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Inhibition of mPGES-1 as therapeutic strategy in inflammation and cancer

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THESIS FOR DOCTORAL DEGREE (Ph.D.)
that is publicly defended for doctoral degree at Karolinska Institutet in Lecture Hall Germinal Center, CMM L8:00 on

Monday, June 10, 2019, at 09:00

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To my parents,

and everyone that believes in determination and hard work.

ABSTRACT

Prostaglandin E_2 (PGE_2) is an inflammatory and oncogenic lipid mediator. It is mainly formed via metabolism of arachidonic acid by cyclooxygenases (COX-1 and COX-2) and the terminal enzyme microsomal prostaglandin E synthase-1 (mPGES-1). Widely used non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-1 and/or COX-2, resulting in decreased PGE_2 production and reduced inflammation. However, NSAIDs block the production of many other lipid mediators that have important physiological and resolving actions, and these drugs cause gastrointestinal bleeding and/or increase the risk for severe cardiovascular events. Selective inhibition of downstream mPGES-1 for reduction in only PGE_2 biosynthesis is therefore an anticipated therapeutic strategy. This PhD thesis aims to increase knowledge on mPGES-1 and its inhibition in inflammation and cancer.

Cultures of human cells, preclinical animal models, and clinical material from humans were used to study inflammation at the molecular level, specifically after manipulation of prostaglandin production. The main method of analysis was liquid chromatography tandem mass spectrometry (LC-MS/MS).

This thesis showed that prostacyclin and PGE_2 are potentially important mediators in human tendon disease (**Paper I**). Proteomics and lipidomics data suggested differences in cellular protein and lipid profiles upon pharmacological inhibition of mPGES-1 or COX-2 in cancer cells, where inhibition of mPGES-1 potentiated the cytotoxicity of cytostatic drugs *in vitro* (**Paper II**). Daily treatment with an mPGES-1 inhibitor suppressed neuroblastoma tumor growth *in vivo* via decreased angiogenesis, reduced infiltration of cancer-associated fibroblasts, and a shift towards anti-cancer macrophage polarization (**Paper III**). Lastly, characterization of five new mPGES-1 inhibitors in preclinical models showed decreased swelling in a paw edema assay in rats and reduced norepinephrine-induced vasoconstriction in human arteries *ex vivo* (**Paper IV**).

In summary, results from this PhD thesis increase knowledge of prostaglandins in pathology and expand the principle of mPGES-1 as a viable target to treat inflammation and cancer.

LIST OF SCIENTIFIC PAPERS

I. **Bergqvist F**, Carr AJ, Wheway K, Watkins B, Oppermann U, Jakobsson P-J, Dakin SG. Divergent roles of prostacyclin and PGE2 in human tendinopathy. Arthritis Research & Therapy. 2019;21(1):74.

II. **Bergqvist F**, Ossipova E, Idborg H, Raouf J, Checa A, Englund K, Englund P, Emami Khoonsari P, Kultima K, Wheelock CE, Larsson K, Korotkova M, Jakobsson P-J. Inhibition of mPGES-1 or COX-2 Results in Different Proteomic and Lipidomic Profiles in A549 Lung Cancer Cells. *Under review.*

III. Kock A*, Larsson K*, **Bergqvist F**, Eissler N, Elfman LHM, Raouf J, Korotkova M, Johnsen JI, Jakobsson P-J*, Kogner P*. Inhibition of Microsomal Prostaglandin E Synthase-1 in Cancer-Associated Fibroblasts Suppresses Neuroblastoma Tumor Growth. EBioMedicine. 2018;32:84.92.

IV. Larsson K*, Steinmetz J*, **Bergqvist F***, Arefin S, Spahiu L, Wannberg J, Pawelzik S-C, Morgenstern R, Stenberg P, Kublickiene K, Korotkova M, Jakobsson P-J. Biological Characterization of New mPGES-1 Inhibitors in Preclinical Models of Inflammation and Vascular Tone. *Under review.*

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ADDITIONAL PAPERS

The author has contributed to the following publications or manuscripts that are not included in the thesis:

V. Revathikumar P, **Bergqvist F**, Gopalakrishnan S, Korotkova M, Jakobsson P-J, Lampa J, Le Maître E. Immunomodulatory effects of nicotine on interleukin 1β activated human astrocytes and the role of cyclooxygenase 2 in the underlying mechanism. J. Neuroinflammation 2016;13(1):256.

VI. Raouf J, Rafique N, Goodman MC, Idborg H, **Bergqvist F**, Armstrong RN, Jakobsson P-J, Morgenstern R, Spahiu L. Arg126 and Asp49 Are Essential for the Catalytic Function of Microsomal Prostaglandin E2 Synthase 1 and Ser127 Is Not. PLoS One 2016:11(9):e0163600.

VII. Ljungblad L, **Bergqvist F**, Andonova T, Jakobsson P-J, Johnsen JI, Gleissman H, Kogner P, Wickström M. Omega-3 fatty acids DHA and EPA decrease oncogenic PGE2 in medulloblstaoma in vitro and in vivo. *Manuscript.*

VIII. Steinmetz J, **Bergqvist F**, Larsson K, Idborg H, Ossipova E, Lengqvist J, Steinhilber D, Korotkova M, Jakobsson P-J. Characterization of the biosynthesis and metabolism of pro-resolving 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) in macrophages upon mPGES-1 depletion. *Manuscript.*

IX. **Bergqvist F**, Sundström Y, Shang M, Sundström M, Jakobsson P-J, Berg L. Screening of anti-inflammatory properties of chemical probes in human blood: focus on PGE2 and IL-8. *Manuscript.*

X. Kock A, **Bergqvist F**, Korotkova M, Johnsen JI, Jakobsson P-J, Kogner P, Larsson K. Selective inhibition of PGE2 enhances the cytotoxic effect of doxorubicin and vincristine in multicellular tumor spheroids. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

15-PGDH	15-hydroxyprostaglandin dehydrogenase
15d-PGJ$_2$	15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$
AA	Arachidonic acid
AC	Adenylyl cyclase
ADMA	Asymmetric dimethylarginine
AKT	Protein kinase B
AOM	Azoxymethane
CIII	Compound III
CAIA	Collagen antibody-induced arthritis
CIA	Collagen-induced arthritis
CREB	cAMP response element-binding protein
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
cPGES	Cytosolic prostaglandin E synthase
cPLA$_2$	Cytosolic phospholipase A$_2$
DC	Direct current
DP	Prostaglandin D receptor
DSS	Dextran sulfate sodium
EGFR	Epidermal growth factor receptor
EGR1	Early growth response protein-1
ELISA	Enzyme-linked immunosorbent assay
EP	Prostaglandin E receptor
ERK	Extracellular signal-regulated kinases
ESI	Electrospray ionization
EX	Eoxin
FLAP	5-lipoxygenase activating protein
FP	Prostaglandin F receptor
H-PGDS	Hematopoietic prostaglandin D synthase
HETE	Hydroxyeicosatetraenoic acid
IL	Interleukin

IP	Prostacyclin receptor
KO	Knock-out
L-PGDS	Lipocalin-type prostaglandin D synthase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLCCs	Lewis lung carcinoma cells
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPS	Lipopolysaccharide
LT	Leukotriene
m/z	Mass-to-charge ratio
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	Mitogen-activated protein kinases
MDSCs	Myeloid-derived suppressor cells
mPGES-1	Microsomal prostaglandin E synthase-1
mPGES-2	Microsomal prostaglandin E synthase-2
MRM	Multiple reaction monitoring
MRP-4	Multi-drug resistance protein 4
N-ERD	NSAID-exacerbated respiratory disease
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBMCs	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PD1	Programmed cell death protein 1
PE	Phosphatidylethanolamine
PG	Prostaglandin

PGEM	13,14-dihydro-15-keto PGE_2
PGFS	Prostaglandin F synthase
PGI_2	Prostacyclin
PGIM	2,3-dinor-6-keto $PGF_{1\alpha}$
PGIS	Prostacyclin synthase
PGT	Prostaglandin transporter
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMN	Polymorphonuclear cells
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
QC	Quality control
RF	Radio frequency
RT	Room temperature
Rv	Resolvin
SAR	Structure-activity studies
SIR	Selected ion recording
SM	Sphingomyelin
SPE	Solid-phase extraction
SPM	Specialized pro-resolving lipid mediators
TAM	Tumor-associated macrophage
Th cells	T helper cells
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TP	Thromboxane receptor
TQD	Triple quadrupole detector
Tregs	T regulatory cells
TX	Thromboxane
TXAS	Thromboxane A synthase
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 INFLAMMATION AND CANCER

Inflammation is a highly controlled immune response to eliminate the cause of tissue injury or infection and to initiate tissue repair back to homeostasis via resolution (1, 2). However, inflammation is not always terminated. Unresolved inflammation causes persistent pain, tissue degeneration, and loss of function. Inflammatory responses drive many autoimmune diseases (3) and inflammation is a hallmark of cancer (4). There is a great need for drugs that are anti-inflammatory and safe.

Inflammatory processes are divided into acute or chronic. The acute phase includes the rapid influx of neutrophils and monocytes to remove pathogens and damaged cells via phagocytosis. This response subsides within days. The innate reactions trigger the activation of the adaptive responses, which means education and recruitment of lymphocytes that neutralize pathogens or kill dysfunctional cells. If the adaptive responses fail, the inflammation becomes chronic. It is not fully understood how this occurs, but the chronic inflammatory state is characterized by presence of pro-inflammatory components and lack of resolving mechanisms. Inflammatory responses are present in many diseases including rheumatic diseases, multiple forms of cancer, neurodegenerative diseases, and cardiovascular diseases.

The complex signaling at the site of inflammation involves several distinct cell types and molecules. This complexity does also change throughout the course of inflammation (and resolution). Of particular interest are potent bioactive lipids of the prostanoid family, especially prostaglandin E_2 (PGE_2). PGE_2 is a central mediator of pain, edema, and cartilage erosion typically observed in the joints of rheumatoid arthritis patients (5, 6). At the same time, PGE_2 is a promotor of the immunosuppressive tumor microenvironment with major impact on tumor progression (4, 7, 8). Multiple non-steroidal anti-inflammatory drugs (NSAIDs) exist in clinical practice that unselectively decrease PGE_2 production, but these drugs are all associated with severe adverse effects. Selective inhibition of PGE_2 production with small molecule inhibitors is therefore a highly anticipated therapeutic strategy in inflammation and cancer. This is the topic of this thesis.

1.2 PROSTANOID METABOLISM

Prostanoids belong to the lipid class eicosanoids, which comprises all lipid metabolites produced from polyunsaturated fatty acids (PUFA) with 20 carbon in chain length. The eicosanoid family therefore comprises a diverse population of bioactive lipids including prostanoids, hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), eoxins (EXs), and resolvins (Rvs). Eicosanoid research traces back to the 1930s when Ulf von Euler first coined the name "prostaglandin" for an unknown substance in seminal fluid. Sune Bergström and Bengt Samuelsson later characterized this molecule and many more prostaglandins, for which they together with Sir John Vane were awarded the Nobel Prize in Physiology or Medicine in 1982. Ever since, increasing amount of research have demonstrated that many different types

of cells produce eicosanoids and these mediators have numerous important physiological and pathological roles.

The first reaction in the biosynthesis of prostanoids (**Figure 1**) is the release of arachidonic acid (AA) from the phospholipid cell membrane. This is catalyzed by phospholipases, in particular cytosolic phospholipase A_2 ($cPLA_2$), which is activated and translocated to the cell membrane upon phosphorylation and increased Ca^{2+} concentration. This enzyme specifically cleaves fatty acids from position two (sn-2) of phospholipids. The next step is catalyzed by the cyclooxygenases (COX-1 and COX-2). These enzymes convert AA to unstable PGH_2 via PGG_2. This occurs by abstraction of a hydrogen on carbon no. 13 followed by lipid peroxidation on carbon no. 11 and carbon no. 15. The peroxide group on carbon no. 11 binds with carbon no. 9 and the peroxide group on carbon no. 15 is reduced to a hydroxyl group, yielding PGH_2 (9). The lower case "2" stands for the number of double bonds in the acyl chain.

The final step in the biosynthesis is the conversion of PGH_2 by terminal synthases into the main prostanoids: PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane A_2 (TXA_2) (10). PGE_2 is generated by cytosolic PGE synthase (cPGES), microsomal PGE synthase (mPGES)-1, and mPGES-2. PGD_2 is generated by hematopoietic PGD synthase (H-PGDS) and lipocalin-type PGD synthase (L-PGDS). $PGF_{2\alpha}$ is generated by PGH_2 9,11-endoperoxide reductase and PGE_2 9-keto reductase (collectively named PGF synthase, PGFS). Prostacyclin is generated by prostacyclin synthase (PGIS). Finally, TXA_2 is generated by TX synthase (TXAS). The terminal enzymes display a preferential, but not exclusive, coupling to the respective COX enzymes. COX-1 couples to cPGES, PGFS, and TXAS, while COX-2 couples to mPGES-1, mPGES-2, and PGIS (11). In addition, PGH_2 can be generated by one cell and further metabolized by neighboring cells (transcellular biosynthesis) (12).

Synthesized prostanoids are secreted from cells via passive diffusion or multidrug resistance protein-4 (MRP4) (13). Once released from cells, prostanoids signal through distinct G-protein coupled receptors in autocrine or paracrine fashion (14), presented in **Figure 2**. The endogenous concentration is dependent on the activity of $cPLA_2$, the expression of COX, and the expression of terminal synthases, which vary depending on cell type and cell state. The estimated affinities of prostanoids to the prostanoid receptors are in the nanomolar range (0.3-25 nM) (15). Prostanoids are fast acting and short-lived, as they are taken up by cells via prostaglandin transporter (PGT) and primarily oxidized by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) to generate inactive forms. Prostacyclin and TXA_2 are unstable at physiological and aqueous conditions and are non-enzymatically hydrolyzed into 6-keto $PGF_{1\alpha}$ and TXB_2, respectively. Prostanoid metabolites are then excreted into the blood stream and filtered out in the kidneys. In summary, the identity and concentration of prostanoids are cell and tissue specific, which is evident by the complex set-up of expression for the enzymes implicated in prostanoid catabolism and anabolism.
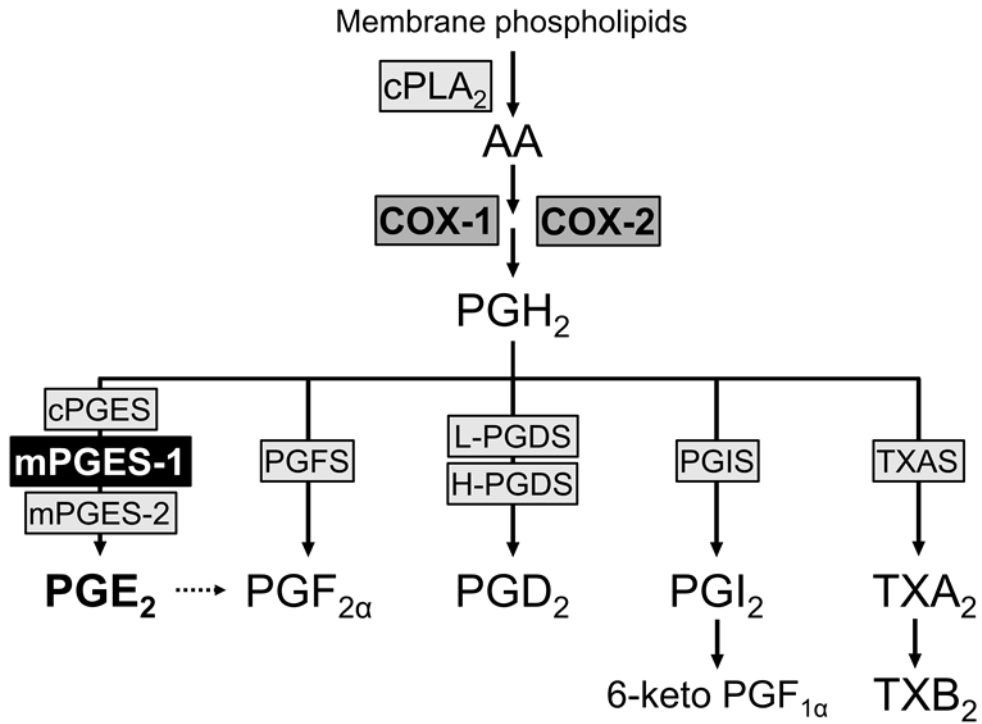
Figure 1. Prostanoid biosynthesis. The COX enzymes convert AA into PGH_2 that is further metabolized by the terminal synthases into the main prostanoids. Prostacyclin (PGI_2) and TXA_2 are rapidly converted into inactive metabolites 6-keto $PGF_{1\alpha}$ and TXB_2, respectively. $PGF_{2\alpha}$ can be generated from PGH_2 or PGE_2. Highlighted in black is the inducible terminal synthase mPGES-1.
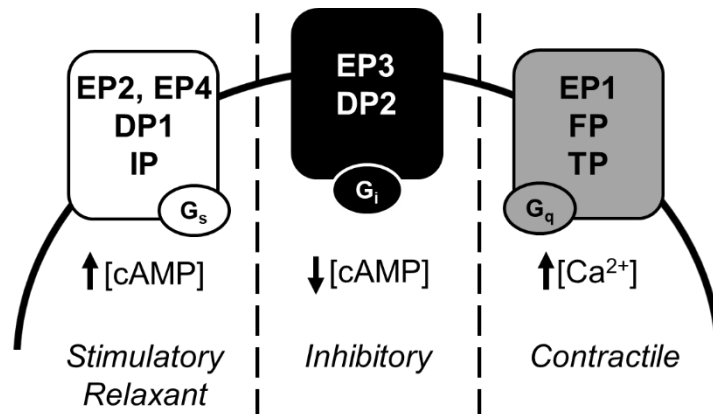
Figure 2. Prostanoid signaling. Each of the five prostanoid receptors (EP, DP, FP, IP, and TP) display a preference for one of the main prostanoids; however, all prostanoids display binding to all receptors with varying affinities. The figure shows the overall classification based on the main effect on smooth muscle cells (relaxant/contractile) or action on cAMP production (stimulatory/inhibitory). Signaling via EP3 and DP1 can promote calcium release, depending on receptor isoform.

1.3 PROSTANOID FUNCTIONS IN HEALTH AND DISEASE

Many types of cells produce prostanoids that can act on even more types of cells. This section will present each of the five main prostanoids separately in terms of their role in physiology, inflammation, and cancer. The molecular structures of prostanoids are presented in **Figure 3**.

PGE$_2$

PGE$_2$ is a regulator of blood pressure (16) and blood flow (17), a protector of mucosal integrity in the gastrointestinal tract (18), a component in renal homeostasis (19), an essential bioactive lipid in female fertility (20), and a mediator of wakefulness (21). As for pathophysiology, PGE$_2$ is involved in all processes leading to the signs of acute inflammation, *i.e.* heat, redness, swelling, and pain, (8). These features are driven by PGE$_2$ (and also prostacyclin and LTB$_4$), causing neutrophil and macrophage infiltration via vasodilation and enhanced vascular permeability (22). The initial burst in PGE$_2$ production is essential for the resolution of inflammation, as PGE$_2$ turns on transcription of enzymes implicated in the generation of pro-resolving lipid mediators (23). Additionally, PGE$_2$ can act on a wide variety of immune cells to modulate immune responses associated with chronic inflammation and cancer. PGE$_2$ promotes tumor-favoring M2 polarization of tumor-associated macrophages (TAMs), attracts immunosuppressive myeloid-derived suppressor cells (MDSCs), enhances the immunity inhibitory function of regulatory T cells (Tregs), decreases amount and maturation of infiltrating antigen-presenting dendritic cells, inhibits anti-tumor activity of natural killer (NK) cells and cytotoxic T cells, and promotes inflammatory functions of T helper (Th) 17 cells (7, 24, 25).

PGE$_2$ signals through four receptors named EP1-4. EP1 is G$_q$ coupled, where binding ultimately results in increase in intracellular Ca^{2+} concentration and activation of protein kinase C (PKC). EP2 signaling is G$_s$ coupled, resulting in activation of adenylyl cyclase (AC), production of cAMP, and activation of protein kinase A (PKA). EP3 is classified as G$_i$ coupled (but may signal via G$_q$ depending on EP3 isoform), resulting in inactivation of PKA by reduction in cAMP production via inhibition of AC. EP4 is coupled to G$_s$ that results in increased cAMP concentration by activation of AC (like EP2). Signaling via EP4 also activates phosphoinositide 3-kinase (PI3K). EP signaling will ultimately influence activation and translocation of transcription factors, including cAMP response element-binding protein (CREB) (26), protein kinase B (AKT) (27), extracellular signal-regulated kinases (ERK) (28), and p38 mitogen-activated protein kinases (MAPK) (29). This translate into PGE$_2$ having the ability to influence survival, proliferation, and migration of cells (30, 31).

Mouse lacking any of the EP receptors have distinct phenotypes (32). For example, deletion of EP1 results in decreased PGE$_2$-induced tactile pain response (allodynia) (33). EP2 knockout mice are less fertile (34), sensitive to salt-induced hypertension (34), and show no bronchodilation in response to PGE$_2$ (35). Deletion of EP3 leads to impaired febrile response to interleukin-1β (IL-1β) or lipopolysaccharide (LPS) (36), reduced acetic acid-induced writhing after LPS treatment (37), and worse allergic response to ovalbumin-induced asthma

(38). Mice deficient in EP4 display reduced ear swelling in a model of contact hypersensitivity (39) and develop more severe colitis induced by dextran sulfate sodium (DSS) (40).

The COX/mPGES-1/PGE$_2$ pathway is induced in several forms of cancer (41-48). The increased expression of COX-2 and mPGES-1 is linked to decreased survival rate in cancer (49, 50). Not only is the biosynthesis of PGE$_2$ important but also the secretion, the uptake, and the hydrolysis of PGE$_2$ are collectively determining the active concentration in the tumor microenvironment (51). A pro-tumorigenic role has been demonstrated for PGE$_2$ in multiple studies, either by injection of PGE$_2$, use of EP antagonist, or pharmacological inhibition or genetic deletion of COX or mPGES-1 (reviewed in (25)). This will be discussed at a later stage, but a recent publication nicely concluded the significance of PGE$_2$ in cancer. Zelenay *et al.* reported that tumor COX activity, namely PGE$_2$ production, promoted immunosuppression in xenograft models of breast (Braf^{V600E} and 4T1) and colon (CT26) cancer, and that COX deficiency in the cancer cells induced a less immunosuppressive microenvironment (52). The authors showed that inhibition of COX by NSAIDs (aspirin or celecoxib) had no effect alone on tumor growth in immunocompetent mice but the inhibitors enhanced the effect of immunotherapy (anti-PD1 antibody).

Increasing amount of literature has identified the COX/mPGES-1/PGE$_2$ pathway in other inflammatory diseases. This includes rheumatic diseases such as rheumatoid arthritis (53), osteoarthritis (54, 55), and idiopathic inflammatory myopathies (56) along with neurological diseases such as Alzheimer's disease (57) and multiple sclerosis (58), and cardiovascular diseases such as atherosclerosis (59, 60), aortic aneurysm (61), and ischemic stroke (62).

In summary, PGE$_2$ is an important mediator of many inflammatory and immunoregulatory processes in multiple diseases.

PGD$_2$

PGD$_2$ and PGE$_2$ are isomers. They differ in the functional groups of carbon no. 9 (hydroxyl group for PGD$_2$, ketone group for PGE$_2$) and carbon no. 11 (ketone group for PGD$_2$, hydroxyl group for PGE$_2$). PGD$_2$ signals through DP1 (G$_s$ coupled) and DP2 (G$_i$ coupled), which affects cAMP production. This prostanoid is recognized for regulating sleep (63) and reproduction functions (64). PGD$_2$ is the main product of mast cells in allergic responses and it is found elevated in bronchoalveolar lavage fluid in patients with severe asthma (65), where it causes bronchoconstriction and vasodilation, increases mucous production, and enables influx of immune cells by increasing capillary permeability (66). PGD$_2$ can also bind TP receptor, causing smooth muscle constriction and platelet aggregation, which contributes to the effects seen in asthma.

Eguchi *et al.* reported that mice lacking L-PGDS were insensitive to PGE$_2$- or bicuculline-induced allodynia but the mice responded normally to thermal hyperalgesia (increased sensitivity to pain, in this case heat) (67). Allodynia was restored with injections of PGD$_2$ in the KO mice, highlighting a role a PGD$_2$ in neuropathic pain. Rajakariar *et al.* showed that mice lacking H-PGDS displayed a more severe acute phase of inflammation and impairment

of resolution in a model of peritonitis (68). They demonstrated that absence of PGD_2 production coincide with decreased concentration of IL-10, increased concentration of tumor necrosis factor-α (TNF-α), and increased concentration of monocyte chemoattractant protein-1 in the exudates. This resulted in elevated levels of polymorphonuclear cells (PMN) in the acute phase with decreased draining of macrophages from the peritoneum to the lymph nodes in the late phase. Moreover, PGD_2 is metabolized to 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) that can modulate inflammatory responses via peroxisome proliferator-activated receptor gamma (PPARγ) (69). Injection of 15d-PGJ_2 in the peritonitis study described above reversed the lack of resolution caused by deletion of H-PGDS (68). 15d-PGJ_2 and other cyclopentenone PGs can act anti-viral *in vitro* and *in vivo* through inhibition of viral protein synthesis via induced synthesis of heat-shock proteins (70).

The immunomodulatory effects of PGD_2 have implications in cancer. PGD_2 signaling decreased the cytotoxic activity of NK cells *in vitro* (71). However, treatment with PGD_2 or L-PGDS decreased proliferation rate of multiple gastric cancer cell lines and tumor growth in a xenograft mice model of gastric cancer (72). In addition, treatment with 15d-PGJ_2 induced cell death of cancer cells *in vitro* (73-77) and decreased tumor growth *in vivo* (78). Genetic deletion of H-PGDS increased while overexpression of H-PGDS decreased intestinal tumorigenesis in $Apc^{Min/+}$ mice (79), again highlighting anti-cancer properties of PGD_2. This translates into that PGD_2 has immunosuppressive effects and at the same time anti-cancer properties, for example via suppression of tumor angiogenesis (80).

These studies conclude that PGD_2 is a mediator of both inflammation and resolution with implications in tumor progression.

$PGF_{2\alpha}$

$PGF_{2\alpha}$ is an important mediator in childbirth and a regulator of blood pressure. It is also implicated in inflammation and cancer (8). This prostanoid signals through the FP receptor, which exist in the two isoforms FP_A and FP_B. The FP receptor is G_q coupled (like EP1), where signaling increases intracellular Ca^{2+} and activation of PKC. In addition, FP signaling can activate Rho pathway via G_q-independent mechanism that regulate intracellular actin dynamics (81). $PGF_{2\alpha}$ has hydroxyl groups on both carbon no. 9 and carbon no. 11, making it very similar in structure to PGE_2 and PGD_2. Both PGE_2 and PGD_2 can bind to the FP receptor, but with lower affinities (15). $PGF_{2\alpha}$ affects blood pressure via the salt balance in the kidneys and via direct vasoconstriction of vascular smooth muscle cells (82).

Genetic deletion of the FP receptor is reported protective in some mice models where it decreases LPS-induced tachycardia (increased heart rate) (83), limits pulmonary fibrosis post-microbial invasion (84), and reduces hyperlipidemia-induced atherosclerosis (85). Basu *et al.* reported that both $PGF_{2\alpha}$ metabolite 15-keto-dihydro-$PGF_{2\alpha}$ and oxidative stress marker 8-iso-$PGF_{2\alpha}$ are increased in plasma in a model of septic shock in pigs (86). They have also showed that elevated levels of 15-keto-dihydro-$PGF_{2\alpha}$ correlated with elevated levels of 8-iso-$PGF_{2\alpha}$ in serum and synovial fluid from patients with arthritis, including rheumatoid arthritis (87). The group of Henry N. Jabbour has studied $PGF_{2\alpha}$ in cancer. $PGF_{2\alpha}$ can stimulate migration and

invasion of colon (88) and endometrial (89) cancer cells *in vitro*. In addition, $PGF_{2\alpha}$ can induce proliferation (90) and increase secretion of vascular endothelial growth factor (VEGF) (91) in endometrial cancer cell lines. The same group has showed that stable expression of the FP receptor on endometrial cancer cells in a xenograft model increased neutrophil infiltration, but this did not influence tumor growth (92).

Prostacyclin

Prostacyclin is a regulator of vascular homeostasis and a mediator of edema and pain. It is mainly produced by endothelial and smooth muscle cells, where upon it functions as a vasodilator and inhibitor of platelet aggregation (94). The vasodilative effect of prostacyclin is evident in pulmonary atrial hypertension and Renaud's phenomenon (episodes of reduced blood flow in fingers and toes), where infusion of prostacyclin analogues are used as treatments (95, 96). Prostacyclin signals through the IP receptor that is G_s coupled. Moreover, prostacyclin is reported as the main prostaglandin found in synovial fluid of patients with rheumatoid arthritis (97). Honda *et al.* demonstrated that mice lacking the IP receptor had reduced disease score in the collagen-induced arthritis (CIA) model, and that IP deletion did not affect the production of autoantibodies. (98). Chen *et al.* showed reduced arthritis score after IP deletion in the K/BxN serum-transfer arthritis model in mice (99).

Work by Murata *et al.* demonstrated that mice lacking the IP receptor had decreased paw edema, reduced pain sensation when challenged with carrageenan, and smaller number of writhing responses in the acetic-induced writhing model (100). However, the mice displayed increased tendencies towards thrombosis. The same group later showed that both IP and EP3 receptor signaling mediate pain following pre-treatment with LPS in the acetic-induced writhing model (37). Moreover, IP receptor antagonists relieve pain in multiple pain models (101, 102). Sasaki *et al.* showed that genetic deletion of PGIS in mice leads to suppressed inflammatory reactions (thioglycollate-induced peritonitis and acetic acid-induced writhing) but accelerates azoxymethane (AOM)-induced colon carcinogenesis (103). Treatment with prostacyclin analog iloprost resulted in accumulation of macrophages in the tumor tissue and decreased tumor formation in the lungs, and this effect was equally great in mice lacking the IP receptor (104). This suggests that the anti-cancer effect from iloprost was not mediated via IP receptor signaling. The authors showed that iloprost increased $PPAR\gamma$ activity in A549 lung cancer cells and epithelial H661 cells. The protective effect of prostacyclin signaling in cancer is also suggested to be mediated via $PPAR\beta/\delta$ (105), but the literature is inconclusive (106, 107).

Collectively, prostacyclin is attributed detrimental (inflammatory and pain mediating) or beneficial (vasodilating, anti-thrombotic, and anti-cancer) actions depending on the biological context.

TXA$_2$

TXA_2 is a potent platelet activator and vasoconstrictor. It signals through the G_q-coupled TP receptor that exists in two isoforms (TP_α and TP_β). While platelet are the dominant source of TXA_2 production, macrophages and neutrophils can also produce TXA_2. Mice lacking the TP

receptor are fully viable but display cardiovascular alterations. Thomas *et al.* reported increased bleeding time and delayed aggregation response in TP deficient mice (108). The authors showed that these mice resisted arachidonic acid-induced shock, which resulted in cardiovascular collapse and sudden death in WT mice. Francois *et al.* reported less cardiac hypertrophy caused by hypertension in mice lacking the TP receptor (109, 110). In inflammation, deletion of the TP receptor protects mice from LPS-induced acute renal failure (111). TXA_2 is also implicated in cancer progression (112). For example, increased TXAS protein expression correlated with tumor progression in prostate cancer (113) and metastasis in lung cancer (114) but TXAS mRNA (*TBXAS1*) expression was reported lower in high-grade tumors compared to low-grade tumors in breast cancer (115).
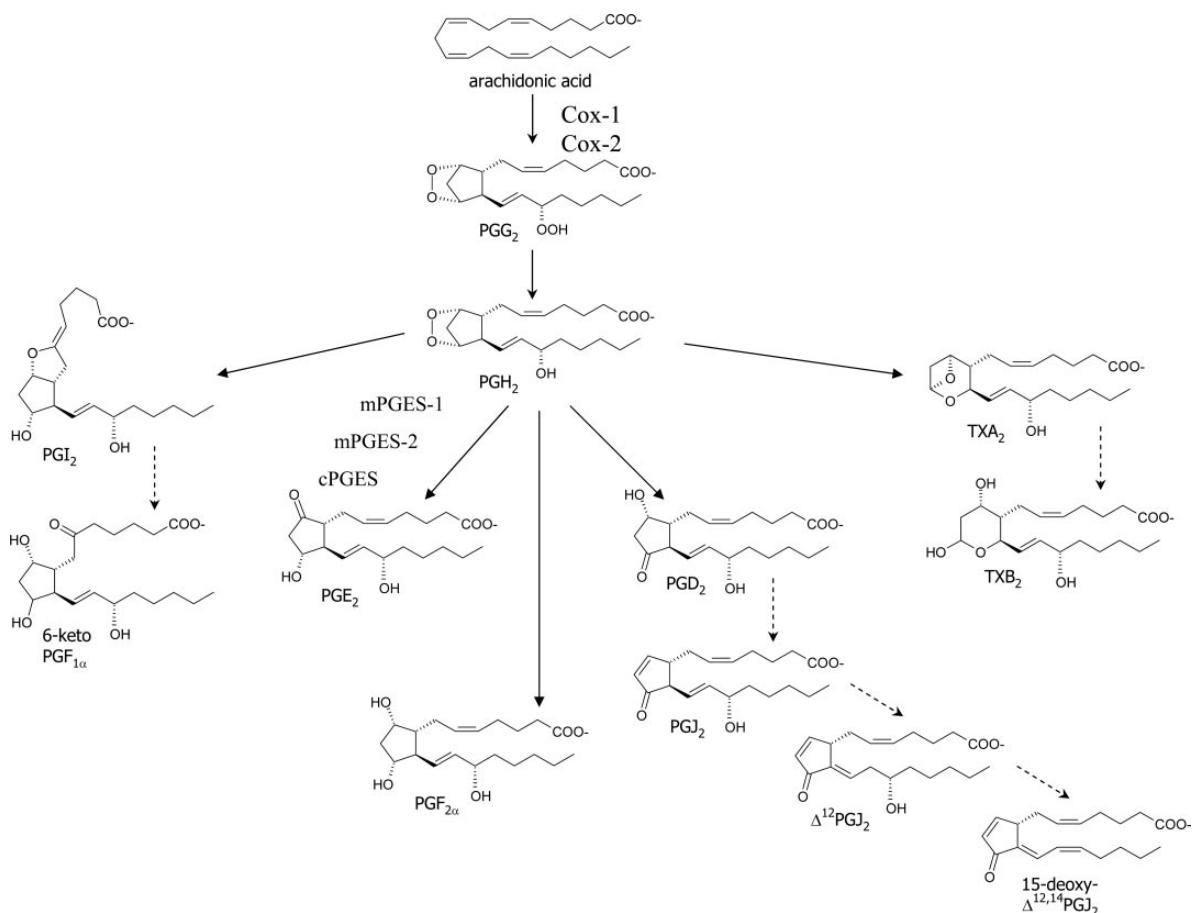

Figure 3. Biosynthesis of the main prostanoids. Reprinted with permission from (116).

1.4 CLINICAL USE OF NSAIDS

The biological set-up of terminal synthases and receptor expression patterns enables complex signaling with prostanoids. This has major implications in inflammation and cancer, where small molecule inhibitors can manipulate prostanoid biosynthesis. NSAIDs, like aspirin and ibuprofen, are widely used drugs to treat pain and inflammation. Their analgesic and anti-inflammatory effects are mainly mediated by non-selective blockage of PGE_2 production via inhibition of COX-1 and/or COX-2 (117), a mechanism that Sir John Vane was awarded the Nobel Prize in Physiology or Medicine for in 1982. This section will introduce the reader to the COX enzymes and the clinical use of NSAIDs.

1.4.1 COX-1 and COX-2

The human COX enzymes are similar in size, structure, and catalytic function (10). COX-1 is 576 amino acid long (71 kDa) and COX-2 is 581 amino acid long (73 kDa), and they have 60% sequence homology. The sequence homology for each isoform between different mammalian species is about 90% (118). There is a lack of interspecies difference in the active site, meaning NSAIDs with activity towards human COX also inhibit for example rat and mice enzymes. Both COX enzymes have a catalytic site for the generation of PGG_2 from AA and a peroxidase active site for the conversion of PGG_2 to PGH_2. COX-1 and COX-2 function as homodimers; although, only one COX monomer is needed for PGH_2 production and the other monomer appears to have allosteric functions (119). While COX-1 is regarded as constitutively expressed for basal prostanoid synthesis, COX-2 is normally absent in most cells and tissues but induced during pro-inflammatory stimuli and therefore viewed as the key isoform for the induced prostanoid production. However, this is a simplified view.

It was early concluded that COX-1 and COX-2 are expressed on the mRNA level at physiological condition but with different tissue-specific expression (120). The isoforms have different tissue distribution and cell localization on the protein level. COX-1 is expressed in nearly all tissues with a preferential protein localization to blood vessels, smooth muscle cells, and platelets. COX-2 is mainly expressed in brain, kidney, lung, and thymus (121).

The isoforms are differently regulated at transcription, translation, and protein levels (122, 123). Indeed, *PTGS2* (gene encoding COX-2) is transcribed upon inflammatory and proliferative stimuli, and the mRNA carries an instability sequence in the 3'-untranslated region. This suggests that COX-2 is the dominant isoform during inflammation and cancer, which is also concluded in many studies. However, mice lacking COX-1 showed decreased inflammation in a model of ear edema (124). COX-2 deficiency was not protective in carrageenan-induced paw edema in rats, where inhibition of both COX-1 and COX-2 was needed to reduce paw swelling (125). Genetic deletion of COX-1 decreased severity while genetic deletion of COX-2 increased severity in a mice model of neuroinflammation (126, 127). Deficiency in COX-1, and not COX-2, is protective in the autoantibody-driven K/BxN serum-transfer arthritis model in mice (99). In contrast, genetic deletion or pharmacological inhibition of COX-2, and not COX-1, is protective in the CIA model in mice (128, 129). In cancer models, COX-1 or COX-2 deletion decreased tumors in small intestine to the same degree in the

Apc$^{Min/+}$ mice model (130), while deletion of either COX-1 or COX-2 had no effect on tumor incidence in a mice model of colitis-associated colon cancer (AOM/DSS model) (131). Nonetheless, repeated injection of AOM alone caused tumor formation in COX-1 deficient mice but not in COX-2 deficient mice. These studies highlight that both isoforms are important during inflammation and cancer depending on the disease model.

1.4.2 Drug history: From aspirin to coxibs

Aspirin has been a product for over a century. It is not only used to treat fever, pain, and inflammation but low-dose aspirin is a successful strategy to prevent cardiovascular events (myocardial infarction, stroke, and vascular death) (132) and colorectal cancer (133). Aspirin is an irreversible inhibitor of COX enzymes, as it acetylates Ser-530 on COX-1 and Ser-516 on COX-2. Acetylated COX-1 has no activity while acetylated COX-2 has altered substrate specificity and activity. Acetylated COX-2 can metabolize AA into 15(R)-HETE (134), which is an agonist for PPARβ/δ (135) that controls cell differentiation and lipid metabolism with implications in inflammation and cancer (136). In addition, acetylated COX-2 produces specialized pro-resolving lipid mediators (SPMs) including lipoxin A$_4$, resolvin D1, and resolvin D3 that are pro-resolving and anti-cancer (137-139). Low-dose aspirin mediates anti-thrombotic effect via the irreversible inhibition of COX-1 in platelets, which suppresses TXA$_2$ biosynthesis without affecting prostacyclin production (140, 141). This shifts the balance between vasoconstrictive TXA$_2$ and vasodilative prostacyclin. Aspirin is rapidly hydrolyzed into salicylic acid and acetic acid *in vivo*, where salicylic acid is equally potent in inhibiting PGE$_2$ production in pouch exudate in rats but less potent in blocking TXA$_2$ biosynthesis in human clotting blood (142). The primary effect on platelet COX-1 by low-dose aspirin is due to the presence of aspirin in portal blood (from stomach and small intestine to the liver) before hydrolysis by plasma esterases and liver carboxylesterase-2 (first-pass effect) (143, 144).

Two landmark articles in 1991 concluded that regular use of aspirin correlated with decreased incidence of colorectal cancer (145, 146). These were the first epidemiological associations that aspirin is protective in cancer. Subsequent epidemiological studies have showed that aspirin intake correlated with decreased incidence and mortality in lung cancer (147), and recently a large meta-analysis showed that regular aspirin users have a lower risk for overall cancer (148). Low-dose aspirin also increases survival following treatment of lung cancer and rectal cancer (149, 150). However, studies in the 1980s showed an increased risk of Reye´s syndrome (a progressive and lethal brain disease that starts in connection to recovery from a viral infection) in children taking aspirin during a fever (151), and this has restricted the use of aspirin in children. In addition, low-dose aspirin increases the risk for gastrointestinal bleeding (132, 152-154).

Multiple NSAIDs entered the market during the 20th century such as phenylbutazone in the 1940s, fenamates in the 1950s, acetic acid derivatives (*e.g.* indomethacin, diclofenac) in the 1960-1970s, propionates (*e.g.* naproxen, ibuprofen) in the 1970s, oxicams in the 1980s, and coxibs (*e.g.* celecoxib) in the 1990-2000s. In the early 1990s, several landmark papers on NSAIDs were published. As mentioned above, regular use of aspirin was observed protective

against colorectal cancers (145, 146). However, aspirin was well-documented to cause gastric damage since many decades due to early work by Arthur Henry Douthwaite, and it was concluded by Langman *et al.* in 1994 that all traditional NSAIDs caused gastrointestinal bleeding (155). Two laboratories identified the gene encoding COX-2 in 1991 (156, 157), which was found induced in several types of cells upon pro-inflammatory stimuli (reviewed in (158)). Selective inhibition of COX-2 quickly became an anticipated therapeutic strategy, which was envisioned to spare the gastrointestinal adverse effects thought to be mediated via COX-1 inhibition, and be more efficient than existing NSAIDs to treat inflammation (159-162).

Indeed, the selective COX-2 inhibitor celecoxib (100 mg twice daily) showed good analgesic and anti-inflammatory responses in treatment of osteoarthritis and rheumatoid arthritis with reduced incidence of gastrointestinal events associated with dual COX-1/2 inhibitor naproxen (163). This was confirmed in a large clinical trial (n=8069), where high dose celecoxib (400 mg twice daily) showed lower incidence of gastrointestinal toxicity compared to ibuprofen (800 mg three times daily) or diclofenac (75 mg twice per day) in patients suffering from osteoarthritis or rheumatoid arthritis (164). Celecoxib became the first selective COX-2 inhibitor (coxib) to be approved by the FDA in 1998. In a clinical trial on colon cancer, celecoxib inhibited growth of adenomatous polyps in familial adenomatous polyposis (165). The coxib rofecoxib (50 mg daily) was equally efficient as naproxen (500 mg twice daily) in treating rheumatoid arthritis as assessed by global disease activity score and modified health assessment questionnaire, where patients receiving naproxen where twice as likely to suffer from gastrointestinal events (166). However, the incident rate for myocardial infarction was four times higher in patients receiving rofecoxib compared to naproxen, although there was no difference in rate of death from cardiovascular causes between the two groups.

Rofecoxib was used as prevention treatment in a clinical trial on colorectal adenoma but this was terminated when the increased rate of cardiovascular events (myocardial infarction and ischemic cerebrovascular events) was detected (167). Another study demonstrated increased risk for cardiovascular events (myocardial infarction, cardiac arrest, stroke, and pulmonary embolism) after receiving the selective COX-2 inhibitors parecoxib or valdecoxib compared to placebo in patients that had undergone cardiac surgery (168). Moreover, increased cardiovascular risk (myocardial infarction, stroke, and cardiac arrest) was observed with celecoxib used as prevention treatment in colorectal adenoma (169). The increased risk for cardiovascular events led to the withdrawal of many coxibs and FDA issued a boxed warning for the use of celecoxib and other NSAIDs in 2004 (170). Multiple observational studies (171-175) and meta-analyses (176-180) have confirmed that coxibs, diclofenac, indomethacin, and to less extent ibuprofen, increase the risk for cardiovascular events. Naproxen is the safest non-aspirin NSAID in terms of cardiovascular risk, although naproxen doubles the risk of heart failure (176). A large cohort study (181), a large case-control study (182), and multiple meta-analysis (183, 184) collectively show that ibuprofen has low, naproxen and diclofenac have intermediate, and indomethacin has high gastrointestinal toxicity.

One additional concern with NSAIDs that act on COX-1 is that a subset of asthmatic patients is sensitive to these drugs and develop non-allergic reactions (185), called NSAID-exacerbated respiratory disease (N-ERD). The pathogenesis is multifactorial, as these patients have decreased basal concentration of protective PGE_2 in the airways (186), increased expression of LTC_4 synthase (for biosynthesis of bronchoconstrictor LTC_4) in bronchial biopsies (187), and increased basal concentration of LTE_4 (LTC_4 metabolite) in urine, saliva, induced sputum, and *ex vivo* blood (188). Patients with aspirin-intolerant asthma have increased concentration of LTC_4 in bronchoalveolar fluid (187) together with increased concentration of urinary LTE_4 and urinary PGD_2 metabolite $9\alpha,11\beta$-PGF_2 upon aspirin intake (188), compared to patients with aspirin-tolerant asthma.

Lastly, NSAIDs are associated with renal complications. This includes peripheral edema and hypertension from sodium retention (189) and increased risk of renal failure (190). The increased risk for any severe adverse effects can be considered small for the general population, when NSAIDs are administered at low doses and for short periods. Yet, the risks may be greatly increased in certain patient groups with high age, systemic disease, and other ongoing treatments such as patients suffering from rheumatoid arthritis (191), where high NSAID doses are used.

Apart from the many protective associations, there are two main lessons learned from observational studies, clinical trials, and meta-analyses on NSAIDs: first, dual COX-1/2 inhibitors cause primarily gastrointestinal bleeding and second, selective COX-2 inhibitors increase the risk of serious cardiovascular events (**Table 1**).

NSAID	COX selectivity	Therapeutic uses	Severe adverse effects
Indomethacin	COX-1 > COX-2	Analgesic, anti-pyretic, anti-inflammatory	Gastrointestinal bleeding (high), N-ERD
Aspirin	COX-1 > COX-2	Analgesic, anti-pyretic, anti-inflammatory, anti-thrombotic (low-dose), prevent cancer	Gastrointestinal bleeding, Reye's syndrome, N-ERD
Naproxen	COX-1/2	Analgesic, anti-pyretic, anti-inflammatory	Gastrointestinal bleeding (intermediate), N-ERD
Ibuprofen	COX-1/2	Analgesic, anti-pyretic, anti-inflammatory	Gastrointestinal bleeding (low), stroke, N-ERD
Diclofenac	COX-1 < COX-2	Analgesic, anti-pyretic, anti-inflammatory	Gastrointestinal bleeding (intermediate), myocardial infarction, stroke, N-ERD
Celecoxib	COX-1 < COX-2	Analgesic, anti-inflammatory, prevent cancer	Myocardial infarction, stroke, gastrointestinal bleeding
Rofecoxib	COX-1 <<< COX-2	*Withdrawn since 2004*	Myocardial infarction, stroke

Table 1. Overview of NSAIDs. Please see the main text for references.

1.4.3 Adverse effects: The relationship between COX-1 and COX-2

Early research on COX deficient mice showed that mice lacking COX-1 display minor prostaglandin production throughout tissues but are fully viable (124). The mice showed decreased platelet aggregation and no signs of gastrointestinal damage. This supports the beneficial role of low-dose aspirin (irreversible inhibition of COX-1), but it does not support that gastrointestinal damage is caused by COX-1 deficiency. Instead, this points towards compensatory effect of COX-2 in these mice as the dual COX-1/2 inhibitor indomethacin induced gastrointestinal erosion in WT and COX-1 deficient mice (124). This effect of indomethacin on gastric erosion was later confirmed in rats, where the authors also demonstrated that neither celecoxib nor selective COX-1 inhibitor SC-560 alone caused gastrointestinal damage, but they did in combination (192). The many PG-mediated mechanisms for NSAID-induced gastrointestinal damage include reduced blood flow, decreased cell proliferation, increased gastric acid secretion, decreased bicarbonate secretion, and decreased mucus production (reviewed in (193)). The gastroprotective role of PGE_2 is evident from clinical studies where misoprostol (a PGE_1 analog that can bind EP2, EP3, and EP4) prevented NSAID-induced gastrointestinal damage (194-196). Then again, PGE_2 concentration in stomach tissue was decreased with SC-560 but not further decreased with celecoxib in the rat study above (192). This suggests that alternative non-PG mediated mechanisms may contribute to NSAIDs-induced gastric injury (197, 198), such as redirection of AA from prostanoids to LTs. Reduction in gastroprotective PGE_2 worsen gastrointestinal damage, where it is has been proposed that NSAIDs themselves may drive the initiation of the damage via biochemical effects including increase in intestinal permeability and intestinal inflammation (199, 200).

COX-2 deficient mice display failure in several reproductive events in early pregnancy (201). In addition, genetic deletion of COX-2 disrupts postnatal kidney development (202, 203), causes cardiac fibrosis (203), and increases blood pressure and accelerates thrombosis (204, 205). As presented above, multiple NSAIDs and coxibs increase the risk for serious cardiovascular events. The established dogma behind the side effects imposed by NSAIDs is reduction in vasodilative prostacyclin from endothelial-derived COX-2 while vasoconstrictive TXA_2 production continues from platelet-derived COX-1 (206). Indeed, healthy volunteers taking the coxibs nimesulide (207), celecoxib (208), or rofecoxib (209) have reduced urinary prostacyclin metabolite 2,3-dinor-6-keto $PGF_{1\alpha}$ (PGIM) but not urinary TXA_2 metabolite 11-dehydro TXB_2. High-dose aspirin (207) and normal dose ibuprofen (208), indomethacin (209), naproxen (210), and diclofenac (211) reduce both PGIM and 11-dehydro TXB_2, suggesting that the balance and not the absolute production of prostacyclin and TXA_2 is important in the increased risk of cardiovascular events. Nonetheless, the literature suggest that naproxen is not associated with major vascular events while indomethacin, ibuprofen, and diclofenac are (176, 179).

The relative contribution of COX-1 and COX-2 for endothelial prostacyclin production in human is debated in the literature (212, 213). There may be additional mechanism(s) for the cardiovascular hazard seen with NSAIDs. For example, Ahmetaj-Shala *et al.* proposed a role

of COX-2 in the kidneys that affected vessel tone via regulation of asymmetric dimethylarginine (ADMA) (214), which is an endogenous inhibitor of endothelial NO synthase. The aortic vessels from mice lacking COX-2 were less responsive to acetylcholine-induced relaxation compared to control mice in an *ex vivo* model. The authors showed increased levels of ADMA in plasma from COX-2 KO mice, parecoxib treated mice, and from humans taking celecoxib or naproxen. Increased levels of ADMA in plasma is in turn linked with all-cause mortality in man (215). In other words, since naproxen reduces urinary PGIM concentration (210) and increases ADMA concentration in plasma (214), this should favor cardiovascular hazard in man with both the prostacyclin hypothesis and the ADMA hypothesis. Then again, there is little or no evidence that naproxen increases the rate of cardiovascular events. Even if the causes for increased cardiovascular events with NSAIDs are not fully understood, the consensus is that non-aspirin NSAIDs (except naproxen) are cardiovascular toxic.

Both COX isoforms are important for the generation of prostanoids in health and disease. As described earlier, there is little evidence for compensatory mechanism in knockout studies. Replacing *PTGS2* with *PTGS1* (encoding COX-1) does not fully restore prostacyclin production nor does it rescue reproductive function or renal complications (hypertension) in knock-in studies (216, 217). However, complete deletion of either COX-1 or COX-2 had no effect on pain transmission in hot plate assay in mice but COX-1 deficient heterozygous showed less pain (218). This suggests that COX-1 can compensate for COX-2 but COX-2 cannot compensate for partial loss of COX-1 in rapidly transmitted pain. At the same time, the number of writhing response following acetic acid was lower in COX-1$^{-/-}$ and COX-1$^{+/-}$ male mice and COX-1$^{-/-}$, COX-1$^{+/-}$, and COX-2$^{+/-}$ female mice. This suggests that COX-1 can compensate for COX-2 in slowly transmitted pain in male mice but not in COX-2 deficient heterozygous female mice.

One important note is that AA is not the only substrate for COX enzymes. For example, endocannabinoids and other free fatty acids such as eicosapentaenoic acid (20:5, ω-3), alpha-linolenic acid (18:3, ω-3), gamma-linolenic acid (18.3, ω-6), and linoleic acid (18:2, ω-6) can all be metabolized by COX-1 and/or COX-2, where the selectivity is isoform specific (219). This adds to the complexity between the relative roles of the two enzymes, as metabolism of each substrate will generate distinct products that mediate different physiological and/or pathological responses.

It is evident that NSAIDs cause gastrointestinal and/or cardiovascular adverse effects, especially at high doses used in treatment of rheumatoid arthritis. Inhibition on the COX level is a complex approach (220), as this blocks the production of multiple prostanoids that have both beneficial and detrimental effects depending on the biological context, and the inhibition may redirect AA from the COX pathway to the LOX (lipoxygenase) pathway. This may cause compensatory effects or introduce new effects, which is difficult to predict. In addition, the therapeutic uses and adverse effects associated with different NSAIDs are not simply explained by their relative selectivity towards the COX enzymes. Drug structure, bioavailability, half-life, and degree of inhibition over time are some of the important

parameters to consider in pharmacokinetics and pharmacodynamics studies when dissecting the cause(s) of adverse effects (221). In conclusion, targeting the COX pathway is an important and widely used therapeutic strategy but the many detrimental effects limit the use of NSAIDs. Selective inhibition of downstream mPGES-1 may be a safer option.

1.5 MPGES-1 AS THERAPEUTIC TARGET

1.5.1 Structure and catalytic function of mPGES-1

The human terminal enzyme mPGES-1 was first cloned and characterized in 1999 (222). The rat and mouse mPGES-1 were described the year after (223). This is a 152 amino acid long and 16 kDa large membrane protein, typically upregulated by inflammatory stimuli including IL-1β, TNF-α, and LPS (222-227). A first low-resolution crystal structure was reported in 2008 (228) and a high-resolution structure was reported in 2013 (229), showing that mPGES-1 is a homotrimer where each monomer consist of four transmembrane alpha-helices. Site-directed mutagenesis studies have concluded that Asp-49 and Arg-126 are important residues for the catalytic function of human mPGES-1 (230-232).

1.5.2 Genetic deletion of mPGES-1

The first mPGES-1 KO model was reported in 2002, in which mPGES-1 deficient peritoneal macrophages failed to produce PGE_2 in response to LPS (233). Since then, genetic deletion of mPGES-1 has been the topic of numerous research investigations. Multiple subsequent studies have demonstrated that mPGES-1 is the key enzyme for the induced PGE_2 production in peritoneal macrophages (103, 234-239). Mice lacking mPGES-1 are fully viable without altered phenotype at normal conditions (240). The basal PGE_2 production was reported lower in stomach, spleen, brain, and kidney (234); however, unchanged basal production of PGE_2 in spleen and brain in mPGES-1 KO mice has been reported (236).

Effect in arthritis models. Arthritis can be induced in mice via immunization with collagen or administration of autoantibodies towards collagen. This is monitored using the degree of paw swelling (thickness) and the clinical arthritis score, which includes redness and number of joints affected. Mice lacking mPGES-1 had reduced incidence and severity of disease in the CIA model (240, 241), as these mice failed to produce autoantibodies (241). Mice receiving intraperitoneal injection of stable PGE_2 analog misoprostol had earlier onset and higher arthritis score in the CIA model (242). This is in line with that genetic deletion of COX-2 (129), pharmacological inhibition of COX-2 (128), and antagonist for EP2/EP4 (98) are reported protective in the CIA model. Treatment with EP4 antagonist was protective in the CIA model and in the glucose-6-phosphate isomerase-induced arthritis model in mice (243). It was reported in the same study that EP4 antagonism decreased complete Freund's adjuvant (CFA)-induced inflammatory pain in rats. While these studies conclude that PGE_2 mediates arthritis in many models, contradicting results have been reported for mPGES-1 deletion in the collagen antibody-induced arthritis (CAIA) model. Kamei *et al.* reported that mice lacking mPGES-1 had 50% reduced PGE_2 production in paws and decreased disease

severity but similar incidence as WT mice (238). In contrast, Frolov *et al.* showed complete blockage of induced PGE_2 production in the arthritic paws from mPGES-1 KO mice that coincided with increased neutrophil infiltration and increased incidence and higher arthritis score (244). In the K/BxN serum-transfer arthritis model in mice, mPGES-1 deletion decreased PGE_2 production by >90% in joint tissues but this had no impact on the disease development (99). In addition, deletion of mPGES-1 did not affect bone loss or osteoarthritis but caused impaired fracture healing in a mice model of skeletal disorders (245). Apart from differences in mice strains and arthritis models, the difference in absolute PGE_2 synthesis may be of importance for disease incidence and severity. The role of other prostanoids (like prostacyclin) needs to be considered. This is especially apparent in the K/BxN serum-transfer model, where prostacyclin (IP signaling) plays a role (99). In conclusion, experimental models suggest that mPGES-1 is important for development of autoimmune arthritis.

Effect in cancer models. Genetic deletion of mPGES-1 is protective in multiple cancer models. The laboratory of Daniel W. Rosenberg has investigated the contribution of mPGES-1 in intestinal tumorigenesis. They reported that $Apc^{\Delta14/+}$ mice developed fewer and smaller intestinal and colonic polyps upon mPGES-1 deletion (246). The KO mice had decreased PGE_2 production in the small intestine and colon without altered production of other prostanoids. In addition, mice lacking mPGES-1 had increased amount of endothelial ($CD31^{pos}$) cells in polyps but this was not associated with formation of new vessels, suggesting that PGE_2 is important for vascular tube formation. The authors showed in the same study that KO mice had 50% decreased amount of aberrant crypt foci in AOM-induced colon carcinogenesis model. This was repeated in another study, where the anti-cancer effect from mPGES-1 deletion coincided with an increase in cytotoxic T cells ($CD8^{pos}$) and MDSCs ($Gr-1^{pos}CD11b^{pos}$) with a decrease in Tregs ($CD4^{pos}Foxp3^{pos}$) in colon-draining mesenteric lymph nodes, suggesting modulation of immunoregulatory responses (247). The group has recently demonstrated that although lack of mPGES-1 is protective in intestinal cancer, deletion of mPGES-1 caused more severe DSS-induced injury (colitis) and impaired recovery in $Apc^{Min/+}$ mice (248). The KO mice had fewer tumors in the small intestines and unaltered tumor burden in the colon at baseline. Following DSS treatment, mice lacking mPGES-1 had even fewer tumors in the small intestine but not in the colon. These mice had altered eicosanoid production at baseline and upon DSS treatment, both in the small intestine and colon. Most notable alterations in the small intestine were the reduction in PGE_2, 6-keto $PGF_{1\alpha}$, and $PGF_{2\alpha}$ at baseline. Following DSS treatment, PGD_2 was increased and $PGF_{2\alpha}$ remained decreased compared to WT mice in the small intestine. This suggests that induction of damage (inflammation) decreases tumor load in the small intestine in mPGES-1 deficient mice, perhaps via increased PGD_2 formation. Contradicting results have been reported by Elanders *et al.*, who showed that $Apc^{Min/+}$ mice lacking mPGES-1 had more and larger tumors in the small intestine despite also displaying a redirection of PGH_2 from PGE_2 to PGD_2 in the tumor tissues (249). While these studies were performed on the same mice background, the study length (10 weeks vs. 18 weeks) may contribute to the differences in prostanoid

22

profiles and consequently tumor progression. It is possible that lack of mPGES-1 is protective in the early-mid phase of tumorigenesis but detrimental in the late phase.

Sasaki *et al.* confirmed that mPGES-1 deficiency decreased the total amount and size of colorectal polyps in AOM-induced colon cancer (48, 103), whereas PGIS deficiency increased the amount of large polyps (103). The authors showed increased formation of prostacyclin in the tumors from mPGES-1 KO animals, highlighting anti-cancer properties of prostacyclin. Kamei *et al.* demonstrated that Lewis lung carcinoma cells (LLCCs) deficient in mPGES-1 formed smaller tumors in WT mice (250). They also showed that mice lacking mPGES-1 had slower growing LLCC tumors in a xenograft model and less metastasis (lung weight). The expression of VEGF was reduced in tumor and lung tissue in the different models, suggesting less angiogenesis to be a main mechanism for the protective effects upon mPGES-1 deletion. Kamata *et al.* used bone marrow chimeric mice to demonstrate that mice lacking mPGES-1 in bone marrow-derived cells had reduced LLCCs tumor growth with less VEGF expression and angiogenesis in the tumors (251). The anti-angiogenic effect upon mPGES-1 deletion was reproduced in a model of lung metastasis formation in prostate cancer (252) and Howe *et al.* showed that mice lacking mPGES-1 had reduced angiogenesis and tumor growth in a transgenic mouse model of breast cancer (253).

Isono and co-workers investigated bone cancer growth and cancer-related pain upon injection of LLCCs in the bone marrow (254). Mice lacking mPGES-1 had slower tumor growth and less bone cancer pain. Moreover, tumors of A549 lung cancer cells or DU145 prostate cancer cells lacking mPGES-1 grow slower in xenograft mice (255). Olesch and colleagues demonstrated that mPGES-1 deficiency decreased tumor burden (weight and number of tumors) in transgenic PyMT mice that spontaneously develop breast cancer (256). They showed that these tumors had increased amount of CD80^{pos} and decreased amount of CD206^{pos} resident macrophages, suggesting a change in macrophage polarization towards anti-cancer M1 phenotype in the tumors. Zelenay *et al.* deleted mPGES-1 and mPGES-2 in Braf[V600E] melanoma cells and showed that these cells were spontaneously rejected in immunocompetent mice but grew in immunodeficient mice (52). While transgenic mice overexpressing COX-2 and mPGES-1 developed tumors in the gastric tract (257, 258), targeted overexpression of mPGES-1 in lung epithelial cells with increased PGE_2 production was not sufficient to induce lung cancer (259). These studies conclude that PGE_2 production from both stroma cells and cancer cells mediate tumor growth via several mechanisms, and that blocking of COX/mPGES-1/PGE_2 pathway is a therapeutic strategy in cancer.

Redirection of PGH_2. Deletion of mPGES-1 can cause an increase in other prostanoids. This is dependent on the biological context. Lack of mPGES-1 results in decreased urinary 13,14-dihydro-15-keto PGE_2 (PGEM) and increased urinary PGIM in mice (60, 61, 205). Peritoneal macrophages from mice lacking mPGES-1 showed increased prostacyclin, TXB_2, and/or PGD_2 when challenged with LPS (103, 234-239). Mouse embryo fibroblasts from mice lacking mPGES-1 had enhanced activation of PPARγ at basal and IL-1β induced conditions, which can be explained by redirection of PGH_2 from PGE_2 biosynthesis to 15d-PGJ_2 production via PGD_2 (260). Moreover, dendritic cells from mPGES-1 KO mice also showed

increased production of PGD_2 upon LPS treatment (261). In a model of acute inflammation (air pouch), mPGES-1 KO mice showed no induced production of PGE_2 but elevated formation of TXB_2 in pouch exudates, although this had no impact on pouch volume or cell infiltration (262). Mice lacking mPGES-1 display reduced inflammatory pain in the acetic acid writhing assay (238) and reduced mechanical allodynia and thermal hyperalgesia following nerve damage in a model of neuropathic pain (263). However, mPGES-1 deletion cause no change in nociception in the hot plate assay or the formalin assay (237, 240). This can be due to increase in prostacyclin after mPGES-1 deletion. It is therefore important to monitor the general prostanoid production after mPGES-1 deletion, as other prostanoids may increase in concentration and compensate for PGE_2 or even introduce new phenotypes.

Models of cardiovascular diseases. The increase in prostacyclin following mPGES-1 deletion is of special interest for the cardiovascular system. The laboratory of Garret A. FitzGerald has investigated the role of mPGES-1 in models of cardiovascular disease. In contrast to COX-2 deficient mice, mice lacking mPGES-1 have no altered blood pressure and no promotion of thrombosis (205). In addition, mPGES-1 deletion retarded atherosclerosis (60), protected against angiotensin II-induced abdominal aortic aneurysm formation and oxidative stress in hyperlipidemic mice (61), and decreased proliferation and migration of vascular smooth muscle cells following vascular injury (264). The protective effect in atherosclerosis is specific for myeloid cells since removal of mPGES-1 in vascular smooth muscle cells or endothelial cells had no effect in this model (265). In the model of vascular injury, deletion of mPGES-1 in vascular smooth muscle cells or endothelial cells caused worse response (intimal thickening, vascular stenosis, and leukocyte infiltration) while deletion in myeloid cells was protective (239). The group has also showed that while mPGES-1 deletion resulted in redirection of PGH_2 from PGE_2 to other prostanoids in the lungs, this was neutral in regards to bronchial tone during baseline or ozone-induced airway inflammation (266).

A protective response upon mPGES-1 deletion was reported in a model of stroke (62). In addition, Wu *et al.* showed that mPGES-1 KO mice displayed no increase in ischemic myocardial damage after myocardial infarction, which was in contrast to inhibition of COX-2 in WT mice (267). The same authors demonstrated that the use of an IP receptor antagonist blocked the protective response from mPGES-1 deletion in acute cardiac ischemic damage after coronary occlusion in mice (268). However, Degousee *et al.* reported that mPGES-1 deficient mice had more severe complications (left ventricular dilation and impaired left ventricular contractile function) after myocardial infarction, but no increase in mortality (269). Deletion of mPGES-1 worsened the outcome in a model of intracranial aneurysm (270) and a protective role of mPGES-1 in maintaining cardiac function after angiotensin II infusion has been reported (271). These studies highlight that mPGES-1, for the most part, drives many detrimental processes in models of cardiovascular disease.

Consequences on renal functions. Given the adverse effects in renal functions observed with NSAIDs, the laboratory of Tianxin Yang has investigated the role of mPGES-1 in the kidneys. Mice deficient in mPGES-1 showed no altered renal functions (normal water and

sodium balance) at baseline but altered responses in different disease models. They reported that genetic deletion of mPGES-1 has detrimental impact on sodium balance and blood pressure after salt loading and angiotensin II infusion (272) or treatment with deoxycorticosterone acetate (273). Treatment with sodium-retaining compound aldosterone had no effect on WT mice but KO mice displayed increased sodium balance with weight gain (274). KO mice were unable to excrete water following acute water loading (275), and the mice were unable to excrete sodium after dehydration (276). Moreover, removal of mPGES-1 made mice resistant to lithium-induced polyuria (277). In a mice model of chronic kidney disease that is characterized by loss of functional renal mass (or glomerular filtration rate), deletion of mPGES-1 resulted in deficient waste excretion (278). The KO mice had milder inflammation but worse anemia compared to WT mice after chronic renal failure. These studies highlight a significant role of kidney mPGES-1 in handling of sodium and water balance after induced challenge but not at baseline.

Consequences on wound healing. The reduced angiogenesis upon mPGES-1 deletion in cancer models (250-253) is not exclusive for tumors since mPGES-1 deficient mice have delayed wound healing of gastric ulcers after challenge with acetic acid (279). Similar to EP4 deficient mice (40), mPGES-1 KO mice developed more severe ulcerative colitis upon DSS treatment (280). In addition, spontaneous ulcerations have been reported in one study with mice lacking mPGES-1 (247). These studies support a protective role of mPGES-1 in gastric ulceration.

Role of mPGES-1 in additional models. Genetic deletion of mPGES-1 blocks induction of fever (281, 282), specifically via brain endothelial cells (283). Mice lacking mPGES-1 displayed no negative feedback in IL-1β and TNF-α production upon repeated LPS injections in a model of neuroinflammation (284), suggesting that mPGES-1 derived PGE_2 in the initial phase of inflammation is needed for limitation of inflammation in later phases. Moreover, deletion of mPGES-1 is protective in mice models of Alzheimer's disease (285, 286) and Kihara *et al.* reported that mPGES-1 mediated disease features in experimental autoimmune encephalomyelitis (EAE), with implications in multiple sclerosis (58). In addition, while tumor growth was not reduced, two studies have investigated the role of mPGES-1 in cancer-induced anorexia in mice. One study showed a protective effect while the other showed a neutral effect upon mPGES-1 deletion (287, 288). Matsuda *et al.* concluded that mPGES-1 increased lymphangiogenesis via VEGF in a model of peritonitis, where its deletion resulted in less lymphangiogenesis and slower draining rates (289). Lastly, mPGES-1 deletion increased survival of mice following hypoxia (290).

Role of mPGES-2 and cPGES in animal models. While studies have investigated the physiological and pathological roles of mPGES-2 and cPGES, these enzymes are up to date not considered as druggable targets compared to mPGES-1. Mice lacking mPGES-2 have no altered phenotype or reduction in basal PGE_2 production in analyzed tissues, and mPGES-2 is not regulated by inflammatory stimuli (one exception is increased expression in colorectal cancer (291)). Deletion of cPGES decreased basal PGE_2 production and loss of cPGES is perinatal-lethal in mice (reviewed in (280)). Expression of cPGES is mostly unaffected by

inflammatory stimuli, but active downregulation of cPGES expression results in reduced nociceptive response in zymosan-induced thermal hyperalgesia and formalin assay in rats (292). The general lack of disease associations for these enzymes may be both a cause and effect of that these enzymes are understudied compared to mPGES-1. Future studies will reveal whether these terminal synthases could be druggable.

Summary of mPGES-1 deletion. The protective effects of mPGES-1 deletion depends on the biological setting. Lack of mPGES-1 limits inflammation and decreases tumor growth. In addition, deletion of mPGES-1 is neutral or even beneficial in cardiovascular aspects compared to COX-2 deficiency. However, consequences on renal homeostasis and wound healing (gastric ulceration) should be taken into account. These results are the fundaments for the development of mPGES-1 inhibitors.

1.5.3 Development of mPGES-1 inhibitors

Merck reported the first selective mPGES-1 inhibitors in 2005 (293). Selectivity is based on the degree of inhibition of recombinant mPGES-1 over other terminal synthases and/or COX enzymes in activity assays. The compounds inhibited recombinant mPGES-1 with IC_{50} values of 0.003-0.007 µM, and also decreased PGE_2 production in IL-1β induced A549 cells with IC_{50} of 0.27-0.49 µM (2% serum) and 5.8-8.0 µM (50% serum). However, the inhibitors showed no inhibition of PGE_2 biosynthesis in LPS-induced human whole blood.

The first selective mPGES-1 inhibitor that showed activity in human whole blood was MF63, first disclosed in 2007 (294) and later fully characterized in 2008 (295). It inhibited recombinant human mPGES-1 (activity assay) with IC_{50} of 0.001 µM and decreased induced PGE_2 production in IL-1β treated A549 cells (2% serum) with IC_{50} of 0.046 µM. MF63 blocked induced PGE_2 production in LPS treated human whole blood with IC_{50} of 1.3 µM. Moreover, guinea pigs treated with MF63 had decreased fever (LPS-induced pyresis) and decreased pain (LPS-induced thermal hyperalgesia and monosodium iodoacetate-induced incapacitance, which is a model of osteoarthritis pain). While MF63 is a potent inhibitor towards human and guinea pig mPGES-1, it lacks activity towards rat and mice enzyme. Nonetheless, the authors showed in human mPGES-1 knock-in mice that MF63 decreased LPS-induced hyperalgesia and PGE_2 formation in an air pouch model. MF63 decreased stomach PGE_2 formation and this did not cause gastrointestinal toxicity. This is a landmark publication, demonstrating that selective inhibition of mPGES-1 is a feasible therapeutic strategy to relive inflammatory pain.

Several mPGES-1 inhibitors have since been described. Merck followed-up on the success of MF63 and reported two substituted MF63-like compounds (named 26 and 44) with increased potency in human whole blood (IC_{50} of 0.14 µM and 0.20 µM) that also reduced hyperalgesia in guinea pig (296). Bruno *et al.* reported compound AF3442 to inhibit recombinant human mPGES-1 (IC_{50} of 0.06 µM) and it displayed activity in human whole blood (IC_{50} of 0.41 µM). AF3442 also decreased PGE_2 formation in LPS-induced human monocytes with no redirection towards other prostanoids (297), which is in contrast to the

effect observed upon genetic deletion of mPGES-1 in mouse peritoneal monocytes (103, 234-238). Pfizer disclosed multiple mPGES-1 inhibitors in 2010 (298). The most potent was PF-9184, which inhibited recombinant human mPGES-1 with IC_{50} of 0.17 µM but it was less potent in human whole blood with IC_{50} of 5 µM (299). Merck disclosed additional mPGES-1 inhibitors in 2010 and 2011, which potently inhibited recombinant mPGES-1 (IC_{50} of 0.001 µM) but the inhibitors displayed large decrease in potency in human whole blood (IC_{50} of 1.6-2.1 µM) (300, 301).

Finetti and colleagues were the first to demonstrate that inhibition of mPGES-1 replicated genetic deletion of mPGES-1 in terms of anti-cancer activity, specifically via less proliferation and angiogenesis (302). Treatment with mPGES-1 inhibitor AF3485 decreased epidermal growth factor receptor (EGFR) signaling and VEGF expression *in vitro* and *in vivo*, which limited tumor growth in mice xenografts of skin cancer A431 cells. Compound 7 (FR20) and derivatives thereof were reported to inhibit mPGES-1 in broken cell assay from human HeLa cells, mouse RAW 264.7 cells, and mouse NIH cells (IC_{50} values of 0.13, 8.4, and 10.7 µM, respectively) (303). However, FR20 displayed poor inhibition in human whole blood (51% residual production of PGE_2 at 100 µM).

In 2013, several publications with new mPGES-1 inhibitors were published. Pfizer characterized PF-4693627 that inhibited recombinant human mPGES-1 with IC_{50} of 0.003 µM (304, 305). This compound was equally efficient to naproxen in reducing PGE_2 production in an air pouch model in guinea pigs when administered at the same dose. However, it was about 100-fold more potent than naproxen since the plasma concentration was 1.2 µM for PF-4693627 and 154 µM for naproxen. AstraZeneca in collaboration with Uppsala University disclosed AZ'0908 as an mPGES-1 inhibitor but they presented only pharmacokinetics and no pharmacodynamics results (306). Dainippon Sumitomo Pharma reported multiple imidazoquinoline derivatives, where the most promising candidate was compound 39 that inhibited recombinant mPGES-1 with IC_{50} of 0.0041 µM and PGE_2 production from A549 cells with IC_{50} of 0.033 µM (307, 308). Hanke *et al.* reported several benzenesulfonamide derivatives with activity towards both human and murine enzymes (309). Compound 28 decreased PGE_2 formation (%-inhibition at 5 µM) in broken cell assay from human HeLa cells (76%, IC_{50} of 13.8 µM) and mice RAW 264.7 (44%) and NIH cells (52%). This compound showed no inhibition of recombinant human COX-1 or COX-2 at 10 µM. Then again, Compound 28 showed poor inhibition in human whole blood (70% residual production of PGE_2 at 100 µM).

Two additional compounds that displayed activity towards both human and rodent mPGES-1 were reported in 2013. Compound II inhibited recombinant human and rat mPGES-1 with IC_{50} of 1.8 µM and 0.62 µM, respectively (310). This compound displayed no inhibition towards COX-1, COX-2, mPGES-2, or PGIS but partial inhibition of both PGDS isoforms and TXAS at high concentrations. Compound II inhibited PGE_2, TXB_2, and prostacyclin production by 50% in peritoneal macrophages from rat, without affecting the cell viability. General prostanoid reduction also occurred *in vivo*, where Compound II decreased both PGE_2 and PGD_2 in air pouch exudates in rats. In addition, Compound II (100 mg/kg) slightly

reduces paw swelling in an adjuvant-induced edema model in rats. The follow-up inhibitor, Compound III (CIII), showed improved potency towards recombinant human mPGES-1 with IC_{50} of 0.09 µM (262). CIII was highly selective and only displayed partial inhibition of L-PGDS at high concentrations. Moreover, CIII had an IC_{50} of ~10 µM in human whole blood. In contrast to AF3442 (297) and Compound II (310), CIII caused a shift from PGE_2 to prostacyclin production in rat peritoneal macrophages. Neither genetic deletion of mPGES-1 or treatment with CIII (10-100 mg/kg) affected exudate volume in an air pouch model, but CIII decreased the number of pouch cells (262). In another study, CIII decreased PGE_2 in LPS induced peripheral blood mononuclear cells (PBMCs) and this caused no increased formation of other prostanoids (256).

Glenmark Pharmaceutical Ltd. disclosed multiple potent inhibitors in 2014, where Compound 48 was reported as highly selective against recombinant mPGES-1 (IC_{50} of 0.005 µM) and with activity in human whole blood (IC_{50} of 0.376 µM) (311). Several additional human mPGES-1 inhibitors have been described. One example is dimethylcelecoxib, which is a derivative of celecoxib that lacks activity to COX, inhibited mPGES-1 in recombinant assay (IC_{50} of 15.6 µM), HeLa cells (IC_{50} of 0.64 µM), and A549 cells (IC_{50} of 0.83 µM) (312). However, the reduction in PGE_2 production is largely driven by inhibition of mPGES-1 promotor activity via dimethylcelecoxib-mediated blocking of the transcription factors early growth response protein 1 (EGR1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (313). This explains the increase in potency in intact cells compared to recombinant activity assay.

Multiple dual mPGES-1 and 5-LO inhibitors have been reported (314-327). Many of these displayed IC_{50} values around or below 1 µM in recombinant enzyme assay or cell-based assay, but most have not been tested in human whole blood or *in vivo*. One of the best characterized is YS-121, which inhibited PGE_2 production in human whole blood (IC_{50} of 2 µM). YS-121 (1.5 mg/kg) decreased PGE_2, LTB_4, and prostacyclin formation along with exudate volume and number of pouch cells in an air pouch model in rats (323). Moreover, natural compounds like curcumin (328) and arzanol (329) can inhibit mPGES-1, although these compounds have numerous other anti-inflammatory effects. One honorable mention is MK-886 (330) that is an early inhibitor with activity towards human and rat mPGES-1 (331), but it binds to several other enzymes such as 5-lipoxygenase activating protein (FLAP) and COX-1 (332). FLAP and mPGES-1 belong to the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) enzyme family. They have similar structure and it is likely that an inhibitor towards one enzyme may also inhibit the other enzyme.

The research approach used by Merck in the characterization of MF63 and its derivatives (294, 295) is the key methodology in the development of mPGES-1 inhibitors. These papers also highlighted two main hurdles. First, seemingly potent inhibitors towards recombinant mPGES-1 can display great loss in potency in human whole blood and low bioavailability *in vivo*. The second hurdle is that inhibitors designed for human mPGES-1 typically lacks or

have heavily decreased potency towards mouse and rat enzymes. Structure-activity relationship (SAR) studies have identified key amino acids in the inhibition of human mPGES-1 (333, 334), and these are not conserved in mouse or rat enzymes. Mapping of MF63 and other mPGES-1 inhibitors' binding sites using hydrogen/deuterium exchange mass spectrometry revealed binding to the active site of mPGES-1 (335). Recently, crystallization of human mPGES-1 in complex with four inhibitors (including MF63) was published (336). The inhibitors showed a general binding mode with critical interactions with amino acid residues that are not conserved between human and rat/mouse mPGES-1.

As presented, there are a limited number of studies with selective mPGES-1 inhibitors in relevant disease models *in vivo*. The results are promising (**Table 2**), but there is a demand for further studies with potent mPGES-1 inhibitors that lacks interspecies differences to study efficacy and safety in preclinical settings.

1.5.4 Clinical use of mPGES-1 inhibitors

The development of mPGES-1 inhibitors for clinical practice have been halted due to at least three reasons. *First*, as already mentioned, there is sufficient interspecies difference in the structure between human and rodent mPGES-1 that makes it challenging to develop a potent inhibitor that function in both preclinical and clinical models. *Second*, there is a guilt-by-association to target the prostanoid pathway as all NSAIDs have boxed warning and should be used with care. This risk is too large for major pharmaceutical companies to take as it is of outmost importance that mPGES-1 inhibitors are safe in cardiovascular aspects. *Third*, it is unclear which disease(s) or disease event(s) that would be the primary target. NSAIDs are successfully used during a limited time to treat pain and inflammation, which means the increased risk for cardiovascular event is rather small. One might argue that this in turn means that there is no great need for a safer compound targeting the same pathway. However, the small increased risk in cardiovascular events makes a large difference as NSAIDs are used on a population scale. In addition, as mentioned earlier, the many adverse effects limit the use of NSAIDs for long-term therapy. It is possible that inhibition of mPGES-1 would be a better strategy for long-term treatment of inflammation and pain in rheumatic diseases or as long-term adjuvant therapy for cancer. The primary use of mPGES-1 inhibitors remains to be elucidated, where compounds should be tested in various Phase II clinical trials to unravel both their efficacy in treating various diseases and potential side effects.

Based on the aforementioned research it becomes reasonable to suggest that targeting mPGES-1 over COX-1/2 is a better therapeutic strategy. Multiple reviews support this conclusion (116, 337-344). Nevertheless, there is no approved mPGES-1 inhibitor for clinical use. Up until 2015, two compounds have reached Phase I clinical trials (www.clinicaltrials.gov), namely GRC 27864 (ID: NCT02179645 and NCT02361034, Glenmark Pharmaceuticals Ltd. India) and LY3023703 (ID: NCT01632579, NCT01849055, and NCT01872910, Eli Lilly and Company). There is still a need for additional research using preclinical models of inflammation and cancer to demonstrate that mPGES-1 inhibitors will replicate findings observed with genetic deletion of mPGES-1. Such studies using potent inhibitors will add much

needed proof-of-principles to boost the drug development. Absolute quantification of PGE$_2$ and other prostanoids is vital in these type of studies to confirm drug efficacy and to observe potential redirection of PGH$_2$.

Inhibitor (year)	Species activity	Recombinant mPGES-1 (IC$_{50}$)	Human whole blood (IC$_{50}$)	Results from *in vivo* models	Ref.
MK-886 (2001)	Human, murine	1.6-2.0 µM (human), 3.2 µM (rat)	~30 µM	Not tested	(225, 293, 326)
23 and **30** (2005)	Human*	0.003 µM and 0.007 µM	Not active	Not tested	(293)
MF63 (2006)	Human	0.001 µM (human), >30 µM (mouse/rat)	1.3 µM	Analgesic, anti-pyretic (guinea pig); analgesic (knock-in mice)	(294, 295)
26 and **44** (2009)	Human*	0.001 µM	0.20 µM and 0.14 µM	Analgesic (guinea pig)	(296)
AF3442 (2010)	Human*	0.06 µM	29 µM	Not tested	(297)
PF-9184 (2010)	Human	0.017 µM (human), 1080 µM (rat)	5 µM	Not active in air pouch model (rat)	(299)
YS-121 (2010)	Human* (dual mPGES-1 and 5-LO)	3.4 µM	2 µM	Decreased exudate volume and inflammatory cells (air pouch, rats)	(323)
AF3485 (2012)	Human	2.6 µM	Not tested	Anti-angiogenic, anti-proliferative (mice xenografts)	(302)
FR20 (2012)	Human. murine	0.13 µM (human), 9 µM (mouse) #	>100 µM	Not tested	(303)
PF-4693627 (2013)	Human*	0.003 µM	0.109 µM	Decreased PGE$_2$ (air pouch, guinea pig)	(304)
Compound 28 (2013)	Human, murine	13.8 µM (human), ~5 µM (mouse) #	>100 µM	Not tested	(309)
Compound II (2013)	Human, murine	1.8 µM (human), 0.62 µM (rat)	Not tested	Decreased PGE$_2$ (air pouch, rat), decreased paw swelling (rat)	(310)
CIII (2013)	Human, murine	0.09 µM (human), 0.9 µM (rat)	~10 µM	Decreased PGE$_2$ and cells (air pouch, rat)	(262)
Compound 48 (2015)	Human*	0.005 µM	0.376 µM	Not tested	(311)

Table 2. Summary of selected mPGES-1 inhibitors reported up until 2015. *Inhibition towards other species was not tested. #Broken cell assay of human HeLa or mouse RAW 264.7 cells.

2 AIM

This PhD thesis aims to improve understanding about mPGES-1 and its inhibition in inflammation and cancer. The results will expand our knowledge of PGE_2 and mPGES-1 inhibitors in translational pharmacology. The objectives are:

Paper I. To investigate prostaglandins as inflammatory mediators in human tendon disease.

Paper II. To investigate differences in protein and lipid profiles after inhibition of mPGES-1 versus COX-2 *in vitro*.

Paper III. To demonstrate that inhibition of mPGES-1 is a potential therapeutic strategy in treating neuroblastoma *in vivo*.

Paper IV. To characterize five new mPGES-1 inhibitors in preclinical models of inflammation and vessel tone.

3 RESEARCH APPROACH

An essential part of eicosanoid research is the need to identify and quantify lipid mediators. The principal method for this, and used throughout this thesis, is liquid chromatography tandem mass spectrometry (LC-MS/MS). The Research approach section includes an introduction to LC-MS/MS, a presentation of prostanoid profiling, selected protocols used by the author, data presentation and statistical analyses, and ethical approval. Additional methods applied by the author, such as cell culture experiments and phospholipid profiling, or methods applied by co-authors of the studies, such as animal experiments and flowcytometry analyses, are described in the papers included in this thesis.

3.1 AN INTRODUCTION TO LC-MS/MS

This section is dedicated to future students that are about to start their research journey using mass spectrometry.

LC-MS/MS methodology is extremely diverse in terms of what to analyze and how to analyze. For this reason, the general methodology will be presented and the illustrations will be based on prostanoid analysis. A detailed introduction to the molecular theories and instrumentation of mass spectrometry is not covered and curious readers are referred to excellent textbooks (*e.g. Mass spectrometry* by Jürgen H. Gross, Springer, 2011).

Mass spectrometry is a highly complex and versatile technique. In the most comprehensive sense, a mass spectrometer is a very accurate and sensitive scale. For many people, however, it is literally just a black box. Operating this sensitive scale typically requires a skilled analytical chemist that has the option to ask two fundamentally different but equally important research questions. The first one is: What does my sample contain? We call this type of analysis *untargeted*. The answer to this question will always be biased, as samples are typically processed prior to the analysis and there is always a detection limit to what a mass spectrometer can pick up as a signal. The second question is: How much of molecule X does my sample contain? This requires that the molecular weight (mass-to-charge ratio, m/z) and preferentially the fragmentation pattern (mass spectrum) of molecule X is known prior to the analysis. The mass spectrometer is optimized to specifically analyze molecule X and we therefore call this type of analysis *targeted*. The problem with the detection limit is still apparent in this type of analysis, but typically less of a problem as the mass spectrometer is set to exclusively look for the molecule(s) of interest. This means that we are more certain in finding what we look for but we have no idea what other molecules the sample might contain. In the first scenario (untargeted analysis), the analyte list is generated *after* the analysis while in the second scenario (targeted analysis), the analyte list is generated *before* the analysis. Modern mass spectrometers are able to answer both of these questions at the same time, although there is a trade-off in the amount of molecules that can be detected and at what concentration. This depends on the *acquisition speed*, which is how long time the instrument needs to spend to collect ions that results in a quantitative signal, and the *dynamic range*, which is the detection range of the smallest to the largest detected signal.

One can appreciate that determining what a sample contain and/or how much of something a sample contains is useful in multiple disciplines ranging from characterizing protein structures and identifying drugs to discovering biomarkers in clinical samples and quantifying pesticides in water. A mass spectrometer on its own is a very powerful tool and it is even more powerful when coupled to an LC system.

3.1.1 Separation in the LC instrument

The LC system enables separation of analytes before the mass spectrometer. A liquid sample is injected onto an analytical column that is chosen to retain molecules of interest. The column is composed of porous beads that will differ in material, diameter, and pore size. In addition, the column itself will differ in length and diameter. The column is the *stationary phase* and the liquid that flows through the column is the *mobile phase*. Several chemical or physical parameters are available to vary for the column, depending on the nature of the molecules of interest. Analytes can be separated based on polarity, size, or charge. We separate prostanoids (and other lipids) based on polarity where the column is relatively hydrophobic and contains beads with tails of 18 carbons in a row (C18 column). This is called *reversed-phase chromatography*, when the stationary phase is hydrophobic. *Normal-phase chromatography* is when the stationary phase is hydrophilic.

Prostanoids are injected onto the column in a mobile phase that is relatively polar (80% water, 20% acetonitrile). The mobile phase is acidic (0.05% formic acid, pH 3.0) to protonate the carboxylic group on prostanoids (pKa ~4.5), making the analytes less polar. This means that the prostanoids are retained on the column. Separation and elution are achieved by gradually increasing the organic proportion of the mobile phase (by going from 20% to 95% acetonitrile). The analytes will elute in the order of increasing hydrophobicity. This is dependent on which phase a molecule preferentially interact with. In other words, there is always an equilibrium in the interaction between the stationary phase and the mobile phase, and the equilibrium is pushed depending on the polarity of the mobile phase. A molecule will be retained in the stationary phase as long as the mobile phase is more polar than the stationary phase (the opposite is true when running in normal-phase). The analytes are separated in time and we say that they have different *retention time*.

The retention time is dependent on the nature of the column, the gradient, and the composition of the mobile phase compared to the analyte of interest. In addition, the temperature and the flow rate will influence the separation. Temperature (thermal energy) affects the viscosity of liquids and thereby the diffusion coefficient between mobile and stationary phases. Increasing the temperature (40-50 °C) increases the column efficiency. Operating at a constant temperature (>25 °C) also leads to stable retention times since the effect of local room temperature fluctuations are removed. Higher temperature reduces the backpressure in the column, which enables higher flow rates, sharper peaks, reduced retention times, and shorter gradients. The concerns with increased temperature is that the column lifetime may be reduced, the molecule(s) of interest may not be thermally stable, and the peaks may be too narrow to enable quantification in the mass spectrometer.

The mobile phase can be designed with endless possibilities as one can elaborate with type of solvents, additives (salt), pH, and the gradient itself. Varying these parameters changes the peak shapes, the peak order, and the time between peaks (the *resolution*). There are two prime goals with separating analytes in the LC: it reduces the sample complexity prior to the mass spectrometer and it enables identification of analytes based on their retention time.

3.1.2 Detection in the mass spectrometer

All mass spectrometers operate based on three distinct features: *ionization*, *separation*, and *detection*. Molecules can be ionized in multiple ways, where *electrospray ionization* (ESI) is one commonly used strategy. ESI was first reported in 1984, and John B. Fenn and Koichi Tanaka jointly received half the Nobel Prize in Chemistry in 2002 for the analysis of biological macromolecules using mass spectrometry (the other half of the Nobel Prize was awarded to Kurt Wüthrich for his work on determining 3D structures of biological macromolecules using magnetic resonance spectroscopy).

The constant flow of liquid from the LC system passes through a thin needle and is sprayed in small droplets. The fine spray occurs in the ion source where the droplets are quickly evaporated and ions are formed as the chamber is kept at high temperature and there is a high voltage applied between the needle and the source cone (interface to the mass spectrometer). The exact mechanism of ionization is beyond this thesis. We are satisfied with ions being formed in the aerosol under the influence of high voltage and the solvent is evaporated, generating ions in gas phase that are attracted into the mass spectrometer. Neutral molecules are mainly lost due to that these are not affected by electric (magnetic) fields. The ion spray is perpendicular to the source cone to avoid that neutral molecules will enter the mass spectrometer. The analytes *flying* in a mass spectrometer are therefore ions and which ions to analyze is selected by choosing the current direction. Positive ions are analyzed in positive mode (ES+) and negative ions are analyzed in negative mode (ES-).

Ions pass through the source cone and via a hexapole (six metal rods to focus the ion beam) into the mass spectrometer, in this case a triple quadrupole detector (TQD). This is where the next separation happens. A TQD consists of three quadrupoles in a row followed by a detector. A quadrupole is composed of four metal rods, creating a path for the ions in the middle along the rods. Currents flow through the rods. By changing both the direction and the amplitude of the currents, selection of ions is enabled due to the change in the magnetic field in the quadrupole. A direct current (DC) voltage and a radio frequency (RF) voltage are applied. The selection of ions is based on that only ions that have a stable trajectory (movement) through the quadrupole can pass. Unstable ions will collide with the rods. The alternated RF will mainly affect smaller m/z as these are more sensitive (change trajectory quicker) than larger m/z. This means the RF enable discrimination of smaller m/z from medium or larger m/z. The DC will mainly affect larger m/z. In other words, the low mass cutoff is dependent on the RF and the high mass cut off is dependent on the DC. By increasing the DC and RF linearly, different m/z will be filtered out. Only ions that are large enough to constantly be refocused (or saved) by the RF will pass through. Ion size in terms of m/z is of course a relative concept. The key

concept is that a quadrupole is not performing one positive selection but two negative selections. Wolfgang Paul was awarded the Nobel Prize in Physics in 1989 for his work on quadrupoles and how to trap ions.

As the currents are changing in the first quadrupole (Q1), certain ions will pass through. The second quadrupole (Q2) uses only RF current and this is where the fragmentation takes place. Ions are fragmented by collision-induced dissociation that occurs in a flow of gas (Ar, He, or N_2). The fragmentation is not random and is influenced by the voltage (energy) applied. Each analyte of interest will fragment differently at different voltages, and just the right amount needs to be tested. Fragments then enter the third quadrupole (Q3) that operates just like the first: it will filter out ions. The combined selection of m/z in the first and third quadrupoles is called a *transition*. A TQD will typically cycle over a defined set of transitions, which is called *multiple reaction monitoring* (MRM). The selected ions (one species, one transition) hit the detector, generating a signal. It should be noted that a TQD can operate using other methods, including *product ion scanning*, *precursor ion scanning*, and *neutral loss scanning*. This is defined by whether Q1 and Q3 will cycle over defined transitions or scan over an interval of m/z. In product ion scanning, the Q1 is fixed at certain m/z while the Q3 scans for generated fragments. The results generated translate into which product ions a certain precursor is fragmented into. Precursor ion scanning is the opposite, *i.e.* when Q1 is scanning and Q3 is fixed at certain m/z. This type of results can be used to identify from which precursor ion(s) certain product ions are generated. Neutral loss scanning is when both Q1 and Q3 are scanning, but Q3 is scanning at a defined offset in m/z compared to Q1. In addition, one can chose to use only Q1 with fixed m/z or scanning without fragmentation in Q2 and selection in Q3.

3.1.3 The detector: What is a signal?

The signal in a mass spectrometer corresponds to the amount of ions measured. Multiple types of detectors exist but they can be divided into two main groups depending on the detection method: destructive or non-destructive. The first one relies on ions hitting a detector (metal plate), creating an electric current as the ions are neutralized. The signal is typically amplified with the use of electron multiplier. In the second detection method, ion motion is recorded. Ions oscillate back and forth in a magnetic field between two metal plates, where the motion is dependent on the m/z. This is called image current detection. The ion identity (m/z) is related to the motion frequency and the ion counts is related to the motion intensity. The first detection method was used in this thesis for prostanoid profiling.

In untargeted analysis, the currents generated in the detector is translated into mass spectra. A *mass spectrum* is a plot with m/z on the x-axis and signal intensity on the y-axis. Mass spectra can be collected before or after fragmentation, generating distinct information. The first dimension (MS1 spectrum) contains precursor ions. The second dimension (MS2 spectrum) contains product ions. When an instrument scan over a defined m/z range, mass spectra are collected at a certain speed and this will generate a large amount of data. Each mass spectrum can be used for identification and/or quantification, depending on the research question and experimental design. When running targeted analysis using a TQD, no mass

spectra are recorded as the instrument is only recording signals at predefined MRM transitions.

As mentioned above, the nature of the signal is different depending on the mass analyzer method used. But what are the parameters influencing the signal intensity? This comes down to *ionization efficiency* and *transmission efficiency*. The ionization efficiency depends on the ionization method applied, the analyte of interest, and matrix effects. For example, molecular weight, volatility, charge, and polarity are molecular parameters that influence how efficient a molecule ionizes. Matrix effects is the presence of co-eluting molecules from the column that cause interferences with the ionization in the ion source. Although the voltage is high to ensure ionization of eluting molecules of interest, co-eluting molecules can compete for the available energy and/or modify droplet formation. The lower ionization efficiency caused by matrix effects can be reduced by considering the sample preparation (extraction, enrichment, and purification) and the separation on the column in the LC system. For example, the sample can be diluted prior to analysis, lower sample volume can be injected, or the flow rate and gradient can be altered. Transmission efficiency is the fraction of ions X generating a signal compared to the total amount of ions X generated in the ion source. This is dependent on the nature of the analyte (how stable the ion species is and how well it fragments) and the hardware of the instrument (how efficient the lenses and quadrupoles are in focusing and maintaining the ion beam).

The absolute signal intensity is important, but it is a difficult term to relate to given the many parameters influencing the signal. Instead, *signal-to-noise* (S/N) is used to describe the certainty of the signal in a quantitative way. This is simply a relative term of how much stronger the intensity of a peak is compared to the background (noise) signal. Noise comes from many sources and can be electronic or chemical. There will always be noise in a mass spectrometer but there are different ways to increase the S/N. For example, increasing the acquisition time and increasing the number of spectra that are averaged. Targeted analysis typically has higher S/N as the mass spectrometer cycles through a defined set of transitions. Using a TQD, the time spent on each transition is longer than if the instrument would scan over a wide range of m/z. Another important parameter is the selectivity. The selection in the two quadrupoles enables confirmation in identity and improves S/N, as fewer possible molecules exist that has the same precursor and product ion compared to only the same precursor m/z.

The S/N is what determines the *limit of detection* (LOD) and the *limit of quantification* (LOQ). There is no exact definition but typically a signal is detected if S/N > 3 and quantified if S/N > 10. These are guidelines to have a cut-off for a positive signal. It should be noted that the certainty in quantification is not improved by increasing the certainty in identification, *i.e.* by confirming a peak via multiple MRM. Certainty in quantification and identification are different entities, and quantification under S/N > 10 should be avoided.

3.1.4 MRM method development

There are several parameters to consider when introducing an analyte into your LC-MS/MS platform. First of all, one needs to guess (or test) if the analyte will ionize and "*fly*" better in positive or negative mode. An analyte can be measured or fly with similar performance in both modes. The actual m/z is determined in the first step. It is not crucial that the m/z is in perfect agreement with the nominal mass, since every TQD will have minor differences effecting the observed m/z. Given that the instrument is calibrated, the observed m/z will not differ more than fractions of Da from the nominal value. The observed m/z can be obtained by performing a narrow scan, about ±4 Da from the molecular mass. The instrument then cycles over the defined range, resulting in a signal that is plotted against time in a total ion chromatogram (TIC). Within the TIC, a peak distribution of m/z values is obtained. The next step is to optimize the cone voltage. The high capillary voltage for ionization is preset depending on ES+ (*e.g.* 3 kV) or ES- (*e.g.* 2.7 kV) while the cone voltage (*e.g.* 10-100 V) is altered to extract ions of interest into the mass spectrometer. Finding the optimal cone voltage is tested by varying it within an injection or by injecting the analyte of interest in a sequence with different cone voltages for each injection. Fragmentation is the next step to test. This is performed by fixed m/z in Q1, applying energy (*e.g.* 20 V) in the Q2, and scan over a range (m/z 100-360, if the analyte of interest is m/z 350 and adduct ions are not of interest) in Q3. This will generate mass spectra covering m/z 100-360. If fragments are not observed, higher voltage can be applied. After this, one or several fragments are selected and the collision energy is noted or further optimized for. This is performed at fixed values in the Q1 and Q3, resulting in a transition, and varying the collision energy in Q2.

3.1.5 Pros and cons with LC-MS/MS

It is now clear that the many parameters and settings used within LC-MS/MS makes it a complex and flexible technique. Almost any molecule of interests can be measured in any type of sample matrix – at least in theory. LC-MS/MS is often the method of choice for the great selectivity, enabling certainty in the identification of the measurements. This is the main advantage over antibody-based methods, such as enzyme-linked immunosorbent assay (ELISA), which are dependent on the antibody specificity. In addition, LC-MS/MS can generate in-depth data in terms of structure, identity, and molecular modifications that is very difficult to observe or distinguish using ELISA. Both LC-MS/MS and ELISA can be used for multiplexing, which is to analyze multiple molecules of interest at the same time within the same injection/sample. However, ELISA has the advantage over LC-MS/MS in many applications as it is easy to use, cheaper, typically requires less sample preparation, shorter time-to-results, higher throughput, and can have a very low LOQ. Compared to gas chromatography (GC), LC can be used for any soluble analyte while GC requires volatile analytes. LC is therefore preferred when working with biological matrices with relatively hydrophilic analytes of interests. Separation in GC is typically quicker due to the increased movement of molecules in gas phase compared to liquid phase, which can enable more narrow peaks and shorter run times. Analytes that are less volatile can be derivatized by

coupling of a functional group, although the resulting molecule still needs to be thermally stable as GC operates at higher temperature than LC. Although derivatization can be time-consuming and costly, it may enable quantification of otherwise undetectable analytes.

3.2 PROSTANOID PROFILING

Prostanoids, or any eicosanoid, are typically produced at low picomolar to nanomolar concentrations in highly complex biological matrices. This makes them challenging to quantify. There are also multiple lipids, or biomolecules in general, that have the exact same molecular weight and very similar structures. This places a high demand on the selectivity in the analysis to ensure correct identification. This section will introduce the reader to sample preparation and the LC-MS/MS workflow used for prostanoid profiling.

The sample complexity needs to be reduced and the prostanoid content needs to be enriched. This is performed by extraction. Liquid-liquid extraction is the use of two solvents with different polarity. For example, prostanoids can be extracted from cell culture supernatant (or tissue homogenate) with ethyl acetate. Another option is to use solid-phase extraction (SPE). This is based on capturing of analytes in a column followed by washing of the matrix and elution of the analytes. Prostanoids are fatty acid derivatives with a carboxylic group that is protonated at low pH. The use of a SPE column containing long-chain carbon (C18), which is fairly hydrophobic, will retain protonated prostanoids that can be eluted in MeOH that is easily evaporated. Despite the selection of extraction method, it is vital to spike each sample with an internal standard mix. In the best case, the mix should contain ^{13}C isotope-labeled variants of *each* analyte of interest. The second best option is deuterated variants. The internal standard will compensate for storage effects, extraction recovery during sample preparation, and matrix effects in the mass spectrometer if added early in the sample preparation. However, the internal standard does not overcome loss in sensitivity due to poor extraction efficacy and/or interfering matrix effects. In other words, using an internal standard does not improve the LOQ but rather compensates for differences in extraction recovery and matrix effects between samples. The deuterated variant have almost identical chemical properties as the non-deuterated molecule. It will therefore be extracted at the same efficacy and elute at approximately the same retention time as the endogenous molecule. This helps confirming the identity of the endogenous prostanoid in the sample.

Prostanoids are quantified in absolute concentration. This is done by internal standard normalization and quantification to an external standard curve. Ideally, the standard curve should be prepared in the same type of matrix as the samples to replicate the loss in signal due to extraction recovery and/or matrix effects. This is difficult when the sample matrix is tissue homogenate or when all the matrices (samples) are positive for the analytes of interest. The most important part with the standard curve is that it will give the dynamic range where the response vs. injected amount is linear, *i.e.* the response factor. The linearity is assumed to be the same for an analyte despite the sample matrix when using an internal standard, but the LOQ will be different depending on the sample matrix. This means that a standard curve in solution enables reliable quantification. The loss in recovery is estimated by spiking a sample before

and after the extraction and calculating the ratio. The interference of matrix effects during ionization in the ion source is estimated by comparing the signal in a spiked extracted sample with the signal in a solution blank sample. LOQ for each analyte is given by the lowest quantified standard with S/N > 10.

Every extraction is evaluated by using technical blanks and double blanks. The technical blank is treated just like a sample but it does not contain the actual sample matrix, and this is a positive control for the sample preparation and to control for false positive signals (contamination) during the sample preparation. The double blank is treated like the blank but it lacks the internal standard. This is to evaluate that the internal standard gives a signal in the technical blank. The performance of the LC-MS/MS analysis is evaluated with a quality control (QC) sample, containing analytes of known concentrations. This will yield a response that can be recorded and compared to each previous analysis. In addition, the peak shape, the retention time, and the peak resolution is checked at this stage. The LOQ is evaluated with the standard curve, which is prepared fresh from a stored stock for each analysis. This is typically analyzed first and last in the analysis or in between long sample lists to observe loss in performance during the analysis. QC samples in solution or a pooled true sample are injected every 10-20th injection to observe changes in retention times or responses during the analysis.

Prior to the analysis the sample cone (interface to the mass spectrometer) is cleaned, fresh solutions for mobile phases are prepared, the LC instrument is primed with solutions, the column is flushed and equilibrated, and the backpressure in the instrument is monitored. When ready, the samples are queued and the instrument will run the sequence. Hopefully the run will finish and the instrument goes into shutdown. However, multiple errors can arise: backpressure reaches instrumental limit due to injection of particles or build-up of unwanted material from the samples, leakage due to column or tube fittings, loss in signal due to accumulation of material on the needle in the electrospray, and (yes, this has happened!) loss of vacuum due to turbo pump failure.

The extraction takes 2-10 hrs, depending on how many samples and what type of samples. The extracted samples are then evaporated for at least 2 hrs. Starting the LC-MS/MS analysis takes 2-4 hrs, depending on preparations. The actual analysis takes 15 min per sample for prostanoid profiling. A normal run with 20-100 samples plus QC samples, standard curve, and blank injections takes 10-30 hrs. Peak integration and data analysis takes at least 2 hrs. This means that the time-to-results is a minimum of two days but can be as long as one week. The throughput is about 200 samples per week, if samples are extracted in 2x96-well plates at the same time. However, this is when everything already is optimized and standardized for (and no errors occur).

The LC-MS/MS method used in this thesis is specifically developed and optimized for to cover the main prostanoids, as the research questions and disease models used throughout the different studies involved prostanoids. However, the LC-MS/MS method contains a broad spectrum of eicosanoids including 6-keto PGF$_{1\alpha}$, PGE$_3$, TXB$_2$, PGF$_{2\alpha}$, PGE$_2$, PGD$_2$, LTB$_4$, RvD1, RvD2, 5-HETE, 12-HETE, 15-HETE, 13-hydroxyoctadecadienoic acid (13-HODE),

15d-PGJ$_2$, protectin DX, and 17-hydroxy-decosahexaenoic acid (17-hydroxy DHA) (**Table 3**). These lipids were individually optimized for in the mass spectrometer and the recovery efficiency during extraction have been evaluated. Nevertheless, analytical performance of all eicosanoid classes where not controlled for with deuterated internal standards to account for extraction recovery and matrix effects in each specific biological sample. Eicosanoids are chemically diverse molecules and there is no one-size-fits-all regarding the extraction procedure. In addition, eicosanoids are likely to have different stability at different conditions affected by temperature, light, oxygen, and pH. This means that the only correct procedure to report absolute concentrations of eicosanoids is to spike samples with deuterated (or ^{13}C isotope-labeled) internal standards for *each* analyte of interest, as mentioned earlier. This of course comes with a price tag and increases the number of MRM transitions. In addition, this will require additional time if multiple extraction protocols and/or gradients are used.

Different LC-MS/MS based methods can be deployed to specifically investigate other classes of eicosanoids. However, many of these lipids are found in extremely small quantities, placing a large demand on the instrumental set up. As discussed above, several instrumental parameters will affect the signal in the mass spectrometer. The instrumental set-up used throughout this thesis is not able to deliver better performance in terms of analytical sensitivity. It must be acknowledged that there are lower LOQ for eicosanoid analyses reported in the literature (345-349), and that analyses can be performed with lower sample amount and in shorter gradients. Nevertheless, it is unclear in some studies if the reported LOQ is the concentrations measured of spiked standard in un-extracted solution or concentration measured of spiked standard in (extracted) biological sample. The lack of deuterated standards, the reported low pg/mL in complex biological matrices, and the cutoff of S/N > 3 or 5 for quantification of peaks place a question mark behind the endogenous presence of some lipids. PGE$_2$ is the product of mPGES-1 during inflammation and cancer, and it is reasonable to include at least the main prostanoids in any type of research regarding COX or mPGES-1 and their inhibition. This is to observe any shunting or perturbation in the prostanoid profile.

Analyte	Item No.*	Retention time (min)	Precursor (m/z)	Fragment (m/z)	Cone voltage (V)	Collision energy (V)
6-keto PGF$_{1\alpha}$	15210	3.2	369.1	245.2	53	21
6-keto PGF$_{1\alpha}$-d4	315210	3.2	373.1	249.2	53	21
CIII (1st ion)	N/A	4.5	383.6	174.1	55	50
CIII (2nd ion)		4.5	383.6	309.3	55	35
PGE$_3$	14990	4.7	349.1	269.3	45	20
TXB$_2$	19030	5.0	369.2	169.2	30	20
TXB$_2$-d4	319030	5.0	373.2	173.2	30	20
PGF$_{2\alpha}$	16010	5.7	353.3	309.3	45	23
PGF$_{2\alpha}$-d4	316010	5.7	357.3	313.3	45	23
PGE$_2$ (1st ion)	14010	6.0	351.1	271.3	38	19
PGE$_2$ (2nd ion)		6.0	351.1	315.4	38	14
PGE$_2$-d4	314010	6.0	355.1	275.3	38	19
PGD$_2$	12020	6.2	351.1	271.3	38	19
PGD$_2$-d4	312020	6.2	355.1	275.3	38	19
RvD2	10007279	6.4	375.5	175.2	35	30
RvD1	10012554	7.0	375.5	141.0	30	20
LTD$_4$-d5	10006199	8.1	500.3	177.2	40	31
LTC$_4$-d5	10006198	8.2	629.4	272.2	50	31
Protectin DX	10008128	8.4	359.3	153.1	33	19
LTB$_4$	20110	8.5	335.3	195.2	40	17
LTB$_4$-d4	320110	8.5	339.3	197.2	40	17
15d-PGJ$_2$	18570	8.8	315.2	271.2	17	24
15d-PGJ$_2$-d4	318570	8.8	319.2	275.2	17	24
13-HODE	38600	8.9	295.4	195.3	40	20
15-HETE	34720	8.9	319.4	219.1	30	15
15-HETE-d8	334720	8.9	327.4	226.2	30	15
17(S)-HDoHE	10009794	9.0	343.3	201.2	30	16
12-HETE	34570	9.0	319.4	179.1	35	17
12-HETE-d8	334570	9.0	327.4	184.1	35	17
5-HETE	34230	9.1	319.4	115.0	30	18
5-HETE-d8	334230	9.1	327.4	116.0	30	18

Table 3. Experimental details for quantification of eicosanoids and CIII by LC-MS/MS. *Cayman Chemical.

3.3 PROTOCOLS

3.3.1 Whole blood assay

Preparation: Inhibitors were reconstituted at 10 mM in DMSO (D2250-100ML, Sigma-Aldrich), aliquoted in Eppendorf tubes or 96-well plates, and stored at -20 or -80 °C. A fresh aliquot was used at each experiment. LPS (L6529-1MG, Sigma-Aldrich) was reconstituted in PBS (D8537-500ML, Sigma-Aldrich) to a final concentration of 0.25 mg/mL and kept at +8 °C.

The assay: Inhibitors, positive controls (diclofenac and NS-398), and vehicle control (DMSO) were prepared in PBS at room temperature (RT) with no direct light on. The treatments were prepared in 25 µL portions to a U-shaped 96-well plate and 200 µL of freshly drawn heparin blood (<2 hrs stored at RT) was added to the plate. The plate was incubated at 37 °C for 30 min and then 25 µL of 0.1 mg/mL LPS in PBS was added followed by pipetting up and down 3 times (final concentration of LPS was 10 µg/mL). The plate was incubated for 24 hrs at 37 °C and then centrifuged at 3000 g for 10 min at 4 °C. Working on ice, 100 µL plasma was recovered to a new plate, sealed with aluminum foil, and stored at -80 °C.

3.3.2 Extraction of prostanoids

0. Preparations: Organic solutions (MeOH, MeCN, EtOH) were of LC-MS grade. Only fresh, highly pure water was used (18.2 MΩ at 25 °C, ppb TOC < 3.0). Solutions for extraction were prepared fresh daily. The pH was checked when applicable (0.05 % formic acid solution, pH < 2.0). The internal standard mix contained 340 ng/mL 6-keto PGF$_{1\alpha}$-d4 (#315210), 160 ng/mL PGF$_{2\alpha}$-d4 (#316010), 240 ng/mL PGE$_2$-d4 (#314010), 160 ng/mL PGD$_2$-d4 (#312020), 160 ng/mL TXB$_2$-d4 (#319030), and 160 ng/mL 15d-PGJ$_2$-d4 (#318570) prepared in 100% MeOH and stored at -20 °C. The standard curve contained 11 stock solutions with 24 to 0.023 pmol/µL of 6-keto PGF1α (#15210), PGF$_{2\alpha}$ (#16010), PGE$_3$ (#14990), PGE$_2$ (#14010), PGD$_2$ (#12020), TXB$_2$ (#19030), and 15d-PGJ$_2$- (#18570) prepared in 100% EtOH and stored at -80 °C. All prostanoid standards were from Cayman Chemical.

1a. Cell supernatant samples: Cell supernatants (400-1000 µL) were thawed on ice, diluted with water to 1 mL, and spiked with 50 µL deuterated internal standard mix (above). The samples were acidified with addition of 0.5-2 µL 100% formic acid (pH < 4.0) and left to equilibrate on shaker for 30 min on ice prior to extraction using solid-phase extraction (SPE).

1b. Plasma samples: Plasma samples (100 µL) were thawed on ice and spiked with 50 µL deuterated internal standard mix (above). Protein precipitation was performed by addition of 800 µL 100% MeOH, vortexing, and centrifugation at 3000 g for 10 min at 4 °C. The supernatants were collected in a new plate and evaporated under vacuum for 4 hrs, until 100-200 µL remained. These were then diluted to 1 mL with 0.05% formic acid in water and subjected to SPE.

1c. Tissue samples: Frozen tissues (tumor, spleen, brain) were used as whole or pieces were excised and weighed (50-500 mg). Tissues were kept on dry ice and spiked with 100 µL

deuterated internal standard mix (above). Additional MeOH was added (500-1000 µL). Homogenization was performed using a pellet pestle (art# 749515-1500, Kontes) for 1-2 min or a tip-sonicator (Sonoplus, Bandelid) 2-4 times 30 sec at 35% power. Homogenates were incubated at -20 °C for 30 min and then centrifuged at 20.000 g for 10 min at 4 °C. The supernatants were collected and extraction was repeated once by addition of 500 µL of 100% MeOH, vortex for 30 sec, centrifugation at 20.000 g for 10 min at 4 °C, and collection of supernatant. Pooled supernatants were evaporated for 4 hrs until 100-200 µL remained. These were diluted to 1 mL with 0.05% formic acid in water and subjected to SPE.

2. SPE: Acidified samples (about 1 mL) were loaded onto Oasis HLB 1cc 30mg single-use column or 96-well plate (Waters Corporation) that had been pre-conditioned with 1 mL of 100% MeOH and 1 mL of 0.05% formic acid in water. The column or plate was washed with 10% MeOH, 0.05% formic acid in water and prostanoids were eluted with 100% MeOH. The eluates were dried under vacuum (at least 4 hrs, but typically over-night) and stored at -20 °C.

3. LC-MS/MS analysis: Dried samples were reconstituted in 50 µL of 20% MeCN in water, vortexed (5-10 sec), allowed to dissolve at +8 °C (30 min), centrifuged at 20.000 g for 10 min at 4 °C (if plate: 3000 g, 10 min, 4 °C), and then transferred to vials or collection plate. Prostanoids were quantified in negative mode with MRM method, using a triple quadrupole mass spectrometer (Acquity TQ detector, Waters) equipped with an Acquity H-class UPLC (Waters). Separation was performed on a ACQUITY UPLC BEH C18, 130Å, 50 x 2.1 mm, 1.7 µm column (Waters) with a 12 min stepwise linear gradient (20-95%) at a flowrate of 0.6 mL/min with 0.05% FA in MeCN as mobile phase B and 0.05% FA in water as mobile phase A. The analytical column was equipped with a ACQUITY UPLC BEH C18 VanGuard, 130Å, 1.7 µm, 5 mm x 2.1 mm pre-column (Waters). The samples were kept at 10 °C. The injection volume was 10 µL and the column was kept at 40 °C. The source temperature was 150 °C, desolvation temperature was 500 °C, cone gas flow was 50 L/h, and desolvation gas flow was 1000 L/h.

4. Data analysis: Data were analyzed using MassLynx software, version 4.1, with internal standard calibration and quantification to external standard curve. Only analytes with peaks intensities of signal-to-noise greater than 10 (S/N >10) were considered in our data analysis. The lower LOQ was typically 0.05 pmol (~20 pg) injected on column for the main prostanoids. This corresponds to quantification limit of 0.25 nM (~100 pg/mL) if extracting 1 mL of sample or 2.5 nM (~1000 pg/mL) if extracting 100 µL sample.

3.4 DATA PRESENTATION AND STATISTICAL ANALYSES

Readers are referred to each paper and the Results section in this thesis for details regarding data presentation and statistical analyses. Statistical analyses were performed using Graphpad prism v. 5-7 (Graphpad Software Inc., USA). The level of significance was set to $P < 0.05$ if not stated otherwise.

3.5 ETHICAL APPROVAL

Paper I included collection of tendon biopsies of supraspinatus tendons, Achilles tendons, and hamstring tendons. Ethical approval for these procedures was granted with references 14/SC/0222 (South Central Oxford B, UK), 14/NI/1063 (Northern Ireland Research Ethics Committee, UK), and 09/H606/11 (Oxford Musculoskeletal Biobank, UK). Full informed consent according to the Declaration of Helsinki was obtained from all patients. **Paper II** involved work on commercially available cancer cell line and no ethical approval was required for this work. **Paper III** included work on human tumors from neuroblastoma patients and two preclinical mouse models of neuroblastoma. Ethical approval and relevant informed consent was obtained according to the references 2009/1369-31/1 and 03/736 (Research Ethics Committee at Karolinska University Hospital, Sweden). Ethical approvals for mouse experiments were granted with references N231/14, N26/11, and N42/14 (Regional Ethics Committee on Animal Experiments, Sweden). **Paper IV** included work on human material, two preclinical rat models, and one preclinical mouse model. Ethical approval for collection of blood from healthy donors was granted by reference 02-196 (Research Ethics Committee at Karolinska University Hospital, Sweden). Ethical approval for collection of biopsies for *ex vivo* myography experiments was granted with reference 273/94 (Research Ethics Committee at Karolinska University Hospital, Sweden). Full informed consent according to the Declaration of Helsinki was obtained from all subjects. Ethical approval for air pouch experiments in mice was granted with reference N86/13 (Regional Ethics Committee on Animal Experiments, Sweden). Pharmacokinetics study in rats was approved by Cerep Institutional Animal care and Use Committee (IACUC), USA. Paw swelling study in rats was approved by Institutional Animal Ethics Committee of Anthem Biosciences, India.

4 RESULTS

4.1 PROSTAGLANDINS AND MPGES-1 IN TENDINOPATHY

We have investigated the role of prostaglandins in human tendon disease (**Paper I**). The results showed that tendon tissues from diseased supraspinatus and diseased Achilles expressed elevated levels of COX-2 and the two terminal synthases PGIS and mPGES-1 compared to healthy hamstring tendon tissue. We isolated cells from tendon biopsies and cultured them *in vitro*. These cells are termed tendon-derived stromal cells and they do not express markers for leukocytes (CD45) or endothelial cells (CD34). We found that cells from diseased shoulder and diseased Achilles tendons produced prostacyclin and PGE_2 while cells from healthy hamstrings only produced PGE_2. COX inhibitors naproxen and NS-398 terminated the prostaglandin production, while mPGES-1 inhibitor CIII reduced PGE_2 and increased prostacyclin formation. In an attempt to study prostaglandin production in tendon disease in connection to pain, we used tendon biopsies from patients with resolved or persistent pain post-surgical treatment and quantified mRNA levels of prostaglandin synthetic enzymes. We found increased expression of *PTGIS* in patients with resolved pain.

We have also studied phospholipid profiles in tendon-derived cells by LC-MS/MS. This was performed on tendon cell pellets from *in vitro* experiments and we quantified 52 phospholipids in each sample: 7 lysophosphatidylethanolamines (LPEs), 9 lysophosphatidylcholines (LPCs), 21 phosphatidylcholines (PCs), 6 phosphatidylcholine (PEs), and 9 sphingomyelins (SMs). Phospholipids are named based on head group with total number of carbons and total number of saturation in the two acyl chains. Relative changes in phospholipids upon treating cells with IL-1β was investigated. Six phospholipids were altered with IL-1β treatment despite disease state or anatomical location: LPE(20:5), LPC(14:0), LPC(20:5), and PC(30:0) where increased while PC(38:5) and PC(38:4) were decreased (**Table 4**). We next investigated if COX or mPGES-1 inhibition altered the IL-1β induced phospholipid profiles. This was performed on cells from diseased shoulder and healthy hamstring (**Figure 4**). Our results show that treatment with CIII reversed the effect observed with IL-1β on three lipids in cells from healthy hamstrings: LPE(16:0), LPE(20:4), and LPC(20:5). This was not observed with naproxen or NS-398. In cells from diseased supraspinatus, CIII decreased LPE(16:0) and increased PC(30:0). Our results show that IL-1β induced alterations in the cellular phospholipid profile and that inhibition of mPGES-1 or COX-1/2 affect the cellular lipid profile differently in tendon-derived cells.

Phospholipid	Healthy hamstring (n=8)		Diseased supraspinatus (n=8)		Diseased Achilles (n=6)	
	Ctrl	IL-1β	Ctrl	IL-1β	Ctrl	IL-1β
LPE(16:0)	5.8 ± 1.2	**4.9 ± 1.2***	4.7 ± 1	**3.9 ± 1.1***	7.2 ± 1.5	6.8 ± 1.2
LPE(18:2)	2.0 ± 0.6	2.1 ± 0.6	2.0 ± 0.4	2.0 ± 0.4	0.7 ± 0.2	0.8 ± 0.2
LPE(18:1)	18.6 ± 3.4	**19.9 ± 3.5***	15.6 ± 2.9	16.6 ± 3.2	19.2 ± 3.2	20.1 ± 2.8
LPE(18:0)	10.9 ± 2.3	9.9 ± 2.7	8.7 ± 2.6	**6.6 ± 1.5***	16.0 ± 8.7	16.5 ± 5.4
LPE(20:5)	2.6 ± 1.2	**3.3 ± 1.3***	5.0 ± 3.2	**5.9 ± 2.7***	2.2 ± 0.9	**2.9 ± 1.1***
LPE(20:4)	40.0 ± 5.7	39.6 ± 4.5	49.0 ± 5.6	49.3 ± 4.2	38.5 ± 8.1	37.3 ± 7.5
LPE(22:6)	20.0 ± 5.2	20.3 ± 4.7	15.0 ± 2.4	15.7 ± 2	16.2 ± 4.9	15.6 ± 3.9
LPC(14:0)	0.6 ± 0.3	**1.0 ± 0.3***	0.5 ± 0.2	**0.7 ± 0.2***	0.7 ± 0.1	**0.9 ± 0.1***
LPC(16:1)	1.7 ± 0.5	**2.7 ± 0.6***	1.5 ± 0.5	**2.4 ± 0.6***	2.1 ± 0.4	2.4 ± 0.4
LPC(16:0)	51.9 ± 2.6	48.8 ± 4.9	50.8 ± 2.4	44.4 ± 8.6	32.2 ± 0.5	32.3 ± 1.6
LPC(18:2)	3.7 ± 0.9	3.1 ± 0.4	3.6 ± 0.8	3.4 ± 0.6	0.9 ± 0.2	1.0 ± 0.3
LPC(18:1)	9.7 ± 1.2	**11.5 ± 2.3***	8.7 ± 0.7	**11.2 ± 2.8***	16.4 ± 1.4	16.9 ± 2.1
LPC(18:0)	29.6 ± 4.2	28.9 ± 4.0	31.0 ± 4.0	31.1 ± 4.8	44.4 ± 2.6	41.8 ± 2.7
LPC(20:5)	0.2 ± 0.1	**0.4 ± 0.2***	0.4 ± 0.3	**0.8 ± 0.5***	0.2 ± 0.1	**0.4 ± 0.2***
LPC(20:4)	2.2 ± 0.5	3.1 ± 1.0	3.0 ± 0.9	**5.1 ± 1.9***	2.6 ± 1.0	3.2 ± 1.1
LPC(22:6)	0.4 ± 0.1	**0.6 ± 0.2***	0.4 ± 0.1	**0.9 ± 0.3***	0.7 ± 0.2	1.0 ± 0.5
PC(28:0)	0.4 ± 0.2	**0.5 ± 0.2***	0.3 ± 0.1	0.4 ± 0.3	0.3 ± 0.1	**0.4 ± 0.1***
PC(30:1)	9.1 ± 2.6	8.9 ± 2.4	10.7 ± 2.9	**9.5 ± 2.3***	7.8 ± 0.7	7.9 ± 1.0
PC(30:0)	4.4 ± 1.0	**5.0 ± 0.9***	3.5 ± 0.5	**4.1 ± 0.8***	3.2 ± 0.6	**3.7 ± 0.4***
PC(32:3)	0.2 ± 0.1	**0.4 ± 0.2***	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.1
PC(32:2)	2.7 ± 1.0	2.8 ± 1.1	1.9 ± 0.6	2.8 ± 2.2	1.3 ± 0.4	1.4 ± 0.6
PC(32:1)	12.9 ± 2.3	**15.6 ± 3.4***	12.1 ± 3.5	13.4 ± 3.6	11.3 ± 1.6	11.3 ± 1.5
PC(32:0)	5.9 ± 2.1	7.2 ± 2.9	6.9 ± 1.8	8.1 ± 3.7	8.1 ± 1.6	**9.1 ± 1.3***
PC(34:4)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.04	0.2 ± 0.03
PC(34:3)	2.0 ± 0.4	2.4 ± 0.8	1.6 ± 0.3	2.4 ± 1.6	0.8 ± 0.2	0.9 ± 0.2
PC(34:2)	16.6 ± 4.1	15.0 ± 2.3	14.4 ± 1.4	15.6 ± 2.1	7.0 ± 1.2	6.7 ± 1.2
PC(34:1)	20.2 ± 5.7	20.8 ± 4.9	21.4 ± 4.3	20.3 ± 6.3	21.6 ± 1.5	21.4 ± 0.7
PC(34:0)	2.2 ± 0.6	2.3 ± 0.6	2.7 ± 0.6	2.2 ± 0.6	2.9 ± 0.3	3.0 ± 0.3
PC(36:8)	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	**0.8 ± 0.2***	1.5 ± 0.1	1.6 ± 0.1
PC(36:4)	4.6 ± 2.1	3.9 ± 1.1	4.6 ± 1.0	4.7 ± 0.7	3.6 ± 0.6	**3.3 ± 0.6***
PC(36:3)	4.2 ± 1.0	3.4 ± 1.2	4.1 ± 0.7	3.7 ± 0.9	2.8 ± 0.1	2.7 ± 0.1
PC(36:2)	5.6 ± 1.9	3.7 ± 1.8	5.3 ± 1.4	**3.8 ± 1.1***	8.5 ± 0.8	8.4 ± 0.7
PC(36:1)	2.2 ± 1.0	2.3 ± 1.1	3.0 ± 1.0	2.5 ± 1.4	7.1 ± 0.9	7.3 ± 0.4
PC(36:0)	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
PC(38:6)	1.2 ± 0.5	1.0 ± 0.3	1.1 ± 0.2	1.1 ± 0.3	1.4 ± 0.2	1.4 ± 0.1
PC(38:5)	2.0 ± 0.7	**1.6 ± 0.4***	2.2 ± 0.5	**1.8 ± 0.3***	4.5 ± 0.4	**4.0 ± 0.4***
PC(38:4)	1.8 ± 0.6	**1.4 ± 0.4***	2.1 ± 0.7	**1.6 ± 0.7***	4.9 ± 0.8	**4.4 ± 1.0***
PE(32:1)	9.0 ± 3.4	8.0 ± 2.3	7.0 ± 2.6	8.2 ± 5.1	4.9 ± 0.8	4.6 ± 1.0
PE(34:2)	16.8 ± 6.6	17.7 ± 6.8	12.9 ± 3.4	**15.2 ± 4.6***	8.2 ± 2.1	8.4 ± 1.8
PE(34:1)	24.8 ± 5.6	24.6 ± 6.8	25.8 ± 6	24.8 ± 7	26.1 ± 4.3	29.5 ± 4.2
PE(36:4)	7.8 ± 3.9	7.4 ± 2.2	7.8 ± 1.3	10.7 ± 4.5	6.3 ± 0.7	6.6 ± 1.1
PE(36:2)	22.0 ± 8.7	21.4 ± 6.8	19.9 ± 5.9	18.1 ± 7.8	29.3 ± 1.7	28.6 ± 2.7
PE(38:5)	19.6 ± 6.5	20.9 ± 7.1	26.6 ± 4.7	23.0 ± 4.8	25.3 ± 3.4	22.3 ± 2.4
SM(32:1)	6.8 ± 2.2	5.4 ± 1.5	6.4 ± 1.9	6.6 ± 3.3	3.5 ± 0.4	3.4 ± 0.7
SM(32:0)	1.0 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.4	0.4 ± 0.1	0.4 ± 0.1
SM(34:2)	7.7 ± 3.4	6.3 ± 2.3	6.7 ± 2.6	7.0 ± 4.4	5.4 ± 1.7	6.2 ± 1.1
SM(34:1)	52.8 ± 4.3	53.2 ± 5.6	57.9 ± 4.3	55.1 ± 9.4	58.1 ± 5.4	57.2 ± 6
SM(34:0)	9.1 ± 2.4	8.5 ± 2.2	7.3 ± 0.7	7.2 ± 0.7	9.9 ± 0.5	9.8 ± 0.9
SM(36:2)	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.3	1.5 ± 0.2	1.5 ± 0.1
SM(36:1)	4.8 ± 1.2	5.1 ± 1.6	5.0 ± 1.3	5.4 ± 1.7	5.2 ± 0.6	5.2 ± 0.7
SM(36:0)	14.4 ± 2.2	**17.3 ± 2.9***	12.5 ± 4.1	14.5 ± 3	15.8 ± 3.8	16.2 ± 3.2
SM(40:7)	1.2 ± 0.4	1.2 ± 0.5	1.2 ± 0.4	1.2 ± 0.4	2.3 ± 0.4	**2.6 ± 0.2***

Table 4. Treatment with IL-1β alters phospholipid profiles in tendon-derived cells. Cells were cultured and treated with medium or medium containing 10 ng/mL of IL-1β for 24 hrs. Phospholipids were extracted from cell pellets and analyzed by LC-MS/MS. Data is presented as relative abundance (area-%, mean ± SD) of phospholipid species within phospholipid classes. Phospholipids altered in the same direction in the three tendon groups are shown in grey. Statistical significance was calculated using paired t-test (*P < 0.05 toward Ctrl).
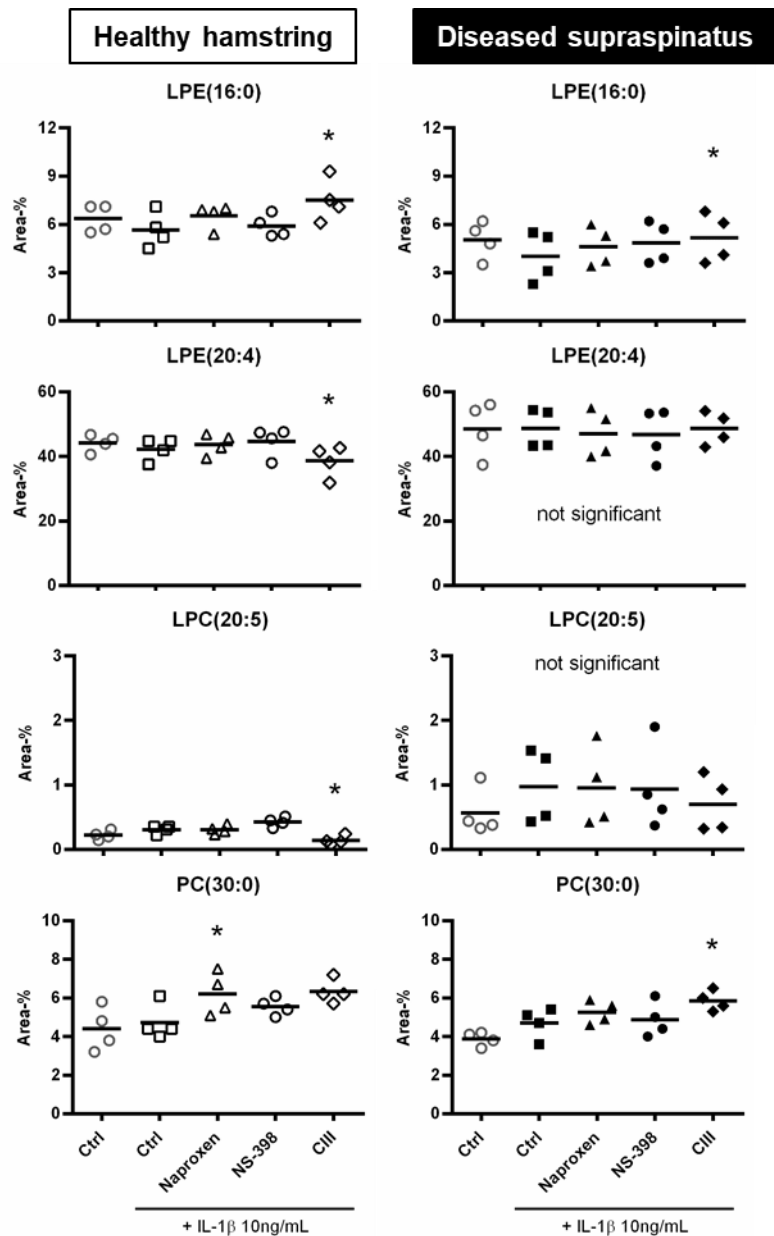
Figure 4. Effect of mPGES-1 or COX inhibitors on phospholipid profiles in tendon-derived cells. Cells were cultured and treated with medium or medium containing 10 ng/mL of IL-1β in presence or absence of 10 μM of naproxen, NS-398, or CIII for 24 hrs. Phospholipids were extracted from cell pellets and analyzed by LC-MS/MS. Data is presented as relative abundance (area-%) of phospholipid species within phospholipid classes. The bars show mean values. Statistical significance was calculated using repeated measures ANOVA followed by paired t-test with Bonferroni correction (*P < 0.05 towards IL-1β).

4.2 INHIBITION OF MPGES-1 IN CANCER

We have studied the difference in protein and lipid profiles upon inhibition of mPGES-1 or COX-2 in cancer cells *in vitro* (**Paper II**). CIII reduced PGE_2 production and increased $PGF_{2\alpha}$ and TXB_2 formation while NS-398 reduced the production of all three prostanoids. Our proteomics analysis suggested changes in individual proteins that we used to translate into cellular functions. The pathway analysis predicted increased cell death with CIII and decreased cell death with NS-398. Our lipidomics data supported this effect, where CIII increased two sphingolipids (sphinganine and dihydroceramide-C16:0) that are implicated in apoptosis. We tested our hypothesis on cell death in a live cell monitoring system but there was no difference in cell death in general or apoptosis in specific between the two inhibitors. We then investigated the effect on cell proliferation and if the inhibitors would alter the cytotoxicity of cytostatic drugs. Our results showed that CIII decreased the proliferation and potentiated the effect of cisplatin, etoposide, and vincristine, resulting in enhanced anti-proliferative effect and increased cell death.

We show that inhibition of mPGES-1 reduced tumor growth in two preclinical models of neuroblastoma (**Paper III**). The xenograft model represents injection of SK-N-AS cells, which are derived from human high-risk neuroblastoma, into the flank of mice with deficient immune system. The transgenic model spontaneously develops tumors that resembles human high-risk neuroblastoma. The expression of mPGES-1 is exclusive for stromal cells (fibroblasts, positive for platelet-derived growth factor receptor β) in both the xenograft and the transgenic model. Daily treatment with CIII (50-100 mg/kg) slowed down the growth of established tumors in the xenograft model. This yielded 40% smaller tumors (weight) after nine days of treatment. Early treatment with CIII (CIII-ET), *i.e.* receiving CIII at the day of inoculation with cancer cells, did not decrease the tumor weight further. Daily treatment with CIII (50 mg/kg) for ten days resulted in 50% smaller tumors (weight) in the transgenic model. Moreover, we showed that treated transgenic mice had a shift in macrophage polarization towards anti-tumor M1 phenotype in the tumors. We also observed decreased tumor angiogenesis and decreased infiltration of cancer-associated fibroblasts with CIII treatment in the transgenic model.

The PGE_2 concentration was not reduced in the treated tumors from the xenograft mice, sacrificed 24 hrs after the last injection of CIII (mean ± SD pmol/mg extracted tumor; Ctrl: 0.021 ± 0.019, n=13; CIII-ET: 0.026 ± 0.029, n=8; and CIII: 0.024 ± 0.018, n=11). We therefore aimed to investigate the presence of CIII in tumor and plasma. An LC-MS/MS method was established and the extraction performance was evaluated based on extraction efficiency and matrix effect in mouse plasma. Our method enabled us to quantify CIII and PGE_2 simultaneously. We performed a kinetics experiment where xenograft mice were sacrificed 2, 4, and 6 hrs post-injection of CIII (50 mg/kg *i.p.*). The concentration of CIII was highest at 2 hrs post-injection in both plasma and tumors. This coincide with an 80% reduction in PGE_2 concentration in the tumor. Other prostanoids were not detected in tumor tissues. We also quantified CIII and prostanoids in brains and spleen from the same animals (**Figure 5** and **Figure 6**). CIII followed the same profile in the brain as in plasma and tumors, whereas CIII seemed to accumulate in the spleen. PGE_2 or other prostanoids were mainly unaffected in

brains or spleens following this single injection. This experiment confirmed distribution of CIII with decreased PGE$_2$ concentration in the tumors at 2 hrs post-injection.
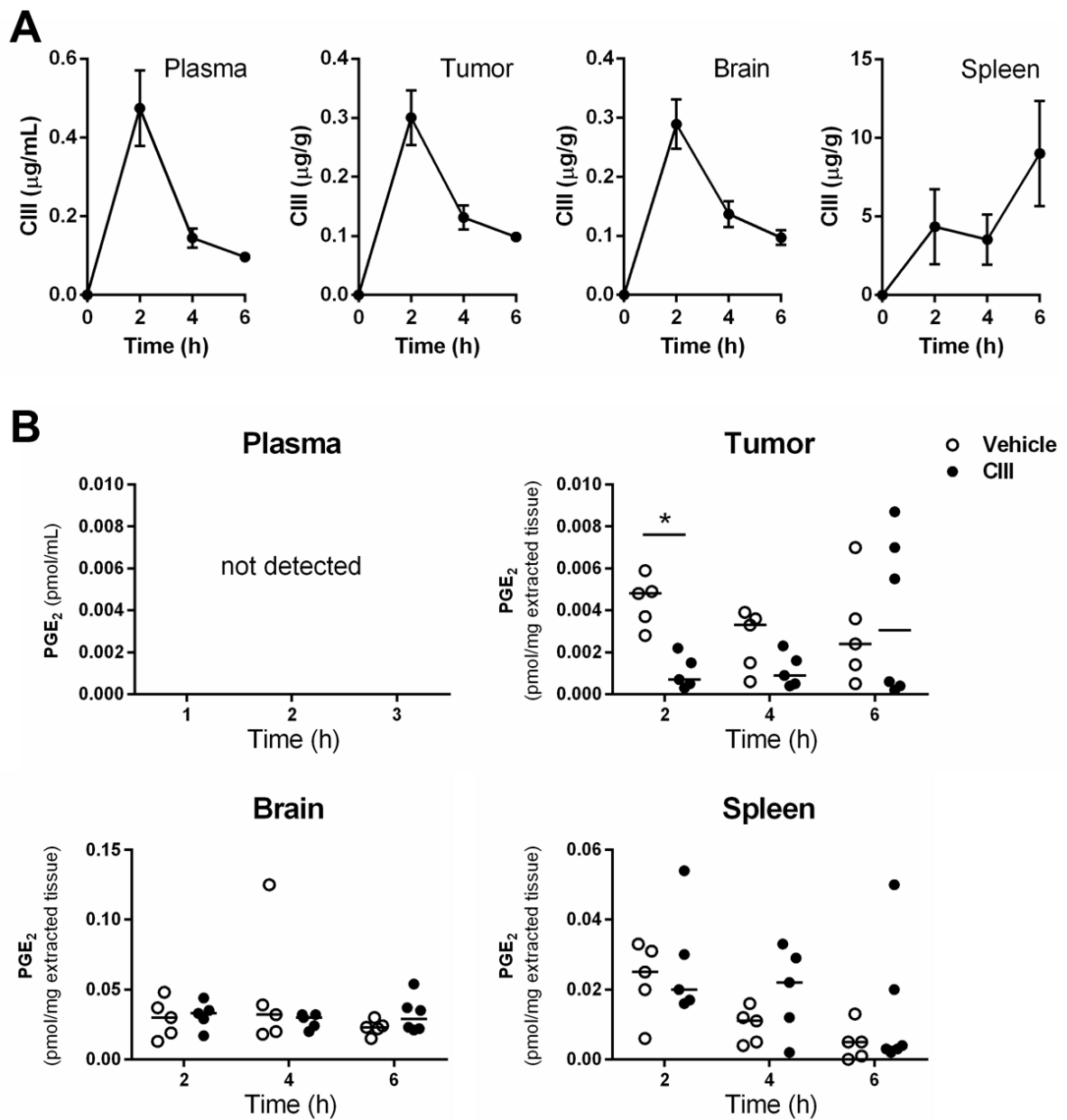

Figure 5. Quantification of CIII and PGE$_2$ in plasma, tumor, brain, and spleen from neuroblastoma tumor-bearing xenograft mice. Animals were sacrificed 2, 4, or 6 hrs post-injection of CIII (50 mg/kg, *i.p.*). Tissues were extracted and analytes were quantified by LC-MS/MS. **A**, Data is presented as mean ± SEM, n=5-6 per time-point. **B**, The bars show median values. Statistical significance was calculated using Mann-Whitney U test (*P < 0.05).
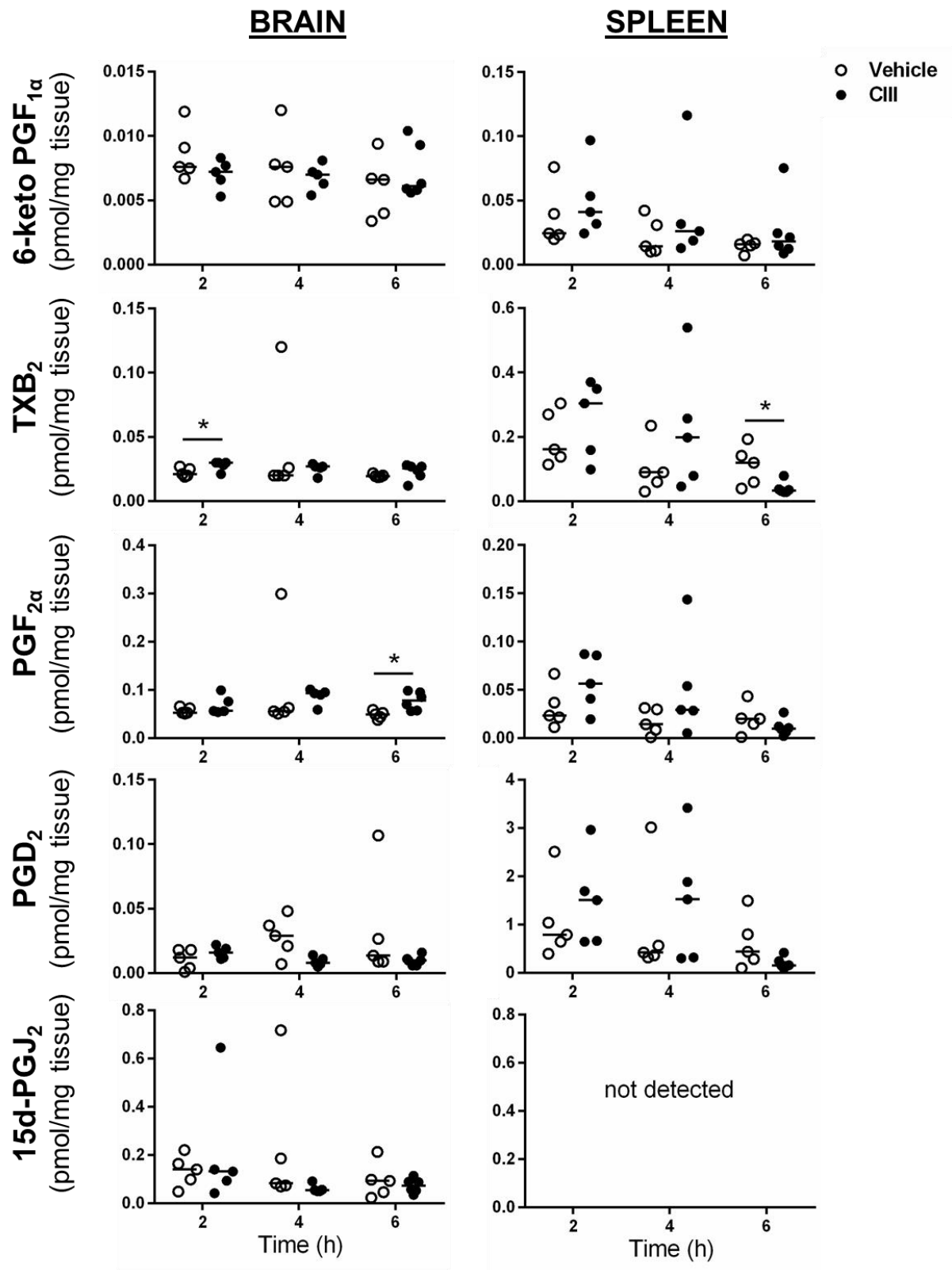
Figure 6. Quantification of prostanoids in brain and spleen from neuroblastoma tumor-bearing xenograft mice. Animals were sacrificed 2, 4, or 6 hrs post-injection of CIII (50 mg/kg, *i.p.*). Tissues were extracted and prostanoids were quantified by LC-MS/MS. The bars show median values. Statistical significance was calculated using Mann-Whitney U test (*P < 0.05).

4.3 CHARACTERIZATION OF NEW MPGES-1 INHIBITORS

We have demonstrated efficacy of five new mPGES-1 inhibitors in multiple models *in vitro*, *in vivo*, and *ex vivo* (**Paper IV**). The compounds 934, 117, 118, 322, and 323 selectively inhibited mPGES-1 in an activity assay against recombinant human and rat mPGES-1. The compounds reduced PGE_2 production in A549 cells, human whole blood, and in an air pouch model in mice. In a paw edema model in rats, the compounds decreased swelling already at 1 mg/kg. Lastly, the compounds 934 and 118 reduced norepinephrine induced vasoconstriction in human small arteries.

The whole blood assay was developed to screen for changes in multiple eicosanoids. Each eicosanoid and corresponding deuterated variant were individually optimized for in the LC-MS/MS analysis. A dilution curve containing 6-keto $PGF_{1\alpha}$-d4, PGE_2-d4, PGD_2-d4, $PGF_{2\alpha}$-d4, TXB_2-d4, 15d-PGJ_2-d4, LTB_4-d4, LTC_4-d5, LTD_4-d5, 5-HETE-d8, 12-HETE-d8, 15-HETE-d8, and undeuterated variants of 13-HODE, RvD1, RvD2, 17-hydroxy DHA, and protectin DX was spiked into 100 µL plasma at different stages throughout the extraction. A dilution curve was spiked in water at the same step. The dilution curve ranged from 0.006-1.5 pmol injected on the column in the LC-MS/MS analysis. This enabled us to investigate the lower LOQ, recovery efficacy, and matrix effect for each eicosanoid. The lower LOQ injected on column was considered as great (0.02-0.05 pmol), good (0.1-0.2 pmol), or poor (0.4-1.5 pmol). Eicosanoids with great LOQ were PGE_2, $PGF_{2\alpha}$, TXB_2, RvD1, RvD2, LTB_4, protectin DX, and 13-HODE; good LOQ were 6-keto $PGF_{1\alpha}$, PGD_2, 5-HETE, 15-HETE, and LTD_4; poor LOQ were 15d-PGJ_2, 12-HETE, 17-hydroxy DHA, and LTC_4. The extraction recovery rates were 33-125%. The response in plasma compared to 20% MeCN were 52-116% (due to matrix effects). The estimated lower LOQ in 100 µL plasma was around 1000 pg/mL for the best performing eicosanoids including PGE_2, TXB_2, $PGF_{2\alpha}$, RvD1, RvD2, and protectin DX. We conclude that the method gave similar quantitative performance in plasma for many eicosanoids.

LPS increased PGE_2 and TXB_2 production in human whole blood (**Figure 7**). All other eicosanoids were below detection limit. We chose 10 µg/mL of LPS as our final concentration. The prostanoid production was blocked with diclofenac (10 µM). High concentration of DMSO (0.1%) slightly decreased PGE_2 production. The intra-assay coefficient of variation (CV, n = 20 technical replicates) was 12% and 11% for PGE_2 and TXB_2, respectively. The inter-assay CV for control material (CV, mean, n = 3 donors) was 20% for PGE_2 and 30% for TXB_2. This was performed on blood that was drawn, incubated, extracted, and analyzed at separate occasions. The suppression in signal due to matrix effects and/or recovery efficiency varied between donors and experiments, ranging from 10-70% suppression compared to signal in extracted blank (mean $\pm$ SD, n=6 donors, PGE_2: 45$\pm$25%, TXB_2: 40$\pm$20%). This assay was used to determine IC_{50} values of the new mPGES-1 inhibitors in human whole blood.
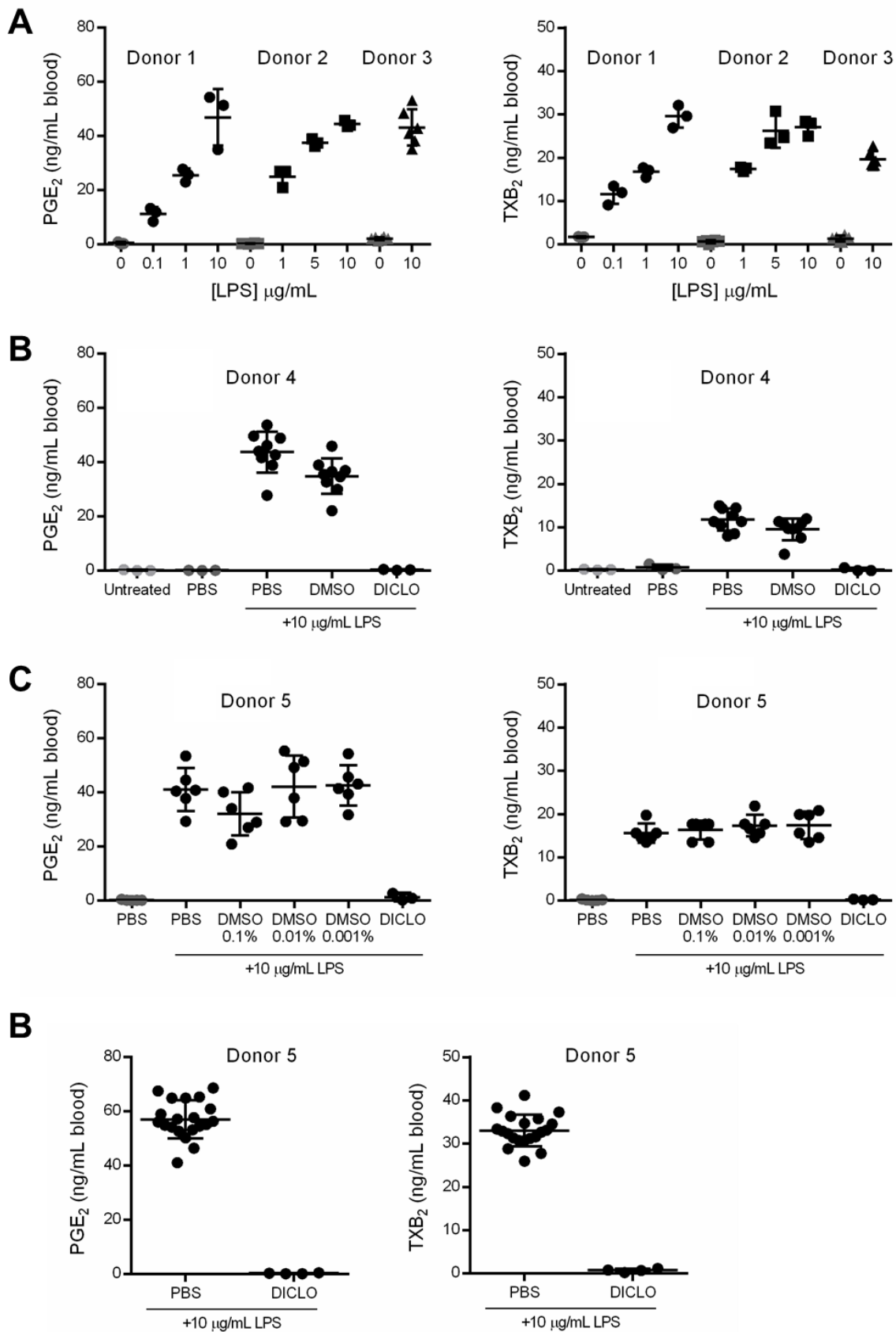
Figure 7. Set up of human whole blood assay. Heparin blood was incubated for 24 hrs with 10 µg/mL of LPS (if not stated otherwise) to induce PGE$_2$ and TXB$_2$ production, as measured by LC-MS/MS. **A**, LPS induced PGE$_2$ and TXB$_2$ production. **B**, Diclofenac (10 µM) blocked induced prostanoid production. **B** and **C**, High DMSO concentration affected PGE$_2$ production. **D**, Inter-assay CV was 12% and 11% for PGE$_2$ and TXB$_2$, respectively. DICLO, diclofenac.

52

5 DISCUSSION AND FUTURE PERSPECTIVES

Preclinical work has demonstrated that inhibition of mPGES-1 has many clinical possibilities. Then again, the drug development of mPGES-1 inhibitors have been halted due to absence of potent inhibitors lacking interspecies differences, fear of severe adverse effects when targeting the COX pathway, and no clear strategy which diseases to primarily target. This thesis aimed to address some of these issues, and this is reflected in the diversity of the papers included.

Inhibition of mPGES-1 in inflammation: prostacyclin and cardiovascular safety

Tendinopathy belongs to the diseases of musculoskeletal soft tissues, which are a major cause of pain and impaired physical function (350). Inflammation was long considered to not play a role in tendinopathy, mainly because there was no infiltration of neutrophils or macrophages at the site of injury. This was attributed by the relatively late presentation to the clinics, and it is now evident that inflammatory processes contribute to the onset and the progression of tendon disease (351, 352). However, the precise mechanisms are poorly understood.

Based on previous literature that describes prostacyclin as a mediator of pain and edema (37, 98-102), our initial results suggested that prostacyclin contributes to these responses in tendon disease (**Paper I**). We therefore hypothesized that the expression of *PTGIS* would be lower in patients that had resolved pain post-surgical treatment compared to those that were still in pain. Since our results showed the opposite, we rejected the hypothesis that *PTGIS* expression (= prostacyclin production) correlated with pain in tendon disease. Instead, based on prostacyclin as a vasodilator and anti-platelet activator, we speculate that prostacyclin may be a defensive response to enable vascularization and limit thrombosis at site of tendon injury. For example, PGIS and not mPGES-1 is increased upon hypoxia during inflammation in vascular smooth muscle cells and endothelial cells as a potential protective response (353). We acknowledge that there is a limitation in measuring the mRNA and not the actual PGIS protein abundance or prostacyclin concentration in this setting. However, these tendon biopsies were very small (especially from patients that had resolved pain) and only qPCR analysis was estimated sensitive enough.

NSAIDs use is heavily debated due to the many adverse effects and these drugs are regarded as "resolution toxic" (23), given the inhibition of SPM biosynthesis required in the resolution of inflammation. NSAIDs are specifically controversial in tendon disease as they affect tendon mechanical properties negatively and impair tendon healing (354, 355). This could be due to inhibition of SPMs and/or potentially protective prostacyclin production. Moreover, diseased tendon tissue expressed mPGES-1, although the functional role of PGE_2 in tendon biology is inconclusive. Studies have showed increased concentration of PGE_2 in connective tissue after mechanical load exercise in healthy humans (356) and in *in vitro* models of fibroblast cultures (357, 358). The use of aspirin or indomethacin blocked baseline and induced PGE_2 production, which decreased the otherwise increase in blood flow in connective tissue of human subjects following exercise (356). In the same study, celecoxib blocked only the induced production of PGE_2. This suggests that an initial burst of PGE_2 is important to initiate inflammation

(increased blood flow) and subsequent resolution. Repeated injection of PGE_2 was found to improve mechanical properties of tendons in rats (359). However, similar injections are reported to cause degenerative processes in rabbits (360). Given the massive amount of literature that describe COX/mPGES-1/PGE_2 as a key pathway in preclinical models of inflammation or in patients with rheumatic diseases (5, 53-56, 98, 238, 240-243, 295, 296, 310, 361-363), we speculate that inflammation (edema and pain) in tendon disease is at least partly driven by the excessive production of PGE_2. Inhibition of mPGES-1 would then be superior to NSAIDs, as this reduces the production of seemingly detrimental PGE_2, increases the production of potentially protective prostacyclin, and spare the inhibition of COX for generation of SPMs.

If mPGES-1 inhibitor would be analgesic in tendon disease is difficult to predict, mainly because pain can come in many flavors. While mPGES-1 deficient mice display less pain in some models but not all (237, 238, 240, 263), Sugita *et al.* reported that simultaneous targeting of prostacyclin and PGE_2 signaling or production was needed for analgesic effect in pain models in mice, since celecoxib exhibited this effect while mPGES-1 inhibition or IP receptor antagonist alone did not (364). Inhibitors of mPGES-1 typically demonstrate analgesic effect in preclinical models of inflammatory pain (295, 296, 362, 363). The significant difference in disease characteristics between human tendinopathy and experimental tendinopathy in animal models (365) makes it difficult to test mPGES-1 inhibition in a preclinical and functional setting in tendon disease.

Inhibition of mPGES-1 is likely to be as efficient as inhibition of COX in terms of reducing PGE_2 production, where the selective inhibition of mPGES-1 will spare the production of other prostanoids. As described earlier, this is important from the cardiovascular point of view. We showed that diseased tendon-derived cells treated with CIII produced more prostacyclin, in line with that CIII increased prostacyclin formation in mouse peritoneal macrophages (262). Increased prostacyclin formation has been reported in mPGES-1 deficient mice as measured in urine (60, 61, 205, 366), and multiple studies support that genetic deletion of mPGES-1 is safer compared to deletion or inhibition of COX-2 in terms of hypertension and evoked thrombosis (205, 239, 367). The systemic shift from PGE_2 to prostacyclin, as measured by urinary PGEM and PGIM concentrations, has been confirmed with an mPGES-1 inhibitor in man (368). However, recent work suggest that urinary PGIM could be derived from kidney prostacyclin without contribution from systemic prostacyclin production (369), which then places a question mark on the urinary PGIM levels as a reflection of endothelial derived prostacyclin biosynthesis in the cardiovascular system. On the other hand, it was recently demonstrated in an *ex vivo* model of human vascular tone that CIII decreased norepinephrine-induced vasoconstriction via prostacyclin formation (370), and we conclude that CIII and our new mPGES-1 inhibitors replicated this effect in human vessels (**Paper IV**). Recent mechanistic data in mice support that mPGES-1 drives vascular remodeling, stiffness, and endothelial dysfunction in hypertension via oxidative stress (371). Inhibition of mPGES-1 is therefore suggested cardioprotective, where the potential shunting towards prostacyclin *in vivo* needs to be further evaluated.

It would be interesting to investigate the clinical significance of an mPGES-1 inhibitor in tendon disease, especially since NSAIDs are associated with decreased tendon healing. As mentioned above, animal models do not fully replicate human tendon disease. Our *in vitro* experiments could be expanded to investigate the effect of mPGES-1 or COX inhibition on for example markers of fibroblast activation, in line with recent work showing that two species of SPMs altered inflammatory signatures in stromal cells from Achilles tendons (372).

For the cardiovascular safety, we plan to investigate the protective mechanisms in human arteries further. Apart from demonstrating actual increase in prostacyclin formation upon mPGES-1 inhibition in this setting, it is important to demonstrate that the use of an IP receptor antagonist renders the effect on vasoconstriction. It would therefore be essential to investigate expression patterns of IP and EP receptors in these vessels. Additionally, experiments where vessels are treated with different concentration (or ratios) of PGE_2 and prostacyclin analogs are suggested to elucidate the direct actions of these PGs in different types of vessels.

Inhibition of mPGES-1 as adjuvant therapy in cancer

Multiple studies have demonstrated that genetic deletion of mPGES-1 results in slower growing tumors *in vivo*, but few studies have been reported using pharmacological inhibition of mPGES-1 in cancer models. Our proteomic and lipidomic data suggested that mPGES-1 inhibitor CIII promoted a pro-cell death state *in vitro* (**Paper II**). Although CIII alone did not induce apoptosis in specific or cell death in general in A549 cells, CIII decreased proliferation rate and increased the cytotoxicity of cytostatic drugs. In other words, CIII potentiated cell death at 10 µM of cisplatin, 10 µM of etoposide, or 0.01 µM of vincristine. This means that a lower dose of cytostatic drugs could be used to achieve the same efficacy in cell death, and this may have important implications on mPGES-1 inhibitors as adjuvant therapy in cancer. Decreasing the cytostatic drug dose without compromising its efficacy translate into potentially less adverse effects from cancer therapy in patients.

Daily treatment with CIII reduced neuroblastoma tumor growth *in vivo* (**Paper III**). Our *in vivo* results fit well into the literature on mPGES-1 and cancer. Especially the reduction in tumor angiogenesis has been demonstrated in mPGES-1 KO mice (250-253) and in mice treated with an mPGES-1 inhibitor (302). The shift in macrophage polarization from M2 to M1 in the tumor microenvironment has been reported in a transgenic breast cancer model using mice lacking mPGES-1 (256). Despite that our *in vivo* study focused on neuroblastoma, we believe that mPGES-1 inhibition is a beneficial therapeutic strategy for multiple cancer. This is supported by the presence of COX/mPGES-1/PGE_2 pathway in multiple forms of cancer (41-48) and the anti-cancer effects observed in mice lacking mPGES-1 (48, 52, 103, 250-256). However, NSAIDs or mPGES-1 inhibitors alone are not resulting in complete halt in tumor growth. We acknowledge that mPGES-1 inhibitors are not intended as single treatment, much like the current use of NSAIDs treatment of cancer in clinical practice. Instead, the selective reduction in oncogenic and immunoregulatory PGE_2 is likely to replicate observations with COX inhibition or deletion in cancer models and at the same time spare the many adverse effects (25, 342).

Resistance to celecoxib has been reported for colon adenomas patients, and this correlated with low expression of tumor 15-PGDH (373). The authors demonstrated that tumor-bearing 15-PGDH KO mice were resistant to celecoxib treatment. 15-PGDH expression is repressed in some of the most common types of cancer including colon (374, 375), gastric (376), lung (377), and breast (378). Targeted overexpression of 15-PGDH in tumor tissue slowed down tumor growth via a shift in macrophage polarization from M2 to M1 and induction of T cell immune response in a mice model of colon cancer (379). These studies highlight that both the machinery for anabolism and catabolism of PGE_2 are important for therapeutic use of NSAIDs and likely mPGES-1 inhibitors. The issue with potential resistance to mPGES-1 inhibition when the expression of 15-PGDH is low may be circumvented via the potential redirection from PGE_2 production towards PGD_2 and 15d-PGJ_2 formation in macrophages (236), where 15d-PGJ_2 treatment was recently shown to increase the expression of 15-PGDH in breast cancer cells *in vitro* (380).

Meta-analysis showed that intake of NSAIDs in cancer pre- or post-diagnosis decreased metastasis (381), in line with results from *in vivo* models using mPGES-1 KO mice (250, 252). This suggest that mPGES-1 inhibitors may not only control primary tumor growth but also metastasis formation. NSAIDs potentiate chemotherapy *in vitro* (382) and immunotherapy *in vivo* (52, 383). Celecoxib can enhance the response to conventional treatment or even overcome treatment resistance in cancer (384-388). Specifically, celecoxib enhanced potency of chemotherapy, such as vincristine and doxorubicin, in neuroblastoma *in vitro* and *in vivo* (389). Recent data using RNAi towards mPGES-1 or inhibition of EP4 signaling sensitized oxaliplatin-resistant HT29 cells to oxaliplatin (390). Knock-down or inhibition of mPGES-1 with MF63 increased the efficacy of EGFR inhibitor erlotinib in prostate cancer cells *in vitro* and *in vivo* (391). Kim *et al.* recently showed that high expression of mPGES-1 in stage III melanoma tissue correlated with low infiltration of CD8^{pos} T cells and shorter survival (392). They demonstrated that deletion of mPGES-1 in a syngeneic melanoma mouse model increased the infiltration of CD8^{pos} T cells, slowed down tumor growth, and enhanced the efficacy of anti-PD1 treatment. Inhibition of mPGES-1 is therefore likely to have anti-carcinogenic effects via several mechanisms including decreased cancer cell proliferation, decreased tumor angiogenesis, promotion of anti-cancer macrophage polarization, re-activation of cytotoxic T cells, and increased effect of conventional therapy.

The next step is to further investigate the synergistic or additive effects of mPGES-1 inhibition with cytostatic drugs, targeted therapy, or immunotherapy *in vitro* and *in vivo*. These experiments can be performed with the new mPGES-1 inhibitors and additional COX inhibitors in parallel, in order to both validate the effect of CIII and to distinguish therapeutic opportunities between targeting mPGES-1 over COX enzymes in cancer.

Translating lipidomics data

Multiple differences in phospholipids were found upon IL-1β treatment and/or inhibition of mPGES-1 or COX enzymes (**Paper I** and **Paper II**). Despite studies on the associations of distinct phospholipid species with for example lung cancer tissue compared to healthy tissue (393-395) or altered membrane phospholipids in macrophages based on polarization phenotype *in vitro* (396), lipidomics is an underdeveloped field of research compared to other omics. The amount of publications on PubMed (www.ncbi.nlm.nih.gov, accessed on 2019-03-18) containing the word genomics, proteomics, metabolomics, or lipidomics during last year (2018) were 20922, 7415, 4307, and 574, respectively. Lipidomics is most often viewed as a subgroup to metabolomics, and lipids as biomolecules have been extensively studied for a long time with an ongoing effort in standardizing the analysis and the reporting of lipidomics data (Lipid Maps, www.lipidmaps.com). However, there is a lack of knowledge regarding the biological significance of distinct lipid species and lipid perturbation. This is a key problem, nicely described by Anthony D. Postle: "One conceptual problem about phospholipids is that, with a few notable exceptions, it is very difficult to claim a unique function for any individual molecular species, defined by a combination of headgroup and fatty acyl chains esterified the sn-1 and sn-2 positions of the glycerol backbone" (397). This is in contrast to assigning functions to a specific gene or protein. Generating relevant lipidomics data is demanding but it is not the bottleneck. Instrumentation, methods, and appropriate samples are in place. The challenge is to interpret and translate the phospholipid data into biological relevance.

The ambition would be to create a library of phospholipids and their relative changes in different cellular responses. A starting point is to use cell lines (*e.g.* A549 cells) to first characterize the basal lipidome and perturbations induced by different standard stimuli, such as LPS, cytokines, staurosporine, or rapamycin. It would then be possible to assign a stimulus (or cell state) to a certain phospholipid profile. This would generate a library of fingerprints. A new stimulus, such as an inhibitor, can then be added to the cells and any change in phospholipid profile can be compared to the library to see if the response is towards any of the standard stimuli. The library can be expanded with multiple cell lines and complemented with data from fatty acid analysis, secreted factors (prostanoids, cytokines), and proteomics. Even if it would still be difficult to determine if changes in specific phospholipids are a mere consequence of the cell state or actual contribution to the cell state, this knowledge would increase the biological association of phospholipid species.

Development of mPGES-1 inhibitors

Several mPGES-1 inhibitors have been reported in recent years, including many patents (398). Despite the many advances, there is a lack of selective mPGES-1 inhibitors with high potency and good bioavailability in rats or mice to facilitate preclinical studies.

The main breakthrough since 2015 was the publication of the first Phase I trial on an mPGES-1 inhibitor (368). As mentioned earlier, the Eli Lilly compound LY3023703 decreased urinary PGEM and increased urinary PGIM and 11-dehydro TXB_2, and there was no increase in TXB_2 formation in serum. LY3023703 dose-dependently inhibited PGE_2 production in human whole

blood *ex vivo*. Regardless of the promising results, one subject developed drug-induced liver injury as determined by elevated levels of serum alanine aminotransferase levels upon taking 30 mg/day of LY3023703 for 28 days. A follow-up compound, LY3031207, was tested in a Phase I trial but the study was terminated when several subjects developed drug-induced liver injury (399). The authors concluded that this is unlikely an mPGES-1/PGE$_2$ driven adverse effect but rather caused by the conversion of the inhibitors into toxic metabolite(s) (400).

Eli Lilly disclosed a SAR study in 2016 wherein they highlighted two compounds for clinical investigations (401). Compound 8 inhibited recombinant mPGES-1 with an IC$_{50}$ of 1 nM and PGE$_2$ production in human whole blood with an IC$_{50}$ of 12 nM, but it displayed poor solubility. The follow-up compound 26 was less potent in recombinant assay and human whole blood but showed good pharmacokinetics profile in dogs. Eli Lilly described additional inhibitors including compound 14 (402) and compound 16 (403). Both were reported as highly selective and potent, where compound 16 displayed an IC$_{50}$ of 1 nM in recombinant assay and 6 nM in whole blood assay. In pharmacokinetics studies in rats and dogs, compound 16 was found in plasma above the IC$_{80}$ value measured in human whole blood assay (24 nM) for up to 24 hrs following a single oral dose.

Sugita *et al.* reported that Compound A blocked PGE$_2$ production with an IC$_{50}$ of 13 nM in A549 cells, and that this caused an increase in other prostanoids (361). Compound A decreased PGE$_2$ biosynthesis in rat peritoneal macrophages (IC$_{50}$ of 1.9 µM) that coincide with an increase in PGF$_{2\alpha}$ formation. Moreover, Compound A was anti-pyretic in LPS-induced pyrexia model and anti-inflammatory in adjuvant-induced arthritis models in rats (30 mg/kg oral gavage, *p.o.*). Glenmark Pharmaceutical Ltd. recently disclosed two SAR studies. Inhibitor 17d displayed potency in human whole blood and showed analgesic effect in guinea pigs (362). The company presented several highly selective mPGES-1 inhibitors with good potency against recombinant enzyme (IC$_{50}$ down to 4 nM) but the inhibitors displayed loss in potency in A549 cells (IC$_{50}$ >250 nM, at 2% serum) (363). The best performing inhibitor was Compound 9, which displayed poor performance *in vivo*.

A few new dual mPGES-1 and 5-LO inhibitors have been disclosed since 2015. Svouraki *et al.* presented a SAR study on natural-derived compounds with low potency towards recombinant enzymes (IC$_{50}$ values of 1-5 µM), although the most potent compound slightly decreased inflammatory cell infiltration in zymosan induced peritonitis (10 mg/kg *i.p.*) (404). Moreover, Compound 47 caused a shift in eicosanoid production from prostanoids and leukotrienes to SPMs in human macrophages (405). This inhibitor displayed activity *in vivo* with decreased production of PGE$_2$ in peritonitis and air pouch models in mice.

Multiple computational studies to facilitate the development of mPGES-1 inhibitors have been performed recently. For example, molecular docking was used to identify lead structures from natural compounds (406) and to develop Compound 8n that inhibited PGE$_2$ production in RAW 264.7 cells with IC$_{50}$ of 4.5 nM (407). Gupta *et al.* performed comprehensive molecular docking and SAR studies with 127 compounds extracted from the literature up until 2015 (408, 409), highlighting amino acids in human mPGES-1 that are shared between

inhibitors. Zhao *et al.* used virtual screening to identify new compounds that they tested against recombinant mPGES-1 with IC_{50} of >250 nM (410). Di Micco *et al.* used molecular docking and SAR studies to identify new scaffolds that inhibited recombinant human mPGES-1 down to IC_{50} of 180 nM (411). Last year, Ding *et al.* reported the compound 4b with potency in both human and murine models (412). Compound 4b inhibited recombinant mPGES-1 with IC_{50} of 33 nM (human) and 157 nM (mouse), and this inhibitor was reported to decrease PGE_2 production in an air pouch model in mice (>5 mg/kg *p.o*). High dose (up to 1000 mg/kg *p.o.*) did not cause ulcers whereas celecoxib (50 mg/kg *p.o.*) did. These studies expand knowledge of existing inhibitors and speed up the development of future inhibitors.

Our newly characterized mPGES-1 inhibitors are highly selective and potent in recombinant assay and intact A549 cell assay. However, the inhibitors showed loss in potency in human whole blood. This is similar to many other mPGES-1 inhibitors (**Table 2**). Our new inhibitors showed good efficacy in *in vivo* models, which is similar to Compound A and Compound 4n (**Table 4**). Of significance, our inhibitors reduced paw swelling already at 1 mg/kg *p.o.*. Collectively, five additional mPGES-1 inhibitors are described that opens up for opportunities to study mPGES-1 inhibition in inflammation and cancer in preclinical settings.

Inhibitor (year)	Species activity	Recombinant mPGES-1 (IC$_{50}$)	Human whole blood (IC$_{50}$)	Results from *in vivo* models	Ref.
CIII (2013)	Human, murine	0.09 µM (human), 0.9 µM (rat)	~10 µM	Decreased PGE$_2$ and cells (air pouch, rat)	(262)
LY3023703 (2016)	Human	Not disclosed	*"17-fold more potent than celecoxib"*	Analgesic (guinea pig, not disclosed)	(368)
Compound 8 (2016)	Human	0.001 µM (human), 34 µM (rat)	0.012 µM	Bioavailability of 37% (dog)	(401)
Compound 26 (2016)	Human	0.241 µM (human), >1000 µM (rat)	0.744 µM	Bioavailability of 60% (dog)	
Compound A (2016)	Human, murine	0.013 µM (human), 0.002 µM (rat)#	Not tested	Anti-pyretic, anti-inflammatory (rat)	(361)
17d (2016)	Human	0.008 µM (human), 0.011 µM (guinea pig)	0.250 µM	Analgesic at >30 mg/kg (guinea pig)	(362)
Compound 9 (2017)	Human	0.057 µM (human)	Not tested.	Analgesic at 200 mg/kg (guinea pig) Bioavailability of 33% (rat)	(363)
16 (2017)	Human	0.0001 µM	0.006 µM	Bioavailability of 60% (dog)	(403)
Compound 4b (2018)	Human, murine	0.033 µM (human), 0.157 µM (mouse)	Not tested.	Decreased PGE$_2$ (air pouch, mouse), No GI toxicity (mouse)	(412)
934, 117, 118, 322, and **323** (2019)	Human, murine	0.010-0.029 µM (human), 0-067-0.250 µM (mouse)	3.3-8.7 µM	Decreased PGE$_2$ (air pouch, mouse), Reduced paw swelling (rat), Bioavailability of 10-46% (rat)	**Paper IV**

Table 4. Selective mPGES-1 inhibitors, as of March 2019. This list is an extension of Table 2. CIII is included as reference for best performing inhibitor that lacks interspecies differences, as of before this thesis was conducted. #Intact cell assay of human A549 cells or mouse peritoneal macrophages.

6 CONCLUSION

The key inflammatory enzyme mPGES-1 has been an anticipated therapeutic target ever since characterized 20 years ago. A few clinical trials have been performed but no mPGES-1 inhibitor has reached clinical practice. This doctoral thesis provides increased knowledge of mPGES-1 and its inhibition in models of inflammation and cancer. The results are important pieces in the large effort of bringing mPGES-1 inhibitors into clinical practice. This thesis showed that inhibition of mPGES-1 decreased PGE_2 biosynthesis and that this:

- increased the formation of potentially protective prostacyclin in an *in vitro* model of tendon disease,
- resulted in decreased proliferation and increased cytostatic-induced cell death in lung cancer cells *in vitro*,
- decreased tumor growth in two preclinical models of neuroblastoma *in vivo*, and
- reduced paw swelling in a preclinical model of edema *in vivo* and made human vessels less prone to contract *ex vivo*.

7 ACKNOWLEDGEMENTS

At the age of 18, I started studying chemistry at Stockholm University with the aim to one day defend my doctoral thesis. Nearly ten years later, I am close to achieving my goal.

Thank you to my main supervisor *Per-Johan Jakobsson* for your trust to let me design and perform my own experiments and projects, for always being open to new ideas, new collaborations, and discussing science – and for sending me to multiple international conferences. I feel fortunate to have worked in your research group.

Thank you to my co-supervisors: *Per Kogner* for our fruitful collaborations regarding neuroblastoma and medulloblastoma; *Marina Korotkova* for your guidance and valuable input in all the projects; and *Karin Larsson* for our interesting discussions and your support whenever I needed it.

Thank you to my high school teacher in chemistry and biology *Sara Swartling* for advising me to pursue research. Thank you to my friend and mentor *Magnus Wetterhall* for introducing me to mass spectrometry back in 2013 on GE Healthcare, all of our discussions over lunches, and for all your support. Thank you to my friend and colleague *Helena Idborg* for all the input on my work, our nice conversations about hardcore analytical chemistry and goofy observations, and your help in the lab, especially all the time spent troubleshooting our baby (our mass spec). Thank you to *Stephanie Dakin* for the many emails, nice discussions, and our joint effort on the tendon disease paper. Thank you to my friend and colleague *André Ortlieb Guerreiro-Cacais* for our many lunches with conversations about life and research.

Thank you to friends, colleagues, and collaborators all over Karolinska Institutet and Karolinska University Hospital, especially *Cátia Cerqueira*, *Anna Kock*, *Linda Ljungblad*, *Heidi Wähämaa*, and *Julia Steinmetz*. Keep doing great research together.

Family is key to life and I would be nothing without the support from my parents, my siblings, and my extended family – Thank you for everything!

And thank you to my nieces and nephew, who are too young to appreciate my doctoral thesis but you keep reminding me of what is truly important in life.

Filip Bergqvist

Stockholm
April 29, 2019

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