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**Characterization of the PGE₂ Pathway
in Arthritis and Inflammation:
mPGES-1 as a Therapeutic Target**

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To my family and a highly anticipated reunion

ABSTRACT

The inducible prostaglandin (PG) E₂ pathway is defined by the concerted activities of the enzymes cyclooxygenase (COX)-2 and microsomal prostaglandin E synthase (mPGES)-1 in producing PGE₂. PGE₂ has pro-inflammatory and immunomodulatory functions and is involved in an array of diseases with an autoimmune and chronic inflammatory component, including rheumatoid arthritis (RA). In RA, mPGES-1 and COX-2 are up-regulated in the inflamed synovium of patients. COX inhibitors like non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors (COXibs) relieve inflammation and pain in RA, but their respective GI tract and cardiovascular side effects preclude long-term use. Moreover, other effective RA therapies like TNF blockade and B-cell depletion therapy leave the inducible PGE₂ pathway unaffected, suggesting that targeting this pathway could have additional benefits in a combinatorial approach. mPGES-1 is currently investigated as a target that could dissociate the anti-inflammatory benefits of COX inhibitors from their detrimental side effects. However, most inhibitors of human mPGES-1 activity generated so far failed to inhibit the murine enzyme ortholog, which complicates their characterization *in vivo* in relevant disease models.

The first objective of this thesis was to extend the characterization of the PGE₂ pathway in arthritis. The second objective was to investigate mPGES-1 as a therapeutic target. The latter was accomplished in part through the development and evaluation of pharmacological inhibitors active on both human and murine mPGES-1.

To fulfill the first objective, we characterized a new mechanism for the induction of PGE₂ production in RA synovial fibroblasts (RASFs), whereby complexes of the alarmin high mobility group box protein-1 (HMGB1) and the cytokine IL-1 β cause an up-regulation of COX-2 and mPGES-1 expression. We also characterized 15-prostaglandin dehydrogenase (15-PGDH) to co-localize with mPGES-1 and the COX enzymes in the synovium of RA patients, suggesting a concerted activity of the anabolic and catabolic parts of the PGE₂ cascade in the joint. In the same patients, we determined that methotrexate therapy did not interfere with the expression of COXs, mPGES-1 or 15-PGDH, adding that therapy to the list of approaches leaving the PGE₂ pathway unaffected. Lastly, we could not confirm an association between the expression of COXs, mPGES-1 and 15-PGDH and quantitative pain assessment or arthritis development in arthralgic individuals at risk of developing arthritis or in early arthritis patients. In line with the second objective, we developed compounds II and III, which inhibit PGE₂ synthesis *in vitro* in different human and murine cell assays and *in vivo* in the air pouch model of acute inflammation. Compound II also reduced edema in the rat adjuvant-induced arthritis (AIA) model. When the PG profile elicited by mPGES-1 inhibition with compound III was compared to that resulting from mPGES-1 gene deletion in the air pouch model, different results were observed suggesting the two modes of inhibition might not have the exact same outcome. While inhibition of PGE₂ synthesis with both compound II and III did not result in the shunting of PGH₂ to other prostanoids, a shunt to thromboxane (TX) B₂ was observed in the mPGES-1 knockout mouse. We also used the mPGES-1 knockout mouse to investigate the impact of mPGES-1 gene deletion on the eicosanoid and fatty acid profiles in inflammation. We discovered that it resulted in macrophages producing more 15-

deoxy- $\Delta^{12,14}$ PGJ₂ and the spleen containing more eicosadienoic acid (EDA). This suggests mPGES-1 inhibition could not only inhibit the synthesis of the pro-inflammatory PGE₂, but also cause the up-regulation of anti-inflammatory pathways.

In conclusion, this thesis further advances the knowledge about the PGE₂ synthesis cascade in arthritis and describes two new mPGES-1 inhibitors with an *in vivo* activity in native rodent models of disease. The latter constitute new valuable tools for the study of mPGES-1 in whichever pathology it has an involvement.

LIST OF PUBLICATIONS

This thesis is based on the following studies, which will be referred to in the text by their corresponding roman numerals.

I. Expression of prostaglandin E₂ enzymes in the synovium of arthralgia patients at risk of developing rheumatoid arthritis and in early arthritis patients.

M.J.H. de Hair*, Patrick Leclerc*, E.C. Newsum, K.I. Majjer, M.G.H. van de Sande, T.H.Ramwadhoebe, D. van Schaardenburg⁴, L.G.M. van Baarsen, M. Korotkova, D.M. Gerlag, P.P. Tak, P.J. Jakobsson
Manuscript.

II. IL-1b/HMGB1 complexes promote the PGE₂ biosynthesis pathway in synovial fibroblasts.

Patrick Leclerc*, Heidi Wähämaa*, Helena Idborg, Per-Johan Jakobsson, Helena Erlandson Harris, Marina Korotkova
Scandinavian Journal of Immunology. 2013, March 12

III. Limited effect of anti-rheumatic treatment on 15-prostaglandin dehydrogenase in rheumatoid arthritis synovial tissue.

Karina R. Gheorghe, Syed Sadique, Patrick Leclerc, Helena Idborg, Ivonne Wobst, Anca I. Catrina, Per-Johan Jakobsson, Marina Korotkova
Arthritis Research and Therapy, 2012, 14:R121

IV. Characterization of a new mPGES-1 inhibitor in rat models of inflammation.

Patrick Leclerc, Sven-Christian Pawelzik, Helena Idborg, Linda Spahiu, Charlotte Larsson, Patrick Stenberg, Marina Korotkova, Per-Johan Jakobsson
Prostaglandins and other Lipid Mediators. 2013, March 23

V. Effects of pharmacological inhibition and genetic deletion of mPGES-1 in mouse models of inflammation.

Patrick Leclerc, Helena Idborg, Linda Spahiu, Charlotte Larsson, Natalia Nekhotiaeva, Johan Wannberg, Patric Stenberg, Marina Korotkova, Per-Johan Jakobsson.
Manuscript.

VI. Effects of mPGES-1 deletion on eicosanoid and fatty acid profiles in mice.

Helena Idborg, Petter Olsson, Patrick Leclerc, Joan Raouf, Per-Johan Jakobsson, Marina Korotkova
Manuscript.

* These authors contributed equally to the study.

ADDITIONAL PUBLICATION

The author also contributed to the following publication during his PhD education.

- **Anti-inflammatory cytokine profile in early human tendon repair.**
Paul W. Ackermann, Erica Domei-Arverud, Patrick Leclerc, Petra Amoudrouz,
Gustavo A. Nader
Knee Surgery, Sports Traumatology, Arthroscopy. September 2012

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LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
15-PGDH	15-prostaglandin dehydrogenase
AA	arachidonic acid
Ag	antigen
AIA	adjuvant-induced arthritis
cAMP	cyclic adenosine monophosphate
CIA	collagen-induced arthritis
cPGES	cytosolic prostaglandin E synthase
COX	cyclooxygenase
COXib	selective COX-2 inhibitor
CRC	colorectal cancer
DAMP	danger-associated molecular pattern
DC	dendritic cell
DMARD	disease-modifying anti-rheumatic drug
EAE	experimental autoimmune encephalomyelitis
EDA	eicosadienoic acid
EGFR	epidermal growth factor receptor
EPA	eicosapentaenoic acid
GPCR	G-protein coupled receptor
HMGB1	high mobility group box protein-1
HTS	high-throughput screening
IBD	inflammatory bowel disease
IHC	immunohistochemistry
IL	interleukin
LPS	lipopolysaccharide
mPGES	microsomal prostaglandin E synthase
MS	multiple sclerosis
MTX	methotrexate
MUFA	monounsaturated fatty acid
NSAID	non-steroidal anti-inflammatory drug
PG	prostaglandin
PGI ₂	prostacyclin
PPAR	peroxisome proliferator activated receptors
PRR	pattern recognition receptor
PUFA	polyunsaturated fatty acid
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RASF	RA synovial fibroblast
SAR	structure-activity relationship
SpA	spondyloarthritis
Th	T helper cell
TNF	tumour necrosis factor
TX	thromboxane
VEGF	vascular endothelial growth factor

THESIS AIMS

The work presented in this thesis focused on extending the characterization of the PGE₂ pathway in arthritis and on further validating mPGES-1 as a therapeutic target by developing pharmacological inhibitors of its enzymatic activity and by studying the mPGES-1 knockout mouse.

Specific aims:

- Paper I To investigate the involvement of the PGE₂ pathway in the pre-clinical and early clinical phases of arthritis.
- Paper II To characterize a new mechanism for the induction of PGE₂ production in rheumatoid arthritis synovial fibroblasts
- Paper III To characterize the expression of 15-PGDH in the RA synovium and study the impact of methotrexate treatment on the expression of the inducible PGE₂ pathway in RA.
- Paper IV To characterize a new pharmacological inhibitor of mPGES-1 *in vitro* and *in vivo* in rat models of inflammation
- Paper V To characterize a new pharmacological inhibitor of mPGES-1 and compare its effect to mPGES-1 gene deletion *in vivo*
- Paper VI To determine the impact of mPGES-1 gene deletion on the fatty acid and eicosanoid profiles of mice

1 INTRODUCTION TO INFLAMMATION AND THE IMMUNE SYSTEM

1.1 PHYSIOLOGICAL BARRIERS AND SURVEILLANCE

At first glance, the human body surface might be perceived as an armor with several openings, but every surface and every cavity of it is coated with a barrier, either physical or chemical, that protects the tissue beneath from the outside environment and helps it to remain under homeostasis. In the event of an injury or a barrier breach, the body is equipped with a danger-sensing mechanism, which is managed by immune sentinel cells populating most tissues. These cells are equipped with receptors that recognize danger-associated molecular patterns (DAMPs). DAMPs can be endogenous or exogenous in nature and one of them, HMGB1, will be the subject of section 2.1.1. Once induced, this mechanism causes the initiation of the inflammatory and immune reactions which, together, attempt to fight a given noxious condition to allow a return to homeostasis.

1.2 INFLAMMATION

Classically, inflammation has been described using the cardinal signs of inflammation: pain, heat, redness and swelling. Pain serves to alert the host of the abnormal state of an affected tissue. Heat, redness and swelling are caused by the increased blood flow and plasma leakage. When amplified, these cardinal signs inevitably lead to loss of function of the affected area.

A more practical definition to help conceptualize inflammation would be that it is a physiological response with the purpose to create a suitable environment for invading organisms to be fought and damaged tissue to be repaired. More specifically, inflammation coordinates the delivery of blood components (i.e. immune system cells and the tissue repair machinery) to a location requesting it. Both cells of the afflicted organ and cells of the immune system orchestrate the inflammatory reaction. Most importantly, they communicate with each other via a complex regulatory network of mediators. These mediators include cytokines, chemokines, lipid mediators, vasoactive peptides and amines, complement fragments and proteolytic enzymes [1]. The cross talk between cells involved, essentially mediates their differentiation and modulates their activities. It also contributes to the recruitment of additional effector cells of the immune system. An important family of mediators that is part of this network is the prostanoid family. Prostanoids modulate all the cardinal signs of inflammation and are presented in section 2.2 and in figure 1.

Under normal circumstances, inflammation is programmed to resolve when an injury has been repaired and/or a pathogen has been cleared by the immune system. However, in the event of a persistent infection or in cases of autoimmunity, the complex regulatory network of mediators previously mentioned can turn into a self-perpetuating loop that drives inflammation to chronicity. Chronic inflammation is a state of sustained increased metabolism. As such, it can promote neovascularization. The objective of the latter is to answer to the heightened metabolic demand as well as

to optimize the delivery of blood components to the affected tissue. Pathologies with an inflammatory component and in which the prostanoid prostaglandin (PG) E₂ is involved are discussed in section 3.

1.3 THE IMMUNE SYSTEM

The immune system is an effector mechanism that enables the body to maintain homeostasis by dealing with noxious agents and tissue injuries. It has two arms, innate and adaptive immunity, which rely on two completely different approaches to defend the host. While innate immunity relies on the recognition of evolutionarily conserved features of danger (DAMPs), adaptive immunity decomposes assailants and generates a response tailored to their constitution.

The recognition of DAMPs by tissue resident immune sentinel cells, like macrophages, induces innate immunity. Cytokines and chemokines such as interleukins (IL)-1 β , 6 and 8, tumor necrosis factor (TNF)- α and RANTES are released locally by activated tissue cells and immune sentinel cells and in turn recruit and activate neutrophils, monocytes and dendritic cells (DCs) from the blood circulation [1]. The latter are phagocytes; effector cells of innate immunity that engulf and dispose of microorganisms and noxious molecules through a process termed phagocytosis. Innate immunity achieves a rapid and efficient response through targeting evolutionarily conserved molecular patterns. However, it does not provide a tailored immunity should the noxious agent be encountered anew. The latter is a feature of the adaptive immunity.

Phagocytes of the innate immune system serve as a bridge between innate and adaptive immunity. DCs for example, digest phagocytosed material and migrate to secondary lymphoid organs to present it to T-cells in the context of a major histocompatibility complex molecule. When this presentation is supplemented with a co-stimulatory stimulus, it causes the activation and expansion of T-cells that recognize the molecule presented, also known as an antigen (Ag). In this function, DCs are called antigen-presenting cells. The activation of T-cells might lead to a humoral response in which B-cells will produce neutralizing, opsonizing and complement-activating antibodies or a cell-mediated immune response. The nature of the response that ensues is dependent on the noxious agent against which it is mounted and this is mostly dictated again by the regulatory network of mediators through which the cells interact. CD4 T-cells, also called T helper (T_h) cells, are lymphocytes shaping the adaptive immune response to fit the pathogen encountered. There are several functional subsets of T_h-cells, T_h17 is one that will be discussed later. When activated, T_h17 cells secrete cytokines that cause an amplification of inflammation through the activation tissue cells and the recruitment of more phagocytes.

There are billions of T-cells and B-cells each with different receptor specificities. This diversity is accomplished through the random rearrangement of lymphocyte receptor genes. The random nature of the process makes it theoretically possible and likely to generate lymphocytes that recognize self. However, lymphocytogenesis is a tightly regulated process and self-recognizing lymphocytes get eliminated through mechanisms

termed central and peripheral tolerance. Despite the existence of those mechanisms, lymphocytes recognizing self sometimes reach circulation and their activation can lead to autoimmunity.

The above description of a classical immune reaction depicts innate and adaptive immunity as a continuum, an analysis of the regulatory network of mediators through which they interact however, can make one realize that they in fact form a well-integrated defense system. The adaptive immune system can also feedback to the innate immune system by recruiting it, activating in or suppressing it in a manner that gives it an added selectivity [2]. The T_h17 subset mentioned earlier is one example. In the pathology of rheumatoid arthritis (RA) described in section 3.1, T_h17 cells recognizing an autoantigen in the joint are thought to exacerbate inflammation leading to joint destruction through the activation of local tissue fibroblasts [3]. The latter exemplifies the possible contribution of adaptive immunity to chronic inflammation in the case of autoimmunity.

2 MEDIATORS OF INFLAMMATION AND IMMUNITY

2.1 DAMPs

DAMPs can be seen as a set of evolutionarily conserved molecular patterns recognized by cells of the immune system as markers of injury or infection. They are recognized by cell surface receptors termed pattern recognition receptors (PRRs) and act as inducers of inflammation and the immune response. They are divided in two categories: pathogen-associated molecular patterns (PAMPs), which are of exogenous nature and signal the presence of bacteria and viruses and alarmins, which are endogenous molecules normally confined to the intracellular space where they have specific functions, but which can be released to the extracellular space in case of trauma or infection. Common examples of DAMPs are: lipopolysaccharides (LPS), foreign nucleic acids, heat shock proteins and high mobility group box protein-1 (HMGB1) [4]. The latter is more extensively presented below.

2.1.1 HMGB1

HMGB1 is a prototypical alarmin. In a healthy cellular environment, it can be found in cell nuclei, where it regulates gene transcription, chromatin regulation and DNA repair or in their cytoplasm, where it serves as a sensor of exogenous nucleic acid and mediates the anti-viral immune response [5, 6]. However, HMGB1 can also be found in the extracellular environment. It can either be passively released from dying cells or be actively secreted by innate immune cells including monocytes, macrophages and DCs in response to pro-inflammatory stimuli such as cytokines and PAMPs [7-9].

The characterized receptors via which HMGB1 mediates its alarmin functions include TLR2, 4 and 9 as well as the receptor for advanced glycation end products (RAGE). HMGB1 binding to these receptors results in the induction of inflammation and innate

immunity as described in the previous section, essentially through the activation of the NF- κ B pathway [4]. As such, HMGB1 released to the extracellular environment can be inactive, trigger the release of pro-inflammatory mediators or act as a chemotactic agent. Alternatively, HMGB1 can mediate inflammation through one additional mechanism: it can form complexes with endogenous and exogenous molecules to enhance signaling through their respective receptors. Reported molecules with which HMGB1 forms complexes include IL-1 α and β and LPS [10, 11]. This latter function is thought to serve in accelerating the initial response of innate immunity to danger. HMGB1 has been implicated in many pathological conditions with an inflammatory or immune component including RA and systemic lupus erythematosus [12-14].

In collagen-induced arthritis (CIA) and in a spontaneous arthritis model, HMGB1 blocking therapies have been shown to alleviate both inflammation and tissue destruction, indicating the potential importance of HMGB1 in arthritis pathogenesis [10, 15, 16].

Furthermore, in RA, HMGB1 is found elevated in the synovial fluid of patients and, *in vitro*, RA synovial fibroblasts (RASFs) can be induced to produce pro-inflammatory cytokines by complexes of HMGB1 and IL-1 β or LPS [10, 13]. Paper II describes the characterization of cytokines and lipid mediators released by RASFs stimulated with IL-1 β /HMGB1 complexes.

2.2 PROSTANOIDS

Phospholipids are commonly recognized as structural monomers forming the cell membrane. However, they also serve as a source of fatty acids, which, once metabolized by cells, become signaling molecules involved in an array of physiological processes. Eicosanoids are one family of signaling lipids predominantly involved in inflammation and immunity. They are issued from the metabolism of arachidonic acid. A major eicosanoid sub-family, the prostanoid family will be described in this section.

2.2.1 OVERVIEW OF THE PROSTANOID PATHWAY

The prostanoid sub-family includes PGE₂, PGD₂ and PGF_{2 α} , prostacyclin (PGI₂) and thromboxane (TX) A₂. By definition, they are formed from the metabolism of arachidonic acid (AA) by the cyclooxygenase (COX) enzymes. There are two COX isoforms found in mammals termed COX-1 [17-19] and COX-2 [20, 21]. Both enzymes are membrane-bound and localized at the endoplasmic reticulum and nuclear membranes [22, 23]. Their expression patterns and functions however, differ. COX-1 is a protein ubiquitously expressed throughout all tissues of the body and, as its promoter suggests, mostly serves housekeeping functions. COX-2 on the other hand, is an inducible protein regulated by a promoter containing typical features of the immediate early gene and responding to classical inflammatory stimuli like IL-1 β , TNF- α and IL-6 [24]. Both enzymes metabolize AA into PGH₂, which then serves as a substrate for terminal prostanoid synthases to produce the five primary prostanoids enumerated

earlier (figure 1). Prostanoids exert their biological activities in an autocrine or paracrine manner by binding to their cognate cell surface receptors. There are five receptors types (EP, IP, DP, FP and TP) corresponding to the five primary prostanoids. Here are the documented receptors for each prostanoid:

PGE ₂ receptors:	EP ₁ , EP ₂ , EP ₃ and EP ₄
PGI ₂ receptor:	IP
PGD ₂ receptors:	DP ₁ and DP ₂
PGF _{2α} receptor:	FP
TXA ₂ receptor:	TP

All but one receptor, DP₂, are part of the G-protein coupled receptor (GPCR) family. Signaling via these GPCRs is achieved through the modulation of intracellular calcium or cyclic adenosine monophosphate (cAMP) levels. All prostanoids are very similar in structure. As a result, prostanoid receptors are not exclusively binding to the prostanoid they are assigned by nomenclature. They can also bind other prostanoids with lower affinities [25]. One functional example of this takes place in the cardiovascular system where a high concentration of PGE₂ can trigger the IP receptor to inhibit platelet aggregation [26]. In some cases, prostanoids can also bind to nuclear receptors such as peroxisome proliferator activated receptor (PPAR) γ with anti-inflammatory effects [27].

2.2.2 PGE₂

PGE₂ can be synthesized by three PGE synthases: microsomal prostaglandin E synthase (mPGES) -1 and 2 and cytosolic prostaglandin E synthase (cPGES). It is involved in a wide array of physiological processes including regulation of vascular pressure, maintenance of the gastro-intestinal integrity, renal functions and female reproduction [24]. Moreover, PGE₂ is a modulator of inflammation and immunity. The latter is predominantly accomplished through the induced expression of mPGES-1. Like COX-2, mPGES-1 expression is inducible under pro-inflammatory conditions [28, 29] and these two enzymes are predominantly functionally coupled to generate the outburst of PGE₂ seen under inflammatory condition [30]. Fibroblasts and vascular endothelial cells of an inflamed tissue, as well as activated innate immune cells like neutrophils, macrophages and DCs, are all sources of PGE₂. They produce it via the up-regulation of mPGES-1 expression [25, 31]. The biological activities of PGE₂ in inflammation basically contribute to all the cardinal signs of inflammation. PGE₂ stimulates vascular smooth muscle cells to cause vasodilatation, which is responsible for the redness and heat caused by the increased blood flow in an inflamed tissue [32, 33]. It also mediates pain hypersensitization both peripherally and centrally by lowering the threshold of activation of nerve endings to pain mediators [34].

As most major cell subsets of the immune system express at least one of the four EP receptors, PGE₂ is also an important immunomodulator of both innate and adaptive immunity [35].

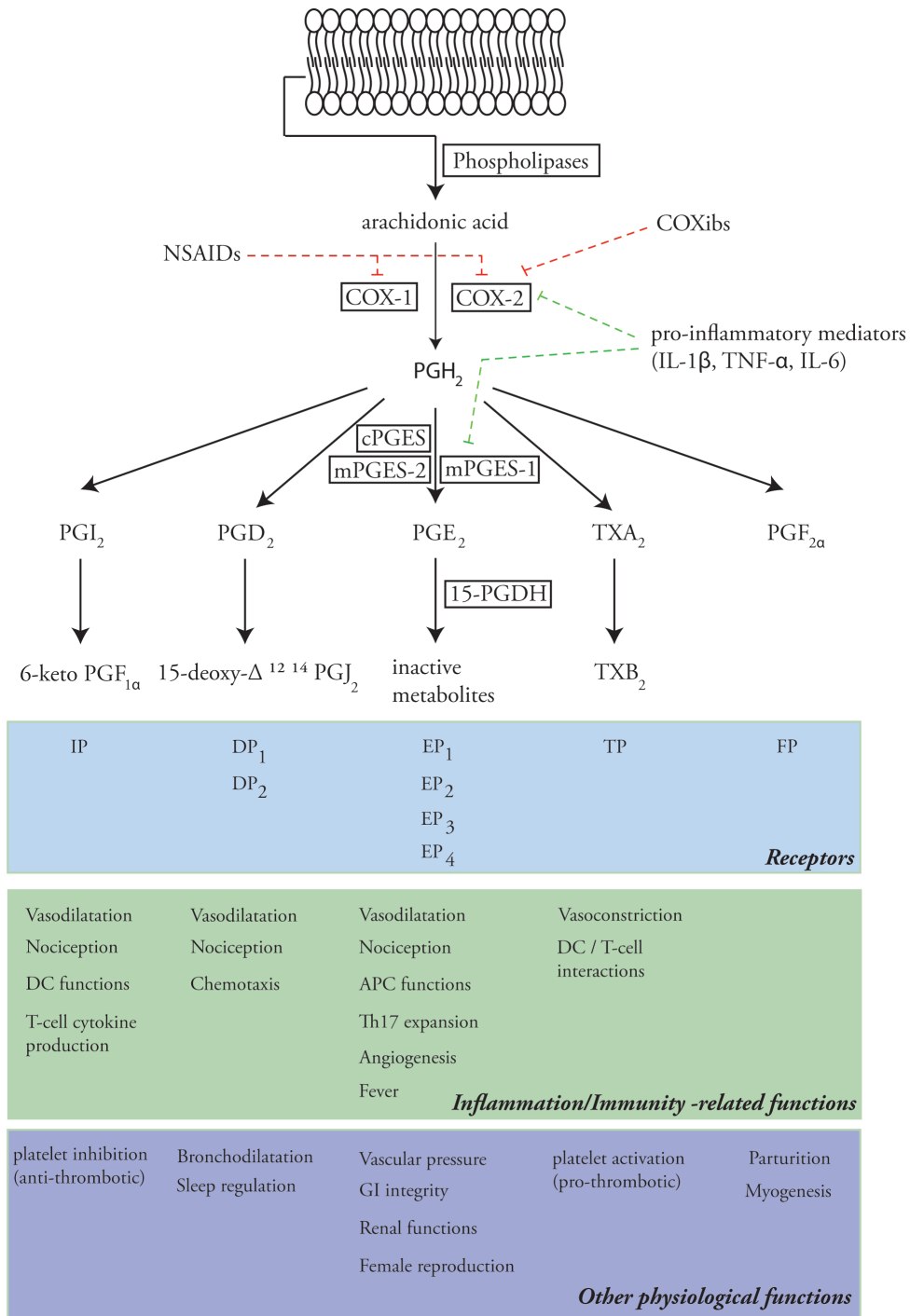


Figure 1: Schematic representation of the prostanoid pathway with emphasis on PGE₂ synthesis enzymes. Below the cascade are lists of receptors and functions for each of the 5 primary prostanoids as presented in section 2.2. Red-dotted lines represent inhibitors of dual inhibitors of cyclooxygenases (NSAIDs) and selective inhibitors of COX-2 (COXibs). Green dotted lines represent pro-inflammatory mediators inducing the expression of COX-2 and mPGES-1.

In innate immune cells, PGE₂ reduces the oxidative burst of activated neutrophils and inhibits TNF- α production while enhancing IL-10 production in activated macrophages and DCs [36-39]. Contrastingly, in macrophages, PGE₂ is part of an autoregulatory loop up-regulating the expression COX-2 and its own production at the site of inflammation [40, 41]. PGE₂ modulates the adaptive immune system with effects on both B-cells and T-cells. In B-cells, it inhibits the lineage development of B-cell precursors and the proliferation of mature B-cells while promoting IgE and IgG1 class switch [42-44]. The results from studies on the impact of PGE₂ on T-cell proliferation are contradictory and point to both inhibitory and stimulatory roles. However, this discrepancy could be explained by the fact that the T-cell receptor and EP2/4 are competing for the recruitment of the same intracellular signalling molecules. Thus, the outcome of stimulation relies on the relative strength of the two stimuli [45]. PGE₂ was also shown to promote IL-23 expression in DCs and to synergize with the latter cytokine to facilitate Th17 expansion [46-48].

Another important role of PGE₂ is the stimulation of angiogenesis. PGE₂ can trigger an up-regulation of vascular endothelial growth factor (VEGF) production in vascular endothelial cells. The latter acts in an autocrine fashion to stimulate angiogenesis [49]. Neovascularization is an important factor in chronic inflammatory diseases and cancer as will be discussed later.

Lastly, PGE₂ also has a central role in inducing the fever response in the brain, which complements inflammation and immunity in creating a hostile environment for pathogens. [50]

PGE₂ is a relatively stable molecule *in vitro*. In contrast, it has a very rapid turnover rate *in vivo* and is quickly eliminated from tissues and circulation. This elimination is carried out by 15-prostaglandin dehydrogenase (15-PGDH), a catabolic enzyme which oxidizes PGE₂ to an inactive metabolite. 15-PGDH and COX-2 are documented to be capable of regulating each other's expression [51].

2.2.3 PGD₂

Two enzymes can produce PGD₂: lipocalin-type and hematopoietic PGD synthases. In the brain, PGD₂ is involved in regulating sleep, temperature and nociception [25, 52]. In the periphery, PGD₂ is produced most abundantly by mast cells, but also by macrophages, DCs and T-cells. Through its action on DP₁, it mediates vasodilatation and bronchodilation as well as inhibition of platelet aggregation. Via DP₂ it mediates chemotaxis of Th2 cells, basophils and eosinophils. The combination of activities just enumerated explains the primary involvement of PGD₂ in allergic reactions [25].

There is a particular interest in the PGD₂ pathway for its potential anti-inflammatory role. More precisely, 15-deoxy- $\Delta^{12,14}$ PGJ₂ a metabolite of PGD₂ produced non-enzymatically has been characterized to inhibit IL-1 β -induced PGE₂ production in neonatal ventricular myocytes through a PPAR γ -dependent mechanism. In the latter experimental setup, 15-deoxy- $\Delta^{12,14}$ PGJ₂ caused a down-regulation of COX-2 and mPGES-1 expression [53].

2.2.4 PGF_{2α}

PGF_{2α} is mainly involved in the female reproductive cycle and in the regulation of myogenesis. It is essential for normal parturition. There is only one receptor for PGF_{2α}, the FP receptor and it is not expressed in immune cells or immune system-related organs such as the spleen or the thymus. There is thus very little evidence of PGF_{2α} involvement in inflammatory or immunological processes.

2.2.5 TXA₂

TXA₂, as its name suggests, is involved in cardiovascular homeostasis. It is produced by platelets and modulates their functions by inducing aggregation. It also triggers vasoconstriction when binding to its receptor on vascular smooth muscle cells [25]. It plays a role in immune responses as well. DCs express the TP receptor. When triggered, it inhibits DC-T-cell interactions [54]. TXA₂ is an extremely unstable molecule that is rapidly hydrolyzed to its inactive metabolite TXB₂ *in vivo*. Measurement of TXB₂ levels is the method of choice to evaluate thromboxane synthesis in biological fluids.

2.2.6 PGI₂

PGI₂ is primarily known for its role in vascular homeostasis. It is produced by endothelial cells and has potent vasodilatory and antithrombotic effects. Basically, PGI₂ opposes the effects of TXB₂ released by platelets to maintain cardiovascular homeostasis [55].

In inflammation and the immune system, PGI₂ has overlapping functions with PGE₂. It causes vasodilatation, just like PGE₂, when binding to the IP receptor on vascular smooth muscle cells and induces pain hypersensitization when acting on peripheral nerve endings [25, 55]. In the adaptive immune system, PGI₂ acts at several levels resulting in both inhibitory and stimulatory effects. It inhibits dendritic cell activation, maturation and T-cell stimulatory functions, having an ultimate immunosuppressive role on Th2 immunity, but promoting Th1 cell differentiation [56, 57]. At the level of differentiated T helper cells, it inhibits the production of both Th1 and Th2 cytokines [58]. Just like TXA₂, PGI₂ has a very short half-life *in vivo* and its production is quantified by the measurement of its metabolite 6-keto PGF_{1α}.

2.2.7 TRANSCELLULAR BIOSYNTHESIS

Biosynthesis of a prostaglandin traditionally occurs in a single cell that contains the complete set of enzymes required for its production. However, it can also happen via a process termed transcellular biosynthesis whereby a prostanoid precursor is transferred from one cell to another during cell-cell interactions. Transcellular biosynthesis of prostacyclin was demonstrated to occur *in vitro* when endothelial cells are co-cultured with platelets or lymphocytes [59].

3 THE INDUCIBLE PGE₂ PATHWAY IN PATHOLOGY

As efficient and well-orchestrated as inflammation and the immune system can be, they do have imperfections, which can cause or contribute to disease. The immune system can break tolerance and turn against self, creating one of the many autoimmune diseases. It can also fail to recognize and destroy cells of the body that have become anarchic, thus allowing cancer to progress. The inducible PGE₂ pathway is involved in several pathologies of these natures as will be discussed here.

3.1 RA

One autoimmune disease in which the inducible PGE₂ pathway is involved and which has been the focus of my work is RA. RA is a disease characterized by an autoimmune response against the joint tissues. The autoimmune reaction triggers chronic inflammation, which eventually leads to synovial hyperplasia and structural damage to the cartilage and bone [60]. The etiology of RA remains unknown. However, genome-wide association scans and epidemiological studies have uncovered several genetic and environmental factors such as HLA subtype, autoantibodies and smoking, which predispose to RA [61-64]. Moreover, the study of individuals with such predispositions has provided some insight into the physiological events leading to disease development. For example, the study individuals with serum autoantibodies against citrullinated peptides and the rheumatoid factor (considered at a high risk of developing RA), has revealed that arthralgia can occur before onset of RA [65].

In healthy articulations, cartilage and a layer of nourishing tissue called the synovium separate adjoining bones. The healthy synovium structure is divided into the lining (intimal) layer, which is mainly composed of type A (macrophage-like) and type B (fibroblast-like) synoviocytes and sub-lining (sub intimal) layer, which also contains scattered blood vessels. In RA patients, a painful inflammation develops in the synovium. It is characterized by the activation of synoviocytes and the recruitment of cells of the immune system. Resident cells and immune system cells recruited to the joint enter a loop of mutual activation, setting up a chronic inflammation in which the hypertrophic synovium tissue invades the cartilage and bone, eventually leading to cartilage destruction and bone erosion.

PGE₂ is found in large amounts in the synovial fluid of RA patients [66] and inducible enzymes of the PGE₂ pathway are expressed in the inflamed RA joint [67]. Accordingly, this pathway has been reported to contribute to multiple features of the disease. The localization of mPGES-1 and COX-2 is characterized in synovial tissue of RA patients and their expression is up-regulated in fibroblasts and macrophages of the lining and sub-lining layers, as well as in vascular endothelial cells [67]. Interestingly, the induction of mPGES-1 and COX-2 expression in the synovium of RA patients was described to remain unaffected by TNF blockade and B-cell depletion therapy, pointing to the potential of targeting PGE₂ synthesis in combination with these two therapies [68]. As discussed previously, PGE₂ activity contributes to inflammation by triggering vasodilatation and pain sensitization in vascular smooth muscle cells and terminal nerve endings respectively. This is the case in the RA synovium. One feature of the synovium

in chronic joint inflammation is hyperplasia. The rapidly growing tissue undergoes neovascularization to respond to the increased metabolic demand. PGE₂ contributes to angiogenesis by stimulating synovial fibroblasts to produce vascular endothelial growth factor [69]. IL-17 and IL-17-producing cells (Th17) are closely related to the pathology of RA, as peripheral blood and synovial Th17 cell populations are increased in RA patients and correlate with disease activity [70, 71]. As mentioned earlier, PGE₂ synergizes with IL-23 to stimulate Th17 cell proliferation. So, the abundance of PGE₂ in the inflamed RA joint could logically contribute to the development of the Th17 immune response, which would in turn stimulate the release of pro-inflammatory cytokines and promotes bone resorption. The contribution of the inducible PGE₂ pathway is very well characterized in animal models of the disease. In mouse CIA, knocking out the mPGES-1 enzyme impairs the adaptive immune response and greatly reduces the incidence and severity of the disease [72, 73]. PGE₂ administration on the other hand, exacerbates the disease with a concomitant increase in IL-17 production in the affected joints [74]. Furthermore, modulation of PGE₂ signaling through the EP₄ receptor has revealed a critical involvement of the latter in CIA, especially in the development of the Th17 immune response [75]. PGE₂ also has a direct influence of bone resorption in RA. Under homeostasis, bone remodeling is a dynamic process involving cells types with opposing functions: osteoclasts, which remove old bone, and osteoblasts, which synthesize new bone. Their functions are regulated by the RANKL-OPG system. In RA, RANKL, which is released by IL-17-stimulated synovial fibroblasts and osteoblasts, initiates osteoclastogenesis and thus tips the balance towards bone resorption [76]. In conclusion, PGE₂ has already been shown to contribute to pain and inflammation in RA and it could potentially have an involvement in angiogenesis, Th17 expansion and bone resorption in this disease. These multiple involvements of PGE₂ in RA make the inducible PGE₂ pathway an attractive therapeutic target.

3.2 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is another disease where inflammation plays an important role. It is commonly considered to be a T-cell mediated disease consisting in an autoimmune response attacking the central nervous system and causing demyelination of neuronal axons. Very little is known about the inducible PGE₂ pathway in MS other than the fact that mPGES-1 is expressed in the macrophages of brain lesions [77]. Interestingly, the disease is believed to have a Th17 immune component as MS patients experience elevated blood and cerebrospinal fluid IL-17 levels, as well as IL-23 expression in DCs and active lesions [78]. Experimental autoimmune encephalomyelitis (EAE) is the animal model of reference for MS. It results from an autoimmune response against myelin antigens and is a T-cell mediated disease, comprising a Th17 component. mPGES-1 is characterized to play a key role in EAE: its expression is up-regulated in macrophages/microglia of brain lesions and mPGES-1 knockout mice experience reduced symptoms in the chronic phase of the disease. The mechanism behind mPGES-1 involvement in EAE was partially elucidated as the presence of active mPGES-1 was shown to aggravate the inflammation and demyelination associated with the disease [77, 79]. Most importantly, mPGES-1 knockout mice experience a lower Th1/Th17 burden [77].

3.3 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a complex group of chronic inflammatory disorders characterized by inflammatory reactions to microbial antigens of the commensal flora. There are two major forms of IBD: Crohn's disease and ulcerative colitis. IBD is one of the most prominent risk factor for colorectal cancer (CRC). As for CRC, the inducible PGE₂ pathway has considerable implications in IBD. An increased amount of PGE₂ can be detected in the mucosa of IBD patients and it correlates with disease activity [80, 81]. This is likely the result of both an increase in PGE₂ synthesis and a reduction in its catabolism, as the expression of both mPGES-1 and COX-2 is up-regulated in colonic epithelial cells of IBD patients while that of 15-PGDH is down-regulated [82-84]. As previously mentioned, PGE₂ also performs physiological functions in the GI tract, including gastric mucosa protection. Thus, in IBD, PGE₂ appears to stimulate both the gastro-protection needed to repair the damage caused by inflammation and inflammation itself [85]. In an experimental model of chemically-induced colitis, the EP4 receptor appears to play a central role in gastro-protection as EP4 knockout mice experienced a more severe disease. Knocking out the other three EP receptors did not have any significant impact in this model [86].

3.4 CARDIOVASCULAR DISEASES

The cardiovascular system is another system where prostaglandins are implicated in both homeostasis and pathological conditions. Concerning physiological functions, as previously mentioned, PGE₂ is involved in the regulation of blood pressure whereas the thromboxane/prostacyclin balance plays a central role in platelet activation. Cardiovascular diseases constitute a broad range of conditions, which, for most, are associated with atherosclerosis. Atherosclerosis is a chronic inflammatory condition. It is characterized by a deposition of lipids into the arterial wall, termed atheroma, which causes progressive wall thickening. Atheromas are found in most people by age 40 and do not pose problem unless they interfere with blood flow by their size or if they rupture and cause thrombosis [87]. mPGES-1 expression together with that of COX-2 is up-regulated in atherosclerotic plaques and PGE₂ production was postulated to contribute to plaque rupture [88]. In mice, PGE₂ is involved in both atherogenesis and thrombosis [89, 90]. Moreover, mPGES-1 gene deletion appears to retard atherogenesis in the hyperlipidaemic mouse model of atherosclerosis and cause a shift in the thromboxane/prostacyclin balance towards the atheroprotective prostacyclin [89]. For the latter finding, urine metabolites of prostacyclin and thromboxane were measured. It is thus a reflection of the systemic levels of the two prostanoids.

3.5 CANCER

Interestingly, COX-2 and mPGES-1 are found elevated, whereas 15-PGDH is down-regulated in a number of cancers [91]. Moreover, the extent of mPGES-1 induction has been correlated to poor prognosis in colorectal neoplasms [92]. One proposed mechanism to explain the involvement of the PGE₂ pathway in this pathology is it drives lymphangiogenic growth through the up-regulation of VEGF production in

stromal cells [92, 93]. Nevertheless, the inducible PGE₂ pathway can potentially be involved in several other processes regulating oncogenesis and cancer progression. Briefly, PGE₂ promotes proliferation and survival in neoplastic epithelial cells. It also modulates tumor immunosuppression and the inflammatory microenvironment creating optimal conditions for tumor progression [91]. PGE₂ contributes to the tumor inflammatory microenvironment via its previously mentioned involvement in vasodilatation and the Th17 response [91]. PGE₂ also favors tumor immunosuppression by inhibiting cytotoxic T lymphocyte activity and antigen presentation in tumor cells and DCs [94, 95]. The inducible PGE₂ pathway is thus contemplated as a therapeutic target in cancer. NSAIDs have already proven to reduce the risk of several types of cancers including those of the breast, colon, lung and prostate [96]. Moreover, studies using mPGES-1 siRNA knock-down in xenograft models of tumorigenesis or mPGES-1 knockout mice in models of intestinal cancer both result in impaired growth and survival of tumor cells [97].

4 RA THERAPEUTIC APPROACHES AND TARGETING THE PGE₂ PATHWAY

There are several therapeutic alternatives used in the treatment of RA. The most efficient approach is undoubtedly the use of disease-modifying anti-rheumatic drugs (DMARDs) as it is the only one able to affect disease progression by slowing down joint destruction. However, kinase inhibitors such as the JAK kinase inhibitor Tofacitinib have recently become available for RA and could potentially provide more disease-modifying treatment alternatives [98, 99]. DMARDs can be divided into synthetic molecules, classically represented by methotrexate, and biological molecules of which TNF blockers are the most prominent players. Methotrexate is characterized to treat RA via two mechanisms. First of all, it inhibits dihydrofolate reductase, which impairs DNA synthesis and thus inhibits cellular proliferation. Secondly, it triggers a rise in adenosine, which has anti-inflammatory properties. The second mechanism is the result of substrate build-up after DHF reductase inhibition [100]. TNF blockers, as the name suggests, are either antibodies (Infliximab, Adalimumab) or chimeric constructs (Ethanercept), which inhibit TNF signaling by binding to the soluble and membrane-bound forms of TNF. In a responsive patient, they reduce inflammation and disease activity and increase joint function [101]. Their most important contribution is certainly the prevention of cartilage and bone destruction [102-104]. B-cell depletion therapy is another biological approach effective in the treatment of RA. Rituximab is a monoclonal antibody against CD20, a B-cell surface marker that mediates B-cell depletion by antibody-dependent cellular cytotoxicity [105]. In responsive patients, Rituximab is suggested to reduce synovial inflammation through the depletion of auto—reactive B-cells [106]. Biological DMARDs typically have limited side effects and they can be used in combination. As a matter of fact, TNF blockers are most efficient at treating RA when combined with methotrexate [104]. Glucocorticoids administered systemically or intra-articularly are another approach for the treatment of RA. They suppress disease activity and reduce inflammation and pain. However, they have considerable side effects including osteoporosis and diabetes, which prevent their long-term use. Another class of therapeutic agents, which are commonly used to treat RA, is COX inhibitors. They were shown to reduce pain and inflammation, but also

have side effects precluding their long-term use. COX inhibitors and other approaches targeting the PGE₂ pathway are presented in detail in sections 4.1 to 4.4.

4.1 COX INHIBITION

Cyclooxygenases were the first enzymes of the PGE₂ pathway to be targeted for therapeutic purpose. In the late 1800s, acetyl salicylic acid, also commercially known as Aspirin, was already being manufactured and marketed as a treatment for fever, pain and inflammation. It is only later in the 1970s that its target was characterized as COX [107]. Aspirin is part of class of drugs called non-steroidal anti-inflammatory drugs (NSAIDs), which all target cyclooxygenase enzymes. They are effective anti-inflammatory drugs. However they have side effects caused by an interference with homeostatic functions of prostanoids. Aspirin is a traditional (t)NSAID, i.e. non-selective COX inhibitor, that has a higher potency for COX-1. During prolonged treatment, it interferes with the protective functions of COX-1-derived PGE₂ in the stomach and is associated with the development of gastric ulcers [108]. As COX-2 is the predominant COX isoform involved in inflammation, selective COX-2 inhibitors (COXibs) were developed in an attempt to inhibit the synthesis of prostanoids in inflammation while sparing their physiological functions. However, these so-called COXibs were found to interfere with platelet functions, for which the PGI₂/TXB₂ ratio is critical. They inhibit COX-2-mediated prostacyclin formation while, sparing COX-1-mediated thromboxane synthesis. Therefore, prolonged treatment with COXibs leads to platelet activation and an increased risk of thrombosis and myocardial infarct [109, 110].

NSAIDs and COXibs are effective treatments for several of the pathologies where PGE₂ is involved. For example, they can relieve pain and inflammation associated to RA, as well as reduce the risk of developing certain cancers [96]. However, most of these conditions require long-term use, which is complicated due to their associated side effects.

4.2 MPGES-1 INHIBITION

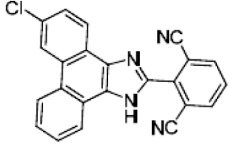
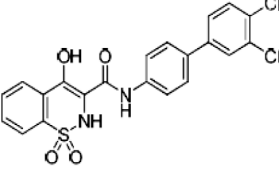
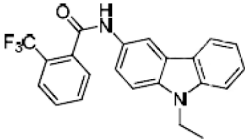
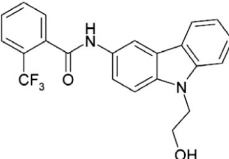
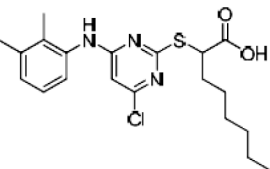
mPGES-1 inhibition has been proposed as an alternative to COX inhibition to dissociate the anti-inflammatory, anti-pyretic and pain killing effects of NSAIDs and COXibs from their gastro-intestinal and cardiovascular side effects. As with COX-2, the inducible nature of mPGES-1 creates a certain therapeutic selectivity for pro-inflammatory environments. Under normal physiological conditions, mPGES-1 expression is low and found in testis, prostate, placenta, mammary glands and the bladder [111]. The protein can also be found constitutively expressed in the lung, spleen, kidney and stomach [112]. As pointed out in the previous section, the therapeutic potential of targeting mPGES-1 is large. As such, several teams are working on the development inhibitors and, as a lesson learned from the failures of NSAIDs and COXibs, a considerable amount of research now addresses the potential physiological consequences of selective mPGES-1 inhibition, to ensure it constitutes a viable therapeutic alternative. Prostanoid profiling and targeted lipidomics are two

techniques used to study the impact of mPGES-1 inhibition on the prostanoid pathway. Prostanoid profiling consists in monitoring the levels of prostanoids and their metabolites upon altering mPGES-1 activity, to determine how the pathway reacts to the change. Targeted lipidomics is like prostanoid profiling, but additionally evaluates the expression of enzymes and receptor of the pathway. The effect of mPGES-1 inhibition on the cardiovascular system was investigated by Fitzgerald et.al. using a mPGES-1 knockout mouse in a model of thrombotic carotid artery occlusion. As opposed to selective COX-2 inhibition or IP receptor gene deletion, mPGES-1 gene deletion did not accelerate thrombogenesis [113]. This is an indication that mPGES-1 inhibition is unlikely to cause the cardiovascular side effect seen with COXibs.

Almost 20 years after the discovery of mPGES-1 and the realization it constitutes a therapeutic target, a great deal of literature that has been generated about molecules capable of inhibiting its activity or its expression. Naturally, publications characterizing endogenous lipids, such as LTC₄ or 15-deoxy-Δ(12-14)-prostaglandin J₂, inhibitors of other prostaglandin/leukotriene pathway enzymes, like sulindac or MK-886 and PGH₂ analogs came first as they were readily available for assessment. However, those were not optimal for therapeutic mPGES-1 inhibition as they either had intrinsic bioactivities or lacked enzyme specificity [29, 114, 115]. Later came molecules issued from rational drug design i.e. high-throughput screening (HTS) hits or inhibitors of other enzymes of the same family or pathway, which were optimized through structure-activity relationship (SAR) studies. Several laboratories attempt to develop potent and selective mPGES-1 inhibitors. Table 1 details the best-characterized mPGES-1 inhibitors in the literature.

There are two major hurdles in the development of mPGES-1 inhibitors: the hydrophobic nature of the target and a species selectivity, which causes most inhibitors of the human enzyme to undergo a profound loss of potency with murine mPGES-1. Both will be discussed later on.

Table 1: mPGES-1 inhibitor characterized *in vitro* in cellular assay and/or *in vivo*

Compound Structure	IC ₅₀ mPGES-1 (human/murine)	Whole blood (IC ₅₀)	Evidence of benefit to pathology	Refs
 MF-63	0.001 μM / >30 μM	1.3 μM	<ul style="list-style-type: none"> • ↓ Fever • ↓ Pain • ↓ Acute inflammation • no GI toxicity 	116, 117
 PF-9184	0.016 μM / inactive	5 μM	<ul style="list-style-type: none"> • Good PG profile in RA synovial fibroblasts 	120, 121
 AF-3442	0.06 μM / not determined	29 μM	<ul style="list-style-type: none"> • Good PG profile in monocytes and whole blood 	123
 AF-3485	0.438 μM / inactive	not determined	<ul style="list-style-type: none"> • ↓ Tumor growth in xenograft model 	124
 YS-121	3.4 μM / not determined	2 μM	not determined	125

4.2.1 MF63

MF-63 is an mPGES-1 inhibitor produced by scientists at Merck-Frosst. It is the product of a HTS campaign, which identified a JAK kinase inhibitor as a hit. The hit was then subjected to SAR studies and a lead optimization phase, to yield MF63 (Table 1). MF63 has an IC₅₀ of 0.001 μM on human recombinant mPGES-1 and a >1000-fold selectivity over the other enzymes of the prostanoid cascade [116, 117]. It can inhibit

PGE₂ synthesis in IL-1 β -induced A549 cells but undergoes a considerable loss of potency when the assay is performed in high serum concentration [117]. Like most mPGES-1 inhibitors published, MF63 is virtually inactive against rat mPGES-1 [116]. To be able to study mPGES-1 inhibition *in vivo* in relevant disease models, Merck-Frosst has generated a human mPGES-1 knock-in mouse. They also studied MF63 in guinea pig models, since it is active against guinea pig mPGES-1. MF63 was found to inhibit PGE₂ synthesis both centrally (brain) and peripherally, to relieve acute inflammation, fever and pain [117]. Moreover, it did not cause gastro-intestinal toxicity [117]. In conclusion, the characterization of MF63 validates mPGES-1 as a therapeutic target and differentiates it from NSAIDs regarding gastro-intestinal side effects. Additional HTS campaigns and SAR studies were conducted at Merck-Frosst, which yielded compounds equally potent and in some cases with optimized pharmacokinetic profiles [118, 119]. Their *in vivo* efficacy, however, remains to be characterized.

4.2.2 PF-9184 and PF-4693627

An mPGES-1 inhibitor was also generated at Pfizer [120]. PF-9184 has an IC₅₀ of 0.016 μ M [121]. Its selectivity however, was only tested against recombinant COX enzymes. It was mainly used to characterize the impact of mPGES-1 inhibition on the prostanoid profile in cultures of synovial fibroblasts from RA patients, in which it causes a shunt of PGH₂ to the prostacyclin pathway in a short incubation assay, but a reduction in both PGE₂ and 6-keto PGF_{1 α} in a long incubation assay [121]. An inhibition of prostacyclin synthase can thus be ruled out, but it remains unclear whether PF-9184 has an inhibitory potential on the other prostanoid synthases. PF-9184 has poor aqueous solubility and attempts to improve this property unequivocally led to loss of potency. Like MF63, PF-9184 is inactive on rat mPGES-1. Pfizer has a second mPGES-1 inhibitor selected as a clinical candidate: PF-4693627. It is also species selective and only inhibits human mPGES-1 (IC₅₀ of 0.003 μ M). PF-4693627 inhibits PGE₂ synthesis in a human whole blood assay without interfering with PGD₂, thromboxane or leukotriene synthesis. It was shown to inhibit PGE₂ synthesis *in vivo* in the guinea pig air pouch model of acute inflammation [122].

4.2.3 AF3442 and AF3485

A collaborative effort between industry and academia yield two other mPGES-1 inhibitors: AF3442 and AF3485. AF3442 has an IC₅₀ of 0.06 μ M on recombinant human mPGES-1 and it remains to be determined if it can inhibit mouse mPGES-1 [123]. It was mainly characterized in human monocyte cultures and whole blood assays to investigate the impact of mPGES-1 inhibition on COX-1 and COX-2-derived prostanoid synthesis. In a 24h LPS-stimulated human monocyte assay, it inhibited PGE₂ synthesis in a concentration-dependent manner without interfering with the production of prostacyclin, TXB₂ or PGF_{2 α} up to 10 μ M. At 100 μ M however, AF3442 partially inhibited TXB₂ synthesis [123]. This could be due to thromboxane synthase inhibition, as the cross-reactivity of the compound against other terminal synthases of the prostanoid cascade was not characterized. AF3442 can also inhibit PGE₂ in LPS-

stimulated whole blood, albeit at a higher concentration due to plasma protein binding ($EC_{50} = 29 \mu\text{M}$) [123]. In both systems, mPGES-1 inhibition could be achieved without repercussions on the production of other prostanoids. This group developed a second mPGES-1 inhibitor. AF3485 has an IC_{50} of $0.438 \mu\text{M}$ in microsomal fractions prepared from IL-1 β -stimulated A549 cells. It does not cross-react with 5-lipoxygenase (5-LO) or other PGES and was further characterized in the context of cancer. AF3485 inhibition of mPGES-1 in A431 cells resulted in a reduction of epidermal growth factor receptor (EGFR) signaling *in vitro* (EGFR is the receptor for VEGF). This effect translated into a reduction in tumor growth when the same cells were used in a xenograft model and AF3485 was administered to mice i.p. It further validates mPGES-1 as a target in cancer [124].

4.2.4 DUAL INHIBITORS OF MPGES-1 AND 5-LO

A German group is also working on dual inhibitors of mPGES-1 and 5-lipoxygenase, the hub enzyme in the leukotriene synthesis pathway. They recently published a series of natural compounds and pirinixic acid derivatives capable of doing so. Two of these compounds named YS121 and 7a have respective IC_{50} of $3.4 \mu\text{M}$ and $0.6 \mu\text{M}$ on human recombinant mPGES-1 [125, 126]. They were also characterized *in vivo* in a rat model of acute inflammation, but it remains unclear whether they have any specific activity on rat mPGES-1. Several pirinixic acid derivatives including YS121 are characterized to be agonists of PPAR, which inhibit inflammation [127].

4.3 EP RECEPTOR AGONISM / ANTAGONISM

EP receptors have been considered as therapeutic targets for several decades. As such, there is an extensive list of agonists and antagonists of EP receptors 1 to 4, which have been used as tools to characterize the functions of each receptor as well their potential for therapy [128]. One problem with EP receptors as therapeutic targets is that they have overlapping functions and their involvement in pathologies is often contrasted by homeostatic functions. For example, EP₃ receptor stimulation promotes tumor angiogenesis, but it also has gastro protective functions making it an unlikely candidate target [128]. Nevertheless, EP receptors are still contemplated as therapeutic targets. For example, Merck-Frosst has developed an EP₄ antagonist, which relieves pain and inflammation in models of arthritis without causing gastro-intestinal toxicity [129].

4.4 15-PROSTAGLANDIN DEHYDROGENASE EXPRESSION

As previously mentioned, 15-PGDH expression is down-regulated in several cancer [91]. As it has a potential tumor suppressor activity via PGE₂ elimination, approaches are being developed to induce its expression to reverse carcinogenesis. Interestingly, thiazolidinediones, an epidermal growth factor receptor tyrosine kinase inhibitor, TGF β and certain NSAIDs have all been shown to stimulate the expression of 15-PGDH in

cancer cells [130] [131-133]. Modulation of 15-PGDH expression also constitutes an interesting therapeutic approach for other pathologies where PGE₂ is involved.

5 METHODOLOGY

5.1 SYNOVIAL BIOPSY MATERIAL AND RASFs

In the first part of this thesis, the prostanoid cascade was studied to further characterize its involvement in arthritis as well as to evaluate how it is affected by MTX treatment. To achieve these objectives, we used synovial biopsy material and cultured synovial fibroblasts isolated from RA patients. As previously mentioned, the inducible PGE₂ pathway is up-regulated in synovial biopsies of RA patients and their cellular localization includes synovial fibroblasts, making the following two biological materials relevant to our studies [67].

The study of synovial biopsy specimens is an established and trusted method to evaluate disease mechanisms, response to treatment and prognosis, as the immunohistological features in synovial tissue consistently reflect disease status [134]. We used synovial biopsy specimens in paper I to verify whether the PGE₂ pathway was up-regulated in individual considered at risk of developing arthritis. We also used biopsies in paper III to characterize the synovial expression of 15-PGDH in arthritis patients and to investigate the impact of MTX treatment on the synovial expression of the enzymes of the PGE₂ pathway.

Synovial fibroblasts isolated from RA patients are one of the reference cell systems used to study the molecular pathways involved in this disease. Synovial fibroblasts mediate both cartilage and bone destruction in RA. Moreover, they specifically contribute to the chronic inflammatory loop that characterizes RA via the production of numerous cytokines and chemokines and prostaglandins [135]. In paper II, we used RASF cultures to characterize a potential new trigger for the induction of PGE₂ synthesis in RA. We also used RASFs in paper III to characterize their expression of 15-PGDH after IL-1 β stimulation and to investigate the impact of MTX treatment on enzymes of the PGE₂ pathway in that cell type. In paper IV, RASFs were also used in the characterization compound II, because they up-regulate the expression of mPGES-1 when stimulated with IL-1 β and TNF- α .

5.2 MPGES-1 INHIBITOR CHARACTERIZATION IN CELLULAR ASSAYS

In the second part of the thesis mPGES-1 inhibitors were developed. After the characterization of their potency on recombinant mPGES-1 and cross-reactivity on other enzymes of the prostanoid cascade, compound II and III were assayed in relevant cellular systems.

5.2.1 A549 SHORT AND LONG INCUBATION ASSAYS

A549 cells are a lung carcinoma cell line reported to express mPGES-1 and COX-2 at high levels after IL-1 β stimulation. The expression of mPGES-1 is functionally relevant for A549 cells. mPGES-1 knock down has been reported to impair their clonogenicity *in vitro* and their tumorigenic potential *in vivo* [136]. They were also previously used in the development of mPGES-1 inhibitors [116, 126]. In papers IV and V, we used A549 cells in an assay to address the on-target/off-target effects of our mPGES-1 inhibitors. We first investigated whether compounds II and III could inhibit PGE₂ synthesis in an assay where they are present for the entire time of the cytokine stimulation (long incubation assay). Then, to ensure that a reduction in PGE₂ synthesis happens specifically through the inhibition of mPGES-1, we perform a short incubation assay where compounds are only in the presence of mPGES-1 expressing cells for 30 mins. The latter allows us to eliminate the hypothesis that PGE₂ synthesis inhibition in the long incubation assay happens through an off-target side effect interfering with the up-regulation of the inducible PGE₂ pathway enzymes.

5.2.2 MOUSE AND RAT PERITONEAL MACROPHAGES

Macrophages are innate immune cells characterized to induce the PGE₂ pathway when stimulated with LPS. As previously mentioned, there is a species selectivity issue with most mPGES-1 inhibitors and we used peritoneal macrophages isolated from mice and rats to investigate the activity of compound II and III in mouse and rat cell cultures.

5.2.3 BLOOD AND BLOOD-DERIVED ASSAYS

To investigate how compounds II and III performed in the presence of serum proteins, we used the whole blood assay developed by Brideau et. al. in the development of NSAIDs [137]. Also, as we did not have access to enzymatically active recombinant thromboxane synthase, we instead isolated platelets from whole blood as a source of COX-1-derived TXB₂ to investigate the cross-reactivity of compounds II and III on thromboxane synthesis. Worthy of mention, platelets do not express mPGES-1 [123].

5.3 MPGES-1 INHIBITOR CHARACTERIZATION *IN VIVO*

5.3.1 AIR POUCH MODEL

The air pouch model of acute inflammation was developed by Edwards, et. al., as an *in vivo* model to study the functioning of the synovium [138, 139]. Once mature, air pouches have a lining that is similar in composition to the synovial membrane. The air pouch model was used in the development of COXibs [140]. We used this model with

carrageenan as a inflammatory trigger to determine whether compound II and III administered intra-peritoneally could inhibit PGE₂ synthesis.

5.3.2 AIA

The AIA model was the first experimental model of arthritis developed [141, 142]. It has a clinical onset and course resembling that of reactive arthritis in humans and essentially starts with a synovitis leading to cartilage and bone destruction.

The AIA model can be performed in two different ways with the complete Freund's adjuvant being injected either at tail base or in one limb. The second variant was used in paper IV as it allowed us to study an acute (ipsilateral paw) and a delayed (contra-lateral paw) inflammatory reaction. These two reactions were relevant for the characterization of compound II as mPGES-1 and COX-2 are up-regulated in the injected paw throughout the course of inflammation, but they are not up-regulated in the inflamed contra-lateral paw in AIA [143].

5.4 QUANTIFICATION OF PROSTANOIDS AND EVALUATION OF THE ENZYMES INVOLVED IN THEIR SYNTHESIS

The methodologies described up to this point were selected for the common feature that they allowed the study of the prostanoid pathway and more precisely the PGE₂ pathway and the enzymes involved in its synthesis. To quantify prostanoids, we used commercially available antibody-based enzyme immunoassays or a prostanoid profiling protocol by mass spectrometry developed in our lab. To characterize the expression of enzymes of the PGE₂ pathway, we used immunohistochemistry (IHC) and Western blotting.

6 RESULTS AND DISCUSSION

The first section of the results and discussion serves as a further characterization of the PGE₂ pathway in RA. The second section investigates mPGES-1 as a therapeutic target in inflammation through the characterization of novel selective mPGES-1 inhibitors and the use of the mPGES-1 knockout mouse.

6.1 INVOLVEMENT OF THE PGE₂ PATHWAY IN RA

Effective RA therapies such as methotrexate, TNF blockers and B-cell depletion have revolutionized the care of patients living with RA and tremendously improved their quality of life by slowing down disease progression. Nevertheless, these therapies don't bring disease progression to a complete halt, nor do they reverse its course. Therefore, there must remain inflammatory and/or immune pathways left unaltered which continue to drive disease progression. The inducible PGE₂ pathway is a likely candidate, as it is up-regulated in the synovium of RA patients [144] and the expression of mPGES-1 remains unchanged following treatment with TNF blockers or B-cell depletion agents [67, 68]. This section of the thesis serves as a further characterization of the PGE₂ pathway in RA, supporting the hypothesis that mPGES-1 is a relevant therapeutic target with potential benefits through combination with anti-rheumatic therapeutic agents leaving its induction unaltered.

6.1.1 THE PGE₂ PATHWAY AND THE PRE-CLINICAL AND EARLY CLINICAL PHASES OF ARTHRITIS.

The etiology of RA remains elusive. However, decades of research have helped to define genetic susceptibility traits, biomarkers and prodromal symptoms that, together, constitute a set of features useful in identifying individuals with an increased risk of developing the disease. In paper I, we assembled and studied a cohort of individuals at an increased risk of developing arthritis based on two criteria:

1. Prodromal arthralgia and/or positive family history of RA
2. Circulating serum autoantibodies recognizing rheumatoid factor and/or citrullinated peptides.

The rationale behind the choice of those two criteria is as follows: autoantibodies have been characterized as biomarkers representing an on-going systemic pre-clinical phase of disease [145-147] and prodromal arthralgia has been described to occur in individuals several years before the first clinical manifestations of arthritis [65]. The molecular mechanisms from which prodromal arthralgia originates however, remain to be characterized, but PGE₂ constitutes a prime candidate. As previously mentioned, PGE₂ functions as a pain mediator both centrally and peripherally. Additionally, the up-regulation of the inducible PGE₂ pathway has previously been reported in the clinical phase of RA and other forms of arthritides [66, 144, 148, 149].

The first part of paper I describes the study of the PGE₂ pathway in a cohort of individuals with an increased risk of developing arthritis. We investigated the hypothesis

that the synovial expression of enzymes of the PGE₂ pathway would either be correlated to pain sensation or predict disease development. Interestingly, the rationale behind the elaboration of the hypothesis came from experimental arthritis model studies, in which pain sensitization [150] and synovial mPGES-1 up-regulation [151] were characterized to take place before onset of disease.

We evaluated the expression of mPGES-1, COX-1, COX-2 and 15-PGDH in subjects synovial biopsies by IHC. The hypothesis could not be confirmed, as no correlation could be established between the expression of the aforementioned enzymes and quantitative pain assessment or arthritis development. However, a trend towards higher expression of COX-1, COX-2 and 15-PGDH was detected in individuals with arthralgia (data not shown and paper I, figure 1).

One limitation with the study of the pre-arthritis cohort was the design of the synovial biopsy sampling procedure. Due to the intrinsic thinness of the synovial membrane in joints bearing no clinical sign of inflammation, a large joint (the knee joint) was designated for biopsy sampling; that regardless of its symptomatic status.

Since the PGE₂ cascade can mediate pain sensitization both centrally and peripherally, two possible mechanisms of action had to be considered when addressing our hypothesis:

1. The PGE₂ cascade would be part of a systemic pre-clinical phase of disease, in which case modulation of enzyme expression could be expected in symptomatic and asymptomatic joints alike.
2. The PGE₂ cascade would be part of a local pre-clinical phase of disease, in which case modulation of enzyme expression would be expected in symptomatic joints only.

When addressing our hypothesis with the latter mechanism in mind, the arthralgia patient sub-groups had to be further stratified into two groups i.e. those for which biopsy sampling occurred in an symptomatic knee and those for which biopsy sampling occurred in a asymptomatic knee (figure 2) While the inclusion criteria had already limited the number of individuals recruited to this cohort, the division of the arthralgia patient sub-group unfortunately left us with a very limited number of individual per group to perform our analyses. However, subjects are constantly being recruited to the pre-arthritis cohort and this study could very well be extended to include a greater number of subjects in the future.

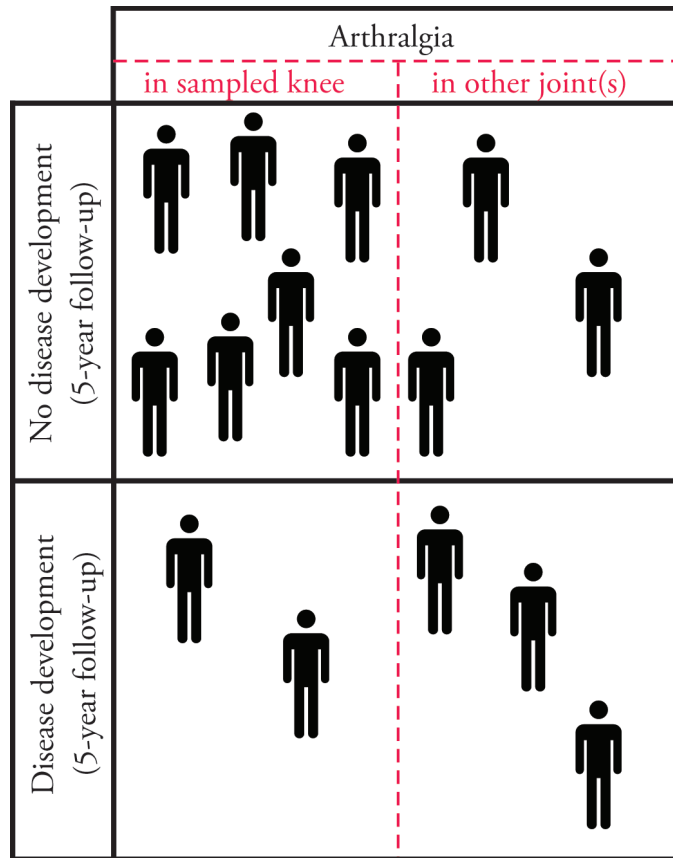


Figure 2: The arthralgia sub-group from the cohort of individuals at risk of developing arthritis. Red dotted line: the division necessary to investigate our hypothesis if we assume the PGE₂ cascade has a local involvement in disease.

In the second part of paper I, we investigated PGE₂ pathway enzymes in an early arthritis cohort and whether their expression correlated with pain sensitization at this stage of disease development. The early arthritis cohort was composed of patients recruited less than a year after arthritis diagnosis and which, two years later, received a definite diagnostic classification of undifferentiated arthritis, spondyloarthritis (SpA) or RA. No correlation could be found in this cohort between pain sensitization and PGE₂ pathway enzyme expression at baseline (paper I, table 3). We did however report a correlation between mPGES-1 and COX-1 and the number of macrophages present in the synovium. (paper I, supplemental table 1) This could be expected, as PGE₂ is a mediator of vasodilatation and is therefore contributing to cellular infiltration.

When the cohort was stratified according to definite diagnostic classification after two years however, baseline mPGES-1 and COX-1 levels were found higher in the SpA group compared to the RA group ($p=0.001$ and $p=0.003$ respectively), consistent with previous findings and supporting the idea that PGE₂ produced via mPGES-1 plays a role in SpA through an effect on osteoblastogenesis [148, 152].

Although the study in paper I does not point to an involvement of the PGE₂ pathway in pre-clinical or early clinical arthritis peripheral pain sensitization, it does not refute the hypothesis that the PGE₂ pathway could be involved in pain sensitization centrally, or in arthritis initiation through a completely different mechanism. As an example, the PGE₂ pathway is reported to be involved in Th17 cell differentiation and could be affecting arthritis pathogenesis through this process [47, 48].

6.1.2 THE MECHANISMS OF UP-REGULATION OF THE INDUCIBLE PGE₂ PATHWAY IN RA

The characterization of mPGES-1 and COX-2 expression in RA revealed the two were co-expressed in the synovial lining and sub-lining [67]. When RASFs are studied *in vitro*, they can be stimulated to induce COX-2 and mPGES-1 expression by several different pro-inflammatory cytokines [28, 144]. IL-1 β is one cytokine involved in RA, able to induce mPGES-1 expression [28, 153, 154]. Interestingly, IL-1 β was also demonstrated to mediate inflammation through an additional mechanism, which involves the formation of complexes with the alarmin HMGB1 [10]. The expression levels of HMGB1 and mPGES-1 in the RA synovium are similarly affected by RA therapies with no impact following anti-TNF- α therapy but a reduction following intra-articular glucocorticoid treatment [67, 155, 156]. This evidence suggests a potential link between HMGB1 and mPGES-1.

In paper II, we investigated whether IL-1 β /HMGB1 complexes could modulate the PGE₂ cascade in RASFs. We found out that IL-1 β /HMGB1 complexes induce PGE₂ synthesis in RASF (paper II, figure 1), and that this induction happens through activation of the IL-1 receptor I (paper II, figure 6), causing an up-regulation of mPGES-1 and COX-2 expression (paper II, figure 3).

In a characterization of the kinetics of release of PGE₂ and cytokines, we also demonstrated that PGE₂ was produced at an earlier time point compared to the cytokines IL-6, IL-8, MCP-1 and RANTES (paper II, figure 2) and that inhibition of COX-2-derived prostaglandin synthesis with NS-398 reduced the release of IL-6 and IL-8 (paper II, figure 4). This suggests an involvement of COX-2-derived prostaglandins in the cytokine response. PGE₂ and PGI₂ were the two prostanoids up-regulated by IL-1 β /HMGB1 complexes in this system (paper II, figure 5). PGE₂ has already been reported to contribute to the IL-6 response in IL-1 β -stimulated RASFs. However, the experimental setup used in the previous investigation could not help clarify whether PGI₂ was also involved [69]. Regardless, this characterization constitutes a new and additional mechanism by which the PGE₂ cascade can be up-regulated in RA. Given their previously reported similar expression patterns in RA following treatment, mPGES-1 and HMGB1 represent therapeutic targets, which could confer additional benefits when combined with the current therapies of choice.

6.1.3 PGE₂ CATABOLISM AND THE IMPACT OF METHOTREXATE (MTX) THERAPY IN RA.

The anabolic part of the PGE₂ pathway is very well characterized in RA, especially the tissue distribution of mPGES-1 and COX-2 and how their expression remains unaffected by TNF blockers and B-cell depletion agents [67, 68]. Much less is known, however, about 15-PGDH, the enzyme that degrades PGE₂, in the same disease or the effect of MTX therapy on the PGE₂ pathway.

In paper III, we attempted to characterize the expression and tissue distribution of 15-PGDH in the healthy and arthritic synovium using IHC. We also investigated the impact of MTX treatment, another effective therapeutic agent, on 15-PGDH, COXs and mPGES-1 expression in the arthritic joint.

15-PGDH localized to the lining macrophages and sub-lining fibroblasts, as well as in the endothelium of blood vessels. This is consistent with the previously characterized localization of mPGES-1 and COX enzymes in RA synovial tissue and suggests a concerted activity of the anabolic and catabolic parts of the PGE₂ cascade into regulating PGE₂ levels in the joint. The promoter of the 15-PGDH gene contains potential AP-1 and CREBP binding sites which suggests its expression can be modulated in inflammation and immunity by factors such as cytokines [157]. In fact, IL-6 and TGF- β are known to regulate its expression in cancer cell lines [158, 159].

In synovial tissue, we found the expression of 15-PGDH to be slightly up-regulated in arthritis patients (paper III, figure 1). MTX, however, did not affect its expression, or that of mPGES-1 or COX-2 in RA patients after 8 weeks treatment, adding to the list of therapies that leaves the induced PGE₂ pathway unaltered.

In conclusion, most of the current RA therapies don't affect the expression of mPGES-1 in the RA synovium. This is most likely due to the redundant nature of pro-inflammatory cytokine networks known to induce the PGE₂ pathway in RA synovial cells [28]. In line with this principle, in paper II we have characterized a new mechanism by which IL-1 β can induce PGE₂ synthesis in RASFs. As redundant networks of mediators regulate its expression, mPGES-1 constitutes an attractive therapeutic target for RA treatment that could either be targeted alone or in combination with other therapies that leave the PGE₂ pathway unaffected.

6.2 MPGES-1 AS A THERAPEUTIC TARGET

As elaborated previously in the introduction, mPGES-1 constitutes a prime target for drug development. It is reported to be involved in a wide range of pathologies, several of which represent markets of sizes large enough to cover development costs and generate profit in the long run.

Several groups are currently developing molecules that target mPGES-1. Most approaches attempt to interfere with its enzymatic activity while one group developed an inhibitor of mPGES-1 expression [116, 120, 123, 124, 160].

There are two major hurdles to overcome in the development of mPGES-1 inhibitors. The first one is the hydrophobic nature of its proposed active site. It is lodged inside the phospholipid bilayer between subunits of the mPGES-1 homotrimer. [161] As a result, the candidate molecules developed also tend to be hydrophobic and perform poorly in assay with high serum concentration or in whole blood [114]. This is most likely due to high protein binding. The second hurdle resides in key amino acid sequence differences within the active site, between human and murine mPGES-1 orthologs. The three amino acids are bulkier in the murine version of the enzyme. One hypothesis is that this bulkiness prevents most human mPGES-1 inhibitors to access the active site of the murine enzyme [162]. Both hurdles compromise the feasibility of *in vivo* studies.

The mPGES-1 inhibitors MF-63, developed by Merck-Frosst, and PF-9184, developed by Pfizer, are good examples of how these hurdles can hinder development [116, 120]. MF-63 and PF-9184 have respective IC_{50} of $0.001\mu\text{M}$ and $0.016\mu\text{M}$ on recombinant human mPGES-1 (Table 1). Nevertheless, they have drastically reduced potency on rat mPGES-1. Moreover, in the human whole blood assay, their EC_{50} rises to $1.3\mu\text{M}$ and $50\mu\text{M}$ respectively [116, 121]. Attempts to increase the solubility of PF-9184 during SAR studies consistently resulted in loss of potency [120]. Other groups have encountered the same hurdles [123, 125]. Merck however, after additional SAR and lead optimization studies, has generated new mPGES-1 inhibitors, which retain a greater fraction of their recombinant enzyme efficiency in serum-containing assays [119, 163].

In Papers IV and V, we describe the *in vitro* and *in vivo* characterization of two mPGES-1 inhibitors, compound II and compound III, which were developed at NovaSAID AB, following a screening campaign that generated hit compounds which were subsequently optimized through SAR and lead optimization studies. Both compounds have inhibitory potential towards human and rat mPGES-1. While compound II inhibits recombinant human and rat mPGES-1 with similar IC_{50} (1.8 and $0.62\mu\text{M}$ respectively), compound III was clearly more potent on the human enzyme ($IC_{50} = 0.09\mu\text{M}$) than on the rat ortholog ($IC_{50} = 0.9\mu\text{M}$). The mode of inhibition of compound III remains unknown and the biochemical characterization revealed that of compound II to be mostly competitive of PGH_2 [164]. There is no report regarding why compound II and III have inhibitory potential on rat mPGES-1, but most likely, their structure allows them to overcome the added bulkiness of the rat enzyme's active site.

As presented in papers IV and V, compounds II and III are also the only mPGES-1 inhibitors reported to inhibit PGE_2 synthesis *in vivo* in native murine models. We addressed their potential in the treatment of inflammation related to arthritis using the air pouch and adjuvant-induced arthritis (AIA) models. The involvement of the inducible PGE_2 pathway is well characterized for both models [143, 165] and they were previously used in the development of NSAIDs [165, 166].

The air pouch model was originally developed as a model to study synovial inflammation [138]. The pouch lining is populated by type A (macrophage-like) and type B (fibroblast-like) cells and scattered blood vessels [138]. Forming a closed cavity, the mature air pouch is very useful to study a local inflammatory reaction.

AIA, in the form we used, involves both an acute (injected paw) and a delayed (contra-lateral paw) inflammatory response. mPGES-1 and COX-2 are up-regulated in the injected paw throughout the course of inflammation, but they are not up-regulated in the inflamed contra-lateral paw [143]. In paper IV, compound II reduced the paw swelling in both the ipsilateral and contra-lateral paws. The inhibition of edema in the contra-lateral paw suggests an impact of compound II on general mechanisms of inflammation away from the paw. As mentioned previously, PGE₂ can synergize with IL-23 to enhance Th17 expansion. Interestingly, in AIA rats an IL-17 response is detectable in serum before disease onset [167]. Thus, the effect seen with compound II on contra-lateral paw inflammation could be through inhibition of the IL-17 response.

In reaction to the report that cardiovascular complications associated to long-term COXibs treatment were likely caused by a modulation of the blood TXB₂/PGI₂ ratio [168], scientists working on mPGES-1 as a therapeutic target have been trying to determine the impact of mPGES-1 inhibition on the prostanoid profile.

Reports about the faith of PGH₂ in systems where the mPGES-1 gene has been deleted or after treatment with an mPGES-1 inhibitor, point to an assay-dependent outcome where cell type, activation state and genetic background can all have an impact [72, 112, 117, 121, 123, 169]. This is most likely due to the relative expression of PG synthases, which differs from one cell type to the next. Additionally, heterogeneity in the expression of prostanoid receptors in different cells should also be considered, as there exist regulatory feedback loops in the prostanoid pathway [41]. Interestingly, considering the complexity of the arachidonic acid cascade, Kihara et. al. developed a targeted lipidomics and transcriptomics approach to monitor eicosanoid levels as well as the expression of enzymes and receptors of the arachidonic acid cascade simultaneously in a given tissue [77].

Using several of the *in vitro* and *in vivo* inflammation assays reported in paper IV and V, we have investigated the impact of mPGES-1 inhibition with compound II and III on the prostanoid profile. The results, in line with the previous investigations mentioned above, turned out to be dependent on experimental conditions. Compound II triggered a general down-regulation of prostanoid synthesis in rat peritoneal macrophages while it caused a significant reduction of 6-keto PGF_{1 α} and PGD₂ levels in the rat air pouch model. Compound III on the other hand was associated with a shunting of PGH₂ into the prostacyclin pathway in the A549 short incubation assay and a trend towards general down-regulation of prostanoid synthesis in the mouse air pouch model.

In light of the heterogeneity of results concerning the impact of mPGES-1 inhibition on the prostanoid profile, a conclusion could be that a characterization can only be useful and conclusive if it is performed in the exact system where the outcome is of interest. If the cardiovascular side effects of mPGES-1 inhibition are in focus, a

prostanoid profile should be performed in the blood circulation of treated animals. If the outcome of mPGES-1 inhibition on the inflamed synovium is investigated, a prostanoid profile should be performed in the air pouch model, as presented for compound II and III in papers IV and V. With the same rationale, if we want to compare the data we generated on the impact of compound II and III on the prostanoid profile at the site of inflammation with the data generated with other mPGES-1 inhibitors, it should only be done if the exact same system was used. The A549 long incubation assay we used for the characterization of compound III was also used by Merck-Frosst for MF63. Both compounds inhibited PGE₂ synthesis in a concentration-dependent manner, causing a shunt of excess PGH₂ into the PGF_{2α} pathway [117] (figure 3).

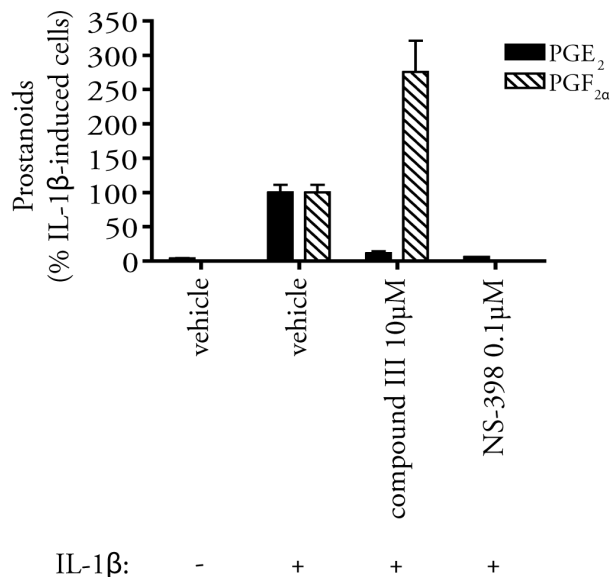


Figure 3: Inhibition of mPGES-1 with compound III in A549 cells stimulated with IL-1 β for 24h exposed a shunt of PGH₂ to the PGF_{2 α} pathway. This confirms results obtained by Merck with MF63.

If the impact of mPGES-1 inhibition on the PG profile in a given system should be consistent from one inhibitor to the next, provided they are specific, one should be careful when comparing gene deletion and enzyme inhibition results. Gene knockout of one enzyme of the arachidonic acid pathway has previously been shown to alter the expression pattern of other enzymes. In the brains of COX-2 knockout mice, the basal expression of cPLA₂, sPLA₂ and COX-1 are up-regulated while that of mPGES-2 is down-regulated, highlighting a compensatory mechanism developed in that genetically modified animal [170]. Moreover, in the case of pharmacological inhibition of mPGES-1, an impact different from that seen with mPGES-1 gene deletion can be expected. In contrast to gene deletion, pharmacological inhibition is to a certain extent partial, and it leaves the mPGES-1 enzyme expression unaltered. In paper V and VI we have therefore investigated mPGES-1 inhibition as well as genetic deletion in LPS-induced peritoneal macrophages, to verify if they had equivalent effects on the PG profile.

While mPGES-1 knockout macrophages experienced a clear redirection of PGH₂ into thromboxane synthesis, compound III caused a shunt into the prostacyclin pathway. We have also compared the two in the mouse air pouch model using mPGES-1 wild type and knockout mice. While mPGES-1 gene deletion was associated with shunting of PGH₂ into the thromboxane pathway, mPGES-1 inhibition led to a trend of general down-regulation of the other prostanoids present. One explanation for the differences could be an alteration in the expression of enzymes of the prostanoid pathway, as characterized for COX-2 knockout mice or simply that mPGES-1 pharmacological inhibition is partial and not sufficient for shunting to occur. Therefore, in the study of mPGES-1 as a therapeutic target, it will be very important to complement results obtained from studies using knockout mice with investigations using mPGES-1 inhibitors in the wild type system.

Revisiting the issue of the PG profile resulting from mPGES-1 inhibition at the site of inflammation, one scenario has been proposed by several scientists: the redirection of PGH₂ into the PGD₂ pathway. One metabolite of PGD₂ is 15-deoxy- $\Delta^{12,14}$ PGJ₂, an anti-inflammatory molecule which exerts its actions in part through modulation of the PPAR γ pathway [171]. Therefore, according to this scenario, if mPGES-1 inhibition would not only attenuate pro-inflammatory pathways, but importantly also redirect excess PGH₂ into an anti-inflammatory pathway (see figure 1). In paper VI, to investigate the potential repercussion of mPGES-1 gene deletion on the arachidonic acid cascade, we have performed the eicosanoid profile of wild type versus knockout mouse peritoneal macrophages between 4 and 16 hours after LPS-induction. 15-deoxy- $\Delta^{12,14}$ PGJ₂ was found elevated in knockout compared to wild-type macrophages at 8 and 12h post-induction. This result suggests a redirection of PGH₂ into the PGD₂ pathway is indeed occurring in this system.

If mPGES-1 inhibition and gene deletion can cause an alteration of the prostanoid profile, another plausible scenario to be considered is that it could also affect the levels of precursors of PGE₂ and COX enzyme substrates such as arachidonic acid and eicosapentaenoic acid (EPA) in the phospholipid bilayer, and thus eventually alter the lipid composition of systems where it is normally active. Such an outcome has already been reported for cPLA₂ and COX-2 knockout mice. These altered lipid profiles, together with gene compensatory mechanisms, led to differences in the arachidonic acid metabolism of knockout animals [172, 173].

In paper VI, we therefore investigated if mPGES-1 gene deletion also altered the fatty acid profiles of the brain and spleen under homeostatic and pro-inflammatory conditions. We specifically investigated brain and spleen fatty acid profiles, since the brain was the subject of the previous reports and the spleen has a constitutive expression of mPGES-1 [112]. Under homeostatic conditions, the brain fatty acid profile of mPGES-1 knockout mice was not different from that of wild type animals. Their spleen fatty acid profile, however, exposed a significant reduction in the total levels of monounsaturated fatty acids (MUFA) and an increase in the Polyunsaturated fatty acids (PUFA) eicosadienoic acid (EDA). The latter has previously been shown to alter the responsiveness to inflammatory stimuli [174]. Together, the results presented in paper VI point to several mechanisms by which mPGES-1 inhibition could

contribute to both a reduction of inflammation and an enhanced contribution of anti-inflammatory pathways. It will be of primary interest to validate these results obtained in mPGES-1 knockout mice with corresponding mPGES-1 inhibitor studies.

7 FUTURE PERSPECTIVES

This thesis has contributed to the characterization of the PGE₂ pathway in the pathology of arthritis and to the validation of mPGES-1 as a therapeutic target in inflammation. More importantly, it has generated the next series of questions to be answered in this field of research and produced tools, in the form of compound II and III, which will be instrumental in addressing some of them.

The study presented in paper I could not establish a correlation between the expression of enzymes of the PGE₂ pathway and pain or disease development in pre-clinical and early clinical arthritis. Nevertheless, it would be interesting to investigate whether the PGE₂ pathway is associated to other disease parameters. For example, VEGF-induced angiogenesis and Th17 development are two processes known to occur in RA and for which mPGES-1 plays a role [69-71]. It would therefore be useful to determine if mPGES-1 expression can be associated with markers of these two processes in RA patient material. In the event of a correlation, compound II and III could then be used to investigate the impact of mPGES-1 inhibition on the two processes in CIA, a model in which VEGF-derived angiogenesis [175] and Th17 cells [74, 75] are both reported to influence disease progression.

The results presented in paper III highlight an incapacity for MTX treatment, despite its efficacious treatment of RA, to impact the up-regulation of mPGES-1 and COX-2 in the RA synovium. This phenomenon also applies for two other RA therapeutic approaches, TNF blockers and B-cell depletion therapy, making three of the most effective RA therapies powerless against the inducible PGE₂ pathway [67, 68]. One way to further characterize the therapeutic potential of mPGES-1 inhibition would be to evaluate the effect of compounds II and III in combination with MTX, TNF blockers or B-cell depletion therapy in a relevant model of arthritis i.e. a model in which MTX, TNF blockers or B-cell depletion therapy also leave the induction of mPGES-1 and COX-2 unaffected.

Alternatively, in paper II we characterized a novel mechanism by which mPGES-1 and COX-2 are up-regulated in RASFs and which involves HMGB1. The therapeutic potential of HMGB1 has already been evaluated in animal models of arthritis using monoclonal antibody therapy. It was shown to reduce both inflammation and tissue destruction. It would be important to determine if HMGB1 blocking therapy reduces the expression of mPGES-1 and COX-2. Such a finding would make it a candidate for a combinatorial approach with MTX, TNF blockers or B-cell depletion therapy.

Compounds II and III described in papers IV and VI are some of the first mPGES-1 inhibitors with a reported activity *in vitro* and *in vivo* on murine mPGES-1. This activity is crucial in the study of mPGES-1 as a therapeutic target in the various pathologies

presented in section 3. mPGES-1 inhibition has already been investigated in models for a number of those diseases, including CIA and EAE, using the mPGES-1 knockout mouse. However, as pointed out by the PG profile of air pouch exudates in paper V, the partial reduction in PGE₂ synthesis achieved by pharmacological inhibition does not necessarily have the same impact as the complete reduction achieved by mPGES-1 gene deletion. It will therefore be very interesting to evaluate the impact of compound II and III in disease models where mPGES-1 is implicated including those for which the question was already investigated using the mPGES-1 knock out mouse.

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