

Importance of bacterial hydrogen sulfide in the pathogenesis of periodontal diseases

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Gothenburg, Sweden, 2017



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ISBN 978-91-629-0270-4 (print)
ISBN 978-91-629-0271-1 (PDF)
<http://hdl.handle.net/2077/52841>

Printed by Ineko AB in Gothenburg, Sweden 2017

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Abstract

Hydrogen sulfide (H₂S) is one of many end-products of the proteolytic activities in the subgingival microbiota in patients with periodontal diseases, such as gingivitis and periodontitis. Although H₂S is generally regarded as toxic, the mechanisms that underlie its production and its effects on human cells and tissues are poorly understood. Therefore, the role of H₂S in the pathogenesis of periodontal diseases was investigated. Two colorimetric methods, the bismuth test (BT) and the methylene blue (MB) method, were used to estimate the amounts of H₂S produced by the bacteria *in vitro* and *ex vivo* (**Papers I, II and V**). Oral bacteria, e.g., *Fusobacterium* spp., *Porphyromonas gingivalis* and *Treponema denticola*, were found to have strong capacities to degrade cysteine and produce H₂S *in vitro* (**Paper I**). The *Fusobacterium* spp. were found to express several enzymes that are involved in the production of H₂S. The expression patterns of the different enzymes varied among *Fusobacterium* subspecies and strains (**Paper III**). In an *ex vivo* experiment using BT, we showed that the subgingival plaques of subjects (N=43) with poor oral hygiene had the capacity to produce H₂S (**Paper II**). High levels of periodontitis-associated bacteria were detected, and the BT values reflected the proteolytic activities of the bacteria and gingival inflammation rather than disease progression and periodontitis. A correlation between a positive BT and gingival inflammation was confirmed in **Paper V**, where H₂S-producing bacteria were significantly more prevalent in the subgingival pockets of periodontitis patients (N=32) than of healthy controls (N=32), which indicates potent bacterial proteolytic activities in the untreated deep periodontal pockets. **Paper IV** described how the peripheral blood mononuclear cells (PBMCs) of blood donors and a monocytic cell line increased their secretion of the pro-inflammatory cytokines IL-1 β and IL-18 *in vitro* when exposed to the H₂S-donor sodium hydrosulfide (NaHS). This secretion was shown to be mediated by the NLRP3 inflammasome. These results were verified in **Paper V**, where the PBMCs of periodontitis patients and healthy controls secreted significantly higher levels of IL-1 β and IL-18 when exposed to NaHS. In addition, both unexposed and exposed PBMCs of the periodontitis patients secreted higher levels of the two cytokines than the corresponding cells of healthy controls. These results suggest that the susceptibility of the host to develop disease can be attributed in part to enhanced secretion of pro-inflammatory cytokines following exposure to bacterial metabolites, such as H₂S. In summary, toxic bacterial metabolites, such as H₂S, may play an important role by affecting the cells of the host immune system, thereby inducing and sustaining gingival inflammation.

Sammanfattning på svenska

Tandlossning är en vanligt förekommande inflammatorisk sjukdom där bakterier i tandköttsfickan spelar en viktig roll. Grava former av sjukdomen drabbar ca 10 % av världens befolkning och kan leda till tandförlust. Trots bakteriernas roll är det fortfarande till vissa delar oklart hur sjukdomen uppstår och fortlöper samt varför vissa individer är mer benägna att utveckla sjukdomen än andra.

I denna avhandling har svavelväte (H_2S) och dess roll vid tandlossningssjukdom studerats. Svavelväte är en toxisk produkt som bakterier i tandköttsfickan bildar vid nedbrytning av aminosyran cystein. Bakteriernas förmåga att bilda svavelväte samt påverkan av svavelväte på kroppsegna celler har studerats i relation till inflammation i tandköttet. Resultaten i avhandlingen visar på att svavelväte kan bildas av bakterier som sedan tidigare är förknippade med tandlossningssjukdom (**delarbete I**). Vidare att en typ av bakterie, i detta fall *Fusobacterium*, har flertalet proteiner som deltar vid bildningen av svavelväte (**delarbete III**).

I **delarbete II** och **V** mättes bakteriernas förmåga att bilda svavelväte i prov från tandköttsfickan. Prov från individer med inflammation i tandköttet eller med tandlossningssjukdom hade förmågan att bilda mer svavelväte jämfört med prov från individer med friskt tandkött. Bakterier, som är förknippade med tandlossningssjukdom samt som har en förmåga att bilda svavelväte, kunde också påvisas i tandköttsfickan hos deltagarna i studierna.

Avslutningsvis har avhandlingen visat att svavelväte kan påverka kroppsegna celler att bilda substanser som kan bidra till inflammation (**delarbete IV och V**), samt att bildningen av dessa substanser sker via NLRP3 inflammasomen i blodceller vid närvaro av svavelväte. Blodceller från olika individer hade varierande förmåga att bilda inflammationsframkallande substanser. Celler från de individer som hade tandlossningssjukdom bildade en större mängd substanser jämfört med celler från individer med friskt tandkött.

Sammanfattningsvis visar resultaten från denna avhandling att bakterier, genom produktion av olika metaboliter så som svavelväte, kan påverka våra kroppsegna celler och därmed bidra till inflammation i tandköttet.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals:

- I. **Basic A**, Blomqvist S, Carlén A, Dahlén G
Estimation of bacterial hydrogen sulfide production *in vitro*.
Journal of Oral Microbiology 7. 2015.
- II. **Basic A**, Dahlén G
Hydrogen sulfide production from subgingival plaque samples.
Anaerobe 35:21-27. 2015.
- III. **Basic A**, Blomqvist M, Dahlén G, Svensäter G
The proteins of *Fusobacterium* spp. involved in hydrogen sulfide production from L-cysteine.
BMC Microbiology 17. 2017.
- IV. **Basic A**, Alizadehgharib S, Dahlén G, Dahlgren U
Hydrogen sulfide exposure induces NLRP3 inflammasome-dependent IL-1 β and IL-18 secretion in human mononuclear leukocytes *in vitro*.
Clinical and Experimental Dental Research 3:115-120. 2017
- V. **Basic A**, Serino G, Leonhardt Å, Dahlén G
Induction of interleukin (IL)-1 β and IL-18 secretion by hydrogen sulfide in periodontitis patients and healthy controls: a clinical cross-sectional study
In manuscript

*Reprints of **Paper I** were made with the permission of the publisher. **Papers II, III and IV** are published in Open Access journals and are distributed under the terms of the Creative Commons Attribution (-NonCommercial) ver. 4.0 International License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial use, distribution, and reproduction, provided that the original work is properly cited.*

List of abbreviations

ASC	Apoptosis-associated speck-like protein
BoP	Bleeding on Probing
BT	Bismuth sulfide test
CAL	Clinical Attachment Level
CARD	Caspase recruitment domain
CBS	Cystathionine- β -synthase
CO	Carbon monoxide
CSE	Cystathionine- γ -lyase
DTPA	Diethylenetriaminepentaacetic acid
GCF	Gingival crevicular fluid
H ₂ S	Hydrogen sulfide
IL	Interleukin
LPS	Lipopolysaccharide
MB	Methylene blue method
MPO	Myeloperoxidase
MS	Mass spectrometry
Mw	Molecular weight
NaHS	Sodium hydrosulfide
NF- κ B	Nuclear factor-kappa B
NLRP3	NOD-like receptor pyrin domain-containing 3
NO	Nitric oxide
NOD-like	Nucleotide-binding oligomerization domain-like
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI	Plaque Index
pI	Isoelectric point
PLP	Pyridoxal 5'-phosphate
PMN	Polymorphonuclear leukocytes
PMMVF	Princess Mother Medical Voluntary Foundation
PPD	Probing Pocket Depth
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SRB	Sulfate-reducing bacteria

TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor-alpha
VSC	Volatile sulfur compounds
3MST	3-Mercaptopyruvate sulfurtransferase

Introduction

Periodontal diseases are common diseases of the oral cavity and severe forms can lead to edentulism. They are classified as inflammatory diseases because inflammation and destruction of the supporting tissues of the teeth are consequences of the host inflammatory response. As this host response is partly due to an oral bacterial challenge, treatment strategies are oriented towards addressing this challenge. While much research has been performed on this topic, the bacterial role in periodontal disease development and progression are still largely unknown, and predicting future disease progression is problematic. Despite many new findings, the anatomy of the tissues around teeth (where a part of the tooth is in a sterile environment and another part is heavily colonized by bacteria), the composition of the biofilm, the interplay between host cells and bacterial cells, and the role of the host immune response all contribute to the complexity of periodontal research.

This thesis addresses one aspect of the host-microbe interplay that may be involved in the pathogenesis of periodontitis, which is the more severe form that involves the destruction of tissues. We focus on the degradation of the amino acid L-cysteine by the microbes present in the oral cavity and the subsequent production of hydrogen sulfide (H_2S), and examine the possible role of H_2S in the host inflammatory response that is characteristic of periodontal diseases. We show how one end-product (H_2S) of the bacterial activity in the subgingival pocket can affect host cells and thereby contribute to an inflammatory response. This is a part of a complex and dynamic interplay between different bacterial species, the products of these bacteria, and the host immune cells. It is the net effect of the outcomes of all these interactions that most likely determines and defines the different periodontal diseases.

Periodontal diseases

Definition and classification

Periodontal diseases afflict the periodontium, which defines the tissues that surround and support the teeth. They are diagnosed on the basis of clinical findings. As a consequence, it is not known if these are different diseases or one disease with different clinical manifestations. According to the classification proposed by Armitage [1] the periodontal diseases can be divided into:

Gingivitis

Gingivitis entails inflammation of the gingiva without any destruction of the periodontium. This is a reversible process in which the tissues can be restored to their initial status after treatment. Gingivitis is induced by plaque accumulation, although there are also forms with viral or fungal etiology. Long-standing gingivitis, as the name suggests, involves gingivitis over an extended period of time, but without clinical signs of tissue destruction.

Chronic periodontitis

Chronic periodontitis involves inflammation of the gingiva and clinical signs of destruction of supporting tissues (Figure 1, top panels). The destroyed tissues cannot be fully restored after treatment, which means that the process is irreversible. Periodontitis can be subdivided into:

- Localized, in which only a part of the dentition (up to 30 % of the teeth) is affected [2]; and
- Generalized, in which > 30% of the teeth are affected by the disease

Aggressive periodontitis

In cases of aggressive periodontitis there is inflammation of the gingiva and clinical signs of destruction of supporting tissues, similar to chronic periodontitis but the destruction is more rapid (Figure 1, bottom panels). The age of onset is often earlier than the chronic form and the pattern of destruction can differ [2, 3]. This form of

periodontitis may have fewer clinical signs of inflammation and lower levels of plaque and calculus. It is subdivided into:

- Localized aggressive periodontitis; and
- Generalized aggressive periodontitis

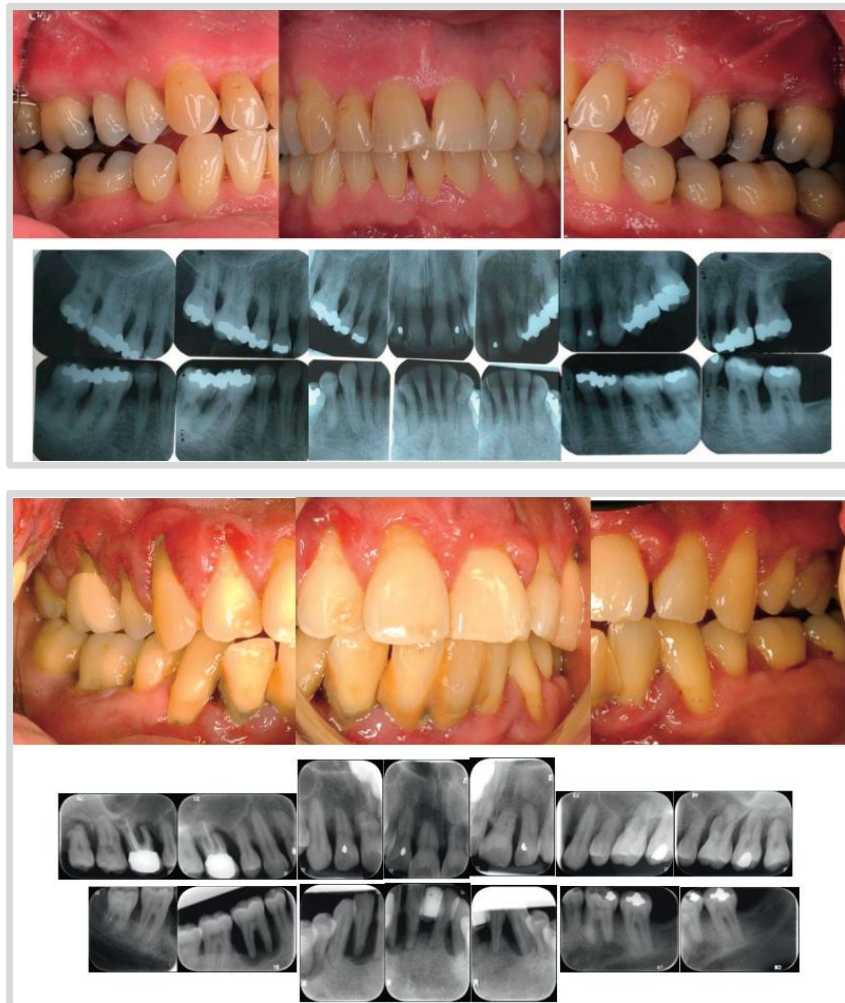


Figure 1 Intraoral photographs and radiographs of two patients with periodontitis. For the two 45-year-old males, the first case (top panels) was diagnosed with generalized chronic periodontitis, while the second case (bottom panels) was diagnosed with generalized aggressive periodontitis.

Photographs by courtesy of Giovanni Serino

The three above-mentioned periodontal disease types are addressed in this thesis. In addition, periodontitis can be a manifestation of systemic disorders (i.e., those associated with hematologic or genetic disorders, such as neutropenia, leukemia and Papillon-Lefèvre syndrome), necrotizing periodontal diseases, abscesses of the periodontium, periodontitis associated with endodontic lesions, and developmental and acquired deformities and conditions.

Clinical signs

The clinical signs of periodontal diseases include the typical signs of inflammation, such as swelling and redness of the gingiva. Bleeding on Probing (BoP) is commonly used to assess the extent of gingival inflammation. Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL) are used to disclose the degree and extent of earlier disease [4]. In addition, the Plaque Index (PI) is commonly used to define the amount of plaque that lies alongside the gingival crevice, as an indicator of oral hygiene efforts. Currently, there are no commonly used tools or strategies to evaluate disease activity or to predict periodontitis development and progression. Thus, based on initial clinical findings of gingivitis it is not possible to predict whether the patient will develop long-standing gingivitis or periodontitis, or indeed revert to a healthy gingiva. Only the progression of periodontitis and the consequences of earlier disease experience can be measured by PPD, CAL or radiography.

Incidence and prevalence

Gingivitis, which is inflammation of the gingiva without destruction of supporting tissues, is believed to result from insufficient oral hygiene. Therefore, it can affect all individuals and probably does so for at least a part of the dentition. Periodontitis, including the loss of connective tissue and alveolar bone around the teeth, is also common, although not as prevalent as gingivitis. The severe form of periodontitis was ranked as the sixth-most prevalent condition in the world in the Global Burden of Disease 2010 [5]. In that study, severe periodontitis was classified as PPD ≥ 6 mm, CAL >6 mm or PPD >5 mm. The prevalence of severe periodontitis was reported as 11.2 % between 1990 and 2010, and the incidence was estimated as 701 new cases per 100,000 persons/year in 2010. The peak incidence was at 38 years of age [5]. In the U.S., the prevalence of severe periodontitis in the same year was reported as 8.5 % [6].

While severe periodontitis is a common disease worldwide, recent studies have shown intra- and inter-country variations [5, 7], for which socioeconomic status, race, and ethnic group tend to be of importance. Severe periodontitis is one major cause of tooth loss. Severe tooth loss (having < 9 teeth) was reported in 2,4 % of the world population in 2010 [8]. The prevalence of total edentulism in persons aged 65–74 years has been reported to be in the range of 10 % – 35 % depending on the country being studied [7].

Associations with age, habits and systemic diseases

Periodontal diseases are associated with oral hygiene status. The bacterial load, including the total number and composition of the microbiota, in the oral cavity is of importance [9], and the treatment of all types of periodontal diseases includes the suppression of bacterial numbers through improved oral hygiene. In addition to the presence of microbes, periodontitis is associated with a variety of genetic, environmental, and behavioral factors [7, 9], the most important of which are described below.

Age

The prevalence of periodontitis increases with age [5], with a substantial increase between the third and fourth decades of life. The aggressive form of periodontitis has historically been associated with onset at an early age, in contrast to the chronic form, which was believed to develop later in life. The classification used today relies less on age and more on the clinical parameters.

Genetics

There is a linkage between periodontitis and genetics. Some systemic diseases and genetic disorders, such as neutropenia, Down's syndrome, Papillon-Lefèvre syndrome, Chédiak-Higashi syndrome, Leukocyte adhesion deficiency syndromes, and Ehlers-Danlos syndrome are associated with certain forms of periodontal diseases [2], as mentioned earlier. Many of these syndromes result in suboptimal functions of the granulocytes of the polymorphonuclear leukocytes (PMN).

Chronic and aggressive periodontitis have not yet been linked to specific genetic changes or abnormalities, although heredity is assumed to play a role [10-12]. The

general consensus in the scientific community is that some individuals are more prone than others to develop periodontitis. This notion is based on the fact that some individuals with poor oral hygiene do not develop periodontitis, despite long-standing gingivitis [13, 14] while others are more susceptible. The reason for this individual susceptibility and the mechanisms involved are still largely unknown, although genetic polymorphisms have been suggested to play a role [15]. However, the results of studies of polymorphism, gene sequence variants, and susceptibility to develop periodontal diseases are contradictory and inconclusive [9, 15-24].

Race and ethnic groups

A difference in the distribution of periodontal diseases has been reported among different populations and ethnic groups [6, 7]. The JP2 clone of *Aggregatibacter actinomycetemcomitans* has been shown to be of importance in the etiology of aggressive periodontitis in children and adolescents in north-west Africa [25]. There are also differences in the frequencies of specific gene polymorphisms among the different ethnic groups [18, 20, 22]. Nevertheless, the prevalence of periodontitis is remarkably similar worldwide, regardless of socioeconomic, and ethnic factors, oral hygiene efforts, and access to oral healthcare [13].

Behavioral factors

Tobacco smoking is strongly associated with the prevalence of periodontitis, including its more severe forms [9]. Consumption of tobacco products has been shown to impair the outcome of periodontal treatment [9]. Tobacco smokers are over-represented in populations that have a high prevalence of periodontitis, as illustrated for the periodontitis group studied in **Paper V**.

Heavy consumption of alcohol is also associated with increased severity of periodontitis [7]. In addition, betel chewing has also been suggested to be associated with periodontal diseases [26].

In addition to the factors mentioned above, periodontitis is associated with systemic disorders, such as diabetes [7, 9] and possibly HIV infection [9]. Furthermore, malnutrition and emotional stress have been suggested to be associated with periodontal diseases [7, 9].

Bacteria and bacterial biofilms

Bacterial cells and other microorganisms, live in symbiosis with human cells in the oral cavity, the intestine, and on the skin [27, 28]. There is a rich diversity of the microorganisms and in the human body they are more numerous than the human cells themselves [29]. One milliliter of saliva harbors approximately 10^8 microorganisms [30]. The human oral cavity is estimated to be colonized by up to 700 different bacterial species [31, 32]. The majority of the bacterial cells in the oral cavity are $0.6 - 4.0 \mu\text{m}$ in diameter [33]. They can be found on the mucosal surfaces, on the tongue and within its fissures, on the teeth and gingiva, as well as in the periodontal pockets. The composition of the microbiota on the oral surfaces is stable but the various sites differ in composition. This composition is dictated by the surrounding environment and available sources of nutrients [34, 35].

Bacterial biofilms

On oral surfaces, microorganisms co-aggregate, interact, and sometimes form biofilms [36]. A biofilm, which is often described as a community of bacterial cells, comprises a mixture of different bacterial species that live, interplay and compete, while embedded in a matrix of polysaccharides and proteins [37]. In biofilms that contain different bacterial species, different environmental niches within the biofilm favor specific microorganisms [34]. A biofilm, when one considers the symbiosis between the microbiota and the host, provides a protective barrier against external pathogens [31]. It also stimulates the host immune system to recognize harmless commensals and to distinguish them from pathogenic microorganisms [27, 29]. The biofilms that form on teeth, in the form of dental plaque, develop directly after cleaning of the tooth surface and the formation of a pellicle, which is followed by the adherence of surrounding bacteria. The bacterial cells in a biofilm are better protected from host defense mechanisms, changes in temperature and pH, anaerobiosis, and antimicrobial agents than the planktonic bacterial cells [38]. The microorganisms can communicate (*via* quorum sensing) and cooperate in food chains. Thus, the nutrients in the biofilm are used in the most efficient way for the overall community [37].

Bacterial homeostasis

Homeostasis is the state in which the host cells and the bacterial cells coexist in balance, and the composition of the microbiota remains stable over time [34]. Homeostasis is associated with health. The current paradigm, based on the ecological plaque hypothesis [34], is that disruption of homeostasis is associated with disease, e.g., in caries and periodontitis [31, 37]. This state is called dysbiosis [31]. It is unclear how disruption of homeostasis occurs in periodontitis, although it is clear that environmental factors, such as access to nutrients, anaerobiosis, and a neutral or alkaline pH, are implicated [34, 35]. It is also unclear whether disruption of homeostasis leads to disease development or if this is a result of the environmental changes induced by the diseased state that has already developed.

The homeostasis in the oral cavity can be disrupted by refraining from tooth brushing. Since there is no shedding of the tooth, the formed biofilm, which at healthy sites is composed mainly of Gram-positive cocci and rods, will remain on the tooth surface adjacent to the gingiva. With time, the biofilm will diversify to include also anaerobes and Gram-negative bacteria, including *Fusobacterium* spp. and spirochetes. This will result in a higher and more diversified bacterial load at the gingival crevice and gingivitis [39].

The growth of the biofilm is dependent upon bacterial growth and activity. Therefore, a growing biofilm is surrounded by nutrients for the bacteria. The bacterial species that are best adapted to the prevailing circumstances will be favored for growth in different environments [35]. The environment of the subgingival pocket in patients with periodontitis is reduced [40], slightly alkaline [41], and favors the bacteria that are associated with a disrupted microbiota, predominantly Gram-negative bacterial species that are anaerobic and have proteolytic activity [34, 42, 43].

Periodontal pathogens

Over the years, certain bacterial species have been classified as periodontal pathogens, as they have been shown in different studies to be associated with periodontal diseases. Arguably, the most famous classification is that of Socransky's colored complexes [44]. In this classification system, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* comprise the prominent red complex, while *Campylobacter* spp., *Fusobacterium* spp., *Prevotella* spp., *Parvimonas micra*, and some other species make up the orange complex, the members of which are frequently found in the deeper periodontitis-affected pockets. These periodontal pathogens

are part of the resident oral microbiota but are typically found in low numbers in healthy subjects. In patients with periodontal diseases, these pathogens are found more frequently and in higher numbers, as illustrated in **Papers II** and **V**. In addition, *A. actinomycetemcomitans* is another classic oral colonizer, being usually associated with the aggressive form of periodontitis. The invasion of bacteria is suggested to be a key event in the initiation of periodontal disease, since many of the bacterial species associated with periodontitis have been shown to invade gingival epithelial cells *in vitro*, and *P. gingivalis* has been reported in the tissue when studying gingival biopsies *ex vivo* [45]. In addition to the classical color-based classification, more recent studies have reported a more diversified flora and have added species, including *Desulfovibrio*-like species, *Desulfobulbus* spp., the not-yet-cultivable phylum *Candidatus saccharibacteria* (TM7), and many others [46-49]. Apart from bacteria, the *Archaea*, which represent a distinct group of prokaryotes, have been detected in the subgingival pockets of periodontitis patients [46, 48].

Virulence factors

Bacteria express virulence factors, which enable them to escape host defenses, to multiply, and to damage host cells. Virulence factors can facilitate colonization, multiplication, invasion, immune activation, and tissue destruction [33]. Among the most frequently studied virulence factors of various bacterial species are different toxins and enzymes.

Exotoxins, which are secreted from the bacterial cells, and endotoxins, which are part of the cell envelope, have been studied extensively. Leukotoxin, which is an exotoxin that damages leukocytes, can be produced by *A. actinomycetemcomitans* [50]. Lipopolysaccharide (LPS) is an endotoxin that is a component of the cell envelope of Gram-negative bacteria [33]. LPS affects many different host cells [51] (and **Paper IV**) and is involved in the induction of inflammation and fever. The LPS of different bacteria differ in structure and composition, as well as in their abilities to induce inflammatory responses and cytotoxicity. Similar to LPS, the lipoteichoic acid of Gram-positive bacteria acts as an endotoxin [52].

Enzymes are proteins that facilitate different chemical reactions. Virulence factors that have enzymatic activities include proteases, such as cysteine proteases [53], metalloproteases, and collagenases.

Metabolites

Metabolites are the products of cellular metabolic processes. They can be scavenged and used by bacteria for the construction of new macromolecules and are in many cases essential for bacterial growth. Alternatively, they can be by-products that have minor impacts on bacterial cell growth.

In the periodontal pocket, the metabolism that is taking place is predominantly proteolytic. The gingival crevicular fluid (GCF; serum exudate) contains proteins that can be degraded by the oral microorganisms, resulting in different metabolites. This bacterial degradation of serum proteins generates hydrogen sulfide (H_2S) as a metabolite [54], along with other metabolites that are dependent upon the specificity of the enzyme involved. H_2S is frequently detected at periodontitis-affected sites [55-59] (and **Paper V**). H_2S has been detected at concentrations of up to 1.9 mM in the GCF obtained from periodontal pockets [60].

Other metabolites that are often detected at sites of periodontitis include various carboxylic acids, such as butyric acid, acetic acid, propionic acid, isovaleric acid, isobutyric acid, phenylacetic acid, and valeric acid, as well as ammonia and indole [33, 61, 62]. The presence of ammonia is believed to contribute to the slightly alkaline pH in the periodontal pockets [35].

It is reasonable to assume that, by measuring the levels of metabolic products, the proteolytic activity of the entire biofilm (and not just that of certain bacterial species) can be assessed indirectly. Thus, the higher the proteolytic activity of the dynamic biofilm, e.g., during rapid bacterial growth or the bacterial shift that takes place during the disruption of the homeostasis, the more end-products and bacterial substances become available to interact with the host. H_2S is one of many products of the subgingival microbiota and is a potential marker to use for measuring the proteolytic activity of the microbiota. In addition to the search for markers of disease activity or progression, examination of the metabolites is important for describing the consequences of a dysbiotic microbiota, as well as for investigations of the mechanisms involved in the pathogenesis of periodontal diseases.

Based on this notion, the overall goal of this thesis was to test the hypothesis that H_2S plays a role in the pathogenesis of periodontal diseases. More specifically, the aims were to: (i) estimate the subgingival levels of H_2S in various disease states, compared to healthy controls. For this, a simple chair-side method was developed; (ii) measure the capacities of oral bacterial species to produce H_2S ; (iii) investigate the bacterial enzymes involved in the production of H_2S ; (iv) evaluate the presence and production of H_2S in relation to the composition of the microbiota in patients with gingivitis or periodontitis; (v) examine the consequences of H_2S contact with

cells of the host innate immune system; and (vi) determine whether the effects of H₂S on host cells differ between periodontitis patients and healthy controls, so as to explore the potential involvement of H₂S in the susceptibility to develop periodontitis.

Methodological considerations

Different methods exist for the isolation and identification of oral bacterial species. These methods have their advantages and certain weaknesses. Thus, the choice of method is dependent upon the question that is being addressed. A combination of different methods can offer a valuable approach to avoid methodological biases.

Sampling

The choice of sampling technique is of great importance when conducting clinical studies. In the periodontal pocket, the bacteria are sampled with a curette or sterile paper points. With the use of the curette, the bacteria in the biofilm that covers the tooth are sampled. Paper points, however, are more likely to adsorb the non-adherent plaque [63-65]. We used curette in **Paper II** and the paper point technique in **Paper V**.

Culture techniques

The most frequently used method for the isolation and identification of oral bacteria involves the culturing of bacteria from clinical samples. The distinctive morphology of the colonies of bacteria grown on solid media allows them to be identified, and even the odor arising from growing cultures can be of assistance in this identification. There are selective media available to facilitate the isolation and growth of species of interest, e.g., the TSBV media for the isolation of *A. actinomycetemcomitans* [66]. The colonies of interest can be subjected to Gram staining [67], and various tests can be performed for the presence of different enzymes and substances, such as catalase, indole, and H₂S [68] (and **Paper I**). While culturing is reliable, it has its limitations, especially when one is dealing with unculturable bacteria. The method is also time-consuming and requires skilled and experienced microbiologists for both the culturing and subsequent identification of the bacteria [47].

Checkerboard

Checkerboard DNA-DNA hybridization [69, 70] is a rapid and rational method for screening many samples. The method is based on bacterial DNA, whereby whole genomic DNA probes derived from species of interest are run against the bacterial DNA samples. Where there is a match, hybridization occurs. By labeling the probes with a marker (digoxigenin), the amount of DNA of the species of interest in the bacterial sample can be calculated through the use of standards. This method is best suited to field-based studies, studies that involve many samples or simultaneous screening for many different species, and samples with a high number of bacteria, as described in **Paper II**. However, there are drawbacks, e.g., cross-reactions between similar species and strains. The reproducibility of the method varies for different bacterial species [71], resulting in somewhat unreliable outcomes and difficulties in interpreting the results. Other disadvantages are the pre-selection of microorganisms to be studied and the high detection threshold of approximately 10^4 bacteria/ml.

Proteomics

Proteomics is used for protein detection and identification [72-74] (and **Paper III**). After bacterial culturing, the bacterial proteins are extracted and separated based on isoelectric point (pI) and molecular weight (Mw) using gel electrophoresis. The proteins are visualized by gel staining (Figure 2) and can be extracted and identified using mass spectrometry (MS) and peptide/protein databases. There are also non-gel based proteomics methods where the gel separation step is skipped [75]. The proteomic profiles change depending on the culture medium used. Thus, the method is good for comparing the changes in the proteome due to environmental modifications, and to pinpoint and identify proteins of interest. Disadvantages of the method include the difficulties encountered in separating proteins of similar size and in visualizing and identifying low-abundance proteins or proteins with very low or high pI or Mw values. In clinical samples, the cells of the oral cavity interfere with bacterial signals in the MS. In addition, a certain number of bacteria must be present in the sample to generate strong peptide signals in the MS and the identification process is highly dependent upon the quality of the bacterial proteome databases. Moreover, the different stages in proteomics analyses are technically advanced and require expensive equipment.

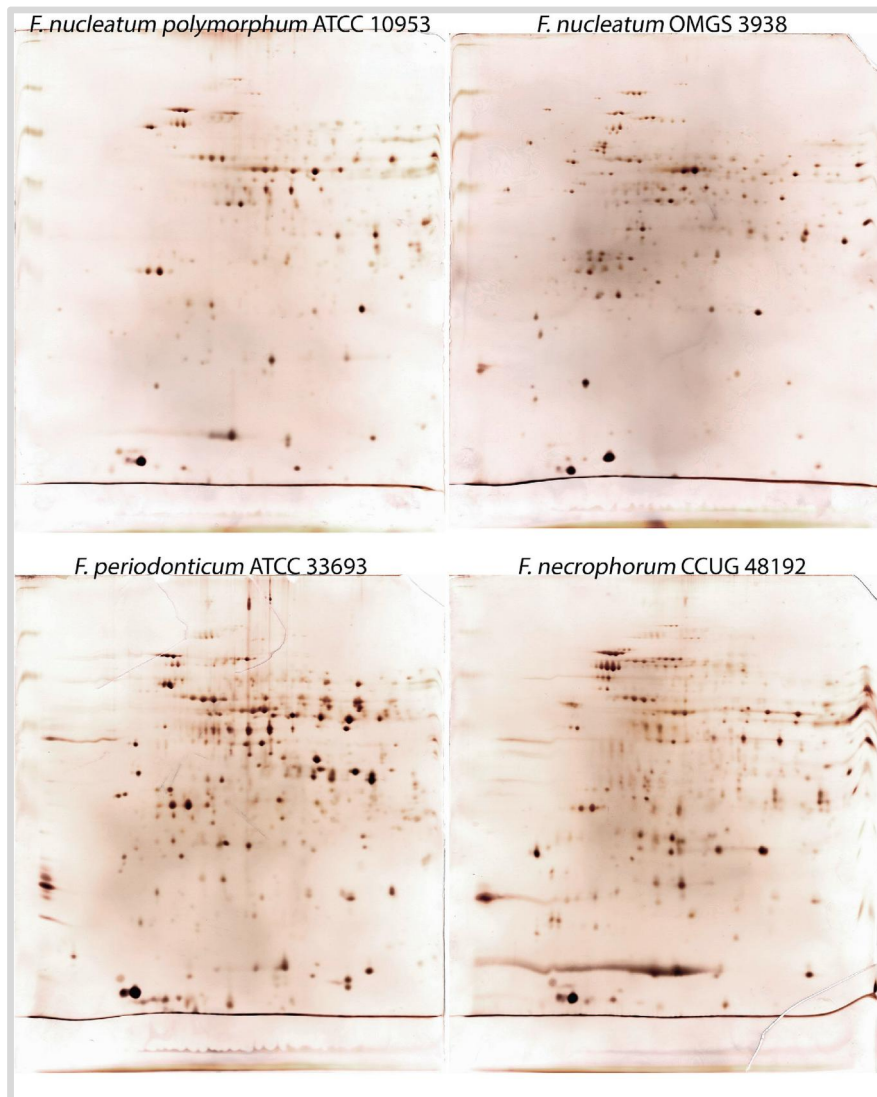


Figure 2 The proteins of *Fusobacterium* spp.

Two-dimensional silver-stained gel electrophoresis of *Fusobacterium* spp. grown under the same culturing conditions. The figure illustrates the significant difference in protein expression pattern between different subspecies and strains. Each spot shows at least one protein. The gels were prepared in connection with the work described in **Paper III** and have not previously been published.

PCR

Polymerase chain reaction (PCR) is used to amplify targeted DNA sequences from clinical samples [76]. The numbers of bacterial cells in the samples can be calculated using standards and DNA-binding dyes or probes labeled with fluorescent molecules in quantitative PCR (qPCR) [77] (and **Paper V**). Depending on the bacterial species of interest, 16S ribosomal RNA gene primers, which are species-unique, can be used. The advantage with this method is the low detection limit and the relatively fast processing. There is, however, some work involved in preparing the protocols for different species, and some methods detect non-specific reaction products. In addition, contaminants in the clinical samples that interfere with the PCR are often a problem. Moreover, PCR does not discriminate between DNA from live and dead bacteria, which means that the viability of the bacteria in the sample cannot be assessed.

High-throughput sequencing technologies

Next-generation sequencing enables the amplification of the DNA and identification of multiple species in a short period [28, 31, 32, 47]. These technologies include 454 pyrosequencing [78] and Illumina [79]. Furthermore, metagenome sequencing allows the simultaneous sequencing and identification of different microorganisms. The method of metatranscriptomic sequencing [79], however, monitors the gene expression patterns of the microorganisms. As large volumes of data are generated using these methods, advanced skills in bioinformatics are required to filter and interpret the essential data.

Despite these advancements in sequencing technologies the periodontal pathogens that have been identified using earlier methods are the same as those associated with periodontitis in newer studies. However, some bacterial strains that have not yet been successfully cultured have been added to the list of bacterial species associated with periodontitis using new technologies [48, 79]. Although, the identification of oral bacterial species is of interest, the composition of the microbiota is not considered to be as important as the consequences of the presence of the bacteria regarding disease initiation and progression. Thus, the functions of the different species are crucial [62]. Therefore, the various tests for different enzymes and the properties of cultured bacteria, proteomics analyses, and metatranscriptomic sequencing are useful tools to elucidate the roles of the specific species of interest.

Hydrogen sulfide (H₂S)

Hydrogen sulfide (H₂S) is a volatile and colorless gas that consists of one sulfur atom and two hydrogen atoms (Figure 3). It belongs to the group of volatile sulfur compounds (VSC), along with methyl mercaptan (CH₃SH), dimethyl disulfide ((CH₃S)₂) and dimethyl sulfide ((CH₃)₂S) [80, 81].



Figure 3 Schematic of the H₂S-molecule

Properties of H₂S

The molecular mass of H₂S is 34.08 g/mol. It has a boiling point of -60°C and a melting point of -82°C. The solubility of H₂S in water is in the range of 0.21 – 0.08 mol/L depending on the temperature (0°–37°C) [82]. The pK_{a1} is approximately 7 and it has a pK_{a2} value of around 19 [82].

Given the properties of H₂S, it is a complicated molecule to study. For instance, there is a difference between using and measuring H₂S gas and soluble compounds that dissolve into HS⁻/S²⁻ and form H₂S. An aqueous solution of H₂S has a pH of approximately 4 [82]. The more acidic the solution, the more of the compound is present as H₂S and less as HS⁻/S²⁻. It is, however, more volatile at low pH than at higher pH [82]. At the physiologic pH, only approximately 20 % is present as H₂S at 37°C with the majority being present as HS⁻. At lower temperatures, a higher fraction is present as H₂S [82, 83]. Given that both forms are usually present, it is difficult to determine if the effects of H₂S that have been reported are due to the gaseous form of H₂S or due to its ion forms of HS⁻/S²⁻, or both.

The toxic effects of H₂S

H₂S has an unpleasant smell resembling that of rotten eggs. In air, the odor is detectable at a concentration of 0.010 ppm, although loss of the sense of smell and irritation of the eyes occur at 100 ppm H₂S (~ 4 μM in air) [82, 84]. At 200 ppm, H₂S affects the respiratory system, leading to loss of consciousness at 300 ppm, and respiratory failure and death upon extended exposure or exposure at levels above 700 ppm.

H₂S is thus a toxic gas [84, 85]. The exact mechanisms involved in the toxic interaction between H₂S and host cells have not been fully elucidated, but are attributed to the ability of H₂S to inhibit cytochrome *c* oxidase in the cell mitochondria [85, 86], split disulfide bonds in host proteins and react with metals [84].

Production of H₂S by host cells

As well as being a product of the bacterial metabolism, H₂S is produced endogenously in the human body. H₂S has been classified as a gasotransmitter, similar to nitric oxide (NO) and carbon monoxide (CO) [87, 88], and is believed to work as a signaling molecule in different processes [89, 90]. The concentration of H₂S in the blood is estimated to be in the nM to μM range [82, 83, 91]. The gas crosses cell membranes [92] and is believed to have major biological effects on host cells at concentrations as low as those found in the blood, whereas it is toxic at higher concentrations. H₂S is involved in several biological processes [93, 94], such as vascular regulation [83, 87], and neurotransmission [95]. Furthermore, both endogenously produced H₂S and bacterially produced H₂S in the colon have been linked to colon cancer [96-98]. Diabetes, atherosclerosis, and asthma are other diseases in which the activities of H₂S are of importance [99].

Three human enzymes are predominantly involved in the degradation of cysteine, as well as homocysteine, and the subsequent production of H₂S by host cells: cystathionine-β-synthase (CBS); cystathionine-γ-lyase (CSE); and 3-mercaptopyruvate sulfurtransferase (3MST). In addition, cysteine lyase is a H₂S-producing enzyme [90]. These enzymes are expressed differently in various tissues, such as the brain, liver, kidneys, and blood vessels [89, 93]. Apart from H₂S, other products that are generated simultaneously from the reactions include pyruvate, serine, lantionine, and ammonia [87, 99-102]. In addition to its enzymatic production, for which new pathways are continuously being discovered [91], H₂S is produced by non-enzymatic reactions, such as the reduction of sulfur [87, 99, 103].

The multiple effects exerted by H₂S in acting as a signaling substance are linked to; its ability to modify proteins by sulfuration (sulfhydration) [94, 104, 105], thereby regulating the functions of ion channels and transcription factors [99]; the fact that it reacts with reactive oxygen species (ROS) and reactive nitrogen species (RNS); and its ability to targets metal centers [105]. Previous studies have also reported additional capabilities of H₂S, such as its ability to modify and release hemoglobin from erythrocytes [106], to stimulate angiogenesis [97, 107], and to increase cellular apoptosis [85].

H₂S and inflammation

Inflammation is the immediate response of the host to injury. H₂S is a mediator of inflammation [108]. The pro-inflammatory effects of both endogenous H₂S production and H₂S exposure have been reported in acute pancreatitis, sepsis, burn injury, and chronic obstructive pulmonary disease, among others [108-110]. Most of these conditions can be improved by the administration of a CSE inhibitor that reduces the production of H₂S. The inflammatogenic properties of H₂S are partially mediated by its ability to trigger the production of cytokines, chemokines, and to modulate the expression of adhesion molecules on host cells [108]. Previous studies using monocytes/macrophages have reported increased protein levels of the pro-inflammatory interleukins (IL)-1 β , IL-6, and IL-18, and also tumor necrosis factor (TNF)- α , after exposure to H₂S [111, 112] (and **Papers IV** and **V**). These responses are mediated in part by the extracellular signal-regulated kinase nuclear factor-kappa B (ERK-NF- κ B) pathway [111, 113]. In addition, we showed that H₂S induced the secretion of IL-1 β and IL-18 via activation of the NLRP3 inflammasome (**Papers IV** and **V**), as will be discussed later.

The induction of anti-inflammatory activities have also been reported for H₂S [94]. H₂S decreases leukocyte adhesion to vascular endothelium [114], and inhibits myeloperoxidase activity (MPO) that is used by PMN to process ROS during inflammation [115]. H₂S has also been implicated in the resolution of inflammation in arthritis [116]. Thus, the modulation of inflammatory responses mediated by H₂S contributes to both anti- and pro-inflammatory effects on host cells [90, 117, 118]. This duality of the effect on inflammation can be explained in part by the concentrations of H₂S applied since low concentrations have been suggested to have anti-inflammatory effects and high concentrations pro-inflammatory or cytotoxic effects [119, 120].

Bacterial production of H₂S

There are numerous oral bacterial species with the ability to produce H₂S and there are various pathways that lead to H₂S production [121-123] (and **Paper I**). This thesis is focused on the degradation of the amino acid L-cysteine and the subsequent production of H₂S as a metabolite. Notwithstanding, there are multiple oral microorganisms that use homocysteine [122], glutathione [124], and cysteine-containing peptides [124-126] to produce H₂S.

Another bacterial route to H₂S is exemplified by the sulfate-reducing bacteria (SRB), which use sulfate to generate sulfide [127]. While SRB have been isolated from various sites in the oral cavity [128], they are found mainly in the periodontal pockets of periodontitis-diseased persons [129], along with other periodontitis-associated species [130]. *Desulfovibrio* and *Desulfomicrobium* species have been identified in plaque samples taken from periodontitis patients [131], although the level of H₂S produced through this pathway is estimated to be low due to the limited concentrations of sulfate in the periodontal pockets [132].

Microorganisms degrade amino acids for energy and to scavenge materials to build new proteins. Pyruvate, for instance, can be metabolized to acetate, which yields energy [62]. The fate of H₂S is somewhat less clear, since its effects on bacterial cells have not been studied as extensively as those on host cells. Nevertheless, some studies have reported on the toxic effects of H₂S on some SRB, whereby inhibition of bacterial growth and altered bacterial composition were noted after exposure to sulfide [133-135]. Interestingly, it has been shown that H₂S can be used by the bacteria to protect themselves against ROS and antibiotics, at least *in vitro* [136-138].

It can be assumed that the H₂S produced by the bacteria passes through the bacterial cell membrane or HS⁻ is transported out of the cell *via* a hydrosulfide ion channel [139]. Since H₂S is volatile, it diffuses to the surroundings and passes through the cell membranes of host cells. It seems likely that some of the H₂S is used by the bacterial cells as a reducing agent or is oxidized to sulfur, to be incorporated into the amino acids synthesized by the bacterial cell. Some toxic effects of H₂S on oral bacteria are also possible since we showed that the presence of a high concentration of the substrate L-cysteine in a culture of *Fusobacterium* spp. reduced the expression of H₂S-producing enzymes (**Paper III**).

H₂S production by *Fusobacterium* spp.

One of the most intensively studied and prominent bacterial species that produces H₂S by desulfuration of L-cysteine *in vitro* is the *Fusobacterium* spp. [121, 140, 141] (and **Papers I** and **III**). *Fusobacterium* spp. have multiple and different sets of enzymes that are involved in the desulfuration of L-cysteine [141-146] (and **Paper III**), which results in rapid and high-level production of H₂S for the majority of the strains that have been tested [121, 122] (and **Paper I**). The enzyme Fn1220 of *F. nucleatum* is 37 % identical to human CBS [143].

Fusobacterium spp. are anaerobic rods that are frequently detected in the subgingival plaque samples from patients with gingivitis. *Fusobacterium* spp. are known for their ability to bind to many different microorganisms and have therefore been attributed as the link between early and late colonizers during dental plaque formation [36]. *Fusobacterium* spp. are also found in deep infections and abscesses [147], and it has been suggested that the ability to produce high levels of H₂S has biological impacts in infections that involve *Fusobacterium* spp. Moreover, *Fusobacterium* spp. have been studied in the gastrointestinal tract in relation to colorectal cancer [148, 149]. In addition, one of the subspecies, *Fusobacterium necrophorum*, causes Lemierre's syndrome [150]. The predominant species of *Fusobacterium* that colonize the oral cavity are *Fusobacterium nucleatum* and *Fusobacterium periodonticum* [151].

It is not known to what degree the H₂S detected *in vivo* is produced by *Fusobacterium* spp., although persons infected with anaerobic bacteria, and *Fusobacterium* spp. in particular, exude a characteristic foul smell, similar to H₂S [152, 153].

Other H₂S-producing species related to periodontitis

Of the remaining bacterial species that have the capability to degrade L-cysteine and produce H₂S, many are associated with periodontitis, such as the red complex bacteria. Among these, *P. gingivalis* has been shown to have both high [122, 154] and low [121] levels of H₂S production from L-cysteine in *in vitro* studies (and **Paper I**). This discrepancy can be explained by differences in the methodologies used in the studies and, as suggested by Persson and coworkers [121], the preference of *P. gingivalis* to use peptides rather than amino acids as a source of nutrients [126, 155, 156]. This may also be true for *Porphyromonas endodontalis*, which shows positive H₂S production after incubation in serum and broth [121] (and **Paper I**). Interestingly, in terms of H₂S production, in line with the strain differences observed with

Fusobacterium spp. [141], differences between *P. gingivalis* strains have also been reported [154] (and **Paper I**). *T. denticola*, is another red complex species that has been reported to express enzymes that can digest L-cysteine and liberate H₂S [157]. This H₂S-producing ability was reported when serum was used as the substrate [121], and more recently when L-cysteine was present in the medium (**Paper I**). Approximately 0.26 mM of L-cysteine has been measured in serum and a similar concentration is expected to be present in the GCF [158]. The H₂S production results for the third member of the red complex, *T. forsythia*, are contradictory [121, 154] (and **Paper I**), and those for *Filifactor alocis* (formerly *Fusobacterium alocis*), are inconsistent, in that high levels were measured with gas chromatography [121] but not with the newer colorimetric methods (**Paper I**). Variations in the expression of enzymes that rely for their production on specific culture conditions may be one explanation for the diverse findings reported in different studies. Other periodontitis-associated species, such as *Prevotella tannerae* and *Parvimonas micra*, also exhibit this H₂S-producing ability [121] (and **Paper I**).

Oral bacterial species that are not associated with periodontitis are usually not as proteolytic or as prolific at producing H₂S. *Lactobacillus* spp. produce negligible amounts of H₂S, as do the majority of the *Streptococcus* spp. [121, 154] (and **Paper I**). However, some *Streptococcus* spp., such as *S. anginosus*, have been shown to produce H₂S [121, 122] (and **Paper I**). The production of H₂S tends to vary between species within the same genus and even between isolates that belong to the same strain.

Although many oral species, especially those associated with periodontal diseases, have the notional ability to degrade L-cysteine, this is of minor concern if the genes that encode these enzymes are not expressed. It is the *in vivo* expression, the activity, and the production level of the entire subgingival microbiota, and not just those of the tested cultivatable species, that are of importance when studying H₂S in periodontal diseases.

Methods to detect H₂S

Owing to its distinctive smell, H₂S has been thoroughly investigated in halitosis research [80, 159, 160]. The most frequently used methods for H₂S detection include organoleptic analysis (to smell) and gas chromatography [80, 160].

The methods used in studies on periodontitis are listed below.

The lead acetate method

The most frequently used method for H₂S detection, both *in vivo* and *in vitro*, is the blackening of paper that has been soaked in lead(II)acetate [55-57, 68, 137, 138]. Sulfide from H₂S binds to lead, leading to the formation of lead sulfide, manifested as a black precipitate on the paper (Figure 4). The more of the precipitate the darker the color and the higher is the concentration of H₂S. This method is approximate and poorly distinguishes differences in the amount and production rate of H₂S.



Figure 4 The lead acetate method

Paper strips were soaked in saturated lead acetate and inserted in the culturing tubes above the broth containing the bacteria. The strips were prepared in connection with the work described in **Paper I**.

The methylene blue method (MB)

H₂S can also be detected through the formation of methylene blue (Figure 5), [161-165] (and **Paper I**). This method has been used to measure H₂S as a product of enzymatic activity [143], the presence of H₂S in aqueous solutions [163, 165], and the production of H₂S during wine production [166]. Methylene blue is formed in an acidic solution that contains N, N-dimethyl-*p*-phenylenediamine in the presence of HS⁻. The reaction requires the presence of an oxidizer, such as Fe³⁺ [165]. Besides the disadvantage of the substances used in the test being toxic, the MB method has been questioned regarding its accuracy and precision when it comes to the measurement of the blue color that is formed [82, 101, 167, 168]. In addition, it proved to have a high detection limit in our *in vitro* investigation in **Paper I**.

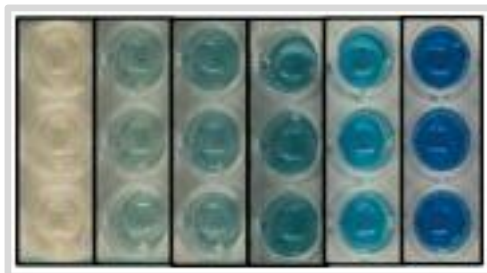


Figure 5 The methylene blue method

Source: **Paper I**

The bismuth sulfide test (BT)

The use of bismuth sulfide to detect and estimate H₂S production in the oral cavity is an old method [169], which has been modified on different occasions [122, 141] (and **Papers I, II, III** and **V**). In the presence of H₂S, a black bismuth sulfide precipitate is formed, where the color intensity is related to the amount of H₂S. BT can be used for semi-quantification of H₂S (Figure 6). The solution contains the coenzyme pyridoxal 5'-phosphate (PLP), which catalyzes the reaction to yield H₂S. Therefore, the bacterial strains that are positive in the BT but not with other methods may use certain enzymes, the PLP-dependent desulfhydrases, for cysteine degradation. The BT is a simple method for the estimation of H₂S levels in periodontal pockets.



Figure 6 The bismuth sulfide test.

Samples from the subgingival pockets of periodontitis patients and healthy controls were transferred to the bismuth solution and the precipitate was read after 2 hours in **Papers II** and **V**

Gas chromatography and other analytical systems

Other methods that use more complex equipment for the detection of H₂S can be employed. Gas chromatography [60, 170] is the most common, although there is a loss of H₂S due to the sampling technique used for the periodontal pocket. The Diamond Probe/Perio 2000 system, a sensor to use in the subgingival pocket, showed promising results and was claimed to measure H₂S concentrations in the range of 0.10 μM–10 mM [58, 59, 171, 172]. Unfortunately, this device has disappeared from the market for reasons unknown to the author. Other electrochemical H₂S sensors [154, 167, 173, 174] have low detection limits but measure S²⁻, which is found only in small concentrations at physiologic pH. Furthermore, these analyses require frequent calibrations, are pH-dependent, and can only be used *in vitro*.

H₂S in periodontal pockets

Previous studies have reported the levels of H₂S production measured with lead acetate-impregnated strips in the periodontal pockets of periodontitis patients [55-57]. All of those studies have noted a higher concentration of precipitate at the apical part of the pocket [55-57]. The presence of H₂S in periodontal pockets has been confirmed using other methods, such as gas chromatography [60], the Diamond Probe/Perio 2000 system [59, 171], and BT (**Papers II and V**).

Although up to 1.9 mM H₂S has been measured from GCF [60], it is debatable whether this correctly reflects the total amount of H₂S in the periodontal pocket. The technique used [60] measured H₂S after the addition of acid, which lowered the pH of the solution and converted HS⁻/S²⁻ to H₂S. The H₂S that was already present in the periodontal pocket and/or on the filter paper strip used for sampling most likely evaporated during the sampling procedure. In the periodontal pocket, which has a temperature of approximately 37°C and a slightly alkaline pH, approximately 20 % is present as H₂S. Consequently, the highest value of 1.9 mM of HS⁻/S²⁻ reported previously appears to be 2.3 mM of HS⁻/S²⁻ and H₂S combined. This concentration is high, as compared to the levels measured in other studies. An earlier study by Morhart and coworkers [56] reported that the degrees of precipitation of samples taken from periodontal pockets were similar when the strips were exposed to H₂S in the range of 1–50 ppm (~ 0.4–20 μM in air, or ~ 0.03–1.5 mM in water). If these values are representative of the 20 % in the form of H₂S, the remaining 80 % existing as HS⁻/S²⁻ still does not add up to the concentrations reported by Persson and coworkers [60]. Most of the newer studies have not reported the maximal levels of sulfide detected in periodontal pockets but have used a low (~ μM range) cut-off point to distinguish sulfide-positive sites from sulfide-negative sites [59, 171]. One study reported 0.1 mM as the highest level measured [58]. Given the discrepancies noted between these studies, the actual concentrations of H₂S in periodontal pockets remain uncertain.

The effects of H₂S on cells present in the periodontal pocket

PMN are the first cells to be recruited during the inflammatory host response to a microbial challenge, as occurs in periodontal diseases. The effects of sulfide on PMN have been studied *in vitro*. Exposure to 1 mM sulfide for 1 hour had only minor effects on the cells, whereas with exposure to 2 mM sulfide decreases in the

respiratory burst and MPO activity were observed [175]. The same study reported lysis of PMN that were exposed to 5 mM sulfide. In addition, chemotaxis and degranulation of PMN were essentially unchanged when the cells were exposed to 1–2 mM of sulfide [176]. However, inhibition of the ability of the PMN to kill capsulated bacteria, by interfering with the opsonization process, was noted in the presence of sulfide [177].

The effects of H₂S on the cells of the periodontium have also been studied. H₂S concentrations of ≥ 5 ppm (~ 0.2 μM in air) proved to be toxic for epithelial cells *in vitro* [56]. H₂S has been shown to increase the permeability of the oral mucosa [178]. Furthermore, H₂S can induce apoptosis of human gingival fibroblasts and periodontal ligament cells [179, 180]. H₂S produced by *P. gingivalis* has been shown to induce the secretion of pro-inflammatory IL-8 from epithelial cells [154]. In addition, the endogenous production of H₂S by periodontal ligament stem cells is inhibited by exposure to exogenous H₂S [181]. A metatranscriptomic analysis of the cells in the subgingival pockets found enhanced sulfur compound-related metabolic processes at baseline for the subjects that at the follow-up had developed periodontitis [79].

The use of sodium hydrosulfide (NaHS)

Sodium hydrosulfide (NaHS) is frequently used as a source of H₂S/HS/S²⁻ when studying the effects of H₂S, both *in vitro* and *in vivo* [85, 109, 137, 182, 183] (and **Papers I, IV and V**). NaHS is easy to use because it is simple to calculate its concentration in solution compared to bubbling H₂S gas [182]. However, the substitution of NaHS for H₂S is controversial [173]. H₂S evaporates or is oxidized to sulfur in aqueous solutions [184]. Other oxidation products of H₂S in solution include polysulfides, sulfites, and thiosulfites [105]. These can contribute to lower concentrations of sulfide being present than when the samples were measured. A way to solve this problem is to use anhydrous NaHS, which is less susceptible to aerobic oxidation, and the metal chelator diethylenetriaminepentaacetic acid (DTPA), which binds to metal ions, thereby blocking their catalyzing activities and preventing further oxidation of sulfide [82] (and **Paper I**). Another approach is to increase the pH, such that almost all the NaHS is in ion form (HS⁻/S²⁻) and evaporation can be avoided.

Despite these preventive strategies, there may be a discrepancy between the obtained concentrations and what the levels would have been if the gas had been used as the HS⁻ source. Thus, the detection limits used for the tests described in **Paper I** might be somewhat lower. In addition, the concentrations of hydrogen

sulfide ions used in **Paper IV** and **V** may also be somewhat lower than 1 mM, especially after 24 hours of incubation when the concentration is expected to decline. A previous study has reported that NaHS contributes to rapid release of a high dose of H₂S, while a H₂S-donor (GYY4137) contributes to a slow release [185]. The results from studies that used NaHS have shown it to have a pro-inflammatory character [108]. In aforementioned study [185], the rapid release of NaHS also proved to have a pro-inflammatory effect, while the slow-releasing H₂S donor had an anti-inflammatory effect. The authors claimed that the use of GYY4137 mimicked more accurately the *in vivo* situation. This reasoning is reasonable and warrants further investigation in future studies.

In summary, H₂S is produced by various host cells and by the microbiota, and it has been extensively studied. Nevertheless, the properties of the H₂S molecule, the important parameters that should be considered when conducting studies with H₂S and the limitations of the methodologies currently used, all add to the difficulties associated with elucidating the roles played by H₂S in various systems. In periodontal research, there is currently no trustworthy *in vivo* method to measure the concentration of H₂S below the subgingival margin, despite numerous efforts to create such tests. While, the concentration of H₂S is of interest, the levels that can be estimated *ex vivo* with the BT (**Papers II** and **V**) are also useful and, due to its simplicity, the test is easily adapted to clinical settings. Therefore, this method is the preferred option for use in future clinical studies on subgingival H₂S.

Monocytes/macrophages

Monocytes are mononuclear white blood cells. When they exit the bloodstream and enter the tissues they can differentiate to macrophages or, in some instances dendritic cells [186]. These cells are part of the innate immune system and central cells for modulation and control of inflammation. They are part of the inflammatory response in periodontal diseases. Up to approximately 5 % of all the cells at periodontitis-affected sites have been found to be macrophages [187]. Macrophages use the CSE enzyme for H₂S production [112, 113]. They are phagocytic cells that, upon stimulation, secrete a wide range of cytokines, including IL-1 β . This is a key cytokine that carries out a number of pro-inflammatory functions [188]. It is involved in fever and bone metabolism [189, 190].

In vitro studies of the behaviors of monocytes usually involve different cell lines, such as the human THP-1 monocytic cell line, in combination with the same cell line that lacks the ability to produce the protein of interest. Thus, the significance of that protein can be studied by comparing the behaviors of the two cell lines in various test situations. Alternatively, an *ex vivo* approach is used in which the peripheral blood mononuclear cells (PBMCs) are purified from the blood samples of donors, usually by density gradient centrifugation. Using this method, the blood is centrifuged over a density gradient medium, which separates the different cell types into layers based on their density. Thus, the fraction that contains the monocytes (and the lymphocytes) can easily be collected, and the cells can be cultured for the intended purpose.

IL-1 β and IL-18

Cytokines are small signaling proteins that orchestrate different processes in the body, such as inflammation. Their main roles are as inducers of chemotactic, pro- or anti-inflammatory, and antiviral activities in cells. They are produced following the activation of different transcription factors in the cells. NF- κ B is one transcription factor that upon activation stimulates the production of pro-inflammatory cytokines, such as IL-1 and TNF- α .

Monocytes/macrophages are the main producers of the pro-inflammatory cytokines IL-1 β and IL-18, which can also be produced by other cells, such as

neutrophils and epithelial cells [188]. While the functions and effects of IL-18 have not been studied as extensively as those of IL-1 β , it is believed to be immunoregulatory, and to play an important role in atherosclerosis [191].

Increased levels of both IL-1 β and IL-18 have been reported in the GCF of periodontitis patients [192, 193], and are implicated in the pathogenesis of periodontal diseases [194, 195] and (**Papers IV and V**). We have showed that the PBMCs of periodontitis patients secrete higher levels of the two cytokines when exposed to H₂S *ex vivo* compared to healthy controls (**Paper V**).

Polymorphisms of the *IL-1* genes have been studied in relation to periodontal diseases. *IL-1* gene polymorphisms have been shown to be associated with the severity of periodontitis [15, 16, 22]. Nevertheless, the genotype does not discriminate between healthy and diseased persons [16, 23]. Meta-analyses have reported both significant associations with chronic periodontitis [19, 20] and a lack of such associations [21]. Apart from the *IL-1* gene polymorphisms, polymorphisms in other genes have been investigated [15, 17, 19, 24], including those in the genes for NLRP3 [23] and IL-18 [196, 197]. In similarity to the *IL-1* gene polymorphisms, no convincing associations have been reported to date.

Inflammasomes

Inflammasomes, which are protein complexes that are formed in cells after exposure to various stimulatory substances, are a part of the innate immune system. When some inflammasomes undergo activation, pro-inflammatory cytokines are secreted from the cells [195]. Inflammasomes are also able to induce pyroptosis, which is a type of inflammatory cell death [191, 198]. The nucleotide-binding oligomerization domain-like (NOD-like) receptor pyrin domain-containing 3 (NLRP3) inflammasome is one of several known inflammasomes [198, 199]. Aberrant formation of inflammasomes, primarily in case of the NLRP3 inflammasome, are linked to many diseases, such as Familial Mediterranean fever multiple sclerosis, Alzheimer's disease, Parkinson's disease, atherosclerosis, diabetes, and obesity [188, 191, 198].

The NLRP3 inflammasome

The NLRP3 inflammasome is an intracellular complex that consists of: NOD-like receptor protein (NLRP3); apoptosis-associated speck-like protein (ASC), which contains a caspase recruitment domain (CARD); and pro-caspase-1 [198]. Upon

stimulation, the NLRP3 inflammasome is activated and induces the secretion of the pro-inflammatory cytokines IL-1 β and IL-18.

The production of IL-1 β and IL-18 requires two signals. The first signal, the priming signal, results in the production of pro-IL-1 β and pro-IL-18 in the cell [199]. This priming signal is typically mediated through Toll-like receptor 4 (TLR4) and the transcription of NF- κ B. TLR4 is the receptor for LPS, which is a known contributor to the priming signal and is frequently used in *in vivo* studies [112, 113, 183, 200]. After the priming event, various stimuli can induce the formation of the NLRP3 inflammasome, including H₂S [195, 198, 199] (and **Papers IV** and **V**). Following the formation of the NLRP3 inflammasome, pro-IL-1 β and pro-IL-18 are processed by caspase-1 to generate IL-1 β and IL-18, respectively, and the two cytokines are secreted from the cell (Figure 7). A prerequisite for the formation of the NLRP3 inflammasome is the efflux of potassium from the cell, i.e., a lowering of the intracellular K⁺ ion concentration [191, 199]. Using cell lines that are unable to form the NLRP3 inflammasome (ASC-deficient or NLRP3-deficient THP-1 monocytic cell lines), we showed that the second signal, which is activated by H₂S, is mediated through formation of the NLRP3 inflammasome in a non-deficient cell line (**Paper IV**). In addition, increased secretion of IL-1 β and IL-18 has been observed in experiments in which cells were exposed to H₂S without pre-exposure to LPS, indicating that H₂S may substitute for the LPS-induced signal (**Paper IV**). The mechanisms involved have not been fully investigated.

The expression of the NLRP3 gene has been reported to be enhanced in gingival tissue from patients with periodontal diseases, as compared to healthy controls, although no differences in expression were seen between different periodontal diseases [201]. NLRP3 gene expression has also been reported to be increased in monocytic cells stimulated with *P. gingivalis* and *A. actinomycetemcomitans* [201, 202], as well as with other periodontitis-associated pathogens [195]. Furthermore, the leukotoxin of *A. actinomycetemcomitans* is able to activate caspase-1 [203].

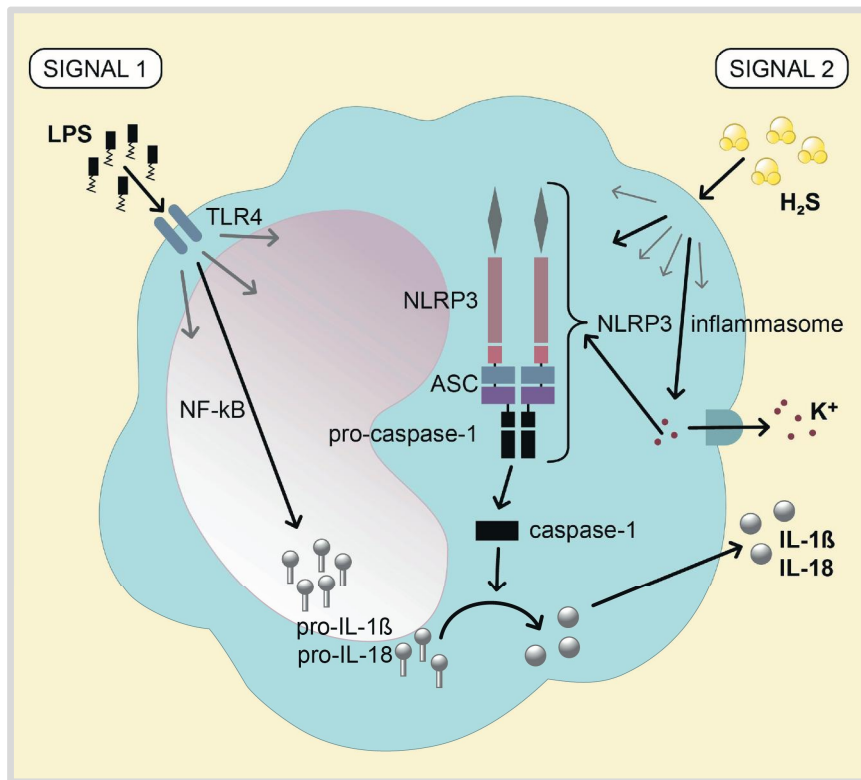


Figure 7 Schematic of the pathways for the production and secretion of IL-1 β and IL-18 in cells. Signal one stimulates the cell to produce pro-IL-1 β and pro-IL-18. The second signal leads to the formation of the NLRP3 inflammasome, cleavage of pro-IL-1 β and pro-IL-18 by caspase-1, and the subsequent secretion of mature IL-1 β and IL-18. While H₂S induces the formation of the NLRP3 inflammasome, it is also suggested to substitute for Signal 1 (**Paper IV**).

Note that although the genes are expressed in the nucleus, the production of the pro-IL-1 β and pro-IL-18 proteins takes place in the cytoplasm.

Schematic drawing by My Erwander

H₂S in the pathogenesis of periodontal diseases

The major results from the studies of this thesis are:

- Periodontal pathogens, such as *Fusobacterium* spp., *T. denticola*, *P. tanmerae*, *P. micra* and *P. gingivalis* are able to produce H₂S from cysteine (**Paper I**).
- Bacterial H₂S production *in vitro* can be estimated using the MB method or BT (**Paper I**).
- *Fusobacterium* spp. use different enzymes to rapidly produce high levels of H₂S (**Papers I and III**).
- The expression of the H₂S-producing enzymes of *Fusobacterium* spp. varies among different subspecies and strains and is dependent upon environmental conditions, such as pH and the presence of cysteine (**Paper III**).
- PBMCs and the monocytic cell line THP1 secrete higher levels of IL-1 β and IL-18 when exposed to NaHS (**Paper IV**).
- The NaHS-induced secretion of IL-1 β and IL-18 is mediated by the formation of the NLRP3 inflammasome (**Paper IV**).
- In the absence of priming by LPS, exposure of PBMCs to NaHS increases the secretion of the two cytokines (**Paper IV**).
- The PBMCs of periodontitis patients secrete higher levels of IL-1 β and IL-18 compared to healthy controls (**Paper V**).
- When exposed to NaHS, the secretion of IL-1 β and IL-18 by PBMCs is enhanced in both groups, although the levels are significantly higher in the periodontitis group than in the healthy subjects (**Paper V**).
- The capacities of bacterial plaque samples to produce H₂S can easily be estimated using the BT (**Papers II and V**).
- Plaque samples from subjects with long-standing gingivitis and periodontitis are capable of producing more H₂S than the plaque samples from healthy subjects (**Papers II and V**).
- Diseased subjects harbor higher numbers of periodontitis-associated bacterial species and species that are able to produce H₂S, as compared to healthy controls (**Paper V**).

The importance of H₂S in periodontal diseases

A schematic of the hypothesis and the main findings of this thesis are presented in Figure 8. The microbiota of the subgingival pocket degrades proteins, peptides, and amino acids, including cysteine, to produce H₂S, along with many other metabolites (**Papers I and III**). H₂S is able to activate the NLRP3 inflammasome in monocytes/macrophages, which leads to the secretion of IL-1 β and IL-18 (**Papers IV and V**). These proteins are pro-inflammatory and exacerbate the inflammatory host response, which is a typical feature of periodontal diseases.

In addition to the pro-inflammatory functions of H₂S shown in this thesis (**Papers IV and V**), H₂S is suggested to scavenge ROS [104, 105] and inhibit MPO [115], both of which result from the functions of PMN, the cells that are part of the first line of defense in periodontal diseases. ROS is important in the host defense against infectious microorganisms, although this killing function is not as effective in anaerobic environments. However, H₂S increases oxidative stress in host cells [85]. Thus, H₂S appears to be able to reduce oxidative stress that is harmful to the bacteria, while at the same time increasing the oxidative stress in the host cells. However, it has not been definitively demonstrated in *in vitro* studies that the functions of PMN are inhibited by exposure to sulfide at the levels that are expected to be present in periodontal pockets [175-177]. Moreover, besides being toxic at high concentrations [84], H₂S traverses cell membranes [92], is oxidized to polysulfides [91, 204], sulfites, and sulfur, and is also involved in sulfhydrylation [105]. H₂S and/or its derivatives operate as gaseous mediators, similar to NO and CO, in the human body and exert multiple influences on host cells. Accordingly, depending on the cell type, differences in the environmental conditions, including variations in the pH, redox potential and oxygen levels, and concentrations of H₂S (endogenously produced, bacterially produced or derived from H₂S-donors) as well as the concentrations of other substances, such as NO and various enzyme inhibitors, the cellular responses, and the consequences of H₂S differ. Given this complexity, investigations of the functions and the effects of H₂S are complicated. Nevertheless, emerging methods and techniques, along with new findings add to our knowledge. For periodontal diseases, there are methods available to estimate in the clinical setting the concentrations of H₂S (**Papers I, II and V**).

It is currently unclear as to whether the concentrations of H₂S measured *in vivo* can be used as (bio)markers for gingivitis and/or periodontitis. Studies have suggested that H₂S measurements can be used to: pinpoint which of the shallow pockets to monitor; detect disease severity; and evaluate the treatment outcome [57-59]. More research is needed to evaluate the levels and relative importance of H₂S in relation to the various periodontal diseases. Furthermore, methods to estimate

the susceptibility of the host to develop periodontitis are needed for clinical risk assessment. In these respects, the outcomes of the experiments involving monocytes exposure to H₂S (**Papers IV and V**), in combination with the previous studies of *IL-1* gene polymorphisms and our advanced knowledge of inflammasomes will be of value and interest.

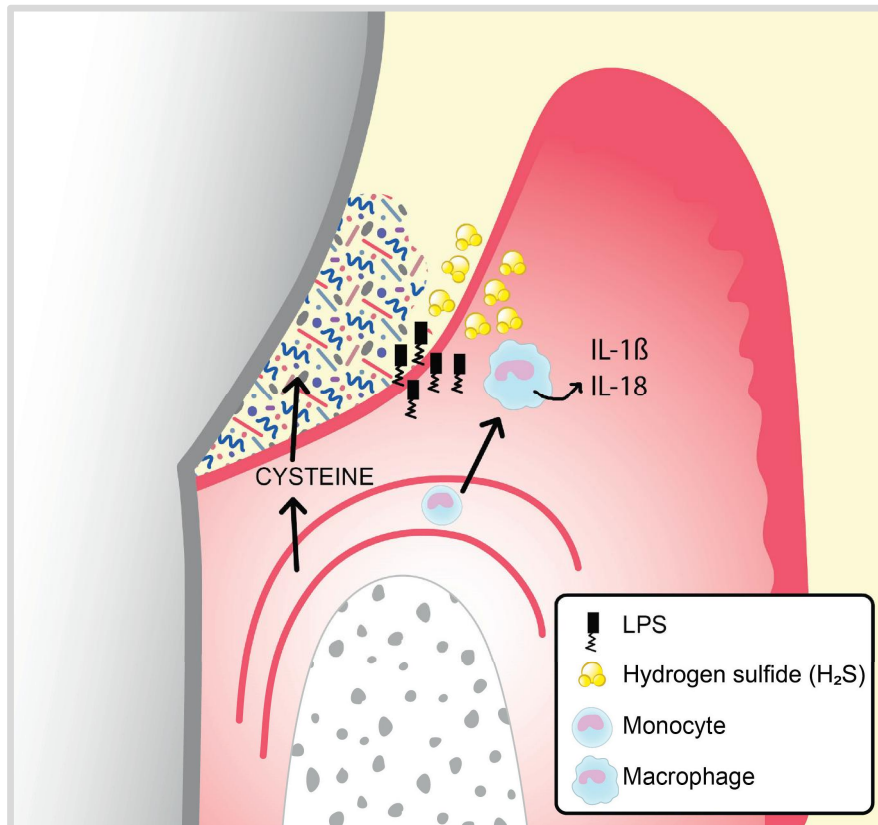


Figure 8 Schematic of an inflamed gingival pocket that summarizes the work of this thesis. The periodontal pocket is colonized by proteolytic microorganisms that can degrade the cysteine in the GCF. The metabolite H₂S is produced. H₂S can promote the secretion of the pro-inflammatory cytokines IL-1β and IL-18 from monocytes/macrophages, thereby enhancing the host inflammatory response that is characteristic of periodontal diseases.

Schematic drawing by My Erwander.
Source: **Paper IV**

A complex interplay between bacteria and host cells

The pathogenesis of periodontal diseases is believed to result from the interactions between the microbiota and host cells that occur in the periodontal pocket, as modeled by Page & Kornman in 1997 [205]. This model remains valid today, although it has been refined based on new findings, such as the behaviors of PMN, dendritic cells, and natural killer cells at diseased sites, the role of the microbiota, the ecological plaque hypothesis, and the presence of microRNAs [206, 207].

The microbiota of the periodontal pockets, its composition, its virulence factors, and the waste products emanating from their activities all contribute to the interplay with host cells and are essential for the development of periodontal diseases [30]. The host cells, including those of the innate immune system and their products, play crucial roles in host susceptibility to periodontal diseases. Under healthy conditions in the mouth, there is a health-promoting interplay between the microbiota and the host cells [31]. Various microbial species, substances, and gaseous mediators regulate the behaviors of the host cells, and the interactions between these actors create the dysbiosis and promote disease development [79, 208], as summarized in Figure 9.

The implicated host factors are: heredity (including race and ethnic group, and gene polymorphisms); age (since the destruction of the supporting tissues of teeth is cumulative); diseases (e.g., neutropenia, diabetes, other inflammatory diseases); smoking; stress; the anatomy of the teeth; and oral hygiene. In addition, the temperature, pH level, redox potential, and the level of access to nutrients determine the environmental conditions for the microbiota in the subgingival pocket.

The microbial factors include the composition and genotype of the bacteria. Nevertheless, the phenotype, growth, and activity of the microbiota are the key factors, as the consequences of these factors include virulence factors and metabolites that interact with the host and have immunomodulatory functions [209].

The role of bacterially produced H₂S and the subsequent secretion of pro-inflammatory cytokines are part of the host-microbe interplay that forms the basis for our current understanding of the development of periodontal diseases. In periodontal diseases, numerous interactions contribute at different levels and result in the activation of various signaling pathways. It is the overall net effect of all these interactions and pathways that determines the fate and well-being of the periodontium.

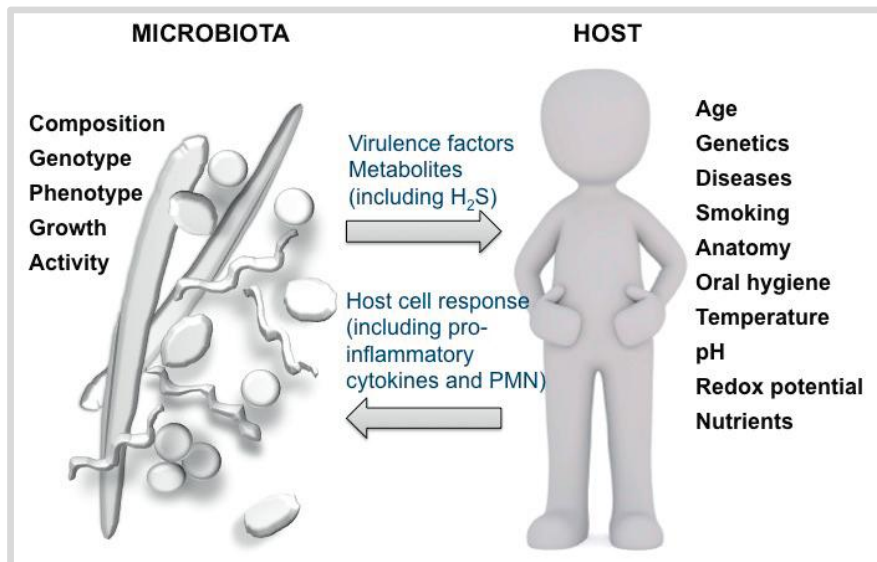


Figure 9 Proposed model for the pathogenesis of periodontal diseases
 Adopted from Page & Kornman [205] and Johansson & Dahlén [210].
 The interplay between the microbiota and the host is dependent upon both environmental and behavioral factors. It is the overall net effect of all these interactions that determines the outcome in terms of the well-being of the periodontium.

Concluding remarks and future perspectives

The activities of the subgingival microbiota and the consequence of their growth, i.e., metabolites such as H₂S, contribute to the immunomodulatory effects on host cells, at least *ex vivo*. Therefore, estimation of bacterial activity, through measurement of H₂S may be used as a marker of disease activity, such as inflammation. Furthermore, the susceptibility of the host to disease development may be estimated by examining the behaviors of the host cells *ex vivo*.

The notion of the oral cavity as a part of the holobiont [31], which encompasses the host cells and the commensal microorganisms that live in symbiosis with the host, is emphasized in this thesis. As is the case for some other diseases, such as inflammatory bowel disease and Crohn's disease, in periodontitis there is disruption of the homeostasis between the host cells of the immune system and the colonizing microorganisms. It is not known whether this disruption in periodontitis is caused by a change in the biofilm or by a change in the response of the host, as in Crohn's disease, or if these two events occur simultaneously.

The fact that H₂S is a signaling molecule in the body and is discovered to be part of a multitude of basal and more specific and complex cellular mechanisms that relate to the interactions between cells, both endogenous cells and exogenous colonizers, is illustrative of the oral cavity as part of the holobiont.

An imbalance in the activities of inflammasomes tends in a similar manner to play a role in disease development and progression. The role of inflammasomes in inflammation and their potential associations with disease are other aspects that warrant further study.

The future perspectives and strategies for dental research, and periodontal research in particular, should be interdisciplinary and more tightly linked to research that is being conducted on other inflammatory processes. The oral cavity should be viewed as a part of the holobiont and not as an isolated bodily compartment. This strategy of integrating research on inflammatory processes with dentistry raises the possibility for resolving the unknowns and creating explanatory models of the pathogenesis of periodontal diseases.

Future studies on the pathogenesis of periodontal diseases and the importance of bacterial metabolites should include the effects of H₂S on other host cells, such

as PMN and epithelial cells. Since the mechanisms employed by H₂S are not yet defined unambiguously, it will be interesting to determine whether the consequence of H₂S exposure is primarily the enhancement of the inflammatory host response, as suggested in this thesis, or if it is instead the impairment of the functions of the host immune cells, so that they are unable to perform their tasks in a proper and sufficient way. Both modes of action may be involved in the pathogenesis of periodontal diseases, and it is possible that H₂S possesses both attributes. Furthermore, the potential effects on host cells of other metabolites produced through the proteolytic activity of the bacteria in the subgingival pocket, including various carboxylic acids and ammonia, should be studied.

The role of H₂S in other organs of the body, including the intestine and the association between *Fusobacterium* spp. and colon cancer development, are other questions that could be addressed in future studies.

The oral cavity and the oral diseases are tightly linked to the host responses. Therefore, it is not surprising that the mechanisms involved in the pathogenesis of periodontal diseases may also participate in other inflammatory diseases. It may also be that the genome and the susceptibility of an individual to develop periodontal diseases relate to other inflammatory diseases [12, 211]. Furthermore, therapeutic strategies developed for other diseases may be applicable to the treatment of oral diseases.

Ethical considerations

The main ethical issues related to the work were considered during the preparation of **Papers I, II and V**. In **Paper I**, ethical concerns were taken into account in the choice of method for the estimation of H₂S with the intention that the test would be used in clinical settings. Therefore, the most challenging part was to identify substances, chemicals, metals or materials that could be used *in vivo* without causing harm to the patients, given that the best chair-side method developed to date for the estimation of H₂S in subgingival pockets is the lead acetate method. This method involves the exposure of patients to lead, and is therefore not suitable for ethical reasons. Given these challenges, the *ex vivo* BT was prepared and used. Although the BT solution contains a heavy metal that is an environment pollutant, the *ex vivo* setting eliminates any potentially harmful exposure to the patients.

The work described in **Paper II** was conducted in remote parts of Northern Thailand and ethical approval was obtained from the Princess Mother Medical Voluntary Foundation (PMMVF) in Bangkok, Thailand. The subjects that were examined volunteered to participate in our study. We examined the participants and sampled their oral bacteria. When pathological conditions were detected treatment was offered by the dentists working at the PMMVF. Although subjects in remote areas of our world are interesting to study due to their diet and often poor oral hygiene, there are ethical issues to address. While the main intention of PMMVF is to educate people to prevent the development of oral diseases, it also offers treatments to those in need. Besides conducting the research project, our role was to report of the oral healthcare needs of the study population.

In **Paper IV**, blood cells from anonymized blood donors were received from the Sahlgrenska University Hospital in Gothenburg, Sweden with permission for the blood to be used for research purposes.

The work described in **Paper V** was approved by the Regional Ethical Review Board (Dnr 871-15) in Gothenburg, Sweden. Periodontitis patients who had been referred to the specialist clinic were asked to participate. Registration and plaque sampling were included in the examinations of the subjects in this group of patients. The subjects were also asked to donate blood samples at Södra Älvsborgs Hospital, Borås, Sweden. The subjects who volunteered for the healthy group were mainly staff members at the Institution of Odontology, Gothenburg University.

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Acknowledgments

Thanks to:

Gunnar Dahlén, you have been an excellent supervisor. It has been an interesting and educative period in my life and I am very grateful to you for giving me this wonderful opportunity. Thank you for sharing your knowledge and for all our stimulating discussions.

My co-supervisors: **Anette Carlén** for introducing me to research, and **Åsa Leonhardt** for help with the logistics and gaining access to examination rooms for the participants in the last study.

Gunnel Svensäter for the introduction to the world of proteomics and for enjoyable visits to the lab in Malmö. You are such a wonderful person!

Susanne Blomqvist for all the support. In addition to your expertise in the lab, you are a wonderful colleague and friend!

My co-authors, **Madeleine Blomqvist**, **Sara Alizadehgharib**, **Ulf Dahlgren** and **Giovanni Serino**, for your valuable contributions.

Anna-Karin Östberg for providing the answers to all my questions and your valuable help with the laboratory work.

Gunilla Hjort and **Lisbeth Bengtsson** for assessments of the color changes in the colorimetric tests.

Marie Arntzen for all your help with administrative issues.

Vincent Collins for the proofreading of Paper V and this thesis

All of you who proofread and discussed the content of this thesis with me

My colleagues of the **Department of Oral Microbiology** and other co-workers on the **5th floor** for enjoyable *fika* moments ☺

Bankeryds Folk tandvård and **Särö Tandvård** for allowing the adjustments to my clinical work schedule so that I could complete the projects of this thesis

My friends and family for all your support.

/Amína

The work of this thesis was supported by grants from **TUA (TUAGBG-67191, 89621, 89621, 365041 and 538271)**, the **Swedish Dental Society (STS)**, and **Gothenburg Dental Society (GTS)**.

