Photodynamic therapy and laser-based diagnostic studies of malignant tumours

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February 1999

ISBN 91-628-3352-9

To my beloved family

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ABSTRACT

Two non-thermal regimens of light interaction with biological tissue, that are in a natural way connected, are discussed in the present thesis. These are *photodynamic therapy* (PDT) for the treatment of malignant tumours and *laser-induced fluorescence* (LIF) for tissue characterization.

In PDT, a photochemical reaction is induced by non-ionizing electromagnetic irradiation in tissue where photosensitizing agents and oxygen are present. A subsequent tissue damage is generated. Porphyrin derivatives have traditionally been used for this purpose. A new way of sensitization was exploited, utilizing a porphyrin precursor, δ-aminolevulinic acid (ALA), for the induction of endogenous porphyrins, mainly protoporphyrin IX (PpIX). Photodynamic therapy was used in the treatment of non-melanoma malignant skin tumours, mainly basal cell carcinomas (BCCs). A randomized, clinical trial was conducted in the treatment of BCCs, where PDT was compared to cryosurgery.

Laser-induced fluorescence (LIF) can be used for non-invasive, real time spectroscopic characterization of biological tissue. Laser light in the near-ultraviolet or ultraviolet region is utilized for fluorescence excitation. The fluorescence signal in pre-malignant and malignant tissue exhibits some characteristics that deviate from those of normal tissue. In addition, the contrast can be increased by administering fluorescent tumour markers. Laser-induced fluorescence measurements were performed in malignancies of the skin and benign, pre-malignant and malignant lesions in the head-and-neck region. As a photoactive agent, ALA-induced PpIX was utilized, both for PDT and LIF. The kinetics of the PpIX build-up and photodegradation was also studied.

Another non-invasive, real time method for tissue diagnostics is laser-Doppler imaging (LDI). Laser-Doppler imaging detects the blood flow in superficial tissue. In the present work, the modality has been used in connection with ALA-PDT of non-melanoma malignant skin lesions, for diagnosing lesions prior to treatment and to identify residual or recurrent tumours during the follow-up period. It was also used to characterize the vascular effects induced by the ALA-PDT. In connection with the clinical trial that was conducted, LDI was used as an objective measure of tissue healing after treatment.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. I. Wang, S. Andersson-Engels, G.E. Nilsson, K. Wårdell and K. Svanberg, Superficial blood flow following photodynamic therapy of malignant skin tumours measured by laser Doppler perfusion imaging, *Br. J. Dermatol.* **136**, 184-189 (1997).

II. I. Wang, B. Bauer, S. Andersson-Engels, S. Svanberg and K. Svanberg, Photodynamic therapy utilising topical δ-aminolevulinic acid in non-melanoma skin malignancies of the eyelid and the periocular skin, *Acta Ophthalmol. Scand*. **77**, in press (1999).

III. K. Svanberg, T. Andersson, D. Killander, I. Wang, U. Stenram, S. Andersson-Engels, R. Berg, J. Johansson and S. Svanberg, Photodynamic therapy of non-melanoma malignant tumours of the skin using topical δ-amino levulinic acid sensitization and laser irradiation, *Br. J. Dermatol.* **130**, 743-751 (1994).

IV. I. Wang, L. Pais Clemente, R.M.G. Pratas, E. Cardoso, M. Pais Clemente, S. Montán, S. Svanberg and K. Svanberg, Fluorescence diagnostics and kinetic studies in the head and neck region utilising low-dose δ-aminolevulinic acid sensitisation, *Cancer Letters* **135**, 11-19, (1999).

V. I. Wang, N. Bendsoe, C. af Klinteberg, A.M.K. Enejder, S. Andersson-Engels, S. Svanberg and K. Svanberg, Photodynamic therapy versus cryosurgery of basal cell carcinomas; results of a Phase III randomized clinical trial, Manuscript for Cancer (1999).

VI. C. af Klinteberg, I. Wang, A.M.K. Enejder, S. Andersson-Engels, S. Svanberg and K. Svanberg, δ-Aminolevulinic acid-induced protoporphyrin IX fluorescence in basal cell carcinomas of the skin, Manuscript for Photochem. Photobiol. (1999).

VII. C. af Klinteberg, A.M.K. Enejder, I. Wang, S. Andersson-Engels, S. Svanberg and K. Svanberg, Kinetic fluorescence studies of δ-aminolevulinic acid-induced protoporphyrin IX in basal cell carcinomas, Submitted to J. Photochem. Photobiol. B (1999).

VIII. A.M.K. Enejder, C. af Klinteberg, I. Wang, S. Andersson-Engels, N. Bendsoe, S. Svanberg and K. Svanberg, Blood perfusion studies on basal cell carcinomas in conjunction with photodynamic- and cryotherapy employing laser-Doppler imaging, Submitted to Acta Derm. Venereol. (1999).

In addition to the above Papers, material is also presented in:

Clinical photodynamic therapy

1. I. Karu, S. Pålsson, I. Wang, C. af Klinteberg, N. Bendsoe, S. Andersson-Engels, S. Svanberg and K. Svanberg, Photodynamic therapy using δ-aminolevulinic acid and intensity modulated diode laser light, Submitted to Lasers Med. Sci. (1999).
2. C. af Klinteberg, I. Wang, I. Karu, T. Johansson, N. Bendsoe, K. Svanberg, S. Andersson-Engels, S. Svanberg, G. Canti, R. Cubeddu, A. Pifferi, P. Taroni and G. Valentini, Diode laser-mediated ALA-PDT guided by laser-induced fluorescence imaging, Submitted to Lasers Med. Sci. (1999).
3. I. Karu, I. Wang, C. af Klinteberg, S. Andersson-Engels, S. Svanberg and K. Svanberg, Evaluation of pain and dysesthesia in connection with diode laser mediated ALA-PDT, Manuscript for Br. J. Dermatol. (1999).

Clinical fluorescence diagnostics

4. I. Wang, K. Svanberg, S. Andersson-Engels, R. Berg and S. Svanberg, Photodynamic therapy of non-melanoma skin malignancies with topical δ-amino levulinic acid: diagnostic measurements, in *5th International Photodynamic Association Biennial Meeting*, ed. D.A. Cortese, Proc. SPIE vol. **2371**, 243-252 (1994).
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Experimental tumour studies

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15. D.L. Liu, K. Svanberg, I. Wang, S. Andersson-Engels and S. Svanberg, Laser Doppler perfusion imaging: New technique for determination of perfusion and reperfusion of splanchnic organs and tumor tissue, *Lasers Surg. Med.* **20**, 473-479 (1997).

1. INTRODUCTION

The basis of this thesis is that the interaction of light with tissue and certain cancer-specific agents can be exploited and utilized in the enormous human struggle against cancer diseases. Today we live in a world of high technology, also within medicine. We have 100 years of experience with the powerful tool of ionizing radiation, and we are standing on the border of a revolution in diagnostics and therapeutics of many diseases with the help of new knowledge within the fields of immunology, molecular biology and genetics. It is truly fascinating that such a seemingly subtle and harmless phenomenon as visible light rays can contribute in this armamentarium.

The laser action was demonstrated in 1960. An important principle of the laser that separated it from any other light source at that time, was the possibility of choosing in principle only one single wavelength, *i.e.* colour, or frequency. The coherent nature of laser light made it possible to introduce it into optical fibres for transportation over longer distances. Understandably this became an invaluable tool in many fields of science. In medicine, the advantages of the laser were early understood and exploited.

The utilization of lasers in medicine has reached an overwhelming extent during the last decades. Due to the possibility of having its biological impact varied, through the selection of wavelength, output power and illumination time and mode, the laser has found its way into many clinical-, pre- and paraclinical specialities. It is mainly the thermal interaction of laser light with tissue that has been exploited. From being a tool in the hands of the pioneers in ophthalmology, it is now a cutting tool in most surgical specialities and is used for all kinds of incisive procedures. Still though, for quite obvious practical reasons, lasers have far from taken over for the traditional "cold knife". The latest news is the possibility of performing microscopical laser surgery on single cells with the so-called microbeam, together with optical tweezers. Other examples of thermal tissue interaction, are the ablation of superficial tissue layers and the selective destruction of certain parts of the tissue. These are widely used by dermatologists in, for instance, the treatment of haemangiomas and pigmented lesions. Laser light is also used for inducing hyperthermia.

This thesis considers two non-thermal regimens of utilizing light within medicine that are, in a natural way, connected. First, *photodynamic therapy* (PDT)[*] in the treatment of malignant tumours, is discussed. Secondly, *laser-induced fluorescence* (LIF) for tissue characterization, one modality of what often is termed *"optical biopsy"*, is discussed.

In PDT, tissue is sensitized to light by supplying photosensitizing agents, *i.e.* photosensitizers. Following the non-ionizing electromagnetic irradiation, a photochemical reaction is induced. The modality can be compared to the photosynthesis in green plants, the photosensitizers used, being similar compounds to chlorophyll. As opposed to the photosynthesis which works in a surrounding specially

[*] A comprehensive list of abbreviations used in this thesis can be found on page 98.

designed to handle the mechanisms involved in the energy transfer (the photosynthetic reaction centre), the photosensitizers are intruders in biological tissue. When excited by light, the photochemical reaction of the photosensitizers will, therefore, generate tissue damage. Oxygen is a prerequisite for obtaining this effect. Eradication of malignant tumours can be obtained by several mechanisms. The optimal treatment light wavelength for PDT is dependent on several factors; the absorption profile of the photosensitizer and how translucent the tissue is at the wavelength chosen, are the most important. Red light penetrates deeper than blue light, and light matching an absorption maxima of the photosensitizer in the red wavelength region, is most often used. Relatively large photosensitizing molecules, mainly for intravenous administration, have been used for this purpose, the first photosensitizers being porphyrin derivatives. A new method of inducing endogenous porphyrins, mainly protoporphyrin IX (PpIX), by supplying a porphyrin precursor, δ-aminolevulinic acid (ALA), has been exploited clinically for the last 8 years.

Laser-induced fluorescence provides non-invasive, real time monitoring of biological tissue. The technique is based on measurements of signals from fluorescent molecules within tissue. These signals are related to molecular concentration and micro-environment. Laser light in the near-ultraviolet or ultraviolet region is most often used as an excitation light source, and the fluorescence signal achieved from the tissue is subject to spectroscopic analyses. The fluorescence signals recorded from pre-malignant and malignant tissue exhibit some characteristics that deviate from those of normal tissue. This endogenous tissue fluorescence is often called the autofluorescence, and the difference between normal and diseased tissue is due to an alteration in the composition of various naturally occurring tissue fluorophores, *i.e.*, fluorescent molecules. In addition, increased contrast can be obtained by fluorescent tumour markers, which are highly fluorescent molecules that to a certain degree accumulate selectively in pre-malignant and malignant tissue. These tumour marking agents are mainly the same substances as the photosensitizers used for PDT, but the doses are considerably lower.

Taken into account the plurality of conventional diagnostic and therapeutical modalities for oncological diseases, one question that will naturally arise, is whether there is a need for alternative methods. The question will partly answer itself, when considering the fact that cancer is still a disease that claims many lives, often at an early age. In addition to the clinical concepts, also the question of cost effectiveness has to considered. Some specific features of PDT and LIF are described below.

Concerning PDT, it is a local treatment modality, which in its nature has several potential benefits. The main advantage is the fact that PDT is comparably selective. There are two aspects behind this. One is that photosensitizers preferably accumulate in malignant tissue, and the other is the spatial confinement of the light irradiation to the vicinity of the malignant tumour. The result is less scarring; something that is particularly important when treating epithelial lesions. So far, in contrast to the conventional treatment modalities of, *e.g.*, ionizing radiation and chemotherapy, one has no strong reason to believe that PDT has any major mutagenic capability. Since

normal tissue remains almost undamaged, there is no real limit to how many times PDT can be repeated. There is also the possibility to utilize PDT in the treatment of primary or recurrent tumours in patients who already have received maximum treatment of surgery, ionizing radiation or chemotherapy. The future niches for PDT have not yet been fully established. In certain cases PDT might be the "treatment of choice", and in other situations it can be used as an adjuvant or palliative treatment. This thesis will discuss one niche where PDT has already proven to be particularly useful, namely, in certain skin cancers.

Looking at the diagnostic modality of LIF, this is primarily a method for investigation of the surface of the skin and body cavities. This is where the major part of all malignancies arises. In connection with conventional endoscopic examinations, LIF can provide guidance for the biopsy sampling for subsequent histopathological analyses. The method is therefore often referred to as "optical biopsy", being exploited mainly for early tumour detection. Ideally, the combined imaging techniques, of ordinary examination under white light and LIF, can increase the value of the investigation concerning sensitivity and specificity. There is also a potential of being able to reduce the number of biopsies in vulnerable organs, for instance in the vocal cords.

This thesis pays special attention to the use of ALA-induced PpIX as the photosensitizing agent and fluorescent tumour marker, both for PDT and LIF. The background and discussion focuses, therefore, on this mode of tissue sensitization. Less emphasis is put on the description the other types of photosensitizers, that are not produced from precursors. These agents will, for the sake of simplicity, be termed pre-formed photosensitizers. Another common term for PDT is photochemotherapy (PCT). Although this designation may have a wider definition (see Section 3.1.2), it is often preferred, partly because it better reflects the actual mechanism involved, *i.e.*, the photochemistry. However, throughout this thesis, the term photodynamic therapy will be used.

Another non-invasive, real time method for tissue diagnostics is discussed, namely, *laser-Doppler perfusion imaging* (LDI). Laser-Doppler imaging monitors the superficial tissue perfusion. In the present work, the modality has been used in connection with PDT, utilizing ALA-induced PpIX photosensitization (ALA-PDT), of non-melanoma malignant skin tumours. The measurements were performed prior to treatment, to characterize the perfusion of the lesions. The method was also used to follow the healing process and to evaluate if it was possible to identify residual or recurrent tumours. The type of vascular effects induced by ALA-PDT, was also monitored by means of LDI.

One main part of this thesis is constituted of a Phase III clinical trial in the treatment of basal cell carcinomas (BCCs). The therapeutic potential of ALA-PDT, was in this trial evaluated in a objective and clinically controlled way, by randomizing the BCCs to treatment with either ALA-PDT or with cryosurgery. In connection with the treatments, LIF measurements were performed to follow the build-up of PpIX from ALA, and the photodegradation during illumination. Laser-Doppler imaging was here

also used as an objective measure of the tissue healing, when comparing the two treatment modalities.

The introduction of new techniques into medicine is always a challenge. The overall goal is, of course, to consider their usefulness in view of the medical situation into which they are to be implemented. When working with laser-based methods, a certain spectrum of knowledge of the biological, biophysical as well as the physical aspects, is also required. In the following chapters, some information deemed useful from a physician's view, is presented. After this, eight papers with original scientific material can be found.

2. LIGHT INTERACTION WITH BIOLOGICAL TISSUE

The interaction of light with such complicated matter as biological tissue is a growing field of research.[1-4] Knowledge about the basic principles of light-tissue interaction is important in all situations where laser-based techniques are utilized within medicine. In this chapter, the basic aspects of this field are presented in a simplified manner with a few examples of applications.

2.1. Light

Visible light constitutes a narrow band of the electromagnetic radiation spectrum that can be perceived by the human eye, with a bandwidth of in total about 400 nm (1 nm = 10^{-9} m). The electromagnetic spectrum ranges from wavelengths less than 10^{-13} m to more than 10^{5} m. The visible part of the spectrum lies in the range of 400-780 nm; the numbers are approximate since there are individual variations. The eye can distinguish the various wavelengths in terms of bands with a width of about 20-160 nm, *i.e.*, the colours. Thus, colour is not a property of the light itself, but a subjective human physiological and psychological response. Colours in a certain band can also be evoked by mixtures of frequencies/wavelengths lying in other colour bands. Values for the colour wavelength bands are given below.[5]

Colour	Wavelength range (nm)
Violet	400-455
Blue	455-492
Green	492-577
Yellow	577-597
Orange	597-622
Red	622-780

The visible wavelength region borders ultraviolet (UV) in the lower part and the near infrared in the upper part. The term, "light", is also at times used when referring to irradiation in the UV and the infrared region. The wavelength of light (λ), is inversely proportional to the energy (E) of individual light photons, *i.e.*, the quanta of light energy:

$$\lambda = h \times c/E \qquad h=\text{Planck's constant}$$

and to its frequency (ν):

$$\lambda = c/\nu \qquad c=\text{the speed of light}$$

2.1.1. LASERS AS LIGHT SOURCES

The designation, LASER, is an acronym for ″light amplification by stimulated emission of radiation,″ which is a short description of the concept of this source of radiation. Laser light is characterized by being monochromatic, coherent and concentrated. The monochromatic quality implies that the light contains only one, or a narrow range of wavelengths. This is in contrast to conventional light sources, that emit light over a much broader wavelength range. For instance, a normal light bulb emits over much of the visible and infrared spectrum. The light waves coming from a laser are in phase, a phenomenon called coherence. This is a unique quality for a laser beam. This beam is concentrated, *i.e.* it has a minimal angle of divergence, and laser light can easily be focused into optical fibres and transported over long distances. Various types of lasers have been utilized for medical purposes. A short description of some lasers used in photodynamic therapy (PDT), together with a description of other possible light sources, can be found in Section 3.4.

2.2. Basic physical aspects of light-tissue interaction

Upon irradiation with light, several processes can occur when the photons pass through the tissue, as illustrated in Figure 2-2. The various processes of absorption, reflection, scattering, transmittance and fluorescence can be utilized for medical purposes. In connection with the following description of these optical phenomena, some examples of their exploitation within medicine are given.

2.2.1. ABSORPTION

If an incoming photon carries an energy that matches the gap between two energy levels in a molecule, it can be absorbed. A *chromophore* is a molecule absorbing visible light resulting in a coloured compound. Here we will extend the definition to molecules absorbing in the near-UV, visible and near-IR region. The absorption coefficients in various tissue chromophores, are shown in Figure 2-1. The visible wavelength region is located in a band where the strong absorption of water is almost absent. The main chromophores in biological tissue are haemoglobin (Hb) and melanin. The “tissue optical window” is the wavelength range between about 630 nm and 1300 nm. In this range, the blood absorption has fallen off, and the penetration of light into biological tissue is at its deepest, with an increased penetration at longer wavelengths. Within this range the scattering coefficient (se below) is dominant, being considerably larger than the absorption coefficient for most tissues.

The stable ground state of most molecules is, in molecular physics nomenclature, called the singlet state (S_0 in Figure 2-2). This state is characterized by the lowest possible energy in that particular molecule. This is obtained by configuring all electrons in such a way that all electron spins are paired, *i.e.* no unpaired electron spins exist. The triplet state (T*), is for most molecules, an unstable excited state with an increased internal energy, characterized by two unpaired electron spins. Oxygen is one of the rare substances that is in the electronic triplet state in its normal ground

state. Excited molecular oxygen in its singlet state, is important in the photochemical reaction occurring during PDT (see Section 3.6.2).

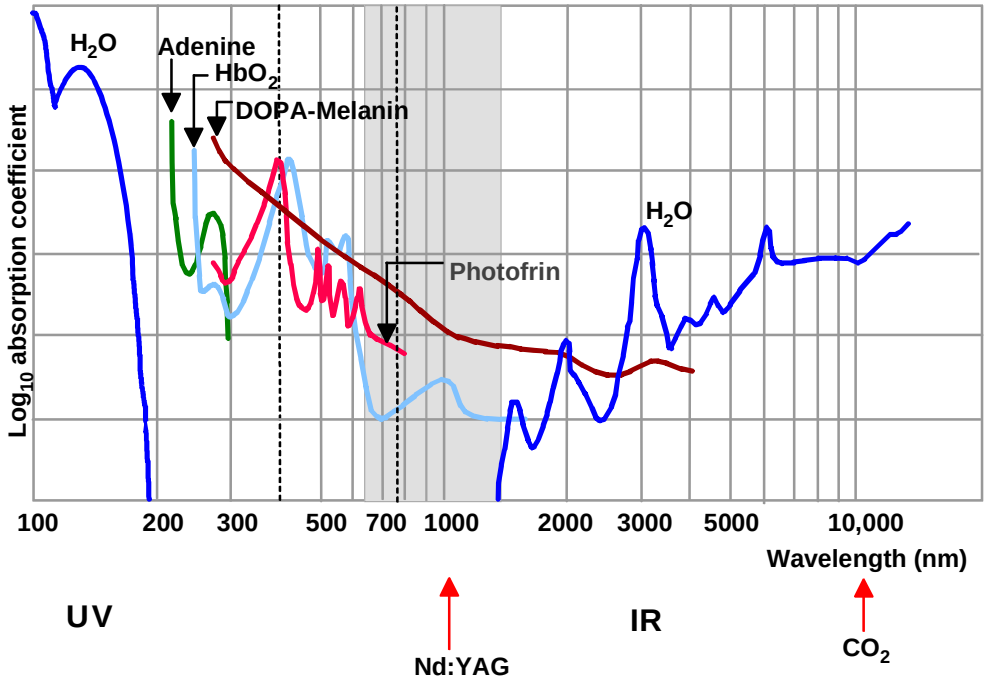

Figure 2-1 *Diagram showing absorption coefficient versus wavelength for major chromophores, present in biological tissue. The visible wavelength region covers the area between the dotted lines. The area marked with grey corresponds to the tissue optical window. In this region the water absorption is very low and the blood absorption falls off, but the melanoma in the skin still absorbs rather much. The absorption curve for Photofrin with its 5 peaks, is also shown. Emission wavelengths for some lasers used within medicine are inserted; the Nd:YAG laser (λ_{em} = 1064 nm) and the CO_2 laser (λ_{em} = 10 600 nm) used for surgical procedures and tissue ablation. (Adopted from J.-L. Boulnois).*[6]

As illustrated in Figure 2-2, after the absorption of a photon and the subsequent excitation of the absorbing molecule to a higher energy level (S_2*), the molecule relaxes down to a lower energy level (S_1*) by a non-radiative process. The singlet state is short-lived, generally much less than 1 μs. After the generation of the lowest excited singlet state, several processes can take place, namely, non-radiative decay, fluorescence, and intersystem crossing.

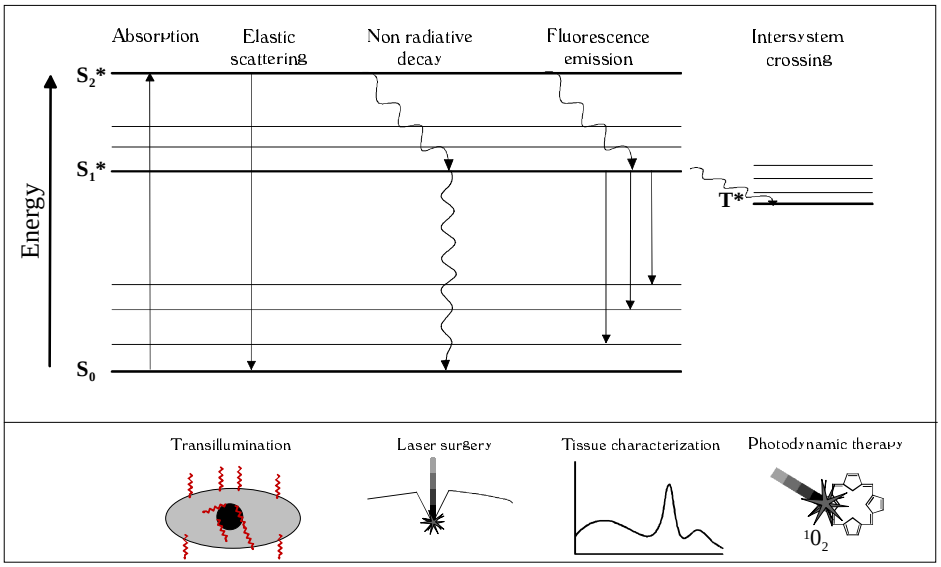

Figure 2-2 *An energy-level diagram of a molecule with a presentation of the happenings that might occur after the absorption of a photon (upper panel). S_0 corresponds to the ground state energy level of the molecule, whereas, S_1* and S_2* are excited, higher energy levels. T* corresponds to a triplet state. Some possible medical applications of the various light-tissue-interaction phenomena are illustrated (lower panel).*

Excited molecules will frequently return non-radiatively to the ground state in collision processes, yielding heat to the tissue. This is utilized in thermal laser therapy modalities. An example is the selective destruction of haemangiomas or of pigmented skin lesions that occurs when tissue is irradiated with high intensity light at a wavelength with strong absorption in blood or in melanin, respectively.

2.2.2. REFLECTION, SCATTERING, AND TRANSMITTANCE

Light scattering is an important process for light transport in tissue. A certain percentage of a photon beam that impinges onto a tissue surface, will be re-emitted from the surface. This phenomenon includes true, specular reflection from the surface and light diffusely back-scattered from the sub-surface tissue layers. In PDT, some of the treatment light will, therefore, be lost from the tissue by diffuse scattering. However, due to back-scattering from deeper tissue layers, the light intensity at the outer layers of biological tissue subject to irradiation at wavelengths used for PDT, will be higher than the intensity of the incoming photon beam. This is particularly true if the tissue has an intact epidermal compartment, which is an example of a highly scattering tissue.[7]

Only part of the light that is transported into the biological tissue, will be transmitted. The rest will either be absorbed or scattered, leading to attenuation, *i.e.* diminution of the light intensity. The attenuation of the beam is, therefore, dependent

on the absorption (μ_a) and the scattering (μ_s) coefficients. Scattering in the tissue is due to several optical effects:[8]

i. reflection or refraction from interfaces between histologic materials having different refractive indices
ii. reflection of light by discrete particles in the tissue ranging from organic molecules to whole cells
iii. absorption of light rays by atoms and molecules and re-radiation at the same wavelength but in other directions.

These scattering processes are called elastic, indicating that light only changes direction in the scattering process, thus, no energy loss is involved. Inelastic processes, such as Raman scattering, will not be considered in this thesis. However, it should be mentioned that the scattering processes involved in laser-Doppler measurements, utilized for detecting tissue perfusion, as described in this thesis (see Chapter 2), involve a small change of energy. In laser-Doppler measurements, a laser beam is spectrally broadened, *i.e.* the frequency is slightly shifted (Doppler shifted), when diffusely back-scattered from the moving blood cells. This energy change is very small, with a frequency shift of typically 10^{-11} times that of the incident photon beam,[9] and this type of scattering is often termed quasi-elastic scattering.

The diffusely back-scattered light escaping the tissue, is lost for treatment purposes, but can be utilized for tissue diagnostics. Elastic-scattering spectroscopy (ESS) is a modality for tissue characterization detecting light that is back-scattered from sub-surface tissue layers. Differences in the back-scattered light can be seen between normal and diseased tissue. The differences are partly due to changes in scattering properties and partly a result of altered absorption properties in the diseased tissues. These variations are partly caused by architectural changes at the cellular and sub-cellular level, induced by pathological conditions.[10,11]

Another diagnostic modality utilizing light scattering in tissue, is optical tomography with time-resolved spectroscopy. Due to the high degree of scattering in biological tissue, transillumination of an organ does not create sharp shadows when detecting the light that has been transmitted. However, if a fast detector is used, it is possible to analyse the light with the shortest pathlength through the medium, *i.e.* the light that has passed straight through the tissue without being much scattered, the "early light". If a tumour with scattering and absorption properties that are altered from those of the surrounding normal tissue, is present in the transilluminated volume, a "shadow" of the tumour can be seen in an image that only visualizes the early light. The modality has mainly been examined for use in connection with mammography, and is then termed optical mammography.[12,13]

2.2.3. FLUORESCENCE

Emission of fluorescence is one of the possible processes that can occur when light is absorbed in molecules. As mentioned earlier, following the absorption of a photon and

the subsequent excitation of the absorbing molecule, the molecule relaxes down to the lowest energy level within the S_1 state, by a non-radiative process (Figure 2-2). From here, it can release its extra energy by emitting a photon as it relaxes down to the lower S_0 energy state. This emission is called fluorescence. Due to the energy loss in the non-radiative part of this process, the fluorescence photon will carry a lower energy and, thereby, have a longer wavelength than that of the absorbed photon. The relaxation from the higher level can end in any level in the lower S_0 energy band, leading to a total fluorescence emission composed of several wavelengths. This explains why fluorescence is characterized by a wide wavelength distribution and not by a sharp peak. The shorter the excitation wavelength, *i.e.* the more towards the ultraviolet region the fluorescence excitation occurs, the more of the visible spectrum can be contained in the resulting fluorescence signal.

A *fluorophore* is a molecular structure that gives rise to fluorescence. Such molecules are frequently characterized by the presence of a series of conjugated double bonds, *i.e.*, alternating single and double bonds.

In addition to the emission wavelength, fluorescence is also characterized by its lifetime. The lifetime is dependent on the surroundings of the fluorophore. The lifetime for endogenous and exogenous fluorophores, is in the order of nanoseconds (ns).

2.2.4. EXCITATION OF PHOTOSENSITIZERS

Photosensitizers are molecules that upon irradiation with light in the visible region can induce damage, if present in biological tissue (the biological aspects of photosensitizers are discussed in Section 3.2). It is the longer-lived triplet state, T* populated through intersystem-crossing from an excited singlet state, that is the photoactive state involved in the photochemical generation of cytotoxic species during PDT. The excitation efficiency of a photosensitizer is defined as the triplet state quantum yield (ϕ_T), which is the probability of the triplet state formation per photon absorbed. Ideally, this should be one for a photosensitizer.

2.3. Effects of ultraviolet light on the skin

The wavelength range of UV radiation (UVR) lies between 280 nm and 400 nm. In older terms, the range 200-300 nm was called the far UV and the range 300-400 nm was called the near-UV. Due to more knowledge about the physical and biological effects of UV light, it is now common to divide UV light into three areas, namely, 200-280 nm, 280-320 and 320-400 nm, termed UVC, UVB and UVA, respectively. When discussing the effects of UVR on the skin, it is particularly the solar UVR range (295-400 nm) that is relevant.[14] Nordic summer sunlight contains about 5% UVR out of which about 95% is UVA and 5% UVB.[15]

Ultraviolet radiation affects the skin in several ways, most of which are harmful. The physiological effects induced by UVR are erythema, pigmentation, hyperkeratosis, and D-vitamin production. Erythema is a reaction indicating that damage has been induced. Pigmentation and development of hyperkeratosis are

defence mechanisms, whereas production of D vitamin is purely beneficial. Ultraviolet radiation also causes the pathological effects of immuno-suppression and skin cancer. In addition, UVR can cause therapeutic effects on certain skin diseases due to an anti-inflammatory effect.

An action spectrum is a representation of the efficiency of different wavelengths to induce certain biological effects. These effects vary for the three wavelength ranges. The best known reaction of the skin is the erythema reaction, which is a well-known reaction to UVB radiation, particularly in the region 280-300 nm.[15] The action spectrum of non-melanoma skin malignancies in humans, as estimated on the basis of measurements performed on mouse skin, has been shown to resemble the action spectrum of erythema.[16] The potential of UVA radiation to induce erythema is considerably smaller. However, it seems that UVA is important in the development of malignant melanomas,[17] something that has been recognized relatively recently and has possible implications for the use of sunlamps[18] as well as the use and the choice of sunscreens.[19]

A strong chromophore in the skin is DNA, that has its major absorption at about 260 nm (UVC), a lower one at about 300 nm (UVB) and a weak absorption peak at about 350 nm (UVA).[14] Photoproducts induced by UVR in the skin, such as pyrimidine dimers, might subsequently be followed by mutations. Other chromophores in the skin are melanin and its intermediates. They have been related to the photocarcinogenesis of malignant melanomas by acting as photosensitizers within the melanocytes when irradiated by UVA, with subsequent effects on the DNA.[14,20]

3. PHOTODYNAMIC THERAPY - BACKGROUND

Photodynamic therapy (PDT) is an investigational modality for local treatment relying on the presence of a photosensitizer and oxygen. The subsequent irradiation of photosensitized tissue with non-ionizing radiation generates tissue damage. Besides the non-ionizing nature of the treatment, the main advantage with the modality is considered to be its local and selective damage of tumour tissue. Two aspects pertain to the selectivity; a selective accumulation of the photosensitizer in malignant tissue and the spatial confinement of the irradiation to the vicinity of the tumour. This chapter will briefly describe the historical development of PDT. Thereafter, the basic knowledge of photosensitizers, light sources and delivery modes, as well as the mechanisms behind the tissue damage achieved, will be presented.

3.1. Historical aspects

The use of light for therapeutic purposes can be traced far back into ancient times. Ambient sunlight has been a universal light source, utilized in many old cultures and throughout the history to treat all sorts of medical conditions varying from vitiligo to psychosis. Sometimes light irradiation has been used in combination with plant extracts. The specificity of the treatment has increased in parallel with the refinement of the light sources and knowledge about the chromophores and mechanisms involved. Today, treatment modalities that are dependent on light, are often categorized as phototherapy or photochemotherapy. Photodynamic therapy belongs to the latter group.

3.1.1. PHOTOTHERAPY

In phototherapy, the treatment consists solely of light within various wavelength bands and intensities. A heterogeneous group of mechanisms are involved. In this case, the light interacts directly with the tissue without prior administration of an activating drug.

About 3000 years ago, the Greeks practised full body sun exposure, later termed *heliotherapy*. At the end of the last century, Niels Finsen started the Finsen Institute in Copenhagen. He treated a substantial number of patients suffering from Lupus vulgaris, *i.e.* cutaneous tuberculosis, with near-UV and UV light exposure.[21] He received the Nobel Prize in medicine in 1903 and was acknowledged as the founder of modern phototherapy.

Today, phototherapy is mostly used within dermatology[22] for certain types of dermatitis and for psoriasis. Within paediatrics, it is important in the treatment of jaundice in new-borns. A new and expanding field is within psychiatry, where encouraging results have been obtained treating seasonal affective disorders (SAD)[23] and sleep disturbances.[24] In this case, the treatment affects the central nervous system, and the mechanisms involved are probably quite complicated and diverse. Light reaches the retina and signals pass through *tractus retinohypothalamicus* to *nucleus*

suprachiasmaticus, which is essential in the regulation of the circadian rhythms (pertaining to the endogenous biological rhythms with approximately 24-hourly repetition). Effects of light exposure have been observed also in blind individuals. The effect on depression is partly ascribed to a subsequent upgrading of the serotonin level of the brain. Exposure to light leads to an arousal of the reticular activating system and an interference of the circadian rhythm. If the light exposure is given at the right point in the sleep-awake rhythm, a beneficial dislocation can be obtained and used in the treatment of sleep disorders and to overcome jet-lag. The hormonal control systems are also affected, and the release of the sleep-awake hormone melatonin is considered to play a major role in the treatment effect.

3.1.2. PHOTOCHEMOTHERAPY AND PHOTODYNAMIC THERAPY

In photochemotherapy (PCT), the treatment consists of two parts; the administration of an exogenous agent, which sensitizes the tissue, and a subsequent irradiation, *i.e.* chemically enhanced phototherapy. Among the oldest known drugs for photochemotherapy are psoralens, which in combination with UVA irradiation constitute what is known today as PUVA therapy, frequently utilized within dermatology, mainly for the treatment of psoriasis. In the psoriatic plaques, the PUVA treatment affects the DNA and reduces the cell turnover in the epidermis. Psoralens can be extracted from a variety of plants. It is known that the treatment was utilized in old cultures. In India and Egypt, psoralens were obtained from *Psoralea corylifolia* and *Anmi majus*, respectively, and used for re-pigmentation of vitiliginous skin.

The expression, photodynamic therapy (PDT), is often used for a type of photochemotherapy which is dependent on the presence of oxygen. The introduction of the term photodynamic, and several important discoveries within the field, occurred around the turn of the century. In 1898, the medical student Oscar Raab discovered by chance that the toxicity of the dye acridine to paramecia was dependent on the ambient light when performing the experiments, rather than to the concentration of the drug. The work was performed in the laboratories of Herman von Tappeiner in Munich. Von Tappeiner continued the work, and together with Jodlbauer, it was reported in 1904 that the presence of oxygen was required to achieve an effect. With this, he first minted the term photodynamic therapy to describe the phenomenon of oxygen dependent photosensitization. Together with the dermatologist Jesoniek, he was the first to perform PDT on humans, reported in 1903, treating skin cancer, lupus of the skin and genital condylomas, with eosin as a photosensitizer. In 1908, the first studies of the biological properties of hematoporphyrin were performed by Hausmann in Vienna who sensitized mice. The first report on sensitization in humans was in 1913, when the German Meyer-Betz injected himself with 200 mg of hematoporphyrin and remained photosensitive to light for two months. Together with a German chemist named Fischer, Meyer-Betz investigated the importance of the porphyrin structure on the PDT effect. Fischer continued the research on porphyrin chemistry, and much of his work was connected to patients with porphyria. From patients suffering from the porphyrias, photosensitization of the skin was well-known due to the accumulation of

various porphyrins and their precursors (δ-aminolevulinic acid, porphobilinogen, porphyrinogens) in the skin (see Sections 3.1.3 and 3.3.1). The selective localization of porphyrins in tumours was observed in Lyon in 1924 by Policard, who excited fluorescence in a tumour with a Woods lamp with UV emission and attributed it to the accumulation of endogenous porphyrins. As in the case of many fields, World War II halted further development for some time. In 1948, Figge showed selective retention and recognized hematoporphyrin derivative (HPD) as an important substance for cancer detection.[25] Pioneering work on the localizing properties of various porphyrin mixtures was performed by Schwartz *et al.* with the original interest to study the protective/sensitizing effect of porphyrins in connection with ionizing radiation therapy. He found the acetic acid-sulfuric acid derivative of hematoporphyrin hydrochloride (HPD) to be a good tumour localizer. Lipson *et al.* performed much work in the 1960's with HPD. He tried to adopt endoscopic systems for tumour localization by HPD fluorescence[26] and succeeded in the treatment of a human breast cancer with HPD.[27]

The history of PCT and PDT has been presented in review papers, where references to the above mentioned work can be found.[22,27,28]

3.1.3. THE PORPHYRINS

Porphyrins constitute a group of pigments with a cyclic tetrapyrrole macrocycle[29] as the essential part, and the metal complexes are of fundamental importance in the ontogenesis of terrestrial life, due to their role as mediators in biological oxidation reactions. The chlorophylls are Mg^{2+}-porphyrin complexes that are central in the transformation of solar energy in bacteria, algae and plants. The haems are Fe^{2+}-porphyrin complexes responsible for the oxygen carrying properties of haemoglobin and myoglobin. Haem is also the prosthetic group in the cytochromes, responsible for electron transport, reduction of oxygen and hydroxylation reactions. Vitamin B_{12} is a Co^{3+} complex of a compound related to haem.

There is a natural link between the research in the field of PDT and the research on the various porphyrias.[30,31] Porphyria is a hereditary disease with a heterogeneous panorama of expressions and symptoms. The common factor in the different types of porphyrias is an erratum in one of the enzymatic steps of the haem biosynthetic pathway, mainly due to the expression of a genetic defect. The liver and erythropoietic tissue are the two main locations responsible for most of the haem synthesis in the human body. The porphyrias are usually classified as either hepatic or erythropoietic, according to the principal site of expression of the specific enzyme defect. In patients with erythropoietic protoporphyria, the fluorescence of porphyrins is well-known, since the discovery in 1953[32] of strongly fluorescent erythrocytes in such patients. This is due to an accumulation of protoporphyrin IX (PpIX) in the erythrocytes secondary to a defect in the enzyme ferrochelatase (see Section 3.3.1).

3.2. Photosensitization of tissue

The term photosensitization covers all reactions in which a substance activated by light causes a biologic effect. Much of the work on photosensitizers originates from the work on porphyrins. Most of the early work on photosensitization was performed using porphyrins and their relatives. However, other and quite different molecular structures have been investigated in search of substances which best meet the demands of the ideal photosensitizer. Most photosensitizers are large, relatively complex molecular structures, that usually have to be administered by intravenous injection. An exception to this is δ-aminolevulinic acid (ALA), which is a precursor of the photosensitizing compound, PpIX. This mode of sensitization will be separately described in Section 3.3.

3.2.1. COMMONLY USED PHOTOSENSITIZERS

The field of research on various photosensitizers is now quite extensive. Only some main groups of photosensitizers which have been utilized in pre-clinical and clinical studies will be mentioned here. The main feature of photosensitizing agents is light absorption in a wavelength range where the surrounding biological tissue is relatively transparent. Most photosensitizing compounds absorb light in several bands within the visible wavelength range. To reach a deeper penetration, the absorption peaks in the red region are used. The most common photosensitizer within the field of PDT, haematoporphyrin derivative (HPD), has an absorption peak in the red wavelength region at about 630 nm. The research within the field aims at finding compounds which absorb far out in the red wavelength region, towards the borders of the infrared, where the penetration depth of the treatment light is at its maximum (Figure 2-1). Larger tumour volumes can then be treated. Another important feature to consider is that the triplet states energies are lower for dyes absorbing in this region, and that there is an upper wavelength limit for the generation of singlet oxygen via the Type II mechanism (see Section 3.6.2). This means that dyes absorbing at longer wavelengths than approximately 800 nm cannot be used. In addition, a useful photosensitizer should have a rather narrow absorption band, with little absorption at other wavelengths within the solar spectrum. This is to decrease the side effect of skin photosensitivity.

Other basic properties that characterize an ideal photosensitizing compound, besides the absorption wavelength, are a high quantum efficiency, selective accumulation in malignant tumour tissue, a fast clearance and a low toxicity. The quantum efficiency is, apart from the photophysical properties, also dependent on the aggregation state of the dye, its localization at the time of light activation and on the localized concentration of endogenous quenchers. The fast clearance rate should ideally include all normal tissues, also the liver, kidney and spleen, but the clearance from the tumour tissue should be slow. The toxicity of a photosensitizer is partly connected to the selectivity of the agent, meaning that photosensitizers which

accumulate mainly in tumour tissue lead to less effects on normal tissues. Photosensitizing compounds can be classified into three major groups:[33]

i. hydrophobic photosensitizers, that bear no charged peripheral substituents. They have negligible solubility in water or alcohol
ii. hydrophilic photosensitizers which have three or more charged substituents and are freely soluble in water at physiological pH
iii. amphiphilic photosensitizers which have two or less charged substituents and are soluble in alcohol or water at physiological pH

The structure of the porphyrin main core is a relatively flat, tablet-like molecular structure with a width of about 7 Å.[34] A spatium in the middle of this structure can accommodate a metal ion. Partly due to this structure, the agents easily aggregate by piling on top of each other. One main challenge when synthesising photosensitizers, has been to produce compounds which stay mainly in the monomeric form in solution. The haematoporphyrins were the first photosensitizers studied more in detail. The second generation of photosensitizers includes compounds from other chemical groups. A brief description of several of these groups, with some examples of individual agents, are given as follows.

Porphyrins The main core of a porphyrin compound can be seen in Figure 3-1. The principal porphyrin-based drug used in both pre-clinical and clinical work is Photofrin . Photofrin is a refined form of HPD, but still it contains an inhomogenous distribution of monomers, di- and oligomers. The absorption spectrum for Photofrin has five peaks, the strongest at about 400 nm (the Soret band) and the weakest at about 630 nm.[34] Protoporphyrin IX is, presently, one of the most utilized photosensitizers. Following the administration of its precursor δ-aminolevulinic acid (ALA), PpIX will accumulate in malignant tumour tissue as described in detail below.

Chlorins Chlorins constitute a group of molecules very similar to porphyrins, as shown in Figure 3-1, illustrating the main core of a porphyrin and a chlorin. Chlorophyll belongs to this group. Chlorins can also be synthesized from porphyrins. In contrast to porphyrins, chlorins have the strongest absorption peaks in the red part of the spectrum, which give the compounds a green colour.[34] In comparison to the porphyrin structure, the chlorin has at least one double bond missing in the pyrrole rings. Benzoporphyrin derivative (BPD) is, despite the name, a chlorin, synthesized from protoporphyrin. Besides the beneficial absorption wavelength at 690 nm, it has a rapid accumulation in the tumour, allowing light irradiation the same day as injection. It also has a relatively short half-life, with skin photosensitization lasting for less than a week. Chlorin e_6 is a compound that has a strong absorption peak at 664 nm. Like BPD, it has a very short-term phototoxicity. Meso-Tetrahydroxyphenylchlorin (m-THPC) is the most potent photosensitizer available for clinical use at present. It has a

strong absorption peak in the red, at 652 nm. Drug and light doses utilized in clinical trials, are in the order of 10 times lower than for Photofrin .

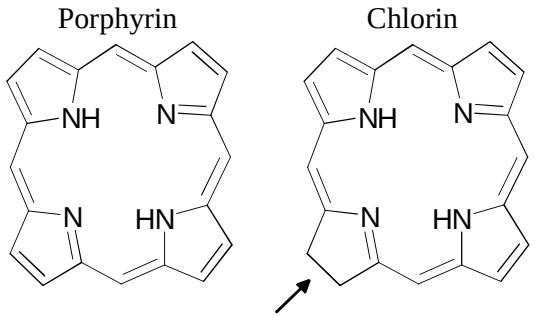

Figure 3-1 *The main core of the porphyrin- and chlorin-type photosensitizers. As can be seen, the difference between the compounds is the lack of at least one double bond in one of the pyrrole rings in the chlorin (arrow).*

Purpurins Purpurins are similar in structure to chlorins, in the respect that they have one reduced pyrrole group. The basic purpurin structure has a strong absorption in the wavelength region 630-714 nm. Purpurins are insoluble in aqueous media.[35]

Phtalocyanines and Naphthalocyanines Phtalocyanines and naphthalocyanines strongly absorb red light with maxima around 670 nm and 770 nm, respectively. When chelated with metal ions, such as, Zn and Al, they exhibit high triplet yields and longer triplet lifetimes. The dyes can be sulphonated to various degrees to enhance the water solubility. The central macrocycle consists of a tetrapyrrole unit, but in contrast to porphyrin, the pyrrole sub-units are linked by nitrogen atoms rather than by methine bridges.[36]

Porphycenes Porphycenes are porphyrin isomers with high absorption peaks between 550 and 650 nm.

Texaphyrins Texaphyrins are expanded, porphyrin-like macrocycles which complex large metal cations. The dyes have a high absorbance peak in the near infrared, 730-770 nm. Due to the absorption profile, treatment light at these longer wavelengths can be used to obtain an increased penetration depth. These photosensitizers can be used for thicker lesions and potentially also for pigmented lesions, such as, melanomas due to the decreased absorption of melanin in the near infrared.[37] One compound that has reached clinical trials, is lutetium texaphyrin (LUTRIN).

Other photosensitizers that can be mentioned, are Pheophorbides and Nile blue derivatives, the latter being an example of a non-tetrapyrrolic agent.

3.2.2. INTRAVENOUS DRUG ADMINISTRATION - DISTRIBUTION, SELECTIVITY AND DRUG DELIVERY REGIMENS

Intravenous injection is the traditional way of administering photosensitizers when they are utilized in the clinical situation. There exist several theories to explain how photosensitizers with a relative selectivity accumulate in pre-malignant and malignant tissue, and several studies have been performed that support the various aspects of them. When discussing this topic, the selectivity for the malignant cells towards the surrounding normal tissue should be emphasized, since many other tissues in the organism also accumulate high levels of photosensitizers, particularly organs with large reticulo-endothelial compartments, such as the liver, spleen and kidneys.[38] However, this accumulation is of less importance in PDT, since these organs will not be irradiated with treatment light. The aspect of selectivity is presumably represented both in the uptake and in the retention of the agents in the malignancies. Most of the mechanisms believed to be responsible for the selectivity are general morphological and physiological features of malignant tissue, as will be discussed below. The full pharmacokinetic description of photosensitizing agents is not discussed.

Uptake Photosensitizing compounds are thought to be taken up in the malignant tissue both by active and by passive paths. In general, the fast angiogenesis of tumour tissue results in vessels of a lower quality that have a tendency to leak, allowing macromolecules to pass into the interstitial compartment, which often is larger in malignancies. In the case of malignant tumours growing in the brain, this is particularly important. The photosensitizer can easily access the tumour area where the normal blood-brain barrier is broken down, while an intact barrier keeps it out from the normal tissue, leading to a rather high selectivity.[39] The various photosensitizers differ in lipophilicity, and thereby, solubility in the aqueous part of the biologic compartments. Lipophilic sensitizers are known to bind preferentially to lipoproteins and hydrophilic sensitizers to serum proteins mainly albumin, after systemic administration.[40] There seems to be an increased selectivity with lipophilic sensitizers.[41] The lipophilic sensitizers bound to lipoproteins distribute within the various types of lipoproteins.[42,43] Tissue with high mitotic activity, including malignant tumour tissue, expresses an increased amount of low-density lipoprotein (LDL) receptors on the cell surface. The uptake of sensitizer molecules associated with the receptor-mediated endocytosis of LDL can therefore be of importance. The sensitizer molecules presumably solubilise within the lipid moiety of the lipoprotein particle and should thereby not interfere with the receptor-mediated uptake process. From this, it appears useful to enhance the fraction of the sensitizer carried by LDL and the administration of liposomal vesicles as a vehicle for drug administration has been tried.[44] This is particularly important with hydrophilic sensitizers, where the drug can be delivered to LDL via the sensitizer-liposome complex.

Due to a less efficient oxygen supply in fast growing malignant tumours, a lowered pH can be found in these regions. This is a result of the production of lactic acid in the anaerobic conditions resulting from a restricted blood perfusion. In an

environment with lowered pH, hematoporphyrin derivatives can be protonated, leading to an increased lipophilicity and cellular uptake. This has been shown in animal tumour models.[45] Producing hyperglycaemia during administration of the drug prior to PDT utilizing Photofrin®, has also been shown to lead to an increased treatment effect and the need for lower light doses in a mice tumour model.[46] In this case, glucose was also administered immediately after irradiation in order to take advantage of the fact that a sudden hyperglycaemia can affect the vasculature and lead to hypoxia.

Tissue localization When sensitizers are carried by albumin or globulins in an aqueous solution, the PDT effect is dominated by vascular effects, while direct tumour cell killing dominates when the drug is encapsulated in liposomes or LDL's.[40,44] Malignant tumours often have a large interstitial space where signs of inflammation are present. Much of the photosensitizers can be found interstitially, particularly the hydrophilic dyes,[47] and in the mono-nuclear inflammatory cells, preferably in the macrophages.[48] The diffusivity of the photosensitizer after it has passed into the tissue, is also important. Studies with several systemically administered photosensitizers did show that the cellular level of the agent and the direct killing of tumour cells decreased with increased distance from the blood supply.[49] An inhomogenous distribution of the photosensitizer was registered in most of these cases. The rate of diffusivity was not dependent on the degree of lipophilicity. BPD and ALA exhibited the most homogenous distribution of the compounds studied. For topical administration, this feature is particularly important.

Intracellular localization The intracellular localization of photosensitizing agents has been studied mainly by biochemical methods involving differential ultracentrifugation of homogenised cells, with examination of the obtained fractions, and by fluorescence microscopy of morphology.[50] In general, lipophilic, anionic photosensitizers localize in membrane structures, *i.e.* the plasma membrane and the membranes of mitochondria, endoplasmatic reticulum and nucleus. Aggragated and hydrophilic photosensitizers can be taken up by endocytosis and can accumulate in lysosomes.[51,52] Some cationic photosensitizers preferentially accumulate in mitochondria due to the electrical potential gradient across the mitochondrial membrane. *In vitro* studies with some of these photosensitizers, namely rhodamine and cyanine dyes, led to highly selective damage of the mitochondria in tumour, as compared to normal, cell lines.[53]

Retention In order to take advantage of the preferred retention of photosensitizers in malignant tissue, one waits for some time after administrating the agent, before performing the light irradiation. For HPD, the period most commonly applied is 2-3 days. For newer sensitizers, this period is significantly shortened. For certain second-generation photosensitizers it is just a couple of hours. The mechanisms behind a selective retention of active substances, are not fully known. One suggested explanation is that malignant tumour tissue often has a poor lymphatic drainage. The elimination of photosensitizers along the lymphatic route might therefore slow down.

3.3. Endogenous photosensitization with δ-aminolevulinic acid

When the mode of tissue photosensitization with endogenous porphyrins was introduced, it started in certain aspects a new era within the field of PDT. In this case, the target tumour tissues can themselves produce the photoactive protoporphyrin IX (PpIX) for sensitization following the administration of the precursor, δ-aminolevulinic acid (ALA). This is basically a different concept requiring other types of knowledge about the porphyrin biochemistry. Much of this knowledge has been gained through studies related to porphyric diseases (see Section 3.1.3). In 1956, Berlin *et al.* studied the pharmakokinetics of ALA in humans (including the authors) and rats by radio-labelled ALA.[54,55] Malik *et al.* induced PDT in leukemic cells by ALA-induced PpIX sensitization in 1987.[56] In 1990, El-Far *et al.* discovered a selective tumour localization of endogenous porphyrins induced by ALA.[57] The full treatment potential of the method was recognized by Kennedy and Pottier, who started to treat malignant tumours in the skin of humans by administering the ALA topically, and published their initial results in 1990 and 1992.[58,59] After this pioneering work, the field of PDT experienced a new uprising due to the many advantages of this modality, as discussed below.

The work performed in Lund within this field started in 1991 and as such was quite early. The number of groups that today are working with ALA-mediated PDT is continuously increasing and a substantial number of papers has been published on the topic.

3.3.1. BASIC PRINCIPLES

ALA is a naturally occurring, 5-carbon, straight-chain amino acid. It is easily dissolved in water, and in a large variety of solvents and/or emulsions. At physiological pH, it takes the form of a zwitter-ion. At a pH around 5 and even lower, ALA is chemically stable, but can react irreversibly and non-enzymatically to several condensation products at higher pH values.[60]

ALA is a precursor in the synthesis of haem, which is the porphyrin molecule PpIX with an iron atom in its ferrous (Fe^{2+}) state incorporated into its core. The haem biosynthetic pathway takes place partly in the mitochondria and partly in the cytosol as illustrated in Figure 3-2. All mammalian, nucleated cells can synthesize haem, the haem cycle being expressed to various degrees. Cells in a proliferative state express the cycle to a higher degree, the principal production sites being the haematopoietic system and the liver. In this process, a total of eight straight ALA molecules are transformed to the tetrapyrrolic macrocycle of PpIX.[61] ALA itself is formed from the condensation of glycine and succinyl-coenzymeA (CoA), catalysed by δ-aminolevulinic acid-synthetase (ALA-S). This enzyme is located in the inner mitochondrial membrane and has been considered to have a main regulatory function in the synthesis. Thereafter, the formation of a ring structure takes place in the cytosol. In the first step, two ALA molecules form one pyrrole ring structure, porphobilinogen. Four of these molecules are in turn polymerised, forming the tetrapyrrole macrocycle

uroporphyrinogen III. Several steps of decarboxylations and oxidations then occur when the molecule re-enters into the mitochondria, where the protoporphyrinogen is finally oxidised to PpIX by the enzyme protoporphyrinogen oxidase. Protoporphyrinogen can also diffuse into the cytosol and there be converted to PpIX by non-specific oxidation processes. The last step has a major rate-limiting function, which is the incorporation of Fe^{2+} into PpIX by the enzyme ferrochelatase. Ferrochelatase spans the inner mitochondrial membrane with its active site towards the matrix. In situations with iron depletion (for instance when iron-chelators are added to induce PpIX accumulation), ferrochelatase can instead incorporate zinc into the PpIX molecule, yielding the fluorescent Zn-protoporphyrin. All enzymatic steps in the process are irreversible. There is a negative feedback in the system, accomplished by the final product, namely haem. Haem exhibits a negative feedback on the enzyme ALA-S at several stages. There is an action directly on the final protein product, as well as on the stages transcription, translation and transport into the mitochondria.

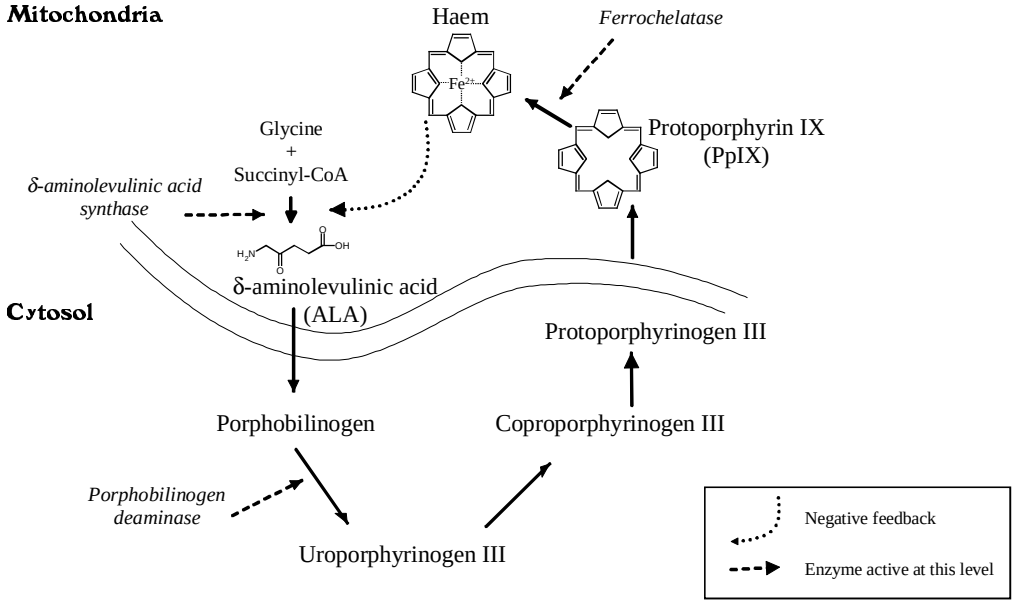

Figure 3-2 *The synthesis of haem: The enzymatic steps that are important for the accumulation of PpIX in cancerous tissue are shown. The molecular structure of ALA and a sketch of the tetrapyrrolic main-core of PpIX, are shown. The negative feedback performed by haem is also marked.*

When supplying ALA in excess, the negative feedback in the system is bypassed. One also exceeds the capacity of the ferrochelatase. In addition, in some pre-malignant and malignant lesions, the enzyme catalysing the third step, porphobilinogen deaminase (PBGD), has been shown to exhibit an increased activity,[62-64] and ferrochelatase has been shown to have a reduced activity.[62,64-67] The

result is a relative selective accumulation of porphyrins, mainly PpIX, in malignant tissue.[68,69] Protoporphyrin IX has a natural "clearance mode" by its subsequent transformation to haem, but this is restricted to the PpIX available as substrate for ferrochelatase, *i.e.* mainly PpIX localized in the mitochondria. Chemically, PpIX is best characterized as an lipophilic structure. In an aqueous environment, it exists as dimers and aggregates.[32] The PpIX-related fluorescence in tissue has a dual peak at about 635 and 705 nm (Papers IV, VI and VII). When dissolved in human plasma one of the absorption peaks of PpIX is in the red wavelength region, at about 635 nm.[70]

3.3.2. KINETICS

One of the main advantages with ALA-induced PpIX for photosensitization besides the possibility of topical administration, is its fast clearance. This is also the case when ALA is given systemically. Much effort has been put into finding the best timing for both PDT and laser-induced fluorescence (LIF) for tissue diagnostics.

Berlin *et al.* found that radio-labelled ALA, given in an oral dose of 35 mg/kg b.w. to a human, was subject to rapid excretion through the kidneys, with about 50% excreted during the first 8 hours.[55] In four patients receiving an oral dose of 60 mg/kg b.w., peak plasma levels of PpIX were reached after 8-12 hours.[71] The elimination showed first-order kinetics with a half-life of 8 hours. After various routes of ALA administration, Rick *et al.* found a peak plasma level of PpIX to occur 6.7 hours after an oral dose (40 mg/kg b.w.), 4.1 hours after inhalation (500 mg) and 2.9 hours after intravesical installation (1 g).[72] After oral administration, the PpIX level was a 100-fold elevated, after the inhalation and instillation it was comparable with reference values. Neither ALA nor PpIX was detectable in the plasma after ALA application on the skin. Plasma ALA peaked 30 minutes after the oral dose and was back to baseline levels in less than 8 hours. Measured by fluorescence, the PpIX level in the skin following the oral dose peaked between 6.5 and 9.8 hours.[72] When administering treatment doses of ALA (30-60 mg/kg b.w.), PpIX levels as detected by fluorescence, peaked later than 4 hours.[68,73] Utilizing low doses of orally administered ALA (5 and 15 mg/kg b.w.), we found a peak PpIX level, as detected by LIF, to occur after 3-4 hours in normal skin and in mucosa of the ear-nose-throat region (Paper IV). Measurements showed that a squamous cell carcinoma (SCC) in the oral cavity, showed higher levels for a longer period of time. After 24 hours, in all tissues, PpIX was hardly detectable with the system used.

The PpIX detected in tissue is considered to be mainly generated *in situ*. This is based on several observations: When ALA is applied topically on the skin, a certain level of PpIX is also built up in normal epidermis (Paper VI). PpIX is generated so quickly in malignant and normal tissues after oral administration, that a transport from other sites of production can hardly be possible on this timescale (Paper IV). Furthermore, the tumour-to-normal ratio of PpIX fluorescence does often not vary significantly between oral[73] as compared to topical administration (Paper VII), but the plasma levels of PpIX are significantly different in these two situations.[72]

3.3.3. TISSUE SELECTIVITY

With ALA-PDT, the situation regarding uptake and retention of the photosensitizer is different from when utilizing traditional photosensitizers. This is due to the fact that the administered agent, the small amino acid molecule of ALA, is significantly different from the large pre-formed photosensitizers, and thus might take other routes to reach the malignant tissue. In addition, the uptake seems to be of less importance. What is important for the accumulation of PpIX, is to what degree the haem cycle is active in cells and the degree to which it is altered. As discussed, the generation of PpIX can be seen in proliferative cells. Besides the liver[74] and haematopoietic system, the normal tissues shown to produce PpIX following ALA administration are the tissue lining the internal organs and the skin. Most epithelial tissues have been shown to generate some levels of PpIX, *i.e.* the epidermis,[75] the urothelium,[76,77] and the gastrointestinal tract.[73] However, it is always restricted to these covering tissue layers superior to the basal membrane.

In the case of topical application of ALA to skin malignancies, the penetration of ALA into the tumour is usually simplified. This is due to a tumour-induced damage of the epidermal component of the skin, particularly the outermost protective keratin layer, which is the main barrier for the penetration of ALA. This is often referred to as the "leaky roof" phenomenon, and it is important in the selective build-up of PpIX in skin lesions. Protoporphyrin IX can be found in the mitochondria, where it is generated, making this organelle one of the primary sites of damage.

3.3.4. PORPHYRIN GENERATION IN MICROORGANISMS

When supplying ALA, several bacteria can generate porphyrins and also be subject to subsequent photoinactivation. Haemophilus parainfluenza, in contrast to Haemophilus influenza, was shown by van der Meulen *et al.* to produce porphyrins with a maximum fluorescence at 617 nm, following incubation with ALA. Illumination with red light led to bactericidal effects.[78] Seen in the perspective of a growing bacterial resistance against antibiotic treatment, there are possible clinical implementations in this field.

3.4. Light sources

Both coherent (see Section 2.1.1) and non-coherent light sources can be utilized for the therapeutic PDT irradiation. The laser is a coherent light source, while filtered lamps and light emitting diodes (LEDs) are non-coherent. As will be described below, the different sources have both advantages and disadvantages.

Lasers The laser has been considered the ideal light source for PDT. This is due to its monochromatic and coherent nature. The monochromacy permits the light to match exactly an absorption band of the sensitizer in the area where the penetration in tissue is at its largest (see Section 2.2.1), and thus avoid heating the tissue with light that is not active in the photochemical reaction. The coherence allows the light to be easily focused into optical fibres and transported into the body cavities or through the lumen

of needles into tissue for interstitial illumination. Traditionally, lasers operating in the red wavelength band needed for HPD-PDT have been originally produced for scientific or industrial purposes, *i.e.* argon-ion or copper-vapour lasers pumping dye lasers, or gold-vapour lasers. The disadvantages with these lasers are the size and complexity of management. The advantage with dye lasers is the possibility of changing the dye, and thereby also the emission wavelength within a certain region, making it possible to use the same laser in combination with various photosensitizers. Later, frequency-doubled Nd:YAG (Neodynium: Yttrium-Aluminum-Garnet) lasers have been used as pumping lasers for dye lasers. Thus, such a system acquired in 1991 (repetition rate 5 MHz, pulsewidth 100 ns), has been extensively used in Lund, mainly for ALA-PDT employing an emission wavelength of 635 nm. Still, this was a relatively expensive and voluminous laser with a weight of about 300 kg. It has generally been acknowledged that regarding the light source, much smaller and cheaper sources are needed before PDT can become a treatment modality for common use.

As for monochromatic light sources, the diode lasers are the most interesting. Diode lasers have been on the market for many years, but just very recently, units with the right wavelength and sufficient output power for ALA-PDT have become commercially available. The first of these diode lasers emitting at 633 nm for PDT, was acquired to Lund in January 1998. This is a continuous wave (CW) laser with a total output power of 1.5-2 W, weighing only 15 kg (Ceralas PDT635, CeramOptec, Bonn, Germany). For the practical, clinical situation this is a significant improvement. Besides having a convenient size, diode lasers are also reliable and easy to use.

Lamps and LEDs Filtered lamps are other possible light sources in PDT, which have already proved to be very useful, particularly in the treatment of skin lesions with ALA-PDT.[59,79,80] The advantages are a low price and small size. In principle, lamps can only be used for superficial illumination. Although filtered, the wavelength band achieved is also quite broad. Bands of 50-130 nm have been utilized.[79,80] The emitted light outside the absorption band of the sensitizer, was earlier thought to have no other purpose than inducing hyperthermia. This hyperthermia might give an increased tumouricidal effect as has been shown with HPD-PDT in animal models.[81] In Lund, we have mostly tried to avoid the induction of simultaneous hyperthermia during PDT in order to be able to separate the effects of PDT from hyperthermic effects. However, more and more focus is now put on the photoproducts generated during the illumination, particularly in the case of ALA-PDT (see Section 3.6.6). Utilizing a lamp as a light source makes it possible to simultaneously excite also the photoproducts and utilize their possible photosensitizing capacity. In Lund, a filtered lamp has been available since 1994. This lamp can be filtered in the blue (bandwidth 380-450 nm), green (bandwidth 520-590) and red (bandwidth 580-720 nm) regions, with an additional irradiation in the IR (1200-1700 nm) region for the green and red filtered modes. The IR band can be used for adding hyperthermia, but can also easily be removed with a simple water filter. The red band has been used for clinical ALA-PDT,

but no controlled comparison has been performed between the lamp and the laser as light sources.

Light emitting diodes (LEDs) are also non-coherent light sources that can be used for PDT; however, until now they have not been widely used. They emit in wavelength bands that are much wider than those from a laser, typically around 25 nm.[82]

3.5. Dosimetry

Penetration depth The limited penetration depth of the treatment light has usually been seen as one of the most important limiting factors in PDT. For light in the red region around 630 nm, a "rule of thumb" is that about 2-5 mm of tissue can be treated, depending on the optical properties of the tissue, and the dosimetry used. On the other hand, there are situations in which a limited penetration depth of the light is exactly what is needed. One example is in the treatment of the superficial, intraepithelial lesions of dysplasia and carcinoma *in situ* (*cis*) in hollow organs, such as the urinary bladder or the colon. In these organs, there is a risk of extensive damage with subsequent shrinkage, and also always a small risk of penetration of the organ wall. In these cases, treatment with green light might be sufficient. When ALA-PDT is applied, the sensitization of the tissue itself sets the limits, as only the tissue over the basal membrane is sensitized. Due to the light scattering in tissue (see Section 2.2.2), the fluence rate in the outermost cell layers, can be several times the fluence rate originating from the light source. Predicted values of light penetration in skin tumours at 635 nm, utilized for ALA-PDT, indicate that treatment effects can be reached to a depth of at least 3 mm.[7] When ALA is applied topically for 3-4 hours, fluorescence microscopy studies of BCCs have revealed that the deeper layers of the lesions, are not sensitized.[75,83] With such regimens of topical application with ALA, this means that the limitations in the treatment are due to the penetration of ALA, rather than to the light. A mathematical definition of penetration depth is where the light has been reduced to 1/e = 2.72 of the incident level.

Pulsed versus continuous light The efficacy of continuous wave (CW) light has been compared with that of pulsed laser light with high peak power. Some experimental work has been performed utilizing HPD as photosensitizer. Cowled *et al.* found no differences *in vitro* and *in vivo* between pulsed and CW irradiation.[84] Shikowitz found *in vivo*, that pulsed irradiation gave a better initial tumour response, but did not improve the overall cure rate. Theoretical analysis performed by Sterenborg *et al.*, predicted that pulsed excitation with peak fluence rates below 4×108 Wm^{-2}, is identical to CW irradiation.[85]

Split dose therapy There are results indicating a possible superior treatment effect by splitting up the irradiation procedure. Various explanations are possible, partly depending on the length of the dark periods. Re-oxygenation of areas where the oxygen was depleted by the photochemistry is probably the most important effect. The

re-oxygenation is partly due to diffusion of oxygen from the capillaries and partly by relaxation of vasculature that has been subject to vasoconstriction, the latter taking longer time than the first. In a tumour model, light delivery with 30 seconds on-and-off exposure, gave increased PDT effects.[86] Messmann *et al.* found that in the normal rat colon, sensitized with ALA-induced PpIX, the introduction of a break of about 150 seconds markedly increased the volume of necrosis, and the total light dose given could be reduced.[87] A redistribution of the photosensitizer has also been discussed as a contributing factor. Another explanation in the time-scale of minutes, can be that of re-perfusion injury.

Fluence rate The use of lower fluence rates has been shown in several studies to increase the treatment effect. With high fluence rates, there is a risk to deplete the oxygen in situ, when it is consumed faster than it can be replenished and thereby stop the photochemical reaction. This is avoided by using lower fluence rates. Mathematical modelling of oxygen consumption and diffusion during PDT with fluence rates of 50 mW/cm^2, estimates that cells sufficiently remote from a capillary, may reside at oxygen levels low enough to preclude or minimise damage mediated by singlet oxygen. This effect will be more pronounced with higher fluence rates.[86] *In vitro* studies with Photofrin® showed a significant improvement when reducing the fluence rate from 200 to 25 mW/cm^2.[88] *In vivo* studies of ALA-PDT on normal mouse skin, showed that substantially more tissue damage was induced by 5 mW/cm^2 than by 50 mW/cm^2.[89]

Total light dose The total light dose measured in J/cm^2, given during PDT, is calculated as the product of the fluence rate and time:

$$\text{Total light dose (J/cm}^2\text{)} = \text{fluence rate (W/cm}^2\text{)} \times \text{treatment time (sec)}$$

For PDT in general and particularly for ALA-PDT, a wide range of light doses has been implemented, both when utilizing lasers and filtered lamps, ranging from 30 J/cm^2 to 540 J/cm^2.[58,90] Utilizing filtered lamps, the dose should be adjusted so that the part of the light that actually can excite PpIX, is sufficient. In Lund the most frequently used total light dose has been 60 J/cm^2. With a fluence rate of 0.1 W/cm^2, this implicates a typical treatment time of 600 seconds for one area. The general idea is that one should illuminate as long as there are photosensitizer molecules left. The presence of photosensitizer molecules in the superficial tissue can be detected by LIF measurements. In Lund, we have followed the PpIX level at the surface with this method during ALA-PDT (Paper VI-VII). Utilizing a total light dose of 60 J/cm^2, the PpIX-related fluorescence intensity after illumination was quite persistently 8-10% of the initial level, regardless of the level before the illumination. From this, we know that the dose is probably not unnecessarily high, but the measurements do not include information on the PpIX level in the deeper layers.

Application modes The most common light application mode is by illumination from the surface, either on the skin (Figure 3-2-a) or in the body cavities. In the body cavities, there are several ways of delivering the light, depending on the geometry of the organ. For localized treatment, spot irradiation can be used, as for the skin (Figure 3-3-b). Tube-formed structures, like the oesophagus, are suitable for side illumination utilizing diffusing fibre tips or specially designed diffusers (Figure 3-3-c). For organs with an approximately sperical shape, spherical illumination with a microsphere at the end of the fibre might be used (Figure 3-3-d), but should often be exchanged for specially designed light applicators (see below). Interstitial illumination through several fibres allows illumination of larger tumour volumes (Figure 3-3-e).

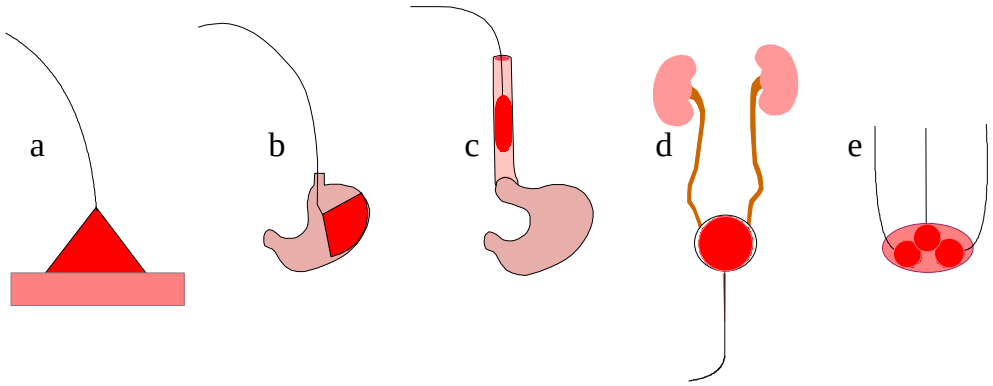

Figure 3-3 *Various light application modes for PDT. a) Superficial (microlens). b) Intracavital superficial. c) Intraluminal superficial. c) Intracavital superficial. e)Interstitial.*

An even illumination over the whole treatment field, *i.e.* "top hat" distribution, is necessary to be able to perform a reasonable dose planning. For focal illumination, this can be obtained by utilizing fibres with a microlens on the tip. Another way is to image a polished fibre tip onto the tissue through a microscope objective. The latter has been utilized in Lund with an objective of 20× magnification. Dose-planning with the other variants depicted in Figure 3-3 (b,c,d,e) is more complicated. This is mainly due to the back-scattering from the opposite organ walls which means that lower total light doses are needed. Another complication is that the body cavities are far from being perfectly cylindrical or spherical, but are often rather asymmetrical and folded. Several applicators shaped after the cavities and with systems for diffusing the light, have been produced, in order to get a more homogenous light distribution.[91] Such applicators can facilitate illumination in, for instance, the oral cavity, the uterus and the bladder. In the latter case, it might also keep the organ distended, avoiding an uneven light distribution in a folded mucosa.

Understandably, the dosimetry can be quite complicated in many cases.[92] This is also true for interstitial treatment. To solve these problems, various systems for detecting the in situ fluence rate and/or light dose, have been developed.[91,93] They

allow real time adjustment of the illumination, so that all areas receive the same planned treatment dose.

3.6. Mechanisms in photodynamic therapy

There are three essential constituents in the PDT reaction; the photosensitizer molecule, light with appropriate wavelength and the presence of oxygen. The interaction among these three constituents is the main part of the treatment effect. The mechanisms involved when utilizing ALA-induced PpIX as the photosensitizer are the same as when utilizing pre-formed agents. In the following, the treatment effects will be divided into direct cell damage and secondary effects, mostly for didactic purposes, since the mechanisms are dependent on each other and presumably interact in a complex way. The various effects may also be different for the various classes of photosensitizers.

3.6.1. MOLECULAR ASPECTS OF THE PHOTOSENSITIZER

Sensitizer molecules can be found as either monomers or in aggregated states. The relative proportions vary and can be detected by measuring the fluorescence lifetime that decreases in the aggregated state.[94] The photochemical properties of monomers and aggregated states of the photosensitizers are different. The monomeric fraction is the one that can participate in most photochemical reactions, since in this case, all the molecules can easily come in contact with the surrounding oxygen.

3.6.2. PHOTO-OXIDATION AND GENERATION OF TOXIC OXYGEN SPECIES

The most important messengers in the processes causing cell damage during photodynamic therapy, are oxygen species in various toxic states. *In vitro* studies have shown that during de-oxygenation of the surroundings, photoinactivation is totally halted.[95-97] From this aspect, PDT can be paralleled with ionizing radiation therapy, where well oxygenated tissue is more prone to destruction. Limitation in oxygen content can occur during treatment, either as a result of vascular damage or by depletion of the local oxygen concentration in the tissue during PDT (see Section 3.6.4).

Molecular oxygen in an excited state is the species considered to play the most important role. This is generated in a process usually referred to as Type II photosensitization, where the photosensitizing molecule in its excited triplet state (T*) transfers energy to molecular oxygen resulting in singlet molecular oxygen ($^1O_2$). To acquire this, the triplet state energy of the photosensitizing compound should be higher than the excitation energy of the triplet state of O_2 (7900 cm^{-1}). Experimental evidence for the predominance of the Type II mechanism is *in vitro* studies, showing that singlet oxygen scavengers suppress the photocytotoxic effect.[98,99] Singlet molecular oxygen reacts rapidly and indiscriminately with electrophilic biomaterials (R), such as unsaturated lipids, proteins, nucleic acids etc. The sequence of reactions will be as follows:

Type II reaction $T^*+O_2 \rightarrow S_0+{}^1O_2$ (1)

${}^1O_2{}^*+R \rightarrow$ photo-oxidation (2)

A highly efficient photosensitizing compound has a singlet oxygen yield that approaches the triplet state yield. After the transfer of energy from the triplet state to oxygen, the photosensitizer may go back to its ground state, and the cycle can be repeated as indicated in (1).

The diffusion length of singlet oxygen before it is quenched or reacts in a biological tissue has been estimated to be about 0.01-0.02 μm, corresponding to a lifetime in the order of 0.01-0.04 μs.[100] This value corresponds to less than one percent of a red blood cell diameter (approximately 7 μm). The lifetime and solubility of singlet oxygen is considerably shorter in a cellular compartment as compared to in solutions.[101] From these considerations, the localization of the sensitizer in a surrounding where it can cause lethal damage is probably as important as the total concentration of sensitizer molecules.

Type I reaction A Type I reaction is the other possible way of energy transfer from the photosensitizer. In this type, the energy is also transferred from the metastable triplet state of the photosensitizer molecule, but not via oxygen. In this reaction, there is a direct interaction between the photosensitizer and the substrate, which can involve hydrogen abstraction or electron transfer.

3.6.3. SUB-CELLULAR AND CELLULAR DAMAGE

The degree of lipophilicity of the photosensitizers determines localization, and therefore site and type of damage of the cells. In general, lipophilic photosensitizers accumulate in the membranes of the cell and its organelles. Photooxidation of lipids and proteins in the cell membranes triggers an activation of membranous phospholipases, leading to a fast degradation of damaged phospholipids, changes in fluidity and a rapid loss of cell integrity. There is cross-linking of amino lipids and polypeptides, and inactivation of membrane-associated enzyme systems and receptors. The cell membrane depolarises with a fast collapse of the ionic balance.[102,103] Hydrophilic, as well as aggregated states of photosensitizers, are likely to be taken up by endocytosis and pinocytosis with subsequent localization in lysosomes and endosomes.[51,52] Following light exposure, lysosomal enzymes will be released to the cytosol. Photosensitizers localized in the cytosol can bind to tubulin, where photodamage leads to a de-polymerization of the microtubuli with accumulation of cells in mitosis, and subsequent cell death.[51] Inhibition of microtubuli formation is also the mechanism involved for some chemotherapeutic drugs. The probability of cell inactivation per quantum of absorbed light, is generally lower for hydrophilic photosensitizers than for lipophilic ones, indicating that the membranous structures are the most vulnerable.[104]

3.6.4. SECONDARY EFFECTS

Foci of viable tumour cells that escape the direct cell toxicity during PDT may be subject to secondary effects of photodamage. The mechanisms usually attributed significance are effects on the blood supply of the tissue, initiation of apoptosis, inflammation and induction of an immune reaction towards the tumour.

Vascular effects PDT causes major effects on the microvasculature of the tumour, usually leading to vascular stasis.[97] Since the photochemistry is dependent on oxygen supply, the induction of tissue hypoxia/anoxia may theoretically "cut both ways". On one hand, it can increase the PDT effect by hypoxic cell damage, while on the other hand, the photochemical process can be halted due to insufficient oxygen supply resulting in sublethal damages only.

With systemic administration of the photosensitizing compounds, the first site for accumulation seems to be the endothelial cells. After systemic administration of most photosensitizers apart from cationic compounds, the major determinant of vascular photosensitivity seems to be the level of circulating photosensitizer.[105] Cell studies utilizing ALA-induced PpIX as photosensitizer have shown that endothelial cells are sensitive to PDT-induced cytotoxicity when proliferating, as is the case in the tumour micro-environment.[106] In the same study, vascular smooth cells produced enough PpIX to be sensitive to PDT. When studying human umbilical vein endothelial cells (HUVEC), Yang *et al.* have shown that several tumour cell lines secrete angiogenic factors that increase HUVEC proliferation and sensitivity to PDT with aluminum-sulfonated phtalocyanine (AlSPc).[107] Damage to the endothelial cells results in thrombogenic sites along the vessel walls, with exposed basement membrane, initiating a cascade of physiological responses leading to vascular stasis. Permeability and leakage of albumin are increased over the damaged vessel wall,[108] which lead to oedema in the treated area. Reduced perfusion in PDT treated tissue with systemic photosensitizer administration, is due to thrombosis formation.[74,109] It is also due to genuine vasoconstriction, which mainly takes place in the arterioles, and only at higher doses of photosensitizer (Photofrin®) in the venules.[108] Release of a number of vasoactive substances has been associated with PDT.[110] Fingar *et al.* have studied the eicosanoid substances, and shown a raised thromboxane B_2 level in connection with PDT with Photofrin , in humans and rats.[111] In rats, a raised leukotriene B_4 level was also seen. In the rats, thromboxane inhibitory agents, indomethacin and imidazole, led to a decreased treatment effect in tumours.[111] Microvascular observations in rats have also shown that indomethacin can inhibit the PDT-induced vasoconstriction.[108] From a treatment point of view, this might be considered if anaesthetic agents are to be given, avoiding to use the non-steroidal anti-inflammatory drugs (NSAIDs).

By utilizing laser-Doppler imaging (LDI) to detect the blood flow of tumour tissue, we have observed a difference between systemic and topical administration of ALA for PDT (see Section 7.2.1). In a rat liver-tumour model sensitized systemically, the perfusion decreased,[74,112] whereas in BCC lesions in humans with topical

application, the blood-flow increased, in tumour and surrounding tissue immediately after PDT (Papers I and VIII).

Apoptosis In addition to direct cell death through necrosis, the fast induction of apoptosis, *i.e.* programmed cell death, is thought to play an important role in PDT.[104] During apoptosis the genetic apparatus of the cell starts a program to destroy damaged cells without involving the inflammatory system. The injuries leading to apoptosis are usually less pronounced than those initiating necrosis. Morphologically, there is a condensation of the cytoplasm with subsequent disintegration of the cell into membrane-bound vesicles that subsequently will be phagocytised by neighbouring cells. The degradation process is characterized by an intact function of the membranes and the organelles, which means that minimal amounts of inflammatory mediators, *e.g.* lysosomal enzymes, are released. During apoptosis, there is a typical fragmentation of DNA, which is usually detected by gel electrophoresis showing a typical ″ladder″ pattern. Cancer cell populations that through mutations have defects in the apoptotic programme, are known to be resistant towards chemotherapy and ionizing radiation.[113] An apoptotic response after PDT was first shown by Agarwal *et al.* in 1991,[114] and has been observed for PDT both *in vitro*[114-116] and *in vivo*.[117] There is an association between photodamage of the mitochondria and an apoptotic cell response.[115] In ALA-PDT, where the production site of the photosensitizing agent PpIX is the mitochondria, apoptosis is expected. This has also been shown *in vivo*,[117] and *in vitro*.[118] In the latter studies by Noodt *et al.*, the apoptotic response was shown to be cell-line dependent.

Inflammation/immunology PDT usually initiates a strong inflammatory reaction in the tissue treated. This is visualised in Paper III, Figure 4, showing an ALA-PDT treated BCC lesion on the abdominal wall of a patient. Directly after the treatment, there is erythema and oedema also affecting the tissue outside the illuminated area and being limited by the dermatome in which the treated lesion is situated. The phototoxic damages in the cell membrane lead to the release of metabolites of membrane lipids that are strong activators of the inflammatory reaction. Macrophages are one of the important cells in the immediate inflammatory reaction, and in a tumour model in mice, the PDT effect was increased when localized treatment with GM-CSF (granulocyte-macrophage colony-stimulating factor) was performed prior to PDT.[119]
The treatment also starts an extended immune reaction that probably is important in the long-term control of the tumour, through a sensitization of the immune system towards the tumour cells. Transplantation studies of immunoactive tissue between immunocompetent and immunodeficient mice,[120] have shown that an intact immune system is necessary for achieving a full tumour cure after Photofrin®-PDT. Furthermore, memory cells of the immune system, *i.e.* lymphocyte sub-populations, could be recovered from distant lymphoid tissues after PDT. Sensitization of the immune system towards even less immunogenic tumour types, theoretically gives the opportunity of systemic cancer treatment at distant metastatic locations.

3.6.5. HYPERTHERMIA IN CONNECTION WITH PHOTODYNAMIC THERAPY

When performing PDT, the question of simultaneous hyperthermic effects will arise naturally, since the treatment light can be adjusted to fluence rates sufficient for light-induced hyperthermia (in biological tissue corresponding to a temperature rise to about 41-47°C). In general, adding hyperthermia to the treatment regimen would be interesting as long at it leads to an additive or synergistic effect. Due to the dependence of PDT on oxygen, there is a limitation in the system at the level where tissue perfusion is compromised by hyperthermia.

To be able to discriminate between photochemical effects and hyperthermic effects and to perform controlled studies, many groups like ours, have utilized fluence rates that are below the threshold of hyperthermia. At 514 nm, it has been demonstrated that for power densities less than 150 mW/cm^2, no hyperthermic effects are contributing.[121] Warloe *et al.* detected the skin surface temperature during ALA-PDT with laser light at 630 nm. With a fluence rate of 100-150 mW/cm^2, a temperature rise to 39-40°C occurred during the first minutes, followed by a stabilization at 37-38°C.[122] Delivering light of 100 mW/cm^2 from a non-coherent light source including approximately 25% infrared light to human skin and skin tumours, did lead to a temperature increase to 39.5-42.5°C in tumour and to 42-43.5°C in normal tissue.[123] These temperatures can possibly add an effect of mild hyperthermia. When irradiating at high fluence rates, there is a possibility that the excitation of photosensitizers overrides the available level of oxygen. The photosensitizers can then be photodegraded (see Section 3.6.6) without contributing to the photodestruction of the tissue. Considering this fact, it has been proposed that when performing a combined treatment regimen, photoactivating light of a moderate intensity should be used together with wavelengths incapable of exciting the photosensitizer, preferably infrared irradiation. The hyperthermic light should not be given before, but together with or directly after the PDT light, in order not to compromise the oxygen supply needed for PDT.[124,125]

3.6.6. PHOTOBLEACHING AND ROLE OF PHOTOPRODUCTS

Several studies utilizing laser-induced fluorescence *in vivo*[126-128] (Paper VI) and *in vitro*[129] have demonstrated that the drug is degraded during the PDT procedure, resulting in a decreased drug-related fluorescence, a phenomenon called photobleaching or photodegradation. In parallel, when utilizing porphyrins as sensitizers, agents fluorescing at other wavelengths have been noticed to appear transiently during the illumination procedure. These agents are usually termed photoproducts. The therapeutic inactivation of the sensitizer molecule and its transformation to photoproducts is oxygen dependent and results from the interaction of the sensitizer with activated oxygen species, mainly $^1O_2$.[130,131] Also, the photoproducts are thought to be degraded in the same way.[131,132] Photoproducts emit fluorescence in the red visible region and are thought to be of a chlorin type.[129,131] When utilizing HPD, a photoproduct absorbing about 640 nm has been observed,[129]

and during PDT with ALA-induced PpIX as sensitizer, an evident photoproduct emitting fluorescence at around 670 nm has been reported.[127]

The photobleaching effect has traditionally been considered to play a protective role in normal tissue in close vicinity to the tumour that is illuminated. A certain amount of lethal/sublethal incidents are necessary to induce cell death. In a normal cell where the amount of sensitizer molecules present are insufficient to generate cell damage, photobleaching can clear the cell for further damage. Subsequent illumination to deliver enough light to the deeper layers will then have no further effect on the normal tissue. To what extent the photoproducts can be photoactive and contribute to the treatment effect, is still not clear. What has been verified is that they seem to be quite short-lived before they again are subject to photodegradation. In the case of ALA-induced PpIX sensitization, much interest has been paid to the photoproducts due to the fact that many groups are using broadbanded light sources for PDT in skin lesions. Various lamps (see Section 3.4) are used that cover the wavelength regions where the photoproducts absorb, and could potentially simultaneously excite several agents. During illumination with 635 nm of PpIX in tissue, we have observed photoproducts appearing in-between the two peaks of PpIX, at about 670 (PaperVI).

3.6.7. MUTAGENICITY

The question of possible mutagenic effects induced by PDT is of extreme importance as with all treatment modalities. This question is also important since the treatment potential of PDT is under investigation for non-cancerous diseases. Comparable treatment modalities besides surgery, for various malignant diseases, are ionizing radiation and also to a certain extent, chemotherapy. Both these modalities have the potential to inducing secondary malignancies in patients who live for several decades after their primary disease is cured.

First of all, PDT is favoured by being a local treatment modality, meaning that small volumes of normal tissue are affected, generally, considerably less than those affected by treatment with ionizing radiation. In general, the risk of inducing mutagenic effects by PDT is considered to be low, but should be taken into consideration. Most of the photosensitizers used are lipophilic and anionic and are thereby not retained in the cell nucleus, bound to the DNA. Due to the short diffusion length of the active species generated during PDT, the possibility of direct action on the cell DNA is limited. Studies of the genotoxic potential with such photosensitizers indicate a less genotoxic potential as compared to ionizing radiation, partly depending on the cell type and DNA repair capability.[133,134] Fiedler *et al.* have in cell studies shown a genotoxic potential due to dark toxic effects after ALA-induced PpIX sensitization, which can be ascribed either to PpIX or to ALA. The latter is thought to be mediated through auto-oxidation of ALA, which generates reactive oxygen species (ROS), $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$.[135] Porphyric patients experience an increased risk of developing malignancies and parallels have been drawn between an increased level of ROS in cells with an altered haem synthesis and the initiation of carcinogenesis.[31] However, compared to the porphyric diseases that are characterized by elevated levels

of the various products in the haem synthesis, for longer periods of time, photosensitization in PDT is a transient “porphyric-like” event.

Concerning the treatment light only, the possible risks of DNA damage by UV light are avoided since preferably light in the red region is used due to the tissue penetration considerations, instead of in the UV/near-UV region, where several photosensitizers have strong absorption peaks.

4. NON-MELANOMA MALIGNANT SKIN TUMOURS

The main skin lesions treated with photodynamic therapy in Lund have been non-melanoma malignant skin tumours, out of which the majority was basal cell carcinomas (BCCs). In addition, squamous cell carcinomas (SCCs) of the in situ (SCC in situ and Mb. Bowen) and the micro-invasive type have been treated, as well as some cutaneous T-cell lymphomas and extramammary Mb. Paget lesions. In this chapter, a concentration on BCCs will be made, since they constitute the focus for PDT in this thesis.

4.1. Basal cell carcinoma

Basal cell carcinoma, synonyms basalioma or basal cell epithelioma, is the most common cancer of the skin with its appendages.[136] Due to the indolent behaviour, BCC is the only malignancy that is not reported to the cancer registry in most countries. Still it can cause significant problems due to its local invasive growth or multifocal existence in a single patient. The old terminology of the disease was "rodent ulcer", which describes a lesion that has been growing for a longer period of time. Even today some lesions can become quite large before they are correctly diagnosed and treated, mainly due to patient delay. In some patients the treatment can cause a significant problem with high morbidity.

4.1.1. CLINICAL APPEARANCE

Clinically, BCCs are usually divided into five classes according to their morphological appearance[137]; 1) ulcero-nodular type, 2) pigmented type, 3) superficial type, 4) morphea-like, sclerotic or fibrosing type, and 5) fibroepithelioma.

The ulcero-nodular type is the most common, and in this group we find the classical BCC appearance; a lesion with a demarcated pearly, elevated border with teleangiectatic vessels on the surface and central ulceration. At times it can be less typical with some parts of the lesion elevated, some parts flat and some parts ulcerating. The superficial lesions are easily misdiagnosed as eczema with their red, slightly scaly surfaces. This is also the case when the lesions are highly inflamed, but not ulcerated and even on histopathological examination the BCCs can be wrongly diagnosed and allowed to develop into quite large lesions. The morphea-like BCCs are the most difficult to treat since their borders can extend both vertically and laterally far beyond what can be observed visually, due to an infiltrative growth pattern. These lesions typically present with an indurated and shiny, pale surface due to epidermal atrophy.

All BCCs can have periods with higher activity, when they present transient ulceration, crust formation and itching, followed by a phase with fewer symptoms. For this reason, it can be wrongly diagnosed as an eczema or a psoriatic plaque. A residual or recurrent tumour can often be recognized by its irritative itching. Metastasizing BCCs are extremely rare and are from a practical point of view non-existing, though it

always has to be kept in the mind of the physicians. The incidence of metastasis has been reported to be 0.0028-0.55% depending on whether the statistics are from a dermatology patient population or a surgical centre.[138] Once the BCC turns into a metastasizing tumour, its behaviour becomes extremely aggressive.

4.1.2. HISTOPATHOLOGY

Histopathologically, the BCCs grow in nests with quite uniform cells that show few anaplastic features. They resemble the basal cells of the epidermis but have a higher nucleus-to-cytoplasma ratio and lack desmosomes. An idiosyncrasy for BCCs is the palisading phenomenon, *i.e.* that the cells at the periphery of the tumour nests tend to form palisades radially to the centre of the tumour nodule. Another characteristic is the production of mucins that causes an artificial retraction phenomenon after fixation. Undifferentiated BCC growth is the most common and usually denoted as solid growth BCC. In superficial lesions, small nodules of BCC cells are seen attached to the inner surface of the epidermis. In ulcero-nodular lesions, the nodules are larger and penetrate deeper into the dermis. The BCC growth pattern can also show differentiation of growth, usually into three types of patterns, named after the structures they simulate. Those are the cystic, keratotic, and adenoid, resembling sebaceous glands, hair structures or apocrine/eccrine glands, respectively. Pigmented BCCs are nodulo-ulcerating lesions, where abnormal melanocytes are seen interspersed in the lesion. There seems to be a resistance in the tumour cells to take up melanin, resulting in an accumulation of melanin granules in tumour-related melanocytes. Such lesions can in some cases be mistaken clinically as melanomas. As opposed to the ulcero-nodular lesions, morphea-like BCCs grow as sheets of tumour cells, often only two cell layers thick, infiltrating into the surrounding tissue and it can resemble cutaneous mammary cancer.[137] In the fibroepithelioma a similar pattern as in the morphea-like lesions, can be seen, but here the strands of tumour cells are thicker, there are more nodules and a palisading phenomenon can exist along the strands.

The growth of BCCs is episodically progressing and regressing, causing variable activity in the surrounding connective tissue and various degrees of activity can even be observed simultaneously within the same lesion.[139] This causes the clinical appearance of lesions that sometimes ulcerate and sometimes heal.

4.1.3. TUMOUR BIOLOGY

BCCs are believed to arise from a pluripotent stem-cell population of the basal cell layer of epidermis and the outer root sheath of the hair follicles. The lesion is believed to arise de novo, since no precursor lesion has been identified. This is in contrast to SCCs, where actinic keratosis is considered a pre-malignant stage of the disease.

The interaction between tumour cells and the connective tissue stroma seems to be important in the growth of BCCs. Tumour growth is always seen as nests going into the dermis or having contact with the dermis. Autotransplants will not survive unless they are transplanted together with parts of their stroma.[140] This lack of autonomy of the tumour cells is also probably the reason why BCCs in principle never metastasize.

4.1.4. AETIOLOGY AND PREVALENCE

The best known etiologic factor in the development of BCCs is radiation with ultraviolet (UV) light. Supportive for this statement, is the high prevalence of BCCs in preferably fair-skinned Caucasians in sun exposed skin areas such as the head/neck region, the trunk and the legs. Also the prevalence of BCCs has been shown to increase with decreasing latitude.[136] Individuals with higher levels of skin pigmentation are less prone to develop BCCs.[136] In African populations, BCCs can behave rather aggressively when present, and they are over-represented in individuals with albinism.[141] Exposure to UV light, particularly in the UVB spectral range, has also been shown to induce BCCs in experimental animal models. The UV irradiation induces DNA damage. In sun-damaged skin, the level of the p53 protein rises, participating in the cell repair of DNA damages. Often, the result is apoptosis of the damaged cell to hinder such a cell to proliferate. Mutations in the p53 gene are well-known in BCCs,[140,142] leading to unrestrained growth of the altered cell line. If cells in the close proximity to a clone of such altered cells are subject to additional UV irradiation with subsequent cell death, the development of the BCC cell line is promoted. This means that both the mutation and the cancer cell promotion, is caused by the same stimulus, namely UV irradiation.[143]

Changes in DNA leading to subsequent development of BCC can be induced in skin that has been exposed to ionizing radiation used as therapy for other diseases, such as malignancies, haemangiomas etc. The time span after the radiodermatitits until BCC develops is similar to that after heavy sun exposure. In traumatised skin, there is an increased incidence of BCC. This is especially the case with burn scars.

A new group of patients that is arising these days, are the patients in whom the immune system has been suppressed for a longer period of time. The transplanted patients constitute the major part of this group. Apart from BCCs they can also develop large numbers of SCC and viral warts.

4.1.5. HEREDITY

Some syndromes carry with them an increased risk of developing high numbers of BCCs starting in young age. The Gorlin syndrome (nevoid basal cell carcinoma syndrome) is an autosomal dominant disease with low penetration named after the American physician Robert J. Gorlin.[144] Sceletal changes are also often present in these patients, primarily jaw cysts. Other extremely rare diseases where BCCs are present since birth or early childhood, are the linear unilateral basal cell naevus with one-sided clustering of lesions, and the Basex syndrome with generalized disturbances in the follicles of the skin.

Xeroderma pigmentosum is a disease characterized by a defective DNA repair following exposure to UV radiation. These patients will also tend to develop BCCs following comparably low doses of UV irradiation.

4.1.6. CONVENTIONAL TREATMENT MODALITIES

Many local treatment modalities have been used in the management of BCCs. Often the choice of treatment is based on the type and localization of the tumour and the resources and expertise available. Many lesions can be handled by a general practitioner.

The traditionally most widespread method is probably surgery with a simple excision of the lesion. This method can be sufficient for patients with few lesions, but in patients with multiple lesions, the scarring can be significant if they are all to be removed in this way. In some cases the excision has to be followed by a skin transplant. It is also necessary to perform reconstructive surgery after removal of lesions in, for instance, the area close to the eye. Particularly during excision of the morphea-like BCCs, it is advisable to examine the resection borders histologically to ensure that they are free of tumour growth. This is performed during surgery by frozen section control. The most refined of these methods is the so called Mohs surgery, named after the American surgeon Frederic E. Mohs[145,146]. In this method, the borders of the excised tissue are examined microscopically in a systematic way until the resection borders are free of tumour growth. Obviously, Mohs surgery is time consuming and expensive and has to be performed by specially trained surgeons.

Radiation therapy has primarily been performed for BCCs that are large and of the morphea-like type. The treatment dose is typically 45 Gy delivered in 10-20 fractions depending on the location of the lesion. The fractionation is necessary to achieve an acceptable sparing of normal tissue, resulting in typically 2-3 weeks of daily treatment.

Other techniques reaching a widespread use are methods that to a higher degree spare the normal tissue and are faster to perform. This is of great value for patients who have multiple lesions and continuously develop new ones. These methods are electrocoagulation and cryosurgery, where the tissue is damaged by either heating it up with an electrically heated metal tip or freezing it with liquid nitrogen. Sometimes curettage is added, *i.e.* debulking the tumour by a curette. Simple cryosurgery is probably the fastest technique. The low temperature of the liquid nitrogen has an anaesthetic effect on the tissue, which implies that local anaesthesia prior to the procedure seldom is provided. Of all local treatment modalities, Mohs surgery is the one with the lowest recurrence rate, being less than 2% [146,147].

Among the systemic treatment modalities, the most used is the oral administration of retinoids (A-vitamin derivatives). This is used for patients who develop high numbers of BCCs, especially those with the hereditary forms. The treatment will not remove the lesions but can prevent new lesions from developing. However, due to its toxicity it is not widely used. In cases of metastasising BCCs, chemotherapy in the form of, *e.g.*, 5-fluorouracil, has been given systemically.

It is within these settings that PDT has found its place. The advantages with ALA-PDT in the treatment of BCCs, and the lesions for which this might be the treatment of choice, are described in Section 5.2.8.

5. PHOTODYNAMIC THERAPY - CLINICAL APPLICATIONS

There is a variety of possible applications for photodynamic therapy (PDT) It can be used as a primary treatment modality, utilizing superficial or interstitial illumination. As an adjuvant modality, it can for instance be added to surgery for "sterilizing" the surgical field for micrometastases or remaining tumour cells. In connection with surgery, it can also be considered for unresectable tumour growth localized in the vicinity of vital anatomic structures. In addition to oncological diseases, several non-oncological applications have been identified. A brief overview of the clinical status of PDT is given in the following section, in which the main focus is put on PDT utilizing δ-aminolevulinic acid-induced protoporphyrin IX photosensitization (ALA-PDT).

5.1. Clinical status of photodynamic therapy with pre-formed photosensitizers

5.1.1. ONCOLOGICAL APPLICATIONS

At present, the most exploited pre-formed photosensitizer in the clinic is hematoporphyrin derivative (Photofrin). Intravenous injection of Photofrin has been utilized in the treatment of basal cell carcinomas (BCCs),[148-150] including the handling of multiple BCCs in patients with the Gorlin syndrome,[151] and good results have been obtained. However, due to the prolonged skin photosensitization, the modality has never reached any widespread use in the treatment of this disease. It is for the oncological diseases with a more life-threatening potential, that this type of PDT has been most exploited. The first health agency approval for PDT, was obtained in 1993 in Canada for prophylactic treatment of bladder cancer with Photofrin . At present, Photofrin has been registered for several indications in the United States, Canada, Japan, and Europe, as outlined in Table 5-1.

Table 5-1 *Regulatory status of Photofrin (1998).*[104]

Country	**Indication**
Canada	Prophylactic treatment of bladder cancer Early stage lung cancer
The Netherlands	Advanced oesophageal and lung cancer
France	Advanced oesophageal and lung cancer
Germany	Early stage lung cancer
Japan	Early stage lung, oesophageal, gastric and cervical cancer Cervical dysplasia
The United States	Advanced oesophageal cancer

Several of the second-generation photosensitizers have reached clinical trials Phase II-III. All these agents have absorption maxima located further out in the red wavelength region as compared to the absorption peak at 630 nm utilized for Photofrin® (see Table 5-2).

Table 5-2 *Overview of photosensitizers which have reached clinical trials Phase II-III. The absorption maximum in the red wavelength region, utilized for treatment, is also shown.*

Agent short name	**Agent full name**	**Agent tradename**	**Company**	**Absorption wavelength**
HPD	Hematoporphyrin derivative	Photofrin	QLT PhotoTherapeutics Inc., Vancouver, Canada	630 nm
BPD-MA	Benzoporphyrin Derivative-monoacid Ring A	Verteporfin	QLT PhotoTherapeutics Inc., Vancouver, Canada	690 nm
mTHPC	Meso-tetrahydroxyphenyl chlorin	Foscan	Scotia Pharmaceutical, Great Britain	652 nm
SnET2	Tin Etiopurpurin	Purlytin	Miravant Inc., Santa Barbara, CA, US	660 nm
Npe6	N-Aspartyl Chlorin e6			664 nm
Lu-Tex	Lutetium texaphyrin	LUTRIN	Pharmacyclics, Sunnyvale, CA, US	732 nm

Several reviews concerning the clinical status of PDT in general[104,152] and within various clinical specialities,[153-155] have been published during the past couple of years.

5.1.2. TOPICAL APPLICATION MODE

Some work has been performed with topical application of pre-formed photosensitizers, but the reported results are quite few. One important reason for this might be, that as soon as ALA-induced PpIX was introduced for photosensitization, this has been preferred to use for topical application. McLean treated several cutaneous metastasis of breast cancer (ϕ = 0.5-1.5 cm) with intralesional injection of Photofrin® and obtained complete response (CR) after one or two treatment sessions (personal communication).[156] Some pre-formed agents have shown to be capable of penetrating skin malignancies, to such a degree that they can be used as sensitizers for PDT. Sacchini *et al.* have treated BCCs with topical application of tetraphenylporphine sulfonate (TPPS) in a vehicle substance. Lesions with a thickness of less than 2 mm were successfully treated.[157] Recently, the porphycene dye ATMPn has been subject for topical studies. The dye was shown to be capable of penetrating into BCC cells lying in the deep dermis after 6 hours of application. On this time-scale the penetration into the perilesional skin was restricted to the upper epidermis.[158]

5.1.3. NON-ONCOLOGICAL APPLICATIONS

The pronounced effect of PDT on vessels (see Section 3.6.4) can be exploited in the treatment of age-related macular degeneration ("wet" type) and diabetic retinopathy. These conditions are characterized by a leaky neovascularization in the retina, and are common causes of blindness. Presently, Phase II trials have been completed in the United States and Europe in the treatment of age-related macular degeneration.[159] Low-doses of BPD-MA were utilized and the light irradiation was performed very shortly after injection, at a time when the dye still was confined to the vessels.[104]

5.2. Clinical status of photodynamic therapy utilizing δ-aminolevulinic acid-induced protoporphyrin IX

Before PDT was clinically introduced as an alternative treatment modality for cancer, naturally multiple studies of the efficiency, toxicity, mechanisms etc., were performed in cell lines and animal tumour models. However, when ALA-PDT was introduced, it found its way more easily into the clinical setting. There are several explanations for this. In addition to the pre-clinical studies on ALA, a large amount of pre-clinical testing had already been performed on PDT with pre-formed photosensitizers and ALA-PDT was assumed to be similar in these aspects. Also, ALA is considered as a relatively harmless agent since it is a naturally occurring substance in the body. Information about plasma levels, toxicity etc. already existed, partly from the research on porphyrias. Furthermore, the topical administration was introduced from the very beginning, and with this application mode the risks of possible systemic side-effects and photosensitivity of the skin are small. The possibility of utilizing a filtered lamp as a light source meant that ALA-PDT of skin lesions could be performed in hospitals where traditional PDT treatment lasers were not available, and thus a more widespread use was possible.

5.2.1. APPLICATION MODES

A variety of application modes has been utilized for the administration of ALA. This includes the administration of ALA in treatment doses intended primarily for PDT as well as the administration of ALA for diagnostic purposes (see Section 6.4). Since, in principle, all body cavities can be reached by topical administration, this application mode has been extensively exploited. Solutions of ALA have been instilled in the bladder,[160,161] and the uterus.[162] For lesions in the oral cavity, ALA solution can be gurgled. Inhalation has been performed for fluorescence diagnostics in the bronchial tree.[163] The fact that ALA is easily absorbed from the gastrointestinal tract makes oral administration a very attractive way of supplying ALA systemically.[164,165] The process of absorption, distribution and PpIX build-up is very fast. When administering ALA orally in low-doses for diagnostic purposes, we detected a build-up of PpIX by means of LIF already after 15 min (Paper IV). Intravenous administration has also been performed.[69]

5.2.2. TREATMENT OF NON-MELANOMA PRE-MALIGNANT AND MALIGNANT SKIN TUMOURS

Actinic keratosis Not too seldom, patients presenting with squamous cell carcinomas (SCCs) and BCCs, also show pre-malignant lesions of actinic keratosis (AK). Several authors report on the treatment of AK with PDT. Complete response rates in the order of 80-100% have been obtained,[58,90,166,167] with a follow-up of 3-36 months. Fink-Puches *et al.* treated AK lesions with various modes of non-coherent light. At long-term follow-up of 36 months, there was a significant difference between treatment with filtered light and treatment with full-spectrum visible light, in favour of the latter.[168] Due to the frequent presence of multifocal disease in the face and scalp, and the superfical nature of these lesions, a specially designed lamp has been produced that can simultaneously treat this whole region with blue light after ALA application.[82]

Basal Cell Carcinoma (BCC) The emphasis in the efforts within the field of ALA-PDT, on skin malignancies, has been placed on the treatment of BCCs. Multiple pilot studies have been presented, and the results have been variable but overall very promising.[58,59,80,90,122,167,169](Paper III) Peng *et al.* have presented a review of 12 clinical studies in the treatment of BCCs with ALA-PDT. The cure rates, given as weighted averages, were 87% for superficial lesions and 53% for nodular lesions (follow-up time 2-36 months).[70] From the initial study at our clinic, we experienced a 100% CR in superficial lesions and 64% CR in nodular lesions after one treatment session. After one additional treatment session, 100% CR was also obtained in the nodular lesions (Paper III). A subgroup of patients had lesions on the eyelids and in the periocular region (Paper II). Following in median 3 treatment sessions, complete response was seen in 42% (8/19), partial response in another 42% (8/19) and no response in 16% (3/19). Minimal scarring and loss of cilia were induced by the treatment of the eye-close lesions, which is particularly important in this anatomic site.

Morphea-like BCCs have shown a considerably lower response to HPD-PDT than the other BCC subtypes.[150] We have had the same experience when treating some of these lesions with ALA-PDT. Fluorescence microscopy has revealed that after topical ALA application, morphea-like BCCs exhibit a spotty and inhomogenous PpIX-related fluorescence after 12 hours of application.[75] Whether this is due to a lower potency of generating PpIX, or that the uptake is hindered by the fibrous stroma characterizing these lesions is not clear.

Squamous Cell Carcinoma (SCC) Most squamous cell carcinomas of the skin treated with ALA-PDT, have been of the in situ type (SCC in situ and Mb. Bowen). Our experience with these lesions is that they respond equally well to the treatment as BCCs of comparable thickness, and can often clear after one treatment session. In a small series, complete response was seen in 90% (9/10), with a follow-up time of less than 14 months (Paper III). Other studies of in situ or early invasive SCC, including 5-20 lesions each, have also shown comparable results, with CR rates in the range of 75-100%.[58,90,166,170] Follow-up periods were 2-36 months.

Cutaneous T-cell lymphoma lesions In Lund, we have experience with the treatment of three patients with this diagnosis. All patients had several lesions. In one patient, all lesions have resolved on 1-3 treatment session (parts of these result are presented in Paper III). The lesions of the second patient were considerably thick, 7-9 mm, and did not respond to the treatment (Paper III). The third patient had lesions located in the eye-close region, which resolved on 1-4 treatment sessions (Paper II).

5.2.3. RANDOMIZED CLINICAL TRIALS

At present, only two randomized, prospective clinical trials have been performed with ALA-PDT. Both trials concern the treatment of malignant skin lesions with topical ALA-PDT and cryosurgery, as the treatment arms. Morton *et al.* conducted a trial in patients with Mb. Bowen. In total 40 lesions were included. A filtered lamp was used as light source (70 mW/cm^2 and 125 J/cm^2). With cryosurgery a single freeze-thaw cycle technique was used. They found that the probability for the lesions to clear after one treatment session, was greater with PDT. Twenty-five percent of the lesions in the PDT group had to be re-treated with PDT. At one year follow-up, no PDT treated, but 10% cryosurgery treated lesions recurred. There was more pain in connection with cryosurgery, as well as occurrence of the adverse effects of ulceration and infection.[170]

We conducted a Phase III clinical trial in the treatment of superficial and nodular BCCs (Paper V). In total 88 patients were included, all on the basis of one lesion. The other treatment arm was cryosurgery with a dual freeze-thaw cycle technique. About one fourth of the lesions in the PDT group had to be re-treated once (2 lesions needed more than one re-treatment). In conclusion, the preliminary evaluation of the clinical study showed that the efficacies of ALA-PDT and cryosurgery are statistically comparable with a tendency of a small advantage for the latter modality. However, the healing time and the cosmetic outcome strongly favoured ALA-PDT.

5.2.4. TREATMENT OF BENIGN SKIN LESIONS

Psoriasis Psoriasis is a quite common skin disease. There is not so much written in the literature, but ALA-PDT has been performed and been shown to give an effect in psoriatic plaque.[171] We have experience with a few patients in which we could obtain partial response and thereby reduce the symptoms in a similar way as with the conventional treatment modalities.

Acne In 1942, Figge noticed that comedones in medical students showed an intense red fluorescence when excited with UV light. He predicted the presence of porphyrins in the material within these structures.[172] Orenstein *et al.* detected endogenous PpIX fluorescence in acne with a fluorescence imaging system.[173] The normal skin bacterial flora, for instance propione bacterium acnae, constituting parts of the bacterial component of acne, stores porphyrins and treatment of acne with blue light has been performed.[174]

5.2.5. SOME TREATMENTS PERFORMED WITHIN VARIOUS CLINICAL SPECIALITIES

Gynaecology Dysplasia and carcinoma in situ (*cis*) are quite common in the lower, female genital tract, primarily in younger women. One of the main etiologic factors is viral infection. Martin-Hirsch *et al.* reported on the treatment of high-grade cervical and vulval intraepithelial neoplasias (CIN and VIN, respectively) with topical ALA-PDT. With one treatment, the cure rate was 50% in the CIN group and 37% in the VIN group. Simultaneously performed fluorescence microscopy studies showed PpIX fluorescence throughout the whole epithelial layer after 4 hours of application.[175] We have treated some patients with extramammary Mb. Paget, in which other treatment modalities had failed or could not be performed. The treatment resulted in partial response of the lesions and a significant reduction of the symptoms. Fluorescence studies after ALA installation in the uterus, have revealed a rather selective build-up of PpIX in the endometrium as compared to the myometrium,[162] and ALA-PDT has been proposed as an alternative modality for endometrial ablation. We have found that oral administration of ALA in low doses induced very high levels of PpIX fluorescence in condylomas of the female genital and such lesions can thereby be a possible indication for ALA-PDT.[176]

Urology Stenzl *et al.* reported on a 10-16 months tumour-free follow-up, in 5/6 patients receiving ALA-PDT for recurrent *cis* of the bladder. The treatment was performed after instillation of ALA for 30 minutes together with 20 min of electromotive diffusion.[161] Initial results in Lund in the treatment of *cis* of the penis are promising.

Otorhinolaryngology The ear-nose-throat region is easily accessible for PDT. Squamous cell carcinomas of the oral cavity are often multifocal and for this reason PDT has been considered a very interesting modality. Fan *et al.* treated pre-malignant and malignant lesions in the oral cavity after oral administration of ALA. They found it to be an efficient treatment for the pre-malignant, dysplastic lesions.[177] Stender *et al.* treated a few patients with actinic cheilitis, a pre-malignant condition of the lips and obtained CR (follow-up 6-12 months).[178] In Lund, some treatments have been performed of *cis* of the vocal cords, and the results have also here been promising.

Surgery Barrett's disease is a condition with metaplasia of the epithelium, from squamous to columnar epithelium, in the lower oesophagus, with subsequent high risk of developing dysplasia and adenocarcinoma. Barr *et al.* treated five of these patients with ALA-PDT (oral administration). High-grade dysplasia could be eradicated in all patients. Squamous epithelium regenerated, but sometimes on top of columnar epithelium, which required continuous surveillance of these patients. No recurrence of dysplasia was seen at follow-up of 26-44 months.[179] Only superficial necrosis was obtained after treatment of benign and malignant tumours in the gastrointestinal tract, with orally adminstrated ALA.[68,165]

Hematology Some of the very early work in the field of ALA-PDT, showed that erythroleukaemic cells could be destroyed *in vitro*.[56]

5.2.6. SIDE-EFFECTS

Dysesthesia and pain ALA-PDT of skin lesions is usually performed on an out-patient basis and very seldom in the operating theatre during general anaesthesia. One of the major obstacles towards the treatment has been identified as an intense pain that occurs in some patients and in certain anatomical locations. During light exposure, most patients have some sort of dysesthesia on the treatment site. The character of these sensations is usually described as a feeling of prickling, stinging or itching that can turn into a really painful sensation in its crescendo.

Another major component is the sensation of warmth that varies on the scale from a comfortable warm feeling to a burning sensation. This should be seen in the context that almost all treatments are given with light fluence rates well below the threshold for hyperthermia. Fijan *et al.* noticed an increased pain requiring local anaesthesia, when utilizing possible hyperthermic fluence rates of 200-250 mW/cm^2 from a filtered lamp.[167] Orenstein *et al.* illuminated both normal skin and ALA pre-treated skin tumours with 100 mW/cm^2 from a non-coherent light source. A temperature increase to 39.5-42.5°C in tumour and 42-43.5°C in normal tissue was observed. The patients noticed a burning sensation in the tumours, but not in the normal skin only subject to irradiation. They concluded that the light itself could not be a probable source of the dysesthesia.[123] Pressure in the tissue is another sensation we have noticed. There is a large inter-patient variation on the degree and character of the dysesthesia. Treatment in well innervated anatomic regions is more prone to give rise to dysesthesia and pain, *i.e.* the face, hands and the perineal region. Open wounds in the treatment area always lead to more pain, something that partly can be explained from the generation of more PpIX due to the easy access of ALA to the tissue. Furthermore, we have experienced that a few patients treated in the head and neck region and on the chest report referred pain. Often, it can be quite difficult to predict the degree of dysesthesia. When present, the dysesthesia or pain, is more intense in the beginning of the treatment session, declining throughout the illumination procedure.

After seven years of experience with topical ALA-PDT for skin lesions in Lund, utilizing light fluences below the threshold for hyperthermia, the general impression based on empiri and organized registrations, is that it is rather seldom that dysesthesia takes the character of unbearable pain and becomes a problem for the patient and the physician. However, most patients have some sort of dysesthesia (Papers II,III and V).[180,181] Several efforts have been undertaken to reduce the pain when present. All sorts of local anaesthesia, *e.g.*, infiltrating and spraying with lidocain, or applying EMLA cream for transdermal delivery of lidocain and prilocain have surprisingly not given any significant improvement. For these reasons, local anaesthesia has not been routinely used. This indicates that the pain induction from the photochemical reaction is of quite another character than that, from for instance, a surgical procedure. What we have found to be the most efficient approach on the treatment site, is to spray with

isotonic saline or water. In order not to compromise the oxygen supply by causing vasoconstriction, a temperature of 15-20°C has been used. This added stimuli reduces the conception of the dysesthesia induced by the treatment. The effect of the water stimuli is shown in Figure 5-1. In this investigation, the patients were instructed to indicate the feeling of pain on a 10 cm visual analogue scale (VAS) during the light exposure. Zero on the scale indicated ″no pain″ and 10 cm indicated ″unbearable pain″. Treatment was performed with two different diode lasers, one continuous wave (CW) at 635 nm and one at 652 nm utilized in two different light delivery modes, a CW and a pulsed (PU). In general, the conception of pain was not severe, but acceptable for the patients, being < 3-4 cm (3-4 cm corresponds to ″slight pain″).[181] Light exposure at the wavelength of 652 nm, which is off the main absorption peak of PpIX generated less pain, but also had a lower treatment efficacy.[182] The same effect of decreased pain when switching to wavelengths that are off the various absorption peaks for PpIX, was experienced by Fink-Puches *et al.*[168] One explanation might therefore be a less pronounced photochemistry. In our case, the water had an immediate effect, reducing the sensation of pain by two units, from moderate to slight pain.

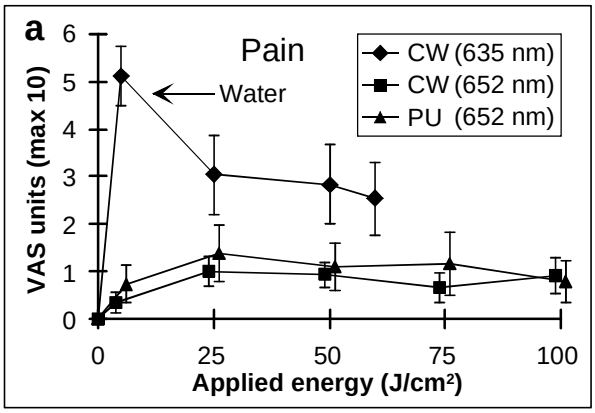

Figure 5-1 *Comparison of pain sensation in patients (shown as mean values ± SEM according to a 10 cm visual analogue scale) with different wavelengths and light irradiation modes (635 nm n=13, 652 nm pulsed n=19, 652 nm CW n=17). In all three groups there were lesions with the diagnosis of BCC, SCC in situ and actinic keratosis. During irradiation at 635 nm water was applied after a light dose delivery of 5 J/cm^2 in 10/13 lesions, whereas at 652 nm it was not needed neither with continuous nor pulsed light delivery.*[181]

Photodynamic therapy can also be performed with anaesthetic nerve blocking. When performing anaesthesia on a higher sensory level, as for instance digital nerve blocking and blocking of the penile nerve roots, satisfactory anaesthesia has been obtained. It can also be mentioned, that when anaesthetic agents are needed in connection with or after PDT, it has generally been anticipated that such agents as the non-steroidal anti-inflammatory drugs (NSAIDs) should be avoided. This is because the inflammation caused by the treatment is considered important for tumour

eradication. In addition, such agents have been shown to decrease the PDT-induced vasoconstriction and the treatment effect in connection with systemic photosensitization (see Section 3.6.4).[111]

A very interesting finding is that the treatment with topically applied ALA pro-drugs, *i.e.* esterified ALA molecules for increased lipophilicity, leads to less pain during ALA-PDT.[70]

Systemic effects after systemic administration Acute intermittent porphyria is a disease characterized by elevated ALA levels in the blood. Well-known manifestations of the disease are attacks of abdominal pain, vomiting, tachycardia, and hypertension. It is also characterized by peripheral or sometimes central neuropathy, and psychiatric manifestations.[30] Only a few of the less serious of these symptoms, have been observed in patients receiving single, high doses of ALA. In a group of patients receiving oral administration of ALA in doses of up to 60 mg/kg b.w., transient nausea and vomiting was noticed in 15-30% of the patients, and 25-30% developed temporary changes in various liver function tests.[68,183]

Cutaneous photosensitivity When kept in subdued light, patients receiving up to 60 mg/kg b.w. ALA, did not develop any cutaneous photosensitivity.[183] In Lund, we have the same experience with patients receiving 45 mg/kg b.w, who were exposed to ordinary indoor light levels, but kept away from strong lamps and sunlight. When given in low doses (≤ 15 mg/kg b.w.) for diagnostic purposes during winter time, no light restrictions were applied and there were no complaints of itching or other symptoms of skin photosensitivity. Very low levels of PpIX could be detected in the skin by means of laser-induced fluorescence and after 24 hours it was hardly detectable (Paper IV).

5.2.7. TREATMENT REGIMENS FOR IMPROVED CLINICAL OUTCOME

Several approaches for improving the clinical outcome of ALA-PDT have been introduced in the clinic. The aim is to increase the bio-availability of ALA by increased penetration and uptake in cells, and to stimulate to increased production and accumulation of PpIX. Improved ALA penetration following topical administration, can lead to a significant reduction in the interval between application and light exposure, due to the fast bio-conversion of ALA to PpIX. The time aspect is of major importance, for practical reasons, in the clinical situation. When administering ALA systemically, a more homogenous distribution of PpIX is obtained in tumours[83] and might be the best administration mode in certain selected cases, as for instance when there are thicker or multiple lesions.

ALA penetration and PpIX accumulation Prior to topical ALA application, malignant skin lesions are usually prepared by removal of crusts and debris and also of the stratum corneum of the skin (Figure 6-1), if it still is intact. For thicker lesions, debulking of the tumour mass has been performed by curettage.[70] Furthermore, lipids

can be removed from the superficial tissue by cleansing with luke-warm ethanol. The application time of ALA is of outermost importance. Theoretical modelling[7] and clinical experience[184] have shown the benefit of a longer application time. The various methods described in the following can probably reduce the application time.

Drugs that potentially work as porphyrin production inducers are the iron ion chelators ethylenediamine tetraacetic acid (EDTA) and desferrioxamin. Both decrease the amount of PpIX transformed to haem by depleting the intracellular level of Fe2+. The effect of EDTA on PpIX accumulation has been evaluated when supplied together with dimethylsulphoxide (DMSO), where an increased level of PpIX was achieved at a deeper level in the tumour.[83] The EDTA molecule alone has a rather shallow penetration in skin as shown in mouse skin.[185] When ALA was applied together with desferrioxamine, a more intense red fluorescence was achieved in skin tumours as compared to supplying ALA alone.[167]

Increased penetration of ALA has been reported after chemical and physical manipulation and is usually evaluated as the intensity of PpIX fluorescence in the tissue layer of interest. DMSO is a well-known vehicle for increased skin permeability. In addition, it is supposed to induce cell differentiation and initiate haem biosynthesis. A significantly increased penetration of ALA in skin tumours was achieved when adding DMSO to the ALA formulation,[83] and it gave a significantly better cure rate in thicker lesions.[70] Similar results have been achieved *in vitro* both with DMSO + ALA alone and in combination with a porphyrogenic drug, allyl-isopropyl-acetamide (AIA).[186] A new and very interesting drug-formulation is obtained by manipulating the ALA molecule to increase its lipophilicity. This is done by adding for instance various esters to the chemical structure. Such compounds have sometimes been termed ALA pro-drugs. Immediately localized intracellularly, esterases will remove the ester-part, leaving the ALA in its original form and thereby increasing the bio-availability. Increased PpIX formation has been reported *in vitro* and in animal tumour models with such pro-drugs.[70,187] In human BCCs, a better selectivity was also observed.[70]

Iontophoresis and phonophoresis are physical methods utilized for transdermal delivery of various drugs. In iontophoresis one utilizes the ionic nature of the ALA molecule in solution. An electric current in the order of 0.1-4 mA can be applied for more than ten minutes without inducing pain or skin damage. Typical areas of the delivery chamber and the attachment pad of the other electrode, that are in contact with the skin, are 4 and 12 cm^2, respectively. When coupling a delivery chamber with ALA/sterile water solution to the anode, ALA is driven through the stratum corneum of the skin and further into the tissue. Rhodes and co-workers evaluated the method in normal skin of volunteers, utilizing the PpIX fluorescence intensity detected at the surface, and post-irradiation erythema as a measure of delivered ALA dose. A total charge of up to 120 mC, utilizing a current of 0.2 mA, was used. They found a direct, positive correlation between delivered charge and PpIX fluorescence, as well as between delivered charge and erythema after irradiation with a filtered lamp.[188] For thicker tumours in the skin, particularly when treating some cutaneous metastasis of

breast cancer, we have used currents of 1-4 mA for 10-15 min and total delivered charges in the range of 600-3600 mC (total charge, mC=current, mA × time, seconds). A similar regimen was applied in connection with ALA-PDT in the bladder after ALA instillation for 30 min. A iontophoresis electrode was placed in a catheter and 20 mA current was applied for 20 min.[161] The theories behind phonophoresis are that the vibrations induced by the applied diagnostic ultrasound (1-3 MHz) induce growth and oscillations of air pockets in the keratinocytes of the stratum corneum. The subsequent conformational changes of the lipid bilayers in the stratum corneum facilitate the passage of molecules. With ultrasound of diagnostic and lower frequencies, transdermal delivery in animal models was successful for proteins such as insulin and erythropoietin[189] and the anti-inflammatory drug indomethacin.[190] In Lund, some initial studies have been started to evaluate the effect of applying ultrasound of 3 MHz in connection with ALA-PDT of thicker lesions.

Light delivery Optimizing the treatment light concerning wavelength, fractionation and fluence rate might be beneficial, as discussed in Sections 3.4 and 3.5.

Repeated treatments Treatment with ALA-PDT can be repeated at various time intervals. One of the major advantages with the method is the fact that concerning side effects and toxicity, there are no real upper limits for the number of repeated treatments that can be performed. After PDT, there seems to be a continuous enzymatic conversion of ALA to PpIX. From our fluorescence measurements performed during the first two hours after light irradiation, we have seen that PpIX is re-generated (Paper VII). Theoretically, one can apply a new light exposure after such a re-generation. Orenstein *et al.* have also reported on detecting such re-appearance of PpIX-related fluorescence in malignant skin tumours in humans, after ALA-PDT with topical application. Re-appearance was only seen in the thicker lesions. They performed a second illumination of the lesions where the PpIX fluorescence re-appeared. That an improved treatment outcome can be obtained with such repeated irradiation, have been shown in connection with both topical[128] and systemic[191] ALA administration, in an animal tumour model. Repeated treatment sessions with new ALA application can also be applied at nearly any time after the initial one. At present, there are no real standards for which schedules to use. Practical circumstances also influence to a certain degree.

5.2.8. CONCLUSIONS

Concerning ALA-PDT as a treatment modality for non-melanoma malignant skin lesions, a lot of experience has been gained in Lund as well as in several other clinics. As discussed in the preceding sections, ALA-PDT can lead to treatment results that are comparable to the conventional modalities. Certain lesions need repeated PDT sessions. The treatment spares normal tissue and gives very little scar formation. As discussed in Section 5.2.7, there seem to be many, easily implemented, small improvements for ALA-PDT which can increase the efficacy, and simplify the

treatment regimen. Each of these suggested improvements, has to be studied separately and in combination, to find the optimal treatment protocol.

From these considerations, there is at least one clear niche where PDT plays a very important role as treatment modality. This is in the treatment of superficial tumour growth when it covers larger areas or is situated in areas where function (Paper II) or cosmetics is important. PDT can also be performed when other modalities are excluded, for instance, when a full dose of ionizing radiation has been given, surgery cannot be performed, or the patient has a poor general condition. A good example of a lesion in which ALA-PDT is an ideal treatment modality, can be seen in Paper V (Figure 1b). This is a large superficial BCC, situated in the pre-tibial area, in which both surgery and ionizing radiation are modalities that can be difficult to perform, due to the proximity to the underlying bone, tight skin covering and generally poor blood perfusion.

6. LASER-INDUCED FLUORESCENCE FOR TISSUE DIAGNOSTICS

The term *"optical biopsy"* is frequently used for optical spectroscopy methods, like the fluorescence technique, used to characterize tissue. The methods have mainly been developed and used for oncological applications, though, another field under development is the detection of arterial plaques. This chapter will be restricted to the diagnosis of oncological diseases.

Laser-induced fluorescence (LIF) for tissue characterization is a technique developed as a natural counterpart to PDT. A brief overview of the history of the latter is given in Section 3.1. LIF is a non-invasive method that can be performed in real time. The technique is based on spectroscopic analyses of tissue fluorescence, following excitation with light. In LIF, the fluorescence from either native tissue fluorophores, or from an exogenous fluorescent tumour marking agent, added to increase the contrast between tumour and normal tissue, can be utilized. In the latter case, the exogenous fluorescent tumour marking agents have so far mostly been the same as the photosensitizers used for PDT. This is the main reason why these two techniques have been so closely related. Below, exogenous agents used for LIF will be called fluorescent tumour markers, while the active substances used for PDT will be referred to as photosensitizers, even though they frequently are the same substances.

Compared to the volume imaging based on ionizing radiation and magnetic resonance techniques, fluorescence diagnostics is a method for inspection of surfaces, as the fluorescence excitation light only penetrates the superficial layers of biological tissue. As a majority of malignant tumours, besides skin malignancies, starts in the epithelium of the body cavities, early detection often relies on techniques to detect tissue abnormalities on a surface. This is best performed with highly sensitive endoscopic methods. Today, the most frequently used conventional techniques, are visual inspection using white light illumination and biopsy sampling. The latter is in certain cases, randomly performed. The LIF method is meant as a supplement to the ordinary white light investigation and can be performed together with all endoscopic examinations to increase the diagnostic sharpness. In the early research work, point monitoring was used, but the ultimate goal for carrying the technique into the clinic is to develop imaging systems. A handful of groups have developed and worked with prototypes for imaging. Among these prototypes, a system developed in Lund appeared early.[192-195]

6.1. Clinical context and indications

Point monitoring techniques give the full spectral information at each measured site, as compared to imaging techniques where, for technical reasons, only a small fraction of the spectral information is normally utilized. On the other hand, the latter technique enables the simultaneous investigation of a larger area. The ultimate goal is to develop the technique for full tissue characterization with the possibility to classify the diseased state of tissue, which actually is the true meaning of the term "*optical*

biopsy". At present, this is not a reality, and there is still a need for a histopathological diagnosis for correlation of the fluorescence signal. On the other hand, LIF can at present be a very useful real time guide in the sampling of tissue for histopathology. The method is particularly useful in situations where the alternative is a random biopsy sampling, and in situations where the possibility of removing tissue is restricted. Examples of the first situation include regular examinations of patients with conditions that predisposes for developing malignancies. The number of patients in this category is quite large and includes, for instance, patients with ulcerative colitis, Barrett's oesophagitis, familial adenomatous polyposis, as well as patients with a history of earlier colon/bladder/ENT malignancies. Examples of the second situation are the examinations of the vocal cords and the cervix.

So far, most studies within the field have been focused on evaluating the contrast between known malignancies and normal tissue. In this phase, it is of extreme importance that the fluorescence spectra and images are correlated to the corresponding histopathology, so that the fluorescence characteristics of various types of tissue can be recognized. The next step is to see whether the method can differentiate among the various stages of dysplasia and, maybe even more importantly, to differentiate dysplasia towards benign inflammatory conditions. To be able to find and recognize the fluorescence characteristics of tissue in these various states, a large number of patients has to be investigated.

Another field where LIF might function as a tool for improving the outcome of the procedure, is in the examination of resection borders in connection with surgery. This is particularly interesting in situations with diffuse tumour growth, multiple lesions and when operating in the vicinity of vital structures. An important example of the latter is the removal of malignant tissue in the brain.

Laser-induced fluorescence is also a very useful tool in connection with PDT dosimetry. The level of photosensitizer, its photodegradation and occurrence of photoproducts can be monitored. The timing of the illumination and dosage of treatment light can be evaluated in real time, making it possible to interact and optimize each single treatment procedure. In connection with this, it should be emphasized that detecting the intensity of the fluorescence signal does give a lot of information about the level of photosensitizer present, although it cannot be interpreted directly as the quantity of the photosensitizer in the tissue. This is because the fluorescence is affected by a variety of factors, such as the aggregation state of the photosensitizer, the micro-environment of the fluorescing molecule concerning, for instance, pH and the presence of fluorescence quenchers, as well as the tissue optical properties determining how well light is transported through the tissue.

Much of the initial studies on fluorescence have been performed *in vitro*. Care should be taken when comparing such data with *in vivo* measurements, due to differences in the biochemical environment, structure, and presence and localization of blood, that will eventually affect the signal.

6.2. Fluorescence detection modes

The various aspects of fluorescence that can be studied, are the fluorescence intensity, the spectral shape and the lifetime. Individual excitation and detection modes may be required for each clinical application, and most clinically adapted systems are built to examine one of these parameters.

The fluorescence spectral shape can be studied in several ways. Excitation spectra are obtained by varying the excitation wavelength and keeping the detection wavelength fixed. Differences in excitation spectra for normal and malignant tissue can be utilized for diagnostic purposes. For obtaining emission spectra, one detects at all emission wavelengths, while keeping the excitation wavelength fixed. This method yields a fluorescence emission spectrum as a superposition of fluorescence spectra of all the fluorophores present within the tissue volume probed. For fluorescence diagnostics utilizing the spectral shape, it is usually the emission spectra that are detected.

Variations in fluorescence lifetime can be detected when utilizing fast detectors, capable of recording the fluorescence intensity as a function of time. Tissue fluorescence has a lifetime in the order of nanoseconds (ns).

6.3. Tissue autofluorescence

After excitation, preferably in the UV and near-UV region, several compounds, *i.e.* fluorophores, in normal tissue will fluoresce, constituting the tissue autofluorescence in the visible wavelength region. The main groups of fluorophores are those associated with the structural matrix of tissue and those involved in the cellular metabolism. Collagen and elastin are mainly responsible for the fluorescence from the first group. The strong fluorescence of these compounds is due to the cross-linking of amino acids. In the second group, one finds nicotinamide adenine dinucleotide (NADH) and the flavins. The first of these, NADH has been considered as very important in connection with early development of cancer due to an altered NADH/NAD+ ratio where the reduced form, NADH, is highly fluorescent (see Section 6.10).[196,197] Other fluorophores of importance are the aromatic amino acids, *e.g.*, tryptophan, tyrosine, phenylalanine, the lipopigments, and the endogenous porphyrins. The main excitation and emission peaks of various native tissue fluorophores, are extracted from the references[196,198-200] and shown in Table 6-1.

Table 6-1 *Fluorescence data from some endogenous tissue chromophores. The fluorescence excitation and emission measurements were performed on the various substances in the form of solution or powder.*

Fluorophore	**Main excitation peak (nm)**	**Main emission peak (nm)**
Collagen	340	395
	270	395
	285	310
Elastin	460	520
	360	410
	425	490
	260	410
NADH	350	460
Flavin mononucleotide	440	520
Tryptophan	275	350
Beta-carotene		520
HPD (similar to endogenous porphyrins)	400	610
		675

The localization of the fluorophores varies in normal tissue. This is particularly evident in hollow organs and skin which have a characteristic layered structure, where each layer has significantly different compositions of the various fluorophores. A cross section of intestinal tissue with a typical layered structure can be seen in Figure 6-1. The figure also shows the epidermis of the skin, which, compared to the intestine, has a thicker and stratified epithelium. It is the changes in these structures, connected to malignant transformation, that can be identified by LIF. Alterations in the concentrations of the tissue fluorophores take place prior to major structural tissue changes, and this is where LIF can contribute in detecting very early malignant transformation.

As mentioned above, the fluorescence detected is not only dependent on the fluorophore emission in the tissue volume sampled, but also on chromophores absorbing the light. The excitation light impinging on the tissue surface is scattered and absorbed by the tissue. The properties of the tissue, related to transportation of the excitation light, will determine the volume of the tissue contributing to the fluorescence signal. Fluorophores excited (within this volume) emit fluorescence characteristic for each fluorophore. To be detected, this fluorescence light has to be transported through the tissue back to the surface. During this transportation, part of the light will be reabsorbed by various chromophores within the tissue, *i.e.* filtered. Some chromophores have characteristic absorption bands in the visible wavelength region. The spectral shape of the fluorescence will, due to this absorption, be distorted during its transportation to the detector. The detected signal will thus be a superposition of distorted fluorescence emission spectra of the fluorophores contributing to the tissue fluorescence. An interesting aspect of this is, that some re-

absorption features in the detected tissue fluorescence emission may be useful for diagnostic purposes.

Haemoglobin (Hb) is such a strong absorber and can, therefore, strongly influence the fluorescence spectrum. The presence of Hb can decrease the overall fluorescence intensity by absorbing the excitation light. It can also change the shape of the emission spectrum due to its strong absorption at certain wavelengths, leading to dips in the spectrum[160] and the illusive presence of false peaks. In addition to a strong absorption at about 420 nm, two absorption peaks for Hb in the visible wavelength region for oxygenated HbA (adult human haemoglobin), are situated at 541 and 577 nm and for de-oxygenated HbA it is situated at 554 nm.[201,202]

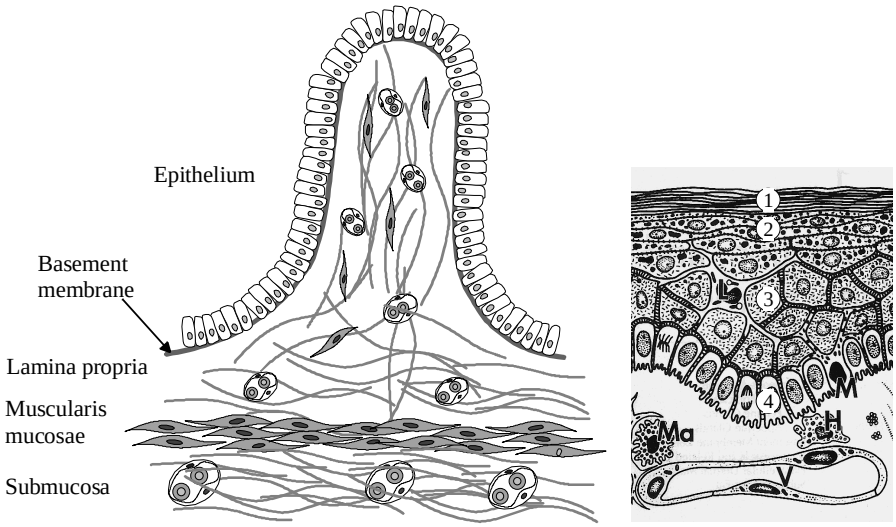

Figure 6-1 *Sketches of two types of epithelial linings that have different optical properties. The one-cell layer lining of the gastrointestinal mucosa (left) is seen together with the underlying structures. In the skin (right) only the multi-layered epithelial lining, the epidermis, is shown, resting on the underlying dermis. The epidermis consists of several layers: stratum corneum (1), stratum granulosum (2), stratum spinosum (3) and stratum basale (4). The outer layer, stratum corneum, contains non-nucleated keratinized cells.*

6.4. Fluorescence diagnostics enhanced by fluorescent tumour markers

As mentioned above, there exists a possibility to increase the diagnostic potential of fluorescence diagnostics by adding a fluorescent tumour marker to the tissue. Frequently, the same substances have been used to enhance the diagnostic sharpness of LIF and for sensitizing the tissue for light during PDT. The possible mechanisms behind the selectivity of these substances in malignant tissue are described in Section 3.2.2. Tumour markers that fluoresce at longer wavelengths, as for instance phtalocyanines,[203] are interesting from the aspect that the peak fluorescence of the dye

overlaps less with the autofluorescence. Its fluorescence can therefore be extracted more easily. To avoid the skin photosensitization connected with the drug administration, low drug doses have been given. Another way of avoiding the skin photosensitization is to utilize fluorescent tumour markers specially designed for LIF, without any photosensitizing properties. The carotenoporphyrins are such agents which have carotenoid polyene groups added to a porphyrin main core.[204,205] The carotenoid part of these molecules can quench the triplet state of the photosensitizer and thereby the generation of cytotoxic singlet oxygen (see Section 3.6.2). This situation mimics what happens in the photosynthetic reaction centres in green plants. Other such tumour localizing fluorescent agents without the cytotoxic potential, are some chlorin derivatives.[206]

For tumour marking in connection with fluorescence diagnostics, δ-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) has also been exploited. PpIX has a characteristic dual-peaked fluorescence emission in the red spectral region, with one high and narrow peak at about 635 nm and one smaller and wider peak at about 705 nm.

In Paper IV, we examined the kinetics of the PpIX build-up, by means of LIF, after oral administration of low doses of ALA given for diagnostic purposes (5 and 15 mg/kg b.w.). The build-up in normal mucosa of the head and neck region was peaking after about 3-4 hours. Skin fluorescence was also measured at the same time, featuring a considerably low overall PpIX fluorescence intensity, but also peaking after about 3-4 hours. The kinetics of the PpIX fluorescence detected in one malignant tumour of the oral cavity was much faster in the build-up and slower in the decay. Similar measurements were performed on basal cell carcinomas (BCCs) of the skin, after topical application of ALA as described in Paper VII. The peak of the PpIX fluorescence level was found to occur after about 4 hours for the nodular BCCs. For superficial BCCs, PpIX fluorescence intensity continued to increase up to the last measurement, 6 hours after application. The build-up was faster in malignant tumour tissue. From these results, it seems advisable to perform the measurements at a quite early stage after the ALA administration, before the normal tissue has started to generate detectable levels, in order to obtain the best demarcation tumour to normal.

6.5. Choice of excitation wavelength

As mentioned previously, most clinically adapted systems for fluorescence diagnostics are based on analysis of fluorescence emission properties. This is mainly due to technical reasons, as such a system is normally less complex than systems based on fluorescence excitation or lifetime characteristics. Thus, when utilizing solely the autofluorescence, a single excitation wavelength in the UV region is usually used. A UV wavelength is often preferable, due to the better excitation of the native fluorophores in this wavelength region.

An excitation-emission matrix (EEM), is a matrix which contains the fluorescence intensity as a function of excitation and emission wavelength. Such matrices can be formed from a series of emission (or excitation) spectra collected for a

range of excitation (or emission) wavelengths. The EEMs can be used to find the excitation and emission wavelengths where the contrast among normal and various diseased states is highest.[207,208] In work performed with Photofrin sensitization of a tumour model, an excitation wavelength of about 370 nm was found to be optimal.[209] There is a lower limit for the choice of wavelength at about 320 nm, due to the potential risk of inducing DNA damage when using such short wavelengths. At the same time, it should be mentioned that the energy in the excitation light utilized for LIF, particularly in the case of point monitoring, is so low, that the risk of inducing such damage is minute. When adding a photosensitizing agent, there is usually a need to use a longer wavelength (typically > 400 nm) to be able to excite the photosensitizer. When excited at this longer wavelength, the autofluorescence mostly constitutes a wide and uncharacteristic fluorescence distribution, and less information can be extracted from the autofluorescence. The main information in such a spectrum is the overall intensity, which is lower in pre-malignant or malignant than in normal tissue. This has primarily been related to an altered NADH/NAD+ ratio (see Section 6.10).

With an excitation wavelength in the UV or near-UV region, the probing depth, *i.e.* the depth of tissue contributing to the fluorescence signal, is rather shallow due to the high absorption in tissue. A depth in the order of a couple of hundreds of μm can be sampled. This corresponds primarily to the epithelial layer, depending on the organ examined. The epidermal layer of the skin, can for instance vary in thickness from 75 to 600 μm, whereas the one-cell layer thick columnar epithelium of the stomach only has a thickness of 20-40 μm (Figure 6-1).[210] When a thinner mucosa is detected, the signal will obviously also be influenced by the underlying structures, where, for instance, highly fluorescent fibrous tissue may be present. The detection in the skin will be affected by the presence of the stratum corneum (see Section 6.10).[211]

6.6. Point monitoring

Frequently, the fluorescence diagnostic methods are divided in point-wise measurements and imaging techniques. Point monitoring, means the analysis of tissue fluorescence from a single position at the tissue surface, usually recorded as a fluorescence emission spectrum. A clinically adapted optical multichannel analyzer (OMA), has been used for the measurements described in Papers IV, VI and VII (some data are also presented in Papers II and III). The system is described in detail by Andersson-Engels *et al.*[212] The set-up of this system is outlined in Figure 6-2. The excitation light source is a compact nitrogen laser emitting at 337 nm and with a repetition rate of 10 kHz. The nitrogen laser can also be used to pump a dye laser when a photosensitizer is used. For the latter purpose, the wavelength of the dye laser can be tuned to the optimal excitation wavelength, which in the case of PpIX, is about 405 nm. At this wavelength, the pulse energy in our system is about 1-2 μJ. The excitation light is transmitted through a 600 μm optical quartz fibre, which is held perpendicular to the tissue under investigation in light contact. The emitted fluorescence light is captured through the same fibre, transported back through a

dichroic mirror transmitting the fluorescence light. Furthermore, it passes a cut-off filter and is focused onto an entrance slit of a polychromator, in which the light is spectrally dispersed. The full fluorescence spectrum, ranging from 450 to 800 nm with a resolution of 5 nm, is then captured by an image-intensified CCD camera, thermo-electrically cooled. The spectra are displayed on a computer screen and saved for further evaluation.

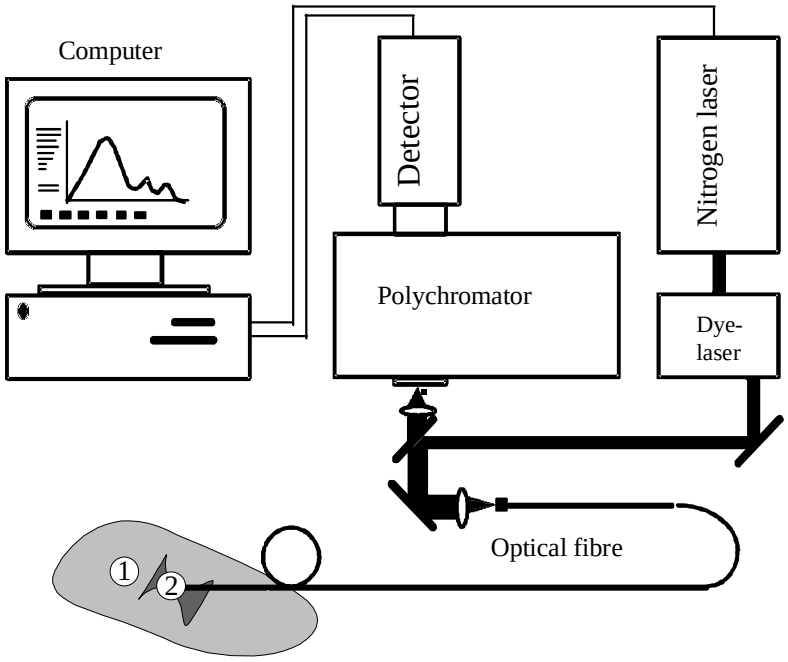

Figure 6-2 *Schematic illustration of the point monitoring OMA system utilized in Papers II-IV, and VI-IIV. A description is given in the running text. The illustration envisions an examination of a resected breast cancer specimen. The two areas marked ① and ② in the sketch correspond to normal breast tissue and infiltrating breast cancer, respectively, after correlation with histopathology. The corresponding fluorescence spectra recorded in the sites ① and ②, are shown in Figure 6-3.*

6.7. Evaluation of spectral information

The primary aim of our fluorescence investigations has been to classify if the tissue is diseased or healthy. One could also have used the fluorescence data to, for instance, obtain information regarding fluorophore concentration in the tissue. In any case, the spectral fluorescence data recorded often have to be condensed to an indication of diseased or non-diseased or to a number. In doing this, both spectral shape and intensity should be considered in the analysis. If measurements are made at one wavelength only, the liability of the data is quite restricted. This is caused by a large inter-patient variation, as well as by variations in detection geometry and in the system itself. Also, even though the spectral information is utilized in an optimal way, some

inter-patient variation often remain. It is thus of interest to evaluate the information relative to something varying in a similar manner. The best "standard" for such measurements, is provided by the patients themselves, *i.e.* it is optimal to use the normal tissue as reference and judge the abnormal tissue in relation to the normal. As mentioned earlier, tissue fluorescence has a rather uncharacteristic shape with contributions from many chromophores. The contribution from each chromophore will also vary with excitation wavelength, and the data evaluation should be adapted to the excitation wavelength, the organ under investigation and the expected malignancies to be found. Much work has been performed, trying to extract as much useful data as possible from measurements where the histopathological diagnosis is available. Several approaches can be used, of which the more advanced cannot be performed in real time, at present.

One way of evaluating data is shown in Figure 6-3. In this case, the measurements were performed *in vitro* on a resected breast cancer specimen.[213] Twenty-four hours prior to surgery, the patient received a low dose of Photofrin®. To be able to excite the porphyrins, an excitation wavelength of 405 nm was used. The porphyrin-related signal can be seen as a fluorescence dual-peak with maxima at about 630 and 690 nm. Prior to evaluation, the spectra were corrected for the spectral sensitivity of the detection system. The emission from a calibrated black body radiator was recorded, and all fluorescence spectra were corrected with the experimental curve. The porphyrin fluorescence is evaluated at its highest peak, 630 nm (denoted A). The pure porphyrin contribution at this wavelength is extracted by lifting off the porphyrin peak. Excited at 405 nm, the autofluorescence constitutes a wide, uncharacteristic distribution. It is evaluated around its peak, in this case at 490 nm (denoted B). A dimensionless ratio can thus be formed, A/B, which is insensitive to the overall intensity and thereby to the possible variations that might occur during the measurements. As can be seen in the figure, tumour tissue is characterized by a lower autofluorescence and a higher sensitizer fluorescence. The ratio will therefore be considerably lower than that in the normal tissue. A full-size histological section of the specimen was later correlated to the obtained fluorescence spectra.

These evaluations have been performed in a specially designed program.[214] When evaluating only autofluorescence, other wavelength pairs can be found to be evaluated in a similar way. Utilizing wavelength pairs that are equally influenced by blood absorption might be beneficial.[201] More complex analysis of such data is possible. The ultimate option is to consider many wavelengths at the same time, performing multivariate analysis,[215,216] or utilizing techniques employing principal component analysis (PCA).[217,218] The optimal diagnostic criteria will vary depending on the various diseases, organs and excitation wavelengths.

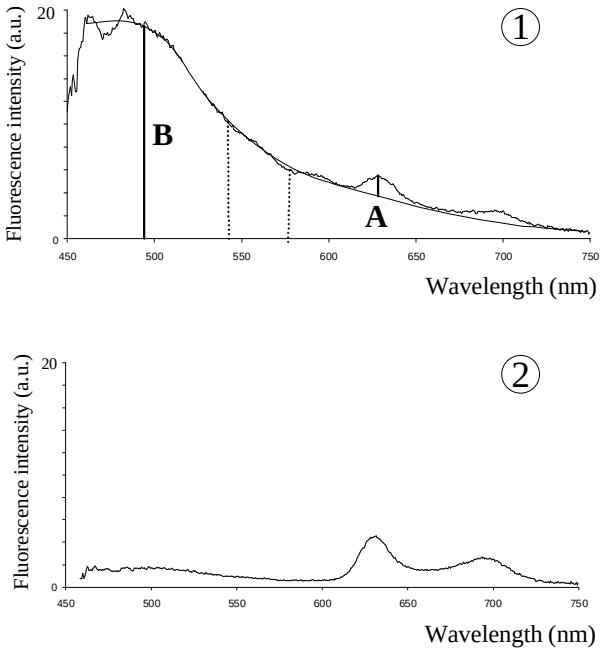

Figure 6-3 *Fluorescence spectra obtained from a resected breast cancer specimen. At 24 hours prior to surgery, a Photofrin® dose of 0.35 mg/kg b.w. was given intravenously. The obtained emission spectra (excitation wavelength: 405 nm) were later correlated to a histopathological section. Spectrum ① was detected on normal breast tissue, whereas spectrum ② was detected on an infiltrating breast cancer. The evaluation of the spectra is illustrated in spectrum ①. The autofluorescence is evaluated as the intensity at 490 nm (B). The porphyrin fluorescence is evaluated as the intensity at 630 nm, lifted off the autofluorescence curve (A). A fitted curve is added to the obtained spectra in order to subtract the background at 630 nm (thin full line). The dotted lines are placed at the wavelengths where blood absorbs, at 540 and 580 nm; particularly at 580 nm a "dip" due to the blood absorption can be seen.*

6.8. Point-monitoring measurements utilizing ALA-induced PpIX photosensitization and detection of autofluorescence

A few series of fluorescence investigations, utilizing ALA-induced PpIX sensitization and autofluorescence, have been performed in various clinical specialities. Point monitoring was used with the OMA system described in Section 6.6. Two excitation wavelengths were used, 337 nm for optimal autofluorescence excitation and 405 nm for optimal excitation of the PpIX.

Twenty-two patients undergoing cystoscopy, were included in a study of the urinary bladder.[160] Most patients had transitional cell carcinoma (TCC). The diagnoses carcinoma in situ (*cis*), dysplasia, cystitis, and a colon cancer perforating to the bladder, were also found. Six patients had no bladder disease. In ten of the patients, an 1% solution of ALA was instilled into the bladder 2-4 hours prior to investigation. Measurements with both excitation wavelengths were performed on the patients that

had received ALA, whereas patients that were not pre-medicated with ALA were only investigated with 337 nm excitation. The data were evaluated by performing ratios of the fluorescence intensity (I) at two wavelengths. With 405 nm excitation, the pair I(635)/I(490) was used, corresponding to the highest PpIX fluorescence peak (with background fluorescence subtracted) and a point near the maximum of the autofluorescence, respectively. Following 337 nm excitation, the ratios I(460)/I(400) and I(431)/I(390) were used. The first ratio was chosen, because a shift in the maximum autofluorescence from about 400 nm to about 460 nm, could be observed between the normal and the tumour tissues. The latter ratio is based on two wavelengths that are equally influenced by the blood absorption of light and therefore independent on the presence of blood in the tissue.[201] A demarcation function, D, was also calculated as $D=(m_n-m_t)/(\sigma_n^2+\sigma_t^2)^{1/2}$, where m_n and m_t are the mean values of the ratios for the normal and tumour tissue, respectively, and σ_n and σ_t are the corresponding standard deviations. A correlation was performed between the evaluated data and the histopathological diagnosis of all lesions (tissue considered as normal was not biopsied).

In conclusion, the best demarcation between normal tissue and tumour tissue was obtained when evaluating the autofluorescence following 337 nm excitation, with both evaluated ratios being higher in tumour tissue. No reliable demarcation could be found following the 405 nm excitation, even though in some patients it worked well. However, one weak point identified in the study was related to the fact that several patients probably did not retain the instilled ALA. The lesions with *cis*, dysplasia and cystitis were too few to draw any conclusions from.[160]

In another study, we investigated various pre-malignant and malignant lesions in the lower female genital tract of 45 women undergoing a routine colposcopy, primarily for investigation of cervical infections and inflammation (cervicitis).[176] Forty of the women were given an oral dose of 5 mg/kg b.w. ALA, 2-4 hours prior to investigation. Also here, the two excitation wavelengths, 337 and 405 nm, were used. Data obtained with the excitation wavelength of 337 nm, were evaluated at 8 wavelengths, 360, 380, 410, 430, 470, 490, 510, and 580 nm. Dimensionless ratios were formed by dividing the intensities at the various wavelengths with each other. Following 405 nm excitation, the ratio I(635)/I(490) was formed, where I(635) represented the background-free PpIX-related fluorescence intensity at 635 nm. Evaluated data were correlated with the histopathological diagnosis of biopsy specimens taken (areas considered by visual inspection as normal were not biopsied).

The results of the histopathological investigations showed that most of the biopsied lesions contained cervicitis, and/or cervical intraepithelial neoplasia grade I (CIN I), or grade I-II (CIN I-II). A few sites showed CIN II-III. Following 405 nm excitation, it was possible to separate the diseased tissue from the normal with a sensitivity of 77% and a specificity of 89%. Following 337 nm excitation, utilizing the two ratios I(380)/I(360) and I(430)/I(410), it was possible to detect the diseased tissue with a sensitivity of 67% and a specificity of 91%. However, with both excitation modes, it was not possible to differentiate between the various grades of CIN and

cervicitis. However, the number of higher grade CIN were few. One weak point in the study, was that the histopathological specimens were large, and some contained both normal and diseased tissue, making the correlation to the fluorescence data uncertain.[176] In comparison, the ordinary colposcopic examination, is characterized by a high sensitivity and a low specificity; the numbers depend on the experience of the doctor. According to a recently published meta-analysis, the sensitivity and specificity for differentiating normal tissue towards viral infection, CIN I-III and cancer, were 96% and 48%, respectively. For differentiating between CIN II-II and the other tissue types of CIN I, viral infection and normal epithelium, the sensitivity was 85% and the specificity was 69%.[219]

In Paper IV, we investigated a number of patients with benign and malignant lesions in the head and neck region. All patients received low doses of ALA orally (5 and 15 mg/kg b.w.) prior to the investigation. Apart from continuous fluorescence measurements of the build-up of PpIX in the normal mucosa and skin of 17 patients, as discussed elsewhere (Sections 3.3.2 and 6.4), fluorescence measurements of the lesions were performed during endoscopy 7-8.5 hours after ALA administration. The background-free fluorescence intensity at 635 nm (A) and the ratio between the fIuorescence intensity at 635 nm and 490 nm (A/B) were evaluated (see Figure 2, Paper IV). One invasive squamous cell carcinoma (SCC) in the oral cavity and four out of five spots with squamous cell dysplasia on the vocal cords, showed values of A and/or A/B that contrasted to the immediately surrounding mucosa. One spot with *cis* failed to demarcate. The benign lesions measured, namely squamous papilloma of the vocal cord and hypertrophy of the false vocal cord, showed almost identical fluorescence as did the normal surrounding tissue.

During the measurements, the switching between 337 nm and 405 nm excitation took time, and the measurements had to be performed in series. First all interesting areas were investigated with one excitation mode, and thereafter, with the other one. The combination of all results from the two modes, tended to increase the diagnostic sharpness. A new system was developed for this reason, with which measurements at 337 nm and in the near UV wavelength range, can be performed in one spot directly following each other with a total accumulation time in the order of 6-7 seconds.[220]

6.9. Fluorescence imaging techniques

Systems that can provide images of a tissue surface are very interesting from a diagnostic point of view. With such a system it is easy to examine large areas. They also automatically provide data from many reference locations adjacent to a suspect area. The challenge from an instrumentation perspective, has been to develop simple systems that can utilize spectral information and not only the fluorescence intensity at a specific wavelength band. Several such systems for fluorescence imaging have been developed.[211,221-227] Recently, reviews have been presented.[228,229] A few examples of various imaging systems which have been used clinically, will be briefly described below.

The very simplest technique for viewing fluorescence, is by the investigator's eyes. Though hardly an imaging technique, it is mentioned in this section. In connection with endoscopic examinations of hollow organs, it is possible to utilize the eye as a detector when the tissue is sensitized with porphyrins and no background light is present. The tissue autofluorescence cannot be easily exploited with this method, due to its low fluorescence yield. Direct visual inspection of red fluorescence, during violet illumination, was one of the first methods in use.[26,230] This was at the time when HPD was investigated for both its fluorescence characteristics and its photodynamic action. After the introduction of ALA-induced PpIX photosensitization, visual fluorescence examination became interesting again due to the simplicity of the system and the sensitization. Following excitation with UV or near-UV irradiation of tissue sensitized with ALA-induced PpIX, a bright red porphyrin fluorescence can be seen. Particularly in the bladder, much work has been performed. Kriegmair *et al.* performed such fluorescence cystoscopy following ALA-instillation in 104 patients with early bladder cancer. A significantly better sensitivity than with ordinary white light examination was obtained, with no improvement of the specificity.[231]

The LIFE system (Light-Induced Fluorescence Endoscopy, Xillix Tech. Inc., BC, Canada) is a system commercially developed for endoscopic applications. A continuous wave (CW) light source is used, emitting in the violet wavelength region. Only tissue autofluorescence is detected and two images, one in the red and one in the green emission band, are synthesized into a composed false-colour image. The system has so far proved to provide valuable diagnostic information when used in certain clinical specialities, giving an improved outcome when utilized together with the ordinary white light examinations. Lam *et al.* have performed extensive studies in the bronchi,[226,232,233] and the system has also been used in the detection of cancers in the head and neck region.[234,235]

Another multi-colour fluorescence imaging system, which can be utilized together with fibre-optical endoscopes, has been developed in Lund.[192,194,195] The system is based on beam-splitting optics, resulting in four images, filtered in different fluorescence emission bands. The images are detected on an intensified CCD camera, utilizing gated detection. Computer processing of the images is performed for viewing a single optimized-contrast-function image. A pulsed light source, an alexandrite laser, tunable in the range of 720-800 nm has been used as a light source in a frequency-doubled mode, emitting at about 390 nm. The pulsed excitation and gated detection allow for performing fluorescence imaging simultaneously with an ordinary white light examination. The system has been studied in connection with investigations of malignancies in several organs.[193] In this context, a photosensitizer (Photofrin or ALA-induced PpIX) has been used, and three of the four wavelength bands were used for the detection. The sensitizer-related fluorescence in the red (A), the autofluorescence evaluated in the blue, around 470 nm (B) and an image in the green yellow region (D) were used. The latter image is needed to be able to subtract the pure sensitizer-related fluorescence from the background autofluorescence. The following function has been used for the image processing

$$\text{Image function} = (A - k_1 D)/k_2 B,$$

where A, B and D are defined above. The two constants, k_1 and k_2, have different values for different applications. After image processing, a false-colour image is shown on the computer screen. This false-colour image can also be superimposed on the ordinary white light image, allowing a direct comparison between the two images. Figure 6-4 shows an example of an image of a BCC, sensitized with ALA-induced PpIX, captured with a modified version of the system described above. In this case, only two images were used to process the final image. Of the two bandpass filters used, one transmitted in the region around the PpIX fluorescence (580-750 nm) and one transmitted much of the autofluorescence (480-580 nm), and the first divided by the second yielded the processed image.

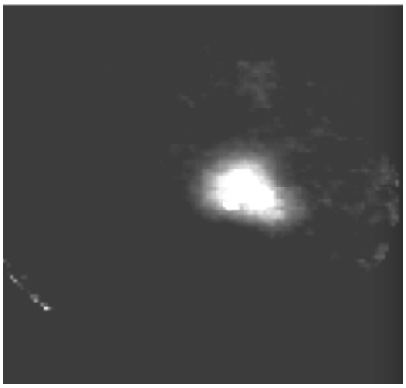

Figure 6-4 *Image of a superficial BCC lesion 6 hours after application of ALA cream (concentration 20% by weight), utilizing a multi-colour imaging system. The same lesion can be seen in a lifetime image, as well as in a photographic reproduction in Figure 6-5.*

Systems detecting fluorescence lifetimes can also be used for imaging. Cubeddu *et al.* have developed such a system. This system is not yet adapted for endoscopic use. Photosensitizers are characterized by a longer lifetime than that of the autofluorescence. In addition, the fluorescence lifetime is dependent on the micro-environment. The balance between the short-lived autofluorescence and the longer-lived porphyrin fluorescence, in addition to possible variations in the lifetime of the sensitizer, are responsible for the image contrast provided by this system. A nitrogen-laser-pumped dye laser is used as an excitation source, emitting at 405 nm. Pulses of 1 ns duration are emitted, and fluorescence is detected by a gateable intensified CCD camera, with a gate rise time of about 2 ns. A cut-off filter is placed in front of the detector, suppressing most of the autofluorescence in the blue-green wavelength region, and transmitting the red fluorescence light. The system is described in detail elsewhere.[236] In Lund, the system has been implemented in the detection of BCCs in

connection with ALA-PDT.[237-239] In Figure 6-5, a BCC is imaged by the lifetime system, 30 minutes after application of ALA in a cream base (concentration 20% by weight). With the time-resolving system used in Lund, the best demarcation was seen after a short application time of less than an hour, before the PpIX built up in the normal, surrounding skin. That the demarcation between the normal skin and the BCCs is better after a short application time, is in good agreement with results of the diagnostic measurements of fluorescence intensity, presented in Paper VII.

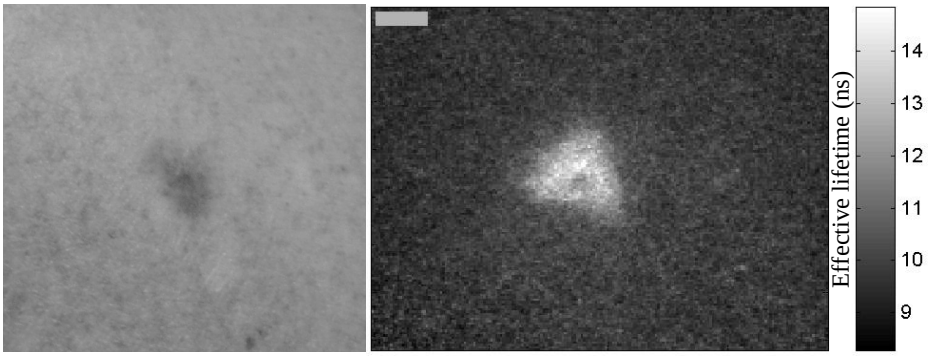

Figure 6-5 *Fluorescence lifetime imaging. A fluorescence lifetime image of a PpIX-sensitized BCC (application time 30 minutes) is seen (right). The grey-scale to the right indicates the lifetime. A photograph of the lesion prior to ALA application is shown to the left.*

A Fourier transform multi-pixel spectroscopy system, as described by Malik et al,[240] allows the full fluorescence spectrum to be recorded in each image pixel. The system can be connected to a microscope, enabling one to visualize for instance the sub-cellular localization of fluorescent dyes. The detailed spectral shape of the dye-related fluorescence, as present in particular cellular environments can also be studied. Alternatively, the system can be applied for imaging of macroscopic surfaces, and Orenstein *et al.* detected skin lesions with such a set-up.[173] A draw-back compared to the multi-colour fluorescence imaging system is, that the sample must be kept fixed during the scan of the Fourier spectrometer mirror.

6.10. Some considerations on tumour-related spectral changes

Tumour growth in mucous membranes is often characterized by a thickening of the epithelial layer. An often identified change in fluorescence in tumour areas, is an overall decreased autofluorescence. This has partly been related to the relatively lower contribution of fluorescence from the underlying connective tissue with higher collagen content.

In the skin, there is a strong fluorescence from the outer keratin layer (approximately 10 μm thick, see Figure 6-1). When stripping off this layer in normal skin, the overall autofluorescence has been shown to decrease significantly.[211] In many

skin tumours, there is a break-down of this epidermal compartment by the tumour growth, which can explain the reduced overall autofluorescence found in these tumours, as described in Papers VI and VII. In contrast, some tumours, for instance at the vocal cords, are characterized by an thickened keratin layer which has a strong autofluorescence. When low doses of ALA are given, PpIX-related fluorescence cannot be seen in such keratinized lesions (C. Eker, personal communication). Furthermore, we investigated several types of normal epithelium also using low doses of ALA (Paper IV). Some PpIX-related fluorescence could be seen in all types of normal epithelium, but the intensity was lower in the skin than in the non-keratinized epithelia of the oral cavity and nose, as well as the tongue with partially keratinized papillae. One explanation for this, related to the optical properties of the tissue, might be scattering and absorption of the excitation and emission light in the keratin layer.

As mentioned earlier, of the other contributing chromophores, the ratio between NADH and NAD+, has been considered to be particularly important in connection with malignant development in tissue. NADH is highly fluorescent with a peak around 460-470 nm, which is not the case for the oxidised form, NAD+. An altered balance between the reduced and the oxidized form in pre-malignant and malignant tumours, with a lower level of the reduced form, has been suggested as an explanation.[196,197] It is also known that malignant tumours can contain endogenous porphyrins.[212,241-243]

7. LASER-DOPPLER PERFUSION IMAGING

Studies of blood perfusion in superficial tissue layers, have been simplified by the introduction of optical techniques utilizing laser-Doppler measurements. Laser-Doppler techniques have been used for a variety of applications, primarily the investigation of the microcirculatory responses to various agents and other stimuli.[244-246] Laser-Doppler perfusion monitoring (LDPM) systems have been developed for clinical use, for point-wise measurements.[247-249] The technique is based on the spectral broadening of monochromatic light after scattering by moving red blood cells in the microvascular network. The spectral distribution of weak Doppler shifted components in the diffusely back-scattered light, can be extracted and used to determine the perfusion state of tissue. However, the spatial variation of blood perfusion can be large, and the local variations of the laser-Doppler signal have been found to be notable.[250] Furthermore, point-monitoring systems, are usually working in a contact mode and those systems are designed for detecting temporal changes in single spots on a tissue surface. There is, however, a risk of inducing perfusion changes by applying pressure on the tissue with the probe.

To be able to analyse the total perfusion state of a superficial tissue area, imaging techniques for non-contact measurements, are needed. Such a system, for non-invasive, real time detection of superficial microvascular blood flow, has been developed and is now fully adapted for clinical use.[251] Some areas of application have been; to follow the perfusion state of skin flaps in plastic surgery,[252] or to follow the perfusion in port-wine stains in connection with laser treatments.[253] The system has also been used for mapping myocardial perfusion in connection with coronary by-pass surgery.[254] We have evaluated this system in the clinical situation in connection with photodynamic therapy (PDT) and cryosurgery of malignancies. The system and our results will be discussed briefly.

7.1. A laser-Doppler perfusion imaging system for clinical use

A commercially available laser-Doppler imaging system (Lisca Development AB, Linköping, Sweden), can be used to scan the superficial blood perfusion. This system is thoroughly described elsewhere.[251] The optical system is composed of a laser emitting light at 633 nm (Helium-Neon or diode laser), and two scanning mirrors yielding a probe beam. The estimated probe depth is a couple of hundred μm.[255] In the skin, this is sufficient for at least probing the blood perfusion in the cutaneous capillaries that are situated in the dermal papillae. The beam is sequentially scanned over the tissue under investigation in a step-wise manner, with a maximum number of measured sites being 64×64 pixels. The area over which the probe beam is scanned is determined by the distance between the light source and the tissue. The reflected light from the moving blood cells is collected by a photo diode situated next to the light source in a scanning and detection box. The resulting Doppler shift distribution, yielding signals proportional to the amount of scattering particles and their velocities,

is deduced and displayed as a colour-coded image. The images can be stored, re-displayed, and printed. In a commercial evaluation program for laser-Doppler images, basic statistical tools are available for image analysis (LDISOFT 1.0, Lisca Development AB, Linköping, Sweden).

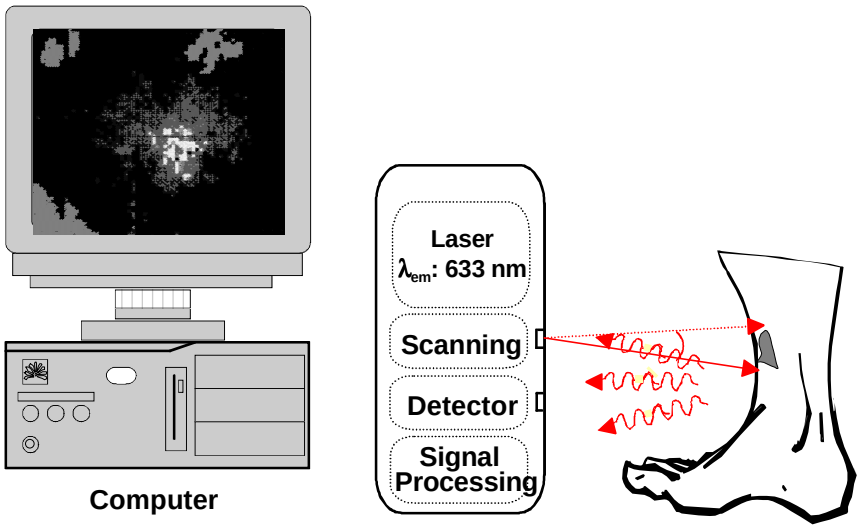

Figure 7-1 *The arrangement of the laser-Doppler imaging system.*

7.2. Laser-Doppler imaging in connection with treatment of skin lesions

Laser-Doppler measurements were performed in connection with the treatment of non-melanoma malignant skin tumours. The lesions were treated with either PDT utilizing topical sensitization with ALA-induced PpIX, or with cryosurgery.

A distance of 16±0.5 cm between the scanning and detection box and the skin surface was maintained during the studies. The number of pixels used were 40×40 or 60×60, which resulted in a scanned area of 6×6 cm^2 or 10×10 cm^2, respectively. With this LDI system set-up, the imaged surface should have its normal parallel with the laser beam. Surfaces with a geometry that is not plane, could not be reliably detected. Often, such geometrical problems could be solved by evaluating only parts of an image. The lesions were often imaged in the centre of the laser-Doppler image, surrounded by normal skin. When images where taken in connection with treatments, untreated tissue outside a treatment safety margin of 1 cm was also included. To achieve this, exceptions to the centrally imaged lesions sometimes had to be made for BCC regions with large surrounding areas of increased blood perfusion.

The images were analysed by taking an average of the resulting blood perfusion signal in the BCC area and the surrounding normal untreated skin, respectively. The areas in the images were correlated to detailed drawings and photos made during the

recordings. The values from the different areas were computed in the evaluation program for laser-Doppler images. Furthermore, a tissue perfusion ratio was formed by dividing the average blood perfusion in the lesion with that in the normal untreated tissue. This was performed in order to compensate for patient-to-patient variations in skin pigmentation and for a possible increase in the tissue perfusion not correlated to the treatment procedures, but due to external factors, such as increased room temperature, activity and stress level of the patient, influencing both tissue regions. A time delay of ten minutes between the cryosurgery and the blood perfusion measurements, allowed most frozen areas to regain more or less normal skin temperature. For some lesions, the central parts did not return to normal temperature when the laser-Doppler image was recorded, resulting in a lower blood perfusion. The average blood perfusion in the BCC area was then based on the peripheral lesion region only, with normal tissue temperature and high blood perfusion. This evaluation approach was also applied when the central lesion was covered with a necrotic crust, preventing the laser light from probing the blood perfusion underneath, due to its low penetration depth.

In an initial study (Paper I), LDI was employed in connection with the treatment of non-melanoma malignant skin lesions with only PDT. Superficial and nodular BCCs as well as a few lesions of Mb. Bowen, cutaneous T-cell lymphoma or psoriasis, were detected. Prior to treatment, all lesions were characterized by an elevated blood flow compared to normal skin. The average ratios between the lesions and the normal skin were about 5:1 for the different malignant skin tumours and 10:1 (only two lesions measured) in the psoriatic plaque. Immediately after treatment, the tissue perfusion increased strongly in the lesion and the immediate surrounding skin. This indicates that ALA-PDT with topical application has a pronounced direct cytotoxicity which primes an immediate inflammation in the tissue with increased perfusion. At follow-up visits more than 3 weeks after the last treatment session, all lesions had a normalized blood flow ratio between tumour and normal skin. A series of LDI images and the corresponding photographs recorded prior to and immediately after PDT, as well as in the follow-up period, can be seen in Paper I (Figure 2).

The LDI studies were continued in connection with a Phase III clinical trial (Paper V). In this trial, the time of healing was one parameter determined when the tolerability of the treatments was evaluated. Monitoring the superficial blood perfusion employing LDI was used as an objective measure on this parameter (Paper VIII). Laser-Doppler images were recorded prior to and immediately after the treatment, as well as during a follow-up period of one year. Some lesions were also recorded at a two years follow-up visit. Before treatment, the lesions exhibited a blood perfusion of 3 (SD=2) times that in the normal tissue. Both PDT and cryosurgery were shown to induce an increase in the blood perfusion in the lesions, which slowly approached normal values in conjunction with successful treatments. The superficial blood perfusion in the lesions successfully treated with photodynamic therapy, was shown to approach normal values at two months post treatments, compared to about

one year following cryosurgery. The tissue perfusion in recurrent lesions was shown not to decrease to normal values after the treatment.

7.2.1. CONCLUSIONS

Non-melanoma malignant skin lesions and psoriatic plaques, are characterized by an elevated blood perfusion as detected by laser-Doppler imaging. In Paper I, we found the average ratios between the different types of lesions and normal skin to be higher than what we found for the BCCs in Paper VIII. In the latter we included a much larger number of lesions, thus, this result might be more representative.

When utilizing topical ALA-induced PpIX as photosensitizer for PDT, the haemodynamic reaction to the treatment was an increased superficial flow. In comparison, when ALA was systemically supplied to rats with inoculated liver tumours, there was a decreased superficial perfusion state following the PDT irradiation.[74] Also, the effect on the tumour vascular supply with subsequent tissue hypoxia has in general been considered a major treatment effect in PDT with systemic administration of the photosensitizer (see Section 3.6.4). In the case of topical application, a hypoxic treatment effect due to immediate vascular effects seems to be less probable and direct cellular effects more likely.

Laser-Doppler imaging can be used to follow the healing process in connection with the treatment of basal cell carcinomas. The technique can also be used to discover possible residual or recurrent tumours in the follow-up period after treatment.

8. SUMMARY OF PAPERS

In Paper I the non-invasive diagnostic method of laser-Doppler imaging (LDI) is discussed. LDI is employed in connection with the treatment of non-melanoma malignant skin tumours with PDT, utilizing ALA-induced PpIX with topical application for photosensitization. Prior to treatment, all lesions were characterized by an elevated blood flow compared to that of normal skin. Immediately after treatment, the tissue perfusion increased strongly in the lesion and in the immediate surrounding skin. This indicates that ALA-PDT with topical application has a pronounced direct cytotoxicity, which primes an immediate inflammatory response in the tissue with increased perfusion. The increased perfusion in the tumour contradicts earlier investigations with systemic administration of both ALA and pre-formed photosensitizers, where an effect on the tumour vascular supply with subsequent tissue hypoxia has been considered a major treatment effect. At follow-up visits more than 3 weeks after the last treatment session, all lesions had a normalised blood flow ratio between tumour and normal skin.

Papers II and III concern the treatment of non-melanoma malignant skin tumours with ALA-PDT. The first patients with non-melanoma skin lesions treated with ALA-PDT in Lund, are described (Paper III). Superficial basal cell carcinomas (BCCs) were resolved with one treatment whilst two treatment sessions were needed for nodular BCCs. The response rate was assessed by visual inspection and palpation, and in 25% of the lesions also by biopsy with histopathology. A good cosmetic result was achieved. A subgroup of patients had lesions on the eyelids and in the periocular region (Paper II). Following, in median, 3 treatment sessions, complete response was seen in 42% (8/19) of BCCs and in 100% (3/3) of cutaneous T-cell lymphoma lesions. In the BCCs, partial response was achieved in another 42% (8/19) and no response in 16% (3/19). Minimal scarring and loss of cilia were induced by the treatment of the eye-close lesions, something which is particularly important at this anatomic site.

In Paper IV diagnostic measurements and pharmacokinetic studies performed in seventeen patients with various kinds of malignant, pre-malignant and benign lesions in the head and neck region, by means of point-monitoring laser-induced fluorescence (LIF), are discussed. For marking different types of tissue, ALA-induced PpIX was used. The PpIX synthesis was monitored by means of fluorescence, at different time intervals after oral administration of ALA in low doses, 5 and 15 mg/kg b.w. Besides the porphyrin-related signal with a peak at about 635 nm, the tissue endogenous fluorescence with a broad fluorescence emission, peaking at about 490 nm, was recorded. The evaluated fluorescence data were correlated to the histopathological tissue investigation. A fall-off in the overall fluorescence intensity evaluated at 490 nm was obtained for all the malignant and pre-malignant areas, as well as an increased red fluorescence peak. A ratio between the red and the blue/green fluorescence intensities was formed. The lower drug dose seems to be sufficient to obtain a useful

demarcation ratio between normal and diseased tissue with a very low PpIX fluorescence intensity in the normal tissue. A PpIX fluorescence maximum seems to occur between 3-4 hours in normal tissues, whereas malignant tumour tissue shows a higher PpIX fluorescence level for a longer period of time.

In Papers V-VIII, data from a prospective, randomised Phase III clinical trial are presented. Patients with BCCs were randomized to treatment with either ALA-PDT or cryosurgery. In total, 88 patients were included in the trial.
In Paper V, the clinical outcome of the treatments is presented, with regard to recurrence rate and tolerability, *i.e.* time of healing, pain in connection with the treatment and final cosmetic outcome. In total 88 patients were included, all on the basis of one lesion. The other treatment arm was cryosurgery. In conclusion, the clinical study showed that the efficacy of ALA-PDT and of cryosurgery are statistically comparable, with a tendency of a small advantage for cryosurgery. On the other hand, ALA-PDT was followed by a significantly shorter healing time and better cosmetic outcome.

Laser-induced fluorescence measurements were obtained in connection with the ALA-PDT treatments, and the data are presented in Papers VI and VII. Protoporphyrin IX build-up and photodegradation were detected by means of LIF as the background-free fluorescence intensity at 635 nm. In 40 patients, the PpIX levels were detected prior to, as well as immediately after, a light dose of 60 J/cm^2 at 635 nm. The ratio of PpIX-related fluorescence inside the lesion to that in the normal adjacent skin was found to be 2.3 (SEM=0.2), six hours after ALA application. Immediately after light irradiation, the percentage of PpIX fluorescence left was calculated as a ratio between the PpIX fluorescence pre- and post-treatment. The values were found to be 8% (SEM=1.2%) in superficial BCCs and 10% (SEM=1.4%) in nodular BCCs. From these results, an effective bleaching fluence, *i.e.* the light dose corresponding to a reduction of PpIX fluorescence to a level of 1/e=0.37 of the initial value, could be calculated to be about 25 J/cm^2. One could not correlate the PpIX fluorescence level prior to illumination with the outcome of the treatment (Paper VI). In 15 of these patients the kinetics of the PpIX build-up was detected at 2, 4 and 6 hours after ALA application, as well as at 2 hours after the light exposure. Superficial BCCs showed a maximum PpIX fluorescence 6 hours after ALA application, while in nodular BCCs, the intensity was at its highest level after 2-4 hours. The increase in PpIX fluorescence was slower in normal skin. Consequently, the contrast in PpIX fluorescence intensity decreases with longer application times, being about 2.5 after 6 hours of ALA application. After light exposure a fluorescence contribution peaking at 670 nm, associated with a light-induced photoproduct was seen, with an intensity of 2% of the pre-PDT PpIX peak fluorescence values. Two hours after light exposure, there was a re-accumulation of PpIX, seen as a uniform distribution of PpIX fluorescence in the lesion and the adjacent normal skin (Paper VII).

The time of healing was one parameter determined when the tolerability of the treatment was evaluated. Monitoring the superficial blood perfusion *in vivo* employing

LDI as outlined in Paper I, gives an objective measure on this parameter (Paper VIII). LDI images were recorded prior to and immediately after the treatment, as well as during a follow-up period of in total 13 to 24 months. Before treatment, the lesions exhibited a blood perfusion 3 (SD=2) times that in the normal tissue. Both PDT and cryosurgery were shown to induce an increase in the blood perfusion in the lesions, which slowly approached normal values in conjunction with successful treatments. The superficial blood perfusion in the lesions successfully treated with photodynamic therapy was shown to approach normal values at two months post treatments, compared to about one year following cryosurgery. The tissue perfusion in recurrent lesions was shown not to decrease to normal values after the treatment. This suggests that the non-invasive method of laser-Doppler imaging also can be used to follow the healing process and to discover possible residual or recurrent tumours in connection with the treatment of basal cell carcinomas.

The contribution of the author to the papers was as follows:
Paper I: performed most of the tissue perfusion measurements, evaluated all data and prepared the major part of the manuscript. Paper II: together with colleagues performed the PDT treatments and clinical follow-up with evaluation of clinical outcome. Performed the fluorescence measurements and prepared the major part of the manuscript. Paper III: participated in most PDT treatments and the clinical follow-up with evaluation of treatment outcome and contributed to the preparation of manuscript figures. Paper IV: performed measurements, evaluated data and prepared the manuscript.

Papers V-VIII: The author took active part in the planning and preparation of the protocol for the Phase III, clinical trial conducted in the treatment of BCCs. The author took also part in the formal monitoring of the trial. Papers V-VIII all present data obtained in connection with this trial. In these papers, the contribution of the author was as follows:
Paper V: in collaboration, performed the PDT treatments, as well as the clinical follow-up of all patients within the trial, with evaluation of clinical outcome, and prepared the manuscript. The inspection and statistical handling of the listed clinical data, were performed by formal monitors. Papers VI-VIII: together with colleagues performed all measurements, correlated the obtained LIF and LDI data with clinical outcome, and contributed to the manuscripts.

ACKNOWLEDGEMENTS

"No man is an island"

Curiosity about the implementation of lasers in medicine brought me to Lund in 1992. I really appreciated to get the opportunity to stay as a Ph.D. student within the framework of the Lund University Medical Laser Centre. The willingness shown from the Norwegian Cancer Society to economically support me was instrumental for the whole project. The work described in this thesis was carried out in a close collaboration between the Department of Oncology, Lund University Hospital and the Atomic Physics Division, Lund Institute of Technology.

Besides the interesting, interdisciplinary projects and the scientific surroundings, the atmosphere of warmth, humanity and enjoyment in the work, created by my supervisors, have made these years truly enjoyable. I am indeed grateful for having had the opportunity to receive this education and guidance in science from Katarina Svanberg, Associate Professor at the Department of Oncology, and Sune Svanberg, Professor at the Atomic Physics Division. In Katarina, my main supervisor, I have also had, with deep respect, a model in the important development of the role as a physician. I am also grateful for a nice and warm friendship.

My co-supervisor Stefan Andersson-Engels, Associate Professor at the Atomic Physics Division, has been an enormous help in many ways, both in all the practical details and in the planning and evaluation of studies, for which I am truly grateful. His impressive knowledge in medicine made it particularly nice and inspiring to discuss various projects.

Professor Dick Killander at the Department of Oncology who I have had the opportunity to have as a co-supervisor, has given very important support, particularly in connection with the clinical trial. This is highly appreciated.

During my stay in Lund, I have had the fortune to learn from and work with an additional number of qualified and nice people. I would like to express my sincere gratitude to:

The people at the Atomic Physics Division where I have had my daily workplace, and particularly my colleagues in the medical group:

- Dr. Annika M. K. Enejder for good collaboration in the clinical trial and a nice friendship.
- Claes af Klinteberg (M.Sc.) for his skills and never-ending patience and help with computer problems and for good collaboration. His interest in language is also appreciated, especially by a Norwegian struggling with the finer nuances of the so-called "similar" Swedish language.
- Dr. David L. Liu with whom I performed most of the early work during my time as a Ph.D. student.

- Dr. Jonas Johansson and Dr. Roger Berg for invaluable help with technical procedures and systems, particularly in the beginning of my stay.
- Dr. Sune Montán, Ingrid Rokahr (M.Sc.), Charlotta Eker (M.Sc.), Dr. Christian Sturesson, Thomas Johansson (M.Sc.), Inga Karu (M.D., Estonian guest researcher), and Sara Pålsson (M.Sc.) for good co-operation and friendliness.
- Physicians at collaborating clinics at the Lund University Hospital, particularly: Associate Professor Birgitta Bauer, Department of Ophthalmology, Niels Bendsøe (M.D.), Department of Dermatology and Venereology, Professor Unne Stenram, Department of Pathology, and Dr. Roland Rydell, Department of Oto-Rhino-Laryngology.
- Eva Gynnstam at the Wallenberg Laboratory and Monica Radnell, Department of Surgery for help with the animal models.

The work as a Ph.D. student has been additionally interesting and educational due to several international co-operative efforts. It has been extremely nice to participate in collaborative work at other European university clinics. I would like to acknowledge our international collaborators and hosts:
- Dr. Aurelija Vaitkuviene, Women's Clinic and Laser Surgery Department, Vilnius University Hospital, Vilnius, Lithuania.
- Dr. Manuel Pais Clemente, Laudelina Pais Clemente (M.D.), Rui M. G. Pratas (M.D.), and Eduardo Cardoso (M.D.), Department of Oto-Rhino-Laryngology, Oporto University Hospital, Oporto, Portugal.
- Dr. Mari-Ange D'Hallewin and Professor Luc Baert, Department of Urology, St. Pieter University Hospital, Leuven, Belgium.
- Professor Johan Moan and Dr. Qian Peng, the Institute of Cancer Research, Helen Heyerdahl (M.Sc.) and Dr. Trond Warloe, Department of Oncological Surgery, The Norwegian Radium Hospital, Oslo, Norway.
- Dr. Heyke Diddens, Dr. Gereon Hüttmann, Lübeck Medical Laser Centre and Professor Dieter Jocham, Department of Urology, Lübeck University, Lübeck, Germany.
- Professor Stephen Bown and Dr. H. Messmann (guest researcher) National Medical Laser Centre, University College London, The Rayne Institute, London, U.K.

All the patients who have given of their time to participate in measurements and answer question formulas are also remembered.

Finally, the support from my family and dear friends is, as always, invaluable. My deepest gratitude goes to my parents. My father Gunnar's enthusiasm for science has always been a rich source of inspiration. Discussing various plans and projects with him is always a pleasure, especially because it is often combined with long walks or jogging in the countryside or in the mountains. My mother Brit has that special gift of creating an atmosphere in which one can be creative and find peace.

Besides the financial support from the Norwegian Cancer Society, generous financial support was achieved from the Medical Faculty at the Lund University. Support was also obtained from the Swedish Cancer Society, the Swedish Research Council for Engineering Sciences (TFR), the Swedish Board for Technical and Industrial Development (NUTEK), Johnson & Johnson Medical GmbH, Germany, Mrs. Berta Kamprad's Foundation for Cancer Research, and the Portuguese Council for Smoking Prevention. The support is gratefully acknowledged.

Lund, January 1999
Ingrid Wang

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ABBREVIATIONS AND SYMBOLS

ALA	δ-aminolevulinic acid
ALA-PDT	Photodynamic therapy utilizing ALA-induced PpIX as photosensitizer
BCC	Basal cell carcinoma (syn. basalioma)
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma *in situ*
CR	Complete response
CW	Continuous wave
λ_{em}	Emission wavelength
Hb	Haemoglobin
HP	Haematoporphyrin
HPD	Haematoporphyrin derivative
LED	Light emitting diode
LDI	Laser-Doppler imaging
LIF	Laser induced fluorescence
Mb.	Morbus, *i.e.* disease
$^1O_2$	Singlet oxygen
OMA	Optical multichannel analyzer
PCT	Photochemotherapy
PDT	Photodynamic therapy
PpIX	Protoporphyrin IX
SCC	Squamous cell carcinoma
UVR	Ultraviolet radiation, *i.e.*, wavelength range 200-400 nm

PAPERS I-VIII