and mouse), inhibition of mPGES-1 (by CII, CIII and compound 4b) significantly reduced pouch exudate PGE_2 and kidney PGE_2 [201, 202, 205]. In the adjuvant-induced arthritis model in rats, inhibition of mPGES-1 with CII and compound A significantly suppressed paw swelling [201, 204]. Further the inhibition of mPGES-1 with compound A was shown to have an anti-pyretic effect in LPS-induced fever [204]. Compared with celecoxib which caused bleeding ulcers in the stomach of mice at 50 mg/kg, the mPGES-1 inhibitor compound 4b showed no adverse effects in the stomach during a 14 day observation period [205]. In a mouse model of hepatic ischemia reperfusion, inhibition of mPGES-1 with CIII resulted in increased liver protection and accelerated liver repair compared with vehicle controls [210]. In a zymosan-induced peritonitis model, inhibition of mPGES-1 with CIII was shown to interfere with the resolution of inflammation [54]. This suggests that mPGES-1 derived PGE_2 contributed to the removal of neutrophils and retention of activated myeloid cells. Further, inhibition of mPGES-1 with CIII in a xenograft neuroblastoma mouse model blocked PGE_2 formation by cancer-associated fibroblast and reduced tumor growth [211].

Deletion of mPGES-1 as well as the pharmacological inhibition *in-vitro* and *in-vivo* are important pre-clinical tools for understanding the role of mPGES-1 and the potential of mPGES-1 as a therapeutic target. However, it should be noted that deletion and inhibition of mPGES-1 may not lead to the same results in terms of prostaglandin profile and modulation of pathological conditions most likely due to adaptation in the KO mice [202, 212]. Therefore, it is critical to characterize the effects of pharmacological inhibition of mPGES-1 on homeostasis, disease development, anti-inflammatory effects, and side effects in experimental models with inhibitors suited for pre-clinical investigations.

1.2 EFFECTS OF MPGES-1 INHIBITION ON INFLAMMATION – FOCUSING ON THE PGD_2 PATHWAY

Selective inhibition of mPGES-1 is a therapeutic strategy to suppress PGE_2 production. Selective suppression of PGE_2 spares other prostanoids or even promotes the increase of other prostanoids such as PGD_2 and PGI_2. The following section summarizes the functions of PGD_2 and its naturally occurring degradation product $15dPGJ_2$.

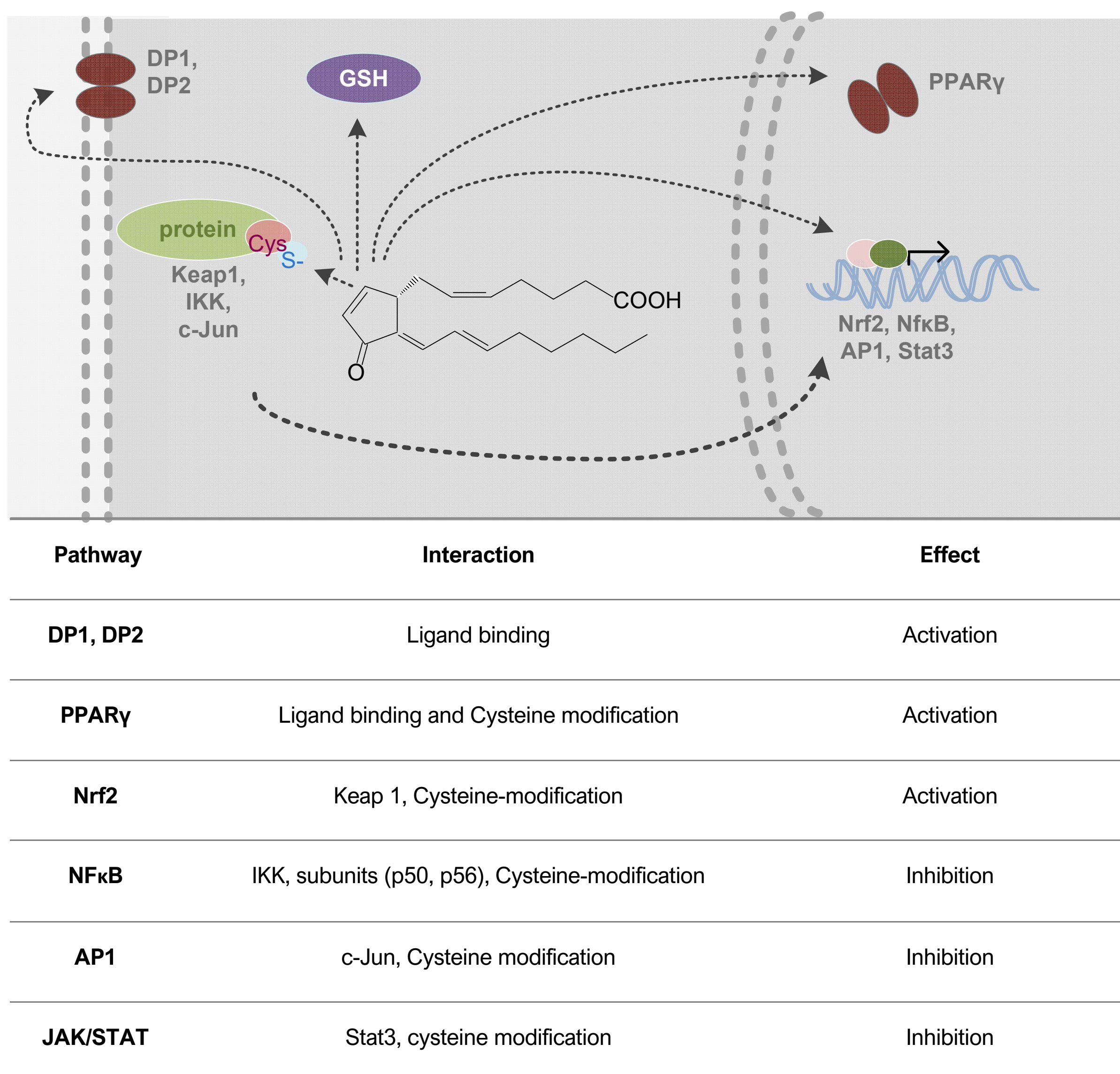
1.2.1 Anti-inflammatory and pro-resolving effects of PGD_2 and $15dPGJ_2$

As described earlier, PGD_2 can act as pro-inflammatory and anti-inflammatory mediator depending on the inflammatory setting. This section focuses on the anti-inflammatory properties of PGD_2 described in the literature. One of the first studies to suggest an anti-inflammatory role for COX-2 and PGD_2 was published by Gilroy and colleagues in 1999 [213]. In a model of CA-induced inflammation in rats, they assessed COX-2 protein expression, prostaglandin formation and inflammatory cell number after 2 and up to 48 hours. After 2 hours, the levels of COX-2 and PGE_2 were upregulated. Exudate cell numbers peaked at 12 hours. Interestingly, they found a second increase in COX-2 after 48 hours that did not correlate with increased PGE_2 levels or number of cells. Instead, an increase in PGD_2 and $15dPGJ_2$ was found. Moreover, inhibition of COX with the inhibitors NS-398 and indomethacin suppressed inflammation at 2 hours but exacerbated inflammation at 48 hours along with a reduction in PGD_2 and $15dPGJ_2$. This study suggested a dual role for COX-2 in the course of inflammation, with the underlying mechanism being a shift in downstream prostaglandin production. The same authors described the resolution phase as a process involving removal of inflammatory stimuli such as bacterial antigens, attenuation of pro-inflammatory signaling and elimination of pro-inflammatory mediators (for example via 15-PGDH that catabolizes PGE_2), followed by clearance of immune cells (remigration to circulation, lymphatic drainage or local cell death via for example autophagy, apoptosis or NETosis) and a post-resolution phase [1, 55]. COX-2 derived $15dPGJ_2$ has been further shown to induce apoptosis of polymorphonuclear leukocytes and macrophages and to exert anti-inflammatory effects through $NF_{K}B$ inhibition contributing to the resolution of inflammation [214-216]. In a mouse model of zymosan-induced peritonitis, PGD_2 levels peaked 2 hours after zymosan injection, whereas levels of $15dPGJ_2$ peaked between 6 and 24 hours [217]. PGD_2 and $15dPGJ_2$ were absent in exudates from WT controls at 0 hours and H-PGDS KOs. The early inflammatory response, measured at 2 hours post zymosan injection, was stronger in H-PGDS KO mice than in WT controls. Reduced IL-10 levels and increased TNFα levels were measured. This effect could be rescued by a DP1 receptor agonist. Furthermore, H-PGDS KO mice exhibited higher accumulation of macrophages in the peritoneal cavity at 24 hours post zymosan injection. A DP1 agonist and $15dPGJ_2$ alleviated macrophage accumulation. In summary, these studies suggested that PGD_2 plays a role in the onset and resolution of inflammation through its metabolite $15dPGJ_2$ [217]. In line, $15dPGJ_2$ has been shown to enhance resolution of inflammation by attenuating leukocyte infiltration and enhancing efferocytosis activity of macrophages mediated via Nrf2 signaling [218]. It has been shown that $15dPGJ_2$ is a ligand of PPARγ [219, 220], acts as a weak agonist for DP1 and activates DP2 with similar potency as PGD_2 in human eosinophils

[221]. In addition, activation of PPARγ by $15dPGJ_2$ has been suggested to result in M2 polarized macrophages [222, 223].

1.2.2 Characteristics of $15dPGJ_2$

The $15dPGJ_2$ molecule contains unsaturated carbonyl moieties in the cyclopentenone ring structure, resulting in high reactivity towards cell nucleophiles such as cysteine sulfhydryl groups. It has been shown that $15dPGJ_2$ binds to the cysteine-containing tripeptide GSH and thiol groups in proteins such as Keap1, AP1, Stat3 or $NF_{\kappa}B$ (illustrated in Figure 2) via Michael addition [224, 225]. Furthermore, it was shown that the primary site of conjugation to GSH and proteins is the electrophilic center at C9 within the cyclopentenone ring of $15dPGJ_2$ [226, 227]. Probably due to the high reactivity of $15dPGJ_2$ and instrumentation limitations the determination of its abundance in biological samples has been challenging and its bioactive role has therefore been questioned [228]. However, as described above, several studies in recent years have reported measurements of $15dPGJ_2$ in its unconjugated form, demonstrating its presence *in-vitro* and *in-vivo*. As reviewed in [229], $15dPGJ_2$ and other cyclopentenone prostaglandins (PGA_2, PGJ_2) have a wide range of functions including suppression of inflammation, induction of apoptosis, inhibition of cell growth, anti-tumor and anti-viral effects. The therapeutic potential of $15dPGJ_2$ was further investigated in different models of inflammation *in-vivo*. In a CIA model in mice, treatment with $15dPGJ_2$ (1mg/kg, daily for 7 days) resulted in improvement of arthritis symptoms as measured by reduced inflammatory cytokines (IL-12, IL-17, TNF-α, INF-γ) in the joints, reduction of edema and pain [230]. *In-vitro* experiments suggested that suppression of T_H17 cells by $15dPGJ_2$ via upregulation of T_{reg} cells is responsible for the protective effects of $15dPGJ_2$ treatment seen in these mice [230]. In a rat model of adjuvant-induced arthritis, intraperitoneal administration of $15dPGJ_2$ after the onset of arthritis (day 7) suppressed the progression of arthritis as measured by paw volume and arthritis score [231]. Injection of 1 mg/kg $15dPGJ_2$ significantly reduced paw volume after 16 days of treatment. In comparison, the effect of PPARγ agonist troglitazone on paw volume reached significance after 14 days of treatment at a much higher dose of 100 mg/kg. Treatment with $15dPGJ_2$ markedly decreased the infiltration of inflammatory cells in the joints as well as the formation of pannus (invasive synovial tissue) [231]. In a mouse model of acute inflammation with CA-induced pleurisy (inflammation of the pleura, tissue layer separating the lungs from chest wall), $15dPGJ_2$ was shown to regulate inflammation through Nrf2 activation [232]. In this study, a persistent inflammation with continuous neutrophile accumulation and a delay in macrophage recruitment were observed in Nrf2 KO mice. Inhibition of COX-2 with the inhibitor NS-398 resulted in a similar persistent inflammatory profile as seen for Nrf2 KO. Administration of $15dPGJ_2$ to the pleural space of mice treated with NS-398 reversed the accumulation of neutrophils and restored normal recruitment of macrophages which was not seen when animals lacking Nrf2 were treated with $15dPGJ_2$. In addition, expression of antioxidant target genes of Nrf2 (heme oxygenase-1, HO-1; peroxiredoxin 1, PrxI) was repressed by inhibition of COX-2 and activated by treatment with $15dPGJ_2$. These results indicated that $15dPGJ_2$ activates Nrf2 and antioxidant target genes, thereby regulating inflammation [232].



Pathway	Interaction	Effect
DP1, DP2	Ligand binding	Activation
PPARγ	Ligand binding and Cysteine modification	Activation
Nrf2	Keap 1, Cysteine-modification	Activation
NFκB	IKK, subunits (p50, p56), Cysteine-modification	Inhibition
AP1	c-Jun, Cysteine modification	Inhibition
JAK/STAT	Stat3, cysteine modification	Inhibition

Figure 2: Selected inflammatory pathways regulated by $15dPGJ_2$-protein interaction. Illustrated and summarized based on [229, 233]. $15dPGJ_2$ can interact with receptors and target proteins through ligand binding or cysteine-modification thereby inhibiting or activating signal transduction. Abbreviations: Nrf2 (Nuclear factor erythroid 2-related factor 2), Keap-1 (Kelch-like ECH-associated protein 1), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), IKK (I kappa B kinase), AP1 (Activator protein 1), PPARγ (Peroxisome Proliferator Activated Receptor Gamma), Stat3 (Signal transducer and activator of transcription 3), JAK (Janus kinase), DP (Prostaglandin D_2 receptor).

GSH is the most abundant cellular nucleophile and glutathionylation is a cellular protection mechanism for electrophile inactivation and excretion. For example, conjugation of $15dPGJ_2$ with GSH was shown to diminish its activity on PPARγ, thereby regulating its bioactivity [234]. In a study overexpressing the efflux transporters MRP1 and MRP3, it was shown that both transporters were able to export $15dPGJ_2$-GS and thereby attenuating the effects of $15dPGJ_2$ on PPARγ target gene expression. This effect was reversed when intracellular GSH was depleted, leaving the reactive free $15dPGJ_2$ able to interact with PPARγ [226]. However, besides the detoxification function, the conjugation to GSH can also be involved in the biosynthesis of bioactive molecules such as the cysteinyl-leukotrienes (LTC4, LTD4, LTE4) [235]. Similarly, extracellular and endogenous $15dPGJ_2$ have been shown to conjugate to GSH in cell culture [226, 236]. Brunoldi and colleagues demonstrated that the $15dPGJ_2$-GS

conjugate was further metabolized to a cysteine-conjugate in HepG2 cells by reduction at the cyclopentenone ring and removal of glutamic acid and glycine (schematic illustration in Figure 3). Although the conjugation reaction of 15dPGJ$_2$ and GSH can occur non-enzymatically, several studies suggest GST to be involved in the formation of cyclopentenone-GS conjugates [227, 234, 237, 238]. The extent to which 15dPGJ$_2$ conjugates are formed within intact cells and whether their formation is enzymatically regulated as well as their biological activity remains to be investigated.

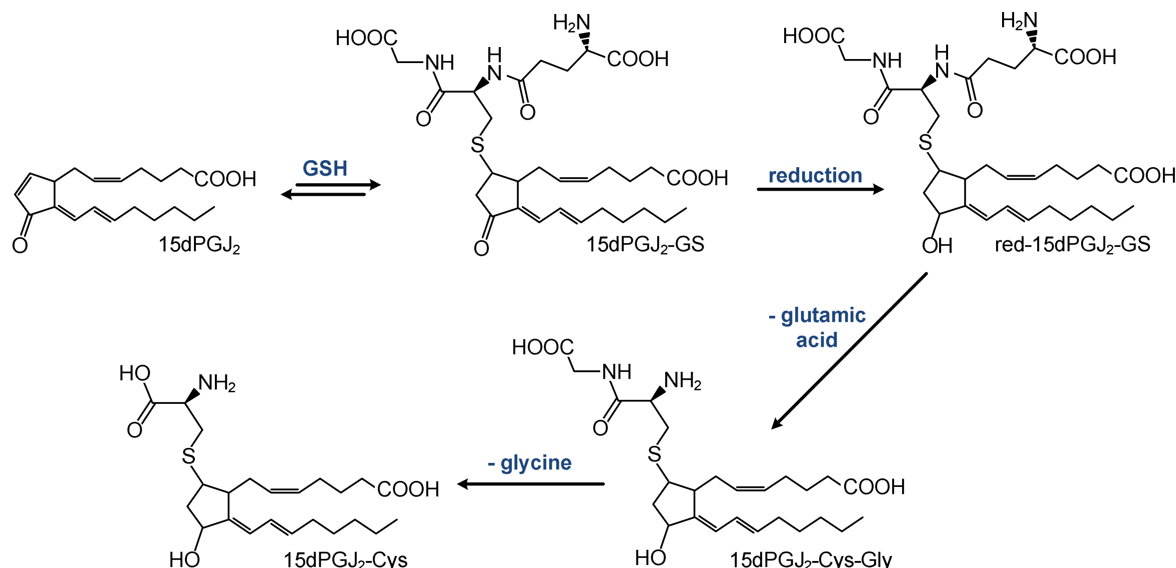


Figure 3: Metabolism of 15dPGJ$_2$ via conjugation to GSH as suggested in [236]. The sulfhydryl group in the cysteine moiety of the GSH molecule is reversibly conjugated to the unsaturated carbonyl at C9 in the cyclopentenone ring. This is followed by reduction of the carbonyl group at C11 (red-15dPGJ$_2$-GS) and removal of glutamic acid (15dPGJ$_2$-Cys-Gly) and glycine, resulting in the 15dPGJ$_2$-cysteine (15dPGJ$_2$-Cys) as the final metabolite.

1.2.3 Effects of mPGES-1 depletion on the PGD$_2$ pathway

Depletion of mPGES-1 may result in a redirection of PGH$_2$ to other anti-inflammatory arachidonic acid metabolites. A shunting towards the PGD$_2$ pathway for example has been described in bone marrow derived dendritic cells from mPGES-1 KO mice [239]. Moreover, in an angiotensin-II induced aortic aneurism mouse model, deletion of mPGES-1 reduced angiotensin-II induced PGE$_2$ formation in urine and enhanced angiotensin-II induced production of PGD$_2$ and PGI$_2$ [240]. This redistribution of PGH$_2$ was associated with a reduction in oxidative stress and a protection from aortic aneurism formation. Furthermore, the levels of the PGD$_2$ metabolite 15dPGJ$_2$ were increased in LPS treated mouse peritoneal macrophages lacking mPGES-1 [241]. The PGD$_2$ pathway could contribute to the resolution of inflammation and shunting into this pathway might occur after pharmacological inhibition of mPGES-1. A better understanding of this pathway and how it is affected by inhibition of mPGES-1 will help to estimate the therapeutic potential of mPGES-1 inhibitors.

1.3 EFFECTS OF MPGES-1 INHIBITION ON INFLAMMATION AND VASCULAR TONE

Inhibition of mPGES-1 selectively blocks the formation of PGE_2 and spares other prostanoids or even promotes their increase. Inhibition of mPGES-1 has been shown to result in a shunting towards PGI_2 in humans [206, 209]. This is of particular interest in view of the feared treatment-related cardiovascular complications associated with modulation of the prostanoid system in the vasculature. The following section gives a brief overview of the cardiovascular system, the vasoactivity of prostanoids, and the role of mPGES-1 in some cardiovascular diseases.

1.3.1 The cardiovascular system

The cardiovascular system is an organ system that circulates blood carrying immune cells, nutrients, oxygen and hormones through blood vessels via the heart to maintain homeostasis and respond to imbalances (a simplified model of blood circulation is shown in Figure 4a). The blood vessel wall consists of three layers, the tunica intima (inner layer containing endothelial cells), the tunica media (middle layer containing smooth muscle cells supported by connective tissue) and the tunica externa (outer layer consisting of connective tissue primarily composed of collagen fibers supporting the vessel). Depending on their location and function, blood vessels can be categorized in arteries (elastic, muscular and arterioles), capillaries, venules and veins (illustrated in Figure 4b and 4c) [242]. Arteries typically have thicker walls and smaller lumen with a rounder cross-sectional appearance than veins. Elastic arteries (typically larger than Ø 10 mm) such as the aorta, pulmonary artery and carotid artery are the largest arteries with a large number of elastic fibers and collagen. Elastic arteries branch further into muscular arteries (Ø < 10 mm) such as the brachial, femoral and radial arteries. Muscular arteries are characterized by a thick tunica media with a large layer of VSMC that allow to constrict and relax in response to sympathetic regulation, shear stress and other vasoactive stimuli. In contrast to elastic arteries, muscular arteries have a reduced number of elastic fibers which limits their elasticity [243]. As the vascular tree branches further, muscular arteries transition through resistance arteries (100-400 µm) into arterioles (<100 µm) and capillaries forming parts of the microcirculation [244]. Resistance arteries as the site of maximal resistance are primarily contributing to the regulation of organ blood flow. Due to their smooth muscle cell layer and maintenance of partial constriction, resistance arteries can adjust blood flow on demand.

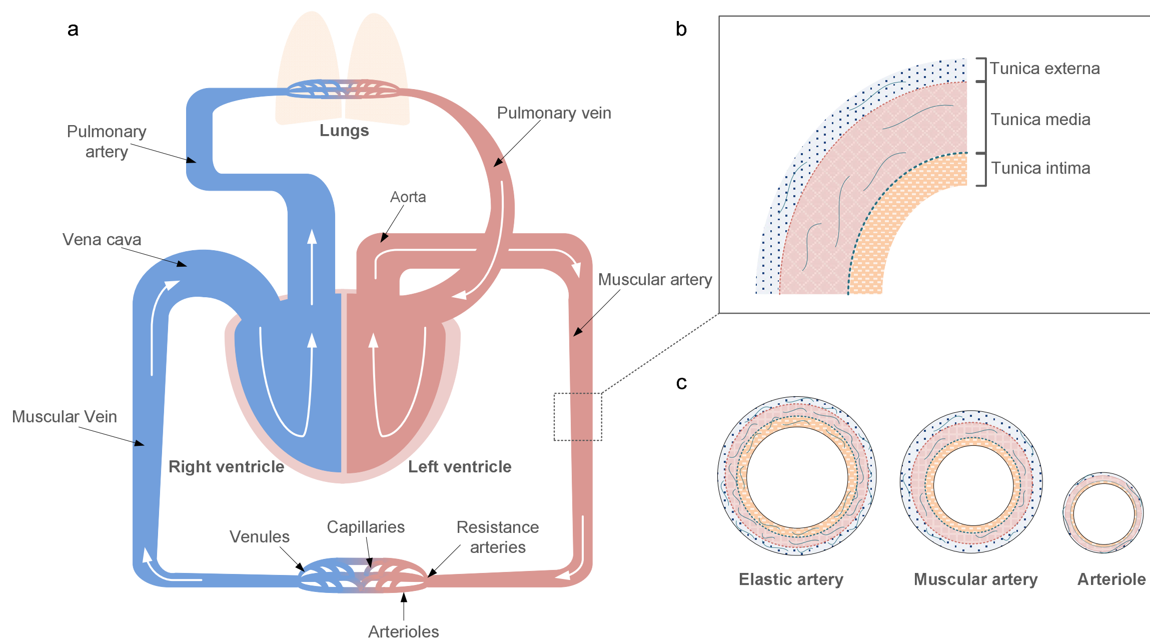


Figure 4: Simplified schematic illustration of the blood circulation and blood vessels. a) Oxygen-rich blood is transported from the heart (left ventricle) through the aorta and smaller arteries to the capillary network where oxygen and other components are released into the peripheral tissue. The blood low in oxygen is then transported through veins back to the heart, through the right ventricle and the pulmonary artery reaching the capillary network in the lungs. In the lungs blood gets oxygenated and circulates back to the left heart. b) The blood vessel wall consists of three layers, the tunica intima (inner layer containing endothelia cells), the tunica media (middle layer containing smooth muscle cells supported by connective tissue) and the tunica externa (outer layer consisting of connective tissue primarily composed of collagen fibers supporting the vessel). c) Artery types, elastic artery (large number of elastic fibers), muscular artery (fewer elastic fibers and more VSMCs) and arterioles.

1.3.2 Prostanoids and the regulation of vascular tone

Vascular tone describes the contractility (degree of constriction based on the contractility of vascular smooth muscle cells) of a vessel. Vascular tone regulates blood pressure and blood flow through the circulation. Constriction of vascular smooth muscle cells occurs in response to different signaling pathways resulting in Ca^{2+} dependent interaction of actin and myosin filaments [245]. An increase in cytoplasmic Ca^{2+} can occur either from channels on the plasma membrane or from the sarcoplasmic reticulum (SR), primarily through the activation of Gq-protein coupled receptors (illustrated in Figure 5). Gq binding causes hydrolysis of IP3 which then opens Ca^{2+} channels in the SR through binding of its (IP3) receptor. Ca^{2+} in the cytosol binds to calmodulin, which activates myosin light chain (MLC) kinase. Phosphorylation of MLC in turn enables interaction of actin and myosin filaments, resulting in constriction [246]. The major Gq-coupled vasoconstrictors are TXA_2, endothelin-1, angiotensin-II, vasopressin and norepinephrine. Phosphorylation of MLC can also be regulated by intracellular cAMP. When intracellular cAMP levels are decreased by Gi-coupled signaling constriction is achieved. Inhibition of dephosphorylation of MLC and thus constriction of VSMC can occur through Rho-kinase activated by Gq-protein signaling [245].

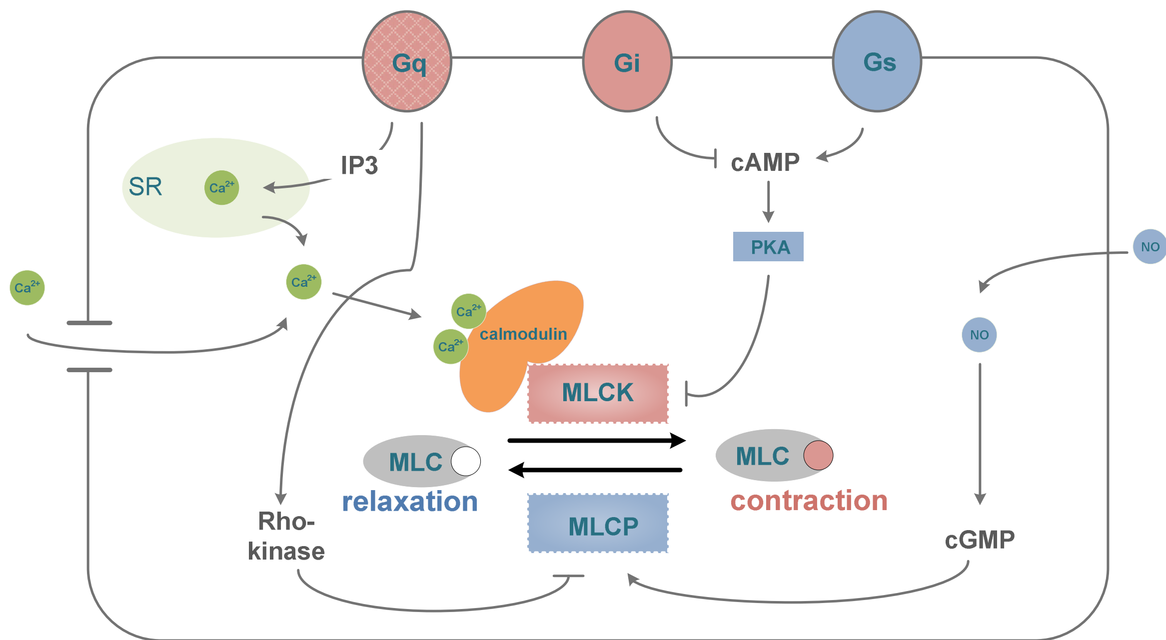


Figure 5: Simplified schematic illustration of VSMC activation by GPCR signaling and NO. Activation of signaling pathways is indicated by ► and inhibition by ┤. Abbreviations: MLCK (myosin light chain kinase), MLCP (myosin light chain phosphatase), SR (sarcoplasmic reticulum), cGMP (cyclic guanosine monophosphate), cAMP (cyclic adenosine monophosphate), IP3 (inositol triphosphate), PKA (protein kinase A), NO (nitric oxide).

Relaxation of VSMC occurs in response to decreased MLC phosphorylation and can be induced by reduced intracellular Ca^{2+} concentrations, inhibition of MLC phosphorylation or activation of MLC dephosphorylation. Inhibition of MLC phosphorylation is mediated by increased cAMP levels and subsequent activation of PKA that phosphorylates MLCK to inhibit its action. Dephosphorylation of MLC and consequent relaxation of VSMC results upon increase in intracellular cGMP levels. PGI_2 is a major endothelium-derived vasodilator that increases intracellular cAMP levels through activation of IP, a Gs-coupled receptor. Besides PGI_2, nitric oxide (NO) and endothelium-derived hyperpolarization factor (EDHF) are mechanisms for endothelium dependent relaxation. NO induces MLC dephosphorylation by increasing cGMP levels in VSMC. NO can be produced enzymatically and non-enzymatically in the vasculature. Enzymatically NO is produced by nitric oxide synthases (NOS), which utilize L-arginine and convert it to L-citrulline and NO, which can then regulate vascular tone in a paracrine or endocrine manner. In the endothelium, NOS (eNOS) is constitutively expressed and regulated by intracellular Ca^{2+} levels which in turn respond to inflammatory mediators [247]. The inactive eNOS is stored in the cell membranes and released to convert L-arginin to NO when Ca^{2+} levels are increased, for instance by NO agonists like bradykinin (BK) or acetylcholine (ACh). NO production is regulated not only by intracellular Ca^{2+} levels but also through phosphorylation of eNOS by PKA, which in turn is induced by shear stress [242]. NO diffuses from the endothelial cell into the VSMC where it binds to guanylyl cyclase, which dephosphorylates GTP to cGMP, resulting in relaxation [248]. EDHF causes vessel relaxation independent on NO and PGI_2. Several factors are suggested as potential EDHF candidates, including e.g., CYP450 products of arachidonic acid (epoxyeicosatrienoic acids), H_2O_2, potassium ions, gap junctions and C-type natriuretic

peptide [249]. Hyperpolarization of VSMC by EDHF results in closing of Ca^{2+} channels and VSMC relaxation [242]. Among the prostanoids, PGI_2 and TXA_2 are mostly involved in regulating vascular tone, however depending on lipid mediator production and receptor expression, PGE_2, PGD_2 and $PGF_{2\alpha}$ also modulate vascular effects (highlighted in Table 1).

Table 1: VSMC activation by G-protein coupled receptors. All prostnoids can act through the other than their primary receptors, however with lower affinity.

Signaling pathway	Second messenger	Primary Receptor	Agonist	Vascular effect
Gq	IP3 → Ca^{2+}↑, Rho-kinase ↑	TP	TXA_2	constriction
		FP	$PGF_{2\alpha}$	
		EP1	PGE_2	
		A1-adrenoreceptor	norepinephrine	
		ET1 receptor	Endothelin-1	
		AT1 receptor	Angiotensin-II	
		V1 receptor	vasopressin	
Gi	cAMP↓	EP3	PGE_2	constriction
		DP2 (CRTH2)	PGD_2	
		A2-adrenoreceptor	norepinephrine	
Gs	cAMP↑	IP	PGI_2	relaxation
		EP2	PGE_2	
		EP4	PGE_2	
		DP1	PGD_2	
		B2-adrenoreceptor	epinephrine	

1.3.3 Effects of mPGES-1 depletion on vascular biology

The severe cardiovascular side effects of NSAIDs are widely ascribed to be caused by an imbalance in pro-thrombotic versus anti-thrombotic mediators resulting in thrombotic events, increased arterial blood pressure and heart failure. While inhibition of COX results in a decrease in PGI_2 levels, platelet derived TXA_2 levels are maintained. COX-2 inhibition is associated with approximately one third increased risk of major cardiovascular events in patients treated with selective NSAIDs and due to the functional coupling of mPGES-1 with COX-2, adverse cardiovascular effects following mPGES-1 inhibition were systematically assumed [250]. However, recent studies indicate a cardiovascular protective profile of mPGES-1 inhibition via shunting towards PGI_2. Today, PGI_2 is considered the most important cardioprotective prostanoid, produced mainly by the endothelial cells in the vessel wall. As with all prostanoids, the functions of PGI_2 are diverse and include maintenance of homeostasis by inhibition of platelet aggregation, and vasodilation of blood vessels, as well as acting as mediator of inflammation. It is not entirely clear, whether the production of PGI_2 in the vasculature is largely driven by COX-1 or COX-2 [251, 252].

Thrombosis and vascular tone. As described above vessel tone depends on the interplay of vasoconstrictors and vasodilators that regulate constriction and relaxation of the VSMC. TXA_2 and PGI_2 are important regulators of vascular tone. In mice lacking mPGES-1, reduced urinary PGE_2 levels, no effects on TXA_2 and increased urinary PGI_2 metabolite production were observed [253]. Furthermore, deletion of mPGES-1 did not increase thrombogenesis in response to thrombogenic stimuli, nor did it increase blood pressure in contrast to COX-2 deletion or inhibition [253]. Thrombosis describes the obstruction of blood flow through blood vessels due to blood clotting. Platelet activation is critical for the coagulation process, which is triggered by disruption of the endothelium and release of for example thrombin or TXA_2. Platelets constitutively express COX-1 and contribute to the majority of TXA_2 biosynthesis in humans [254]. TXA_2, like the other mediators act autocrine on platelets through its receptors multiplying its formation and release. TXA_2 is not only involved in platelet activation but also in platelet-shape, degranulation, and aggregation. PGI_2 in turn counteracts TXA_2 effects and inhibits platelets via stimulation of cAMP formation [255]. It was also shown that platelet activation, and aggregation as well as release of platelet-derived microparticles were reduced in mPGES-1 KO mice treated with LPS compared with WT controls [256]. In addition, the inhibition of mPGES-1 with CIII in human vessels *ex-vivo* showed decreased vascular tone through shunting to PGI_2 [257].

Cardiovascular disease models. In mice with MI due to left coronary artery ligation, global deletion of mPGES-1 did not alter the post-MI cardiac dysfunction observed in WT animals [258]. Moreover, myeloid specific deletion of mPGES-1 improved survival after MI, and no adverse cardiac remodeling was found [258]. Furthermore, deletion of mPGES-1 did not reduce survival after MI, nor did it increase ischemic myocardial damage 24 hours post coronary artery ligation in mice, in contrast to WT mice treated with celecoxib [259]. Expression of COX-2 was attenuated in hearts from mice lacking mPGES-1, suggesting a positive feedback regulation of the two enzymes contributing to ischemic myocardial

damage. However, in other studies, it was shown that in a mouse model of MI, global deletion of mPGES-1 or its deletion in bone marrow derived myeloid cells, impaired cardiac remodeling after MI [260, 261].

In a mouse model of angiotensin-II induced aneurism formation, deletion of mPGES-1 delayed aneurism formation in hyperlipidemic mice associated with reduced urinary PGE_2 and enhanced urinary PGD_2 and PGI_2 metabolites [240]. In mice with injury of the femoral artery deletion of mPGES-1 reduced VSMC proliferation and hyperplasia after vascular injury [262]. Deletion of mPGES-1 has been shown to increase PGIS and also TXS expression in endothelial cells lacking the low-density lipoprotein receptor [184]. In the same study, LPS stimulation of VSMC and macrophages lacking mPGES-1 resulted in a shunting towards PGI_2 and also TXA_2 production [184].

Besides reduction of PGE_2 and shunting to PGI_2, inhibition of mPGES-1 could also be cardioprotective by sparing of the endothelial nitric oxide synthase inhibitor ADMA pathway. In contrast to COX-2 inhibition, plasma levels of PGI_2 were increased in mPGES-1 KO mice and no effect on genes of the ADMA pathway could be observed. PGI_2 and PGE_2 may regulate ADMA levels through IP, PPARβ/δ and the EP4 receptors [263].

2 RESEARCH AIMS

The overall aim of this thesis was to study the effects of inhibition of mPGES-1 on inflammation and cardiovascular disease. Specifically, to gain a better understanding of the underlying mechanisms focusing on the re-direction of PGH_2 after mPGES-1 inhibition into the PGD_2/15dPGJ_2 and the PGI_2 pathways.

Specific aims of individual studies included in this thesis were to:

- characterize new inhibitors of mPGES-1 with cross-species inhibitory activity in pre-clinical models of inflammation and vascular tone **(Paper I)**.

- study the biosynthesis and metabolism of the PGD_2/15dPGJ_2 pathway in immune cells and to investigate how inhibition of mPGES-1 affects this pathway **(Paper II)**.

- assess the effects of mPGES-1 inhibition on human microvascular tone *ex-vivo* under normal and disease conditions **(Paper III)**.

- study the effects of mPGES-1 inhibition on cardiac function after myocardial infarction in mice **(Paper IV)**.

3 METHODOLOGICAL APPROACH

The following section describes the research approach used by the author and includes general aspects of primary methods, statistical analysis, and ethical considerations. More detailed descriptions of the methods and additional techniques applied by the author and co-authors can be found in the respective papers.

3.1 ENZYME ACTIVITY ASSAYS

In-vitro enzyme activity assays can be used to determine the catalytic activity of an enzyme towards product formation. In such assays, a substrate is generally incubated with the enzyme and product formation is evaluated under given assay conditions. In the same assay, the activity of an inhibitory compound can be measured by evaluation of product formation after incubation of the substrate with the enzyme and the inhibitory compound. In the following the two enzyme activity assays that were used in **Paper I** and **Paper II** are described.

mPGES-1 activity assay

The mPGES-1 enzyme activity assay is an *in-vitro* assay to determine the inhibitory potency of a compound towards mPGES-1. PGE_2 is generated by mPGES-1 via isomerization of PGH_2. Classically, the formation of PGE_2 is measured after incubation of the mPGES-1 membrane fraction or purified recombinant mPGES-1 with an inhibitory compound and the substrate PGH_2. Pre-incubation of the mPGES-1 enzyme with the inhibitor test compound is typically carried out at 4°C for 30 minutes. Then the substrate PGH_2 is added, and the reaction at RT is stopped after 60-90 seconds by adding citric acid and $FeCl_2$. The addition of $FeCl_2$ non-enzymatically decomposes any remaining substrate to malondialdehyde (MDA) and 12-hydroxyheptadecatrienoic acid (12-HHT) (see Figure 1). The individual incubations are kept on ice and spiked with internal standard and subsequently processed by solid phase extraction for LC-MS/MS analysis of PGE_2 [171, 264]. Alternatively, enzyme activity can be assessed indirectly by measuring the PGH_2 degradation product MDA, which forms a red 2-thiobarbituric acid conjugate (MDA-TBA), in a colorimetric assay. The latter enables robust, high-throughput screening of test compounds as it can be performed in a 96-well format and does not require time-consuming sample extraction and LC-MS/MS analysis. However, no absolute quantification of product formation is possible and as substrate residues are measured low enzyme activity cannot be measured accurately. The MDA-TBA assay was performed according to a previously established method [265] and applied in **Paper I**.

Briefly, recombinant membrane fraction of mPGES-1 (rat) was pre-incubated with the test compounds at 4°C for 30 minutes in 0.1 M sodium phosphate buffer supplemented with 2.5 mM GSH. The test compounds were prepared in a 96-well plate in DMSO at concentrations ranging from low nM up to µM. The substrate PGH_2 is unstable in aqueous solution and degrades non-enzymatically to PGE_2, PGD_2 and $PGF_{2\alpha}$. PGH_2 was therefore kept on dry-ice and only 5 minutes prior to the assay aliquoted in acetone on wet-ice. PGH_2 was then added to the enzyme-compound mixture (10 µM final concentration) and incubated for 90 seconds at room temperature. To stop the reaction and decompose any remaining PGH_2 to MDA and 12-

HHT an excess of $FeCl_2$ in citric acid pH 3 was added. Pre-heated (80°C) TBA was added, and the plates were incubated at 80°C for 30 minutes. The MDA-TBA conjugate has an absorbance maximum at 535 nm [266] and can be measured using absorbance at 530 nm (subtracting absorbance at 560 nm) or fluorescence with excitation at 485 nm and emission at 545 nm [265]. Each sample was analyzed in duplicates. The reaction without inhibitory compound present (vehicle) served as positive control for PGH_2 to PGE_2 conversion and a reaction without enzyme and with denatured enzyme (boiled for 5 minutes) served as negative controls. In each experiment a reference compound (CIII at 10 µM in activity assays with rat mPGES-1) was tested, and the inhibitory potency of the test compound was expressed as percentage of the reference compound: Inhibition = (Test compound – Positive) / (Reference – Positive) x 100.

MAPEG activity assays

In **Paper II**, enzymes of the MAPEG family, namely MGST1, MGST2, MGST3, LTC4S, mPGES-1 and FLAP were tested for their activity to form $15dPGJ_2$-GS. The substrate $15dPGJ_2$ was incubated with the respective enzymes in the presence of GSH. Product formation was assessed by LC-MS/MS analysis with external standard curve or by HPLC separation and quantification of its UV absorbance relative to a spiked standard.

In brief, recombinant purified enzymes (MGST1, MGST2, MGST3, LTC4, FLAP, mPGES-1) were incubated with $15dPGJ_2$ in reaction buffer containing 25 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.03 % DDM and 0.06 mM GSH. The reaction was carried out at room temperature with gentle shaking for 5 minutes. The reaction was stopped with 0.5 % formic acid and the reaction tubes were placed on wet-ice until solid phase extraction. Samples were spiked with internal standard, extracted, and analyzed by LC-MS/MS for $15dPGJ_2$-GS formation. Solid phase extraction and LC-MS/MS settings are described below. Each reaction was performed in triplicates, and control reactions without enzyme or substrate were included. This approach allowed to measure product formation and simultaneous observation of remaining substrate and $15dPGJ_2$-Cys formation.

In order to determine the apparent Michaelis constant (Km) for MGST3, MGST3 was incubated with $15dPGJ_2$ in reaction buffer containing 25 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.03 % DDM and 0.1 mM GSH. The concentration of $15dPGJ_2$ ranged from 1 µg to 90 µg. Reactions were carried out at room temperature with gentle shaking for 5 minutes. The reaction was stopped with 0.5 % formic acid, PGB_2 standard was spiked, and the reaction tubes were placed on wet-ice until HPLC separation and UV detection. With this approach more samples could be processed, and a faster analysis was possible. $15dPGJ_2$-GS formation was monitored at an absorbance of 306 nm, the λ_{max} of $15dPGJ_2$. The collected area units were subtracted from background (measured in control samples without substrate and enzyme) and quantified relative to the spiked standard, using the correction coefficient of 0.4 (extinction coefficient of $15dPGJ_2$ (ε: 12000) / extinction coefficient of PGB_2 (ε: 30000)). Data were expressed as pmol/µg/min and the apparent Km value was calculated using hyperbolic regression analysis [267]. The apparent Km value describes the substrate concentration at which the enzyme

reaction rate (velocity) has reached half of its maximum (V_{max}). V_{max} represents the maximum enzyme reaction rate (at a given enzyme concentration) at saturated substrate concentration.

3.2 WIRE MYOGRAPHY

Wire myography is a technique used to study the vascular reactivity of isolated *ex-vivo* vessels by recording the force development across the vessel wall. It was developed by Mulvany and Halpern in 1977 for the investigation of functional responses of small arteries less than 500 µm in diameter [268]. Arteries are kept in an organ bath, containing oxygenated physiological salt solution (PSS) to maintain tissue viability, and mounted on two stainless steel wires that go through the lumen. The wires are connected to a transducer and a micrometer to monitor constriction and relaxation of the vessel. The wire myography technique is commonly used in vascular physiology and pharmacology and was applied in **Paper I** and **Paper III**.

Human vessels were dissected from subcutaneous fat biopsies. Biopsies were obtained during operation and immediately placed in ice-cold physiological salt solution which was regularly replaced during the dissection process. The PSS contained NaCl 119 mM, KCL 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.17 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.18 mM, EDTA 0.026 mM and glucose 5.5 mM, maintaining physiological pH and mimicking *in-vivo* ion concentrations. Resistance arteries were isolated and cleaned from surrounding tissue using microdissection forceps and scissors under a stereomicroscope. Two wires (25 or 40 µm) were carefully inserted into the lumen of the artery which was mounted into a specimen holder and placed into the chamber of a Mulvany´s type 4-channel multi myograph system (Danish Myotechnology, Model 610). The organ baths were maintained at 37°C and continuously bubbled with 5% CO_2 and 95% O_2. The organ bath solution was exchanged about every 30 minutes. Arteries were rested for equilibration for at least 30 minutes before normalization and viability assessments were performed. As described before one of the inserted wires was connected to a micrometer, measuring the distance between the two wires. The other wire was connected to a force transducer that again is connected to a chart recorder, displaying the force development in milli-Newton (mN) units. The Lab chart 8 software (AD Instruments, New Zeland) was used for data recording and normalization. In order to guarantee controlled experimental conditions, vessels were normalized to the same initial conditions using a standardized normalization procedure [269]. The vessel was stretched to its internal circumference that reproduces the natural *in-vivo* wall force under transmural pressure (100 mmHg or 13.3 kPa). Therefore, stepwise stretching of the vessel, whereby the force was measured at each step, generated a passive length/tension graph that allowed to calculate the diameter the artery would have in relaxed state under transmural pressure. The artery was then set to 90 % of that diameter as this allows optimal active force development and arteries were washed with PSS and allowed to rest for 30 minutes. Besides normalization it is crucial to assess the viability and endothelial function of the artery to make sure the isolation and mounting did not affect the vessels functionality. Viability assessment included smooth muscle activation and endothelium-dependent relaxation. First the response to high potassium physiological salt solution depolarization (KPSS, equimolar substitution of 125 mM Na+ with K+ of PSS) and a

vasoconstrictor (norepinephrine or phenylephrine) was tested followed by endothelium-dependent vasodilation by acetylcholine (ACh) or bradykinin (BK). Vessels that did not show constriction or >50 % relaxation to ACh or BK were excluded from further analysis.

Wire myography is a useful *in-vitro* technique that makes it possible to study the functional and mechanical properties of small vessels and to monitor the response to pharmacological treatment. Because intact vessel segments are used, the interplay of cells and mediators within the vessel can be studied, allowing better transferability to *in-vivo* situations compared to other methods that focus, for instance on single cell responses. Nevertheless, the technique is limited in mimicking *in-vivo* conditions as the vessels are dissected out of its vascular bed and surrounding cells that contribute to vascular function are removed. Moreover, wire myography does not involve cannulation of the vessel and no intraluminal flow is generated, so the effects of blood flow/shear stress are not considered in the observations.

In this thesis the wire myography technique was applied to study effects of mPGES-1 inhibitors on resistance artery tone development. To study effects of mPGES-1 inhibitors on vasoconstriction, arteries were constricted with increasing concentrations of NE (0.001 – 3 µM) until a stable plateau was reached. Washing the arteries with PSS allowed them to return to basal tone. Arteries were then incubated with the test compound or a combination of test compounds (mPGES-1 inhibitors, COX inhibitors, receptor agonists or antagonists) for 30 minutes and a second NE-concentration-response curve was performed. The concentration response curves were expressed as % of initial high potassium constriction, and the differences between the first and second NE-constriction curves were used to determine effects of the test compounds. To study the effects of mPGES-1 inhibitors or COX inhibitors on ACh-induced relaxation, arteries were pre-constricted with a single concentration of NE (3 µM). After a stable plateau was reached ACh was added cumulatively (0.0001 – 10 µM). The concentration response curves before and after treatment were expressed as % of maximal relaxation (the resting basal tone was set to 100 % and the stable level of constriction to 0%). The differences between the first and second response curves (after 30 minutes incubation) were used to determine effects of the test compounds. Similarly, PGE_2 or PGI_2 (stable analogue Iloprost) were added cumulatively to NE-pre-constricted arteries to evaluate their effects on vascular tone. Control incubations with the vehicle were performed and artery viability was tested after the experiment with NE at 1 µM.

3.3 SAMPLE PREPARATION FOR LC-MS/MS ANALYSIS

In this thesis prostanoids were measured by liquid chromatography (LC) tandem mass-spectrometry (MS/MS). Prior to LC-MS/MS analysis, prostanoids were extracted from various types of samples, including cells and tissue, cell or tissue supernatants, plasma and urine. These matrices contain interfering elements such as proteins, phospholipids, nucleic acids or sugars and commonly only low levels of prostanoids. If these interfering elements are not removed or reduced, they can influence the analyte ionization and detector response during LC-MS/MS analysis [270]. Therefore, prostanoids need to be extracted from the natural matrix, cleaned from impurities and concentrated [271]. This is widely done by solid phase extraction (SPE).

The principle of SPE is based on chemical separation of analytes from matrix components between the solid phase (stationary phase) and the liquid that is passing through (mobile phase). Prostanoids are commonly separated based on polarity using reverse-phase SPE with a non-polar stationary phase (e.g., extraction columns with C18 or hydrophilic lipophilic balanced copolymer (HLB) as functional groups). Prostanoids as the analytes of interest are retained onto the stationary phase, while interfering elements can be washed off. For prostanoids to be retained in the column, samples are diluted in aqueous buffer containing an acid (e.g., formic acid) to protonate the analytes and allow capturing to the functional groups of the stationary phase. Unwanted impurities can then be washed off with a wash buffer containing low percentage of an organic solvent (depending on the strength between the analyte of interest and reverse-phase functional groups, the wash buffer strength may vary). Finally, analytes of interest are eluted from the column with an organic solvent (typically acetonitrile or methanol). Samples were then evaporated and stored at -20°C until LC-MS/MS analysis. In order to account for losses during sample preparation and also to identify and quantify analytes by LC-MS/MS, samples were spiked with 50 µL internal standard (IS) as soon as possible and prior SPE. The internal standard contained if not indicated otherwise the following deuterated analytes (240 ng/mL PGE_2-d4 (Cayman, #314010), 220 ng/mL PGD_2-d4 (Cayman, #312010), 330 ng/mL 6-keto-$PGF_{1\alpha}$-d4 (Cayman, #315210), 170 ng/mL $PGF_{2\alpha}$-d4 (Cayman, #316010), 170 ng/mL TxB_2-d4 (Cayman, #319030), and 330 ng/mL 15-deoxy-$\Delta_{12,14}$ PGJ_2-d4 (Cayman, #318570) in 100% methanol. SPE was used for prostaglandin extraction in **all papers** and the steps of SPE using Oasis-HLB 1cc 30mg columns included:

1) Activation of functional groups in stationary phase with 1 mL 100% methanol
2) Equilibration of the column with 1 mL 0.05% formic acid in MilliQ
3) Sample loading (acidified and diluted with 0.5% formic acid, spiked with IS)
4) Clean-up step with 1mL 5% methanol in 0.05% formic acid/MilliQ
5) Elution of prostanoids with 1 mL 100% methanol

In the following the extraction from different matrices is described where different protocols were used or an additional extraction was needed.

Prostanoid extraction from urine samples

Prostanoids are rapidly metabolized to inactive stable metabolites and excreted by the kidneys to the urine. Urinary metabolites of prostanoids are considered to reflect systemic prostanoid production and can indicate eventual changes related to pathology or induced by drugs. In **Paper IV** levels of urinary prostanoid metabolites were analyzed. Urine samples were thawed on wet-ice and centrifuged at 14000g for 10 min. Supernatants (~250 µL) were collected and spiked with 50 µL IS containing tPGE_2-M-d6 (Cayman # 314840), tPGD_2-M-d6 (Cayman, #10009039), 2,3-dinor-$PGF_{1\alpha}$-d9 (PGI_2-M) (Cayman, #9000462), 2,3-dinor-TXB_2-d9 (Cayman, #10009584) and 6-keto-$PGF_{1\alpha}$-d4 (Cayman, #315210). Good results for the extraction of urinary metabolites were shown to be achieved with reverse-phase/strong anion exchange SPE [271]. Therefore, samples were pH adjusted with 700 µL 10 mM ammonium acetate (AmAc) pH 9.5. Subsequently, samples were loaded on pre-activated (1mL 100 %

MeOH) and equilibrated (10 mM AmAc, pH 9.5) Oasis-MAX cartridges (96-well plate, 30mg, Waters), washed first with 1 mL 10 mM AmAc, pH 9.5 and second with 1 mL 100% acetone. Samples were eluted with 1 mL 3% acetic acid in 100% acetone, evaporated until dryness and stored at -20 °C until LC-MS/MS analysis.

Extracted samples were reconstituted and analyzed for PGE_2 and PGD_2 measured by their urinary metabolites $tPGE_2$-M and $tPGD_2$-M. To improve the chromatographic separation and MS detection of urinary prostanoids and in particular 2,3-dinor-6keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2 that produce tautomeric forms, the samples were derivatized [18]. For the analysis of PGI_2 and TXB_2 by their urinary metabolites 2,3-dinor-6keto-$PGF_{1\alpha}$ and 2,3-dinor-TXB_2, remaining samples were derivatized by the addition of 10 µL methoxyamide-hydrochloride (0.5mg/mL) to generate methoxime derivatives (MO) in particular, 2,3-dinor-6keto-$PGF_{1\alpha}$-MO and 2,3-dinor-TXB_2-MO. The addition of each MO adds 29 mass units to the molecular weight [100].

Prostanoid extraction from plasma samples

Plasma is a complex sample matrix that contains for example proteins, carbohydrates, salts, lipids and amino acids at up to millimolar concentrations. Therefore, a protein precipitation step was performed prior SPE. Plasma samples were thawed on wet-ice and spiked with IS. Protein precipitation was performed by addition of 100% ice cold methanol, vortexing and centrifugation at 3000g for 10 min at 4 °C. Supernatants were evaporated until dryness, subsequently diluted to 1 mL with 0.05% formic acid in MilliQ water and subjected to SPE HLB. Samples were evaporated and stored at -20 ºC until LC-MS/MS analysis.

Prostanoid extraction from tissue samples

For the extraction of prostanoids from whole tissue such as mouse spleen, spleens were weighed and spiked with 100 µL IS. Additional 500 µL methanol was added and spleens were homogenized with a pellet pestle. During the extraction procedure the tissue was kept on ice constantly. Homogenates were then incubated at -20 °C for 30 min, followed by centrifugation at 4000g for 10 min at 4 °C. Supernatants were collected and additional 500 µL 100% methanol was added to the pellets, followed by vortexing for 30 sec and an additional centrifugation at 4000g for 10 min at 4 °C. Supernatants were collected. Pooled supernatants were evaporated until 100-200 µL liquid remained. Supernatants were diluted to 1 mL with 0.05% formic acid in MilliQ and subjected to SPE HLB. Samples were evaporated and stored at -20 ºC until LC-MS/MS analysis.

3.4 LC-MS/MS ANALYSIS

Oxylipids are found in biological matrices at very low concentrations because of poor stability and fast metabolism. Many are derived from the same fatty acid and share similar structures or, in the case of regioisomers (PGE_2, PGD_2), even the same molecular mass. Among the available methods for the detection of oxylipids, LC-MS/MS is the most used method that allows chromatographic separation and quantitative determination [18, 271]. LC-MS/MS was used to measure prostaglandins and inhibitors in **all Papers**.

LC-MS/MS is an analytical technique that combines the separation of analytes in a sample mainly based on hydrophobicity followed by identification by their mass to charge ratio. In Figure 6 the process is schematically illustrated for the analysis with an LC system coupled to a mass spectrometer equipped with a triple-quadrupole detector (TQD). The following description contains methodological details such as column temperature, column material, flow rate, or solvents that are specific to the applied protocol and can vary for other applications. Extracted samples are reconstituted and injected into a mobile phase (containing low percentage organic solvent and 0.05% formic acid) that is delivered by a pressure pump of the LC. The protonated analytes are then transported in the mobile phase passing through the analytical column containing a nonpolar stationary phase for reverse-phase chromatographic separation. The analytical column consists of nonpolar hydrocarbons coupled to silica particles that form hydrophobic interactions with the analytes flowing through. The mobile phase composition varies gradually from low percentage organic to high organic over a set time. The analytes elute from the column based on their hydrophobicity, with more hydrophobic analytes being retained on the column longer and thus eluting later. The gradient length and slope are manually set based on the analytes of interest and their elution profile. The time from injection to elution in a specific gradient is the retention time (RT) of an analyte. The ultra-performance (UP)-LC operates at a constant flow rate of 0.6 mL/min and at high pressure with column temperatures around 40°C. Gradually separated analytes are infused into the mass spectrometer where they are converted to gaseous ions, separated by their mass to charge ratio (*m/z*) and detected.

Analytes that elute from the analytical column under atmospheric pressure are infused to the mass spectrometer, which operates under high vacuum through an interphase that is based on atmospheric pressure ionization (API) such as electrospray ionization (ESI) [272, 273]. In ESI analytes are ionized by spraying the analyte solution (e.g., organic solvent and acid) into an electrical field. This happens in the ion source where the analyte solution is pushed through a fine nozzle into the evaporation chamber, generating a fine spray of droplets. A high voltage is applied at the nozzle tip, which charges the analytes in the solution droplets and electrostatically attracts them towards the inlet of the mass spectrometer, the cone. Dry gas (e.g., nitrogen gas), heat or a combination of both are applied to the droplets before they enter the vacuum. This helps to gradually evaporate the solvent as it forces the charged analytes within the droplets close together which then repel each other eventually generating single charged analytes (free of solvent). The spraying nozzle can be kept at a positive potential or negative potential resulting in charged analytes.

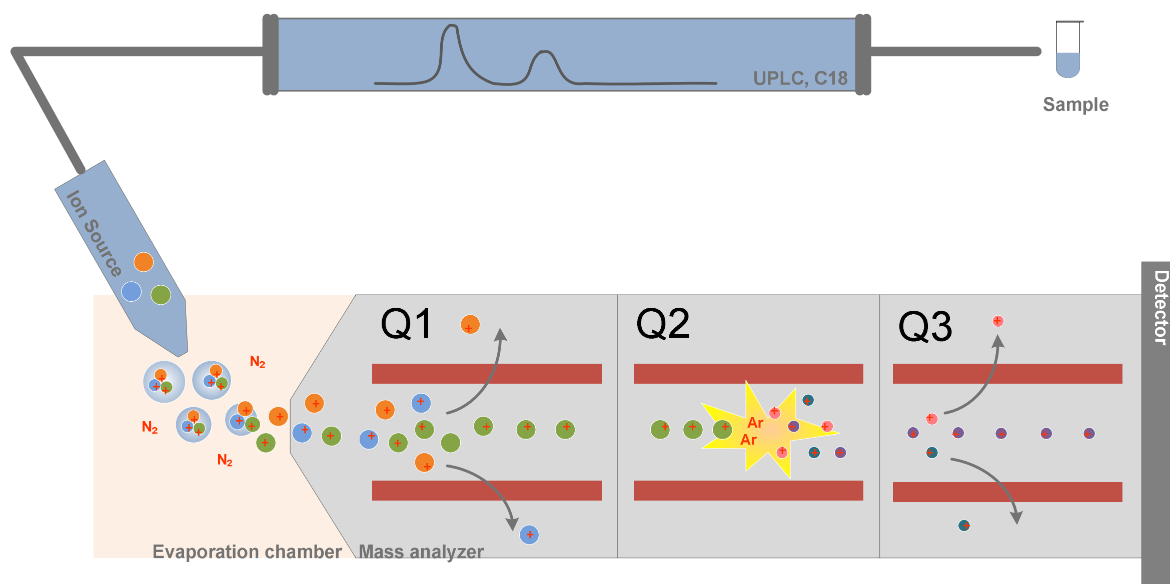


Figure 6: Simplified illustration of LC-MS/MS. UPLC system coupled to a mass spectrometer equipped with an ion source (ESI), a mass analyzer (Q1-Q3) and a detector.

The ionized analytes enter the mass analyser that separates the ions based on their mass to charge ratios. A quadrupole analyser consists of four cylindrical rods which together create a varying electrical field that accelerates ions within it towards the detector or a following quadrupole [274]. The varying electrical field determines which ions are passing through the quadrupole at each time of the scan. In a triple quadrupole mass spectrometer three quadrupoles are sequentially connected. The first quadrupole (Q1) can scan over a wide range of *m/z* or is set to filter only one specific *m/z*. Ions selected in Q1 pass to the second quadrupole (Q2). This quadrupole acts as a collision cell in which voltage and atoms of a collision gas (typically argon) are applied resulting in fragmentation of the selected ions. This fragmentation process is called collision-induced dissociation (CID). The fragment ions produced in Q2 can be scanned in the third quadrupole (Q3) or a specific *m/z* is selected and monitored. Ions that pass Q3 collide onto the detector where their charge is neutralized creating an electric current that is amplified and converted to be processed by the connected computer. Data that is acquired by LC-MS/MS is commonly represented in mass chromatograms where the signal intensity is plotted against the retention time. This can show the total ion current (TIC) chromatogram which is the summed intensities over a *m/z* range or the selected-reaction monitoring (SRM) chromatogram which is the summed intensities for a specific *m/z*. In a mass spectrum the signal intensity is plotted against the *m/z* ratio. Eicosanoid analysis is commonly performed by LC-M/MS using multiple reaction monitoring (MRM), a form of SRM [272]. A precursor ion (or parent ion) representing the charged analyte of interest is selected in Q1, fragmented in Q2 and a specific fragment ion (daughter ion) is selected in Q3. The MRM chromatogram shows the intensity of the selected transition (*m/z* Q1 > *m/z* Q3) over time. Ideally multiple transitions are scanned per analyte. For identification and quantification of analytes their intensity is expressed in relation to a spiked IS. ISs are for example deuterated analogues (resulting in a different mass as the analyte of interest) that have the same RT and almost identical chemical properties as the analyte of interest and are spiked into the sample prior extraction at a defined

concentration. Quantification is then performed based on the peak area of the endogenous analyte in relation to the peak area of the spiked standard. Areas must be large enough to be considered as true signal whereby a signal to noise (S/N) ratio of ≥10 is accepted. Concentrations are calculated using a standard curve which contains a serial dilution of the endogenous analytes and the same IS concentration as spiked into the sample. Further normalization depending on the origin of the sample (e.g., cell number, protein content, tissue weight, extracted volume) is applied where applicable to estimate the levels of the analyte of interest within individual samples.

Prostanoids were analyzed based on an established method [241] and as described in the respective papers. The analysis was performed on an UPLC instrument (Aquity UPLC, Waters) over a C18 analytical column (Waters) coupled to a triple quadrupole mass detector (TQD or Xevo-TQ-XS, Waters). Prostaglandins were analyzed in negative ionization mode, except of the conjugates of $15dPGJ_2$ and inhibitors of mPGES-1 and COX that were analyzed in positive mode.

3.5 STATISTICAL ANALYSIS

In **Paper I**, data were presented as mean ± standard deviation (SD) or median with inter-quartile range. The IC_{50} values were calculated using nonlinear regression and sigmoidal concentration–response curve fit. In the air-pouch model and in the paw swelling model statistical significance of the treatment compared to the mean of the vehicle group was calculated using one-way ANOVA (normally distributed data) followed by Dunnett's multiple comparison test, with a single pooled variance. Post hoc tests were only run if F achieved $P < 0.05$. Data from wire myography experiments were expressed as % of K+ constriction and to obtain normal distribution log transformed. Differences in pEC_{50} values (before and after inhibitor incubation) were compared by student's t-test. Statistical significance level was indicated as * $p < 0.05$.

In **Paper II**, the results were expressed as mean ± standard deviation (SD). Comparisons between two groups were performed using student's t-test. Statistical significance level was indicated as * $p < 0.05$. Calculations and graphs were prepared using GraphPad Prism version 9.0.

In **Paper III**, the characteristics of ESKD patients and Non-ESKD controls were presented as median with inter-quartile range or total numbers (percentage) and statistical analysis was performed using Mann–Whitney U test for continuous parameters and chi-squared test for categorical parameters. Data from wire myography experiments were presented as mean ± standard error mean (SEM) and as % of K + constriction or % of max. relaxation and log transformed to obtain normal distribution. Paired comparison of concentration response curves (before and after inhibitor incubation) were performed using Two-way analysis of variances (ANOVA) with post-hoc Bonferroni's correction for multiple comparisons if F achieved $p < 0.05$ and there was no significant variance inhomogeneity. pEC_{50} values were calculated using nonlinear regression. Differences in pEC_{50} values, E_{max} values and PG levels were compared

by student's t-test. Statistical significance level was indicated as * $p < 0.05$. Calculations and graphs were prepared using GraphPad Prism version 5.0 and Statistica version 12.0.

In **Paper IV**, data was presented as mean ± standard error mean (SEM). Comparisons of two groups were made using unpaired student's t-test or nonparametric Mann-Whitney U test. Comparison among multiple groups was performed by one-way ANOVA followed by Holm-Šídák's multiple comparisons test, or Kruskal-Wallis test followed by Dunn's multiple comparisons test. Statistical significance was defined as *$p<0.05$. Statistical analysis was performed using GraphPad Prism version 9.0.

3.6 ETHICAL CONSIDERATIONS

The studies in this thesis involve *in-vitro* cell culture experiments as well as mouse *in-vivo* and human *ex-vivo* studies. All animal experiments and studies on human material were performed according to guidelines approved by the regional ethics committees or by the external responsible institutions.

Animal experiments

In **Paper I** a pharmacokinetic study was performed to obtain information on the bioavailability of the tested compounds. The pharmacokinetic experiments were performed in rats by an external contract research organization (CRO) (Cerep) and approved by Cerep Institutional Animal Care and Use Committee (IACUC). The paw swelling model includes oral administration of the compounds one hour before the injection of CA into the sub plantar hind paw of rats. The experiment was performed by Anthem Biosciences and approved by the Institutional Animal Ethics Committee of Anthem Biosciences. Moreover, a CA induced air pouch model in mice was used to study the potency of mPGES-1 inhibitors to reduce inflammation. The CA induced air pouch model is an often used model to study inflammation and includes the injection of air to the back of the anesthetized mouse. After five days stable air pouches are generated, and the compounds were administered followed by injection of CA. Mice were sacrificed for exudate collection. The experiments were performed according to the guidelines of the regional ethics committee for animal research, Stockholm, Sweden. Further in **Paper II** we used mPGES-1 KO mice to study prostanoid production in mouse macrophages. For these experiments the animals were sacrificed and tissue like spleen and bones were collected to generate primary cell cultures. The experiments were approved by the regional ethics committee for animal research Stockholm, Sweden. In **Paper IV** we use a mouse model of MI to study the impact of mPGES-1 inhibition on cardiac remodeling compared to COX-2 inhibition. The mice were subjected to left coronary artery ligation and treated with the compounds intraperitoneally. All experiments were ethically justified and approved by the regional ethics committee, the Institutional Animal Care and Use Committee at Fuwai Hospital, Chinese Academy of Medical Science, China.

Human material

In **Paper I** and **II,** blood samples from healthy donors were collected to assess inhibitor potency in whole blood and to study stability of prostaglandins in plasma. Blood samples were obtained according to the guidelines of the regional ethics committee at Karolinska University Hospital. In **Paper II** cord-blood from volunteers was collected to generate mast cells that were used to study prostaglandin production. Sample collection was approved by the regional ethics committee, Stockholm. In **Paper I** and **III** isolated small arteries obtained from human fat biopsies during operation were studied with wire myography, IHC and LC-MS/MS. Recruitment of the subjects included in these studies and investigations of the biopsies were approved by the the regional ethics committee, Stockholm.

4 RESULTS AND DISCUSSION

This thesis contains four studies addressing the anti-inflammatory and vasoactive effects of mPGES-1 inhibition. The results obtained in these studies are described and discussed in the following section. To investigate the effects of mPGES-1 inhibition, inhibitors suitable for pre-clinical studies are needed. Results from the characterization of five new inhibitors are presented in the first paragraph (**Paper I**). Thereafter, the results of three studies testing inhibitors of mPGES-1 in models of inflammation and cardiovascular disease are described. The focus in these studies was on the metabolism of $15dPGJ_2$ (**Paper II**) and the cardiovascular effects via PGI_2 (**Paper III** and **IV**).

4.1 INHIBITORS OF MPGES-1 FOR PRE-CLINICAL STUDIES

Until a compound reaches FDA approval and market availability, it needs to pass several evaluation stages. Thousands of compounds are commonly screened towards the target, followed by lead-optimization and evaluation in pre-clinical studies, contingently reaching the clinic with an anticipated success rate of about 10 % [275]. Pre-clinical studies are essential in this process to understand the biological effect in different disease settings, toxicity and dosage range, potency and mechanism of action of the lead compound (s). As previously described, many compounds have been reported to inhibit mPGES-1 *in-vitro* but only few of them were eventually suitable for pre-clinical *in-vivo* tests [197]. In the development of inhibitors of mPGES-1, interspecies differences were one of the reasons that had slowed the pre-clinical progress of inhibitor candidates. In **Paper I** we aimed to characterize five new inhibitors with cross-species activity for their use in *in-vitro* and *in-vivo* pre-clinical assays (results are summarized in Table 2).

The five compounds are benzimidazoles, identified during lead-optimization of a series of compounds that originated from a screen of a compound library towards human mPGES-1. During this process an early hit series evolved, to which CIII belongs [202], that also reached submicromolar IC_{50} values for recombinant rat mPGES-1 and served as a basis for further optimizations, leading to the five compounds 934, 117, 118, 322, and 323. All compounds were potent inhibitors of recombinant human and rat mPGES-1. Compounds 117 and 118 inhibited both enzymes with the lowest mean IC_{50} values of 0.017 and 0.023 µM for human mPGES-1 and 0.055 and 0.078 µM for rat mPGES-1, respectively. Compared to other reported cross-species inhibitors of mPGES-1, the compounds showed increased potency in the recombinant enzyme activity assays. For example, CIII inhibited human and rat mPGES-1 with IC_{50} values of 0.09 and 0.9 µM, respectively [202], and the mPGES-1 inhibitor compound 4b inhibited recombinant human and mouse mPGES-1 with IC_{50} values of 0.033 and 0.157 µM, respectively [205]. To assess enzyme selectivity of the studied compounds, their inhibition on other enzymes involved in prostaglandin biosynthesis was tested. All compounds moderately inhibited mPGES-2 at 10 µM. A weak inhibition of COX-1 and COX-2 was found for compound 323. 322 also showed a weak inhibitory effect for COX-2 and PGIS. Overall, no inhibitory effect was found towards COX-1, PGIS, L-PGDS or H-PGDS.

A549 cells are commonly used to study the potency of test compounds to inhibit PGE_2 production in intact cells. Therefore, A549 cells were induced with IL-1β and treated with the test compounds at various concentrations for 24 hours. All compounds inhibited PGE_2 production dose-dependently with IC_{50} values ranging from 0.15 - 0.82 µM. In comparison, CIII was reported to inhibit IL-1β-induced PGE_2 formation in A549 cells with an approximate IC_{50} value of 2 µM [202]. Notably, FBS supplementation varied between the two assays, with 2% for the compounds and 10% for CIII. As previously described, many compounds that show good IC_{50} values in recombinant enzyme and cell based assays, loose their potency in a complex biological matrix such as whole blood. In our study, the compounds inhibited LPS-induced PGE_2 in whole blood with IC_{50} values of 3.3 – 8.7 µM (with 10-15% residual PGE_2 at 20 µM). No effect on TXA_2 production by the compounds was observed. In these experiments, diclofenac completely inhibited the production of PGE_2 and TXA_2 production at 10 µM. In comparison, CIII has been shown to inhibit LPS-induced PGE_2 in whole blood with an approximate IC_{50} of 10 µM [202].

Moreover, the compounds showed potency in the CA air pouch mouse model that was used to study PGE_2 inhibition *in-vivo*. All compounds significantly reduced PGE_2 in pouch exudates at 100 mg/kg and compounds 322 and 323 even at 30 mg/kg. In these experiments, the COX-2 inhibitor celecoxib (used at 50 mg/kg) significantly blocked PGE_2 and PGI_2 production. The tested compounds had no effect on PGI_2 production, except for 117 which increased PGI_2 formation. These results are comparable with previous descriptions for CIII. CIII was shown to reduce PGE_2 in air pouches at 50 mg/kg and 100 mg/kg without affecting PGI_2 or TXA_2 levels [202]. Compound 4b was shown to reduce kidney PGE_2 levels in the air pouch model at 10 mg/kg (p.o. administration) [205]. Monitoring the redirection of PGH_2 connected with the inhibition of mPGES-1 is of great interest regarding the safety of mPGES-1 inhibitors. Depending on the pathological condition shunting to PGI_2, TXA_2, $PGF_{2\alpha}$ or PGD_2 could be detrimental and has been discussed as problematic for the development of selective mPGES-1 inhibitors [191]. Notably, assumptions have been primarily based on investigations with mPGES-1 KO mice although the deletion of mPGES-1 does not exactly mimic the effects of pharmacological treatment. This was demonstrated in the CA-induced air pouch model where an increase in TXA_2 and PGI_2 (measured by their stable metabolites TXB_2 and 6-keto-$PGF_{1\alpha}$ respectively) was observed for mPGES-1 KO mice but not for CIII-treated mice [202]. Moreover, as discussed in this thesis, the shunting to PGI_2 is considered as advantageous regarding cardiovascular safety. To further study the effects of the test compounds on acute inflammation, a CA-induced paw oedema model in rats was used. All compounds significantly reduced paw swelling at all tested doses (1-100 mg/kg) compared to the vehicle control at 1 hour post CA-induction. When paw swelling was assessed over the time course of 4 hours (paw swelling peaked at 4 hours) post CA-induction a significant decrease was observed for 934 (10, 30, and 100 mg/kg), 117 (1, 3, 10, and 30 mg/kg), 118 (10 mg/kg), 322 (3 and 100 mg/kg), and 323 (100 mg/kg). Celecoxib showed significant reduction in paw swelling at 10 mg/kg. In comparison, the mPGES-1 inhibitor CII reduced paw swelling at 100 mg/kg [201] and the compound A was reported to reduce paw swelling at 30 mg/kg [204].

Inhibition of mPGES-1 is thought to have a safer cardiovascular profile beacuse it does not inhibit cardioprotective PGI_2 compared with COX inhibitors. Deletion of mPGES-1 in mice did not result in thrombogenesis and high blood pressure [253] and the underlying mechanism is thought to be a redirection of PGH_2 to PGI_2, counterbalancing TXA_2. The mPGES-1 inhibitor CIII moreover reduced norepinephrine (NE)-induced vasoconstriction in human arteries via shunting to PGI_2, tested *ex-vivo* with human saphenous vein and mammary artery. In comparison, COX-2 inhibition was shown to increase NE-induced constriction *ex-vivo* [257]. From the results of the whole blood assay and the CA-induced air pouch model we saw that the compounds did not affect TXA_2 production and maintained PGI_2. To further investigate effects of the compounds on vascular tone, we tested the compounds on resistance arteries using wire-myography. Similar to previously reported results in larger human arteries, CIII used as reference here, reduced NE-induced vasoconstriction. Of the five new compounds, 934 and 118 were tested based on structural difference and their overall performance in the other assays. Compound 118 reduced NE-induced vasoconstriction at threefold lower concentration than CIII with increased efficacy. Compound 934 showed a tendency towards reduced NE-induced vasoconstriction but presented a high variability, probably due to poor solubility.

Studying the pharmacological inhibition of mPGES-1 *in-vivo* is necessary to understand whether and how inhibition of mPGES-1 might represent a therapeutic strategy. However, this requires inhibitors that can be used in animal models. Inhibitors that do not show activity towards rat or mouse mPGES-1 could alternatively be tested in guinea pigs or in knock-in mouse models with human mPGES-1 [189]. However, these approaches have the disadvantage that most disease models were developed for mouse or rat, and they require a more complex experimental design. The five compounds investigated in this study selectively inhibited both recombinant human and rat mPGES-1 with low nanomolar IC_{50} values. They showed improved potency in intact cells and whole blood compared to other previously described compounds with cross-species activity (e.g. CII, CIII, MK-886, FR20, compound 28) [197]. All compounds blocked PGE_2 production in an air pouch mouse model and reduced paw swelling in rats with similar efficacy as compound 4b and A [204, 205]. Compound 118 moreover significantly reduced NE-induced vasoconstriction in human arteries *ex-vivo*. Compared with other published cross-species mPGES-1 inhibitors, the test compounds show improved potency in *in-vitro* and *in-vivo* assays of inflammation and vascular tone. Although, the characterized inhibitors do not reach the criteria necessary for clinical candidates (e.g. bioavailability F > 30%, half-life $t_{1/2}$ > 4-6 hours or low nM-pM IC_{50} [276]), they still represent refined tools that can be used to investigate the inhibition of mPGES-1 in pre-clinical models. Despite the overall comparable potency and selectivity of the five compounds, compound 118 exhibited the most favorable profile and was therefore used alongside CIII in the following papers of this thesis.

	934	117	118	322	323	CIII [202]
In-vitro [IC_{50} µM]						
Biochemical assays						
mPGES-1 human	0.024	0.017	0.023	0.037	0.031	0.09
mPGES-1 rat	0.17	0.055	0.078	0.27	0.14	0.9
Cellular assays						
A549 cells	0.15	0.26	0.15	0.82	0.66	~2[&]
Whole blood	3.7	3.4	2.5	6.4	4.9	~10
Ex-vivo [sig. reduction of EC_{50}]						
vasoconstriction						
Resistance arteries	n.s. at 3µM	n.t.	$P<0.05$ at 3µM	n.t.	n.t.	n,s at 10µM
In-vivo [mg/kg]						
Paw swelling rat[*]	10, 30, 100	1, 3, 10, 30	10	3, 100	100	n.t.
Air pouch mouse[#]	100	100	100	30, 100	30, 100	50, 100

Table 2: Summary of biochemical assays performed with 934, 117, 118, 322, 323. Modified from figures and tables presented in **Paper I** [277]. Results from CIII in similar assays are presented for comparison [197, 202]. [*]All compounds reduced paw-swelling 1 hour post induction at concentrations ranging from 1-100 mg/kg. CA-induced paw swelling reached its maximum 4 hours post induction. Comparison of the area under the curve (AUC) of inhibitors with vehicle treated CA-induced control resulted in significant reduction for the indicated concentrations. [#]Significant reduction of PGE_2 levels in CA-induced air pouch after treatment with indicated doses compared to untreated CA-induced air pouch. [&]A549 cells treaded with CIII were incubated in 10% FBS instead of 2%. n.s. = not significant. n.t. = not tested.

4.2 INHIBITION OF MPGES-1 PRESERVES THE PGD_2/15DPGJ$_2$ PATHWAY – CHARACTERIZATION OF THE 15DPGJ$_2$ METABOLISM IN MACROPHAGES AND MAST CELLS

The therapeutic potential of inhibiting mPGES-1 lies in the sole reduction of PGE_2 compared to NSAIDs, which block all prostanoids. It is hypothesized that maintaining the other prostanoids will result in fewer side effects and improved efficacy. One important aspect of mPGES-1 inhibition is its effect on the PGD_2 pathway. We have previously found that 15dPGJ$_2$, a metabolite of PGD_2, is upregulated in macrophages from mPGES-1 KO mice [241]. 15dPGJ$_2$ has been shown to interact with several signaling pathways leading to an anti-inflammatory and pro-resolving response. 15dPGJ$_2$ acts as a receptor ligand or Michael acceptor, modifying target proteins. However, the relevance of 15dPGJ$_2$ as an endogenous bioactive lipid has been questioned as very low levels have been detected *in-vivo* which contrast with the high concentrations used *in-vitro* to achieve significant activation of for example PPARγ [228]. It is therefore important to note that, besides its interaction with cellular proteins, 15dPGJ$_2$ can bind to the cysteine within the tripeptide GSH. Primarily this was described as a detoxification process to avoid unselective protein binding and removal of 15dPGJ$_2$ from the cell. However, the description of the metabolism of 15dPGJ$_2$ in HepG2 cells proposed a major alternative biosynthetic pathway which could account for the low levels that were detected of the free 15dPGJ$_2$ *in-vivo* [236]. Metabolism of 15dPGJ$_2$ occurred in 15dPGJ$_2$-treated HepG2 cells via conjugation to GSH, followed by reduction of the C11, removal of glutamic acid and glycine resulting in a cysteine-conjugate. We hypothesized that the metabolism of 15dPGJ$_2$ occurs via GSH conjugation in immune cells and gives rise to bioactive metabolites that may be upregulated upon mPGES-1 inhibition and therefore may contribute to its anti-inflammatory effects.

We studied the metabolism of 15dPGJ$_2$ by analyzing the 15dPGJ$_2$-GS and 15dPGJ$_2$-Cys conjugates by LC-MS/MS in the monocyte-macrophage RAW264.7 cell line, primary mouse macrophages derived from bone marrow (BMDM) and primary human mast cells derived from cord-blood (CBMC). Macrophages and mast cells are known to produce high levels of PGD_2 and therefore resemble a good source for downstream metabolites [278]. Mast cells play an important role in allergic inflammation. Upon activation they release inflammatory mediators including LTB_4, LTC_4 and PGD_2. PGD_2 is the primary prostaglandin formed by mast cells and can cause bronchoconstriction, vasodilation, or induction of lung inflammation [279]. We used BMDM and CBMCs as a model system for mouse and human primary immune cells to study the endogenous formation of PGD_2 metabolites.

First, RAW264.7 cells and BMDM were incubated with 15dPGJ$_2$ for various time points and conjugate formation was measured in the supernatants. We observed a similar profile as described for HepG2 cells in mouse macrophages with 15dPGJ$_2$-GS formed first and then the 15dPGJ$_2$-Cys. We incubated the 15dPGJ$_2$ in parallel in cell free culture medium, where we found significantly less conjugates formed. Addition of GSH to the cell free culture medium resulted in equivalent levels of 15dPGJ$_2$-GS but not of 15dPGJ$_2$-Cys. These experiments and additional incubations with reduced serum albumin concentrations excluded a possible

involvement of albumin in the formation of the $15dPGJ_2$-conjugates. It has been previously shown that the conversion of PGD_2 to PGJ_2 and $15dPGJ_2$ is albumin independent, while the formation of Δ^{12}-PGJ_2 from PGJ_2 requires the presence of albumin [78]. However, we observed that reducing serum albumin in the cell culture medium allowed earlier detection of the $15dPGJ_2$-GS conjugate. A possible explanation for these observations could be that serum albumin acts as a carrier for fatty acids in extracellular fluids and may capture $15dPGJ_2$ in an albumin-fatty acid complex or covalently bind $15dPGJ_2$ via Michael addition [280, 281]. Those interactions could delay the cellular uptake of $15dPGJ_2$. In addition, we observed that the inhibition of cellular transcription and translation significantly blocked the formation of $15dPGJ_2$-GS within the first 12 hours of incubation. These results indicated a cell dependence for the formation of $15dPGJ_2$-conjugates.

To further explore possible enzyme involvement in the metabolism of $15dPGJ_2$ we studied the catalytic activity of MAPEG proteins towards the formation of $15dPGJ_2$-GS *in-vitro*. All MAPEG proteins, except of FLAP, possess GST activity, and in analogy with the leukotriene pathway, where LTC4 synthase catalyzes glutathionylation of LTA4, they are possible candidates for the conjugation of $15dPGJ_2$ with GSH. We found that MGST3 significantly enhanced the formation of $15dPGJ_2$-GS compared with the control. mPGES-1, MGST1 and MGST2 showed weak activity without reaching significance and, LTC4S and FLAP did not affect $15dPGJ_2$-GS formation. MGST3 has previously been shown to catalyze the GSH conjugation to LTA4 and to reduce 5-HpETE to 5-HETE via GSH dependent peroxidase activity [267]. MGST3 presents a wide tissue distribution. MGST3 mRNA has been found in human heart, skeletal muscle, adrenal cortex, brain, placenta, liver, and kidney. Low levels of MGST3 mRNA have been described in lung, thymus and blood leukocytes [267]. Human and mouse macrophages were also described to express MGST3 protein [282, 283]. To understand whether MGST3 was present in the cells we studied, and could potentially contribute to the formation of $15dPGJ_2$-conjugate in these cells, we measured mRNA expression of MGST3 in RAW264.7 cells and human CBMCs. MGST3 was expressed in RAW264.7 cells and human CBMCs. We also observed that in RAW264.7 cells LPS reduced the expression of MGST2, MGST3 and LTC4S, and inhibition of COX or mPGES-1 did not affect the expression levels. Similarly, in anti-IgE-treated CBMCs inhibition of COX or mPGES-1 did not alter the expression of MGST3. LPS has been reported to be linked with reduced mRNA levels of LTC4S and MGST3 [284, 285]. Additionally, Kdo_2-lipid A (KLA) treatment of RAW264.7 cells resulted in MGST3 protein levels that tended to decline over time [283]. LPS has been described to reduce the cellular antioxidant capacity through downregulation of PPARγ and Nrf2 [286], and MGST3 has been described as an antioxidant gene regulated by Nrf2 [287, 288] which could be an explanation for the effect of LPS we observed in RAW264.7 cells. To investigate if MGST3 is involved in the conjugation of $15dPGJ_2$ with GSH in intact cells, we incubated RAW264.7 cells that lacked MGST3 with $15dPGJ_2$. Compared to WT cells, the MGST3 KO cells showed significantly reduced formation of $15dPGJ_2$-GS and $15dPGJ_2$-Cys, indicating an essential role of MGST3 in the metabolism of $15dPGJ_2$ with GSH. The expression of MGST3 is most likely induced by oxidative stress through Nrf2 [287, 288]. We found an

upregulation of MGST3 mRNA after 24 hours in RAW264.7 cells incubated with $15dPGJ_2$, which could indicate Nrf2 activation by $15dPGJ_2$. This might also explain the rather slow conjugate formation observed in RAW264.7 cells and BMDM.

The previous experiments established that $15dPGJ_2$ is metabolized via GSH in RAW264.7 cells and BMDM and that this metabolism is dependent on MGST3. However, in these experiments $15dPGJ_2$ was added to the cells at relatively high concentrations. To understand whether $15dPGJ_2$ is endogenously conjugated to GSH and converted to a $15dPGJ_2$-GS and $15dPGJ_2$-Cys conjugate, we incubated RAW264.7 cells, BMDM, and human CBMCs with inflammatory stimuli (LPS and anti-IgE, respectively) and analyzed the cells and supernatant for the formation of $15dPGJ_2$-conjuagtes. We studied the production of PGE_2, PGD_2, $15dPGJ_2$, $15dPGJ_2$-GS, $15dPGJ_2$-Cys in RAW264.7 cells over time. We found that PGE_2 reached highest levels after 24 hours whereby PGD_2 peaked already at 12 hours post LPS treatment. The $15dPGJ_2$ started to increase after 12 hours and peaked at 24 hours. The $15dPGJ_2$-conjugates increased at the later time points (24, 32 hours) and the $15dPGJ_2$-Cys conjugate was significantly increased at the 32 hour time point. The observed time course indicates the conversion of PGD_2 to $15dPGJ_2$ and further to the $15dPGJ_2$-conjugates, which could indicate that they are metabolites of the late inflammatory phase as previously suggested for $15dPGJ_2$ [213]. Levels of $15dPGJ_2$-conjugates in the cell pellets were lower than in the supernatants. Rapid secretion of prostaglandins into the supernatants was previously described for RAW264.7 cells [289], and MRP1 and MRP3 were shown to export $15dPGJ_2$-GS [226]. The $15dPGJ_2$-Cys conjugate was further detected in BMDM and human CBMCs. These experiments show that the $15dPGJ_2$-conjugates were induced upon inflammatory stimuli and formed endogenously in immune cells. Endogenous formation of GSH conjugates with $15dPGJ_2$ or $15dPGJ_2$-like metabolites have so far to our knowledge only been described in vehicle treated MCF7 cells and rat liver respectively [226, 290]. Our data therefore adds insights into the biosynthesis of $15dPGJ_2$ and its metabolism via GSH in mouse macrophages and human mast cells.

Our initial hypothesis included that inhibition of mPGES-1, similar as previously observed in macrophages derived from mPGES-1 KO mice [241], could promote the PGD_2 pathway, including the $15dPGJ_2$ metabolites. We therefore tested inhibitors of mPGES-1 (CIII, 118) and the COX-2 inhibitor NS-398 in RAW264.7 cells. NS-398 blocked the formation of PGE_2, PGD_2, $15dPGJ_2$, $15dPGJ_2$-GS and $15dPGJ_2$-Cys. In contrast, the mPGES-1 inhibitors CIII and 118 inhibited PGE_2 synthesis (40 – 50 % of the LPS control) and preserved the PGD_2 pathway. $15dPGJ_2$, and its GSH conjugates tended to increase upon mPGES-1 inhibition, although the increase did not reach statistical significance. In BMDM from mPGES-1 KO mice a significant shift to increased PGD_2 was observed in cells treated with LPS and GSH compared to WT controls. Blocking COX-1/COX-2 with diclofenac inhibited PGD_2 and $15dPGJ_2$-Cys formation in anti-IgE activated CBMC. Together, these results demonstrate that the GS-and Cys-metabolites of $15dPGJ_2$ are indeed derived from the PGD_2 pathway and blocked by inhibition of the COX enzymes. In contrast, when mPGES-1 is inhibited in murine macrophages the PGD_2 pathway is preserved and even enforced. PGD_2 and $15dPGJ_2$ have been

shown to promote the resolution of inflammation in various models [214, 217, 218, 291] and their blockage might interfere with the resolution process. Thus, maintenance of the PGD_2 pathway could contribute to the therapeutic effects of mPGES-1 inhibitors in situations where PGD_2 is pro-resolving. In opposite, however, the inhibition of mPGES-1 has been described to interfere with the resolution of inflammation [54, 55]. In these studies, the reduction of PGE_2 interfered with the clearance of neutrophils and the activation of pro-resolving lipid mediators in mice. Therefore, it is important to note that the effects we describe on the PGD_2 pathway in macrophages and mast cells, are limited to *in-vitro* observations, and do not reflect the complex *in-vivo* environment during inflammation. Further studies are needed to determine the potential of mPGES-1 inhibition to support the resolution of inflammation. And more specifically to address the potential bioactivity of $15dPGJ_2$-conjugates and their role in the different phases of the inflammation process.

As depicted in Figure 3, the metabolism of $15dPGJ_2$ via GSH includes two intermediate metabolites between the $15dPGJ_2$-GS and $15dPGJ_2$-Cys. Here, we simplified the analysis of $15dPGJ_2$ metabolism and focused on the first and last metabolites in this pathway. The $15dPGJ_2$-Cys metabolite showed superior stability in plasma compared to its precursors PGD_2, $15dPGJ_2$ and $15dPGJ_2$-GS and was previously described as the final metabolite in this pathway [236]. Therefore, measurement of the $15dPGJ_2$-Cys conjugate could be used alone or in addition to measurements of $15dPGJ_2$ to monitor changes in the PGD_2 pathway. However, analysis of the red-$15dPGJ_2$-GS and the $15dPGJ_2$-Cys-Gly conjugate would improve our understanding of the kinetic profile of this pathway. Furthermore, the $15dPGJ_2$-GS represents an unstable intermediate as the carbonyl group allows retro-Michael addition and measuring the red-$15dPGJ_2$-GS could provide more accurate estimations of the amounts produced. Prostaglandin levels in this study were measured by LC-MS/MS. For the analysis of $15dPGJ_2$-GS and $15dPGJ_2$-Cys an MRM method was established, and levels were quantified based on an external standard curve as no deuterated standards are available for internal standard normalization. It is important to note that the commercially available $15dPGJ_2$-GS standard is not stable and breaks down into the free $15dPGJ_2$ and GSH over time and concentrations are not absolute. Additionally, the quantification of the $15dPGJ_2$-Cys was based on an in-house generated standard. The concentration estimation of the generated standard was based on the initial $15dPGJ_2$ concentration, and the reaction efficiency measured by the consumption of free $15dPGJ_2$ after reaction with cysteine. This approach allowed us to identify the formation of $15dPGJ_2$-conjugates in biological samples but is limited in the absolute quantification of these metabolites. Therefore, the levels that we report for the $15dPGJ_2$-GS and $15dPGJ_2$-Cys must be considered as semiquantitative. Still, in agreement with reports by others [217, 292, 293], our data support endogenous formation of $15dPGJ_2$ and show its GS- and Cys-metabolites in activated macrophages and mast cells.

4.3 INHIBITION OF MPGES-1 HAS VASOACTIVE EFFECTS

Inhibition of mPGES-1 is a promising therapeutic target for inflammatory diseases. Besides equivalent inflammation control, targeting mPGES-1 could overcome the adverse effects seen for COX inhibitors. However, especially the cardiovascular complications that limit the use of NSAIDs are an equally debated concern with mPGES-1 inhibitors. Nevertheless, the general hypothesis is that inhibitors of mPGES-1 may not cause severe cardiovascular side effects. This is based on pre-clinical and clinical observations and primarily includes the shunting to PGI_2.

COX inhibitors block the biosynthesis of all prostanoids by inhibiting PGH_2 synthesis. PGI_2 is thought to be produced primarily constitutively by COX-2 in the vasculature, while TXA_2, a product of COX-1 is produced primarily by platelets. Under physiological conditions vasodilators and vasoconstrictors are balanced to maintain vascular homeostasis. Under pathological conditions pro-inflammatory COX-2 is upregulated. Coxibs target preferably COX-2 to reduce inflammation, and thereby they block the primary source of PGI_2 in the vasculature shifting the balance to an increase in vasoconstrictors resulting in the observed side effects. Not only coxibs but literally all NSAIDs except low dose aspirin have been associated with cardiovascular complications [156]. Selective inhibition of mPGES-1/PGE_2 is anti-inflammatory and spares the other prostaglandins including PGI_2 and is therefore thought to be a safer treatment strategy. For example, pre-clinical studies have shown that deletion of mPGES-1 protects against blood pressure increase and thrombosis [253], the formation of aortic aneurism [240] and the development of atherogenesis [184]. Clinical observations confirmed reduction of PGE_2 and increased production of PGI_2 in human after treatment with mPGES-1 inhibitor [206, 208, 209]. As seen from these studies prostanoids are potent regulators of vascular tone and the mPGES-1 inhibitor CIII has recently been shown to reduce NE-induced constriction via shunting to PGI_2 in human saphenous vein and internal mammary artery *ex-vivo* [257]. In **Paper I** we observed a similar reduction in NE-induced vasoconstriction for CIII, 934 and 118 in human vessels of the microcirculation from healthy volunteers.

In **Paper III** we aimed to follow up on these studies and to characterize the effects of mPGES-1 inhibition on vascular tone in resistance arteries from controls (Non-ESKD) and patients with end-stage kidney disease (ESKD). Chronic kidney disease (CKD) describes the slow and gradual damage and loss of kidney function, eventually leading to kidney failure. The kidney becomes increasingly incapable to clean the blood from waste products which eventually requires dialysis treatment or kidney transplantation. Alterations in macro- and micro-circulation, such as endothelial dysfunction and arterial stiffness, are common features of CKD, and patients are at increased risk for cardiovascular disease [294]. In fact, cardiovascular disease is the leading cause of death in patients with ESKD with a bidirectional relation of both diseases [295]. Low-grade inflammation is considered as a risk factor for CKD and correlates with cardiovascular and total mortality [296] In **Paper III**, resistance arteries, isolated from subcutaneous fat biopsies of Non-ESKD controls and ESKD patients were used as model system to test inhibitors of mPGES-1 under normal and inflammatory conditions.

Inhibition of mPGES-1 by CIII and 118 reduced NE-induced constriction in resistance arteries from ESKD patients and Non-ESKD controls (Figure 7a, results for 118 in Non-ESKD). Moreover, enhanced ACh-induced relaxation was observed for treatment with the inhibitor 118 (Figure 7b). These results were in accordance with our previous findings on reduced NE-constriction tested in Non-ESKD controls (**Paper I**). Initially we hypothesized that the effects of the inhibitors may differ between ESKD and Non-ESKD groups since differences in inflammation and calcification markers were found. Levels of serum high sensitivity C-reactive protein (hsCRP) were doubled but not significantly elevated in ESKD patients compared to Non-ESKD, while triglycerides as well as phosphate were significantly elevated in ESKD patients. The vascular response to NE, KPSS and ACh was however not found to be significantly different between Non-ESKD and ESKD, which could explain the comparable functional responses of the inhibitors observed in ESKD and Non-ESKD. In experiments with larger human arteries (saphenous vein and internal mammary artery) and intrarenal arcuate arteries from mice, inhibition of COX-2 caused enhanced constriction [257, 297]. In our experiments the COX-1/COX-2 inhibitor indomethacin as well as the COX-2 selective inhibitors NS-398 and etoricoxib had no effect on NE-induced vasoconstriction (Figure 7g-i). Indomethacin did furthermore not affect ACh-induced relaxation in Non-ESKD. Moreover, we only found very weak COX-2 staining in arteries from ESKD and Non-ESKD. Thus, our results support the preferential role of COX-1 that was described in different vascular beds [298]. However, these observations could question the role of COX-2 in resistance arteries. COX-2 as the primary source of PGI_2 in the vasculature is controversial and not clearly established yet. It has been shown that deletion of COX-2 in endothelial cells and VSMC leads to a reduction of systemic PGI_2 [251, 299]. On the other hand, a dominant role of COX-1 in PGI_2 production has been demonstrated in mouse aorta [252, 300]. The same authors discuss that severe systemic inflammation is needed to simulate a vascular situation in which COX-2 dominates over COX-1. When we incubated isolated arteries with IL-1β, and treated them with NS-398, PGE_2 and PGI_2 levels were significantly blocked (Figure 7e and f). This indicates that COX-2 could contribute at least partly to PGI_2 formation in resistance arteries. Prostaglandin levels however were not significantly enhanced by the IL-1β treatment which could be because induction of COX-2 already occurred during the isolation and culturing procedure. It was previously suggested that COX-2 is inducible by shear stress and that it is unstable outside the body due to a high turnover rate and posttranslational modification [301]. Moreover, it was demonstrated that COX-2 levels increased in *ex-vivo* tissue cultures of mouse aorta over time and primarily contributed to PGI_2 production from 4 hours up till 7 days [252]. This could explain why we saw high baseline production of prostaglandins, inhibition of prostaglandins by NS-398 in cultured arteries and only weak expression of COX-2 in the tissue. Compared with inhibition of COX-2, inhibition of mPGES-1 by CIII or 118 in the artery cultures reduced PGE_2 levels but spared PGI_2 (Figure 7e and f). These results are in accordance with the reduction of PGE_2 by CIII in cultures of larger human arteries [257], although they do not replicate the increase in PGI_2.

The effect of mPGES-1 inhibition in human saphenous vein and internal mammary artery, was shown to involve shunting towards PGI_2 [257]. This was demonstrated by detection of increasing PGI_2 concentrations in artery cultures and blocking of the IP receptor in functional studies. We performed similar experiments to investigate the effect of an IP receptor antagonist (CAY10441) and antagonists of EP4 (GW627368X) and PPARγ (GW9662) on the resistance arteries incubated in the presence of the mPGES-1 inhibitor 118 or CIII. Overall, we did not find that signaling through the IP receptor was responsible for the observed reduced vasoconstriction, neither signaling through EP4 or PPARγ. However, in Non-ESKD arteries, the maximal constriction (E_{max}) was enhanced compared to incubations with the mPGES-1 inhibitors 118 only, indicating that in those experiments PGI_2/IP signaling contributed at least to some extent to the reduction in maximal vasoconstriction. These results stand in contrast with the previous report of CIII in larger human vessels where a clear shunting to PGI_2 was demonstrated to be the underlying mechanism for reduced vasoconstriction by CIII [257]. Possible explanations for this discrepancy could be the differences in the vascular beds studied and the differences in experimental set-up, as we did not involve incubation with inflammatory stimuli. In addition, biphasic effects of PGI_2 and PGE_2 and their ability to redundantly act through different prostanoid receptors may contribute to the observed effects. For example, it was shown that PGE_2 dilated renal afferent arterioles from rat through the EP4 receptor and caused constriction through the EP3 receptor [302]. PGI_2 was further shown to be able to act via EP1, EP3 and EP4 [303]. In renal arteries from humans, PGE_2 and PGI_2 were shown to cause vasoconstriction through the TP receptor [304]. We could confirm the biphasic effects of PGE_2 where nanomolar concentrations cause relaxation and micromolar concentrations cause constriction in arteries from both ESKD and Non-ESKD (Figure 7d). Furthermore, we analyzed the expression of prostanoid receptors (EP1, EP2, EP3, EP4, IP, and TP) and found all receptors present in endothelial cells and VSMC. Expression of mPGES-1 was mainly found in VSMC. VSMC, endothelial cells as well as fibroblast and surrounding immune cells have been described to express mPGES-1 and possibly contribute to local PGE_2 formation in vessels [299, 305-307]. Another explanation for the effects we see after inhibition of mPGES-1 could be that the combination of reduced PGE_2 and spared PGI_2 creates an antioxidant vascular milieu that promotes dilatation. It has been previously shown that PGE_2 can cause vascular dysfunction by enhancing oxidative stress [308]. PGE_2 produced by VSMC has been shown to induce ROS via EP1 and EP3. This could affect the bioavailability of NO as a decrease in the levels of NO was prevented in angiotensin-II infused mPGES-1 KO mice. Moreover, a correlation of mPGES-1 mRNA and superoxide production was found in human peripheral blood mononuclear cells, and deletion of mPGES-1 resulted in a shunting to PGI_2 and prevention of vascular damage induced by angiotensin-II [308]. PGI_2 was furthermore shown to reduce oxidative stress in patients with coronary artery disease [309].

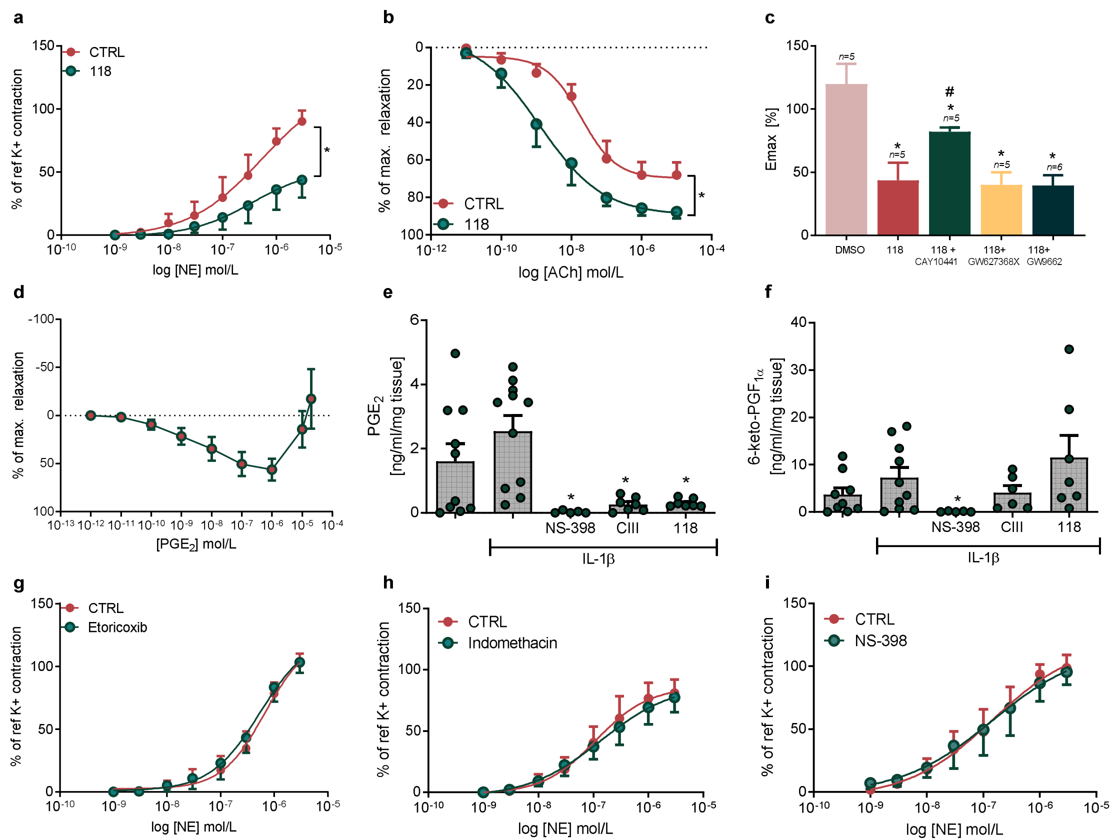


Figure 7: Effects of mPGES-1 inhibition on resistance artery tone from Non-ESKD controls [310]. a) Effect of 118 on NE-induced constriction and b) ACh-induced dilatation. c) E_{max} levels after incubation with the mPGES-1 inhibitor 118 in combination with receptor antagonists. d) Biphasic effects of PGE_2 on vascular tone. e) PGE_2 and f) PGI_2 (6-keto-$PGF_{1\alpha}$) in cultured arteries incubated with IL-1β and COX-2 or mPGES-1 inhibitors. g-h) Effects of COX inhibitors on NE-induced constriction. Comparison between concentration-response curves in a and b were performed using Two-way ANOVA. Differences in E_{max} levels (c, * indicates difference to DMSO control, # indicates difference to 118) and prostaglandin levels were compared by students t-test. Statistical significance level was set to $p<0.05$.

Our results, together with the findings of others mentioned above suggest that the inhibition of mPGES-1 has beneficial vasoactive effects and could lead to improved blood flow and reduced resistance *in-vivo*. It is however important to note that although we used human tissue *ex-vivo*, it does not reflect the *in-vivo* conditions. Components such as surrounding cells and tissues or blood flow are not considered. Additionally, the observed responses could be specific to the contractile and relaxing agonists used. It should also be noted that the patient group consisted of younger and healthier individuals compared to the typical dialysis patient as they underwent living donor transplantation [311]. In addition, the number of patients analyzed in this study was limited and an imbalance of male and females was observed. However, we did not find major differences between males and females regarding the parameters analyzed. Furthermore, it is eventually important to acknowledge that although the effects of mPGES-1 inhibition were similar throughout different vascular beds the underlying mechanisms may vary. We speculate that a combination of retained PGI_2, reduced PGE_2, and possibly modulation of other

prostanoids or vascular factors upon mPGES-1 inhibition caused the responses observed in resistance arteries. Further studies are required to understand and characterize the vasoactive effects of mPGES-1 inhibitors. Future investigations could include co-incubations of COX inhibitors and mPGES1-1 inhibitors to get more insights into the contributions of COX-1 or COX-2 to vascular PGE_2 and PGI_2 formation. Continuative experiments testing receptor antagonists in combination with the mPGES-1 inhibitors should be performed. Combinations of multiple antagonists could help to pinpoint the primary signaling pathway (s) that are possibly responsible for the observed changes in vascular tone. This should include all prostanoid receptors but also PPARα and, PPARβ. To understand possible contributions and interference with the NO-pathway, co-incubations of the mPGES-1 inhibitors with the eNOS inhibitor L-NAME or L-NOARG would be of interest. Remarkably, L-NOARG in combination with CIII in internal mammary arteries did not alter the reduced vasoconstriction, suggesting that the effects of inhibition of mPGES-1 are independent of the NO biosynthesis in these vessels [257]. Moreover, testing of other mPGES-1 inhibitors with structural differences and the assessment of mPGES-1 inhibitors in additional vascular beds would help to expand our knowledge of their vasoactive properties and therapeutic potential.

4.4 INHIBITION OF MPGES-1 IMPROVES CARDIAC FUNCTION AFTER MI

Our observation that inhibition of mPGES-1 has vasoactive effects in resistance arteries of the human microcirculation encourages investigations on its effects in diseases such as pulmonary artery hypertension or MI. MI is a life-threatening condition that is caused by a disabled blood flow to the heart muscle due to occlusion of the coronary artery. Plague rupture in the coronary artery and subsequent thrombotic occlusion leading to necrosis in the myocardial tissue is the most common cause of acute MI. Chronic low-grade inflammation (associated with risk factors such as obesity, diabetes and hypertension) as well as acute inflammation (induced by e.g., plaque rupture) are components of cardiovascular diseases and imply a role for the prostaglandin system. Although being anti-inflammatory, NSAIDs and coxibs can have detrimental effects on the cardiovascular system. Blocking PGI_2 synthesis is considered the primary cause for their cardiovascular side effects and inhibition of mPGES-1 is anticipated to be safer due to the sole reduction of PGE_2. Deletion of mPGES-1 indeed has been shown to augment PGI_2 levels, to prevent from angiotensin-II induced aortic aneurism formation and to retard atherogenesis [184, 240]. In another study, global deletion of mPGES-1 as well as deletion of mPGES-1 in myeloid cells (macrophages) did not affect cardiac function after MI in mice, the latter actually increased post MI survival [258]. Moreover, deletion of mPGES-1 in mice with acute MI led to increased plasma PGI_2 production and the acute cardiac ischemic injury was not worsened compared to WT mice [312]. Additional results from the same authors demonstrated that ischemic myocardial damage did not increase in mPGES-1 KO mice and post-MI survival was not decreased, in contrast to WT mice receiving celecoxib [259] indicating a more favorable cardiovascular profile for blocking of mPGES-1. Nevertheless, other studies showed that global deletion of mPGES-1 or deletion in bone marrow derived myeloid cells impaired cardiac remodeling after MI [260, 261]. These controversies indicate that the role of mPGES-1 in MI is still unclear and that there is a need for investigations on the

pharmacological inhibition of mPGES-1 in this disease. In **Paper IV** we have therefore studied inhibition of mPGES-1 in comparison with COX-2 inhibition in a mouse model of MI.

To study the effect of mPGES-1 inhibition, MI was induced by permanent ligation of the left anterior descending coronary artery (LAD). This is the most common mouse model to mimic MI and eventually results in heart failure with reduced ejection fraction (HFrEF; LVEF < 40%) [313]. LAD ligation causes ischemic injury through cardiomyocyte death and extracellular matrix deposition followed by scar tissue formation. These tissue alterations result in thinning and dilation of the infarcted area which further leads to hypertrophy and reactive fibrosis in the remote myocardium, eventually manifesting in left ventricle dilatation and impaired cardiac function. After LAD ligation (24 h), the mice in our study were treated once daily with the mPGES-1 inhibitor CIII (25 mg/kg and 50 mg/kg), the mPGES-1 inhibitor 118 (25 mg/kg), celecoxib (10 mg/kg) or vehicle (1% Tween80, 0.5% carboxylmethyl cellulose in 0.9% NaCl) and cardiac remodeling was monitored for 28 days. The used drug concentrations were based on a pharmacokinetic study carried out prior to the MI study. Urine from metabolic cages was collected on day 14 for PG analysis. Plasma was collected at day 28. Cardiac function on the anesthetized animals was assessed at day 7 and 28. Scar thickness and fibrosis area were determined by histological analysis of the hearts at day 28.

We observed post-MI cardiac remodeling over a period of 28 days. Measurements of cardiac function at day 7 post-MI showed an increase in ejection fraction after treatment with 50 mg/kg CIII. No significant changes were observed in the other treatment groups compared with the vehicle group. It has been described that it typically takes 4 weeks post-surgery for LAD ligation to result in heart failure, which may explain why we did not observe changes for all groups after only 1 week of treatment [313]. However, prolonged treatment with CIII or 118 showed significant improvement in cardiac function 28 days after MI. Specifically, this was seen for the ejection fraction, fraction shortening, left-ventricular end-diastolic anterior wall thickness and left ventricular end-systolic wall thickness. All parameters were improved after treatment with CIII or 118 compared to the vehicle or celecoxib group. Celecoxib did not improve cardiac function compared to the vehicle group. Exercise intolerance and early exhaustion are features of chronic heart failure and drastically limit the daily activities of patients. We found an improvement in running distance in animals treated with the mPGES-1 inhibitors. Moreover, the inhibition of mPGES-1 had no effect on blood pressure, platelet activation in the peripheral blood, blood coagulation or carotid artery thrombosis. Replacement of dead cardiomyocytes with collagen-based (primarily collagen I) scar tissue is called reparative fibrosis and is essential to prevent cardiac rupture [314]. CIII (50 mg/kg) increased scar thickness and reduced the infarct area in the left ventricle. Moreover, the fibrosis-related genes,Tgfb1 and Timp1 were inhibited by CIII. TGF-β is thought to actively contribute to fibrosis by inducing protease inhibitors such as TIMP1 thereby suppressing matrix metalloproteinase (MMP) synthesis and degradation of extracellular matrix [315]. Our results therefore indicate that inhibition of mPGES-1 reduced infarct size, improved scar healing while diminishing fibrosis. Additionally altered collagen type I/III ratio is associated with reactive cardiac fibrosis and left ventricular dysfunction in MI [314]. The ratio of collagen I/III was

decreased in the CIII group compared with celecoxib and vehicle treatment. This could suggest that CIII promotes extracellular matrix metabolism and protects from cardiac dilatation and fibrosis. Our results are in accordance with reports showing that the deletion of mPGES-1 attenuated cardiac fibrosis in mice and that PGI_2 protected against fibrosis in models of cardiac fibrosis [316-318]. Increased levels of pro-inflammatory cytokines are associated with fibrosis and myocardial pathologies. TNF-α, IL-1β and IL-18 were for example described to be involved in collagen deposition, activation of fibroblasts or recruitment of macrophages [315]. The role for IFN-γ in the heart includes a wide range of actions including inflammatory cell recruitment and development of heart failure [319]. We found that all inhibitors reduced IFN-γ levels and celecoxib and CIII (50 mg/kg) also reduced TNF-α levels. Levels of IL-1β and IL-18 were not affected. Capillary density in the infarct area not in the remote area, based on CD31 staining was highest after CIII treatment and indicated an increase in neovascularization. PGI_2 has previously been shown to be important for regeneration in the cardiovascular system including promotion of endothelial cell proliferation and could be responsible for the observed healing after CIII treatment [320].

We found that the urinary PGI_2/PGE_2 metabolite ratio was significantly increased in the CIII group and correlated with improved ejection fraction, fraction shortening and left ventricular end-systolic anterior wall thickness as well as scar thickness compared to vehicle or celecoxib treatment. Notably, only urinary PGI_2 metabolite was positively correlated with cardiac function, suggesting that preservation and increase of PGI_2 rather than reduction of PGE_2 was responsible for the observed cardiac improvement. From a previous study testing CIII in a neuroblastoma mouse model, we know that despite detectable drug concentrations and effects on tumor growth, the effects on PGE_2 levels were transient and only detectable after two hours in the tumor tissue [211]. Similarly transient reduction of PGE_2 which we may not cover measuring urinary metabolites over 24 hours could still contribute to the profound functional effects we observed. Notably, an increase in urinary PGI_2/PGE_2 metabolite ratio was also observed in the celecoxib group compared with vehicle although to a lesser extent than in the CIII group. This effect was primarily driven by a strong reduction of PGE_2. Inhibition of COX-2 is associated with an increased risk for cardiovascular diseases. In experimental permanent ligation MI models, COX-1/COX-2 inhibition with indomethacin and ibuprofen was shown to increase left ventricular dilatation and infarct size in dogs [321, 322] whereas COX-2 inhibition with NS-398 improved fibrosis and cardiac function in mice [323, 324]. In a study with pigs, celecoxib treatment resulted in increased mortality and impaired cardiac function after MI [325]. In our study we found no adverse effects with celecoxib compared to vehicle treatment.

As mentioned above, results obtained from studies with mPGES-1 or COX KO mice, as well as from studies with COX inhibitors, are discordant. Several studies indicate a protective role of PGE_2 signaling underlying the adverse effects seen with COX-2 but also with mPGES-1 depletion in different experimental models including MI. For example, PGE_2-EP3 signaling was shown to be important for cardiac healing after MI in mice [326]. Disruption of PGE_2-EP3 signaling resulted in reduced cardiac function and increased infarct size. PGE_2-EP3 signaling was found to facilitate infiltration of tissue reparative myeloid cells ($Ly6C^{low}$ macrophages,

M2-like) and suppression of PGE_2-EP3 signaling interfered with angiogenesis. In addition, EP3 was found to enable TGF-β1 dependent activation of vascular endothelial growth factor (*vegf*) genes leading to $Ly6C^{low}$ cell migration and neovascularization. In another study, mPGES-1 was described to be protective through PGE_2-EP4 signaling in mice with acute myocardial ischemia-reperfusion injury [327]. Mice lacking mPGES-1 showed impaired cardiac microvascular perfusion and more infiltrated immune cells. Reduced artery dilatation *in-vivo* and *ex-vivo* and enhanced leukocyte-endothelial cell interaction were observed in the mPGES-1 KO mice. Furthermore, deletion of EP4 was shown to result in impaired microcirculation and increased myocardial infarction ischemia reperfusion (MI/IR) injury. In contrast, EP4 activation with the PGE_2 analogue misoprostol improved microcirculation suggesting that mPGES-1 protected the microcirculation in these mice. It is important to mention that the described studies involved receptor ablation or mPGES-1 KO mice but did not address pharmacological inhibition of mPGES-1. Therefore, and also based on previously discussed differences between mPGES-1 deletion and inhibition, it can be speculated that the pharmacological inhibition of mPGES-1 may result in different outcomes. It is thus important to highlight that the inhibition of mPGES-1 with CIII in our study did not result in reduced urinary PGE_2 metabolite levels compared to the celecoxib group or as described in the studies using mPGES-1 KO mice. We know from previous experiments that the inhibition of mPGES-1 does not universally result in complete blockage of PGE_2. The degree of PGE_2 reduction differs among cell types. For example, in A549 cells [202] CIII reduced ~80 % of PGE_2 levels while in RAW264.7 cells (**Paper II**) a 40-50 % reduction was observed. Inhibition of mPGES-1 primarily reduces inflammatory PGE_2 levels and low levels may remain which could be beneficial for homeostasis and reparative processes as described above. In line with the discussion on the role of PGE_2 in MI, it must be noted that prostanoid levels were measured by their urinary metabolites in this study. As described previously, urinary prostanoid metabolites are widely considered to reflect systemic production. However, recent studies suggest that urinary levels of some prostanoid metabolites reflect renal levels rather than systemic production of prostanoids [156, 328]. With this in mind it would be of interest to additionally assess prostanoid levels in the hearts, comparing infarct and remote area as well as other tissues. It has been previously shown that levels of PGE_2 were about 8-fold higher in the infarct zone compared to the remote area within one week after MI. Moreover, the deletion of mPGES-1 had no effect on the levels of PGD_2, TXB_2, $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ in the heart [260]. It has been further described that the spleen contributes to the production of leukocytes that migrate to the left ventricle and to lipid mediator biosynthesis that aids in the remodeling post-MI [329]. We also measured the levels of prostanoids in the spleen but did not find correlations with cardiac function. Further studies are needed to investigate the underlying mechanisms of CIII treatment and PGI_2 in MI, possibly including ablation of receptors for PGI_2, measurements of plasma ADMA levels, assessment of the inflammatory status and effector cells. Another consideration for future experiments is the timepoint for intervention. In our study we treated the animals with the mPGES-1 inhibitor shortly after MI and whether the inhibitor would have similar effects when admitted at different phases in the model (e.g., prior to MI or during the remodeling phase) remains to be investigated.

We studied the effects of mPGES-1 inhibition compared with COX-2 inhibition in a mouse model of MI, causing HFrEF. LAD ligation is the most common method for inducing MI. The model is reliable for induction of tissue damage and heart failure but does not reflect the clinical situation. Underlying factors like coronary artery disease, atherosclerosis, hypertension or thrombosis are not taken into consideration as well as reperfusion therapy which most patients are subjected to after acute MI [313]. We observed a very high mortality rate after permanent ligation of the LAD artery independent on the treatment. The high mortality rate was mainly due to cardiac rupture and is a general disadvantage of this model compared to for example the MI/IR model [330]. Temporal ligation of the LAD with subsequent reperfusion has a higher survival rate and mimics the clinical scenario more closely. Thus, we tested the mPGES-1 inhibitors CIII and 118 in a model with 30 minutes of ischemia followed by 28 days of reperfusion. After MI/IR injury a non-significant slight increase in cardiac function after CIII (50 mg/kg) treatment and no effect on infarct area or body weight could be observed. The severity of myocardium injury was markedly less in the I/R model compared to the permanent ligation MI model (EF: 72.40±10.21% (Naïve, before surgery), 45.34±13.33% (I/R), and 22.24±6.97% (MI)). This could be a reason for why the effects of the mPGES-1 inhibitors are more pronounced in the permanent ligation MI model. However, compared to acute reperfusion injury where deletion of mPGES-1 worsened cardiac function [327], we did not find adverse effects with the pharmacological inhibition of mPGES-1 in the chronic reperfusion injury model.

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5 SUMMARY AND PERSPECTIVES

The aim of this thesis was to study the anti-inflammatory and vasoactive properties of pharmacological inhibition of mPGES-1.

In **Paper I** we have characterized five new mPGES-1 inhibitors and found them to be well suited for pre-clinical investigations. We concluded that the inhibitors are refined tools with improved selectivity and potency compared to other described compounds. Their use in pre-clinical disease models will hopefully increase our knowledge on the therapeutic potential of mPGES-1 inhibition and help elucidate the underlying mechanisms. Although the inhibitors showed very similar results in most of the assays, there may be differences when applied in other disease models and assessment of multiple mPGES-1 inhibitors (preferably with additional inhibitors of structural difference) will strengthen our understanding of their effects.

Inhibition of mPGES-1 may be as efficient in lowering PGE_2 levels as inhibitors of COX, without inhibiting the other prostanoids. Indeed, maintenance of other prostanoids and redirection of PGH_2 has been observed in various *in-vitro* and *in-vivo* assays. Inhibition of mPGES-1 comes therefore with an uncertainty that has raised concerns about potential side effects but also anticipation of additional beneficial effects. Whether the levels of prostanoids (PGD_2, $PGF_{2\alpha}$, PGI_2, TXA_2) become upregulated or remain the same upon inhibition of mPGES-1 is highly dependent on tissue expression of prostanoid synthases in a pathological condition [189]. Based on numerous pre-clinical studies with overall favorable outcomes, the blocking of mPGES-1 has emerged as a promising therapeutic strategy. And associated shunting, especially shunting to PGI_2 is believed to contribute to the therapeutic effects seen with inhibitors of mPGES-1.

Besides shunting to PGI_2, a shift to the PGD_2 metabolite $15dPGJ_2$ was found in macrophages from mice lacking mPGES-1. Depending on the inflammatory context, PGD_2 and $15dPGJ_2$ are described to aid in the resolution of inflammation and the anti-inflammatory and pro-resolving effects of PGD_2 are thought to be to a large extend mediated through the activities of $15dPGJ_2$ [217]. **In Paper II**, we studied the biosynthesis and metabolism of $15dPGJ_2$ in macrophages and mast cells which are known to produce the precursor PGD_2 and are a good source for downstream metabolites [278]. We found that $15dPGJ_2$-GS and $15dPGJ_2$-Cys are generated via conjugation of $15dPGJ_2$ with GSH in macrophages and mast cells. Moreover, our results suggest that MGST3 plays an important role in the biosynthesis of the $15dPGJ_2$-conjugates in these cells. Inhibition of mPGES-1 with CIII and 118 preserved the PGD_2 pathway with a tendential increase for the $15dPGJ_2$-conjugates. Whether the maintenance or enhancement of the PGD_2 pathway upon inhibition of mPGES-1 is sufficient to contribute to its observed phenotypes remains to be investigated. Future studies with stable and pure standard molecules for $15dPGJ_2$-GS and $15dPGJ_2$-Cys and possible intermediate molecules will be required for more accurate quantification and functional studies on their bioactivity. Whether the metabolism of $15dPGJ_2$ into the $15dPGJ_2$-GS and $15dPGJ_2$-Cys conjugate is primarily occurring for detoxification purposes or if it results in bioactive compounds that have functions in inflammation requires further research. Macrophages are important immune effector cells,

and several studies describe that $15dPGJ_2$ can contribute to macrophage polarization [222]. Evaluation of mPGES-1 inhibitors and $15dPGJ_2$-conjugates in assays of macrophage phagocytosis or efferocytosis would be of interest to gain insight into the potential for macrophage polarization and resolving capacity. Despite numerous studies demonstrating anti-inflammatory and pro-resolving effects of $15dPGJ_2$, its high reactivity with GSH and cellular proteins implies unselective effects and opposing activities have been reported [331]. It remains to be elucidated, whether the conjugation of $15dPGJ_2$ with GSH results in bioactive mediators that more selectively activate for example nuclear receptors such as PPARγ and RXR and thereby resemble novel therapeutic strategies.

Modulation of the prostanoid system is connected with concerns about side effects, especially since the withdrawal of several COX-2 targeting drugs due to severe cardiovascular complications. Pre-clinical studies assessing the effects of mPGES-1 inhibitors in models of cardiovascular disease are therefore of great importance. In **Paper III** we tested inhibitors of mPGES-1 in human resistance arteries regarding their capacity to modulate the vascular tone. The results from this study overall confirm previous functional results in other vascular beds but also highlight beneficial effects on the human microcirculation. We found that inhibition of mPGES-1 reduced vascular constriction and enhanced relaxation in arteries from patients with ESKD and Non-ESKD. We were unable to identify a specific pathway to be responsible for the observed effects. Human resistance arteries expressed COX-1, mPGES-1, PGIS, EP1-EP4, IP and TP. Only very low staining was found for COX-2. Blocking of the IP, EP4 or PPARγ receptors did not restore the reduced constriction. The maximal constriction in Non-ESKD arteries was however enhanced after treatment with 118 and IP antagonist compared to 118 alone, indicating a PGI_2 mediated effect. Our results suggest that multiple pathways possibly contribute to the effects seen upon mPGES-1 inhibition and further studies are needed to understand the underlying factors. We concluded that inhibition of mPGES-1 may not cause an increase in blood pressure and could even reduce vascular resistance in *in-vivo* conditions. This concurs with the results published from phase I clinical trials with inhibitors of mPGES-1 which showed no adverse effects on blood pressure in healthy volunteers [206, 208, 209]. To increase confidence in the cardiovascular safety profile of mPGES-1 inhibitors, studies confirming the effect in different vascular beds would be a next step.

MI is a leading cause of death, and studies report conflicting results on the safety of mPGES-1 depletion in this disease. To evaluate the potential of pharmacological inhibition of mPGES-1, we tested the mPGES-1 inhibitors CIII and 118 in mice with MI. In **Paper IV,** we found that treatment with CIII or 118 for 28 days after MI significantly improved cardiac function. In addition, increased scar thickness, reduced infarct size, reduced collagen I/III ratio, and enhanced neovascularization was found in the mPGES-1 inhibitor-treated animals. In comparison, celecoxib had no effect on these parameters. Improved cardiac function correlated with an increased PGI_2/PGE_2 metabolite ratio in urine. Our results contribute to a better understanding of the connection between the prostanoid system and the cardiac healing process after MI and suggest that inhibition of mPGES-1 could be cardio protective. Future studies will need to consider additional measurements of inflammatory components (cytokines, cell

infiltration), prostanoid receptor ablation, timing of the intervention and clinical relevance of the disease model to prove the therapeutic potential of mPGES-1 inhibition for MI.

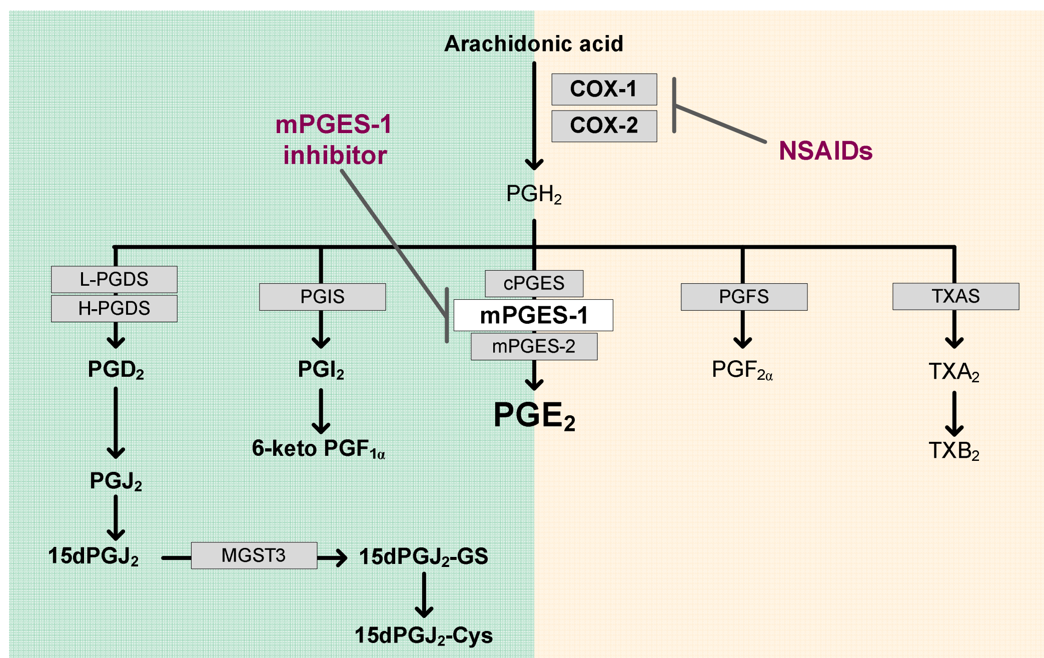

Figure 8: Schematic description of the biosynthesis of prostanoids. Blocking of COX-1 or COX-2 by NSAIDs suppress formation of PGH_2 and other downstream prostanoids, contributing to adverse effects. Inhibition of mPGES-1 blocks the formation of inflammatory PGE_2. A redirection of PGH_2 into other prostanoids may be observed cell type specifically. Shunting to PGI_2 and the PGD_2 pathway potentially contribute to the therapeutic effects of mPGES-1 inhibition supporting the resolution of inflammation and cardiovascular protection. Figure modified from [332].

Inhibition of mPGES-1 is probably superior to COX inhibition and EP receptor targeting because it only reduces inflammatory PGE_2, spares baseline PGE_2 production, and possibly induces a beneficial shunting to prostanoids such as PGI_2 or PGD_2 (Figure 8). However, redirection of PGH_2 to other prostanoids is tissue- and disease-specific and might contribute to reported controversial observations. In addition, in studies with mPGES-1 KO mice and with inhibitors of mPGES-1, effects on the lipid mediators other than prostanoids have been described and suggest profound changes occurring beyond PGH_2 [241, 333]. Future investigations should include the effects of mPGES-1 inhibition on the prostanoid profile and the larger lipid mediator network to estimate consequences for the treatment of pathological conditions.

In summary, in this doctoral thesis, CIII and new inhibitors of mPGES-1 were used to investigate their effects in models of inflammation and cardiovascular disease. We could show that the inhibitors reduced PGE_2 levels and inflammation *in-vivo*. Inhibition of mPGES-1 maintained metabolites of the PGD_2 pathway, and our studies contribute to knowledge on their biosynthesis in immune cells. We found that inhibition of mPGES-1 had vasoactive effects in human resistance arteries under normal and inflammatory conditions and that inhibition of mPGES-1, probably via increased PGI_2 improved cardiac remodeling after MI in mice. Overall, the results support the hypothesis of a beneficial anti-inflammatory and cardiovascular profile of inhibition of mPGES-1 and provide insights into the underlying mechanisms.

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