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Porphyrins and Phototherapy of Oral Bacteria

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

In the mid-1990s researchers became interesting in blue light-based therapies using light-emitting diodes (LED) light. Blue light therapy has been successfully used in treatment of acne, as well as shown wound healing effects. It has also shown significant antibacterial effects, especially on black-pigmented bacteria. Recent studies have shown that a toothbrush equipped with blue light LED emitting in the 412 nm region can reduce plaque and gingival inflammation up to 50 % after 4 weeks of intervention.

According to the World Health Organization gingivitis is common in children and adolescents worldwide, and 5–20% of the adult population have periodontal destructive disease. Persistent gingival inflammation has been reported to be a risk factor for developing periodontitis. Thus, it is of great interest to prevent and treat gingival inflammation. Studies have shown that 85% of adults in Sweden brush their teeth at least twice a day, but it has also been reported that the majority don't use a proper technique or have a low motivation for keeping a sufficiently good oral health. In this context there is a need to emphasize the importance of repeated oral hygiene instructions and to strengthen the motivation for maintaining oral health.

This thesis is based on four papers. In **Paper I** it is demonstrated that the periopathogen *Aggregatibacter actinomycetemcomitans* on its own is able to produce red fluorescence. According to the literature, bacteria that are able to emit red fluorescence can be killed by phototherapy due to their endogenous porphyrins which raised an interest to investigate if *A. actinomycetemcomitans* could be killed by blue light.

In an *in vitro* pilot study it was demonstrated that 410 nm blue light could inhibit the growth of *A. actinomycetemcomitans*. In **Paper II** a new sensitive method using high performance liquid chromatography - tandem mass spectrometry (LC/MS/MS) was developed for chemical analysis of porphyrins in microorganisms and it was used to identify and quantify porphyrins in the oral pathogens *A. actinomycetemcomitans* and *Porphyromonas gingivalis*, as well as in the fungi *Saccharomyces cerevisiae.* In **Paper III** chemical porphyrin profiles of *A. actinomycetemcomitans* and *P. gingivalis* are determined and their changes during bacterial growth investigated. It was shown that the porphyrin content changes drastically during cultivation and thus points out the need for standardized culturing protocols when performing phototherapy experiments *in-vitro*.

In **Paper IV** a randomized controlled trial was conducted with the aim to investigate the phototherapeutic effect on dental plaque and gingival inflammation of toothbrushes with incorporated 450 nm LEDs. In both the intervention and the control groups there were significant reductions in plaque and gingival inflammation ($p<0.001$). There was a larger decrease in all three clinical indices for the blue light intervention group when compared to the control where the amount of plaque was reduced by 62% and 51% respectively. This difference was established at a level of $p=0.058$, and there was no significant difference observed in the GI and BOP indices ($p>0.29$) between the blue light group and the control

group. For the blue light intervention groups there were significant decrease in all four inflammatory markers used in the data evaluation, *i.e.* in IL-1β and IL-8 in GCF, as well as in MMP-8 and TIMP-1 in saliva. This is in contrast to the control group which only showed a statistical significant decrease in the concentration of MMP-8. These differences in clinical indices as well as in inflammatory markers suggest that there could be a weak effect from the 450 nm LED illumination. However, the conclusion must be that at a level of $p=0.05$ there was no significant adjunctive effect to tooth brushing found for the 450 nm LED. Combined with what is stated in the literature a suggestion is to use toothbrushes equipped with 405 nm LED for further studies.

LIST OF SCIENTIFIC PAPERS

This thesis is based upon the following papers which will be referred to in the text by their Roman numerals:

I. **Nadja Bjurshammar**, Annsofi Johannsen, Kåre Buhlin, Sofia Tranæus, Conny Östman.
On the red fluorescence emission of *Aggregatibacter actinomycetemcomitans*
Open Journal of Stomatology 2 (2012) 299-306.

II. Jonas Fyrestam, **Nadja Bjurshammar**, Elin Paulsson, Annsofi Johannsen, Conny Östman
Determination of porphyrins in oral bacteria by liquid chromatography electrospray ionization tandem mass spectrometry
Analytical and Bioanalytical Chemistry 407 (2015) 7013-7023.

III. Jonas Fyrestam, **Nadja Bjurshammar**, Elin Paulsson, Nesrine Mansouri, Annsofi Johannsen, Conny Östman
Influence of culture conditions on porphyrin production in *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*.
Photodiagnosis and Photodynamic Therapy 17 (2017) 115–123.

IV. **Nadja Bjurshammar**, Sebastian Malmqvist, Elisabeth Almer Boström, Jonas Fyrestam, Gunnar Johannsen, Conny Östman, Annsofi Johannsen.
The Effect of Local Phototherapy on Dental Plaque and Gingival Inflammation - A Randomized Controlled Study.
In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
BOP	Bleeding on probing
CAL	Clinical attachment loss
Cdt	Cytolethal distending toxin
CPIII	Coproporphyrin III
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionization
FWHM	Full width at half maximum
GCF	Gingival crevicular fluid
GI	Gingival index
HACEK	A group of fastidious gram-negative bacteria including *Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella* and *Kingella* spp.
HPLC	High performance liquid chromatography
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
LAP	Localized aggressive periodontitis
LED	Light emitting diode
LOD	Limit of detection
LOQ	Limit of quantification
MMP-8	Matrix metalloproteinase 8
MPIX	Mesoporphyrin IX
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
nm	Nanometer
P. gingivalis	*Porphyromonas gingivalis*
PDT	Photodynamic therapy
PI	Plaque index
PPD	Probing pocket depth
PPIX	Protoporphyrin IX
QLF	Quantitative Lightinduced Fluorescence
RCT	Randomized clinical trial
ROS	Reactive oxygen species

RP-HPLC	Reversed phase high performance liquid chromatography
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
TIMP	Tissue inhibitors of metalloproteinase
TNF-α	Tumour necrosis factor α
TPC	Molar total porphyrin content
UPI	Uroporphyrin I
UV/VIS	Ultraviolet/Visible

INTRODUCTION

Periodontal disease and epidemiological studies

Periodontal disease, gingivitis and periodontitis, is one of the most common inflammatory diseases in humans [Van Dyke and Van Winkelhoff 2013]. The most prevalent conditions are chronic periodontitis and dental plaque-induced gingivitis [Armitage 1999, Tatkis and Kumar 2005]. Plaque-induced gingivitis is a reversible superficial inflammation in the gingiva, while periodontitis is an extension of the inflammation into the supporting tissues leading to a progressive loss of tooth attachment and alveolar bone destruction [Cekici et al. 2014].

Data from epidemiological studies shows that on a global level most of the children and adolescents are affected by gingivitis, and periodontitis is found in 5-20 % of the adults [Albandar 2005, Jin et al. 2011, WHO 2012]. Studies of the US adult population performed by the National Health and Nutrition Examination Survey (NHANES) in 2009-2012, showed that the prevalence of periodontitis was almost 46 % of adults aged $\geq$ 30years, and severe advanced periodontal destruction was found in 8.9% of the adults [Eke et al. 2015].

Dental plaque - a microbial biofilm

Dental plaque is a biofilm defined as a complexed microbial community that grows on the tooth surfaces, embedded in a matrix of polymers of both microbial and host origin [Socransky and Haffajee 2002]. Dental plaque biofilm supports the host in its defence against invading microorganisms and according to the prevailing "ecological plaque hypothesis" changes in the environmental conditions can lead to a homeostatic break down and cause periodontal disease. In addition, this hypothesis states that no specific bacteria are the etiologic agent of disease [Marsh 2004]. Dental plaque is a primary etiological factor for the initiation of gingival inflammation, which can be followed destruction of periodontal tissue. However, the expression of the disease is depending on the interaction between the biofilm and inflammatory response of the host [Kornman 2008, Marsh 2011, Marsh 2015], and in a consensus report from the fifth European workshop in periodontology it was stated that disruption of the dental biofilm is a very effective way to prevent and treat gingivitis and periodontitis [Kinane and Attström 2005]. There are several plaque indices and one of the most widely used is the plaque index (PI) developed by Silness and Löe [1964].

Gingival inflammation

Gingivitis is an inflammatory reaction in the gingiva which is initiated by bacterial biofilms (dental plaque). It involves inflammation at the gingival margin with no destruction of the periodontal tissues, and no perforation of the junctional epithelium which have the function to protect the underlying tissues. [Lang et al 2009]. Bacterial components such as lipopolysaccharides, peptidoglycans, lipoteichoic acids and leukotoxin (LtxA) may cause the inflammatory response, [Madianos et al. 2005]. This bacterial challenge can create an innate immune response that will increase the movement of plasma and neutrophils from the blood

vessels, widening the intercellular spaces of the epithelial surfaces and release inflammatory interleukins (ILs) such as, IL-1β, IL-6, IL-8, and tumour necrosis factor α (TNF-α) [Lang et al. 2009]. Innate immunity is the first defence line and the function of the epithelial barriers is to block microbes from entering the tissues. If they succeed to enter the tissues or the circulation they will be attacked by lymphocytes.

Activation of the innate immunity system can lead to an activation of the adaptive immune system, which can lead to a destructive chronic inflammation (periodontitis) [Preshaw and Taylor 2011].

Clinical signs of gingival inflammation are redness of the gingival margin, swelling and loss of texture, and spontaneous bleeding [Armitage 2004, Lang et al. 2009], **Figure 1**. There is also an alteration in the permeability of the blood vessels, resulting in an increased flow of sulcular fluid. There are several indices for quantifying gingival inflammation, of which one of the most widely used is the Gingival Index (GI) developed by Löe and Silness [1963].

Figure 1:   Patient with clear signs of gingivitis. [Photo by Annsofi Johannsen].

According to The Swedish Council on Technology Assessment in Health Care (SBU), bleeding on probing (BOP) is the most reliable tool for diagnosing inflammation in the periodontal tissues [SBU 2004]. Self-performed oral hygiene is the most important factor to prevent gingivitis, especially if it is combined with professional oral instructions [Drisko 2013].

The relationship between gingivitis and periodontitis has been widely discussed, and the most supported theory is that periodontitis must be preceded by gingivitis [Page and Kornman, 1997], and several studies have shown that persistent gingival inflammation is a risk factor for developing periodontitis [Sheiham, 1997, Albandar et al. 1998, Schätzle et al. 2003, Lang et al. 2009].

The influence of periodontopathic bacteria

The influence of periodontopathic bacteria in dental plaque has been extensively investigated. *Aggregatibacter actinomycetemcomitans* is an important periopathogen and LtxA produced by this bacterium is among the most important virulence factors with a capacity to induce a

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massive pro-inflammatory response in monocytes/macrophages [Henderson et al. 2010, Höglund-Åberg. 2015, Hirschfield et al. 2016]. *A. actinomycetemcomitans* is considered to be associated with localized aggressive periodontitis (LAP) [Slots 1999, Fine 2006, Fine 2007, van der Reijden, 2008]. A two year population-based study on 121 adolescents demonstrated that subject harbouring the JP2 clone of *A. actinomycetemcomitans* had a significantly higher clinical attachment loss (CAL) after two years compared to the subjects not harbouring this clone of *A. actinomycetemcomitans*. This highly leukotoxic JP2 clone is most frequent in individuals of African descent [Haubek et al. 2004].

Höglund-Åberg et al. [2015] have addressed that specific bacteria may seem unimportant according to the "ecological plaque hypothesis" and that elimination of specific bacteria not necessarily will cure periodontal disease but they point out the importance to focus on the virulence factors rather than on the bacteria that produce these factors. Suguimoto et al. [2014] have shown that *A. actinomycetemcomitans* also can produce cytolethal distending toxins (CdtA, CdtB, CdtC). This may be an important strategy of this bacterium to thwart an effective immune response, leading to a decrease in phagocytosis. In addition, they suggest that Cdt can indirectly manipulate the production of cytokines from macrophages [Suguimoto et al. 2014]**.** Periodontitis is an infectious disease and periopathogens such as *A. actinomycetemcomitans* have been proposed to be able to invade soft periodontal tissues and the underlying vascular endothelium and migrate to blood vessels and heart tissue [Schenkein 2000]. *A. actinomycetemcomitans* is a member of the HACEK group of microorganisms (*Haemophilus* spp, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella* spp) which are associated with systemic diseases such as bacteraemia, endocarditis, septicaemia, pneumonia, infectious arthritis, skin infections, and various types of abscesses [van Winkelhoff and Slots 1999, Fine 2006, Nakano 2009].

The "red-complex" pathogens *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*) and *Treponema denticola* play an essential role in the pathogenesis of destructive periodontal disease [Darveau 2010, Hajishengallis 2014]. The presence of "red-complex" bacteria can cause a dynamic shift in the dental biofilm which can lead to inflammatory bone loss depending on their capability to interfere with the host defence mechanisms [Honda 2011, Darveau 2010, Darveau 2012]. Studies have shown that the invasive ability is an important virulence factor of *P. gingivalis* which contributes to the degradation of the important proinflammatory cytokine interleukin IL-1β in gingival crevicular fluid (GCF). Such changes in the GCF cytokine profile can have consequences in the pathogenesis of periodontal disease [Stathopoulo et al. 2009]. *P. gingivalis* is also suggested to be involved in several systemic diseases including diabetes, stroke, and atherosclerotic cardiovascular disease [Hayashi 2010].

Inflammatory biomarkers

Periodontal risk can be identified and quantified by measures of certain biomarkers present in dental plaque, saliva and/or in GCF [Taba et al. 2005]. Measurements of IL-1β, IL-6, IL-8, and TNF-α, are often analysed in both saliva and GCF in clinical studies. IL-1β is a potent

pro-inflammatory cytokine mainly produced by monocytes and macrophages, and this cytokine plays a pivotal role in periodontal pathogenesis through its involvement in the regulation of inflammatory responses and bone resorption [Graves 2003], and its expression has been shown to reflect gingival inflammation. IL-1β stimulates cells in the gingiva including immune cells as well as tissue resident fibroblasts and keratinocytes, to produce chemokines, tissue destructive matrix metalloproteinases MMPs and cytokines that activate osteoclasts and thereby induces bone destruction [Kida 2005, Stein et al 2011, Boström 2013]. Sorsa et al. [2011] have reported that MMP-8 play a significant role when determine degree of inflammation. The activity of MMPs is controlled by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) and in disease there is an imbalance of MMPs with their inhibitors [Naesse et al. 2003]. IL-8 is a chemokine with a strong specificity for neutrophils, the most numerous population of immune cells in periodontitis [Bickel 1993], and is associated with periodontal disease, and up-regulated in gingival fibroblasts challenged with inflammatory stimuli [Takigawa 1994].

Oral daily self-care

Tooth brushing and interproximal cleaning

Reducing dental plaque and inhibition of biofilm formation in the mouth has during the years been regarded as one of the major treatment strategies for oral diseases. It is well known that the sub-gingival microbial flora is influenced by the removal of the supra-gingival plaque [Dahlen et al. 1992, al-Yahfoufi et al. 1995, Haffajee et al. 2003]. Today there is a multitude of different toothbrushes on the market. Different kinds of toothbrushes have been manufactured, differing in numbers of filaments, hardness of the filaments and angulations of tufts. There are also electric toothbrushes with circulating or/and oscillating actions. Aspiras et al. [2013] found that power brushing significantly reduced BOP and pro-inflammatory cytokine IL-1β levels compared to manual toothbrushes, while changes in bacterial levels showed non-significant trends between both brushing modalities.

Studies have shown that the majority of the population do not clean their teeth well enough or have improper technique [Haffajee et al. 2001, Claydon, 2008]. A systematic review by Chapple et al. [2015] reported moderate evidence for inter-dental brushes (IDB) providing greater reduction in dental plaque scores than other inter-dental aids such as dental floss. There is so far no evidence that flossing can reduce gingival inflammation or if healthy sites would benefit from daily interproximal cleaning [Chapple et al. 2015]. In a randomized controlled clinical study performed in Sweden, the importance of repeated oral hygiene instructions was pointed out and it was suggested that regular repetitions and check-ups of the dental status might have an effect similar to a professional polishing at a clinic [Hugoson et al. 2007].

The influence of toothpastes

The main purpose with fluoride containing toothpaste has been to prevent dental caries. In later years several other purposes with toothpastes have been introduced such as whitening treatment, prevention/treatment of gingivitis and dentin hypersensitivity, *etc.* [Lippert, 2013]. There are a large number of whitening toothpastes, *e.g.* containing hydrogen peroxide, on the market, and recently Attia et al. [2015] demonstrated that whitening toothpastes contain more abrasive particles. Some of these can increase the roughness on restorative material surfaces, especially if the toothpaste also contains silica, bicarbonate, or a combination of bicarbonate and calcium pyrophosphate. It has also been demonstrated that whitening dentifrices can increase enamel surface roughness when combined with the bleaching agent carbamide peroxide [Amaral et al. 2006]. In a 6-month randomized clinical study it was demonstrated that antimicrobial agents, such as 0.45% stannousfluoride/sodium hexametaphosphate, in toothpastes can significantly reduce gingivitis compared to toothpaste with triclosan/copolymer [Archila et al. 2004]. In a 3-year randomized controlled trial (RCT) two methods for improved infection control in oral homecare were evaluated. The use of an oscillating/rotating powered toothbrush together with a triclosan/copolymer containing dentifrice was compared to using a manual toothbrush in combination with standard fluoride toothpaste. The authors found no significant differences between the two groups with regard to amount of dental plaque, BOP or probing pocket depth (PPD) [Bogren et al. 2007].

Light therapy of bacteria

Light has been used in several publications regarding killing oral bacteria as well as in other medical applications [König et al. 2000, Ashkenazi et al. 2003, Fukui et al. 2008, Cieplik et al. 2013]. Light emitting diodes (LED:s) emit light by electroluminescence, **Figure 2**.
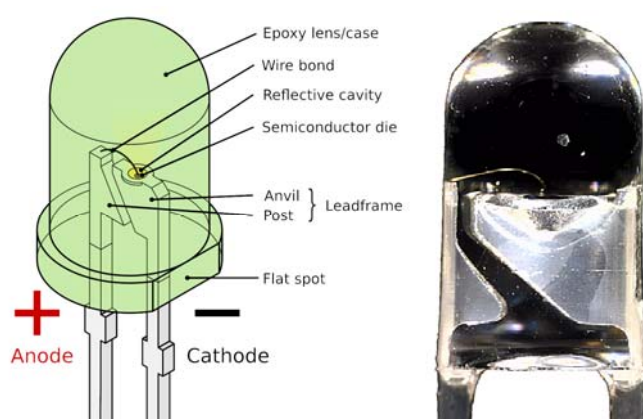

Figure 2:   Light Emitting Diode (LED) describing the different parts and a photo of a LED used in this study. [LED schematic retrieved 2017-01 from http://en.wikipedia.org/wiki/Light-emitting_diode. Photo by the author].

The mechanism for light generation is that in semiconductor diodes, made of materials such as gallium arsenide phosphide, gallium phosphide) the electrons crossing the P-N junction in

the LED dissipate their excess energy in the form of light. Depending on the material used in the P and N layers the LED will emit light of a certain wavelength. The light from the LED is divergent with a full width at half maximum (FWHM) of around 20 nm. The spectral properties of a 465 nm LED mounted in a toothbrush (BlueIllume, Stockholm, Sweden) is shown in **Figure 3**. Maximum emission at 463 nm, FWHM around 22 nm, and total radiation flow around 100 mW [SP 2010].
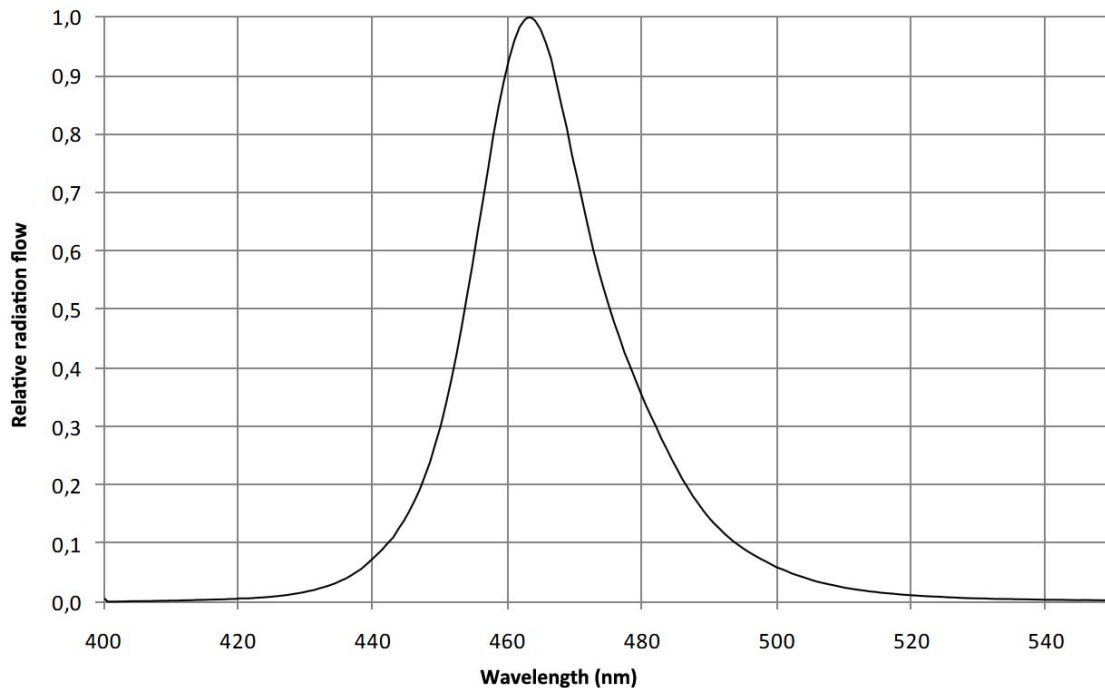

Figure 3: Emission spectrum of a 465 nm LED incorporated at a commercial toothbrush. Maximum emission as 463 nm, bandwidth at half height around 22 nm, and total radiation flow around 100 mW [From SP Rapport MTkPX05459 2010-09-02].

Porphyrins - Chemical properties and their significance in phototherapy

Porphyrins constitute a group of macromolecules involved in the biosynthesis of several essential biological molecules, e.g. the synthesis of haem **Figure 4** [Milgrom 1997]. The haem synthesis pathway is common in most living organisms, and the basic structure of the compounds included in this pathway is porphine, a macrocycle which consists of four pyrrole rings connected with methine bridges at their α-carbons, **Figure 5**.
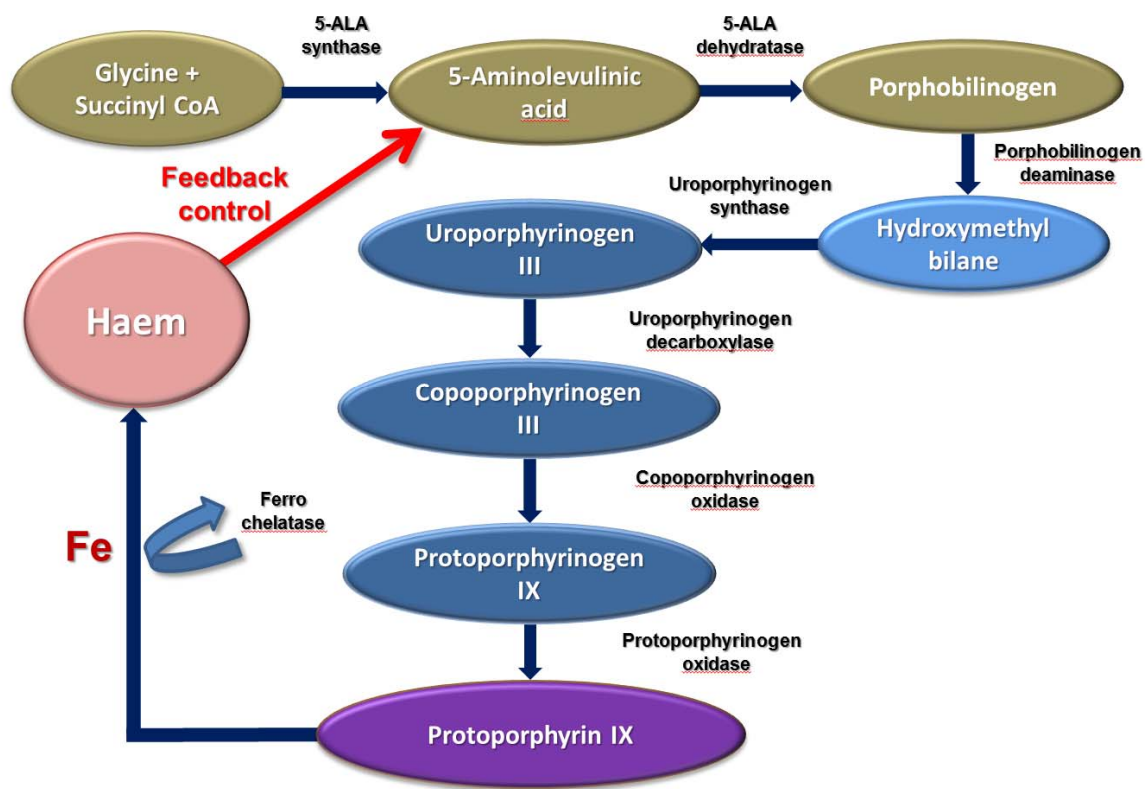
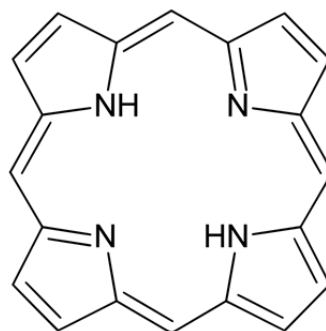**Figure 4:** The biosynthesis pathway of haem.

Figure 5: Porphine, the basic structure of porphyrins, is a macrocycle which consists of four pyrrole rings connected with methine bridges. [Structure drawn with ChemDraw 4.0].

The different porphyrins are specified by their side chain substituents consisting of alkyl, alkene or carboxylic acid groups. Protoporphyrin IX is a porphyrin of special interest since it is an important precursor to biomolecules such as heme, cytochrome c, and chlorophylls, all being biologically essential prosthetic groups. Most organisms are able to synthesize protoporphyrin IX from other basic precursors, among these most of the bacteria present in the oral cavity. Protoporphyrin IX has four methyl, two propionic acid and two vinyl side chains attached to the porphine structure, **Figure 6**.
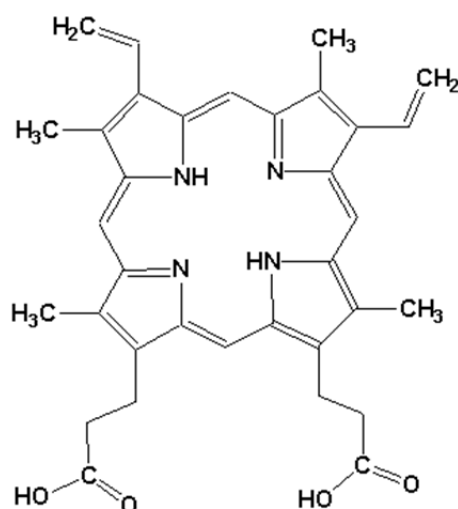
Figure 6: Chemical structure of protoporphyrin IX. The basic structure is porphine, a macrocycle which consists of four pyrrole rings connected with methine bridges. Protoporphyrin IX has four methyl, two propionic acid and two vinyl side chains attached to the porphine structure. [Structure drawn with ChemDraw 4.0].

Due to the common basic structure and their aromatic nature porphyrins show characteristic absorption bands between 390-750 nm, with a strong Soret band in the 380-450 nm region and weaker Q-bands in the 500-750 nm region [Milgrom 1997, Lim 2010]. Most porphyrins also show fluorescence, emitting red light in the visible part of the electromagnetic spectrum. In **Figure 7** the absorption and emission spectra of protoporphyrin IX is shown as an example.

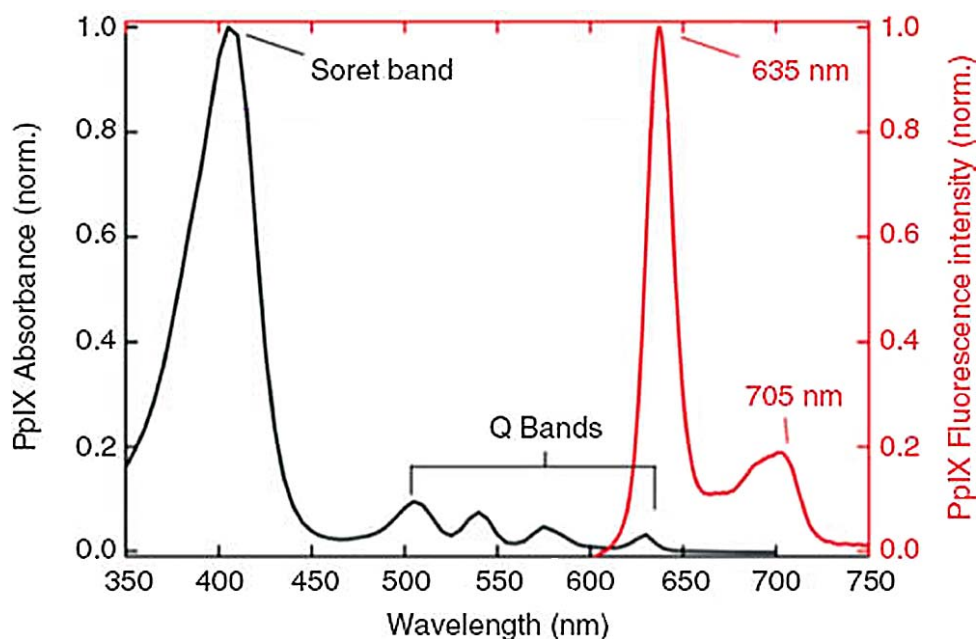**Figure 7:** Spectral properties of protoporphyrin IX. The black line shows the absorption spectrum and the red line the fluorescence spectrum. Protoporphyrin IX can be excited to a higher energy state by illumination with wavelengths in the region of the absorption spectrum with the most efficient and energetic wavelength for excitation at 405 nm. [Figure from Photonics & Lasers in Medicine 4 (2013) 287].

Photodynamic therapy

Bacteria containing porphyrins are able to absorb blue light from LEDs and produce red fluorescence [Brazier 1986, Koenig & Schneckenburger 1994a, Koenig et al. 1994b]. Studies have suggested that these spectral characteristics can be used for treatment of different kinds of bacterial induced oral diseases [Koenig & Schneckenburger 1994a, Koenig et al. 1994b]. A number of studies have shown that light of appropriate wavelength can kill bacteria containing porphyrins, a property utilized in photodynamic therapy (PDT) [Meisel and Kocher 2005, Gomer 2010, Soukos and Goodson 2011]. In PDT a chemical, called a photosensitizer, is placed within the subject to be treated and is illuminated by light of a certain wavelength to get into an excited singlet state. This species can react in two different ways. Either it reacts with a substrate that forms a radical reacting with oxygen to produce cytotoxic products, or the excited photosensitizer reacts directly with oxygen forming a radical that will oxidize biological molecules, leading to cytotoxicity. These radicals are highly reactive and will react immediately with the surrounding cells, meaning that the effect is local. To achieve these effects a variety of different photosensitizers has been used in different kinds of PDT treatments [Gomer 2010, Soukos 2011].

Phototherapy

The basic idea in this project was to use the endogenous porphyrins in oral bacteria as photosensitizers for phototherapy treatment. This eliminates the use of various chemicals such as phenazathionium chloride trihydrate (methylene blue) that are commonly added as photosensitizers in PDT treatments [Betsy et al. 2014].

It has been shown that the wavelength range of 405-470 nm can be used for phototherapy, *i.e.* it has antimicrobial effects without the addition of a photosensitizer chemical [Maclean et al. 2009, Dai et al. 2012, Wang et al. 2016]. The suggested mechanism is illustrated in **Figure 8**. Phototherapy with visible light has great advantages in the sense that no chemicals are needed and visible light is regarded safe to use for treatment [Kleinpenning 2010]. *In-vitro* studies have indicated that blue light may reduce oral microorganisms such as *P. gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Staphylococcus aureus* and *Fusobacterium nucleatum* which are involved in oral diseases [Feuerstein et al. 2004, Soukos et al. 2005, Fukui 2008, Maclean et al. 2009, Chui 2012].

Irradiation with light in the 400-410 nm region has been shown to be efficient to suppress the growth of *P. gingivalis* [Fukui 2008]. The inactivation of a variety of microbial species such as *Escherichia*, *Salmonella*, *Shigella*, *Listeria*, and *Mycobacterium* by the exposure to 405 nm light has also been demonstrated [Murdoch et al. 2012]. Further, it has been shown that blue light has wound healing abilities [McDonald et al. 2011] and clinical studies demonstrated that blue light therapy has antibacterial effects and red light has anti-inflammatory effects in treating acne caused by the bacterium *Propionibacterium acnes* [Papageorgiou et al. 2000]. Phototherapy has today become an accepted form of acne treatment [Gold et al. 2011], and in

this case it has been shown that a combination of red and blue light gave the most efficient result [Papageorgiou 2000].

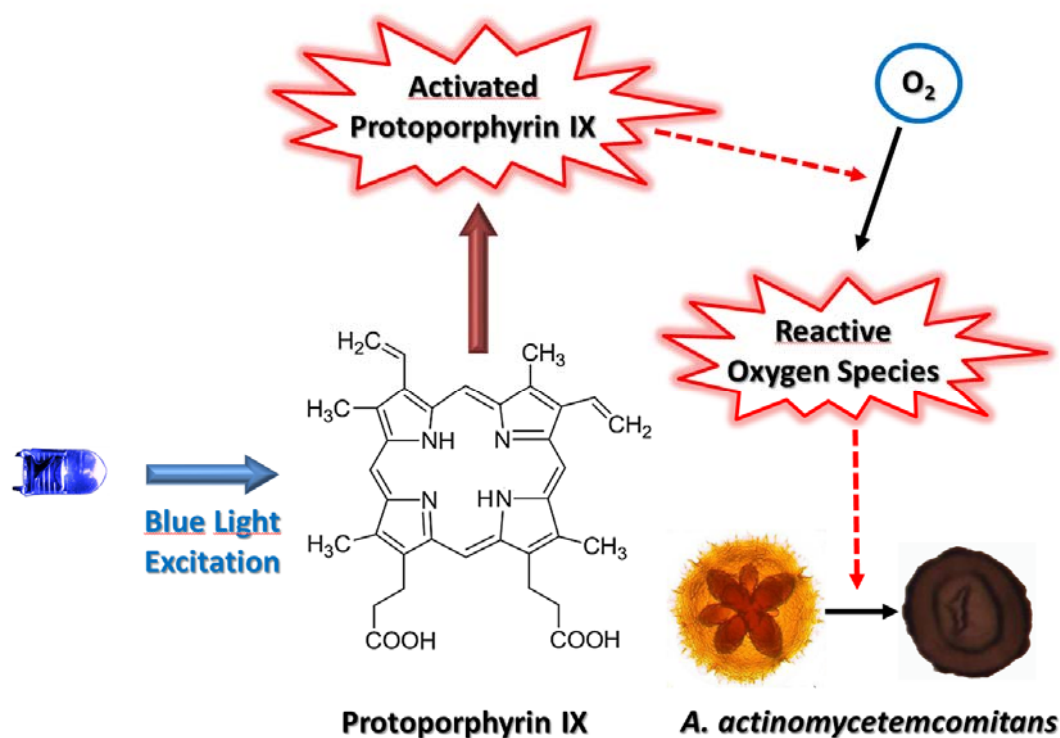**Figure 8:** A schematic illustration of the supposed mechanism of phototherapy. Blue light excite endogenous protoporphyrin IX in the bacterium to an exited sate. By energy transfer from PPIX, oxygen is transformed into reactive oxygen species which is detrimental to the bacterium and kills it from the inside. [Aa photo from MicrobeWiki, https://microbewiki.kenyon.edu/index.php/Actinobacillus_actinomycetemcomitans]

AIMS OF THE THESIS

The general aim of this thesis was to perform studies of porphyrin content and profiles in selected oral bacteria, primarily the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis*, phototherapy in-vitro and the application of phototherapy in-vivo in a randomized clinical trial.

Specific aims

Paper I

To investigate the red fluorescence properties of *A. actinomycetemcomitans* and to investigate if these properties were related to the growth, morphology and size of the bacterial colonies.

Paper II

Develop a method for the sensitive and selective determination of porphyrins in oral pathogenic bacteria by combining an efficient extraction and clean-up method for oral bacteria with efficient separation on RP-HPLC and selective detection with mass spectrometry using selected reaction monitoring (SRM).

Paper III

Elucidate how the porphyrin profile differs by changing the culturing conditions, such as time of culturing, passaging and addition of blood to the growth medium. *A. actinomycetemcomitans* and *P. gingivalis* were selected as model oral pathogens.

Paper IV

The primary aims were to investigate if a toothbrush with incorporated 450 nm LEDs used in daily oral care, could reduce dental plaque and gingival inflammation. Secondary aim was to investigate if there would be any influence from peroxide or non-peroxide toothpastes.

MATERIALS AND METHODS

Paper I

Quantitative Light-Induced Fluorescence

The method used in study 1 was the spectroscopic technique Quantitative Light-Induced Fluorescence (QLF^{TM}, Inspektor Research Systems BV, Amsterdam, the Netherlands). This is an optical method using the principle of fluorescence for visual enhancement of both caries and bacterial activity. With QLF it is possible to detect plaque that is not clinically visible in ordinary white light, but clearly visible as reddish fluorescence in QLF vision, **Figure 9**. *A. actinomycetemcomitans* ATCC 33384 was cultured on Columbia blood agar plates (CB) and on Trypticase Soy agar with Bacitracin and Vancomycin agar plates (TSBV) for 12 days in 37°C in5% CO_2 atmosphere. The agar plates were irradiated with blue light ($\lambda = 370$ nm) and the QLF images of the colonies were captured by a colour micro-CCD sensor, equipped with a yellow high-pass filter ($\lambda > 520$ nm) to exclude scattered light. Images of the colonies were captured using red fluorescence mode and analysed and transformed with the Inspektor^{TM} PRO 2.0.0.38 computer software to obtain the area of the colonies and their fluorescence intensity.

White Light Photographs

White light digital photographs were obtained for comparison with images of selected colonies obtained with the QLF instrument, **Figure 9**. A digital camera (Coolpix 4500, Nikon Corporation, Japan) was installed in a light microscope set at x 6.4 magnification. The white light photo images were adjusted to the same size and direction as the QLF images using an internal reference system. This system used triangular shaped, hard, matt, white card board reference marks placed close to the selected colonies on the surface of the agar substrate. Thus, the area of both types of images could be compared.
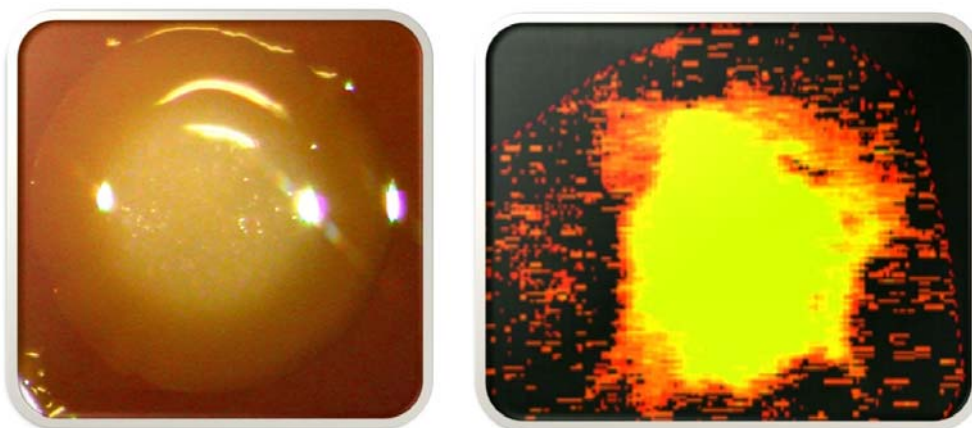

Figure 9:   When certain bacteria absorb blue light (370-450 nm) they will emit red light (590-650 nm) in a process called fluorescence. These pictures show a selected colony of the bacterium *A. actinomycetemcomitans* in white light and as a colour coded image showing the emission of red fluorescence [Pictures by the author].

Time series of Bacterial Growth

TSBV agar plates and CB agar plates were inoculated and the cultures were cultured undisturbed for 96 hours. The culture plates were taken out of incubation, subjected to QLF assessment and white light photography in a dark room and then put back into incubation. Measurements were repeated in 24 hour cycles up to and including the twelfth day of incubation.

Paper II

A quantitative and selective method was developed and validated for the determination of porphyrins in oral bacteria. The method is based on high performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS).

The developed method was as follows: The bacteria were cultivated according to standard methods, **Figure 10**, using a CO_2 incubator and agar plates. After culturing, the bacteria were harvested from the plates using a cell scraper, weighed, and lysed using a Tris-EDTA buffer followed by ultrasonication using an ultrasonication rod. After centrifugation the supernatant was cleaned-up using a C18 Solid Phase Extraction (SPE) cartridge.
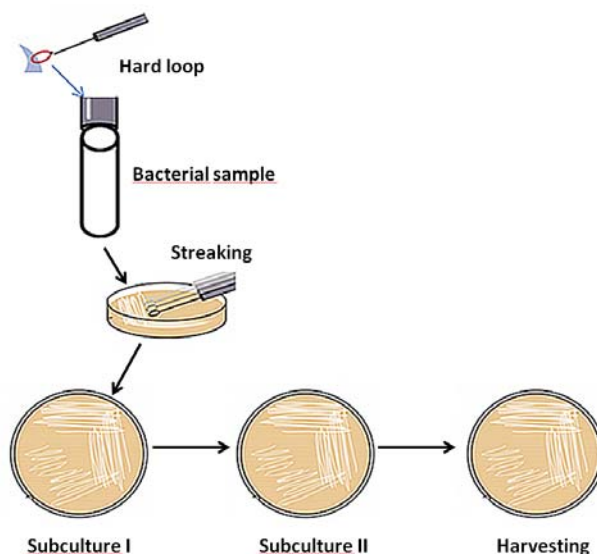

Figure 10: Culturing bacteria [Drawing by J Fyrestam]

The porphyrin enriched fraction eluted from the SPE was analysed with HPLC/MS/MS using a C18-PFP HPLC column (octadecyl pentafluorophenyl silane, ACE 3, 75×2.1 mm, dp=3 µm, Advanced Chromatography Technologies Ltd., Aberdeen, Scotland), **Figure 10**. Separation on the C18-PFP stationary phase column is based on a mixed mechanism with hydrophobic, π-π, dipole-dipole, hydrogen bonding and shape selectivity interactions [Advanced Chromatography Technologies 2012]. This stationary phase have several possible interactions with the different functional groups of the porphyrins.
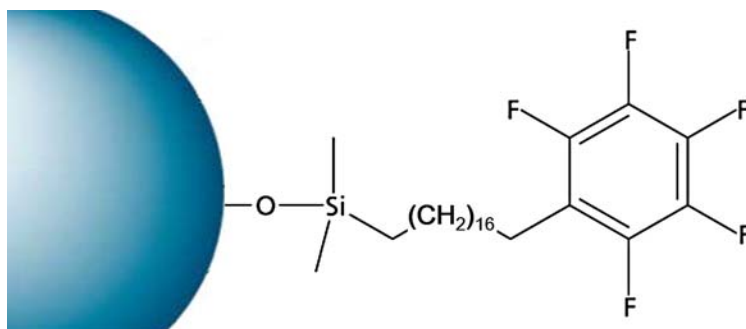
Figure 10: The chemical structure of the C18-PFP HPLC stationary phase [Drawing by C Östman].

The porphyrins are separated with the HPLC system (Agilent 1100 binary solvent delivery system with degasser and autosampler, Wilmington, DE, USA) and the eluate introduced on-line to a mass spectrometer (Sciex API 2000, Toronto, ON, Canada) with an atmospheric electrospray ionization (ESI) interface run in positive mode and a triple stage ion separation system (MS/MS). Ions are produced in the ESI interface and introduced into the first ion separation stage for selected reaction monitoring (SRM) analysis, a quadrupole analyser, were a specific ion of the compound to be analysed is selected. The selected ion is introduced in stage 2, which is a hexapole working as a combined ion guide and collision chamber. The selected ion is collided and fragmented and the fragments introduced into the third stage, the second quadrupole analyser. A fragment ion of the compound is selected and then sent to the detector. A schematic of the HPLC/MS/MS instrumentation is shown in **Figure 12**. The signal from the system is called a mass chromatogram. The specifics of the instrumentation and the method are described in detail in *Paper III*.
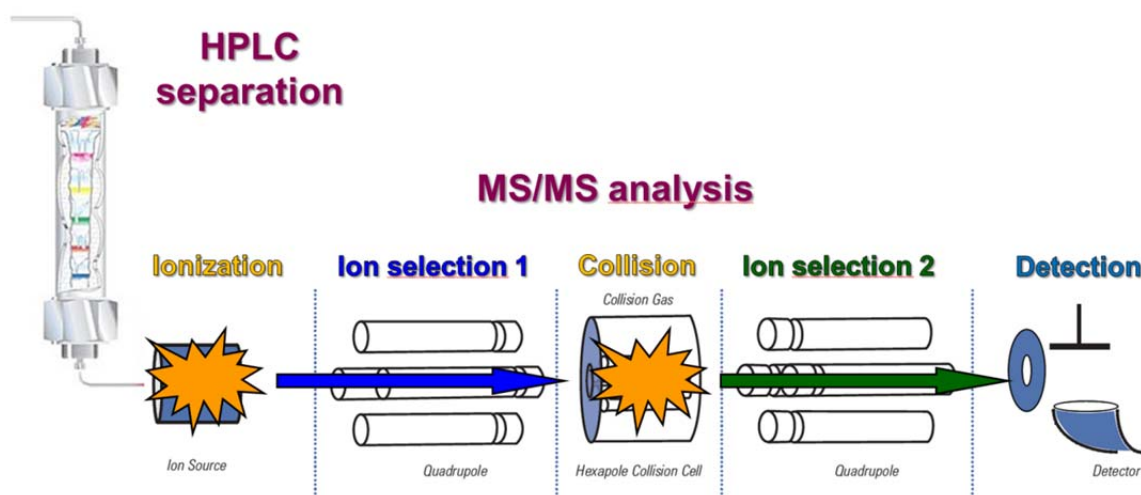

Figure 12: Chemical analysis of porphyrins with HPLC-MS/MS [Original drawing from Agilent Inc, modified by the C Östman].

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Paper III

Profiles of endogenously produced porphyrins as a function of changing variables of bacteria culturing conditions were investigated. Model organisms were the oral pathogens, *A. actinomycetemcomitans,* ATCC 33384*,* and two strains of *P. gingivalis,* ATCC 33277 and ATCC BAA-308. The concentration of porphyrins and heme in the bacteria were determined with the method based on LC/MS/MS developed in study 2. Bacteria were cultivated for different lengths of time, passaging and growth medium. Cultivation and sampling of bacteria, as well as sample preparation and chemical analysis were performed as described above for study 2. The specifics of the instrumentation are described in detail in **Paper III**.

Paper IV

Study design and study population

The clinical study was designed as an 8-week single blinded randomized clinical trial (RCT) comparing toothbrushes with and without 450 nm blue LED light emission, **Figure 13** & **14**.
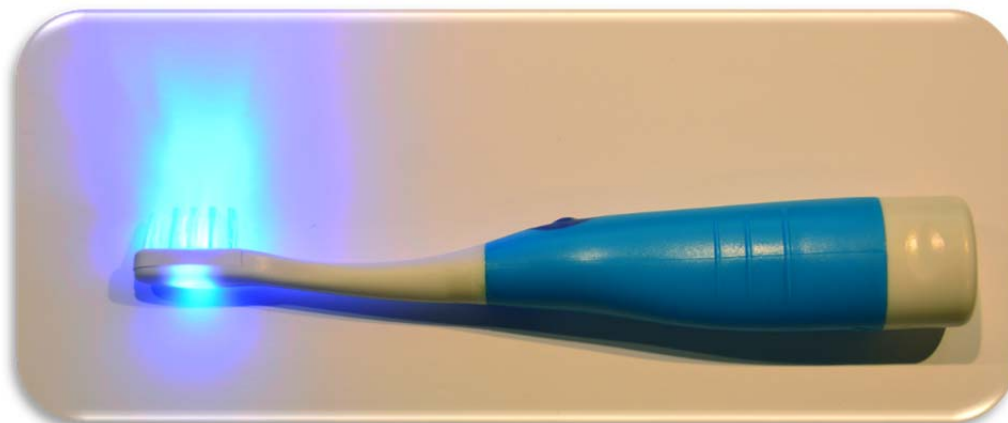

Figure 13: Toothbrush with an incorporated 450 nm blue LED. [Photo by the author].

Figure 14: Using a toothbrush with an incorporated 450 nm LED. [Photo by the author].

The study population was divided into two intervention groups and two control groups. All first year students (n=90) attending the Dental Hygiene Programme at the Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden, in the autumn semester 2014 and 2015 were asked to participate in the clinical study and 68 of the students accepted to participate. Of these subjects 48 completed the study. Inclusion criteria were the presence of gingival inflammation defined by a relative BOP level above 20%. Exclusion criteria for all subjects were: having periodontitis or any systemic diseases, having used antibiotics and/or anti-inflammatory drugs less than three months prior to the investigation or/and being a current smoker.

Clinical examination and prophylaxis

A flow chart of the study's proceedings is shown in **Table 1.** Two weeks before baseline all subjects received a professional supra-gingival scaling and cleaning performed by two blinded examiners. At baseline, all subjects were subjected to a clinical periodontal examination including PI, GI, probing pocket depth (PPD) and BOP. All subjects were then given oral prophylactic information and instruction in oral hygiene procedures, i.e. to brush twice a day during at least two minutes and instruction in flossing technique, as well as to refrain from using antiseptics. After both one and three weeks PI and GI scores were recorded and repeated oral hygiene instructions and a follow-up instruction in oral hygiene were given. After 8 weeks the same clinical parameters and samples were collected in the same manner as at baseline.

Table 1:   Flow chart of the study design.

Week -2	- Scaling, polishing and flossing
Week 0 **Baseline**	- Clinical measurement of PI, GI and PPD/BOP. - Saliva and GCF sampling. - Instructions in tooth brushing and flossing. - Handing out toothbrushes and toothpastes
Week 1	- Clinical measurement of PI and GI. - Re-instruction in tooth brushing.
Week 3	- Clinical measurement of PI and GI. - Re-instruction in tooth brushing.
Week 8 **Follow up**	- Clinical measurement of PI, GI and PPD/BOP. - Saliva and GCF sampling.

Immunological and microbiological analysis

Inflammatory markers were determined using immunological methods. The levels of interleukin IL-1β, IL-6, IL-8, and TNF-α were determined in GCF and saliva using the Luminex bead-based multiplex assay using a High Performance Assay kit according to the manufacturer instructions (R&D Systems Inc, Minneapolis, MN, USA) on a Bioplex Suspension Array System (Bio-Rad Laboratories, Hercules, CA, USA). The levels of MMP-8) and TIMP-1 in saliva were determined by Enzyme Linked Immunosorbent Assay (ELISA) (R&D Systems Inc, Minneapolis, MN, USA).

Microbial analyses were done by sampling bacteria from the mesiobuccal gingival pockets of tooth number 26 and 36, both at baseline and after 8 weeks, and sending the samples to the Department of Microbiology and Immunology at Gothenburg University for microbiological analyses with DNA-DNA hybridization technique. Since the detection limits for this technique not was reached, the method is not further discussed.

STATISTICAL METHODS

In Paper I ANOVA using post hoc t-tests with Bonferroni correction, t-test for equal and unequal variances, and bivariate linear regression were applied using the WinStat 3.0 statistical software (Analytical Chemistry, Stockholm University, Sweden).

In Paper II a validation of the developed method was made according to U.S. Department of Health and Human Services, Food and Drug Administration [FDA 2001]. Calibration standards containing seven porphyrins were prepared at six concentration levels and all calibration standards were injected in triplicate. Instrument response (peak area) was plotted versus concentration for each porphyrin and regression made by the linear least squares method. LOD and LOQ were determined by calculating the signal to noise ratio (S/N) from triplicate injections of standard solutions with 25 fmol of each porphyrin. The noise was defined as the standard deviation of the peak area and the signal was defined as the mean area of triplicate injection. LOD and LOQ were defined as 3 and 10 times the S/N ratio, respectively. Samples of spiked extracts from baker's yeast *Saccharomyces cerevisiae* (*S. cerevisiae)* with a known concentration of each porphyrin analysed in triplicate were used to determine accuracy. It was calculated as the relative difference between the mean of the measured concentration and the true concentration. Endogenously produced amounts of porphyrins in the extracts were subtracted from the spiked samples. A volumetric internal standard (IS_{Vol}), mesoporphyrin IX (MPIX), was used to correct for differences in injection volume and ionization efficiency between analytical runs. Spiked extracts from *S. cerevisiae* were analysed in triplicate during the same day to investigate the intra-day precision of the analysis.

In Paper III analysis was performed using high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) with a C18-PFP column for the HPLC separation and a triple quadrupole mass spectrometer for the detection, as described in study 2 using statistics as described for study 2.

Statistical data analysis in Study 4 was made by testing for normal distribution, F-test for variance, and t-test for two sample means and paired t-test to determine the statistical significance in the changes of clinical indices (GI, PI and BOP) as well as in the concentrations of the six selected inflammatory markers (MMP-8, TIMP, IL-1β, IL6, IL-8 and TNF-α).

RESULTS AND DISCUSSION

The overall goal of our studies was to obtain a scientific basis for the implementation of the concept of phototherapy as a novel tool in everyday oral care by incorporating blue light LEDs in a toothbrush. Experimental data from a pilot study conducted by our research group demonstrated that the growth of the bacterium *A. actinomycetemcomitans* could be inhibited by the irradiation of light in the wavelength region of 410 nm, i.e. in the blue end of the visible electromagnetic spectrum.

The general aims were to determine porphyrin content and profiles in selected oral bacteria starting with the periopathogens *A. actinomycetemcomitans* and *P. gingivalis*, to investigate the relation of these compounds to the efficiency of phototherapy, and to apply phototherapy in-vivo in a clinical study at daily oral care conditions. Our interest in porphyrins was due to the proposed mechanism for phototherapy [van der Veen et al. 2006, Coulthwaite et al. 2006]. Blue light is absorbed by bacterial endogenous porphyrins, which gets to an exited state, subsequently giving of their energy to generate reactive oxygen species which kills the bacteria from the inside. We wanted to apply this selective killing by blue light in daily oral care to investigate if this could influence or reduce human dental plaque in order to reduce gingival inflammation.

Paper I

In this first study we used QLF-technique to investigate the fluorescence of the single capnophilic gram negative bacterium *A. actinomycetemcomitans* with in order to study the bacterium's red fluorescence, **Figure 15**.
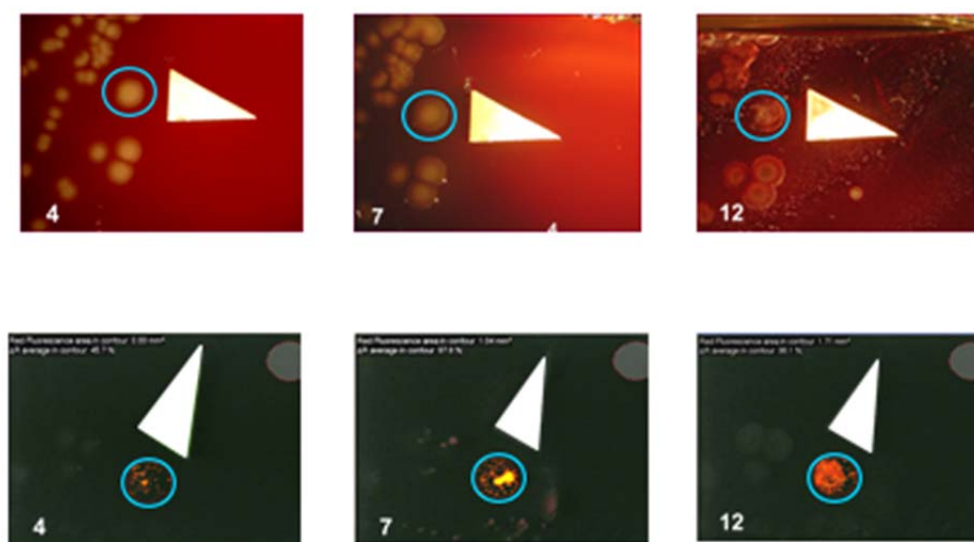

Figure 15:   An *A.actinomycetemcomitans* bacteria colony after 4, 7 and 12 days of growth. Blue circle marks the colony, white triangle is the target mark. **Upper row**: Digital photos. **Lower row**: QLF images.

We showed that this bacterium was able to emit red fluorescence on its own when illuminated with blue light, which is in contradiction with other studies that suggest that the obligate anaerobic black-pigmented bacteria *P. gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Prevotella melaninogenica* are responsible for the red fluorescence [Soukos et al. 2005]. We also showed that the fluorescence changed with growth of the colonies. An increased colony size gave larger fluorescence are but it seemed not to necessarily correlate with the area of the bacterial colonies Red fluorescence has been suggested to be due to emissions from porphyrins [Koenig and Schneckenburger 1994], and a study by König and co-workers [2000] has demonstrated that bacteria which have the ability to produce red fluorescence can be killed by optical excitation.

Pilot study

As mentioned above there were strong indications from the literature that it was possible to kill bacteria with illumination with blue light and that this mechanism involved endogenous porphyrins. Our first study indicated that red-fluorescing bacteria are containing porphyrins which raised the interest to study oral bacteria with respect to their content of porphyrins starting with the periopathogens *A. actinomycetemcomitans* and *P. gingivalis*.

The spectrum below shows the UV/VIS spectrum of PPIX recorded using a standard solution. It shows that the absorbance maximum is around 403 nm, **Figure 16**.
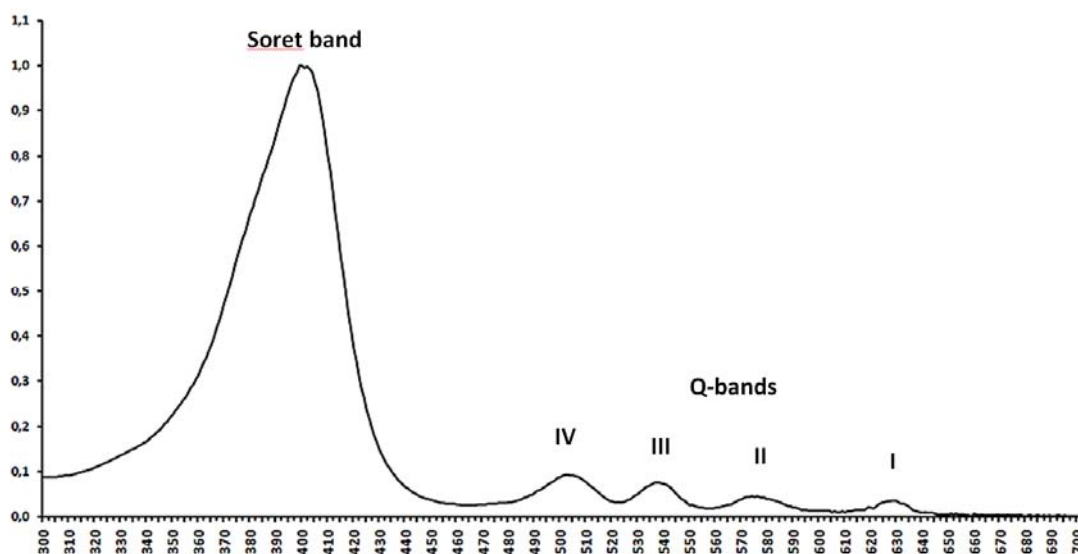

Figure 16: UV/VIS absorption spectrum of a standard solution of protoporphyrin IX showing the characteristic intense Soret band with a maximum at 403 nm, and the lesser intense Q-bands from 480 to 650 nm [Spectrum from J Fyrestam].

A contact was taken with a Swedish company marketing blue light toothbrushes. The brushes were equipped with two 465 nm LED, but the company had made prototypes of 410 nm LED toothbrushes that was to be used for studies of bacterial killing. According to the suggested mechanism of phototherapy were porphyrins acts as endogenous photosensitizers, this wavelength was regarded as close to optimal. In pilot experiment *A. actinomycetemcomitans*

grown on agar plates were illuminated and the experiments showed a clear killing effect of this bacterium **Figure 17**. *A. actinomycetemcomitans* was cultivated on agar plates as described above. Reference plates were treated in the same way as the illuminated plates, *i.e.* they were taken out of cultivation and placed in the fume hood during the same time as the illuminated plates were treated with the light from the 410 nm toothbrush. Illumination was performed during 10 minutes in the morning and in the afternoon to simulate daily oral care.
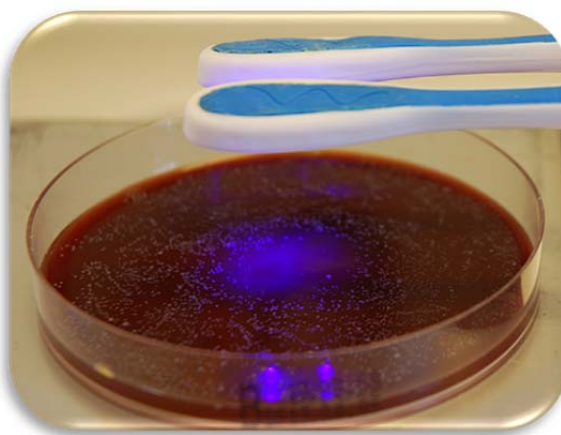

Figure 17:   Pilot study where agar plates were illuminated with a toothbrush having 410 nm LEDs incorporated. [Photo by the author].

Figure 18:   Agar plates from the pilot study. **Left:** Non-illuminated reference plate, **Right:** Plate illuminated in the centre with a 410 nm toothbrush showing only a few bacterial colonies in the illuminated area. [Photo by the author].

The result from the pilot study showed that this 410 nm toothbrush was effective for inhibiting the growth of the *A. actinomycetemcomitans* bacterium (data not published), **Figure 18**.

These results gave us at hand that we needed to develop a method to determine the porphyrin-content in oral bacteria since they are the supposed compounds being the endogenous photosensitizers. Previous methods have mainly used HPLC separation relying only on UV/VIS detection, which gives little information on the identity of the porphyrins present in

the bacteria [Romiti et al. 2000; Ashkenazi et al. 2003, Soukos et al. 2005]. This result, combined with what is stated in the literature also led to a clinical study with this kind of LED toothbrushes.

Paper II

In this paper we present a new analytical chemical method including extraction of porphyrins from the bacteria and using liquid chromatography in combination with tandem mass spectrometry (HPLC/MS/MS) for the final separation and selective detection of porphyrins. The method was validated applied on the oral pathogens *A. actinomycetemcomitans* and *P. gingivalis*, and on baker's yeast *S. Cerevisiae*.

Mass spectrometry (MS) has been used in other studies, but as stated above the determination of porphyrins in bacteria has mostly been performed using UV/VIS detection, utilizing the similarities in spectral properties of the porphyrins. This gives non-selective methods due to the low selectivity of UV/VIS spectroscopy. When determining porphyrins in biological samples, the number of porphyrin species and the complex nature of the matrix demand a selective detection technique which we have achieved by applying tandem mass spectrometry. The developed HPLC/MS/MS method makes it possible to characterize the bacterial porphyrin contents by identifying the individual porphyrins and determine their concentrations in the bacteria. All compounds were well separated, including the isomers CPI and CPIII, **Figure 19**.
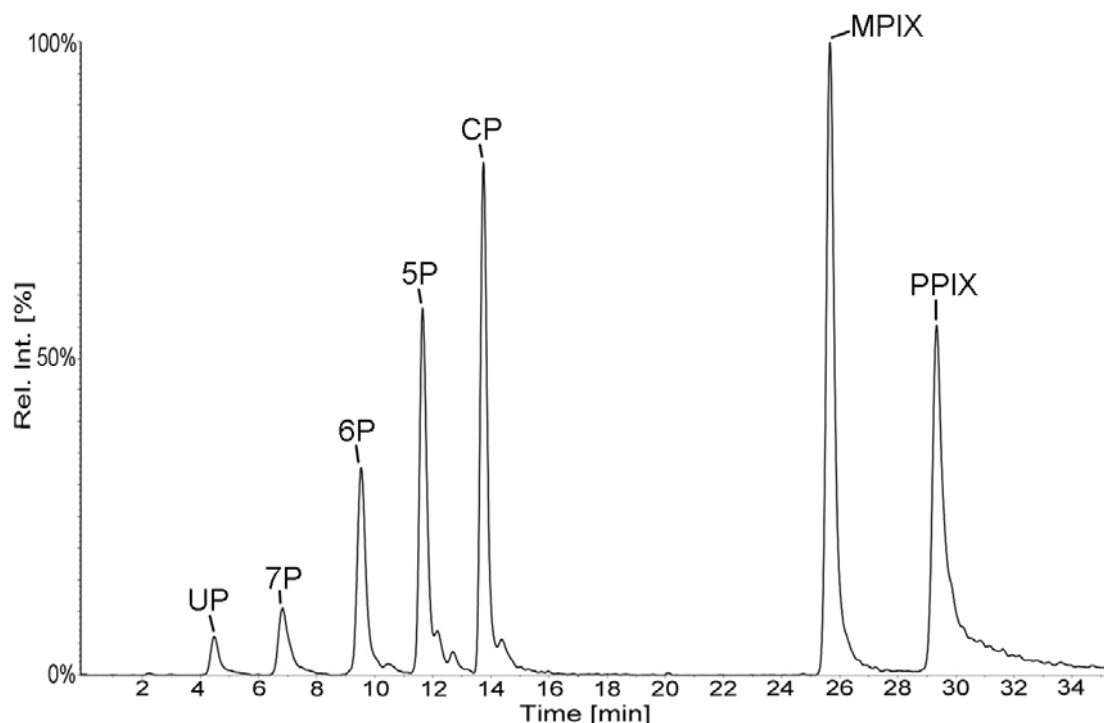

Figure 19: A chromatogram from HPLC/MS/MS analysis of a porphyrin standard mixture. UP = uroporphyrin I, 7P = 7-carboxylporphyrin I, 6P = 6-carboxylporphyrin I, 5P = 5-carboxylporphyrin I, CP = coproporphyrin I, MPIX = mesoporphyrin IX, PPIX = protoporphyrin IX. [Figure from Paper II].

The extraction method was efficient with respect to cell lysis and showed high recoveries and reproducibility. This study demonstrated that the porphyrin profile differs between *P. gingivalis* and *A. actinomycetemcomitans*, but both contained CPI, CPIII and PPIX. It was also revealed that *A. actinomycetemcomitans* could produce UP and 7P. To our knowledge this was the first time the porphyrin profile was determined for *A. actinomycetemcomitans*. Since the analytical method could be applied on both oral bacteria, *A. actinomycetemcomitans* and *P. gingivalis*, as well as on yeast the method is readily extended for use on other oral bacteria. The specific porphyrin content in bacteria can be of large interest if they differ in light absorption of blue light as well as the efficiency to generate reactive oxygen species (ROS) in illuminated bacteria. This may affect the killing efficiency of different bacteria as well as the choice of the light source in phototherapy and PDT.

Porphyrins all absorb light around 405 nm [Milgrom 1997]. If porphyrins in bacterial extracts are measured with UV/VIS spectroscopy it will only give some kind of "total concentration" of the porphyrin content. However, different porphyrins have different extinction coefficients [Milgrom 1997], and they may also have different photosensitizing properties. Studies have suggested that PPIX is the endogenous porphyrin acting as the photosensitizer [Soukos et al. 2005, Wang et al. 2016]. Thus, two different bacteria which have different porphyrin profiles, but exhibit the same total amount of porphyrins are likely to differ in light inactivation efficiency.

Paper III

The aim was to elucidate how the profile of endogenously produced porphyrins differs by changing the variables of bacteria culturing conditions. The contents of porphyrins were analyzed with the analytical method developed in the previous study. The results revealed large variations in porphyrin profiles and concentrations depending on the culturing conditions.

The porphyrin content of *A. actinomycetemcomitans* was highly affected by the age of the culture and whether or not the cultivated colonies were passaged onto a new fresh agar plate. When passage was performed the total porphyrin content increased up to 28 times, and UP and 7P were both present in the porphyrin profile, **Figure 20**. CP III, PPIX and CPI were detected at day 3 with 80%, 16% and 4% respectively of molar total porphyrin content (TPC). At day 9 the profile had changed dramatically. PPIX had increased to be the most abundant porphyrin with 85% of TPC, while CPIII had decreased to 15% and CPI was below limit of quantification (LOQ), **Figure 21**.

Our results also showed that when *P. gingivalis* was grown on blood containing medium higher concentrations of protoporphyrin IX (2.5 times) and heme (5.4 times) were quantified compared to bacteria grown without blood, suggesting an uptake from the blood in the culturing medium. The results from this study clearly points out that the culturing conditions have to be standardized if phototherapy experiments performed in vitro shall be compared.
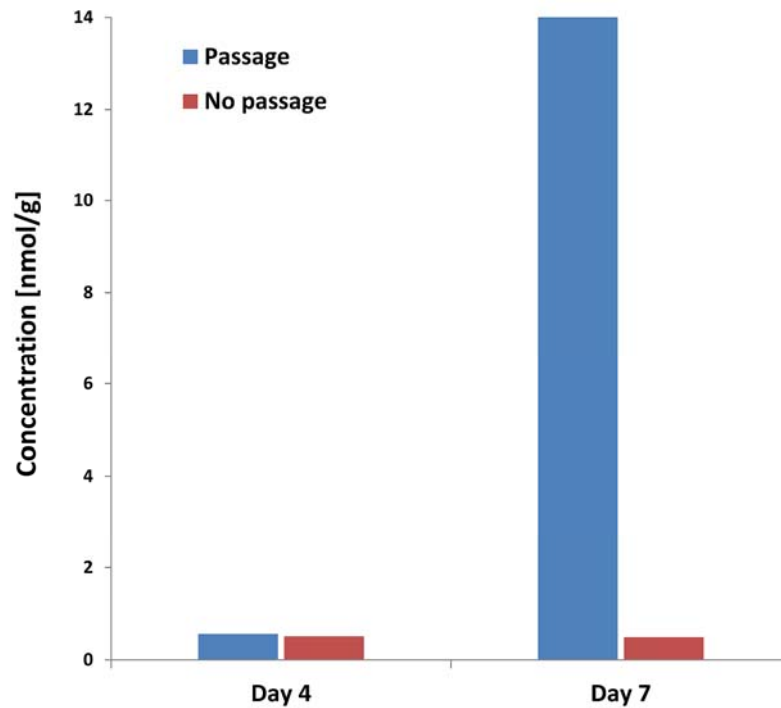
Figure 20: Difference in total porphyrin content without (red) and with (blue) passaging of the bacterial culture. [Figure from Paper III].
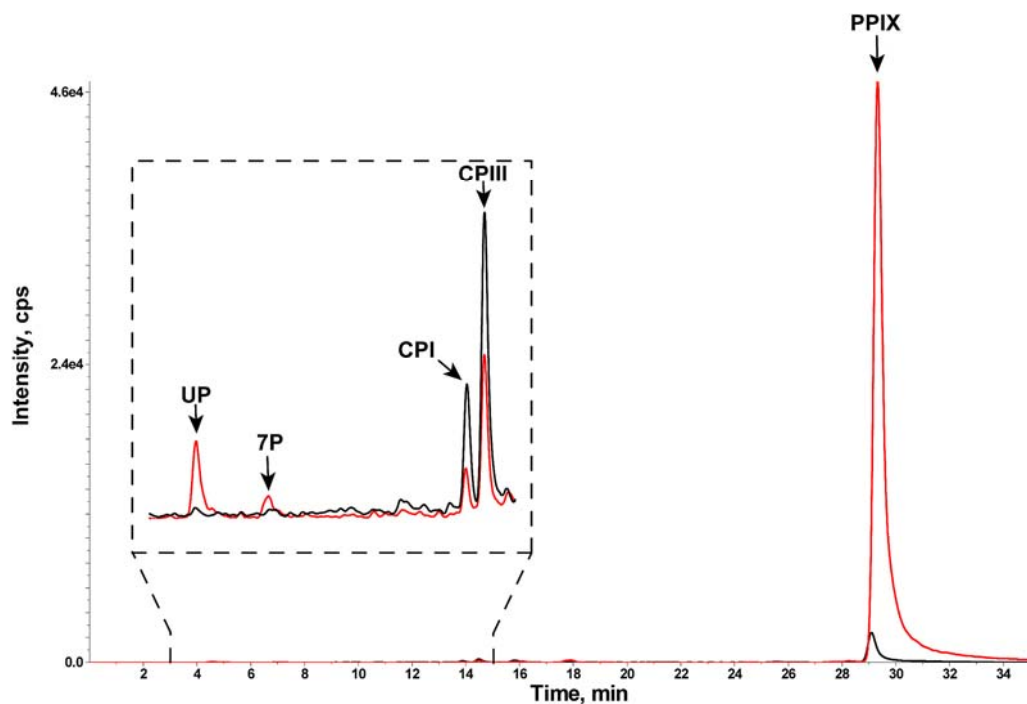

Figure 21: Chromatogram from the HPLC/MS/MS analysis showing the difference in porphyrin profile without (black) and with (red) passaging of the bacterial culture. [Figure from Paper III].

There is a need to develop new effective therapies for plaque control in the prevention of gingivitis which also can decrease the risk for extension of the inflammation into the periodontal tissues [Lang et al 2009, Yang et al 2012]. In a review by Cieplik et al. [2014] it

is stated that it is of a great importance to search for new strategies because of the increasing numbers of antibiotic-resistant pathogens. Several studies have shown that phototherapy, *i.e.* without the addition of a photosensitizer chemical, in the blue light region of the electromagnetic spectrum, 405–470 nm, has antimicrobial effects [Maclean et al. 2009, Dai et al. 2012, Wang et al. 2016].

Paper IV

In an 8-week single blinded RCT-study the outcomes of interest were if a toothbrush incorporated with blue light LED has any effect on the amount of dental plaque and on the degree of gingival inflammation. A drawback in this study was that the company delivering the 410 nm prototype toothbrushes went out of business. A new company was involved that only could deliver toothbrushes equipped with 450 nm LEDs for the first part of the study, but were developing toothbrushes with 405 nm to be used for the second part of the study. However, the 405 nm LED toothbrushes have still not today reached completion and, the 450 nm LED toothbrushes had to be used for the entire clinical study.

Our results showed that a toothbrush with 450 nm LEDs did not yield any statistical significant adjunctive effect to tooth brushing (p=0.05) with regard to reduction in plaque amount and gingival inflammation. However, all three clinical indices decreased more from baseline to the follow-up visit in the blue light group compared to the control, where the difference in PI had a level of p=0.058. This indicates that the blue light may have some adjunct effect with tooth brushing, **Figure 22**.
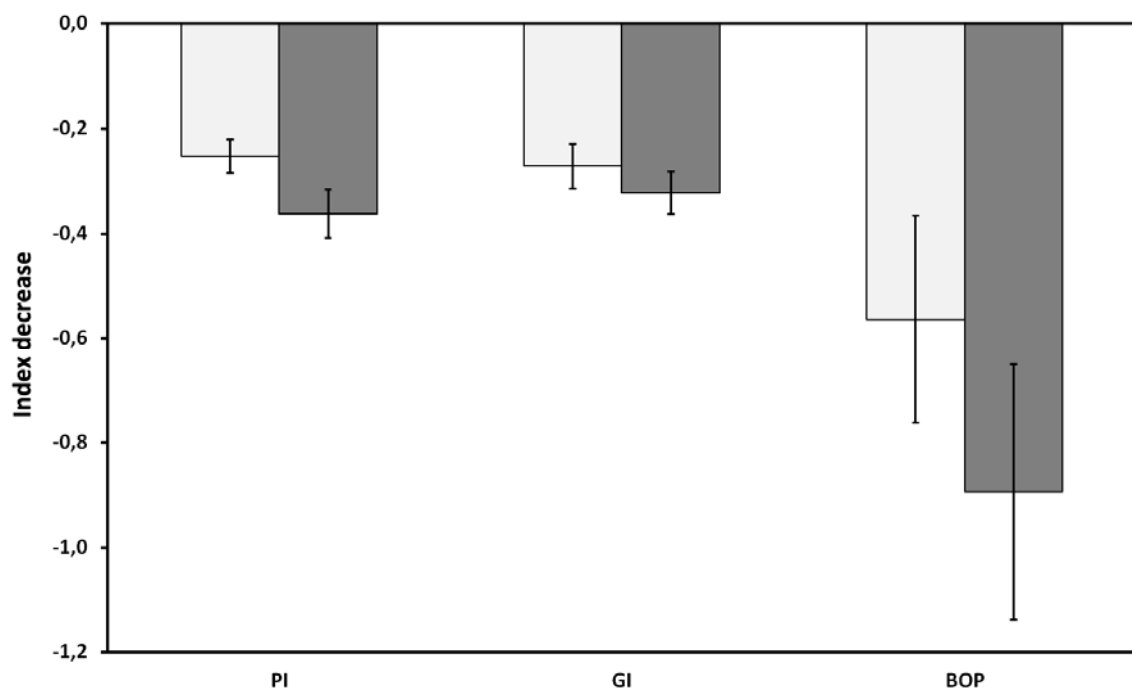

Figure 22: Decrease in the clinical parameters PI, GI and BOP for the control groups [light grey] and the groups using a 450 nm blue light LED toothbrush [dark grey] with the standard error of the mean as the error bars. Observe that the bars for BOP have been multiplied with a factor 0.1 to fit into the diagram. [Figure from Paper IV].

A review study by Boronat-Catalá [2014] showed that the determination of inflammatory mediator levels in biologic fluids is a good indicator of inflammatory activity. They demonstrated that there is sufficient evidence showing that IL-1β in GCF and saliva can be used as markers regarding the degree of gingival inflammation. In our study the degree of gingival inflammation and the levels of inflammatory markers, MMP-8 and TIMP-1 in saliva, IL-1β and IL-8 in GCF, showed a significant reduction within all subjects in all groups from baseline to the follow up visit, but there were no differences between the intervention group compared to the control group.

Furthermore, within the blue light intervention group there was significant decrease in all inflammatory markers from baseline to week 8 which was in agreement with the observed decrease of the inflammatory indices of GI and BOP in the blue light group. This was in contrast to the control group which only showed a statistical significant decrease in MMP-8. These results imply that there might be a possible adjunct effect of the blue light in combination with tooth brushing. A recent study by Genina et al. [2015], had a similar design as our study. They reported that a toothbrush incorporated with blue LEDs emitting with a maximum at 412 nm significantly reduced plaque and gingival bleeding up to 50 % more compared to the control group after four weeks intervention. Studies have demonstrated that the most efficient photo destructive wavelength is in the region 407-420 nm [Achkenazi et al. 2003], where 405 nm specifically has been demonstrated to have the most efficient antibacterial effect [Fukui et al, 2008; Kumar et al. 2015]. This coincides with porphyrins having their absorption maxima around 405 nm, known as their "Soret band", **Figure 16**, and the commonly accepted hypothesis is that endogenous porphyrins in bacteria may act as photosensitizers and lead to a lethal autophotosensitization processes in the bacteria [Cieplik et al. 2014].

When brushing your teeth with a LED equipped toothbrush, each tooth will only receive approximately four seconds of blue light irradiation, based on brushing 28 teeth for 2 min. Recently a clinical study has demonstrated a significant reduction of the proportion of certain black-pigmented bacteria when illuminating the buccal surfaces of premolar and molar teeth on one side of the mouth with a light device of 455 nm with a power of 70 mW/cm^2 for 2 minutes twice day for 4 days [Soukos et al. 2015]. Throughout our study we used a commercially available toothbrush with LEDs having an emission maximum of 450 nm with an effect of 13.5 mW/cm^2. The intention was to use a toothbrush with LEDs having an emission maximum at 405 nm during the second part of the clinical study. However, the company was not able to finish the toothbrush before the end of the project. When considering the hypothesis that endogenous porphyrins in bacteria are the acting photosensitizers that lead to a lethal autophotosensitization processes, the results from our study showed that PPIX is the most prevalent of the porphyrins in *A. actinomycetemcomitans* and *P. gingivalis.* When combining this with the results of the study by Genina et al. [2015] it is most likely that a 405 nm LED toothbrush would give a more effective phototherapeutic effect compared to the investigated 450 nm toothbrush.

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When people are involved in studies, it can often lead to changes in behaviour and this may be due to the effect of compliance or being observed [Gilbert et al. 1998]. A meta-review by van der Weijden and Slots [2015], regarding the effect of different types of toothbrushes, have suggested that long-term studies is to prefer when evaluation the effect of plaque and gingivitis, and they pointed out that there were lot of differences in the design of the investigated studies, *e.g.* the level of repeated oral hygiene instructions, during the intervention periods.

In our study a significant decrease (p<0.001) in the average of all three clinical parameters (PI, GI and BOP) was observed from baseline to the follow-up visit, as well as between each visit, **Figure 23**. The reason for this could be the influence of repeated prophylactic instructions given at every visit during the study. Earlier studies have pointed out the importance of repeated oral hygiene information and instructions for prevention of gingivitis [Hugoson et al. 2007]. The improvements of oral hygiene in the subjects participating in this study can be explained by the increased knowledge, understanding and motivation regarding how to take care of their oral health that they received at each examination occasion. An additional aspect is that all subjects attended the Dental Hygiene Programme, where oral health promotion is highlighted in the education.
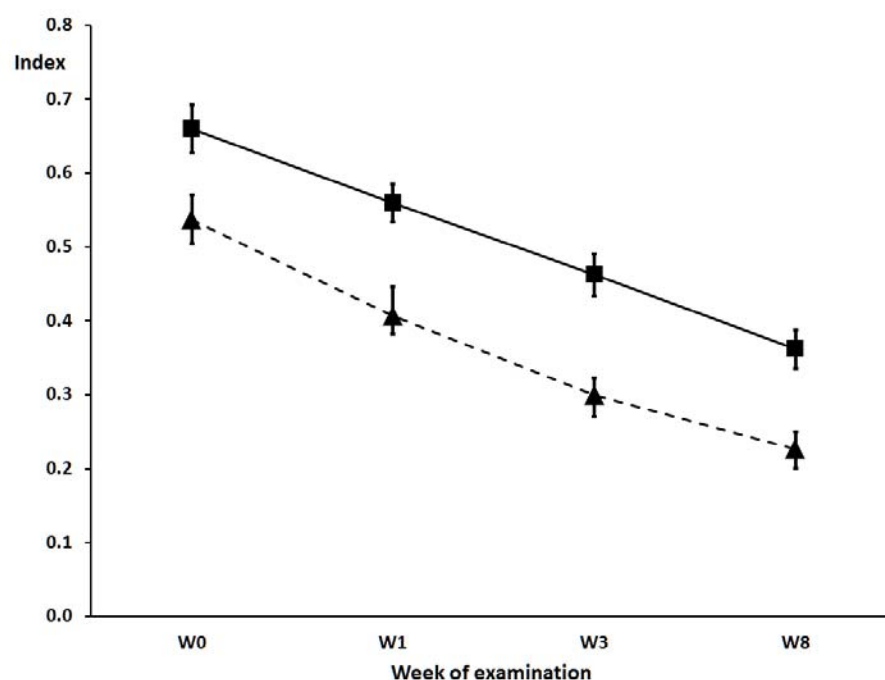

Figure 23: Change in the clinical parameters PI (dashed line) and GI (solid line) for all subjects from Baseline (W0) to the last follow-up visit at week 8 (W8). The decrease between each week of examination was significant with p <0.001, n=48. The error bars correspond to the standard error of the mean for each measurement. [Figure from Paper IV].

In present study we also investigated if toothpaste with and without peroxide had any additional effect. The results showed no differences between the two toothpastes in any of the groups regarding the effect on reducing dental plaque or gingival inflammation. This is

consistent with other studies that suggest that the effect on the gingival condition is primarily due to the blue light exposure rather than to a peroxide toothpaste [Tavares et al. 2003].

Clinical studies have shown reduction of gingivitis through supervised supragingival plaque control and have also shown that the subgingival flora is influenced by reducing the quantity and the composition of the biofilm both above and below the gingival margin [Dahlén et al. 1992, Al-Yahfoufi et al. 1995, Haffajee et al. 2003]. Daily oral care is therefore crucial for dental plaque control [Chappel et al. 2015]. The majority of the population do not clean their teeth in a proper way, and results from a Swedish oral hygiene surveys of 20 to 60 year old individuals showed that few of these performed interdental cleaning on a daily basis [reviewed in Claydon 2008]. However, it has been reported that 85% of the adults in Jönköping, Sweden brush their teeth at least twice a day [Norderyd et al. 2015] and Jensen et al. [2015] emphasized the importance of individual-based oral hygiene instruction and motivation.

GENERAL DISCUSSION

Ethical considerations

Papers I-III are all based on experimental *in-vitro* studies where principles of good laboratory practice was followed, and there were no need for ethical permissions.

The study in Paper IV was a Randomized Clinical Trial that was approved by Stockholm Regional Ethical Review board, Sweden on the 28th of March, 2013 (Dnr 2013/200-31/3), 17th of July, 2014 (Dnr 2014/1197-32), and with amendments approved on the 8th of December, 2016 (Dnr 2016/2348-31). In the work presented in Paper IV the recommendations of the Helsinki declaration [World Medical Association 2013] regarding ethical principles in clinical studies were followed. All included subjects gave informed consent to the study protocol and were informed of their rights to refuse to participate as well as to withdraw consent to participate at any time. The researchers emphasized the voluntary participation in the study, but an ethical aspect may be that students were a part of a research project and could feel that they had to participate because it could affect their educational situation. The blue light used in this study was within the visible region of the electromagnetic spectrum. Tests that were performed by Sveriges Tekniska Forskningsinstitut (SP) which showed that a LED with the same spectral characteristics as the one used in this toothbrush were free from harmful UV radiation [SP 2010] and not connected to damage mammalian cells.

Methodological considerations

Paper I-III

Laboratory work - culturing bacteria

Paper I-III includes laboratory work involving cultivation and harvesting the bacteria *A. actinomycetemcomitans* and *P. gingivalis*. The regulations from the Swedish Work Environment Authority [2005] have been implemented in this work. The risk assessment of the work with these bacteria gave a Risk Group 2 classification [Government of Canada 2009]. The laboratory was inspected and approved as a Class 2 laboratory and Basic Laboratory Safety (BLS), including such aspects as protective equipment, routines for waste management, transport, reporting of adverse events *etc.* was implemented based on the regulatory framework.

The study in Paper I was designed as an experimental in vitro study to determine the ability of the bacterium *A. actinomycetemcomitans* to produce red fluorescence. Bacteria were cultivated on agar plates, and their red light emitting properties were analysed with the QLF-technique during the growth as well as taking digital photos in white light. The considerations here was whether the bacterial colonies or their porphyrin content could be affected when they were taken out of incubation and subjected to QLF assessment and white light photography. According to the literature porphyrins are sensitive to light. Due to this all the

analysis was performed in a dark room, and the colonies were subjected to light exposure for only about 30 seconds when capturing images. Reproducibility of the measurements were performed by letting a second operator capture three images each on two different agar plates, which demonstrated a reproducibility of ≥99%. Tranæus et al. [2002] demonstrated that both repeatability and reproducibility in-vivo of the QLF method are high when analysing caries lesions area, as well as average and maximum changes in caries lesion fluorescence. Several clinical studies have shown that QLF can be used for detecting bacterial activity on tooth surfaces using red fluorescence, however the amount of plaque did not correlate with the red fluorescence area [Pretty et al. 2005, van der Veen et al. 2006, Coulthwaite et al. 2009].

Laboratory work – chemical analysis

In paper II and III a chemical analytical method for the determination of porphyrins in bacteria and fungi was developed, evaluated and validated. The evaluation/validation followed the guidelines for bioanalytical method validation recommended by the U.S. Department of Health and Human Services, Food and Drug Administration [FDA 2001]. This involves parts such as determining yield, LOD, LOQ, linearity, accuracy, precision, repeatability, reproducibility *etc.* Porphyrin standard compounds with a purity of >90 % were obtained from Frontier Scientific Inc. (Logan, UT, USA), a company specializing in synthesizing and selling porphyrin derivatives. The HPLC/MS/MS instrumentation used for most parts of the porphyrin analyses was dedicated for this project and operated by a PhD student specially trained for both running and maintaining the instrument. This PhD student was also the person responsible for developing the entire analytical methodology for porphyrins in bacteria and fungi.

A limitation in Paper III is that due to the instability of the porphyrins in the solutions used for the HPLC/MS/MS analyses could only performed as a single injection of each sample to ensure the accuracy of the analysis. The reason for this was that the cycle time for one single analysis with the HPLC/MS/MS instrument was ~1h. To analyse the time trend study for *A. actinomycetemcomitans*, using seven individual samples, standards, blank samples, and when performing separate porphyrin and heme analyses, the total run time was over 24h. In that time the porphyrin compounds had started to degrade and it was not possible to do repeated analysis of the samples with good results. However, in the evaluation of the method it was shown that the accuracy was high and that intraday precision of the instrument was ≤ 5% for all porphyrins. Further confirmation of the porphyrin profile was made by performing selected experiments at day 4 and 7.

A possible limitation in both paper II and III is that more bacterial strains could have been investigated. However, such studies are in progress. A recent study on porphyrins in solutions have also been made, which have strongly improved their storage stability.

Paper IV

Strengths and limitations

The aim with this study was to determine if a toothbrush with incorporated blue light LEDs could reduce dental plaque and gingival inflammation. It was an intervention/experimental study with a RCT design. Proper randomization and similar intervention and control groups decreased the risk for bias. A double-blinded design, including both examiners and participants, would have been optimal to strengthen objectivity and minimize potential bias. However, the study could only be single-blinded for the obvious reason that it was impossible to hide that one group had a toothbrush with working blue light LEDs, while the other group had the LEDs disabled. Thus, the participants knew what type of toothbrush they had which possibly could have had some influence on the outcome. For example the blue-light group could have brushed better or the non-light group could maybe wanted to compensate for not having blue light and thus brushed more eagerly.

Methodological issues

Selection bias: Allocation concealment and blinding is important to avoid bias. By conducting randomization in a proper way, selection bias problems can be eliminated. In this study the non-blinded research leader was responsible for the randomization process, as well as for distributing toothbrushes and toothpastes to the subjects. A selection bias that can occur is not all the subjects complete the study [Viera and Bangidiwala 2007]. In this study there were 13 subjects that did not complete the study. The optimum is two have a double-blinded study where the investigators and participants not are aware of the upcoming assignment [Viera and Bangidiwala 2007]. As mentioned above, this study could only be single-blinded for obvious reasons. The two investigators performing the clinical examinations were blinded, but the participating subjects could not be blinded. Inclusion criteria are also important. In this study the participants had to have gingival inflammation defined by a BOP index above 20% to ensure that the subjects had sufficient inflammation when the study started.

Information bias: Information bias occurs if you have errors in the data collected during the study [Viera and Bangidiwala 2007]. Before the start of this study, three experienced clinical examiners were calibrated regarding how to perform examinations of the clinical parameters and how to collect saliva and GCF samples to minimize the risk for information bias. One blinded examiner (NB) collected samples of subgingival bacteria and registered PI and GI indices, while the other blinded examiner (SM) performed all measurements of BOP and PPD indices at baseline and at the final follow up visit.

Confounding: Confounding factors can be associated with both outcome and exposure, as well as describe an association not on the causal pathway [Friedmann et al. 1996]. In this RCT study known confounders that could affect gingival inflammation were excluded (see exclusion criteria above). A known confounder is gender. In this study all the subjects were students at the Dental Hygiene programme which always have been predominantly women.

For this reason there were only six males participating in the study. An idea was to exclude male participants, but it was decided not to do so since there was no gender aspect on the study.

Validity: The study was single-blinded which may eventually reduce the internal validity *i.e.* the extent to which a causal conclusion based on a study is warranted. This is determined by the degree to which the study minimizes systemic bias [Turlik 2009]. To strengthen the internal validity of this study, important known confounders that may give rise to erroneous associations were excluded to increase the probability that the outcomes were associated with the exposure to the blue light. In RCT studies the differences between intervention and control group could be expected in a population by chance [Friedmann et al. 1996]. As described above this study has a restriction due to few male participants, a factor that may affect the external validity.

CONCLUSIONS

➢ To our knowledge this was the first time that it was shown that the single gram negative capnophilic bacterium *A. actinomycetemcomitans* is able to produce fluorescence in the red spectral region on its own, which is in contrast to previous suggestions that the presence of other bacteria is necessary. I was also shown that blood agar was necessary to obtain red fluorescence from this bacterium on culture plates, that all bacterial colonies did not show fluorescence and that the fluorescence seems to be unevenly distributed within the colonies on the agar plate.

➢ An *in-vitro* pilot study demonstrated the ability of toothbrushes with 410 nm LEDs to inactivate the growth of *A. actinomycetemcomitans* grown on agar plates.

➢ A method for chemical analysis of porphyrins in oral bacteria and fungi based on HPLC/MS/MS has been developed, evaluated and validated. In contrast to previous methods it separates, identifies and quantifies the individual porphyrins occurring in the biosynthesis of haem.

➢ Chemical analysis was used to identify and quantify the profile of porphyrins occurring in *A. actinomycetemcomitans*, *P. gingivalis*, and *S. Cerevisiae.* It was shown that the porphyrin content in *A. actinomycetemcomitans* differs drastically depending on culturing conditions. This point out the necessity to standardize culturing conditions to be able to compare *in-vitro* phototherapy experiments.

➢ The clinical study showed weak indications of a phototherapeutic effect of a 450 nm LED toothbrush. All three clinical indices decreased more in the intervention group compared to the control group, but the differences were not statistical significant. All four inflammatory markers showed a significant decrease in the blue light group but only one inflammatory marker showed a significant decrease in the control group.

FUTURE PERSPECTIVES

This study has given weak indications that a toothbrush with incorporated blue light LEDs may have a phototherapeutic effect. As discussed above other researchers have stated evidence for phototherapeutic with LEDs in the region of 405 to 412 nm incorporated in a toothbrush. Combined with the several other studies on the phototherapeutic effect of blue light in the region around 405 nm there are indications that a toothbrush with an incorporated LED with a wavelength of 405 nm could be a tool in reducing dental plaque and gingival inflammation when used in daily oral care compared with an ordinary toothbrush.

There is a need to further explore the phototherapeutic effects of a toothbrush with incorporated LEDs. According to the prevailing theory of phototherapy for killing oral bacteria, porphyrins constitute the endogenous photosensitizers forming the ROS species lethal for bacteria. We have shown that protoporphyrin IX is the predominant porphyrin in the investigated bacteria and the wavelength of the LEDs should thus be around 405 nm. That this seems to be the optimal wavelength has also been shown in-vitro by other authors as discussed above.

Further studies are in progress on the phototherapeutic effect, *i.e.* the bacterial killing efficiency, on more oral bacterial pathogens in combination with their content of porphyrins. Studies are also planned for investigating the phototherapeutic effect on human dental plaque cultivated *in-vitro*.

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