Photodynamic therapy for treatment of Acne Vulgaris in clinical studies: dose response and mode of action

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Cover:
The cover picture illustrates a patient with Acne Vulgaris undergoing photodynamic therapy.

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To my family – Hans, Emma and Carl

If we continue to do what we have always done,

we will continue to get what we have always got.

Words from an unknown philosopher

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Abstract

This thesis deals with the use of photodynamic therapy (PDT) for treatment of Acne Vulgaris. Acne Vulgaris is one of the most common skin disorders. Conventional treatments target the pathogenic factors and include a variety of topical and oral medications such as antibiotics. Many patients show no clinical response or experience side effects from these conventional therapies. The wide use of antibiotics leads to bacterial resistance, and hence there is a need for new alternatives in acne treatment. Photodynamic therapy (PDT) is based on an initial photosensitization of the skin, followed by irradiation with visible light producing cytotoxic singlet oxygen. When PDT is applied for treatment of acne it is believed to affect the sebaceous gland and the bacterium *P.acnes*; however, the full mechanism involved in PDT of acne is not clear. The work in this thesis deals with investigating mechanisms of action and the most effective treatment regimen for PDT of acne.

Patients with mild to severe acne have been studied. In a nonblinded dose finding study, patients received aminolaevulinic acid (ALA) PDT at different light doses on the cheeks and on the back. No significant difference in clinical result was found between the different light doses of ALA-PDT, although pain and hyperpigmentation were more common at higher doses. In a split-face placebo-controlled blinded study, patients received two consecutive methylaminolaevulinate (MAL) PDT and placebo treatment. Greater reduction in total inflammatory lesion count was obtained with two consecutive MAL PDTs compared with placebo PDT; however, in another split-face un-blinded controlled study, where single-treatment low light dose MAL-PDT and treatment with red light only were compared, no significant difference between the treatment protocols was obtained. Both MAL-PDT and red light only showed significant decrease in acne score. The studies also showed that there was no significant reduction of *P. acnes* or sebum excretion, neither for ALA-PDT nor for MAL-PDT. Furthermore, fluorescence images revealed poor selectivity of MAL-induced fluorescence to the acne lesions. In a fourth, *in vitro* study the photodynamic effect on the skin bacteria *P.acnes* was investigated. Bacteria suspensions were anaerobically incubated in the presence or absence of sensitizer, i.e. ALA or MAL. The viable counts of *P.acnes* were significantly reduced after illumination with either red or blue light when incubated in the presence of either ALA or MAL; however, long incubation times were necessary (4 to 5 days), confirmed by fluorescence measurements.

Taken together, the results of this thesis suggest that PDT using either ALA or MAL is effective in treatment of acne. Light doses minimizing side effects such as pain and hyperpigmentation should be applied. However, the results also imply that explanations other than eradication of *P. acnes* and destruction of the pilosebaceous unit should be considered for describing the mechanisms behind the treatment.

Key words: Acne Vulgaris, delta-aminolaevulinic acid, fluorescence imaging, methyl-aminolaevulinic acid, photodynamic therapy, porphyrins, Propionibacterium acnes

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PUBLICATIONS

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

I. C. Hörfelt, J. Funk, M. Frohm-Nilsson, D. Wiegleb Edström and A-M Wennberg. Topical methyl aminolaevulinate photodynamic therapy for treatment of facial acne vulgaris: results of a randomized, controlled study, Br J Dermatol 2006; 155: 608-13

II. C. Hörfelt, B. Stenquist, O. Larkö, J. Faergemann and A-M Wennberg. Photodynamic Therapy for Acne Vulgaris: a Pilot Study of the Dose-Response and Mechanism of Action, *Acta Derm Venereol 2007*; 87: 325-329

III. C. Hörfelt, B. Stenquist, C. B. Halldin, M.B. Ericson, A-M. Wennberg. Single low dose red light is as efficacious as MAL-PDT for treatment of acne: Clinical assessment and fluorescence monitoring. Submitted for publication.

IV. C. Hörfelt, N. Karami, J. Faergemann, A-M. Wennberg, M.B. Ericson. Photodynamic effect in Propionibacterium acnes: An in vitro study investigating the effect of red and blue light in the presence and absence of aminolaevulinic acid or its methyl ester. Submitted for publication.

CONTENTS

LIST OF ABBREVIATIONS

AE	Adverse event
ALA	Aminolaevulinic acid
Cp III	Coproporphyrin III
LED	Light emitting diode
MAL	Methyl aminolaevulinate
P.acnes	Propionibacterium acnes
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PDT	Photodynamic therapy
Pp IX	Protoporphyrin IX
UV	Ultraviolet radiation
Up III	Uroporphyrin III
VAS	Visual analogue scale (pain score scale)

INTRODUCTION

Acne Vulgaris

Clinical manifestation

Acne Vulgaris is a very common, chronic skin disorder of the tiny hair follicles which are associated with large grease-producing (sebaceous) glands. Figure 1 shows a schematic picture of a cross-section of a pilosebaceous follicle. These follicles are found in highest amount on the face and upper trunk.

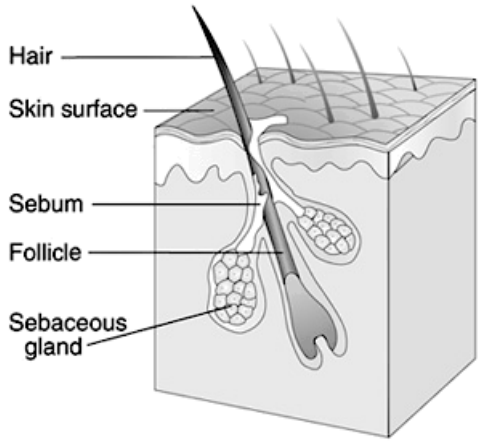


Figure 1: Schematic picture of the pilosebaceous follicle.

The beginning of acne typically appears during adolescence, with the development of non-inflamed spots (whiteheads and blackheads), which often, but not always, are found on the forehead and mid-facial region. They are called *comedones.* These superficial spots can become inflamed and are then called *papules* and *pustules.* Very large, deep inflamed spots are called *nodules*. In the clinic acne is divided into mild, moderate or severe (Figure 2) and the choice of treatment between these differs.

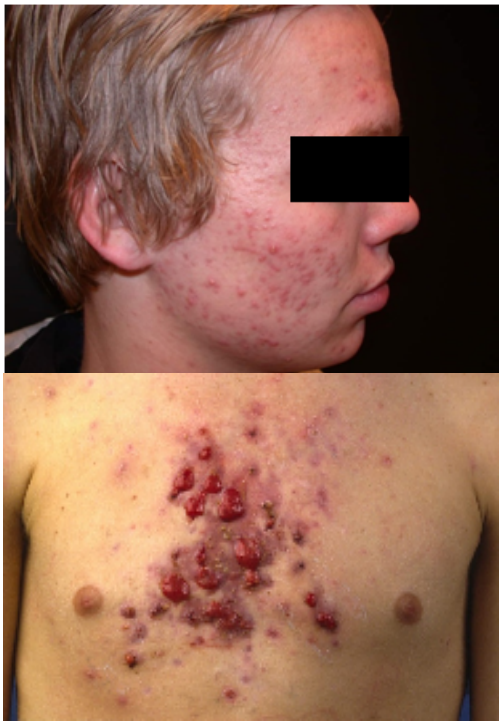


Figure 2: Moderate acne in the face (top) and severe acne on chest (bottom).

Various grading systems are used to assess acne severity, but there is no generally accepted quantitative system in use (Doshi *et al.* 1997). Lesion counts may be acceptable for representing acne severity, but the method is laborious, inaccurate and irreproducible. The Leeds revised acne grading system is the most accepted (O'Brien *et al.* 1998). This grading is based on clinical photos of acne patients from the face and back numbered from 1 to 12 with the highest number representing the worst severity of acne. Pillsbury *et al.* 1961 grades I–IV is an older acne grading system and has been criticised for being too rough. Table 1 shows the definition of the four scoring grades.

Table 1. Acne scoring grades I–IV according to Pillsbury *et al.* 1961

Grade	Definition
I.	Acne with few or a lot of comedones with little or no inflammation.
II.	Acne with comedones and small superficial pustules and inflammatory lesions in the follicle.
III.	Acne consisting of comedones, small pustules and tendency to deeper inflammatory lesions.
IV.	Extensive secondarily infected cystic acne. Confluent lesions with canalised sinuses.

Physical scarring is common (Purdy *et al.* 2006) so that the legacy of acne often remains visible long after active disease has come to an end. Irrespective of severity, acne is often accompanied by psychological effects such as low self-esteem, lack of confidence and depression (Baldwin 2002, Tan 2004, Thomas 2004). Patients with acne have a higher unemployment rate than adults without acne due to depression and anxiety (Cunliffe 1986, Katsambas *et al.* 2004).

Epidemiology

Acne Vulgaris is one of the most common skin disorders with an estimated prevalence of up to 85% in the young population (Dreno *et al.*2003, Wood 1997). The prevalence of the disease is higher among men than women, but women are more likely to seek medical advice for the acne treatment (Stern 1992, Stern 1996). The true prevalence of adolescent acne is difficult to evaluate due to the definition of the disease, which varies in different studies (Dreno *et al.* 2003). In the absence of effective treatment, acne persists for an average of 8-12 years in most sufferers (Cunliffe 1996) before it resolves spontaneously, usually but not always, by the early twenties.

Two main forms of acne in individuals aged over 25 years have been identified: persistent acne and late-onset acne, in which the disease develops for the first time over the age of 25 years (Epstein 1968, Cunliffe and Gould 1979). Persistent and late onset acne is well recognized, and is more common in women than in men (Goulden 1997, Goulden *et al.* 1999, Poli *et al.* 2001).

Aetiology of acne

Acne usually begins at puberty, when the output of sebum by tiny hair follicles in the face and upper trunk increases substantially (Rothman and Lucky 1993). The production of sebum is controlled by male hormones (androgens) in both sexes. Genetic factors play an important role. There are families which have several individuals with severe forms of acne (Zouboulis *et al.* 2005). Between the ages of about six and eight the adrenal glands begin to secrete androgens, in particular testosterone and dehydroepiandrosterone sulphate, into the circulation in both boys and girls (Zouboulis 2004). Androgens act with other hormones, such as insulin-related growth factor 1, to stimulate a gradual increase in size of the sebaceous glands (Thiboutot 2004) and to initiate sebum production.

There are four important pathophysiological factors contributing to the acne development: sebum secretion, comedo formation, ductal colonization with *P.acnes,* inflammation and immunological host reactions (Figure 3).

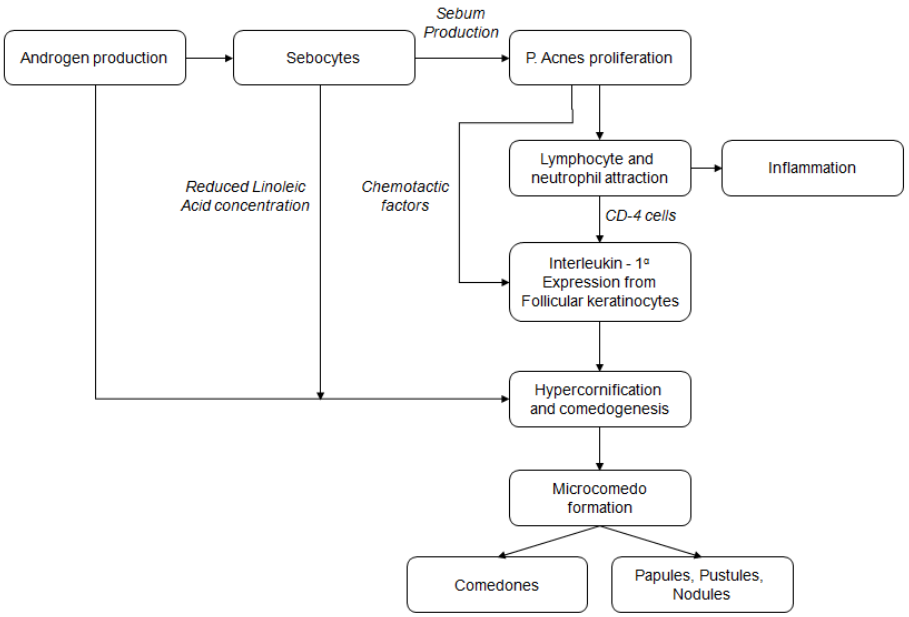
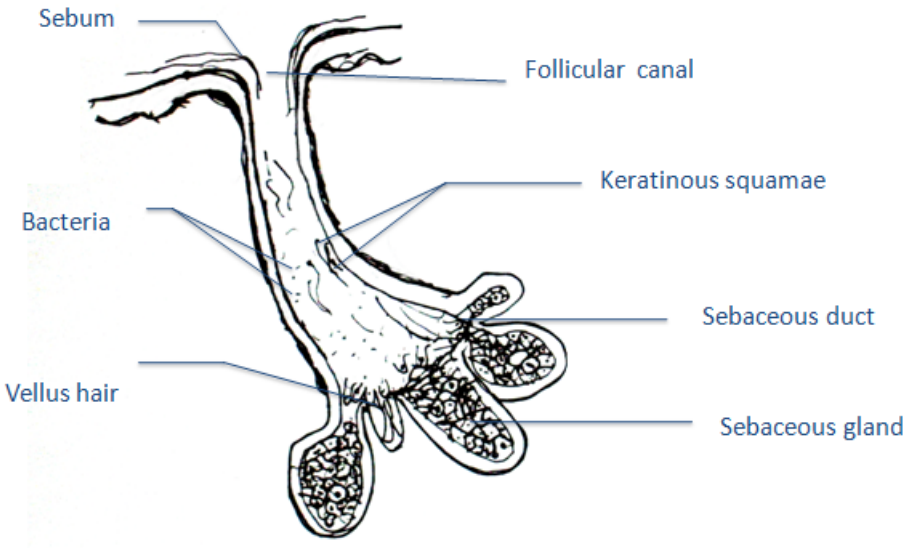


Figure 3: The pathogenesis of Acne Vulgaris. -

The pilosebaceous follicle is the target organ in acne, and the earliest morphological change in the pilosebaceous unit is abnormal follicular epithelial differentiation (Knutson 1974). This process is called comedogenesis, which is illustrated in the schematic picture in Figure 4; the growing comedo eventually blocks the flow of sebum (Cunliffe *et al.* 2004). The sebum acts as a nutrient for *Propionibacterium acnes*, which grows abnormally in follicles whose pores are blocked, and this last step leads to visible inflammation (Eady 2000). The bacterium produces an extracellular lipase that hydrolyses sebum triglycerides to glycerol, used by the bacterium as a growth substrate, and free fatty acids, which have proinflammatory and comedogenic properties (Shalita and Lee 1983).

Sebaceous follicle



Microcomedo

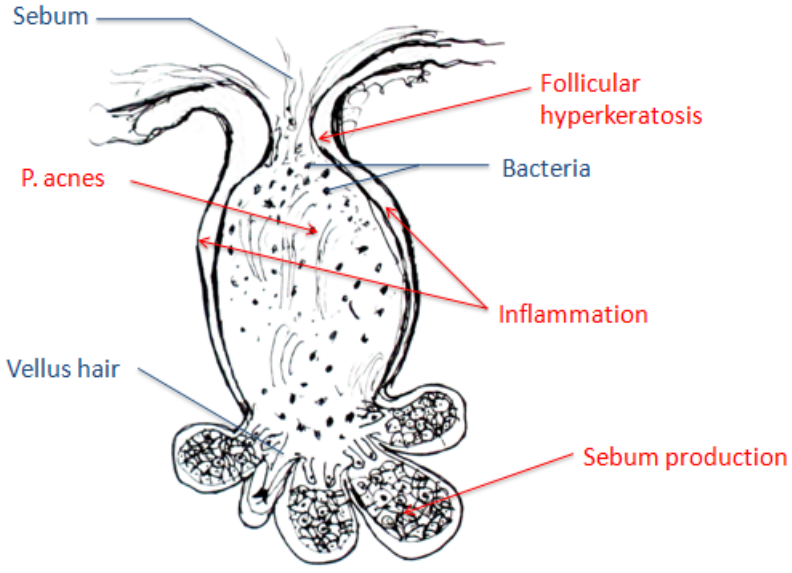


Figure 4: Schematic picture of comedogenesis. Drawing by Carl Hörfelt

Propionibacterium acnes (*P.acnes*)

P.acnes is a Gram-positive anaerobic bacillus and was first isolated from the skin of acne patients (Gilchrist 1901). It is not pathogenic by normal standards because it is present in nearly 100% of healthy persons (Jappe *et al.* 2002)**.** It is isolated from the skin surface, but the multiplication takes place in the duct of pilosebaceous follicles. The bacterium is generally 0.5-4 mm in size, dome-shaped and beige to pink in color (Figure 5). Only 17% of the follicles in normal individuals are colonized (Eady and Ingham 1994)**.** The density varies a lot among individuals and between different sites in the same person. Levels are highest in areas that are rich in sebaceous glands, such as the face and scalp. The bacterial population correlates with the amount of lipids produced in different body regions (McGinley *et al.* 1980). These findings suggest that sebum serves as a substrate for *P.acnes* growth. There is no correlation between bacterial density on the skin surface and acne severity (Simpson *et al.* 2004).

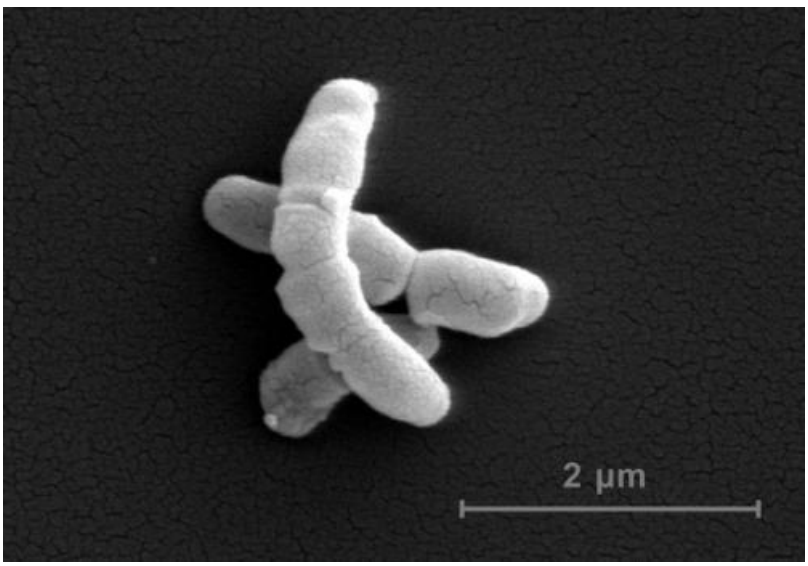


Figure 5: Electron microscopy image of *P.acnes* published with approval from Prof. Dr. Gerhard Gottschalk, Göttingen.

Treatment of Acne Vulgaris

Conventional treatments target the pathogenic factors and include a variety of topical and oral medications such as antimicrobials, anti-inflammatory agents, hormones and retinoids (Katsambas *et al.* 2004, James 2005). Figure 6 shows a schematic illustration of treatment steps. *P.acnes* is the major target of antibacterial treatment, administrated either topically or systemically, although the reduction in bacterial numbers does not correlate with the efficacy of treatment (Leyden *et al.* 1975).

Topical treatment

Topical treatment may act primarily against comedones (comedolytic agents) or inflammatory lesions (antibacterials and antibiotics). Topical retinoids reverse the process of abnormal follicular keratinisation, thereby reducing microcomedo formation. Secondarily, the number of inflammatory lesions resulting from rupture of microcomedones also decreases (Leyden *et al*. 1986). Benzoyl peroxide is an oxidizing agent that is bactericidal for *P.acnes* (Leyden *et al.* 1980). Erythromycin and clindamycin are bacteriostatic for *P.acne*s.

Systemic treatment

Antibiotics have been used for over 40 years and continue to be the most prescribed therapy for acne. A systematic review of clinical trials (1962-2006) on oral tetracyclines (Simonart *et al.* 2008) showed no evidence of superiority for one tetracycline over the other. The antibiotic dosage had no impact on efficacy in inflammatory and noninflammatory lesions. The effect of tetracyclines may be due not only to an antimicrobial effect but also to their ability to reduce neutrophil chemotaxis as well as their inhibitory effect on cytokines and matrix metalloproteins (Sapadin *et al.* 2006). Oral antibiotics should not be used alone but in combination with a topical retinoid (Dreno *et al*. 2004, Gollnick *et al.* 2003).

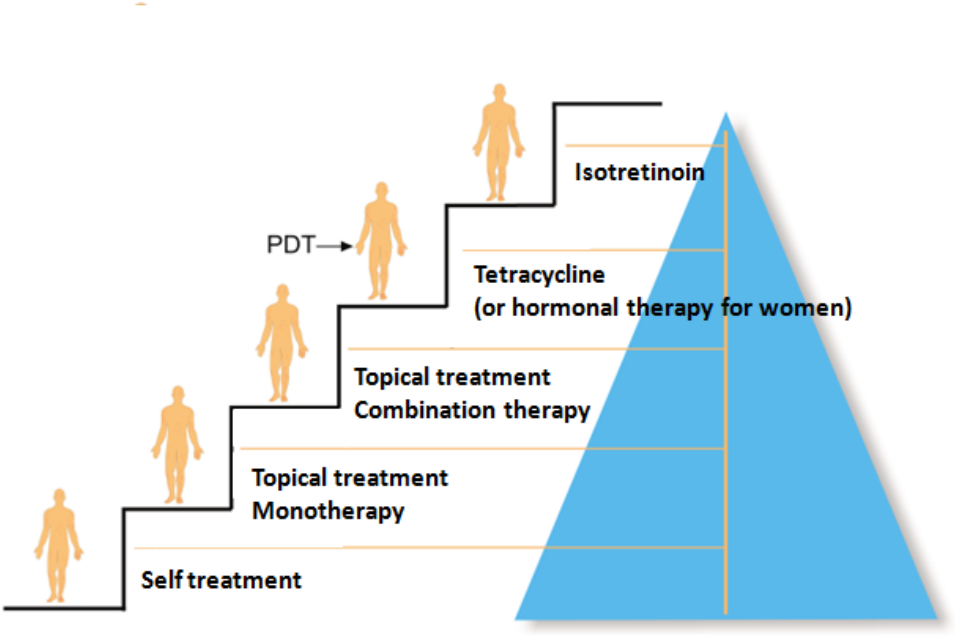


Figure 6: Schematic illustration of treatment steps for acne. Drawing by Morgan Carlsson

Oral retinoids

Oral administration of isotretinoin has been used for over 20 years and is the most effective treatment, with 80–90% reduction of acne lesions (Katsambas *et al.* 2004, Thiboutot *et al.* 2003, Jones *et al.* 1980). Isotretinoin, a synthetic retinoid (13-cis retinoic acid), affects all factors involved in acne pathogenicity: sebum production, follicular hyperkeratinization, microbial colonization, and release of inflammatory mediators into dermis (Goldsmith *et al.* 2004). Side effects of isotretinoin are, most importantly, teratogenic (McClane 2001). Other side effects are: dryness or irritation of the skin and mucous membranes, pain or stiffness of bones, joints and muscles, increased blood lipid levels and, more seldom, low counts of white and red blood cells (Katsambas *et al.* 2004).

Antibiotic resistance in *P. Acnes*

Until the late 1970s the propionibacteria were uniformly susceptible to antibiotics (Leyden 1976). By 1979 the situation had changed. Resistance to macrolide, erythromycin, lincosamide and clindamycin has been reported among cutaneous propionibacteria from Europe, USA, Australia and the Far East (Leyden *et al*. 1983, Cooper 1998, Ross *et al*. 2001). There are few reports of propionibacterial resistance to tetracyclines.

A study from Sweden (Oprica *et al.* 2004), investigating *P. acnes* strains isolated from patients with moderate to severe acne, found 37% resistance to *P.acnes* versus 13% of the non-antibiotic group. Thus antibiotic-resistant *P.acnes* strains were significantly more often isolated from antibiotic-

treated patients with moderate to severe acne than from non-antibiotic-treated patients. A patient can be colonized with different strains with varying degrees of antibiotic resistance.

There also seems to be a relation between the carriage of resistant strains and failure to respond to the treatment with the corresponding antibiotic (Eady *et al.* 1988, Eady *et al.* 1989, Leyden *et al.* 1983). A poor clinical response to treatment can also be explained by bad compliance, inadequate duration of therapy, development of Gram-negative folliculitis resistance or a high sebum excretion rate.

Photodynamic Therapy (PDT)

Basic aspects

The term "photodynamic effect" was first described by Von Tappeiner and Jodblauer (Von Tappeiner *et al.* 1904), who described oxygen-consuming chemical reactions/fluorescence in protozoa after the application of aniline dyes in 1904. Photodynamic therapy is a treatment modality that uses a photosensitizer, visible light and molecular oxygen to selectively kill cells (Dougherty *et al.* 1978). The topical use of δ-aminolaevulinic acid (ALA) in PDT was introduced and evaluated by Kennedy and colleagues (Kennedy *et al.* 1990, Kennedy *et al.* 1992). This enhances the formation of protoporphyrin IX (PpIX), which acts as a photosensitizer in the skin. Today, the topical photosensitizer most commonly used is ALA, or its derivates (Cerburkov *et al.* 2000, Malik 1987), in a cream. But also other photosensitizers can be used, as will be discussed below. The main advantages of topical PDT for treatment of non-melanoma skin cancers are that the treatment is non-invasive and effective, and generally gives good cosmetic outcomes (Peng *et al.* 1997, Salva 2002).

Photosensitization

The first step into PDT is the photosensitization of the abnormal cells (Dougherty *et al.* 1978). This can be achieved by administration of exogenous photosensitizing molecules, e.g. porphyrins, chlorins, or phthalocyanines. The photosensitizer is accumulated in tumour tissue, and the photochemical reaction occurs when the tissue is irradiated at a wavelength matching the absorption spectrum of the sensitizer. Singlet oxygen is produced via energy transfer from the excited sensitizer to molecular oxygen present in the tissue. It is the singlet oxygen that subsequently destroys the malignant cells (Kennedy *et al.* 1992).

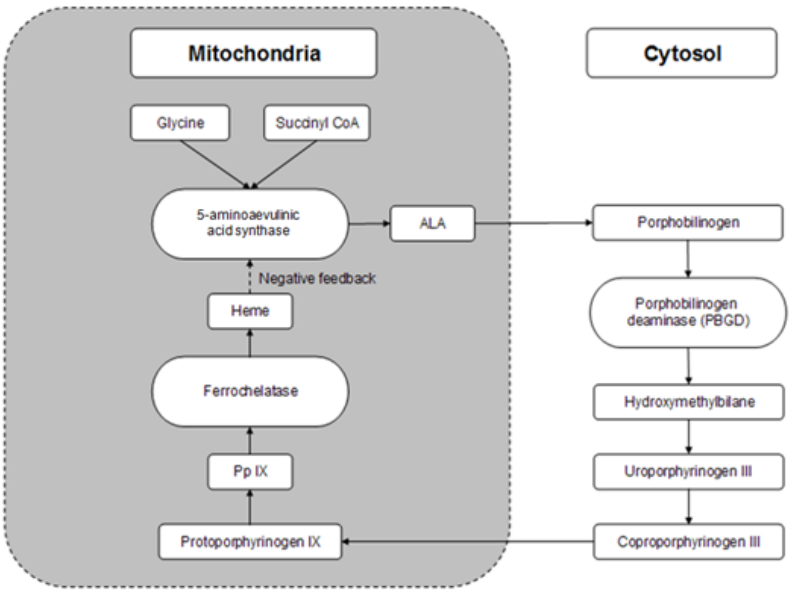
ALA is used clinically for the treatment of skin cancer (Szeimies *et al.* 1996, Cairnduff *et al.* 1994). ALA is a prodrug that is converted to photoactive PpIX in the cells through the heme biosynthesis (see below), and can be delivered topically or systemically. Since ALA is a hydrophilic compound, its penetration through cellular membranes and into the interstitial space of tissues is limited (Kennedy *et al.* 1990, Goff *et al.* 1992).

The methyl ester of ALA, MAL, can also be used. Within the tissue, MAL is de-esterified into ALA by intracellular enzymes. MAL, being an ester, is more lipophilic than ALA. Thus, MAL is suggested to

penetrate more easily and deeper into the target lesion (Peng *et al.* 2001). MAL has also been shown to result in a more selective accumulation of PpIX in actinic keratosis and basal cell carcinoma (Uehlinger *et al.* 2000); however, a recent study implies that there is no significant difference in transdermal penetration of ALA and MAL in tumour tissue (Sandberg *et al.* 2008). The photosensitizers can also be administered intravenously and concentrated in tumour tissue when PDT is used in oncology and ophthalmology (Pass 1993).

Photosensitization using ALA

Almost all types of cells in the human body are able to synthesize heme. Figure 7 illustrates a schematic picture of the heme biosynthesis. ALA is formed from glycine and succinyl CoA under the influence of the enzyme ALA synthase. This step is regulated by the amount of heme in the cell by a negative feedback. At the end of the synthesis, iron is incorporated into PpIX by the enzyme ferrochelatase and heme is produced. The first and the last steps of the heme biosynthesis pathway take place in the mitochondria of the cell.



Figur 7: Schematic picture of the heme biosynthesis.

The formation of ALA and heme by the enzymes aminolaevulinic acid synthase and ferrochelatase are rate-limiting steps. Application of exogenous ALA bypasses the first rate-limiting reaction, which gives an accumulation of PpIX in the cells. Since PpIX is photoactive, this accumulation causes the photosensitization.

Despite the fact that sufficient amounts of ALA are present in both epidermis and dermis following topical application, the dermal cells do not develop significant PpIX levels to become photosensitized

(Divaris 1990).The preferential accumulation of PpIX in tumour cells is due to altered skin barrier (Gilmore *et al.* 2006, Moan *et al.* 2001) and enzymatic differences (Collaud *et al*. 2004) in the tumour compared with normal skin. Since the formation of PpIX in the heme synthesis is localised to the mitochondrial membrane, it has been shown that the cellular localisation of PpIX after ALA application is restricted to the mitochondria (Iinuma *et al.* 1994, Malik *et al.* 1996, Peng *et al.* 1992). Hence, the initial photodynamic damage will be localized to this organelle and the following apoptosis of the cells has been reported to occur within 10 hours (Webber *et al.* 1996).

Photophysics

Chromophores such as porphyrins absorb energy when excited by light of a certain wavelength. There are different absorption spectra for every chromophore. It was early discovered that a free-based porphyrin, e.g. PpIX, exhibits a four-band visible absorption spectrum. This is illustrated in Figure 8.

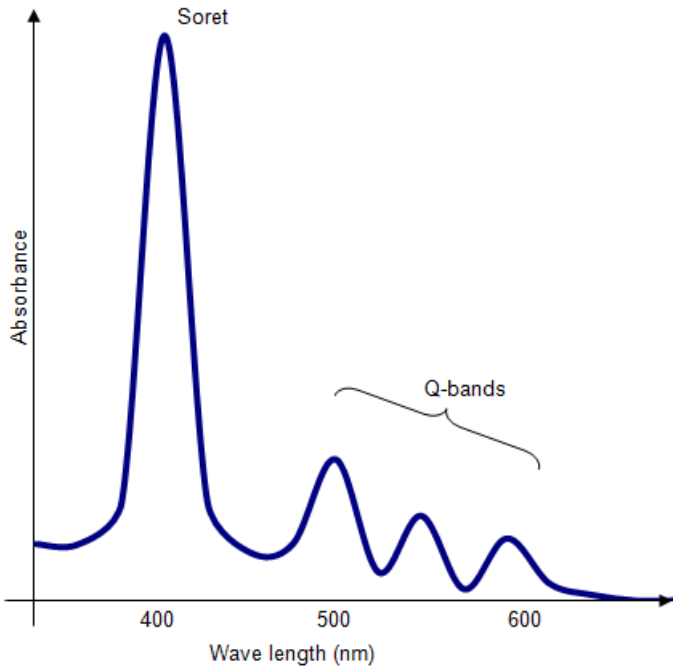


Figure 8: Absorption curve for protoporphyrin IX (PpIX), Soret Band.

The porphyrins generally have an intense absorption in the region around 400 nm, i.e. in the blue and ultraviolet range. This peak in the absorption spectrum is called the “Soret band“. Because the best absorption of porphyrins occurs when using blue light (405 nm), some light sources for PDT are designed to target this absorption peak.

Visible spectra of porphyrins also show several weaker absorption peaks, i.e. Q-bands, at longer wavelengths (450 to 700 nm). Since red light is preferentially used to deliver light deeper into tissue, also these absorption peaks are targeted in PDT – particularly the red peak at 630 nm, which despite this low absorption offers better light penetration into tissue.

Photobleaching

It has been observed that PpIX in solution exposed to light is degraded from a bright red appearance to brownish green, commonly referred to as photobleaching (Cox *et al.* 1982). This process is oxygen-mediated and has been identified as a self-sensitized photo-oxidative reaction producing different photoproducts. The photoproducts formed have been shown to absorb strongly around 670 nm (Dietel *et al.* 1995).

There is a correlation between the fluence-rate-dependent efficiency of the treatment and the oxygen content in tissue (Finlay *et al.* 2001, Boere *et al.* 2003). Higher fluence rates induce oxygen depletion, and hence the photobleaching process will be slowed down. Photobleaching is totally abolished during oxygen depletion (Ericson *et al.* 2003a). There is a direct relation between the bleaching rate and the epithelial damage (Boere *et al.* 2003). The photobleaching rate and treatment outcome are dependent on the fluence rate, where low fluence rates are preferable when performing PDT (Ericson *et al.* 2004).

Basic aspects of fluorescence

When a molecule absorbs a photon of a certain wavelength it becomes excited. This is an unstable condition and the molecule tries to return to the ground state, giving away the excess energy. For some molecules, it is favourable to emit the excess energy as light when transforming back to the ground state. This process is called fluorescence. The term 'fluorescence' was coined by George Gabriel Stokes in a publication in 1852 (James 1983). The name was given as a description of the essence of the mineral fluorite, composed of calcium fluoride, which gave a visible emission when illuminated with "invisible radiation" (UV radiation).

The peak wavelength of the emitted fluorescence is shifted towards longer wavelengths compared to the absorption wavelengths due to loss of energy in the conversion process. This is called the Stokes shift and is illustrated in Figure 9.

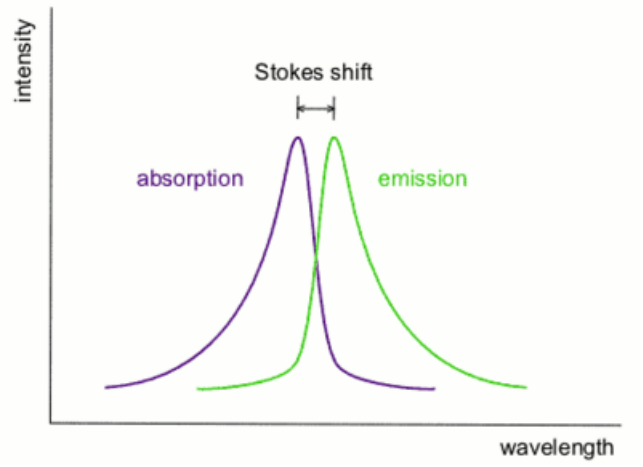


Figure 9: Schematic picture of Stokes shift.

Light sources for PDT

Different light sources are used for PDT at the clinics, which will be reviewed below.

Broadband light. Filtered non-coherent broadband lamps have proven to be very useful, particularly for ALA-PDT of superficial skin lesions (Wennberg *et al.* 1996). One of the most positive aspects of these filtered lamps for PDT is that they are inexpensive and easy to handle, but the wavelength bands are usually quite broad. This might even be of advantage when used as a light source for PDT, since photosensitizing photoproducts with other absorption peaks are formed during PDT and this type of lamp could cover their spectrum (Gudgin Dickson and Pottier 1995), although in later studies no difference in efficacy has been seen when comparing broadband and laser light sources (Clark *et al.* 2003).

Light-emitting diodes (LEDs). The wavelength band is narrower compared to filtered lamps. The first LEDs had problems with low intensity, but later generations of LEDs provide sufficient intensity. This light source is preferred in clinical practice since it is very compact and cheap.

Lasers. Lasers have the advantage of producing monochromatic light, which can exactly match an absorption band of the sensitizer in the area of the spectrum where the penetration in tissue is the greatest. One disadvantage is that the area of irradiation is limited, so that the beam has to be scanned. Furthermore, lasers are generally expensive.

Adverse events of PDT

The normal reactions after topical PDT are local erythema and oedema, usually followed by superficial exfoliation and crusting which will heal in 2 to 6 weeks (Morton *et al.* 2004). The most important adverse event (AE) is pain, which has been reported as a burning, stinging or prickling sensation during illumination. The pain arises within a few seconds to a minute after commencing illumination. It is believed to be a consequence of the photochemical reaction in the tissue, although the mechanism is not yet fully understood. There have been several attempts to reduce the pain associated with PDT (Wang 1999, Pagliaro *et al.* 2004, Sandberg *et al.* 2006). The following

recommendations have been suggested: cold fan, ice water and treating smaller areas at a time. Recently nerve blocks have proved to be effective in reducing the pain during PDT (Paoli *et al.* 2008). MAL-PDT has been shown to be less painful than ALA-PDT in normal skin (Wiegell et al. 2003) and in actinic keratoses (Kasche *et al*. 2006)**.**

Photoactivation of bacteria

Photoinactivation of Gram-positive and Gram-negative bacteria is based on the accumulation of photosensitizers in significant amounts in or at the cytoplasmic membrane, the critical target for inducing irreversible damage to bacteria. In recent years it has been shown that there is a difference in susceptibility to antibacterial PDT between Gram-positive and Gram-negative bacteria (Minnock *et al.* 1996, Nitzan *et al.* 1992). It has been established that Gram-positive bacteria are very sensitive to the photosensitizing action of anionic or neutral photosensitizers absorbing visible light. However, Gram-negative bacteria show a remarkable resistance to antimicrobial PDT (Malik *et al.* 1990, Bertoloni *et al.* 1984, Bertoloni *et al.* 1993). Below, the photoactivation of Gram-positive *P.acnes* will be discussed.

Photoactivation of P.acnes

P. acnes produce small amounts of porphyrins, mostly coproporphyrin III (Cp III) and uroporphyrin III (Up III) (Ramstad *et al.* 1997, Romiti *et al.* 2000, Johnsson *et al.* 1987, Kjeldstad *et al.* 1984 b). Due to this porphyrin production, clusters of *P. acnes* can be seen as fluorescent spots on the skin when irradiated with broadband near-UV light, also called Woods light (McGinley *et al.* 1980, Melo *et al.* 1982). The porphyrin production seems to be influenced by pH (Kjeldstad *et al.* 1984 b).

In vitro irradiation of *P.acnes* colonies with visible blue light leads to photoexcitation of the endogenous bacterial porphyrins. Subsequently, singlet oxygen is produced which results in bacterial destruction (Arakane *et al.* 1996). Ashkenazi *et al*. (2003) could show that *P.acnes* was eradicated by the endogenic porphyrins after illumination with intense blue light at 407-420 nm and a better photodynamic effect was obtained when cultures were illuminated two or three times. In addition, when ALA was added to the cultures before illumination with blue light, the viability of the cultures decreased by seven orders of magnitude (Ashkenazi *et al.* 2003). In contrast, Guffey *et al.* 2006 could not prove any bactericidal effect with 405- or 470-nm light.

ALA and MAL can also be taken up by *P.acnes* and result in an increased concentration of endogenous porphyrins. In some colonies a predominance of PpIX can be observed (Kjeldstad *et al*. 1984); in others the porphyrin pattern includes mostly CpIII and UpIII (Ramstad *et al.* 1997) when *P.acnes* is incubated with ALA or MAL. This formation of porphyrins further increases at higher temperatures (Ramstad *et al.* 2006).

Light therapy and Acne Vulgaris

Sun exposure is reported to have a beneficial effect on acne by up to 70% of the patients (Cunliffe 1989). UV radiation gives the beneficial camouflage of erythema by pigmentation, but it is also likely that other biological effects on the pilosebaceous system are responsible for the therapeutic effect. Sunlight has been reported to have an anti-inflammatory action in acne, possibly by its effect on follicular Langerhans cells (Cunliffe *et al.* 2000). *In vitro* experiments have shown that *P.acnes* can be

inactivated by relatively low doses of broadband near-UV radiation; this phenomenon was found to be oxygen-dependent. There are reports that UV radiation can also induce changes in surface lipids and subsequently enhance comedogenesis (Mills *et al.* 1978). Even if UVB has the capacity to kill *P.acnes in vitro,* this seems clinically irrelevant since its ability to penetrate the skin is low and only high doses resulting in sunburn have been shown to induce improvement (Kjellstad *et al.* 1986, Sigurdsson *et al.* 1997). UV radiation may also have an anti-inflammatory effect and may induce changes in comedonal cytokines (interleukin IL-10, IL-1 receptor antagonist) in patients with acne (Suh *et al.* 2002). The efficacy of UV radiation with *in vitro* experiments has not been supported by clinical improvements *in vivo* studies.

In addition to UV-light, visible light alone has been shown to have positive effects on acne. In order to define the most effective wavelengths for treating acne with visible light, Sigurdsson *et al.* (1997) found that all "full-spectrum" green and violet light sources improved acne, but there was a tendency for violet light to be more effective.

Topical PDT and Acne Vulgaris

Light therapies and topical PDT are proposed as alternatives to traditional acne treatments for patients with recalcitrant acne or those who are intolerant to side effects (Pollock *et al.* 2004). Hongcharu and colleagues (Hongcharu *et al.* 2000) laid the foundation for the use of ALA-PDT in the treatment of acne. In their landmark study in the year 2000, the authors reported statistically significant clearance of acne for 10 weeks after a single treatment and for 20 weeks after four treatments. The authors suggested a mechanism of ALA-PDT by showing that sebum excretion and bacterial fluorescence were decreased, and sebaceous glands were damaged.

These encouraging results stimulated other investigators to further explore the use of ALA-PDT for acne. The mechanism was further investigated by Pollock *et al.* (2004). They used red laser light for photoactivation; however, they found contrasting results, i.e. no reduction in the population of skin surface *P.acnes* and no reduction in sebum excretion. Recent studies have shown the efficacy of MAL-PDT for moderate to severe inflammatory Acne Vulgaris (Wiegell and Wulf 2006a)**.** In a study comparing ALA- and MAL-PDT, Wiegell and colleagues found no differences in the response rate between the two treatments, although ALA-PDT resulted in more prolonged and severe AEs after treatment (Wiegell and Wulf 2006b).

The postulated mechanism of photodynamic therapy of acne is the photodestruction of *P.acnes* (see "Photoactivation of *P.acnes*" previous page). In addition to the natural porphyrins produced by *P.acnes*, externally applied compounds such us ALA or MAL seem to have the ability to selectively accumulate in sebaceous glands, causing a selective photosensitization. A study in mice has indicated that ALA is preferentially taken up by the pilosebaceous unit (Divaris *et al.* 1990). Some of the factors that influence *P.acnes'* photoinactivation are the concentration of porphyrins and the wavelength of the light. The action spectrum for the inactivation of *P.acnes* is highest for shorter wavelengths and decreases with increasing wavelength (Kjellstad 1987). The most common treatment protocols for light treatment of acne are based on either red or blue light.

Red Light Although it has a lower extinction coefficient, red light has better penetration in the skin than blue light. Red light may also have anti-inflammatory properties by influencing cytokinase

release from macrophages (Young *et al.* 1989), and red light phototherapy has been shown effective for mild to moderate Acne Vulgaris when performed twice a day for 8 weeks (Na *et al.* 2007)**.**

Blue Light Theoretically blue light has the most effective wavelength for photoactivation of f *P.acnes*, but blue light has the disadvantage of poor penetration (<2mm) in the skin (Moan J *et al.* 1996). In the clinic the practitioners must sacrifice penetration depth for absorption efficiency and vice versa. There are studies showing a therapeutic effect of blue light in the treatment of Acne Vulgaris (Morton *et al.* 2005, Tremblay *et al.* 2006, Eldman *et al.* 2003).

Combination of Blue and Red light. Phototherapy with mixed blue (450 nm) and red light (660nm) has been proposed to be more effective than blue light alone for treating mild to moderate acne, probably by combining antibacterial and anti-inflammatory actions (Papageorgiou *et al.* 2000, Sadick 2008). No significant short-term AEs were observed**.**

There has been an explosion of new information with regard to light- and laser-assisted PDT of acne (Ross 2005, Ortiz *et al.* 2005, Alexiades-Armenakas 2006, Haedersdal *et al.* 2008a); however, different wavelengths, light doses, and number of treatments as well as varying follow-up times make it difficult to judge which regime is the most effective (Pollock *et al.* 2004, Hongcharu *et al.* 2000, Itoh *et al.* 2000). Evidence from controlled clinical trials indicates a short-term efficacy from phototherapy with the most consistent outcome for PDT (Haedersdal *et al.* 2008b), but the most effective protocol for phototherapy or PDT of acne is not yet established.

Adverse events of PDT and Acne Vulgaris

AEs of PDT when treating acne are generally the same as described earlier in "Adverse events of PDT", page 14.

Fluorescence Diagnostics

Imaging spectroscopy using digital cameras can be used to study fluorescence from tissue. The major advantage of imaging compared to spectral point measurements is that spatial information about skin can be obtained (Svanberg *et al.* 1998, Peng *et al.* 1995, Heyerdahl *et al.* 1997, Moan *et al.* 1998**).**

Autofluorescence is the fluorescence obtained from tissue without presence of an external photosensitizer or other fluorescent markers. There are several studies reporting on the use of autofluorescence for demarcating between normal and neoplastic tissue, e.g. for diagnostics of cancer of cervix uteri, colon and skin cancer (Lohmann *et al.* 1989, Richards-Kortum *et al.* 1991**,** Na *et al.* 2001, Stenquist *et al.* 2006)**.**

Induced fluorophores, i.e. ALA- or MAL-induced fluorescence: this is the fluorescence obtained when ALA or MAL is topically applied to the tissue and the cells accumulate PpIX, as described earlier.

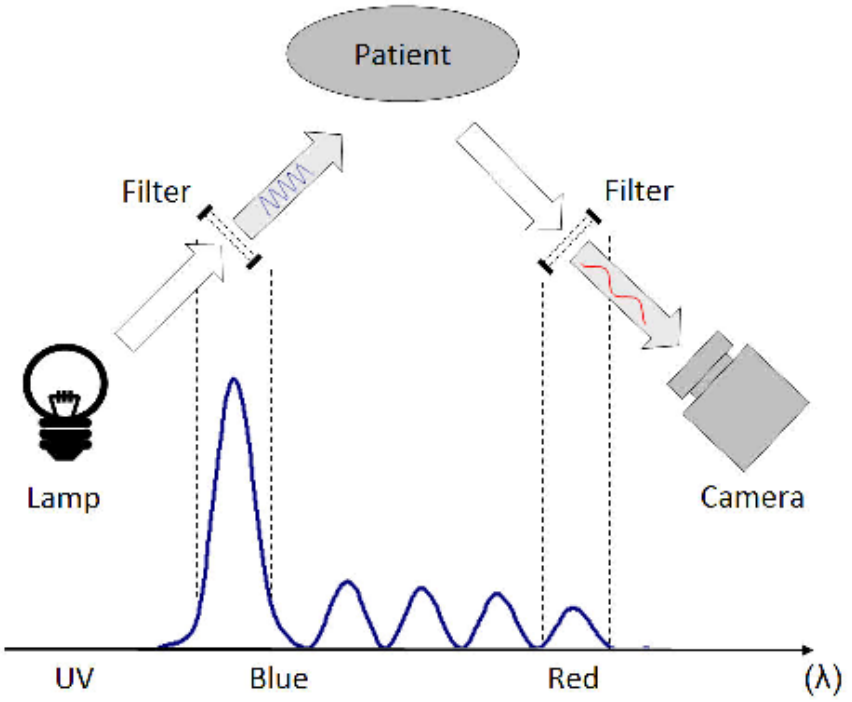


Figure 10: Schematic set-up of fluorescence imaging.

The equipment needed for fluorescence imaging is a light source for excitation, a digital camera for detection and software for data processing, as illustrated in Figure 10. The PpIX fluorescence can serve as a marker for neoplastic tissue. This method is used in the clinic for demarcation of skin tumours, so-called fluorescence diagnostics. The Pp IX absorbs light at 405 nm and fluoresces in the red region around 630 nm. The selectivity of ALA fluorescence has been shown to be dependent on the time after application (Ericson *et al*. 2003 b). Studies have shown that MAL is more selective against abnormal skin lesions than ALA (Fritsch *et al.* 1998, Peng *et al.* 2001)**.**

Fluorescence of Acne Vulgaris

Due to the presence of endogenous porphyrins, colonies of *P.acnes* bacteria are observed as fluorescent spots on the skin when irradiated with near-UV light, which is illustrated in Figure 11.

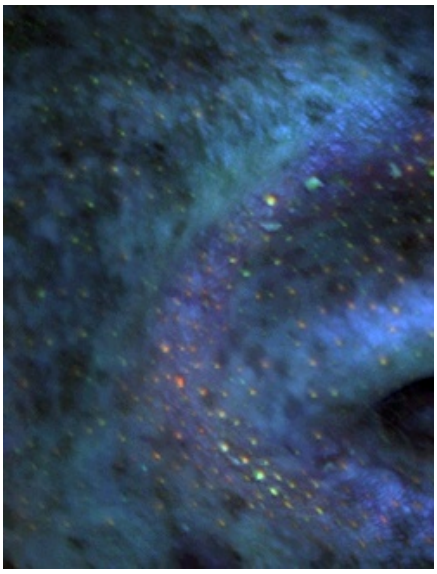


Figure 11: Autofluorescence of the side of a nose. Image provided by M.B. Ericson.

Few studies have been published concerning MAL-PDT and fluorescence of Acne Vulgaris. In one of those available, a correlation between inflammatory acne lesions and high PpIX fluorescence was found (Wiegell and Wulf 2006a). This indicates that inflammation may lead to a high uptake of MAL. Another study comparing ALA versus MAL showed a significantly higher and more uniform PpIX fluorescence when ALA was used, compared to MAL (Wiegell and Wulf 2006b).

AIMS OF THE INVESTIGATION

- To investigate the efficacy and tolerability of ALA and MAL-PDT for moderate to severe inflammatory facial acne *in vivo*.
- To determine the optimal light dose for effective ALA-PDT of acne.
- To investigate whether ALA-PDT or MAL-PDT reduces sebum excretion and the amount of *P.acnes* at treatment locations *in vivo*.
- To study the efficacy of single low light dose PDT using MAL for moderate to severe facial acne compared to treatment using light only *in vivo*.
- To evaluate fluorescence images and skin surface biopsies before and after MAL-PDT.
- To assess the photoactivation of *P.acnes* after incubation with ALA or MAL and irradiation with red or blue light *in vitro*.
- To monitor porphyrin production by fluorescence measurements and viable counting of *P.acnes* before and after illumination.

PATIENTS

Paper I

A placebo-controlled, prospective blinded study including thirty patients (25 males and 5 females, age range 15 -28 years, mean age 18), with moderate to severe facial acne (Leeds score 5-10) were investigated during a ten-month period in two centres in Sweden (male patients only) and one centre in Norway (males and females). Lesions were counted in the whole face except on the nose. Acne treatment was discontinued up to 3 months before the study.

Paper II

A dose-controlled, prospective non-blinded study including fifteen patients (9 males and 6 females, age range 16-44 years, mean age 25), with mild to severe acne grade I-IV according to Pillsbury, were investigated during a ten-month period at one clinic. The acne lesions were localized either to the cheeks (10 patients) or to the back of the patients (5 patients). Lesions were counted within a 10 cm circular area. Acne treatment was discontinued 2 months prior to PDT.

Paper III

A placebo-controlled, prospective non-blinded study including twenty-three patients (9 males and 14 females, age range 19- 37 years, mean age 18) with moderate to severe facial acne, grade II–III according to Pillsbury, were investigated during a seven-month period in one clinic. The acne lesions were counted within a circular area defined by a 10 cm template. All oral acne treatments were discontinued for 3 months and local treatments for at least 4 weeks prior to study. Patients were asked not to wash or use any emollient during the last 12 hours before each visit.

Paper IV

In vitro study and no patients were included.

Ethics

In paper I the study was approved by the ethics committee responsible for each participating centre. Papers II and III were approved by the local ethics committee. In paper IV no approval from the ethics committee was needed.

METHODS

Paper I

MAL treatment

MAL cream 160 mg/g (Metvix® PhotoCure ASA) and matching placebo cream were applied evenly on each side of the face (above the jaw line) in a 1 mm thick layer excluding the nose and a 1 cm periocular area. The application site was covered with an adhesive occlusive dressing. After 3 hours the cream was wiped off from both sides of the face immediately before illumination. Nodular or cystic lesions were prepared using a cannula (1-2 mm) to facilitate cream penetration.

Illumination

An Aktilite© CL 128 lamp (average wavelength 635 nm, light dose 37J/cm^2, 34 mW/cm^2) with non-coherent red light was used. The side of the face not receiving illumination was covered when the other side was illuminated. Patients received a second PDT after 2 weeks.

Pain during treatment was monitored by visual analogue scale (VAS).

Follow-up

Patients were followed up 4 and 10 weeks after the last PDT treatment.

Paper II

ALA treatment

ALA cream (20% in Unguentum Merck®) was applied in a 1 mm thick layer on two circular areas on cheek or back, using a template so that each area could be accurately located at subsequent visits. No preparation of the lesions was done. The application site was covered with an adhesive occlusive dressing. After 3 hours the cream was wiped off immediately before illumination.

Illumination

A Waldman PDT 1200 lamp providing non-coherent red light (wavelength 600-730 nm) was used. Both areas of investigation, i.e. on the back and on the cheeks, were irradiated. The cheek not receiving illumination or the surrounding skin on the back was covered when the other side was illuminated. Patients received only one PDT treatment.

Ten patients were treated with a light dose of 50 J/cm² or 30 J/cm² (fluence rate 50 mW/cm² or 30 mW/cm²) on the right and left cheek respectively. Five patients were treated in two areas on their backs with a light dose of 50 J/cm² or 70 J/cm² (fluence rate 50 mW/cm² or 70 mW/cm²) on the different locations.

Follow-up
The patients with facial acne were followed up after 1, 2, 3, and 10 weeks. The patients treated on their backs were in addition seen after 20 weeks.

Sebum measurement
The sebum excretion was registered in the patients with acne on their cheeks before PDT and at every follow-up. A sample was taken from the same spot 3 cm lateral to the alar rim on both cheeks at every visit. Patients were informed not to wash or use any emollient during 12 h before each visit. The sebum measurements were performed by briefly pressing a plastic film against the skin for 30 s. The samples were then immediately measured photometrically using a Sebumeter Combi (SM810/CM825/PH900). The sebum content is expressed in μg sebum/cm^2.

Counts of P. acnes
The amount of *P. acnes* was measured with the method of Mills and Kligman (1983). A skin surface biopsy was taken by using a quick-setting cyanoacrylate polymer to extract the content of the sebaceous follicles. Microcomedones were cut out of the glass slide with a sterile scalpel and isolated by homogenising the samples in Triton X. Samples were then cultured anaerobically and colonies of *P. acnes* were counted.

Paper III

MAL treatment
Each patient was assigned MAL (Metvix®, PhotoCure ASA) on the right cheek while the left cheek served as control, i.e. only being treated with red light. No preparation of the lesions was done. MAL was applied in a 1 mm thick layer on a circular area on the right cheek, using a template so that the area could be accurately located at subsequent visits. The application site was covered with an adhesive occlusive dressing. After 3 hours the cream was wiped off immediately before illumination.

Illumination
The illumination was performed using non-coherent red light from an Aktilite® CL 128 lamp (average wavelength 635 nm, light dose 15 J/cm^2, 63 mW/cm^2). The right and the left cheek were treated sequentially, while covering the side of the face not receiving illumination. All patients had only one PDT session.

Follow-up
The patients were followed up 1, 10 and 20 weeks after treatment.

Fluorescence imaging
Both cheeks were photographed prior to treatment and fluorescence images were obtained before application of MAL, after incubation with MAL, after illumination and at follow-up visits. Fluorescence imaging was performed using a fluorescence imaging device, Photo Demarcation System 1, Prototype 5. See Fig 10, page 27. The equipment consists of Hg lamps for fluorescence

excitation, 365 ± 5 nm and 405 ± 5 nm, and a filtered CCD (charge-coupled device) camera to record the fluorescence between 610-700 nm, corresponding to the PpIX emission around 635 nm. The size of the images was 512 × 446 pixels with 12 bits grey level resolution.

Sebum measurement

The sebum measurement was performed by the method described in Paper II.

Counts of P. acnes

The amount of *P. acnes* in the skin was determined using a method introduced by Mills and Kligman (1983). This method is described in Paper II.

Paper IV

P. acnes

P.acnes (ATCC 6919) was grown anaerobically on blood-agar plates at 37˚C. Bacterial suspensions were diluted until McFarland was 5, approximately 1.5 x 10^9 cells/ml. The bacteria were suspended in a medium to the final concentration 1.5 x 10^8 cells/ml and incubated for 5 days. Some of the *P.acnes* suspensions were centrifuged to separate the bacteria from the medium, and spectra were obtained from these samples. The survival of *P. acnes* following illumination was monitored by viable counting. This was performed by counting the number of colony-forming units after appropriate dilution on blood agar plates and calculating their number per ml.

MAL and ALA treatment

Stock solutions of 5 mg/ml of ALA and MAL in sterile distilled water were freshly prepared and immediately diluted to a concentration of 100 µg/ml before being added to the bacterial suspension. The bacteria were incubated with or without either ALA or MAL for 5 days.

Illumination

Illumination of the bacteria suspension took place after 5 days incubation with ALA or MAL. Illumination with blue light, 405 nm ± 10 nm, was performed using a LED lamp. The light was delivered at 50 Hz in msec pulses, having a pulse energy of 100 µJ/cm^2. The average light fluence rate was 5 mW/cm^2, and an accumulated light dose of 7.5 J/cm^2 was obtained after 25 min of illumination. The illuminated area was 10 x 20 cm. The illumination with red light was performed using a red LED lamp, having an average wavelength 635 nm, and a total light dose of 99 J/cm^2 was obtained after 23 minutes illumination according to manufacturer's specification.

Fluorescence spectra

The induction of PDT-relevant porphyrins in *P.acnes* was monitored *in vitro* using fluorescence measurements. The excitation was set to 405 nm and the fluorescence emission was monitored in the range of 410-700 nm using a Varian Eclipse spectrophotometer. After incubation with or without ALA or MAL for 1, 2, 4 and 5 days the emission spectra were obtained from the *P.acnes* suspensions.

Statistical Methods

In paper I the differences between MAL-PDT and Placebo-PDT in the change of lesion counts, improvement in global severity and pain score were analysed using the Wilcoxon signed rank test for one sample. A distribution-free method was used when calculating the reduction in inflammatory lesion counts. The investigator's global severity assessment was analysed using McNemar's test. All statistical tests were two-sided. In paper II the statistical analysis was carried out using ANOVA to compare the changes from baseline of acne lesion counts, sebum excretion and *P. acnes*. The Sign test was used to evaluate the effect of PDT from baseline of patients' assessment of their acne. In paper III the differences between MAL-PDT area and control area concerning the change in lesion counts, acne score, amount of *P.acnes*, and sebum content were analysed using Wilcoxon signed rank test for continuous variables and Sign test for ordinal variables. In paper IV the statistical analysis, t-tests and the nonparametric Mann-Whitney and Kruskal-Wallis tests were used.

RESULTS

Paper I

Follow-up

Three patients discontinued the study between the first and second PDT due to adverse events (two had moderate erythema, and one had moderate pain). Therefore 27 out of 30 patients completed the study.

Clinical evaluation

The numbers of inflammatory and non-inflammatory lesions were about the same in regions allocated to MAL-PDT or placebo PDT. The assessment was done by one investigator at each center and was blinded. Global severity assessment of acne was graded as moderate in 23 patients and severe in seven at baseline.

MAL-PDT was significantly more effective than placebo PDT in treating inflammatory lesions, as indicated by a greater median percent reduction in total inflammatory lesion counts at both week 6 (63% vs. 28%) and week 12 (54% vs. 20%). See Figure 1. Paper I.

The non-inflammatory lesions were reduced in both groups, although in this case no statistically significant difference between the groups was observed. The clinical success rate was significantly higher in the MAL-PDT group than in the placebo PDT group at week 12 (30% vs. 10%).

Adverse events

Adverse events were reported for 21 of the 30 patients (70%). The most frequently reported adverse event were pain, erythema and skin swelling. Twenty patients (67%) experienced local adverse events that were considered related to MAL-PDT. All but four local adverse events were of mild to moderate severity. All adverse events were resolved within 7 days, with the exception of two cases lasting a few days more. Patients' assessment of pain by VAS showed that treatment with MAL-PDT was associated with more pain than placebo PDT, although the intensity of the pain with MAL-PDT was reduced at the second treatment. Analysis of pain scores reported by patients at each centre showed that pain scores varied greatly across the centers (median 3.6, 5.0 and 6.9 respectively).

Paper II

Follow-up

In those patients with acne on their cheeks, one patient was lost for follow-up after 3 weeks due to moving from town. Therefore 9 out of 10 patients completed the study. In those patients with acne on their backs, two had to be excluded. One patient was lost for follow-up and the other was treated with isotretinoin because of severe acne on other localizations.

Clinical evaluation

Baseline acne severity was the same regardless of treatment location with different light doses in every patient according to Pillsbury grading. The assessment was done by one investigator and was not blinded. In the nine patients that completed the study there was no difference between 30 J/cm^2 and 50 J/cm^2 at the final evaluation. The improvement of acne lesions was the same for the two light doses. Eight out of nine patients were improved in their acne after PDT.

Also in the patients with acne on their backs, no difference was found between the two doses 50 J/cm^2 and 70 J/cm^2 at the final evaluation. The improvement of the acne lesions after PDT was the same independently of the light dose.

Adverse events

Hyperpigmentation was more common at higher doses of light, and pain was more often experienced when higher doses were used. Hyperpigmentation remained in 3 patients for 10 weeks. All other adverse events resolved after 3-10 days.

Assessment of sebum excretion rate and *P.acnes*

These investigations were only carried out in the 10 patients with acne on the cheeks. *P. acnes* measurements were only done on the right cheek (50 J/cm^2) and there was no significant reduction of *P.acnes* after PDT. The sebum excretion was measured on both cheeks and no significant reduction was seen after PDT.

Paper III

Follow-up

All but four patients attended the follow-up visits. In two of these four patients, the premature discontinuation was related to treatment failure. One patient had to discontinue the study due to military service, and another patient had an accident and required treatment with oral antibiotics. Thus 19 out of 23 patients completed the study.

Clinical evaluation

The patient and lesion characteristics at baseline were similar in both facial regions allocated to either MAL-PDT or serving as control (red light only). The assessment was done by one investigator and was not blinded. Based on the investigator's global severity assessment, acne was graded as moderate in 8 patients and severe in 15 patients at baseline. Significant reductions in acne score ($P<0.01$) were obtained at follow-up visits after 10 and 20 weeks for both MAL and control. No significant difference in acne score was observed between MAL and control sites after treatment. The numbers of papules and pustules were significantly reduced at the two follow-up visits at both the MAL and the control sites. The reduction in number of cysts observed at follow-up was only significant after 10 weeks. Also the number of comedones was found to be significantly reduced at both treatment locations (MAL and control), equally at 10 and 20 weeks.

The assessment of treatment outcome made by the patients themselves at the follow-up visit 20 weeks after treatment with MAL or control showed that a majority of the patients assessed that their acne had improved on both the MAL-treated and control sides, 15/19 and 14/19 respectively.

Adverse events

Erythema was observed in all patients except in one after one week, all being related to the treatment of MAL-PDT. Four patients had more serious erythema and one had serious exfoliation. In 4 of these patients the fluorescence images revealed an apparent photobleaching. Two patients got a hyperpigmentation, which was resolved after 3 months for one patient and after 5 months for the other. Patients experienced moderate to severe pain only on the MAL-treated site.

Assessment of sebum excretion rate and *P.acnes*

Neither the sebum excretion rate nor the amount of *P.acnes* decreased significantly after either treatment protocol.

Evaluation of fluorescence images

Fluorescence images, obtained at baseline and before treatment procedures, showed no significant correlations between the number of highly fluorescent pores (Figure 12), acne severity, and the number of lesions or the amount of *P.acnes.*

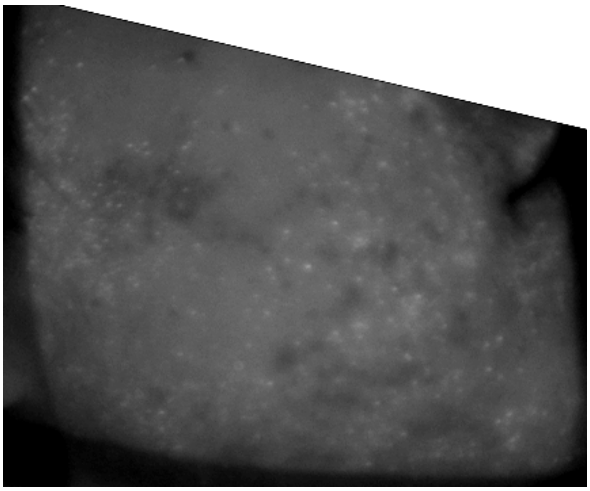


Figure 12: Fluorescence image illustrating presence of highly fluorescent pores on the cheek of one patient with severe acne before treatment.

The fluorescence images showed a lack of selectivity for MAL to acne lesions. Larger and more inflammatory lesions were observed to exhibit a higher fluorescence. After the treatment with red light only, the number of highly fluorescent pores was assessed, as presented in Figure 13.

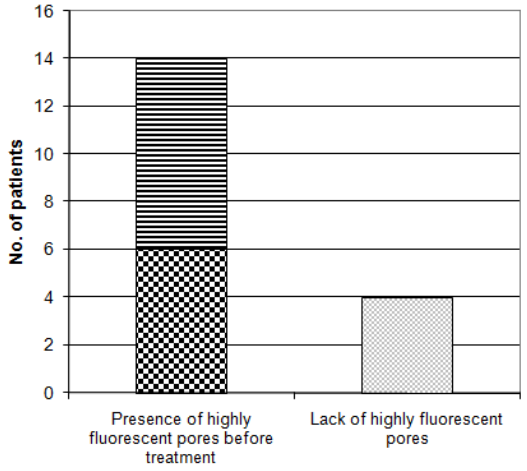


Figure 13: Assessment of number of highly fluorescent pores present in fluorescence images of the treatment area exposed to red light only, before and after treatment.

No correlation between the decreased number of highly fluorescent pores and treatment efficacy was found. Apparent photobleaching of MAL fluorescence after irradiation was present in 13 of 18 patients, as illustrated in Figure 14. No correlation between photobleaching and improvement in acne score was observed on the MAL-treated location.

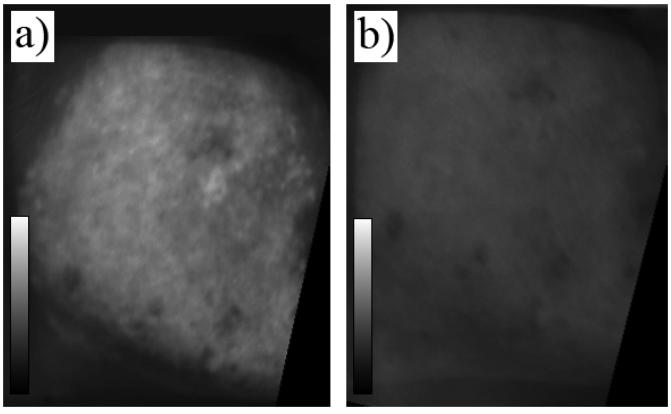


Figure 14: Fluorescence image of MAL-treated side before (a) and after (b) irradiation.

Paper IV

P.acnes without MAL and ALA treatment

No significant porphyrin peaks were observed in bacteria cultured without ALA or MAL. *P.acnes* count without supplementation of ALA or MAL decreased only slightly after illumination with blue light, i.e. one order of magnitude. No significant effect of red light was found. Two illuminations gave only a slightly better effect than one.

P.acnes with MAL and ALA treatment

Almost zero amounts of porphyrins were detected using fluorescence measurements at the onset of the experiment. Characteristic porphyrin peaks were visible in the spectra after 4 to 5 days incubation with ALA or MAL. In general, bacteria suspensions incubated with ALA induced higher porphyrin levels than bacteria with MAL. ALA- and MAL-triggered cultures of *P.acnes* exhibited a better inactivation pattern upon illumination with both red and blue light. *P.acnes* incubated with ALA or MAL showed a significant decrease in *P.acnes* viable count after one illumination and more after two illuminations ($P<0.0005$). The viability of the cultures was reduced by four orders of magnitude after one illumination with blue and red light incubated with ALA or MAL. No significant differences between red light at the dose 99 J/cm^2 and blue light at the dose 7.5 J/cm^2 were found. Two consecutive illuminations at an interval of 24h between treatments caused a decrease in the viable count of the culture by five or more magnitudes. No significant difference between ALA and MAL was found, nor between red and blue light.

Evaluation of fluorescence spectra

Porphyrin amounts were found to increase as a function of growth time. Spectral peaks were found at about 580 nm and 620 nm, and a small shoulder at about 635 nm. The porphyrin peak at about 580 nm (metalloporphyrins) was higher in the supernatant, and the 620 nm peak (CpIII) was higher in the bacteria.

DISCUSSION

Methodological considerations

Paper I

The inclusion criteria were defined as moderate to severe facial acne, Leeds score 5-10. This acne score is one of the most accepted for grading acne, but still it is not a precise scale and the judgment could differ between the three investigators when comparing patients' acne with images of different acne scores.

Patients' acne lesions on each side of the face were counted before the first PDT and at follow-up 4 and 10 weeks after the last treatment. It is not always obvious how to grade an acne lesion appropriately as papule, pustule, nodule/cyst or comedone. The results might have been more reliable with two independent investigators at each center.

The patients were enrolled between October 2004 and May 2005, summer excepted, which could have affected their acne status. There was no control of whether the patients were tanning from sun exposure or using sun beds. The acne patients were asked to quit their acne treatment 3 months before the study, but patient compliance was not monitored.

Each patient was randomly assigned to placebo and MAL cream, to be applied to either side of the face. This was done in a double-blinded manner for both patient and investigator. The MAL cream and placebo cream were similar as to colour and consistency. However, an experienced observer could tell the difference since the MAL cream gave obvious side effects such as pain shortly after onset of illumination.

Split face studies involving light are difficult to assess, as it is difficult to control the effect of cytokines which may be released but not necessarily localized to the treated area only. Thus a systemic immunological effect related to a reaction at the MAL-PDT side could improve the acne at the other side of the face.

Nodular or cystic lesions were prepared using a cannula (1-2 mm) to facilitate cream penetration, and in some patients a small bleeding occurred. This could theoretically decrease the light reaching into the lesion and the photodynamic reaction would be less effective.

Patients' assessment of pain by VAS varied greatly across the centers. None of the patients had previous experience of PDT. Information given to the patients by study personnel is therefore likely to have affected patients' perception of the treatment.

Papers II and III

The scoring of acne in papers II and III was according to Pillsbury grade, Table 1, page 4, used at our department routinely. The Leeds score might have been better to use. However, we chose this scoring because the Leeds score would be too detailed in these small groups of patients.

In both studies there were several dropouts. The reasons for leaving the study were different, but patients with acne belong to that age when life changes a lot. They move to other cities for studies, jobs, military duty etc. A number of the patients had so many things going on in their lives that they had problems with attending the follow-up visits. One has to consider that it could be a selection of patients who dropped out from the study, but one does not know if they are the good or the bad responders to treatment.

The number of patients in both studies was limited. This causes a risk of introducing a type 2 error, i.e. a risk of underestimating treatment outcome and thereby missing the difference between the interventions.

The split face design was not randomized, but the blinding would have been revealed because of the obvious side effects of MAL-PDT. In these split face studies the systemic effect cannot be eliminated, as mentioned in paper I. The study design was chosen because of the limited number of patients. A different study design may yield different results.

These clinical trials were designed with uncontrolled before–after design, which does not take the intrinsic volatility of acne into account. In addition, longer follow-up periods might have been preferable.

The sebum excretion rate did not decrease significantly following ALA or MAL-PDT. It is possible that the sebumeter (Combi SM 810) might not have been as sensitive as other techniques. However, the tool has been used at our clinic earlier with good results, which is the reason why we chose this technique.

The amount of *P.acnes* did not decrease significantly after PDT. It is possible that the sampling method was inadequate, making it difficult to detect changes in *P.acnes.* When using the skin surface biopsy method, *P.acnes* levels are measured at the surface of the skin, and it might be argued that samples from the pilosebaceous ducts would produce a different result. Another possible explanation for the observed lack of effect on *P.acnes* could be that PDT, rather than destroying the bacteria, only causes damage to the bacteria. Subsequently, when the *P.acnes* are cultured in optimal conditions they recover and start to grow normally. The most likely explanation, however, is that even though *P.acnes* might be destroyed by the treatment, the pilosebaceous glands are recolonized by bacteria between measurements.

Paper III

The wash-out period was 4 weeks for systemic antibiotics and 2 weeks for topical treatments. This short period was used because we wanted to include as many patients as possible and patients were eager to receive treatment. None of the patients had been on isotretinoin during the last year before inclusion.

In some cases there was too much background light in the room when the fluorescence images were taken. The window shield was leaking light. When all the images were analyzed, several fluorescence images were overexposed and had to be excluded. The fluorescence images were limited in number and hence there could be a risk of introducing a type 2 error, i.e. not finding the correlation between photobleaching and improvement.

Paper IV

No significant porphyrin peaks were observed in the fluorescence emission spectra of the bacteria without addition of ALA or MAL. Detection limits of the fluorescence spectrophotometer might be one explanation. It is known that autofluorescence can be detected from the porphyrins in a pilosebaceous-rich skin. It is likely that sebum contains some components that are necessary for the porphyrin production. *P.acnes in vivo* are incubated in the sebum for longer time than 5 days and replaced with new bacteria in the skin.

Two illuminations gave only a slightly better effect than one illumination. This is most likely explained by a depletion of porphyrins after the first illumination. Even though porphyrins might have been present during the second illumination, the amounts were expected to be lower. The bacterial suspension we used had pH 6.95. It is possible that a lower pH might have given a greater concentration of porphyrins, since the highest production of porphyrins has been shown to be at pH 6.1.

The peak at 580 nm has been ascribed to metalloporphyrins, which are likely to be formed from free-based porphyrins and metal ions, e.g. Zn, present in the culture medium.

GENERAL DISCUSSION

Acne Vulgaris is the most common skin disease to affect younger humans. Different therapeutic approaches have been introduced into clinical practice. Antibiotics are considered to be the mainstay for treatment of acne, although their success rate varies considerably, due to patient compliance and the increasing antibiotic resistance of *P. acnes*. The emergence of bacterial resistance is a potential problem associated with prolonged antimicrobial use. The excessive use of tetracyclines is also an environmental concern since they remain in nature for a long time before they are eliminated.

The World Health Organization voted in 1998 on a resolution to classify antimicrobial resistance as one of the major threats against human health. During the last decades only a few new antibiotics have reached the market. Furthermore, the many promises of biotechnology and genetics fail to deliver new effective antibiotics. In this context PDT may be an interesting alternative. The renewed interest in antimicrobial PDT originates from two main factors: first, the promising results obtained by PDT in the fields of treatment of skin cancer for which it is now used in routine clinical practice, and secondly, the need for new antimicrobial therapies. An important advantage of ALA and MAL is that they do not pose an environmental risk with limited stability in biological tissues and they are readily biodegradable.

The treatment for severe acne vulgaris is isotretinoin which is the most effective treatment, with 80–90% reduction of acne lesions described earlier in "Treatments of Acne Vulgaris", page 9. MAL-PDT is not as effective as systemic isotretinoin. The acne severity of the patients treated with PDT is less severe and they do not fulfil the criteria for isotretionoin. Instead moderate to severe acne vulgaris is often treated with systemic antibiotics. Different investigators have found a reduction of 45–66 % of inflammatory lesions after 12 weeks of treatment with standard antibiotic therapy (Cunliffe *et al.* 2003, Dubertret *et al.* 2003, Bossuyt *et al.* 2003, Dreno *et al.* 2001, Grosshans *et al.* 1998). We found that the reduction in inflammatory lesions after 6 weeks was 63% and after 12 weeks 54% using MAL-PDT. Our results show that PDT is as effective as oral antibiotics.

Optimal treatment depends on accurate assessment of disease severity and the ability to characterize the specific lesions. To date, most research and clinical practice has relied on visual assessment by the clinician and standard photography. Several investigators, most notably Cunliffe and Burk 1984, have attempted to standardize the assessment process through rating scales; however, these scales are dependent on clinical visualization, with a high degree of subjectivity. Furthermore, the quality of photography can vary greatly.

Fluorescence imaging could be a complement in assessment of acne. Visible fluorescent spots correspond to presence of porphyrins produced by *P. acnes*. Some authors report on the use of fluorescence as an accurate assessment of bacterial presence on the skin. We could not find any correlation between the number of highly fluorescent pores, acne severity and the number of acne lesions or the amount of *P.acnes.* There was no correlation between the decreased number of highly fluorescent pores and treatment efficacy. According to our results the specific quantitative measurement of the amount of *P.acnes* on the skin is more likely to be accurate than the indirect measurement using fluorescence. On the other hand, fluorescence can be used to monitor

photosensitization rather than degree of acne. In Paper III, a homogeneously high PpIX fluorescence was found throughout the treatment area in a majority of the fluorescence images. Selectivity toward inflammatory acne lesions was found in only one third of the images. This observation questions the theory that the sebaceous glands are selectively treated when performing topical PDT**.** We also found that there seems to be a relation between the photobleaching and the photodynamic reaction when performing MAL-PDT. In paper III, four of the patients with apparent photobleaching in the MAL-treated area also reported serious local AEs for the corresponding treatment location.

Pain has been recognized as the major side effect of PDT. This was also one of the dominant side effects reported in the clinical studies included in this thesis. In paper I, the pain scores were found to vary across the different centers. This is probably due to the fact that a tolerance to pain can develop and that the information to the patients from the study personnel differs. Furthermore, we found that the intensity of pain was reduced at the second MAL PDT. The same result has been found by Wiegell *et al.* (2008), showing significantly more pain during the first treatment compared with the second treatment. This was explained by the significantly lower PpIX fluorescence at the second treatment. In addition, we found in paper II that a lower light dose was less painful using ALA-PDT, although the effect was as good as for the higher dose. Also these results are in agreement with the findings of Wiegell *et al*. (2008).

Since the amount of *P.acnes* was unaffected after PDT, as revealed in papers II and III, we decided to study this bacterium *in vitro*. The results from paper IV show that the amounts of endogenously produced porphyrins from *P.acnes* are insignificant to give a photodynamic effect. It was also shown that an incubation time of 3 hours with ALA or MAL is too short to raise the porphyrin levels of *P.acnes in vitro*. Instead, incubation for 5 days was necessary to obtain a photodynamic effect. In addition, the number of bacteria in acne skin is several orders of magnitude lower than the initial bacteria amounts in this *in vitro* study. Thus, photoactivation of *P.acnes* cannot be the sole mechanism of PDT on acne.

In paper I, MAL-PDT was significantly more effective than placebo PDT in treating inflammatory lesions, but no statistically significant difference was observed between the groups in the reduction of non-inflammatory lesions. In paper III no significant differences were obtained by comparing MAL-PDT and placebo PDT treatment sites for either inflammatory lesions or non-inflammatory lesions. The light dose in paper I was more than double the dose used in paper III. Furthermore, MAL-PDT was repeated in paper I. This implies that MAL requires some minimal dose for efficacy, beyond the dose that produces AEs. It is possible that the lower dose might only give an effect on the epidermis with keratinocyte shedding and reduction of follicular obstruction.

In papers II and III we could not find a decrease in sebum excretion after PDT, and this has also been found by other groups (Pollock *et al.* 2004). Divaris *et al.* (1990) found that ALA is preferentially taken up by the pilosebaceous unit, and since then this has been an argument that PDT is selectively treating the acne lesion.

Taken together, an alternative mode of action for ALA- or MAL-PDT other than direct damage to the sebaceous glands or photodynamic killing of *P.acnes* is suggested by the results in this thesis.

It has been proposed that MAL, which is more lipid-soluble, should be more selective, although MAL has not proved to be more efficient than ALA in treating acne (Wiegell 2006 b). Our results in paper III were in disagreement with the above findings since we found a homogeneously high PpIX fluorescence in the MAL-treated area, which implies a lack of selectivity for the MAL to acne lesions.

It cannot be excluded that a general superficial photo-ablation of the treated area is more likely to occur. In addition, the lack of effect on sebum excretion and amount of *P.acnes* could question the theory that sebaceous glands are selectively treated when performing PDT. A more likely mechanism to propose is the combination of photo-ablation, an effect on the epidermis with keratinocyte shedding, reduction of follicular obstruction and an immunological effect on the cytokines in the skin.

CONCLUSIONS

- Both ALA- and MAL-PDT are effective treatments of moderate to severe facial acne. However, MAL-PDT causes more pain than placebo PDT. The pain was reduced at repeated treatments.
- The lower light dose 30 J/cm² is as effective as 50 J/cm² for ALA-PDT in the face. Comparable results were found when treating acne on the back. Pain and hyperpigmentation were more common at higher doses of light.
- No significant reduction of *P. acnes* or sebum excretion was found at any time after ALA or MAL-PDT
- No significant difference was found in treatment efficacy between single treatments of MAL-PDT compared to red light only, when treating moderate to severe facial acne. MAL-PDT was found to be associated with more adverse events.
- MAL-induced fluorescence showed poor selectivity to the acne lesions. No correlations were found between the number of highly fluorescent pores and acne severity, the number of acne lesions or the amount of *P.acnes.*
- No difference between red and blue light, or between ALA and MAL in reducing the *P.acnes* viability count was found.
- Fluorescence emission spectra showed no significant porphyrin peaks of *P.acnes* wi*thout a*ddition of ALA or MAL. No porphyrin peaks were visible until after 4 to 5 days incubation with ALA *or MAL.* The photosensitizers in clinical use, ALA and MAL, are applied for 3-4 hours in clinical practice prior to PDT. Thus photosensitization of *P.acnes* is not the sole mechanism for the photodynamic treatment in acne.

OUTLOOK FOR THE FUTURE

Further work is *warrant*ed in order to optimize the use of ALA- and MAL-PDT for treatment of acne. Light based therapy of this skin disorder is a fast-growing therapeutic modality. In the future it will be even more important to find alternative treatments for acne that do not affect the environment as much as today. In addition there is a problem with increasing bacterial resistance to antibiotics. More clinical studies are needed to elucidate efficacy and safety when applying PDT for different acne symptoms in a larger set of patients and in longer follow-up periods. My opinion is that when performing clinical studies, one has to be aware that patients suffering from acne are at an age when it is difficult to get good compliance.

These future studies should compare conventional therapy with light therapy or combinations. PDT does not necessarily have to be a monotherapy. Perhaps the best therapy will be with light and a topical retinoid or a very short period with oral antibiotics.

PDT of acne is proposed as an alternative for those resistant to conventional treatments or who are intolerant of their side effects. The practical advantages associated with this treatment modality is that it is not a systemic treatment and therefore does not suffer from concerns related to administration of current acne treatments, including patient compliance and systemic AEs.

The treatment itself must therefore not cause any significant harmful effects and the development of postinflammatory pigmentation is of concern. It is important to balance efficacy, side effects and patients tolerance. It is likely that reduced AEs such as pain, inflammation at the treatment site and post inflammatory hyperpigmentation could be obtained by the use of short (0, 25-1 hr) incubation times. Instead multiple treatment sessions would ensure clinical efficacy and patient compliance. Treatment areas of patients with acne are usually big areas and by treating smaller areas at a time could reduce the pain. Reduction of both light dose, concentration of MAL or ALA will also be an alternative.

The most troublesome adverse effect of PDT is the pain during illumination. Recently published papers show promising results when using transcutaneous electrical nerve stimulation (Halldin *et al.* 2008) and nerve blocks (Paoli *et al.* 2008) for pain relief during PDT of actinic keratoses. This may also be alternatives for acne patients undergoing PDT**.** Furthermore, a correlation between the amount of PpIX fluorescence before illumination and the pain experienced during PDT has been shown (Wiegell *et al.* 2008). This finding implies that fluorescence could help us in the future to find which patients would need more advanced pain relief as for example nerve blocks.

In paper III, fluorescence revealed unspecific MAL-induced PpIX accumulation. Parameters which are likely to affect the selectivity are the drug concentration and type of ALA-ester. Perhaps other ALA-esters will be more effective in the future. The acne skin is often inflamed and without hyperkeratosis and because of this the cream could easily penetrate the skin in a general fashion. Further research is needed to create new compounds with more selectivity for inflamed skin.

As discussed in this thesis earlier, sun exposure is reported to have a beneficial effect on acne. Furthermore, low light dose PDT is to be preferred when treating acne since it reduces the pain. These facts together with the ongoing promising study of day-light mediated PDT are hopeful in the future development of acne treatment. So far repeated treatments are necessary for an effective acne treatment and day-light mediated PDT would be a convenient alternative for those young people with acne.

The most effective treatment regimen for PDT of Acne Vulgaris is not yet established. Further studies are needed before photodynamic therapy would be a convenient recommendation for young people with acne.

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