New insights into photodynamic therapy of the head and neck
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Chapter 1

General Introduction
This thesis is focused on photodynamic therapy (PDT) of cancers in the head and neck region. The main aims of this thesis are analyzing the current situation of PDT of head and neck cancer and identify opportunities for potential improvements. Thereto, a critical analysis of clinical use and outcome of the current state of the art PDT treatment is needed. Development of spectroscopic tools can help us understand clinical PDT and help to optimize it. Development of new light delivery methods such as interstitial treatments will broaden the indication range of PDT. This chapter of the thesis gives brief background information about PDT and head and neck cancer.

**HEAD AND NECK SQUAMOUS CELL CARCINOMA**

Head and neck tumors is a collective name given to all tumors located in the head and neck area excluding the tumors of the brain, eyes, the thyroid gland, skin, bones and muscles. It encompasses a broad spectrum of tumors of different histologic origins. The overwhelming majority of these tumors are squamous cell cancers. This thesis concentrates on squamous cell carcinoma of the upper aerodigestive tract or as commonly used: head and neck squamous cell carcinoma (HNSCC).

Globally the incidence of HNSCC is 633 per 100,000 population with mortality of 355 per 100,000 population [1]. In the Netherlands, about 2970 new head and neck cancers were diagnosed in 2011 [2]. This number comprises about 4% of all cancer cases. The incidences are 10.2/100,000 in males and 5.4/100,000 in females. Advanced head and neck cancer is the fifth most common cancer in men in the world [3]. The incidence has an increasing trend. Between 1999 and 2000 the average incidence increase was 3.5% per year [4].

Conventional treatment of HNSCC is surgery, radiation, or a combination of two with or without accompanying chemotherapy [5]. The choice of primary treatment may vary for the primary site of the tumor as well as among different countries and institutions. For example, while the primary treatment of oral cavity cancers is surgical removal alone or combined with (chemo)radiotherapy depending on the tumor stage, the pharynx cancers are usually primarily treated with (chemo)radiation and surgery is a salvage option if (chemo)radiation fails. Alternative treatment methods such as photodynamic therapy (PDT) have not gained popularity and are not incorporated in treatment guidelines. Chapters 2 and 3 of this thesis explore PDT as an alternative to surgery of oral cavity squamous cell cancers.

Around 50% of the patients with HNSCC fail first line of treatment [6]. Majority of these second primary tumors or recurrent/residual tumors are located in the previously treated region. These advanced cancers are hard to manage in the previously treated region. Further surgical resection (salvage surgery) provides 30-40% 5-year survival, and is
usually complicated with functional losses and impaired wound healing [7]. Re-radiation is usually not possible because of increased radiotoxicity to vital structures as a result of the cumulative character of radiation [8]. Complications include osteoradionecrosis of skull, facial skeleton and vertebral column, radiation neuropathies and frequently inability to swallow. Palliative chemotherapy can be administered to these patients provided that their general health condition is favorable to endure the side effects caused by this treatment. Several of these patients are elderly and do not meet the general health condition criteria for palliative chemotherapy. The objective response rates of palliative chemotherapy are reported to be between 10-30% [9]. Newer strategies with targeted therapies combined with chemotherapy fail to increase the overall response beyond 30% [10]. There is a need for a new treatment for these patients who have no treatment options left except for supportive care or hospice [5].

PHOTODYNAMIC THERAPY (PDT)

PDT is based on the interaction of three essential components: photosensitizer(PS), light and oxygen [11,12]. The PS, which is not toxic initially, can be activated by light to react with oxygen to produce reactive oxygen species (ROS) (13). ROS can readily oxidize biomolecules leading to cell death. The treatment effects of PDT against cancer derive from three mechanisms: direct cytotoxic effects on tumor cells [13-16], damage to the tumor vasculature [17,18] and induction of an inflammatory reaction that can lead to development of systemic immune response [19,20].

PHOTOCHEMICAL REACTION

Most PSs in their ground (i.e. singlet) state have two electrons with opposite spins located in an energetically most favorable molecular orbital. Absorption of light leads to a transfer of one electron to a higher-energy orbital. The excited form of the PS either gets rid of the excess energy by emitting fluorescence or it may undergo an intersystem crossing to form a more stable triplet state with inverted spin of one electron. The triplet state can interact with molecules in the environment. Two types of interactions are described [21].

For type I interactions, the PS reacts directly with an organic molecule in a cellular microenvironment, acquiring a hydrogen atom or electron to form a radical. Subsequent autoxidation of the reduced PS produces a superoxide anion radical. Dismutation or one-electron reduction of superoxide anion radical gives hydrogen peroxide, which in turn can undergo one-electron reduction to a hydroxyl radical.
For type II interactions, the PS in triplet state can transfer its energy to molecular oxygen, which leads to the formation of reactive oxygen species and specifically singlet oxygen.

**CYTOTOXICITY**

The main photochemical reaction leading to PDT effect is believed to be a type II reaction rather than a type I reaction. The lifetime of produced singlet oxygen, however, is very short, limiting its diffusion to only approximately 10-55 nm in cells [22]. Therefore the subcellular location of the PS determines the type of damage to the cell. Various photosensitizer have different target organelles and specific patterns of localization may vary also among different cell types. The PS used in this thesis, m-tetrahydroxyphenylchlorin (mTHPC) has been reported to target mostly mitochondria and endoplasmic reticulum [14].

The damage caused by singlet oxygen can lead to one of three kinds of cell death pathways: apoptosis, necrosis and autophagy-associated cell death [15]. Apoptosis is a generally major cell death modality in cells responding to PDT. Apoptosis is programmed cell death caused by activaton of intracellular molecular pathways, which lead to characteristic cell changes (morphology) and death. These changes include cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. The apoptotic cells are removed by phagocytes and do not induce an immune response. mTHPC is situated predominantly in mitochondria and cause photodamage mainly in this organelle. Photodamage to the regulatory proteins of Bcl-2 family located in the mitochondrial membrane triggers caspase related apoptotic pathways [15,16,23]. Other kinds of pathways triggered by various proteases such as calpains are also involved in the apoptotic effects of PDT [15,23].

Necrosis is a disorderly cell death usually caused by extensive damage to the cell membranes, rendering intracellular content exposed to the environment. Especially membrane specific PSs cause this reaction [15]. Necrosis can induce immune responses by exposing normally hidden intracellular antigens to the immune system. Recent evidence suggests that certain form of necrosis can be propagated through signal transduction pathways [24]. The molecular mechanisms underlying programmed necrosis are still elusive, but certain events including activation of receptor interacting protein 1 kinase, excessive mitochondrial ROS production, lysosomal damage and intracellular calcium overload, are recurrently investigated [23]. Severe inner mitochondria membrane photodamage or intracellular calcium overload could promote mitochondrial permeability transition, and favor necrotic rather than apoptotic phototoxicity [15].
Recently PDT was also associated with the stimulation of autophagy [25-27], which is a lysosomal pathway for the degradation and recycling of intracellular proteins and organelles. The breakdown of cellular components can ensure cellular survival during starvation by maintaining cellular energy levels. Autophagy can be stimulated by various stress signals including oxidative stress through ROS [27]. Recent studies suggest autophagy as a means of survival after PDT as well as an additional programmed cell-death [27]. The oxidative stress caused by PDT causes photodamage to the organelles and especially the endoplasmic reticulum (ER), triggering autophagy mechanisms that remove these ROS-damaged organelles and cytosol proteins. The relationship or balance of autophagy and apoptosis is not yet fully understood. Autophagy might be helping the PDT process or helping the malignant cells survive PDT. In cell lines where autophagy is prevented by silencing the Atg-7 gene the photokilling occurred at lower light doses [26]. This result is consistent with the proposal that autophagy can serve as a protective mechanism. There is also data supporting that autophagy contributes to cell death as a result of PDT. In apoptosis-deficient cells, such as murine embryonic fibroblasts (MEFs) doubly deficient for Bax and Bak genes, PDT stimulates a form of caspase-independent cell death, with a necrotic morphotype, for which aberrant autophagy stimulation is required [26].

There are cytoprotective mechanisms present in cancer cells to avoid cytotoxic effect of PDT [15]. The first mechanism identified was based on over-expression of antioxidants in some tumor cells [29,30]. For example superoxide dismutase (SOD) over-expression have been shown to counteract the cytotoxic effect of PDT [28,29]. It has been reported that PDT can induce the release heat shock proteins (HSP), which can protect against the effects of PDT [30].

**VASCULAR EFFECTS**

PDT causes vascular damage leading to a variety of responses: vasoconstriction or vasodilatation, thrombosis, increased permeability of vessels, and bleeding [31,32]. Vasoconstriction and vasodilatation, which are reversible, occur early during PDT. Endothelium appears to be a primary target for PDT. Especially vessels formed by tumor angiogenesis are susceptible to PDT. These vessels do not have the normal structure and have complex geometries and react differently to PDT than normal vessels [18]. As a result of the damage to the endothelium, the subendothelial matrix is exposed and thrombocytes and leukocytes can adhere to the matrix, causing thrombosis. Thrombosis can cause further vasoconstriction through release of thromboxanes from activated thrombocytes. This may lead to shutdown of vessels and subsequent ischemia of the tumor tissue. When the perfusion restarts after vasoconstriction subsides ischemia/reperfusion injury may occur as
well [31,32]. Vessel wall damage and increased permeability causes the typical reaction of edema after PDT.

**IMMUNE RESPONSE**

PDT frequently provokes a strong acute inflammatory reaction easily observed as edema and pain at the treatment site [11]. The acute oxidative stress caused by PDT is perceived as tissue injury by the body and protective actions evolved for dealing with threat to tissue integrity are launched [34]. The main aims of these protective responses are containing the disruption of homeostasis, ensure removal of damaged cells, and then promote local healing with restoration of normal tissue function. This is a non-specific innate immune system reaction. The onset of PDT-induced inflammation is marked by dramatic changes in the tumor vasculature that becomes permeable for blood proteins [33]. The inflammatory cells, led by neutrophils and followed by mast cells and monocytes/macrophages, rapidly and massively invade the tumor [19]. Depletion of these inflammatory cells or inhibition of their activity after PDT was shown to diminish therapeutic effect [34].

Numerous pre-clinical and clinical studies have demonstrated that PDT can either cause a potentiation of adaptive immunity, or immunosuppression. Different regimens and PSs have different effects on the immune system [34-36]. Immune compromised mice show diminished long-term tumor response to PDT, suggesting that immune response may play an important role in PDT efficacy [37]. PDT is shown to induce specific anti-tumor immunity [38,39]. PDT of multifocal angiosarcoma of the head and neck resulted in increased immune cell infiltration into distant untreated tumors that was accompanied by tumor regression [38]. PDT of basal cell carcinoma (BCC) increased immune cell reactivity against a BCC-associated antigen [39]. PDT activates both humoral and cell-mediated anti-tumor immunity. The cell-mediated immune response appears to be CD8+ T cell-mediated and independent of CD4+ T cells and dependent on natural killer (NK) cells [40]. Dendritic cells present taking part in the inflammatory response can play a role in developing anti-tumor vaccines [41]. The specific immune response induced by PDT and development of PDT induced anti-tumor vaccines may transform PDT from a local treatment to a systemic one also effective against metastatic tumors.

**COMPONENTS OF PDT: PHOTOSENSITIZER, LIGHT AND OXYGEN**

**PHOTOSENSITIZER (PS)**

The PS are based on molecules with tetrapyrole structures, similar to that of the protoporphyrin contained in hemoglobin [14]. These agents typically have absorption peaks
in the red light spectrum. Typically the PS are termed as first-, second-, and third-generation.

The first generation clinically used anti-cancer PS is a mixture hematoporphyrin derivatives (HPD, Photofrin), with an activation wavelength of 630 nm [42]. The second generation PS are based on chemically pure and defined PS constructed on chlorins, bacteriochlorins and phthalocyanines, all of which have absorption wavelengths between 600-800nm [14]. These longer excitation wavelengths allow the light to penetrate deeper into tissues, thereby increasing the depth of the PDT effect. The third-generation are specifically targeted PS, which are still under development.

The PS investigated in this thesis is Meta-tetrahydroxyphenylchlorin (mTHPC, temoporfin, Foscan®). mTHPC is a chlorin with activation wavelength of 652 nm [43,44]. In 1980s Bonnet et al screened porphyrins to identify a chemically pure PS and introduced met-tetrahydroxyphenylporphrin (mTHPP) as a PS that is 25-30 times as potent as HPD [43,44]. The drawback of mTHPP was that its excitation wavelength was still 630nm, which did not have any superiority to HPD. mTHPC, the chlorin analog of mTHPP, redshifted the absorption band, enabling deeper treatment effect in the tissue [43]. mTHPC mediated PDT has higher tissue penetration and shorter systemic photosensitivity than HPD-PDT. Since the synthesis of mTHPC, this PS has been used to treat various tumors, and has eventually been registered for the indication of recurrent head and neck squamous cell cancers [43] by European Medicines Agency (EMA).

Typically the PS is administered a time before the light delivery (illumination or irradiation) to allow distribution and accumulation at the tumor tissue. This time interval is called drug-light interval, which is another parameter that can be changed in PDT to obtain optimal results [45-47]]. If the light is given within 12 to 24 hours after the administration, vascular effects are pronounced leading to a larger area of necrosis compared to longer drug light intervals [47]. Longer drug-light intervals allow intracellular absorption of mTHPC and washing from the vascular tissues, leading to better tumor selectivity [16,45-46]. Although not a dramatic difference, the best tumor to normal tissue ratio is reached 96 hours after mTHPC administration. This drug-light interval is the most commonly used interval in the clinic [43].

When the PS is activated it emits light or fluoresces at larger, specific wavelengths. Therefore measuring this fluorescence can reflect the PS content of the target tissues. For instance, the fluorescence of the PS decreases as it is used up during the PDT process, termed photobleaching. Photobleaching is proposed to have direct relationship with singlet oxygen production and the subsequent photodynamic effect [48,49]. Optical fluorescence spectroscopy can detect fluorescence and thus photobleaching of the PS non-invasively [48-56]. Thus, fluorescence spectroscopy is an important opportunity to provide insight
into the treatment process during PDT, which forms part of this thesis. Various optical spectroscopy techniques and the advantages and shortcomings of the technique used in this thesis are discussed in chapters 4 and 5.

LIGHT

The penetration of light in tissues is dependent on many factors such as the wavelength dependent tissue optical properties and the illumination geometry [57,58]. While blue light penetrates moderately, longer wavelengths in the red spectrum can reach deeper tissues. Therefore it is desirable to use light with wavelengths longer than 600nm to achieve higher volumes of treatment with PDT. On the other hand, light with wavelengths longer than 850nm has insufficient energy to produce singlet oxygen [59] and is therefore undesirable. The wavelength of light used for PDT is also dependent on the specific excitation wavelength of the PS [14]. mTHPC, the PS of interest for this thesis, has an excitation wavelength of 652nm, which lies in the visible red light spectrum.

The total energy delivered to the tissue per surface area is referred to as radiant exposure and expressed in joules per cm$^2$. The rate at which this energy is delivered or in other words the “power” is referred to as irradiance (also called intensity) and expressed in watts/cm$^2$. Both of these parameters can be changed to induce maximum effect. Delivering higher doses of light does not necessarily induce more effect; as the PS or the oxygen is depleted the excess light will have no effect. PDT is therefore a complex balance of the simultaneous presence of light, PS and oxygen.

A complicating factor is that the above mentioned radiant exposure and irradiance do not directly represent the more important parameters of the amount of light locally present in the tissue, the fluence (J/cm$^2$) and fluence rate (W/cm$^2$), respectively. As light penetrates the tissues, it decays in intensity. This decay is dependent not only on the specifications of the light delivered but also on the optical properties of the tissues, e.g. the scattering and the absorption coefficients and the scattering anisotropy [60]. These optical properties also depend on the tissue components. The main absorber in the visible part of the spectrum is hemoglobin. Other pigments such as melanin and bilirubin also contribute to the absorption properties of the tissue. Furthermore, the optical properties of the tissue can also change with time for example as the blood content of the tissue changes. The optical properties can and will change during PDT and do influence the therapeutic effect [60,61].

Albeit that the fluence and fluence rate are hard to determine or predict precisely in the tissue, please note that they can be directly influenced by changing the radiant exposure (c.q. output energy) and the irradiance (c.q. the power) of the laser. The fluence rate can be
changed by delivering the irradiance in a longer or shorter time [58]. Lower fluence rates with longer delivery times are associated with better responses. The rationale behind this improved response is that lower fluence rates allow time for replenishing of depleted oxygen and PS in the target tissues. The same replenishing principle can also apply for fractionation of light, where the fluence is delivered in fractions with pauses in between [62].

OXYGEN

The main type of photochemical reaction that drive PDT effect is a type II reaction which involves interaction of the photosensitizer, light and molecular oxygen to produce ROS. Therefore sufficient oxygen is necessary to induce the PDT effect. The amount of oxygen, however is not constant, as it can be depleted as a result of the photochemical reactions or vascular effects during PDT [63,64]. Head and neck tumors can also have low oxygen content [65]. Oxygen is transported to the tissues bound to hemoglobin, which is a pigment with specific light absorption pattern. The absorption pattern is detectable with reflectance spectroscopy, which provides information about the oxygen content of the treated tissues. The spectroscopic technique to measure oxygen related parameters is discussed in chapters 4, 5 and 6 of this thesis.

PDT IN HEAD AND NECK CANCER

The anti-cancer use of PDT started in the late 1970s. After pre-clinical animal studies [66,67] demonstrating the efficacy of PDT, Kelly et al published PDT of five patients with bladder cancer [68]. In 1978, Dougherty reported the first large series of patients successfully treated with PDT (at that time named photoradiation therapy) with hematoporphrin derivatives (HPD) [42]. Complete or partial responses were observed in 111 of 113 malignant lesions of various histopathologies. Of the large variety of tumors examined, none was found to be unresponsive. This initial success led to clinical trials in several clinical disciplines. In the literature more than 200 clinical articles on PDT can be found. Most of these are case series rather than sufficiently powered controlled randomized trials [69]. Comparison or pooling of the data is difficult because of varying PDT parameters used and differences in reporting the outcome [69].

For the treatment of head and neck cancers, data of over 1,300 patients treated with PDT using Photofrin, HPD, ALA, or mTHPC has been published [70], ranging from treatment of small primary tumors and oral/laryngeal dysplasia to advanced and even incurable tumors. Tumors of a large variety of histopathologies were treated with PDT in the head and neck area. The first report is by Keller et al who reported HPD mediated PDT of 3 oropharyngeal carcinomas with complete response [71]. Several case-series of photofrin mediated PDT
of head and neck tumors were published; some of them with few patients and some with large cohorts [70-82]. The clinical success reported is variable and likely dependent on the experience of the center performing the procedures. Unfortunately, no randomized or retrospective study to compare photofrin PDT to surgery is available.

5-Aminolevulonic acid, a protoporphrin precursor, in its systemic and topical form has been used to treat oral leukoplakia. Unfortunately the clinical results were discouraging with low complete response rates and no visible tumor selectivity of ALA [83-86].

With the introduction of mTHPC, a second generation PS that can be activated by light of longer wavelengths, several centers started experimenting with its use in the treatment of HNSCC [77-91]. mTHPC has a deeper PDT effect than photofrin, shorter general photosensitivity period and shorter illumination time, making it more attractive to the clinician [70]. Earlier publications report on the early experience with a very heterogenous indications or with very few number of patients [87-89]. Publications following these reports concentrated on a single indication rather than pooling all tumors and stages, unfortunately resulting in too limited numbers to draw conclusions [90-92]. In 2004, a multicenter phase II trial was carried out, reporting an initial success rate ranging from 84-96% and complete tumor response of 85% of 114 protocol compliant patients [93]. Complete response was maintained in 85% of responders after one year and in 77% after two years.

Parallel to the above-mentioned phase II study, another multicenter phase II study was carried out on PDT of advanced, incurable HNSCC [94]. Of the 128 patients included in the study, 38% of evaluable patients achieved an overall tumor response, and 16% achieved a complete tumor response. Subset analyses revealed two subgroups in which significantly better responses were seen: patients with tumors 10 mm or less in depth and patients with fully illuminated lesions. In patients fulfilling both categories, overall tumor response was 54%, complete tumor response was 30%, and 61% demonstrated significant clinical quality-of-life benefit. This study was a ground breaking development, opening the road to several other trials on recurrent, incurable head and neck cancer [95-97]. They reported much better results of 60-68% complete tumor response with selected group of patients with tumors thinner than 10 mm, which were completely illuminated. These results led to registration of mTHPC mediated PDT for treatment of advanced recurrent HNSCC by European Medicines Agency (EMA).

The challenge with recurrent head and neck tumors is that they often are thicker than 10 mm, which is beyond the penetration of the treatment light. There has been recently efforts to implant light sources to these tumors to expand the treatment arsenal of mTHPC mediated PDT of the head and neck [98-101]. This method is either named interstitial photodynamic
therapy or ultrasound-guided or MRI-guided photodynamic therapy. However, the distribution of light gets even more complex with multiple light sources within the target tissue. The experience of our group and efforts to develop the method is published and forms a part of this thesis (Chapters 7 and 8).

**OUTLINE OF THE THESIS**

**Chapter 1** is an introduction to PDT in general and PDT of head and neck region in specific.

**Chapter 2** is a retrospective matched cohort comparing PDT to surgery of early stage oral cavity cancer.

**Chapter 3** is an analysis of the clinical experience with surface PDT to identify and establish expected clinical success rates.

**Chapter 4** is a review of optical spectroscopy techniques and an early phase introduction to fluorescence differential path-length spectroscopy (FDPS), for analyzing clinical PDT of the head and neck area.

**Chapter 5** provides detailed information about the FDPS technique and its validation in healthy volunteers and patients undergoing PDT of the oral cavity.

**Chapter 6** describes the results of FDPS used to evaluate oxygen and PS related changes in 27 consecutively treated (with PDT) oral cavity squamous cell carcinoma with special focus on trying to identify reasons of clinical failure.

**Chapter 7** critically analyzes clinical experience with interstitial PDT (iPDT) of the recurrent base of tongue squamous cell carcinoma with special focus on clinical results and safety.

**Chapter 8** is a description of a new iPDT treatment algorithm, consisting of the treatment simulation, implantation of light sources, verification, modification of the treatment plan if necessary and illumination.

**Chapter 9** is the general discussion bringing the results of all the chapters together and deliberating on the current status and future strategies on improving the clinical success rates of clinical PDT of the head and neck area.
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