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Expression of Prostaglandin E Synthase-1 in Periodontitis
in vivo and in vitro studies

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EXPRESSION OF PROSTAGLANDIN E SYNTHASE-1 IN PERIODONTITIS - IN VIVO AND IN VITRO STUDIES

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"If we knew what it was we were doing, it would not be called research, would it?"
-Albert Einstein

To my family
Abstract

Periodontitis is a chronic inflammatory disease characterized by destruction of the supporting structures of the teeth, including gingival tissues and alveolar bone. In advanced cases of periodontitis the ultimate clinical outcome is tooth loss. The inflammatory mediator prostaglandin E$_2$ (PGE$_2$) plays a central role in the pathogenesis of periodontitis and elevated levels of PGE$_2$ have been observed in gingival crevicular fluid and gingival tissues from patients with periodontitis. The biosynthesis of PGE$_2$ involves three groups of enzymes acting sequentially; phospholipase A$_2$, cyclooxygenases (COX-1 and COX-2) and prostaglandin E synthases (PGES). The PGES enzymes, catalyzing the terminal step in PGE$_2$ biosynthesis, exists in three distinct isoforms; the inducible microsomal membrane-associated and glutathione dependent PGES (mPGES-1); the constitutively expressed cytosolic PGES (cPGES) and the glutathione-independent, membrane-associated PGES (mPGES-2). The aim of this thesis was to investigate the expression of PGES, especially mPGES-1 in gingival tissues and gingival fibroblasts. Furthermore, we also aimed to identify and study the effect of novel mPGES-1 inhibitors on PGE$_2$ synthesis in gingival fibroblasts, experimental periodontitis in rats and osteoclastogenesis using RAW 264.7 cells stimulated with receptor activator of NF-κB ligand (RANKL).

In gingival tissues collected from patients with periodontitis, we demonstrated protein expression of mPGES-1, mPGES-2, cPGES and the upstream enzyme COX-2 in fibroblasts, endothelial cells, smooth muscle cells, epithelial cells and immune cells. In cell cultures of human gingival fibroblasts, the mRNA and protein expression of mPGES-1 as well as COX-2 accompanied by subsequent PGE$_2$ production was increased by pro-inflammatory cytokines; tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β). To further investigate additional cell types contributing to elevated levels of PGE$_2$, we used cultures of human airway smooth muscle (HASM) cells, human umbilical vein endothelial cells (HUVECs) and mast cells as a model system. Our results showed that mPGES-1 and COX-2 expression, as well as PGE$_2$ production, was increased by the cytokines, IL-1β and TNFα in HASM cells. In HUVECs, only TNFα increased PGE$_2$ production via an up-regulated expression of COX-2. In mast cells, the expression of PGES and COX-2 was not affected by cytokines and PGE$_2$ production was only observed at basal level. Collectively, these findings suggest that gingival fibroblasts together with smooth muscle cells may contribute to elevated levels of PGE$_2$ in inflamed gingiva.

To target mPGES-1 in the context of periodontitis, novel mPGES-1 inhibitors, aminothiazoles, were identified. Studies on PGE$_2$ synthesis in gingival fibroblasts revealed that cytokine-induced PGE$_2$ production was inhibited by the aminothiazoles TH-848 and TH-644. IL-1β-induced mPGES-1 mRNA expression was not affected by aminothiazoles, whereas protein expression was slightly decreased by TH-848 but not by TH-644. In addition, IL-1β-induced expression of COX-2 was not affected by aminothiazoles either at the mRNA or protein level. Similarly, other isoenzymes of PGES, mPGES-2 and cPGES
were not affected either by cytokines nor aminothiazoles in gingival fibroblasts. In an *in vitro* assay for mPGES-1 enzyme activity, TH-848 and TH-644 inhibited mPGES-1 activity without affecting COX-2 activity.

In ligature-induced experimental periodontitis in rats, topical treatment with the aminothiazole TH-848 reduced alveolar bone resorption compared to vehicle-treated controls. Furthermore, *in vitro* studies of aminothiazoles on osteoclastogenesis demonstrated a decreased number of osteoclasts in parallel with decreased PGE$_2$ production in RANKL-stimulated RAW 264.7 cells.

In conclusion, all three PGE synthases were expressed in gingival tissue from patients with periodontitis. The isoenzyme mPGES-1 regulated the cytokine-induced PGE$_2$ production in gingival fibroblasts and smooth muscle cells. The novel mPGES-1 inhibitors aminothiazoles inhibited PGE$_2$ production in gingival fibroblasts, reduced alveolar bone resorption in experimental periodontitis and decreased osteoclastogenesis in RANKL-activated RAW 264.7 cells. This suggested that mPGES-1 is an important regulatory enzyme in inflammation-induced PGE$_2$ production and that inhibition of mPGES-1 might be an attractive treatment target for chronic inflammatory bone destruction, such as periodontitis.
List of publications


*These authors contributed equally to this work.


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<td>complementary DNA</td>
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<td>c-Fms</td>
<td>colony-stimulating factor-1 receptor</td>
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<td>cyclooxygenase</td>
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<td>DEX</td>
<td>dexamethasone</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EIA</td>
<td>enzyme immunoassay</td>
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<td>EP</td>
<td>PGE(_2) receptor</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GCF</td>
<td>gingival crevicular fluid</td>
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<td>GSH</td>
<td>glutathione</td>
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<td>HASM</td>
<td>human airway smooth muscle</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>IL</td>
<td>interleukin</td>
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<td>interleukin-1(\beta)</td>
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<td>lipopolysaccharide</td>
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<td>macrophage colony-stimulating factor</td>
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<td>matrix metalloproteinase</td>
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<td>membrane-associated prostaglandin E synthase-2</td>
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<td>MTT</td>
<td>3-[[4,5-dimethylthiazol-2-yl]-2,5diphenytlazo]lumbium bromide</td>
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<td>NF-(\kappa B)</td>
<td>nuclear factor-(\kappa B)</td>
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<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>PDL</td>
<td>periodontal ligament</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin D&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>PGES</td>
<td>prostaglandin E synthase</td>
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<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RANK</td>
<td>receptor activator of NF-κB</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of NF-κB ligand</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>TH-644</td>
<td>4-((3-fluoro-4-methoxyphenyl)-N-(4-phenoxyphenyl)-1,3-thiazol-2-amine</td>
</tr>
<tr>
<td>TH-848</td>
<td>4-((4-(2-naphthyl)-1,3-thiazol-2-yl)amino)phenol</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinases</td>
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<tr>
<td>TNF&lt;sub&gt;α&lt;/sub&gt;</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate resistant acid phosphatase</td>
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<tr>
<td>TXA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
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Introduction
Inflammation is an essential mechanism that protects us from our environment. This natural defense towards, for example bacterial invasion during tissue damage, helps us to overcome this threat and begin the healing process. However, if the acute phase of inflammation does not resolve, it becomes a chronic pathological condition associated with persistent inflammation and tissue destruction. The chronic inflammatory disease periodontitis is characterized by irreversible destruction of the supporting structures of the teeth. It is generally accepted that periodontitis is initiated by microbial products that activate a host immune inflammatory response leading to activation of immune cells and resident cells such as, gingival fibroblasts. Collectively, these cells overexpress inflammatory mediators, including cytokines and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), which contribute to tissue and bone destruction. The inflammatory mediator PGE\textsubscript{2} is key player in inflammation and inhibition of this mediator is one of the main therapeutic targets for management of chronic inflammatory conditions. The focus of this thesis has been to study the expression and inhibition of the terminal enzymes responsible for PGE\textsubscript{2} production, prostaglandin E synthases (PGES), in periodontal tissues. This section gives a brief background about periodontitis and prostaglandins focusing on the PGES enzymes, especially the inducible isoform of PGES, mPGES-1.
Periodontal disease

Periodontal diseases are inflammatory conditions that affect the supporting structures of the teeth. Besides dental caries, periodontal disease is one of the most prevalent diseases in the oral cavity worldwide. The name ‘periodontal disease’ is a collective name for gingivitis and periodontitis. Gingivitis is common inflammation of the gingiva normally caused by accumulation of dental plaque and in healthy individuals this condition is reversible after improved dental hygiene. Periodontitis occurs when the inflammation extends deeper into the tissue and becomes chronic leading to a non-reversible destruction of the supporting structures of the teeth. The typical characteristics of periodontitis are destruction of periodontal ligaments, gingival connective tissue and alveolar bone, and in advanced cases eventual tooth loss. Mild to moderate periodontitis affects approximately 20-50% of the adult population, whereas severe, generalized periodontitis affects 5-15% of adults depending on the study population, socio-demographics and the definition used to diagnose periodontitis. Periodontitis is not a life-threatening disease and in most cases, especially at early stages, the patients rarely experience any symptoms, but as the disease progresses patients can experience sporadic pain and discomfort, and eventually tooth loss. However, periodontitis is a multi-factorial disease associated with both environmental risk factors as well as genetic predisposition (Figure 1). The most recognized environmental risk factor is smoking, since it has been reported that smokers are much more susceptible to develop periodontitis. It has also been stated that there are associations between periodontitis and other systemic inflammatory diseases including rheumatoid arthritis (RA), cardiovascular diseases, diabetes and obesity.

The pathogenesis of periodontitis is complex and involves interactions between bacterial products, host immune-response and inflammatory mediators as well as environmental and genetic risk factors (Figure 1). Bacterial components of the biofilm initiate an inflammatory cascade, including infiltration of immune cells and production of inflammatory mediators in the periodontal tissue, resulting in soft tissue degradation and bone destruction. Periodontal therapy today is mainly focused on the management of the microbial biofilm, not considering the central role of inflammation as a driver of tissue damage, which makes this therapy only partly effective. It has therefore been suggested that new and more efficient treatment options, based on modulation of the inflammatory response together with direct control of the microbial biofilm, are needed for the management of periodontal disease.
Figure 1. Schematic model of the pathogenesis of periodontitis. Periodontitis is a complex disease that is mediated by microbial challenge and the host immune-inflammatory response. In addition, environmental and genetic risk factors may affect the host immune response as well as connective tissue and bone metabolism resulting in clinical signs of periodontal disease. This illustration is adapted from Page and Kornman 1997.14

Abbreviations: LPS, lipopolysaccharides; MMPs, matrix metalloproteinases; PMNs, polymorphonuclear leukocytes.

Host immune response

The microflora of the oral cavity consists of more than thousand bacterial species.15 On the tooth surface the bacteria grow as complex, mixed, interdependent colonies densely packed against the tooth in the deeper layers, with more motile forms in the superficial layers.1 During the progression of periodontitis the composition of the biofilm in deeper pockets shifts to contain more gram-negative and anaerobic bacteria including the “red complex” pathogens Tannerella forsythia, Porphyromonas gingivalis and Treponema denticola. The presence of “red complex” species in subgingival plaque are strongly associated with the severity of the disease, such as deeper periodontal pockets and bleeding on probing.16,17 The microbial pathogens of the biofilm produce bacterial components, such as lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acids, proteases and toxins, which instigate an inflammatory reaction.18-20 This bacterial challenge activates and stimulates various inflammatory cell types as well as resident cells of the gingival tissue. Bacterial antigens and products, such as LPS and peptidoglycans, are recognized by toll-like receptors expressed on the surface of resident cells as well as leucocytes in the periodontal environment, which initiates an inflammatory response.21

The inflammatory response consists of a series of events in the periodontal tissue (Figure 2). Mast cells are stimulated to release vasoactive amines and pre-formed tumor-necrosis factor α (TNFα), contributing to increased vascular permeability and increased expression of adhesion molecules.22,23 Polymorphonuclear leukocytes (PMNs) are recruited into the tissue, where they release lysosomal enzymes contributing to the degradation of
the connective tissue. Lymphocytes and macrophages begin to invade the tissue and start to synthesize and secrete molecules with pro-inflammatory and catabolic activities, including cytokines interleukin-1 (IL-1) and TNFα, prostaglandins, especially PGE$_2$, and hydrolytic enzymes including matrix metalloproteinases (MMPs). At an early stage, the collagen in the connective tissue is degraded at the affected sites, but the bone is still intact. The condition is reversible at this point and the tissue can be repaired without permanent damage. In individuals with genetic predisposition and/or the influence of environmental factors such as smoking, the inflammation fails to resolve. In these cases, the connective tissue breaks down and irreversible bone loss is initiated. The invading inflammatory cells together with resident cells drive the process of inflammation and tissue destruction (Figure 2).

**Figure 2. Overview of processes involved in the tissue and bone destruction in periodontitis.** 1) Periodontal pathogens release microbial products, such as LPS that are recognized by resident cells including gingival fibroblasts. 2) Resident cells produce pro-inflammatory cytokines and chemokines that recruit immune cells to the periodontal tissue. 3) The immune cell infiltrate together with resident cells results in enhanced production of pro-inflammatory mediators. 4) Activated immune cells as well as resident cells, such as osteoblasts, produce RANKL which binds to its receptor RANK and activates osteoclastogenesis. 5) Increased levels of RANKL in the tissue may lead to an imbalance in the RANKL/OPG ratio resulting in increased bone resorption. 6) Inflammatory mediators produced by immune cells also affect osteoblasts and the process of bone formation to counteract increased osteoclastogenesis. 7) The local chronic inflammatory reaction results in connective tissue breakdown by destruction of the ECM due to increased production of MMPs and decreased levels of TIMPs, inhibitors of MMPs. Reprinted with the permission from the publisher, from Garlet 2010. Abbreviations: ECM, extracellular matrix; LPS, lipopolysaccharides; MMP, matrix metalloproteinase; OPG, osteoprotegerin; TIMP, tissue inhibitor of metalloproteinase; RANK, receptor activator of NF-κB; RANKL, RANK ligand.
The endothelial cells and smooth muscle cells of the vessels are the first to come in contact with circulatory inflammatory cells. Endothelial cells express surface adhesion molecules in response to TNFα, and thereby attract inflammatory cells to the site of inflammation. 

Gingival fibroblasts, the most ubiquitous resident cell type in gingival connective tissue, is primary responsible for the synthesis and remodeling of extracellular matrix (ECM) in the connective tissue. During an inflammatory response, fibroblasts are activated to produce cytokines, chemokines, MMPs and prostanoids such as PGE2, and thereby contribute to the progression and persistence of the disease. Fibroblasts also contribute during the resolution of inflammation by modulating immune cell behavior and normalize the chemokine gradients, and thereby allowing the infiltrating leukocytes to undergo apoptosis or leave the tissue through the draining lymphatic system. The periodontal ligament (PDL) fibroblasts, located between the tooth and the alveolar bone, are in close contact with the biofilm in the periodontal pocket and are also involved in early recruitment of leucocytes in the inflammatory response. The characteristics of PDL fibroblasts are complex including, cementoblast-like and/or osteoblast-like properties, such as expression of bone-associated markers, modulation of osteoclastogenesis, and the ability to form cementum-like tissues. In addition, these cells produce inflammatory mediators, such as cytokines and chemokines contributing to periodontal inflammation and alveolar bone resorption.

Destruction of alveolar bone and connective tissue in periodontitis is mediated by several factors including MMPs and receptor activator of nuclear factor-κB ligand (RANKL). The proteolytic enzymes MMPs contribute to the degradation of ECM components, such as collagen. The regulation and inhibition of MMPs activity is mediated by their endogenous inhibitors tissue inhibitors of matrix metalloproteinases (TIMPs) (Figure 2). RANKL, the key factor in bone destruction, is regulated by inflammatory mediators, cytokines as well as other modulators of bone metabolism including parathyroid hormone and vitamin D3. Inflammatory mediators and bone resorption is further described in the following sections.

**Inflammatory bone resorption**

Bone remodeling is a lifelong process where mature or damaged bone is resorbed by osteoclasts and new bone is formed by osteoblasts. Under normal/healthy conditions there is a balance between bone formation and bone resorption. This system is regulated by macrophage colony-stimulating factor (M-CSF), RANKL, receptor activator of nuclear factor-κB (RANK), and osteoprotegerin (OPG) (Figure 3). In addition, numerous cytokines have been demonstrated to affect, directly or indirectly, the bone metabolism by stimulation or inhibition of osteoclast formation and activity.
Figure 3. Schematic overview of inflammatory-mediated osteoclastogenesis. Increased production of RANKL and M-CSF, and decreased levels of OPG stimulates osteoclastogenesis. M-CSF activates monocytes/macrophages via its receptor c-Fms. The binding of RANKL to receptor RANK activates pre-osteoclasts to differentiate and mature into bone resorbing osteoclasts. This process is stimulated by inflammatory mediators including cytokines (IL-1, TNFα, IL-6) and PGE₂. Modified from Stepieen 2011.36

Abbreviations: c-Fms, colony-stimulating factor-1 receptor; IL-1, interleukin-1; IL-6, interleukin-6; M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; PGE₂, prostaglandin E₂; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TNFα, tumour necrosis factor α.

During inflammatory bone resorption, osteoclast activity is enhanced without a corresponding increase in bone formation, occurring when the resolution of inflammation fails. Pro-inflammatory cytokines and mediators including TNFα, IL-1β, IL-6 and PGE₂, produced by immune cells and resident cells, are involved in bone resorption by amplifying osteoclast differentiation and activation.10,35 Osteoclastogenesis, stimulated by pro-inflammatory mediators, is mediated by cells of the monocyte/macrophage cell lineage. These cells form pre-osteoclasts/progenitors that fuse into polykaryons consisting of 10-20 cells, and thereafter differentiate and mature into bone resorbing osteoclasts.10 Local osteoclastogenesis is mediated by activated T cells, B-cells, fibroblasts and osteoblast precursors that overexpress RANKL and M-CSF.10,31,37,38 M-CSF activates the macrophages/monocytes via colony-stimulating factor-1 receptor (c-Fms) and thereby enhances the proliferation and survival of the progenitor cells.31 RANKL is a member of the TNF cytokine family, normally produced by osteoblast/osteocytes but also by synovial and PDL fibroblasts in vivo.31 When RANKL is recognized by the RANK receptors,
expressed on the surface of osteoclast precursors, these cells starts to differentiate and mature into bone resorbing osteoclasts.\textsuperscript{31,37} Osteoclastogenesis is regulated by the soluble RANKL decoy receptor, OPG.\textsuperscript{39} Increased secretion of OPG neutralizes the effect of RANKL, disabling RANKL to bind the receptor RANK on pre-osteoclasts resulting in reduced osteoclastogenesis.\textsuperscript{39} A schematic illustration of the osteoclastogenesis is described in Figure 3.

Osteoclasts, formed by fusion of cells from the monocyte/macrophage cell lineage, are considered to be the principal cell type responsible for bone resorption.\textsuperscript{35} Typical characteristics for osteoclasts are the high expression of tartrate resistant acid phosphatase (TRAP) and Cathepsin K. TRAP is one of the most renowned histochemical markers for osteoclasts. The function of TRAP in biological processes includes skeletal development, collagen synthesis and degradation, mineralization of bone, cytokine production by macrophages and dendritic cells, macrophage recruitment and dendritic cell maturation.\textsuperscript{40} The primary function of Cathepsin K, also used as a common marker of activated osteoclasts, is to degrade type I collagen, the main component of bone matrix.\textsuperscript{41}

\textbf{Inflammatory mediators}

In the complex process of inflammation, interactions between numerous cell types and inflammatory mediators are central. Several pro-inflammatory mediators including TNF\textalpha, IL-1, IL-6 and PGE\textsubscript{2} have been demonstrated to be involved in the pathogenesis of periodontitis.\textsuperscript{9,20,42-46} Elevated levels of these mediators have been found in gingival crevicular fluid (GCF) and periodontitis-affected gingival tissue,\textsuperscript{42,47-49} which can decrease after periodontal therapy.\textsuperscript{46,50,51} In animal studies, it has been demonstrated that administration of the cytokines TNF\textalpha or IL-1 exacerbates experimental periodontitis in rats.\textsuperscript{52,53} In addition, soluble receptors of IL-1 and TNF have been shown to inhibit the progress of periodontitis in a primate model.\textsuperscript{54,55}

TNF\textalpha is involved at an early stage in the inflammatory cascade, as it is released from mast cells in response to bacterial stimuli such as LPS.\textsuperscript{18} However, both TNF\textalpha and IL-1 are synthesised by many cell types in periodontal tissues, including monocytes/macrophages, PMN cells, fibroblasts, epithelial cells, endothelial cells and osteoblasts.\textsuperscript{56} The up-regulated levels of TNF\textalpha and IL-1 can further stimulate the release of several other inflammatory mediators, such as IL-6, IL-8, MMPs and PGE\textsubscript{2}.\textsuperscript{20} Increased amount of PGE\textsubscript{2} detected in patients with periodontitis is associated with connective tissue destruction and bone resorption.\textsuperscript{9,42-45} The role of PGE\textsubscript{2} in inflammation and periodontitis is further described in the next section.
Prostaglandins

Prostaglandins (PG) were first discovered and isolated from seminal fluid in 1935, and therefore believed to be a part of the prostatic secretion.\[57\] The name 'prostaglandin' derives from the origin of the substances, which was the prostate gland.\[57\] Later it was shown that many tissues produce PGs, in fact almost all nucleated cells produce PGs for different functions.\[58\] The first total syntheses of PGF\(_{2\alpha}\) and PGE\(_2\) were reported in 1969, and in 1971 it was determined that aspirin-like drugs could inhibit the synthesis of PGs.\[59\] In 1982, researchers from Karolinska Institutet, Sune Bergström and Bengt Samuelsson together with Sir John Vane from London University received the Nobel Prize in Physiology or Medicine for their research on PGs.

PGs are members of the prostanoid subclass that also includes thromboxanes and prostacyclines. PGs are described as mediators of inflammatory and anaphylactic reaction; thromboxanes are mediators of vasoconstriction; and prostacyclins are active in the resolution of inflammation. The overall function of the prostanoids is to act as autocrine and paracrine lipid mediators to maintain local homeostasis in the body.\[60\]

The prostanoids are ubiquitously produced by different cell types and usually each cell type produces 1 or 2 dominant products.\[60,61\] During an inflammatory response, both the amount and the profile of prostanoid synthesis change dramatically.\[60\] PG production is generally very low in uninflamed tissues but increases immediately in acute inflammation before the recruitment of leukocytes and the infiltration of immune cells.\[60\] Prostanoid biosynthesis starts with the conversion of membrane phospholipids to arachidonic acid (AA), which can further be metabolized by a range of enzymes to different prostanoids. AA can also be converted to leukotrienes, which are produced mainly in inflammatory cells and are involved in allergy and asthma as well as in sustaining inflammatory reactions.\[62,63\] An overview of prostanoid biosynthesis is illustrated in Figure 4.

PGs are secreted from the cells through the plasma membrane by a PG transporter protein.\[64\] The action of secreted PGs depends on the expressed PG receptors on the surface of the target cells. There are currently nine identified PG receptors. Most of these are G-protein-coupled receptors mainly expressed on the plasma membrane, but can also be localized to the nuclear envelope.\[65\] The most described PG in the literature is the PGE\(_2\). This mediator has been implicated in many different biological processes throughout the human body, such as regulation of inflammatory and immune responses, blood pressure, gastrointestinal integrity and fertility.\[66\] PGE\(_2\) has also been described as a key mediator in chronic inflammatory diseases such as RA, atherosclerosis and periodontitis.\[62,67-69\]
Figure 4. Overview of prostanoid biosynthesis. The metabolite AA derived from membrane phospholipids by PLA$_2$, is further converted to prostaglandins (PGE$_2$, PGD$_2$ and PGF$_{2\alpha}$), prostacyclin (PGI$_2$) or thromboxanes (including TXA$_2$) as well as leukotrienes.

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; PGD$_2$, prostaglandin D$_2$; PGE$_2$, prostaglandin E$_2$; PGES, prostaglandin E synthase; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; PGH$_2$, prostaglandin H$_2$; PGI$_2$, prostacyclin; PLA$_2$, phospholipase A$_2$; TXA$_2$, thromboxane A$_2$; 15d-PGJ$_2$, 15-deoxy-Δ12,14-prostaglandin J$_2$.

Prostaglandin E$_2$

PGE$_2$ is one of the most abundant PGs produced in the body. In inflammation, PGE$_2$ is involved in all processes leading to the classic signs of inflammation such as redness, heat, swelling and pain. This mediator is also involved in the pathogenesis of chronic inflammatory conditions including periodontitis, RA and cardiovascular diseases.$^{42,67,69,70}$ In periodontitis, increased levels of PGE$_2$ have been detected in GCF and gingival tissue from patients with periodontitis compared to periodontally healthy controls.$^{9,42-45}$ Elevated levels of PGE$_2$ in GCF correlate with the severity of periodontitis, measured by attachment loss.$^{47}$ Accordingly, it has been suggested that biomarkers such as PGE$_2$ in GCF may serve as a predictor of periodontal disease.$^{71}$ In experimental periodontitis, PGE$_2$ has been shown to exacerbate the disease, in contrast to the structurally related lipid mediator Resolvin E1, which has been shown to protect from osteoclast-mediated bone destruction and restore tissue homeostasis in a rabbit model.$^{72,73}$

The biological function of PGE$_2$ varies between different tissues of the body. In periodontal tissue, PGE$_2$ is produced by immune cells, fibroblasts and other resident cells of inflamed gingiva and has a wide range of biological effects including stimulation of
inflammation-associated bone resorption. PGE₂ is involved in the stimulation of inflammatory mediators, MMPs and RANKL leading to connective tissue breakdown and bone resorption. The effect of PGE₂ on a specific cell type depends on prostaglandin receptors, EP₁ through EP₄. The receptors EP₂ and EP₄ have been highlighted in the pathogenesis of periodontitis since they are reported to be involved in bone resorption.

PGE₂ has a central role in bone resorption. Elevated levels of PGE₂ alters bone metabolism towards enhanced osteoclastogenesis without a corresponding increase in bone formation. In addition, PGE₂ stimulates osteoblasts/osteocytes to increased RANKL production and decreased expression of OPG. PGE₂ can also directly affect osteoclasts to increase the expression of receptor RANK (Figure 3). Furthermore, it has been reported that PGE₂ synthesis may exert a dual effect on osteoblasts where low doses of PGE₂ increases OPG expression and thereby overwhelm RANKL expression, while higher concentrations of PGE₂ results in the converse effect. In addition to the role of PGE₂ in osteoclastogenesis, it has also been reported that PGE₂ promotes cementoblast-mediated cementoclastogenesis by regulating the expression of RANKL and OPG, further supporting the role of PGE₂ in the pathogenesis of periodontitis.

**Biosynthesis of PGE₂**

PGE₂ synthesis is part of the prostanoid biosynthesis that also includes the production of prostacyclines (PGI₂), PGD₂, PGF₂α and thromboxane A₂ (TXA₂) (Figure 4). The biosynthesis of PGE₂ is regulated by three groups of enzymes acting sequentially. The first group of enzymes, phospholipase A₂, is responsible for release and conversion of membrane lipids to AA. The second group, the cyclooxygenases (COX-1 and COX-2) converts AA to the intermediate and highly unstable PGH₂. The third group, prostaglandin E synthase (PGES), is responsible for catalyzing the conversion of COX-derived PGH₂ to PGE₂. In this thesis the PGES enzymes together with the upstream enzyme COX-2 have been central and are therefore further described in the sections below.

**Cyclooxygenase**

The COX enzymes are responsible for converting AA to PGH₂. This reaction is performed in two steps, each catalyzed by a separate active site of the enzyme. First, the COX active site catalyzes the formation of the intermediate PGG₂, and in the next step the peroxidase active site reduces PGG₂ to PGH₂. PGH₂ can further be converted into PGE₂, PGL₂, PGD₂, PGF₂α or TXA₂ (Figure 4).

There are two distinct isoforms of the COX enzymes described; COX-1 and COX-2. Both COX enzymes are located in the endoplasmic reticulum (ER) and the nuclear envelope, but COX-2 is more concentrated in the nuclear envelope. The two isoforms have 60 % homology in amino acid sequence and their three dimensional structures are
almost identical. A third isoform, COX-3 has been suggested but is controversial since it is a splice variant of the COX-1 gene, retaining an intron, which is not included in mRNA of COX-1. However, information about COX-3 is limited and the relevance of this COX variant in the human context has been questioned.

COX-1 is constitutively expressed and is reported to be unaffected by inflammatory stimuli in several cell types in vitro, including gingival fibroblasts. The main function of COX-1 is to maintain tissue homeostasis and this enzyme is primarily involved in immediate PG production. COX-2 on the other hand, is reported to be induced by inflammatory stimuli and mainly involved in delayed PG production. Conversely, COX-2 may be involved in immediate PG production if its expression has already been increased by inflammatory stimuli. Inflammatory stimuli, such as LPS, TNFα and IL-1β have been reported to increase the expression of COX-2 in several cell types including gingival fibroblasts and the osteoclast precursors RAW 264.7 cells. The importance of COX-2 has been indicated by its increased expression in several chronic inflammatory diseases including RA, cancer and Cohn’s disease. In addition, several reports demonstrate up-regulated COX-2 expression in inflamed gingiva from patients with chronic periodontitis, compared to gingival tissue from healthy subjects. To date, COX-2 is the primary target for clinically approved anti-inflammatory drugs such as non-steroid anti-inflammatory drugs (NSAID) and selective COX-2 inhibitors. Anti-inflammatory drugs targeting the PGE2 synthesis are further described in the section "Inhibition of prostaglandin E2 synthesis".

Microsomal prostaglandin E synthase-1

The terminal enzymes responsible for PGE2 production are the PGES. There are three isoforms of PGES that have been identified and cloned; membrane-associated microsomal PGES (mPGES-1), membrane-associated PGES (mPGES-2) and cytosolic PGES (cPGES).

mPGES-1 was first identified and described in 1999 by researchers at Karolinska Institutet. This PGE2 regulatory enzyme is generally described as an inducible, membrane-associated, microsomal and glutathione (GSH)-dependent enzyme, since it requires GSH as a cofactor for its activity. mPGES-1 is located in the ER and in the perinuclear area and appears to be co-localized with COX-2. This may explain the fact that mPGES-1 couples predominantly with COX-2 and is involved in delayed PGE2 production.

The expression of mPGES-1 is reported to be induced by pro-inflammatory stimuli and considered to be the PGES isoform mainly responsible for inflammation-induced PGE2 production. However, constitutive expression of mPGES-1 in certain tissues and cell types has also been reported. The expression of the mPGES-1 is generally low under normal conditions in most tissues, but the levels of this enzyme increases considerably by inflammatory stimuli, such as TNFα, IL-1β or LPS. Increased expression of mPGES-1 has been observed in various tissues from patients with chronic
inflammatory diseases, including gastric ulcer tissue, RA-affected synovial tissue and in tumor-associated inflammation, highlighting the significance of these enzymes in chronic inflammation and cancers. 

mPGES-1 expression has also been reported in periodontally healthy gingiva and in experimental gingivitis tissue. Thus, the information about PGE synthases in periodontitis is limited.

In cell cultures, mPGES-1 can be induced by cytokines in several cell types, including cancer cell lines, orbital fibroblasts, synovial fibroblasts, smooth muscle cells and gingival fibroblasts. In cells involved in bone metabolism, mPGES-1 expression is upregulated in response to LPS in osteoblasts and mouse bone marrow derived macrophages. Moreover, the enhanced expression of mPGES-1, COX-2 as well as PGE2 production in response to LPS stimulation has been demonstrated in the macrophage cell line RAW 264.7.

In mPGES-1 deficient mice the significance of mPGES-1 has been established. In collagen-induced arthritis, mPGES-1 knockout mice exhibited a reduced incidence and disease severity compared to wild-type controls. In experimental periodontitis, LPS induce alveolar bone loss in wild-type mice but not in mPGES-1 knockout mice. Together, these findings demonstrate that deletion or inhibition of mPGES-1 markedly reduced inflammatory responses and bone resorption in mouse models.

**Cytosolic prostaglandin E synthase**

Cytosolic PGE synthase was characterized in 2000. Similar to mPGES-1, this enzyme requires GSH as cofactor for its activity. cPGES is expressed ubiquitously and in abundance in the cytosol of various cells including gastric and gingival fibroblasts. The expression of cPGES is constitutive and unaffected by pro-inflammatory stimuli in most cases, although some exceptions have been reported in models of neuroinflammation and animal studies showing LPS-induced cPGES expression in brain. However, studies of cPGES in knockout mice have not been informative about the role of cPGES-derived PGE2, since deletion of cPGES resulted in perinatal lethality with poor lung development, delayed skin maturation and growth retardation.

Similar to COX-1, cPGES is localized in the cytosol. The co-localization of these enzymes in ER allows cPGES to couple with proximal COX-1 in preference to the distal COX-2 in the perinuclear envelope. Since cPGES is capable of converting COX-1 but not COX-2 derived PGH2 to PGE2, suggests that the functions of cPGES in vivo overlap significantly, if not entirely, with COX-1 contributing to immediate PGE2 release.

**Microsomal prostaglandin E synthase-2**

The third isoform of PGE synthases, mPGES-2, was cloned and characterized in 2002. This isoform is also a membrane-associated protein and was therefore named mPGES-2. However, spontaneous cleavage of the N-terminal hydrophobic domain of the protein can
lead to the formation of a truncated mature protein that is distributed into the cytosol.\textsuperscript{91} mPGES-2 has distinct structural differences from mPGES-1 and unlike mPGES-1, this enzyme does not require GSH for its catalytic activity.\textsuperscript{101} The GSH-independent mPGES-2 has been reported to contribute to immediate as well as delayed PGE\textsubscript{2} synthesis.\textsuperscript{101,128,129} In contrast to both mPGES-1 and cPGES, this isoform can functionally couple both COX-1 and COX-2.\textsuperscript{91} Expression of this enzyme is mainly constitutive, but the inducibility varies between different tissues and cell types.\textsuperscript{112,124,130,131} In addition, a study of LPS-induced pre-term labor using mPGES-1 knockout mice, revealed that lack of mPGES-1 can be compensated by mPGES-2 resulting in inflammation-induced PGE\textsubscript{2} production in myometrium.\textsuperscript{132} On the other hand, mPGES-2 expression was not affected by LPS treatment in microglia derived from mPGES-1 knockout mice,\textsuperscript{133} highlighting the diverse role of mPGES-2 in different tissues. However, deletion of mPGES-2 gene in knockout mice does not result in decreased levels of PGE\textsubscript{2}, indicating that mPGES-2 is not essential for \textit{in vivo} PGE\textsubscript{2} synthesis.\textsuperscript{128}

**Inhibition of prostaglandin E\textsubscript{2} synthesis**

Since the discovery of aspirin by the Nobel laureate John Vane,\textsuperscript{59} PGE\textsubscript{2} has been one of the main targets for pain relief and anti-inflammatory therapy. Treatment of inflammatory conditions is mostly managed by drugs inhibiting PGE\textsubscript{2} biosynthesis. The COX enzymes have been the main therapeutic target for PGE\textsubscript{2} inhibition for several decades.\textsuperscript{134} In fact, most of the common anti-inflammatory drugs approved for clinical use, such as corticosteroids, NSAIDs and selective COX-2 inhibitors inhibit the COX enzymes.\textsuperscript{100,134-136} Selective COX-2 inhibitors, as well as traditional NSAIDs have been reported to provide symptomatic relief to patients with RA and osteoarthritis.\textsuperscript{137} It has also been demonstrated that inhibition of COX-2 reduces bone loss and cartilage destruction associated with inflammatory joints in rodent models.\textsuperscript{138} In periodontitis, it has been shown that inhibition of PGE\textsubscript{2} by selective COX-2 inhibitors or NSAIDs decreases periodontal disease progression and reduces alveolar bone resorption in humans, highlighting the significance of PGE\textsubscript{2} in the pathogenesis of periodontal disease.\textsuperscript{139-141}

Despite the beneficial effects of NSAIDs targeting COX-1 and COX-2, these drugs cause side-effects in the gastrointestinal tract, such as gastrointestinal ulcers and gastrointestinal bleeding.\textsuperscript{142,143} Inhibition of COX enzymes, specifically COX-1, by NSAIDs results in the inhibition of protective prostanoids resulting in the irritation of gastric mucosa that becomes more sensitive and susceptible for topical attack by endogenous and exogenous factors.\textsuperscript{144,145} For example, inhibition of COX-1 blocks platelet production by TXA\textsubscript{2}, which increases bleeding in active gastrointestinal bleeding sites. On the other hand, PGs derived from COX-2 can be generated at the ulcer margin and appear to play an important role in ulcer healing by triggering cell proliferation, promotion of angiogenesis and restoration of mucosal integrity.\textsuperscript{144} Pain and inflammation are mediated
by COX-2, and gastric protection by COX-1, therefore selective COX-2 inhibitors avoiding gastrointestinal complications associated with non-selective NSAIDs are favorable. In fact, the selective COX-2 inhibitors that were developed had little to no effect on COX-1 and provided effective pain relief with reduced gastrointestinal effects.\textsuperscript{100,144,146} Years later, these COX-2 specific inhibitors, with reduced gastrointestinal toxicity, were reported to cause increased risk for cardiovascular injury.\textsuperscript{143,146,147} In fact, in 2004 the blockbuster drug rofecoxib (sold commercially as Vioxx) was withdrawn from the U.S. market when several studies linked selective COX-2 inhibitors to a higher rate of heart attack and stroke.\textsuperscript{146,148} The mechanism behind these adverse effects was due to a disturbed balance between vasoconstriction and vasodilation in the vascular system. Vasoconstriction and platelet aggregation is mediated by TXA\textsubscript{2} regulated via COX-1, and vasodilation is promoted by PGI\textsubscript{2}, which restrains platelet activation. Therefore, inhibition of PGI\textsubscript{2} and PGE\textsubscript{2} in the blood vessel wall by selective COX-2 inhibitors, without concomitant inhibition of TXA\textsubscript{2}, could result in hypertension and thrombosis, and thereby an increased risk for cardiovascular injury.\textsuperscript{146}

In light of the discovered side-effects associated with NSAIDs and selective COX-2 inhibitors, the inducible mPGES-1 enzyme acting downstream of COX, has been suggested as an attractive drug target for selective inhibition of inflammation-induced PGE\textsubscript{2} production.\textsuperscript{149}

**Prostaglandin E synthase-1 inhibitors**

Despite that the mPGES-1 enzyme was suggested as a drug target already in 1999,\textsuperscript{84} progress in bringing drugs to the market has been very slow. Currently there are no selective mPGES-1 inhibitors available for clinical use,\textsuperscript{150} and several possible explanations may exist for this delay. One reason is that mPGES-1, as a promising therapeutic target, has been questioned because blocked mPGES-1 may allow redirection of the substrate PGH\textsubscript{2} to other PG synthases, and the consequences of this remains to be determined.\textsuperscript{142} Another reason may be that there are essential structural differences in the active site of mPGES-1 between human and rodent mPGES-1 orthologs, which makes it difficult to test potential inhibitors designed for human mPGES-1 in animal models.\textsuperscript{151,152} However, the number of publications of identified mPGES-1 inhibitors is increasing but still none of these have made it to clinical trials.\textsuperscript{153,154} These inhibitors are identified in virtual models and a few have been tested in experimental models.\textsuperscript{155} One of the earliest identified inhibitors of mPGES-1, 5-lipoxygenase-activating protein inhibitor 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid (MK-886), was demonstrated to inhibit mPGES-1 enzyme activity and protein expression.\textsuperscript{124,155} The structure of MK-886 has often been the starting point for identifying more potent and selective inhibitors of mPGES-1.\textsuperscript{156} The substance curcumin from the turmeric plant, with anti-inflammatory
and anti-carcinogenic activities, has been reported to reduce IL-1β-stimulated mPGES-1 expression in \textit{in vitro} studies.\textsuperscript{157,158} In animal studies, curcumin, administrated by oral gavage, was shown to inhibit TNFα, IL-6 and COX-2 expression in gingival tissues of rats with induced experimental periodontitis.\textsuperscript{159} The anti-bacterial agent Triclosan has been demonstrated to reduce PGE\textsubscript{2} production and mPGES-1 expression in gingival fibroblasts.\textsuperscript{160} In addition, brushing with toothpaste containing Triclosan showed beneficial effect on periodontal disease by reducing gingival inflammation.\textsuperscript{161} It has also been reported that toothpaste with Triclosan reduced alveolar bone loss in experimental periodontitis in rats.\textsuperscript{162}

More research is required to develop clinically useful mPGES-1 inhibitors. In this work, we identified aminothiazoles as potential inhibitors of mPGES-1 that may be used in future treatment of chronic inflammatory diseases including periodontitis.
Aims of the thesis

The overall aim of this thesis was to study the expression of PGES, especially mPGES-1, in periodontal tissues and to investigate the effect of novel mPGES-1 inhibitors on PGE\textsubscript{2} synthesis and bone resorption by \textit{in vivo} and \textit{in vitro} studies.

Specific aims

\textbf{Study I}

The aim of the study was to investigate whether mPGES-1 is expressed in gingival fibroblasts and its potential role in the regulation of PGE\textsubscript{2} production.

\textbf{Study II}

The aim of this study was to investigate the cellular localization of PGE\textsubscript{2}-producing enzymes, focusing on the expression of PGE synthases in human gingival tissues collected from patients with periodontitis. In addition, investigate the cellular regulation of these enzymes \textit{in vitro}.

\textbf{Study III}

In this study, the aim was to investigate the effect of aminothiazole derivatives as potential mPGES-1 inhibitors on the regulation of PGE\textsubscript{2} in gingival fibroblasts and on experimental periodontitis in rats.

\textbf{Study IV}

The aim was to further study the effect of aminothiazoles, identified in Study III, on RANKL-induced osteoclastogenesis in the osteoclast precursor cell line RAW 264.7.
Materials and Methods
This section gives an overview of the materials and methods used to obtain the results presented in this thesis. The methods mainly focus on the expression and inhibition of PGE$_2$ synthesis, focusing on the expression of mPGES-1. Methods used in this thesis are \textit{in vivo} studies of gingival tissue, \textit{in vitro} studies using cell cultures and experimental periodontitis studies in rats. For complete protocols, including in-depth details such as primer sequences, concentrations of substances and buffers, or sources of chemicals, please refer to the Materials and Methods sections of the Studies (I-IV) in the Appendix.
Gingival biopsies (Studies I-III)

In this thesis gingival biopsies were obtained to study either the expression of the PGES enzymes *in vivo* by histological and immunohistochemical methods or for isolation of gingival fibroblasts used in the cell culture experiments. Biopsies from gingival tissues were taken during dental surgery as a part of the patients planned treatment and therefore the patients were not exposed to any additional discomfort. All included studies were approved by the Ethical Committee at Karolinska Institutet and all patients gave their informed consent for their gingival biopsies to be used in this research.

For *in vivo* studies of PGES expression in Study II, gingival tissue biopsies were obtained from otherwise healthy adult patients (age 35-68) diagnosed with periodontitis. For the clinical definition of periodontitis, bone resorption measured by clinical attachment loss between 2 and \( \geq 6 \) mm, and pocket probing depth (PPD) ranging from 3 mm to \( \geq 6 \) mm have been suggested.\(^{163}\) The inclusion criteria used in Study II were clinical signs of periodontitis at the site of biopsy collection, including radiographic bone resorption, PPD \( \geq 6 \) mm and bleeding on probing. The biopsies were taken during surgical procedures as part of periodontal therapy.

Gingival biopsies for establishment of primary gingival fibroblast cell lines, used in the cell culture experiments (Studies I-III), were obtained from healthy children (age 6-12) with no clinical signs of periodontal disease. Biopsies were taken during planned surgical treatment, mainly extractions.

Gingival tissue staining (Study II)

The expression of PGES enzymes and other markers of inflammation were studied in gingival tissue sections either by histological staining or by immunohistochemistry. Histological staining was mainly preformed to give contrast to the tissue as well as to highlight particular features of interest, while immunohistochemical analysis was performed using specific antibodies that bind to antigens on the proteins of interest. In Study II, gingival tissues from patients with periodontitis were either formaldehyde fixed, processed and paraffin embedded or immediately snap-frozen in liquid nitrogen.

Histological staining was performed on paraffin embedded gingival tissue biopsies. Serial sections (4 \( \mu \)m) were deparaffinized using xylene and rehydrated through an ethanol series. Sections of each biopsy were histologically stained with haematoxylin and eosin, and Giemsa in order to assess the orientation of the epithelium and the degree of immune cell infiltration. The expression of PGE synthases, COX-2 and IL-1\( \beta \) was analyzed by immunohistochemistry and immunofluorescent double staining was used to confirm the expression of mPGES-1 in different cell types. The presence of mast cells in the tissues was identified by staining with 0.5% Toluidine blue in McIlvaine’s buffer (pH 4) or with anti-human mast cell tryptase.
Immunohistochemical staining

To investigate the expression of PGE synthases, COX-2 and the inflammatory mediator, IL-1β, in gingival biopsies from patients with periodontitis, deparaffinized sections were immunohistochemically stained with primary antibodies using the commercial Cell and Tissue Staining Kit (R&D systems), according to the manufacturer’s instructions. Briefly, sections were incubated with primary antibodies for mPGES-1, mPGES-2, cPGES, COX-2 and IL-1β overnight. To investigate leukocyte infiltration in the tissues, sections were immunohistochemically stained with antibodies for CD45, a common marker for leucocytes. For negative controls, primary antibodies were substituted with an isotype-matched control antibody. After incubation with primary antibodies, the slides were washed and incubated with biotinylated secondary antibodies followed by addition of high sensitivity streptavidin-conjugated horseradish peroxidase (HSS-HRP). The biotinylation on the secondary antibodies enabled the binding of HSS-HRP to the antibody complex. In the final step, the sections were briefly incubated with the HRP substrate 3,3’-diaminobenzidine that formed brown colored products visualizing the protein of interest. The slides were mounted and analyzed under a light microscope.

Immunofluorescence double staining

To confirm the expression of mPGES-1 in fibroblasts, endothelial cells and smooth muscle cells, immunofluorescence double staining was performed. For these stainings, frozen biopsies were cryostat sectioned (7 µm). The frozen sections were fixed, permeabilized and blocked. Thereafter, the slides were incubated with a mixture of two primary antibodies consisting of mPGES-1 and either the fibroblast marker anti-prolyl-4-hydroxylase, the endothelial cell marker von Willebrand factor or the smooth muscle marker smooth muscle actin. After washing, the sections were incubated with a mixture of secondary antibodies. The primary antibody for mPGES-1 (polyclonal rabbit) formed a complex with the secondary antibody conjugated with Alexa 594 (anti-rabbit) displaying a red fluorescence signal. The other three primary antibodies (monoclonal mouse antibodies) were visualized by the secondary antibody conjugated with Alexa 488 (anti-mouse) displaying a green fluorescence signal. The slides were mounted and analyzed using a Nikon fluorescence microscope and image analysis was performed by the software NIS-elements (Nikon). Two pictures visualizing red and green fluorescence were taken of the same area and merged in the NIS elements program. The overlap of green and red signals was displayed as yellow, confirmed co-expression of mPGES-1 and the markers for fibroblasts, smooth muscle cells or endothelial cells. For all experiments, isotype-matched irrelevant antibodies were used as negative controls in the staining procedures.
**Induction and inhibition of PGE\textsubscript{2} (Studies I-IV)**

The protein expression of PGES, especially mPGES-1, was studied in different cell types (described below). The cells were stimulated by different cytokines to induce PGE\textsubscript{2} synthesis (IL-1\textbeta or TNF\alpha) or osteoclastogenesis (RANKL) (Table 1).

To study the expression and regulation of mPGES-1, cPGES, mPGES-2 and the upstream enzyme COX-2, different inhibitors known to inhibit PGE\textsubscript{2} were used for treatment of cell cultures. The inhibitors that were used included the glucocorticoid dexamethasone (DEX), the unspecific COX inhibitor Indomethacin, the specific COX-2 inhibitor Celecoxib, the anti-bacterial and anti-inflammatory agent Triclosan, and the mPGES-1 inhibitor MK-886\textsuperscript{124,160,166-168}. In addition to the known PGE\textsubscript{2} inhibitors, we identified novel inhibitors of PGE\textsubscript{2}, the aminothiazoles (described below), that were also used in cell culture experiments and experimental periodontitis studies. The different cytokines and inhibitors used in the cell culture experiments are listed in Table 1.

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<th>Substance</th>
<th>Effect</th>
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<tbody>
<tr>
<td>IL-1\textbeta</td>
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<tr>
<td>TNF\alpha</td>
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<tr>
<td>DEX</td>
<td>Anti-inflammatory glucocorticoid</td>
<td>X X</td>
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<td>Indomethacin</td>
<td>NSAID</td>
<td>X</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>Selective COX-2 inhibitor</td>
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<tr>
<td>Triclosan</td>
<td>Antimicrobial agent</td>
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<tr>
<td></td>
<td>PGE\textsubscript{2} inhibitor</td>
<td>X</td>
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<td>MK-886</td>
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<td>X</td>
</tr>
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<tr>
<td>TH-644</td>
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<tr>
<td>RANKL</td>
<td>Cytokine for osteoclast differentiation and activation</td>
<td>X</td>
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<tr>
<td>PGE\textsubscript{2}</td>
<td>Inflammatory mediator</td>
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Table 1. Substances used for treatment of cells (Studies I-IV)

Abbreviations: DEX, dexamethasone; IL-1\textbeta, interleukin 1\textbeta; MK-886, 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-\textalpha,\textalpha-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; RANKL, receptor activator of NF-\textkappaB ligand; TH-644, 4-(3-fluoro-4-methoxyphenyl)-N-(4-phenoxophenyl)-1,3-thiazol-2-amine; TH-848, 4-((4-(2-naphthyl)-1,3-thiazol-2-yl)amino)phenol; TNF\alpha, tumor necrosis factor \textalpha.
Figure 5. Molecular docking of substances to the mPGES-1 3D structure. Molecules docked were PGH$_2$ (A), MK-886 (B), Triclosan (C), TH-848 (D) and TH-644 (E).

Abbreviations: MK-886, 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid; PGH$_2$, prostaglandin H$_2$; TH-644, 4-(3-fluoro-4-methoxyphenyl)-N-(4-phenoxyphenyl)-1,3-thiazol-2-amine; TH-848, 4-((4-(2-naphthyl)-1,3-thiazol-2-yl)amino)phenol.

Aminothiazoles (Studies III and IV)

Aminothiazoles were identified as novel inhibitors of PGE$_2$ by docking studies toward the active site of the three-dimensional crystal structure of mPGES-1 (PDB ID code 3DWW). These docking studies, performed by the software Autodock Vina, resulted in the identification of aminothiazole derivatives as potential mPGES-1 inhibitors (Figure 5). The starting point for identification of potential inhibitors was based on the structures of the well-known mPGES-1 inhibitor MK-886 and Triclosan reported to inhibit mPGES-1 (Figure 5B and C). The aminothiazoles 4-((4-(2-naphthyl)-1,3-thiazol-2-yl)amino)phenol (TH-848) and 4-(3-fluoro-4-methoxyphenyl)-N-(4-phenoxyphenyl)-1,3-thiazol-2-amine (TH-644) were identified as potential mPGES-1 inhibitors (Figure 5D and E), and were further evaluated in experimental in vivo and in vitro studies.

Cell cultures (Studies I-IV)

In this work different cell types were used as model systems to study the expression and regulation of PGE synthases. The main cell types used in this thesis were gingival fibroblasts (Studies I-III) and RAW 264.7 cells (Study IV). Therefore, these two cell types are more thoroughly described in the sections below. For the isolation and culture procedures of human airway smooth muscle (HASM) cells, mast cells, human umbilical vein endothelial cell (HUVEC), gingival fibroblasts from mPGES-1 knockout mice and co-cultures with lymphocytes please refer to the Material and Methods section of Study II. For isolation and studies of gingival fibroblasts from Sprague Dawley rats, please refer to the Material and Methods section of Study III.
Gingival fibroblasts (Studies I-III)

Gingival fibroblasts are the dominant cell type in the gingival connective tissue and are therefore relevant cells to use for in vitro studies of periodontitis. Human gingival fibroblasts, used in Studies I-III, were established from gingival biopsies of healthy individuals without clinical signs of periodontal disease. Minced pieces of gingiva were placed in culture flasks with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with penicillin, streptomycin and fetal bovine serum (FBS), and the primary outgrowth of fibroblasts was collected by trypsinisation. The cells were incubated at 37°C in a humidified incubator aerated with 5% CO₂ and routinely passaged by trypsinisation. For the experiments, fibroblasts were seeded in petri dishes, 6, 24 or 96 well plates and cultured for 24 - 48 h. Thereafter, the cell layers were treated with serum-free DMEM containing the cytokines IL-1β or TNFα alone or in the presence of either aminothiazoles or other PGE₂ inhibitors listed in Table 1. The cells were incubated 6 hours for mRNA expression analysis or 24 hours for protein expression, PGE₂ determination or cytotoxicity analyses. Cell culture medium was collected and stored for subsequent analysis of PGE₂ and the cell monolayers were either collected for protein analysis or for total RNA isolation.

RAW 264.7 cells (Study IV)

RAW 264.7 is a macrophage-like cell line derived from male BALB/c mice. These cells can be differentiated into osteoclasts and are therefore commonly used for studies of osteoclastogenesis.¹⁷¹,¹⁷² The RAW 264.7 cells, used in Study IV, were cultured in minimum essential medium alpha supplemented with FBS, L-glutamine and antibiotics at 37°C. For differentiation and histochemical staining of the osteoclasts marker TRAP, the cells were seeded in 24 well plates. For RNA isolation and PGE₂ measurements the cells were seeded in 6 well plates and for cytotoxicity analysis the cells were seeded in 96 well plates. RAW 264.7 cells were treated with recombinant truncated mouse RANKL to induce differentiation into osteoclasts. The cells were treated with RANKL alone or in the presence of the aminothiazoles, TH-848 or TH-644, the COX-2 inhibitor Celecoxib or exogenous PGE₂. For mRNA expression analysis and PGE₂ determination, the cells were incubated for 72 h. To study osteoclastogenesis, evaluated by counting the number of multinucleated osteoclasts, the RAW 264.7 cells were differentiated for a total of four days, during this time the culture medium with all the reagents was replaced once after three days. Differentiated cell layers were fixed in paraformaldehyde and histologically stained for the osteoclast marker TRAP using a commercial ‘Acid Phosphatase, Leukocyte (TRAP) Kit’ (Sigma Aldrich) according to the manufacturer’s instruction. Multinucleated cells containing ≥ 3 nuclei were scored as osteoclasts and counted under a light microscope by two blinded observers.
RNA expression (Studies I, III and IV)

There are several methods to study the expression of genes. The most common technique used in molecular biology is the polymerase chain reaction (PCR). In this method a piece of DNA is amplified generating thousands to millions of copies of a particular DNA sequence. In order to study mRNA expression with this technique the mRNA is reverse transcribed by the enzyme reverse transcriptase into complementary DNA (cDNA), therefore this type of PCR reaction is called Reverse Transcription PCR (RT-PCR). Quantitative RT-PCR (RT-qPCR) is combined technique based on the principal of RT-PCR but with the detection method of quantitative PCR (qPCR) using fluorescent probes to quantitatively determine the amplification of cDNA. Both RT-PCR and RT-qPCR have been used in this work for analysis of RNA expression.

RNA isolation and cDNA synthesis

Cells were cultured as described under “Cell cultures”. For total RNA isolation, cell layers were rinsed with PBS and lysis buffer was added to the cells. The content of the dish or well was transferred to QIAshredder column (Qiagen) for homogenization followed by total RNA extraction according to the protocol of the RNeasy Mini Kit (Qiagen). The amount of total RNA was quantified spectrophotometrically. To obtain first strand cDNA, 1 µg of total RNA was reverse transcribed by the enzyme reverse transcriptase according to the protocol for Superscript II (Applied Biosystems) (Studies I and III) or the commercial iScript™ cDNA Synthesis Kit (BioRad) (Study IV).

RT-PCR (Studies I and III)

mRNA expression was analyzed by RT-PCR. cDNA, reverse transcribed from total RNA, was used as a template in the PCR reactions. For the PCR amplification conditions and primer sequences for mPGES-1, COX-2 and GAPDH please refer to the Material and Method section in Study I and other publications by our group. For each experiment, PCR amplifications were also carried out in samples without cDNA as negative controls. After amplification, the PCR reaction products were separated on a 2-3% agarose gel. In Study I, the gels were treated with ethidium bromide and the bands were visualized by irradiation with UV light. In Study III, GelRed was added to the agarose gel that was detected by the molecular imager ChemiDoc XRS+ System (Bio-Rad). The results were analyzed semi-quantitatively by measuring the intensity and volume of the bands. The volumes were normalized to the reference gene GAPDH and the results expressed as fold change of treated cells relative to untreated control cells.
**RT-qPCR (Study IV)**

In Study IV, the RT-qPCR was performed by amplification of cDNA using either TaqMan Gene Expression Assays (Applied Biosystems) or specific primers together with SYBR® Green dye. TaqMan assays may be considered as more precise methods for detection of specific PCR products since this system is predesigned with specific primers and fluorogenic probe, while SYBR® Green is a dye that binds to double stranded DNA and requires optimization. However, both systems were used in Study IV. Analysis for mPGES-1 and GAPDH was performed by TaqMan Gene Expression Assays together with Taq-Man Universal PCR Master Mix. For expression of TRAP, Cathepsin K, RANK, OPG and GAPDH, RT-qPCR analysis was performed with specific primers and iQ™ SYBR® Green Supermix (Bio-Rad), the optimal conditions for these assays were based on previous reports. All qPCR reactions were analyzed in duplicates or triplicates on the CFX96™ Real-Time PCR Detection System (BioRad) and GAPDH was used as reference gene. Gene expression was calculated according to the $\Delta\Delta^{Ct}$ method, where each sample was normalized to the mean expression of GAPDH.

**Protein expression analysis (Studies I-III)**

Proteins are the main actors within the cell; they perform a vast array of functions encoded by the information in our genes. The function for PGES enzymes is to convert PGH$_2$ to PGE$_2$.

Common methods to study protein expression are based on detection with specific antibodies. Antibodies recognize a unique part of the foreign target, called antigen. Polyclonal antibodies have specificity for multiple epitopes of the same antigen, while monoclonal antibodies are specific for a single epitope of an antigen. Both monoclonal and polyclonal antibodies have been used in the different methods for protein expression in this thesis. In gingival tissues, the protein expression was analyzed by immunohisotchemical staining (described above) and in cell cultures by Western blot and flow cytometry.

**Western blot (Studies I and II)**

Western blot is a widely used technique to detect specific proteins in a sample of cell extract or tissue homogenate. In this work, cells were seeded and cultured as described in the section “Cell cultures” and total protein was isolated from the cell layers. Total protein extraction was done by lysing the cells in lysis buffer containing protease inhibitors. Protein concentrations of each sample were determined spectrophotometrically. For each sample equal amounts of protein were separated by electrophoresis either on 4-15% linear gradient polyacrylamide tris-HCl gels or 10% sodium dodecyl sulphate-polyacrylamide gels according to molecular weight of the proteins. The proteins were transferred from the gel to a nitrocellulose membrane and the membranes were blocked in 5% non-fat dry milk to
prevent unspecific binding of antibodies. The membranes were first incubated over night with primary antibodies for mPGES-1, mPGES-2, cPGES or COX-2, and then washed and incubated with a HRP-conjugated secondary antibody. HRP linked to the secondary antibody was a reporter catalyzing the formation of a light-emitting product (chemiluminescence) that could be recorded on photographic film. Therefore the membranes were developed using enhanced chemiluminescence (ECL) and exposed to hyperfilm-ECL.

**Flow cytometry (Study II and III)**

Flow cytometry is an antibody-based method that provides the user with an ability to identify proteins expressed on surfaces of cells, as well as intracellular proteins. In this work, cells were seeded and grown as described in the “Cell cultures” section. After treatment with different inflammatory mediators alone or in the presence of PGE$_2$ inhibitors, the cells were collected by trypsinization and/or centrifugation. The cells were washed with PBS, thereafter fixed in 2% paraformaldehyde and permeabilized with saponin buffer. Saponin allowed antibodies to pass through the cell membrane, enabling staining of intracellular proteins like the PGES enzymes. Cells were first incubated with primary antibodies against PGE synthases and COX-2 and thereafter washed and incubated with fluorescent labeled secondary antibodies. Flow cytometric analysis was performed using either FACSCalibur™ or FACSVerse™ flow cytometer (Becton Dickinson). For each sample 10 000-20 000 events were acquired. A gated area for the particular cell type was determined using forward scatter (giving information about the cell size) and side scatter (relating to the cell granularity) parameters. The fluorescent intensity was related to the expression of the analyzed protein. In this work, gated cells were analyzed regarding expression of mPGES-1, mPGES-2, cPGES or COX-2. The results obtained were presented either as histograms of cell counts versus fluorescence intensity or mean fluorescence intensity.

**Enzyme activity assays (Study III)**

To study the potency of drugs, enzyme activity assays for evaluation of inhibitory effects towards the target enzyme are necessary to perform. It is also highly relevant to perform activity assays towards related enzymes to evaluate the specificity of the drugs. In this thesis, aminothiazoles were identified by docking studies as potential mPGES-1 inhibitors. Enzyme activity assays were performed to evaluate their effect on the target enzyme mPGES-1. In addition, activity assays towards the upstream enzyme COX-2 was performed to elucidate the selectivity of aminothiazoles.
mPGES-1 activity

The *in vitro* mPGES-1 activity assay was performed using a modification of a previously described method.\(^\text{176}\) Human recombinant mPGES-1 enzyme was mixed with the aminothiazoles TH-848, TH-644 or the known mPGES-1 inhibitor MK-886 and incubated for 15 min. PGH\(_2\) was added to initiate the reaction and after 20 s of incubation the reaction was stopped by FeCl\(_2\) solution. The amount of PGE\(_2\) in the samples was determined using the commercial PGE\(_2\) enzyme immunoassay (EIA) kits. The enzyme activity of mPGES-1 was expressed as recombinant mPGES-1 activity relative to control samples measuring the conversion of PGH\(_2\) to PGE\(_2\) by recombinant mPGES-1 enzyme alone (without inhibitors).

COX-2 activity

COX-2 enzyme activity assay was performed using the COX Inhibitor Screening Assay Kit (Cayman Chemicals) according to the manufacturer’s instructions. In this assay the conversion of AA to PGH\(_2\) by the COX enzymes was studied. Since PGH\(_2\) is highly unstable, all the major PG compounds (the terminal products in the prostanoid biosynthesis) were measured by EIA. The aminothiazoles TH-848, TH-644, mPGES-1 inhibitor MK-886 and COX-2 inhibitor DuP-697 (provided with the kit) were tested and the results expressed as recombinant COX-2 activity relative to control samples.

Analysis of prostaglandins, leukotriene B\(_4\) and cytokines (Studies I-IV)

Since mPGES-1 is responsible for PGE\(_2\) production, the amount of PGE\(_2\) was measured in cell culture supernatants (Studies I-IV), mPGES-1 activity assays supernatants (Study III) and blood plasma from rats (Study III). PGE\(_2\) levels were determined either by radioimmunoassay (RIA) (Study I) or EIA (Studies II-IV) using commercial kits from Cayman Chemicals according to the manufacturers’ instructions. In addition to PGE\(_2\), the levels of Leukotriene B\(_4\) (LTB\(_4\)) and 6-keto PGF\(_{1\alpha}\), the stable breakdown product of prostacyclin, were measured in blood plasma samples from experimental periodontitis rats (Study III) using commercial EIA kits.

In Study III, 15 different cytokines were analyzed in blood plasma from experimental periodontitis rats. Blood samples were collected on the first and last day of experiments from the tail of selected rats. Selection was based on sufficient amounts of blood plasma for analysis at both time points, and whether the ligatures around the teeth remained intact at the end of the experiment. Cytokine measurements were performed using Luminex technology on a Bio-plex Suspension Array System with a commercial Bio-Plex Rat Cytokine Assay (Bio-Rad).
Cytotoxicity and antimicrobial analysis (Studies III and IV)

Assays to measure cytotoxic effects of drugs can be accomplished either by measuring cytotoxicity, proliferation or viability of the cells. The proper choice of an assay method depends on the type and number of cells used as well as the expected outcome.

In Studies III and IV the cytotoxicity after treatment of the cells with aminothiazoles was measured by lactate dehydrogenase (LDH) release using the commercial CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer’s instructions. LDH is an enzyme that is released during tissue damage and is therefore a common marker of injuries and diseases. Viability and cell proliferation after treatment with TH-848 in Study III was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) according to the protocol of the commercial MTT Cell Proliferation Assay Kit (Abnova).

The effects of aminothiazoles on different bacterial species were investigated in Study III. Antimicrobial susceptibility of twelve different bacterial strains to the aminothiazoles TH-848 and TH-644 was performed using the disc diffusion assay as described in detail in Study III. The first group of strains were the facultative anaerobic gram-positive microorganisms S. mutans IB, S. mutans OMZ, S. sobrinus, S. sanguis, S. salivarius, L. brevis, L. casei, A. naeslundii, and A. viscosus. The second group of bacteria consisted of the anaerobic gram-negative microorganisms P. gingivalis, A. actinomyctecomitans and F. nucleatum. All these bacterial strains are known oral pathogens. Circular antibiotic sensitivity discs, containing TH-848 or TH-644 were placed on the surface of the agar plates. Triclosan and chlorhexidine were used as positive controls and the negative control consisted of DMSO diluted in 70% ethanol, which was also used for preparation and administration of the aminothiazoles. The agar plates were incubated at 37°C for 24 h (gram-positive strains) or 72 h (gram-negative strains) in an anaerobic environment and thereafter analyzed.

Animal studies (Study III)
The effects of the aminothiazoles were studied in vivo using the ligature-induced experimental periodontitis model. In this study, Sprague Dawley rats, weighting 270-350 g, were used for the experiments. The Sprague Dawley rat is a strain of outbred albino rats, extensively used in medical research. The rats were kept at the animal facility at Karolinska University Hospital, housed in temperature-controlled rooms, receiving water and food ad libitum. Prior to starting, this study was approved by the Stockholm North Animal Ethics Committee.
Figure 6. Induction of experimental periodontitis. Silk ligatures were tied around the second upper molars of the rats to initiate experimental periodontitis (images 1-4).

Experimental periodontitis and treatment with aminothiazole

Totally 80 rats, were divided into different treatment or control groups consisting of 20 animals in each group. The four groups of rats were: experimental periodontitis groups treated with aminothiazole TH-848 or vehicle, experimental periodontitis group receiving only ligatures and the non-ligated control group. The rats were anesthetized with isoflurane and in the experimental periodontitis groups silk ligatures were tied around the cervical part of the second upper molars on both sides (Figure 6). The purpose of the ligatures was to promote bacterial adhesion and act as a reservoir for microbial pathogens to maintain an ongoing inflammatory process and thereby mimicking the clinical features of periodontitis.

Treatment was initiated by injections with TH-848 or vehicle into the gingiva, buccaly next to the ligature-equipped teeth, immediately after ligature placement. On the following days of the experiment, topical treatment with gel containing TH-848 or vehicle was administered once per day. For the preparation of the gel and injections containing the aminothiazole TH-848 or vehicle please refer to the Material and Methods section of Study III. Blood samples were collected from all rats at start and end of the experimental period. The experiment was terminated after eight days and all rats were euthanized by CO$_2$. The jaws were collected for measurements of alveolar bone levels, and from a few control rats gingival biopsies were taken for establishment of gingival fibroblasts. The experimental design for the experimental periodontitis study is illustrated in Figure 7.
Figure 7. Study design of experimental periodontitis. Day 1, the ligatures were applied and treatment was initiated by injections into the gingiva. Day 2-7, local topical treatment with a gel containing TH-848 or vehicle was administrated once/day. Day 8, all the rats were euthanized and the jaws were saved for measurements of alveolar bone resorption. Blood samples were collected at days 1 and 8.

Measurements of alveolar bone levels

The upper jaws were evenly separated at the intermaxillary suture and dissected. For the assessment of radiological alveolar bone levels, a standard digital intraoral X-ray unit was used. The pieces of jaws were placed with the occlusal surface perpendicular to a Planmeca ProSensor and exposed to 50 kV and 8 mA for 0.16 s. Radiographs were analyzed using the software Planmeca Romexis. Alveolar bone levels were measured from a straight line marking and extrapolating the level of occlusal surface of the second upper molar. From this line, distance to the marginal bone was measured at interproximal sites; mesial and distal of the tooth. The measurements were performed by two blinded dentists. Approximate mean bone degradation was calculated by subtracting the mean bone level of non-ligated control group from the mean bone levels of ligature-treated groups. Teeth that lost their ligatures during the experimental period were excluded from the study. After radiographic analysis, some jaws were defleshed, stained with aqueous methylene blue (1%) and photographed under a light microscope for visualization of the marginal bone.

Statistical Analysis (Studies I-IV)

The main statistical analysis used throughout this thesis was the student’s t-test (two-tailed). This test was applied for multiple comparisons of normally distributed data, which was the case in all cell culture experiments and in vitro assays (Studies I-IV).

In the animal experiments (Study III) statistical analyses were performed using the statistical package IBM SPSS Statistics 19.0 (IBM). Pearson’s correlation coefficient was used to calculate the interexaminer agreement regarding alveolar bone level measurements. One-way variance analysis was adopted for comparisons between groups regarding alveolar
bone level. Pairwise comparisons within groups were performed using Scheffé’s method. For comparisons of cytokines, PGE₂, 6-keto PGF₁α, and LTB₄ in blood plasma before and after treatment within treatment groups the Wilcoxon paired signed-rank test was used, while the Kruskal-Wallis test was applied for comparisons between treatment groups regarding change after treatment. Bonferroni correction was used to compensate for multiple comparisons. In all studies in this thesis, results were considered statistically significant at P < 0.05.
Results and Discussion
In this thesis, the expression of PGES enzymes, especially mPGES-1, was investigated in gingival tissues from patients with periodontitis and various cell types including fibroblasts, smooth muscle cells, endothelial cells and mast cells. In addition, novel mPGES-1 inhibitors, aminothiazoles, were identified and evaluated in gingival fibroblasts *in vitro*. The effect of these inhibitors on alveolar bone resorption was also studied *in vivo* in experimental periodontitis in rats and further investigated in an *in vitro* model of osteoclastogenesis using RAW 264.7 cells. The first three studies have been published in peer-reviewed journals and the fourth study is in manuscript form. All four studies can be found in their entirety in the appendix. This section gives a brief overview of the results from these studies and a discussion in relation to the current literature.
mPGES-1 is expressed in gingival fibroblasts (Studies I-III)

The inflammatory mediator PGE₂ is a potent inducer of bone resorption and plays an important role in gingival inflammation. Elevated levels of PGE₂, detected in gingival tissue biopsies and in GCF from patients with periodontitis, suggest that PGE₂ is an important mediator in the pathogenesis of periodontal disease. The predominant cell type in gingival connective tissues, gingival fibroblasts, produce large amounts of PGE₂ in response to inflammatory mediators and are likely to contribute to the enhanced levels of PGE₂ found in GCF.

The expression of mPGES-1, responsible for the terminal step in PGE₂ biosynthesis, was investigated in gingival fibroblasts in Studies I-III of this thesis. Study I, published in 2004, was the first study to demonstrate the expression and induction of mPGES-1 in human gingival fibroblasts. mPGES-1 expression was induced in response to pro-inflammatory mediators, IL-1β and TNFα, both at mRNA and protein levels and in addition, subsequent PGE₂ production was increased by these mediators. The cytokines TNFα and IL-1β had previously been reported to stimulate PGE₂ production and COX-2 expression in gingival fibroblasts. However the induction of mPGES-1 by IL-1β and/or TNFα, at that time had only been reported in A549 cells, umbilical vein endothelial cells, orbital fibroblasts, rheumatoid synovial fibroblasts, vascular smooth muscle cells, endometrial stromal cells and nonsmall lung cancer cell lines. Since the publication of Study I several Studies, including Studies II and III, have reported induced expression of mPGES-1 in gingival fibroblasts.

The expression of mPGES-1 in gingival fibroblasts in vivo was demonstrated, in Study II, in gingival tissues from patients with periodontitis by immunohistochemical staining. The presence of fibroblasts in the gingival connective tissues was confirmed by immunofluorescent double staining of mPGES-1 and a fibroblast marker. In addition, stimulation of mPGES-1-null gingival fibroblasts, isolated from mPGES-1 knockout mice, by TNFα revealed no induction of mPGES-1 expression in null cells compared to wild type cells analyzed by immunostaining. PGE₂ production was detectable at basal level in null fibroblasts, but at much lower concentrations compared to wild-type control cells. In addition, stimulation by TNFα resulted in a much stronger increase in PGE₂ levels in wild type cells compared to null cells. Our experiments with null gingival fibroblasts demonstrated that mPGES-1 is important for inflammation-induced PGE₂ production.

To study the regulation of mPGES-1 and its role for PGE₂ synthesis, gingival fibroblasts were treated with known anti-inflammatory drugs such as DEX, Celecoxib and Indomethacin. Cytokine-induced mRNA and protein expression of mPGES-1 as well as PGE₂ production was decreased by the glucocorticoid DEX in gingival fibroblasts. Glucocorticoids have a broad effect on inflammatory processes and the inhibitory action of glucocorticoids on the biosynthesis of PGs is mediated by a complex series of pathways.
including decreased COX-2 expression.\textsuperscript{89,105,185} Our results showing that DEX inhibited mPGES-1 expression, in agreement with similar results in A549 cells,\textsuperscript{106} implies that the inhibitory effect of DEX on PGE\textsubscript{2} production is also regulated by mPGES-1. In addition, DEX reduced mRNA expression of mPGES-1 in otherwise untreated control cells, which might be due to a direct inhibition of mPGES-1 at the transcriptional level. Glucocorticoids are potent inhibitors of the nuclear transcription factor NF-κB, which may partly explain the inhibitory effect of DEX on cytokine-induced mPGES-1 expression, since inflammatory mediators IL-1β and TNFα, activate NF-κB.\textsuperscript{186,187} Notably, it has been reported that the signal transduction pathway of NF-κB is positively regulated by the cytokine TNFα assessed by global gene expression profiling, and inhibition of this pathway reduces the expression of mPGES-1 and COX-2, as well as PGE\textsubscript{2} production in gingival fibroblasts.\textsuperscript{116}

The selective COX-2 inhibitor, Celecoxib, also decreased mPGES-1 protein expression followed by decreased PGE\textsubscript{2} production in gingival fibroblasts. Accumulating evidence implies that treatment with specific COX-2 inhibitors as well as NSAIDs reduce the progression of alveolar bone resorption in patients with periodontitis.\textsuperscript{139,141,188} However, the specific COX-2 inhibitor, Celecoxib, and the non-specific inhibitor of COX, Indomethacin, decreased PGE\textsubscript{2} production in gingival fibroblasts at the doses tested. Interestingly, Celecoxib, in contrast to Indomethacin, strongly reduced mPGES-1 protein expression. In agreement with our results, additional studies have shown that selective COX-2 inhibitors decrease the cytokine-induced mPGES-1 expression and subsequently PGE\textsubscript{2} production.\textsuperscript{189,190} The effects of NSAIDs on mPGES-1 expression is however inconclusive. In agreement with our results, no effect of NSAIDs on induced mPGES-1 expression have been observed in other model systems,\textsuperscript{189} and in contrast to our results, inhibited expression of mPGES-1 by non-specific COX inhibitors including Indomethacin have also been reported.\textsuperscript{119,190,191} Altogether, our findings that mPGES-1 is induced by pro-inflammatory stimuli and inhibited by the anti-inflammatory glucocorticoid DEX and selective COX-2 inhibitor Celecoxib in gingival fibroblasts (illustrated in Figure 8), highlights the importance of this enzyme in gingival inflammation, including the chronic inflammatory disease periodontitis.

**PGE synthases are expressed in gingival tissue from patients with periodontitis (Study II)**

The terminal step in PGE\textsubscript{2} biosynthesis is catalyzed by the PGE synthases. Three distinct isoforms of these enzymes have been identified.\textsuperscript{84,101,102} In addition to the inducible mPGES-1, the constitutive isoforms mPGES-2 and cPGES, also contribute to PGE\textsubscript{2} production.\textsuperscript{129} Study II, published in 2011, was the first and to date the only report describing the expression of all three isoforms of PGES in periodontitis, although the expression of mPGES-1 had previously been shown in gingivitis.\textsuperscript{115} In this study, we
demonstrated the cellular localization of all three PGE synthases in gingival tissues from patients with periodontitis. Immunohistochemical analysis revealed that the expression of PGE synthases mPGES-1, mPGES-2 and cPGES, as well as the upstream enzyme COX-2 were localized to vessels (endothelial cells and smooth muscle cells), fibroblast-like cells, immune cells and epithelium. Our results regarding mPGES-1 and COX-2 expression are in agreement with studies reporting the expression of these inflammation-induced enzymes in epithelial, endothelial and fibroblast-like cells in inflamed gingiva.98,115 The expression of PGES has also been observed in other inflammatory conditions such as, RA synovial tissue,113 gastric ulcer tissue,112 and recently in ocular tissue of dry eye disease.192

In gingival connective tissue biopsies from patients with periodontitis, expression of mPGES-1 in different cell types was confirmed by immunofluorescent double staining using cell type-specific markers for fibroblasts, smooth muscle cells and endothelial cells. Co-immunostaining of mPGES-1 was seen in fibroblasts, smooth muscle cells and endothelial cells, indicating that these cells might contribute to the elevated PGE\textsubscript{2} levels detected in periodontal tissue.45,47,177 Immune cells were also positively stained for PGES, and the presence of mast cells in the tissues was confirmed by specific mast cell markers. Mast cells together with fibroblasts, endothelial cells and smooth muscle cells were further studied \textit{in vitro} to investigate the cellular source of the increased PGE\textsubscript{2} levels observed in gingival tissues and GCF from patients with periodontitis.42,177

\textbf{PGE\textsubscript{2} synthesizing enzymes are expressed in different cell types (Study II)}

The significance of PGE\textsubscript{2} in periodontitis has been well established in clinical studies, where increased levels of PGE\textsubscript{2} in GCF correlates with severe clinical features of periodontitis.45,47 In Study II, we showed that PGE\textsubscript{2}-synthesizing enzymes, including all three isoforms of PGES, as well as COX-2, were expressed in gingival tissues from patients with periodontitis. To further investigate the contribution of these enzymes in inflammation-induced PGE\textsubscript{2} production, \textit{in vitro} studies using various cell cultures were performed. Cell types including fibroblasts, smooth muscle cells, endothelial cells and mast cells, which were positively stained for PGES \textit{in vivo} in gingival connective tissues, were further studied \textit{in vitro} in cell culture models.

The results showed basal protein expression of mPGES-1, mPGES-2, cPGES and COX-2 in cultures of fibroblasts, endothelial cells, and for the first time also in smooth muscle cells and mast cells. To study the regulation of these enzymes and their potential contribution to the production of PGE\textsubscript{2} in different cell types, cells were stimulated with the pro-inflammatory cytokines TNF\textalpha{} and IL-1\beta. These cytokines have been reported to induce PGE\textsubscript{2} production in different cell types, and have been implicated in the pathogenesis of chronic inflammatory diseases including periodontitis.56,117,193,194 Protein
expression of mPGES-1 and COX-2 increased in response to TNFα and IL-1β stimulation in fibroblasts and smooth muscle cells, whereas in endothelial cells, only COX-2 expression increased by TNFα treatment. Induced protein expression of mPGES-1 and COX-2 was reflected by PGE₂ production determined in the culture supernatants. Among the cells tested, strong increase in PGE₂ production was observed in gingival fibroblasts and smooth muscle cells treated with the cytokines, TNFα and IL-1β, but only a modest increase in endothelial cells in response to TNFα.

In human gingival fibroblasts, the inflammatory mediators TNFα and IL-1β induced mPGES-1 and COX-2 expression in agreement with our previous reports including Study I. Other PGES isoforms, mPGES-2 and cPGES, remained unaffected by the cytokines in gingival fibroblasts as previously described. Moreover, our group reported that neither siRNA knock-down nor chemical mPGES-1 inhibition affected the expression of cPGES or mPGES-2 in human gingival fibroblasts. Taken together, the results of Study II suggest that fibroblasts may contribute to increased levels of PGE₂ via mPGES-1 and COX-2 in chronic inflammatory diseases such as, periodontitis. An active role of fibroblasts in periodontitis is further supported by previous reports that fibroblasts may be a therapeutic target in chronic inflammatory diseases since these cells contribute to the persistence of inflammation and orchestrate the inflammatory infiltrate.

In the HUVEC cell line, used as a model system for endothelial cells, cytokine treatment with TNFα and IL-1β did not affect the expression of either mPGES-1 nor mPGES-2 or cPGES. In contrast to PGES enzymes, the expression of upstream enzyme COX-2 was increased by TNFα stimulation. Furthermore, PGE₂ production was only enhanced by TNFα treatment suggesting that this increase was rather mediated by COX-2 than PGE synthases, which is in line with previous findings showing the induction of COX-2 expression by TNFα in primary HUVECs. However, neither the expression of mPGES-1 and COX-2, nor the production of PGE₂ was increased in response to IL-1β. This observation was in agreement with similar findings in a study reporting no increase in PGE₂ production or COX-2 expression by IL-1β stimulation in a hybrid cell line between HUVECs and A549 cells.

For smooth muscle cells, also stained positively for PGES and COX-2 in gingival tissues from patients with periodontitis, the expression of PGE synthases was studied in vitro using HASM cells. Treatment of these cells with the cytokines TNFα and IL-1β increased the expression of mPGES-1 and COX-2 followed by increased PGE₂ production. Other PGES enzymes, cPGES and mPGES-2, were not affected by the cytokines. In line with our results, COX-2 expression and PGE₂ production have been reported to be increased by IL-1β in HASM cells, although our study was the first to report the expression of PGE synthases in these cells. However, expression of PGE synthases, as well as up-regulation of mPGES-1, COX-2 and PGE₂ in response to IL-1β and TNFα has been demonstrated in primary human vascular smooth muscle cells. The induced
expression of mPGES-1 and COX-2, as well as PGE₂ production, in smooth muscle cells in response to TNFα and IL-1β suggest involvement of these cells in the elevated levels of PGE₂ observed in inflammatory diseases.⁶⁹,¹¹⁸,¹⁹⁸

Mast cells constitute a part of the inflammatory response that has been poorly studied in regard to the expression of PGE synthases and PGE₂ production. Our results showed that cultured human mast cells expressed PGE synthases and COX-2, as well as produced PGE₂ at a basal level, which was not affected in response to TNFα or IL-1β treatment. However, basal PGE₂ production by mast cells may still contribute to enhanced total PGE₂ production through an additive effect, since an increased number of mast cells has been reported in inflamed gingival tissue compared to healthy gingiva.²²,¹⁹⁹ In the connective tissue from patients with periodontitis, other immune cells were positively stained for PGE synthases and COX-2. In line with our findings, expression of mPGES-1 and COX-2 in immune cells have previously been demonstrated in macrophages from RA-affected synovial tissue,¹¹³ and B-cells from RA patients displayed an up-regulated expression of mPGES-1 and COX-2 compared to healthy controls.²⁰⁰ In addition, in the monocytic cell line, THP-1, the cytokines TNFα and IL-1β increased the expression of mPGES-1 and COX-2 accompanied by enhanced PGE₂ production.²⁰¹

We also investigated the presence of CD45+ cells in the connective tissues. CD45 is a marker for lymphocytes.¹⁶⁴ We hypothesized that these cells may contribute to the elevated PGE₂ levels via cell-cell interactions, since these cells produce inflammatory mediators in vivo during a host-immune response.²⁰ In order to mimic the in vivo situation, we performed co-culture experiments with lymphocytes and the adherent cells; fibroblasts, endothelial cells or smooth muscle cells. In the co-culture experiments, we observed enhanced expression of mPGES-1 and COX-2 paralleled with increased PGE₂ production in cultures of fibroblasts and smooth muscle cells, suggesting a regulatory interplay between these cells contributing to the up-regulation of PGE₂ synthesis. One possible explanation for this may be that lymphocytes release pro-inflammatory cytokines such as IL-1β,²⁰² and thereby stimulate PGE₂ synthesis. This suggestion is further supported by our previous findings that COX-2 expression and PGE₂ production increased in gingival fibroblasts by cell-cell contact with lymphocytes.²⁰³

Among the cells studied in vitro, gingival fibroblasts and smooth muscle cells exhibited a higher basal PGE₂ production, and these two cell types also considerably increased PGE₂ production in response to cytokines, via induced expression of mPGES-1 and COX-2. HUVECs and mast cells produced lower amounts of PGE₂. In HUVECs, PGE₂ production was only increased in response to TNFα, whereas mast cells were not stimulated by the cytokines. In addition to the cells described above, epithelial cells were positively stained for PGES and COX-2 in gingival tissues, although these cells were not included in the in vitro studies since the research was focused on the connective tissues. However, these cells have previously been reported to increase PGE₂ production after stimulation with bacterial components,²⁰⁴,²⁰⁵ and recently it was reported that COX-2 and
mPGES-1 expression was induced in this cell type. Thus, epithelial cells may also contribute to elevated PGE₂ levels in periodontitis.

Altogether, the results of Study II suggest that among the cells analyzed, the most prominent cellular sources of inflammation-induced PGE₂ included fibroblasts and smooth muscle cells. A summary of the different cell types studied and their possible contribution to inflammation-induced PGE₂ production in gingival connective is illustrated in Figure 9. However, one has to be aware that the in vitro findings are not directly transferrable to the in vivo situation. Apart from the gingival fibroblasts, the cell types used in Study II are not primary cells isolated from human gingiva. The results should therefore only be taken as indications of possible in vivo expression and regulation. To confirm our results in the context of periodontitis, future studies using cells isolated from gingival tissues or in situ analysis of periodontitis-affected gingival biopsies using next generation single-cell sequencing would be of great importance.

**Aminothiazoles, novel inhibitors of mPGES-1 (Studies III and IV)**

Several reports, including Studies I and II, have suggested that mPGES-1 together with COX-2 regulates PGE₂ production. Inflammation-induced expression of these enzymes may thereby contribute to the increased PGE₂ levels observed in chronic inflammatory diseases such as periodontitis and RA. The widely used anti-inflammatory drugs, NSAIDs and selective COX-2 inhibitors, are reported to cause side-effects, such as cardiovascular and gastrointestinal injury. In light of the side-effects associated with these drugs inhibiting COX enzymes, mPGES-1 acting downstream of COX has been suggested as an attractive therapeutic target for next-generation anti-inflammatory drugs with possibly fewer and less severe adverse effects. Several novel inhibitors potentially targeting mPGES-1 have been identified in structural computer models, but only a few inhibitors targeting the enzyme mPGES-1 have been reported to also inhibit in vivo activity. Currently, there are no specific mPGES-1 inhibitors approved for clinical use and apart from Study III none of the identified mPGES-1 inhibitors have been tested in models of periodontitis. In Study III, docking studies identified possible mPGES-1 inhibitors that inhibited PGE₂ selectively via mPGES-1; subsequent investigations evaluated the potential of these inhibitors as possible future treatments of chronic inflammation, including periodontal disease. Based on the structural binding of the active site of mPGES-1 enzyme, we identified several compounds, of which seven were aminothiazoles. Thiazoles and their derivatives exhibit a range of biological activities and have been attributed with promising anti-inflammatory properties, including positive effects on both collagen and pristane-induced arthritis in mice. Recently, it was also reported that aminothiazoles reduced vascular permeability and inflammatory cell
infiltration in a mouse peritonitis model. The potential anti-inflammatory activities of thiazoles have previously been attributed to the findings that these compounds inhibit 5-Lipoxygenase enzyme activity and LTB₄ production, and inhibit the synthesis of the inflammatory mediator nitric oxide. However, the effect of aminothiazoles on PGE₂ synthesis in the context of experimental periodontitis was for the first time described in Study III. In this work the two most potent inhibitors, TH-848 and TH-644, selected by their ability to inhibit cytokine-induced PGE₂ production in cell cultures were further investigated in Studies III and IV.

**Aminothiazoles inhibits PGE₂ biosynthesis via mPGES-1 (Study III)**

Human gingival fibroblasts were used as an *in vitro* model to study the effect of the aminothiazoles on PGE₂ synthesis. In gingival fibroblasts, aminothiazoles, TH-848 and TH-644, decreased IL-1β-stimulated PGE₂ production in a dose-dependent manner. Similar to the aminothiazoles, we also showed that the anti-inflammatory drugs DEX, Celecoxib and Triclosan, used as positive controls, also inhibited IL-1β-induced PGE₂ production as previously reported.

In a cell-free enzyme activity assay, mPGES-1 activity was decreased by TH-848 and TH-644 without affecting COX-2 activity. In human gingival fibroblasts, mPGES-1 expression was up-regulated by the inflammatory cytokine IL-1β in agreement with Studies I and II. Up-regulated protein expression of mPGES-1 was not affected by TH-644 but slightly reduced by TH-848. The effect of TH-848 on mPGES-1 protein expression was similar to our results obtained by the known mPGES-1 inhibitor MK-886, used as control, and the previously reported finding that imidazole derivatives also reduced mPGES-1 expression. The biological significance of this reduction by TH-848 on mPGES-1 protein expression is yet to be explored. However, the well-characterized COX-inhibitors that are widely used in clinical therapies are reported to reduce PGE₂ synthesis via mechanisms affecting both expression and activity of the COX enzymes. None of the compounds, TH-848 or TH-644, affected IL-1β-induced mPGES-1 or COX-2 mRNA expression. In addition, COX-2 protein expression was not affected by aminothiazoles. Moreover, neither TH-848 or TH-644 nor IL-1β affected the basal expression of the isoenzymes mPGES-2 or cPGES, in agreement with our previous findings, including Study II showing no increase of these isoenzymes in response to cytokines. In line with our results, it has been reported that thiazoles bind to mPGES-1 protein and decrease IL-1β-induced PGE₂ production in cancer cell lines. In summary, our results showed that the two aminothiazoles inhibited PGE₂ production in gingival fibroblasts by targeting mPGES-1 enzyme activity, indicating that the inhibitory effect of TH-848 and TH-644 on the regulation of PGE₂ was mainly mediated by decreased mPGES-1 enzyme activity (Figure 8).
Alveolar bone destruction is reduced by aminothiazole TH-848 (Study III)

Promising in vitro effects of aminothiazoles as potent mPGES-1 inhibitors lead us to further study their effect in vivo using a model of experimental periodontitis in rats. Experimental periodontitis was induced by silk ligatures tied around the second upper molars of the rats, which is a common model used for experimental studies of periodontitis, including testing of anti-inflammatory and anti-bacterial agents for treatment of periodontal disease. Animals were treated locally by topical application with a gel with or without the aminothiazole TH-848 for one week. Alveolar bone levels were measured on dental radiographs and our results revealed that alveolar bone resorption was decreased by 46% after treatment with the aminothiazole, TH-848, compared to vehicle-treated controls. This reduction may be explained by decreased PGE₂ production in the gingiva in response to treatment with TH-848, which has the capacity to inhibit PGE₂ synthesis. To further explore this assumption, we performed in vitro studies using rat gingival fibroblasts. Similar to human gingival fibroblasts, rat gingival fibroblasts also demonstrated a reduction of cytokine-induced PGE₂ production in the presence of TH-848. These results suggest that TH-848 is not only an inhibitor of human mPGES-1, but is also capable of inhibiting the rat ortholog of the enzyme. Essential structural differences
between human and rodent mPGES-1 enzyme exists. Inhibition of rat and human mPGES-1 by TH-848 is important since one of the main difficulties in developing useful mPGES-1 inhibitors is the preclinical testing of inhibitors designed for human mPGES-1 in animal models.

In line with our results, several studies using substances known to inhibit PGE\(_2\) synthesis have reported beneficial effects on periodontal disease. For example, the selective COX-2 inhibitor, Celecoxib, have shown beneficial effects on the progression of periodontal disease, as well as other diseases associated with inflammatory bone destruction, such as RA. Triclosan, also reported to inhibit mPGES-1 in gingival fibroblasts, reduced gingival inflammation in patients and alveolar bone loss in experimental periodontitis in rats. Notably, Triclosan also has anti-bacterial properties causing undesirable effects, such as environmental toxicity and a potentially increased risk of bacterial resistance, suggesting that this agent should be avoided in commercial use.

In contrast, our results showed no anti-bacterial effects for TH-848 or TH644 for the tested oral bacteria, indicating that these aminothiazoles lack anti-bacterial properties. The substance curcumin has also been reported to reduce IL-1\(\beta\)-stimulated mPGES-1 expression in vitro and cytokine expression in gingival tissues of experimental periodontitis in rats, but this substance did not decrease alveolar bone resorption in ligature-induced experimental periodontitis. However in that study, curcumin was administered systemically by oral gavage, but in our study TH-848 was administrated topically to the ligature-equipped tooth. We believe that local, preferably topical, administration is essential to obtain accurate treatment outcomes, in this case a decrease in alveolar bone resorption. Our results demonstrate that the aminothiazole TH-848 reduced alveolar bone loss in experimental periodontitis in rats, suggesting this compound as a potential drug for future treatment of inflammatory bone resorption.

**Aminothiazoles inhibit osteoclastogenesis in RAW 264.7 cells (Study IV)**

The intriguing results from Study III, demonstrating decreased alveolar bone resorption in experimental periodontitis after treatment with the aminothiazole TH-848, which lead us to further investigate the effects of aminothiazoles on osteoclastogenesis in vitro. For this purpose we used the murine macrophage cell line RAW 264.7, which is commonly used as a model of osteoclastogenesis due to its ability to differentiate into osteoclasts upon stimulation by RANKL or LPS. In this study, RAW 264.7 cells were activated by RANKL and treated with the aminothiazoles, TH-848 or TH-644. The number of multinucleated osteoclast-like cells positively stained for the osteoclast marker TRAP was decreased by the aminothiazoles in RANKL-activated RAW 264.7 cell cultures, suggesting an inhibition of osteoclastogenesis by these compounds (Figure 9).
in cultures of RANKL-stimulated RAW 264.7 cells. Similar to our results, it has been reported that Celecoxib also reduces osteoclast differentiation and decreases RANKL-stimulated PGE$_2$ production in RAW 264.7 cells,$^{226}$ suggesting that the inhibitory effect of Celecoxib on osteoclastogenesis involves PGE$_2$ synthesis. These results are in agreement with our findings that exogenous PGE$_2$ increased the number of osteoclasts in cultures of RANKL-stimulated RAW 264.7, which has also been demonstrated in previous studies.$^{92,226}$ Altogether, this supports our hypothesis that the inhibitory effect of the aminothiazoles TH-848 and TH-644 on osteoclastogenesis may be mediated by inhibition of PGE$_2$.

The inhibition of osteoclastogenesis, assessed by the number of osteoclasts, and the inhibition of PGE$_2$ production occurred at similar concentrations for TH-848 (IC$_{50}$ 0.20 and 0.24 µM respectively) and TH-644 (IC$_{50}$ 12.9 and 10.4 µM respectively). Interestingly, the inhibitory concentrations, in terms of decreased osteoclast numbers and PGE$_2$ production, occurred at much lower concentrations for TH-848 than for TH-644 in RANKL-stimulated RAW 264.7 cells. In human gingival fibroblasts, in Study III, the inhibition of IL-1β-stimulated PGE$_2$ occurred at IC$_{50}$ 1.1 µM and 1.5 µM for TH-848 and TH-644 respectively. A possible reason for the differences in inhibitory concentrations between TH-848 and TH-644 on PGE$_2$ production in RAW 264.7 cells and the similar inhibitory concentrations in human gingival fibroblasts might be due to their different selectivity toward human versus murine mPGES-1 since RAW 264.7 is a murine cell line and the aminothiazoles were identified as inhibitors targeting human mPGES-1.$^{184}$ Our findings that aminothiazoles, especially TH-848, inhibited PGE$_2$ production in murine RAW 264.7 cells, as well as in human and rat gingival fibroblasts (Study III)$^{184}$ indicates that TH-848 might be a potent inhibitor of human, rat and murine mPGES-1. Thus, we suggest that TH-848 may be a valuable mPGES-1 inhibitor in the future quest towards discovering clinically useful mPGES-1 inhibitors, overcoming the structural differences between human and rodent mPGES-1.$^{143,144}$

To further evaluate the inhibitory effect of aminothiazoles on osteoclastogenesis, mRNA expression of the genes TRAP, RANK, Cathepsin K and OPG, involved in bone metabolism, were analyzed. The results showed that only Cathepsin K, predominantly expressed by osteoclasts,$^{227}$ was significantly down-regulated by the two aminothiazoles in RANKL-stimulated RAW 264.7 cells.

These observations, together with the results from Study III, showed that the aminothiazoles, TH-848 and TH-644, inhibited PGE$_2$ production via mPGES-1 enzyme activity in vitro, ameliorated experimental periodontitis and inhibited osteoclastogenesis, suggesting that these compounds may be useful in future treatment strategies of bone resorption in chronic inflammatory diseases, such as periodontitis.
Figure 9. Schematic drawing of the results from Studies I-IV. The figure illustrates the production of PGE$_2$ from different cell types and its possible effect on osteoclast differentiation. Bold texts highlight increased expression of PGE synthases or COX-2 in response to TNF$\alpha$ or IL-1$\beta$. Increased PGE$_2$ levels stimulate RANKL-mediated osteoclast differentiation and treatment of the cells with aminothiazoles TH-848 and TH-644 inhibited PGE$_2$ production and osteoclastogenesis.

Abbreviations: EC, endothelial cell; F, fibroblast; IL-1$\beta$, interleukin-1$\beta$; MC, mast cell; OC, osteoclast; P, osteoclast precursors; PGE$_2$, prostaglandin E$_2$; RANKL, receptor activator NF$\kappa$B ligand; SMC, smooth muscle cell; TNF$\alpha$, tumor necrosis factor $\alpha$. 
Main findings

• The terminal enzymes in PGE$_2$ biosynthesis, PGE synthases, were expressed in gingival tissues from patients with periodontitis. In gingival connective tissues, all three isoforms of PGE synthases, mPGES-1, mPGES-2 and cPGES were expressed in fibroblasts, endothelial cells, smooth muscle cells and immune cells.

• Human gingival fibroblasts, HASM cells, HUVECs and mast cells expressed mPGES-1, mPGES-2 and cPGES as well as the upstream enzyme COX-2 accompanied by subsequent PGE$_2$ production. Up-regulated expression of mPGES-1 followed by increased PGE$_2$ production was observed in gingival fibroblasts and HASM cells in response to the inflammatory cytokines TNF$\alpha$ and IL-1$\beta$. 

• Aminothiazoles were identified as novel mPGES-1 inhibitors by docking studies. The aminothiazoles TH-848 and TH-644 inhibited mPGES-1 enzyme activity but not COX-2 activity in vitro. Cytokine-induced PGE$_2$ production was decreased by the two aminothiazoles in gingival fibroblasts. IL-1$\beta$-induced protein expression of mPGES-1 was slightly decreased by TH-848 but not affected by TH-644. Neither the protein expression of COX-2, mPGES-2 and cPGES nor the mRNA expression of mPGES-1 and COX-2 was affected by the aminothiazoles TH-848 and TH-644.

• In ligature-induced experimental periodontitis in rats, aminothiazole, TH-848, decreased alveolar bone resorption after one week of local daily treatment.

• In a model of osteoclastogeneis using osteoclast precursors, RAW 264.7 cells, the aminothiazoles TH-848 and TH-644 inhibited osteoclast differentiation in parallel with decreased PGE$_2$ production in RANKL-activated RAW 264.7 cells.
Future perspectives

The role of the inflammatory mediator PGE₂ is well established in the pathogenesis of chronic inflammatory diseases including periodontitis. The terminal enzymes in the PGE₂ biosynthesis, PGES, are considered to be an attractive therapeutic target for next-generation anti-inflammatory drugs. Current anti-inflammatory drugs, NSAIDs and selective COX-2 inhibitors are reported to have side-effects such as gastrointestinal injury and increased risk for cardiovascular events due to inhibition of the COX enzymes. Intensive research is therefore underway to develop specific mPGES-1 inhibitors lacking the adverse effects associated with COX inhibition. In this work we identified and described two aminothiazole derivatives, TH-848 and TH-644, as potential inhibitors of mPGES-1 without affecting the activity or expression of COX-2. In a model of experimental periodontitis in rats, we demonstrated reduced alveolar bone resorption after local treatment with the aminothiazole TH-848. Furthermore, the aminothiazoles decreased osteoclastogenesis and PGE₂ production in cultures of RANKL-activated osteoclast precursors RAW 264.7 cells.

Our results show that inflammation-induced PGE₂ production is mediated by increased expression of mPGES-1 and COX-2 in gingival fibroblasts, which might contribute to the increased and sustained PGE₂ levels detected in gingival tissues from patients with periodontitis. Aminothiazoles inhibited PGE₂ production via mPGES-1 enzyme activity in vitro, ameliorated experimental periodontitis in rats and inhibited osteoclastogenesis, suggesting that these compounds may be used for future treatment strategies of bone resorption in chronic inflammatory diseases, such as periodontitis. To fully understand the suitability of aminothiazoles as potential drugs for treatment of inflammatory bone loss and periodontitis, more research is required. Further studies to evaluate the effect of aminothiazoles using other cell types involved in the pathogenesis of periodontitis and bone resorption, such as periodontal ligament cells and osteoblasts, would be of great importance. Additional experimental studies in animal models investigating the expression of mPGES-1 in gingival tissues and levels of PGE₂ in gingival fluid in response to aminothiazoles would further clarify the clinical significance of mPGES-1 inhibitors in periodontitis.
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