New insights into photodynamic therapy of the head and neck
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Chapter 6

Integration Of Fluorescence Differential Path-Length Spectroscopy To Photodynamic Therapy Of The Head And Neck Tumors Is Useful In Predicting Clinical Outcome

Manuscript in preparation

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ABSTRACT

BACKGROUND
Fluorescence differential pathlength spectroscopy (FDPS) is a validated instrument to provide quantitative information about oxygen physiology and photosensitizer content of oral cavity tumors undergoing mTHPC mediated photodynamic therapy (PDT). FDPS by utilizing differential spectra of two adjacently placed optical fibers, interrogates a small volume of tissue; minimizing background scattering. The collected reflectance spectra is used to correct the fluorescence spectra for tissue absorption. The model fit provides quantitative values for oxygen saturation, blood volume fraction (BVF), blood vessel diameter, and photosensitizer fluorescence.

PATIENTS AND METHOD
Twenty-seven oral cavity cancers or dysplasias were evaluated with FDPS, before and after PDT.

RESULTS
The mean tumor center (TC) to normal mucosa (NM) ratio of fluorescence was 1.50 ± 0.66. Expected photobleaching was observed in 24/27 lesions treated. The mean photobleaching was 81% ± 17%. FDPS spectra coupled with clinical results have identified 3 types of correctable errors in PDT technique. Two patients had very low concentrations of photosensitizer in TC, indicating injection error or erroneous distribution of mTHPC. One tumor showed no photobleaching and high BVF, suggesting that the present blood have prevented the treatment light from reaching the target tissue. All 3 of the lesions had no clinical response to PDT. Four patients had less than 50% photobleaching at the tumor margins (TM), suggesting a possible geographic miss of safety margins. One of these patients had a recurrence within 2 months even though there was initial good response.

CONCLUSION
Integration of FDPS to clinical PDT of head and neck area yields relevant and feasible information, and can help identify treatment errors that can be compensated.
1. INTRODUCTION

Photodynamic therapy (PDT) is a targeted treatment modality that involves the administration of (non-thermal) light and a light sensitive drug, termed a photosensitizer, to yield localized tissue destruction with minimal systemic toxicity. When the photosensitizer is activated, light energy is transferred to molecular oxygen, which leads to the formation of reactive oxygen species (ROS). ROS combine with biomolecules, oxidizing them, leading to the destruction of the illuminated tissue through a range of mechanisms that include apoptosis, necrosis, destruction of tumor vasculature and an immune response against tumor cells [1-3].

Several articles have been published outlining the clinical results of meta-tetrahydroxyphenylchlorin (mTHPC) mediated PDT of the head and neck tumors [4-12]. While many report early experiences, a number of these publications report clinical complete responses around 69-95% of carefully selected patients with early stage oral cavity cancer [5-12]. However regardless of the careful selection criteria and standard treatment technique, a small but significant number of tumors do not respond to PDT. There are numerous factors that can cause these failures. Treatment related factors, in other words sub-optimal PDT could play a role that can be improved with better techniques. In this manuscript we have aimed to attempt to unravel some of these treatment related factors.

The local therapeutic effect of PDT is driven by the production of reactive oxygen species (ROS) and depends on the presence of three components: light, photosensitizer and oxygen [1,13,14]. Earlier studies have shown inter- and intra-subject variations in parameters such as photosensitizer pharmacokinetics, tissue optical properties and subsequent differences in delivered fluence (rate), and the ability of the local vasculature to provide sufficient oxygen during therapy [15-18]. Each of these variables can be different for individual lesions, patients, and can be interdependent and change dynamically during, and as a result of therapy [19-20]. These factors can lead to variations in the PDT dose that is delivered to the target tissue and the surrounding normal tissues and may be the source of treatment failures.

Better understanding of these variations can help us analyze treatment failures on individual treatment basis. Optical spectroscopy is a non-invasive method of monitoring these variables [13-21]. Optical spectroscopy is a promising tool to monitor PDT related variables [16-25]. Reflectance spectroscopy utilizes the dominant absorption bands of hemoglobin in oral mucosal tissue to measure parameters that characterize local vascular physiology, such as microvascular oxygen saturation, and blood volume fraction [13,14,21]. Fluorescence spectroscopy fluorescence attributed to optically active photosensitizing compounds, and
can be used to monitor the local photosensitizer concentration and photobleaching during therapy [16-18]. The combination of monitoring reflectance and fluorescence within the same interrogation volume may also provide additional information on the relationship between photobleaching and local supply of oxygen during PDT. When interpreting these types of signals it is important that the measured parameters are representative of the same volume of tissue, are reproducible, and are comparable.

Our group has developed differential path-length spectroscopy (DPS) [22,23] and florescence differential path-length spectroscopy (FDPS) [25] for this purpose. Reflectance spectroscopy (DPS) utilizes the dominant absorption bands of hemoglobin in oral mucosal tissue to measure parameters that characterize local vascular physiology, such as microvascular oxygen saturation, and blood volume fraction (BVF) [16,17,24]. Fluorescence spectroscopy (FDPS) can measure fluorescence attributed to the photosensitizer [23,25]. FDPS makes use of two optical fibers one for delivery of light (white light in case of DPS and 652nm excitation light in case of FDPS), and both for collecting light reflected (or emitted) from the tissue. The difference of the two collected signals limits the contribution of long path-length photons to the signal, enabling sampling the small volume of tissue immediately in contact with the fibers. Therefore FDPS can deal with large variations in background absorption using a simple correction algorithm. This makes FDPS especially valuable for in vivo photosensitizer fluorescence spectroscopy during PDT, when the background absorption can change significantly. Another advantage of FDPS is that the collection volume can be adjusted to match the relevant dimensions of the application. For absolute fluorescence measurements of photosensitizers, it is essential to selectively interrogate the relevant tissue volumes and to avoid averaging drug concentrations over a volume that is either deeper or shallower than the intended sampling location. It is important to note that while FDPS remains dependant on the scattering coefficient of tissue, this is expected to have a relatively small influence on the signals collected particularly in tissues of the same type [23,25].

We have applied FDPS to the clinical setting and to healthy volunteers and reported the initial feasibility data in our previous publication [25]. The results presented in this earlier publication indicated that FDPS could safely be utilized in the clinical setting. The FDPS correctly estimated the absence of mTHPC in volunteers and detected photobleaching in the areas receiving treatment light in patients undergoing PDT treatment. Based on these initial results we have utilized FDPS to analyze optical characteristics of oral cavity tumors being routinely treated with PDT in our institute. The clinical treatment protocol was kept standard to allow evaluation of treatment failures. This manuscript reports the reflectance and fluorescence spectroscopic analysis of these patients and attempt to explain treatment failures based on spectroscopic measurements.
2. METHOD

2.1. PATIENT POPULATION AND CLINICAL METHOD

The patients undergoing PDT for the treatment of oral cavity cancers were included in the project. Both superficial and deep-seated tumors were included. These deep-seated tumors, that were treated using interstitial PDT, were analyzed separately and will be presented in a separate report. All of the patients reported in this manuscript had squamous cell carcinoma (SCC) or carcinoma in situ (CIS) of the oral mucosa, without regional or distant metastasis and not deeper than 5mm as measured by ultrasound (T1N0M0 or TisN0M0 by AJCC staging system). Primary tumors, second primary tumors and recurrences of earlier treated tumors were included. The photosensitizer Foscan® (m-tetrahydroxyphenylchlorin, m-THPC) was injected to a proximal vein in the arm at a dose of 0.15 mg/kg. The surface illumination procedure took place four days after the photosensitizer injection. Depending on the location of the tumor the procedure was either performed under general anesthesia or local anesthesia. FDPS measurements were taken from at least three locations on the center of the tumor (TC), three locations on the adjacent normal appearing mucosa that received treatment light as oncologic safety margin (TM) and three locations in the oral cavity, distant from the tumor, located under shielding (therefore not receiving treatment light) (normal mucosa: NM), immediately before and immediately after the PDT procedure. Therapy was performed with light from diode laser 652nm (Biolitec, Germany), using a microlens diffuser, with an oncologic margin of at least 5 mm around the visible tumor, using a irradiance of 100 mWcm$^{-2}$ to a radiant exposure of 20 Jcm$^{-2}$.

2.2. FLUORESCENCE DIFFERENTIAL PATH-LENGTH SPECTROSCOPY

The clinical application of fluorescence differential pathlength spectroscopy (FDPS) performed in the present study has been described in detail previously [25]. The outline of the method is described briefly for the readers in this manuscript. For a full description of the technical aspects of the measurement, the filter sets used, the quantification of system artifacts and the incorporation of spectroscopy in to the clinical workflow please refer to our previous publication [25]. Figure 1 is a schematic representation of the device setup. The system uses two 800 µm diameter optical fibers located adjacent to each other and encased in a rigid probe (the probe); one fiber is used for delivery and collection of light (dc) and the adjacent fiber is used for light collection only (c). The dc fiber is connected to a white light source, a diode laser delivering 652nm light and the spectrometer, while the c fiber is connected to the spectrometer. Broad band white light and 652nm light from a diode laser are sequentially delivered to the tissue and enable the collection of a white
light reflectance spectrum and the excitation and collection of mTHPC fluorescence. Before light is led to the spectrometer, both fibers pass through a notch filter; this filters the light from the laser source, thus preventing saturation of the fluorescence spectroscopy system. This results in a small wavelength band over which reflectance spectra are associated with significant levels of noise. Each sequence of measurements is combined with an appropriate calibration procedure such that differential reflectance (DR) and differential fluorescence (DF) spectra are recorded from the tissue of interest.

Figure 1. Setup of the FDPS system. The dc (delivery/collection) fiber in the probe excites the tissue with white light and laser light sequentially while both the dc and c (collection) fibers collect and transfer light to the spectrometer.

2.3. DATA ANALYSIS

The DR is fitted to an empirical model that incorporates the presence of a background scattering model that is appropriate for tissue, and quantifies the influence of absorption on the collected differential reflectance. The wavelength dependent absorption coefficient of tissue of the oral cavity is described by components that are related to various absorbers such as oxy-and deoxyhemoglobin and bilirubin. By accounting for the non-homogeneous distribution of blood in tissue, these components are appropriately combined to yield various physiological parameters: the blood volume fraction (BVF); the microvascular oxygen saturation; the average microvasculature blood vessel diameter and the concentration
of bilirubin. A least squares fitting procedure is performed that provides estimated mean values and confidence intervals for each of these parameters.

The calibrated DF (expressed in units of photon counts/ms/mw) is corrected for the effects of absorption by multiplying the DF by the ratio of the DR at the excitation wavelength (652 nm) with and without absorbers present. The value of DR in the presence of absorption is calculated from the fit of data to the empirical model described above while the DR in the absence of absorption is estimated from the background scattering model. This corrected DF is then analysed as a linear combination of basis spectra representing tissue autofluorescence, mTHPC fluorescence, and a small signal that is attributable to fluorescence from the optical components of the system. Basis spectra for each of these components were determined from the average of 5 DF measurements in the oral cavity with and without the presence of mTHPC.

High quality fittings of mTHPC fluorescence and physiological parameters were achieved in the majority of measurements even in the case of relatively low blood volumes. Figure 2 shows a representative example of DR and DF spectra and their components. Spectra that did not fit the model are excluded based on the presence of observable features in the residual between the model fit and the data as shown in figure 2.

**Figure 2.** The DPS (left) and FDPS (right) spectra from a center of the tumor before PDT. The absorption of light by the tissue as seen by the difference between the scattering model and the measured reflectance (DR) is used to calculate oxygen saturation, blood volume fraction and vessel diameter and to correct the measured fluorescence for absorption. The noise at around 652nm is due to the notch filter. The components of the measured fluorescence (DF) are autofluorescence of the tissue (autoFL), fluorescence of the FDPS system used (system FL) and fluorescence attributable to mTHPC (mTHPC). The effect of the filter is not visible on the FDPS spectrum, because the spectrum is a plot of around 675nm and longer wavelengths. The “residual” plot underneath the spectra represents the residual between the model fit and the collected data. Residuals between 0.2 and -0.2 are assumed as acceptable.
The differential fluorescence attributable to the photosensitizer (mTHPC) (this value is expressed as “fluorescence” in the rest of the text), was acquired in 3 sequential measurements by placing, removing and replacing the FDPS probe from 3 different sites of the tumor center (TC), margins of the tumor located in the illumination field as safety margin (tumor margin (TM)), and normal non-illuminated mucosa (normal mucosa (NM)). Measurements at each site are averaged to yield one value and a standard deviation. The photobleaching of mTHPC was quantified by the percentage decrease in mTHPC fluorescence calculated using the average values immediately before and after illumination for each site separately. If the BVF in any single DR measurement was high (<50%) fluorescence measurements were repeated after removing excess blood from the measurement area and the probe tip. The reflectance measurements were considered feasible for analysis if the blood volume fraction was between 0.3 and 50%. The interpretation of individual combinations of sequential co-localized reflectance and fluorescence spectroscopy are discussed further in the results section.

Patients were followed at least 1 year to detect any partial response or recurrence of the tumor at the treated site. The treatment failures (if any detected) were coupled to the measured spectra in an effort to explain the reason(s) of the failure.

3. RESULTS

Twenty-four patients with 27 oral cavity lesions were included in the study. Seventy eight percent of the spectra obtained were suitable for analysis using the criteria described in the methods.

3.1. VARIABILITY IN MTHPC FLUORESCENCE AND TUMOR SELECTIVITY

The fluorescence measured at the TC before PDT ranged from 3.02 to 0.09 photon counts/ms/mw with a mean of 1.33 ± 0.77. The mean fluorescence measured at TM was similar to mean fluorescence at TC before PDT (1.27 ± 0.49 vs 1.33 ± 0.77, respectively), whereas the mean fluorescence measured at NM (0.95 ± 0.47) was slightly lower. It is evident from high standard deviations that there is considerable variation inter-subject. When average fluorescence of TC, TM and NM are plotted per patient/lesion (figure 3), the mean TC/NM ratio of fluorescence was 1.50 ± 0.66. It can be seen from Figure 3 that, although true for the majority, not in all cases the TC contain more photosensitizer than NM. There is no observable difference between TC and TM with a TC/TM ratio of 1.18 ± 0.53. Two lesions (no. 12 and 13) showed very low fluorescence in all measured locations before PDT (Figure 3).
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Figure 3. Fluorescence attributable to mTHPC at tumor center, margin and normal mucosa before PDT in all measured lesions. TC had average 1.50 times more fluorescence than NM but with a high standard deviation (0.66). It can be seen that in some cases NM has more fluorescence than TC.

3.2. PHOTOBLEACHING OF MTHPC

The hypothesized reaction is a decrease in fluorescence as a result of PDT (figure 4), referred to as photobleaching. The fluorescence after PDT at the TC ranged from 0.74 to 0.02. Taking into account the large range of fluorescence observed, we have decided to compare the fluorescence decrease (photobleaching) as a unit of percentage decrease calculated for each individual case. The mean photobleaching was 81% ± 17% with a range of 99% to 7% at TC (figure 5). Twenty four lesions have shown the hypothesized pattern of photobleaching with decrease in fluorescence due to PDT. In addition to two lesions with very low fluorescence, one lesion has shown almost no photobleaching, with only 7% decrease in fluorescence due to PDT. The mean photobleaching of the 24 lesions with the hypothesized reaction is 85% ± 10% with a range of 99% to 66%.
Figure 4. FDPS (above) and DPS (below) spectra measured before and after PDT, demonstrating the anticipated photobleaching. The blue line in the fluorescence spectra (above) represents the fluorescence attributable to mTHPC. It can be observed that the fluorescence peak between 700 and 740 nm wavelength decreases due to breakdown of mTHPC during PDT.

There was somewhat less photobleaching at TM after PDT. The mean photobleaching was 61% ± 25% with a range of 91% to -6%. The photobleaching of -6% is seen along the margins of the tumor that showed negligible photobleaching at the tumor center mentioned in the paragraph above (lesion 8). There are 4 additional photobleaching below 50% at TM (lesions 1, 2, 15 and 24) (Figure 5).

There was no photobleaching effect seen at NM which were not illuminated. The average difference before and after PDT was -5% ± 23 at NM (Figure 5).
Figure 5. Average fluorescence in the measured 27 lesions. Blue bars indicate fluorescence before PDT and red bars after PDT. Lesions number 12 and 13 show very low fluorescence indicating low concentrations of mTHPC. Lesion number 8 shows no photobleaching (difference before and after PDT). Lesions 1, 2, 8, 15 and 24 show less than 50% photobleaching at tumor margins suggesting a geographic miss (top right graph). There is no evident photobleaching in normal mucosa that did not receive PDT (bottom left graph). Missing or not evaluable data are left blank on the graphs. The numbers in all three graphs correspond to the same lesion/patient.

3.3. DIFFERENTIAL PATH-LENGTH SPECTROSCOPY

The weighted mean oxygen saturation was slightly lower at TC than NM, and the vessel diameter was slightly greater in TC than NM (Table 1). However these values are not significant due to the large standard deviations. There was no detectable difference in oxygen saturation, BVF and vessel diameter before and after PDT in any of the measurement sites.

Table 1. Weighted mean (and standard deviation) blood volume fraction, oxygen saturation and blood vessel diameter of TC and TM before PDT.

<table>
<thead>
<tr>
<th></th>
<th>Blood volume fraction</th>
<th>Saturation</th>
<th>Vessel diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>0.0161 (0.0130)</td>
<td>0.872 (0.211)</td>
<td>0.0171 (0.0069)</td>
</tr>
<tr>
<td>NM</td>
<td>0.0157 (0.0061)</td>
<td>0.966 (0.229)</td>
<td>0.0149 (0.0061)</td>
</tr>
</tbody>
</table>
3.4. CORRELATION WITH CLINICAL RESULTS

Out of 27 treated tumors 8 had incomplete response or early recurrence (lesions 1,8,12,13,22,23,25,27). When the spectroscopy data was analyzed three types of possible reasons of failure were identified for 4 of the lesions treated (lesions 1,8,12,13).

**Insufficient photosensitizer content in the tumor:** Significantly low fluorescence was measured in two treated lesions. Lesion number 12 had fluorescence of 0.29 and lesion number 13 had fluorescence of 0.09 compared to mean fluorescence of 1.33 before PDT at TC. Such low values suggest absence of the photosensitizer in the treated lesions. Both tumors (in two patients) had no response to PDT (figure 5). In Figure 6, examples of DPS and FDPS spectra of lesion 12, before and after PDT can be seen. There was no detectable difference in the fluorescence peaks before and after PDT.

![Spectra of lesion 12 before and after PDT](image)

**Figure 6.** FDPS (above) and DPS (below) spectra of lesion 12 before and after PDT. The blue lines in FDPS spectra are almost flat, indicating very small concentrations of mTHPC in the TC, both before and after PDT.

**Insufficient photobleaching:** Lesion number 8 showed no change in fluorescence before and after PDT (0.64 vs 0.60) at TC (Figure 5). This patient had bleeding and hematoma formation.
during PDT which was also detectable with some reflectance spectra of 100% BVF. Figure 7 shows DPS and FDPS spectra before and after PDT. It can be seen that the fluorescence peaks does not change as a result of PDT. The absorption bands of hemoglobin are deep indicating considerable blood content in the interrogated tissue. Hemoglobin can absorb treatment light, acting as a filter, preventing light to reach the tumor. (Figure 7)

**Figure 7.** FDPS (above) and DPS (below) spectra of lesion 8, before and after PDT, demonstrating insufficient (absent) photobleaching. As evident in the FDPS spectra (above), there is no decrease in fluorescence attributable to mTHPC (blue lines), after PDT. This can be explained by the filtering effect of excessive blood in the treated tissue. The presence of excessive blood can be observed in the reflectance spectrum post PDT (below). The absorbance peaks of hemoglobin between 500 and 650nm post PDT are much deeper compared to pre PDT.

**Geographical miss/ insufficient treatment to margins:** Lesion number 1,2,8,15, and 24 showed less than 50% decrease in fluorescence in the treatment margins (TM) with PDT. Of these lesions number 8 had also insufficient photobleaching at the center of the lesion (Figure 5). The others had good photobleaching at TC. Lesion number 1 had good response to PDT but local recurrence within 2 months after treatment. The other lesions had complete sustainable response to PDT.
4. DISCUSSION

FDPS supplied reliable data in a very short time and without compromising the clinical procedure. The FDPS method data were feasible for analysis 78% of the time. The non-feasible data was either due to insufficient contact with the tissue and therefore contamination by ambient light. This kind of aberrant data are easily recognizable by the residual function in the model. If the data did not fit the model it is not representative of the tissue. The measurements with very low BVF (below 0.03%) were excluded from reflectance analysis, because they would not reflect blood circulation related parameters, namely the oxygen saturation, BVF and vessel diameter. However these measurements were representative of the fluorescence of the tissue of interest and were used for fluorescence analysis. The measurements with excess BVF (above 50%) were not considered representative of the tissue but of blood itself. These cut-off points are arbitrary and it can be argued that other cut-off values could be used. By using these cut-off points we were able to get consistent and comparable results.

The fluorescence measured that is attributable to mTHPC had a large range from patient to patient. However the measured values were comparable and consistent among the measurements performed in the same patient/ lesion. Therefore the large range is due to variations in photosensitizer concentration rather than variations in the technique used. Measurement of consistent values of fluorescence at normal mucosa before and after PDT confirmed this observation. Observing such a variation among patients but consistency within the same patient, led to the decision to use percent decrease in fluorescence as the parameter to measure photobleaching (figure 5). Even though the administered dose of mTHPC, technique and drug-light interval is consistent for the whole patient group, there were variations in mTHPC concentration. There could be important variations in drug metabolism and pharmacokinetics from patient to patient. The clinical response was very similar unless very low fluorescence was detected as in lesions 12 and 13, or if there was no photobleaching in lesion 8. As long as the data fits the model, these should be considered as feasible data and real variations [25]. Kiesslich et al reported such a variation in mTHPC uptake in a series of colangiocarcinoma cell-lines [26]. The variation in the present study could also be due to differences in cell biology of the studied lesions, with some tumor cells containing more mTHPC than others. Mitra et al showed heterogenous distribution of mTHPC in murine cancer models [27]. The heterogenous distribution can also account for the variations. However we were able to detect consistent fluorescence values within the same lesion indicating that the heterogenous distribution was not evident in our data set.
Although for majority of the cases the TC contained 1.5 times more photosensitizer than NM, there is variability. In some cases NM contained more photosensitizer than TC. From this data set it is not evident that malignant tissue always contain more photosensitizer (mTHPC) than normal mucosa, which is a desirable situation to destroy tumor while protecting normal tissue. Earlier animal studies have shown higher tumor/muscle and tumor/skin concentration ratios of mTHPC [28]. This tumor selectivity is not evident when tumor is compared to mucosa. Blant et al reported as well that there is no difference between early stage SCC and mucosa [29]. They were able to detect slightly higher mTHPC concentration in advanced stage SCC. It should also be kept in mind that animal models do not correlate well with the distribution in humans [30,31]. The lack of selectivity is not really necessary since the treatment technique itself is selective through illuminating only the target tissue and protecting the normal tissue by shielding. The shielding techniques seem to be sufficient since there was no photobleaching detected at NM sites, which were located under the shields.

The same variation was observed in reflectance spectroscopy. Especially the BVF and vessel diameter showed considerable variation. This variation is not a surprise. If the interrogated tissue contains a larger or denser capillary network the vessel diameter and therefore the BVF would be higher than a tissue that does not contain large or many capillaries. The thickness of the avascular keratin layer can also be a reason of the variations [32]. It might be possible to detect differences if more DPS measurements of the tissues of interest were taken and averaged. TC had lower oxygen saturation than NM but not significant enough to draw conclusions. The data is consistent with earlier articles about DPS measurements of oral cavity malignancies and leukoplakia [32,33], however with more variability in the present data. There was no specific trend of increase or decrease observed in any of the blood related parameters before and after PDT. It should be kept in mind that the data presented in this manuscript is collected from superficial tumors with excellent blood supply as opposed to tumors with larger volumes with aberrant neo-vascularization and necrotic centers. Probably the blood supply is immediately replenished making the vascular effects of PDT undetectable.

By using FDPS it was possible to detect some technical problems with PDT that might lead to clinical failure. In two patients the fluorescence measured at the tumor center, margin and normal mucosa was very low compared to the other measured lesions. These measurements could have been the lower range of the variation observed in fluorescence. However both of the lesions showed none of the usual responses to PDT, such as edema, pain, or sloughing of the mucosa. The tumors were not affected by the treatment. Coupled with the clinical response it can be concluded that there was insufficient photosensitizer concentration in
the tissues to achieve a PDT effect. It is rather speculative trying to explain the reasons for this strange phenomenon. Even though both of the patients had photosensitizer injections exactly according to the protocol, in some way the photosensitizer failed to reach the target tissue. Both of the patients had second degree burns around the injection site and along the vein injected in the direction of the shoulder. This suggests that there might have been either a leak from the vessel walls, or mTHPC was injected in the soft tissue rather than the vein. It is not possible to comment on the photosensitizer concentration threshold where no PDT response is to be expected, just from two observations. However it is reasonable to expect treatment failures when low fluorescence values are observed. Unfortunately when low fluorescence is observed on the day of the illumination it is not possible to compensate for lack of photosensitizer on the spot. The illumination should be carried out, but the clinical team should be planning for possible additional treatments such as surgery, radiotherapy or repeat PDT in short term when applicable. If PDT is to be repeated the photosensitizer should not be injected at the previous injection site. Since there is probably residual extravasated photosensitizer at this site any additional injection would only increase the risk of serious burns.

The second type of error detected by this study is insufficient photobleaching. In one lesion almost no difference in fluorescence was observed before and after PDT. There was diffuse bleeding from the lesion on the day of the illumination. Pressure with gauze was applied to stop the bleeding. When the bleeding was at manageable levels the illumination took place with frequent cleaning of the treatment surface with vacuum suction. However this was not sufficient. The DPS detected large blood volume fraction, in some cases 100% even though there was no visible blood pooling. There was probably a thin film of blood either on the surface or immediately under the mucosa which was detected by DPS. The lack of photobleaching suggests that even such an invisible layer of blood is capable of blocking the treatment light. It is possible that the measured fluorescence is from the blood present at the interrogated volume rather than the tumor itself. In order to examine this possibility only measurements with BVF of less than 50% were taken into account. The mean BVF was 25% in the evaluated samples. In these samples there were still the same amount of fluorescence as before PDT. This conclusion was also confirmed by lack of clinical response to PDT. This problem can be easily corrected on site. If no photobleaching is detected the illumination can be repeated until the photobleaching becomes evident. It is recommendable that the repeat illumination is delayed until the bleeding completely stops. Overtreatment should not be feared, because treatment effect is not observed until there is photobleaching. Repeat illumination was not carried out in this patient for two reasons: First of all it was not known what the clinical implications of this lack of photobleaching could be.
Secondly repeat illumination would be a breach of protocol preventing us to observe the consequences of such a phenomenon.

Third type of error that can be detected with FDPS is geographic miss at the safety margins. What is meant by geographic miss, is insufficient photobleaching at the safety margins, potentially leaving untreated extensions of the tumor that are invisible to the naked eye. The common practice with conventional methods, such as surgery and radiation therapy, is to treat at least 5-10mm of safety margins around the visible lesion to prevent missing these extensions. If the tumor recurs is dependent on the presence or absence of malignant cells in the missed tissue. In the data set presented in this manuscript, only one out of four lesions with less photobleaching of the margins had a recurrence within 2 months, indicating that an extension of the tumor was missed. The others probably did not have tumor extensions in the treatment margins, or even though the photobleaching was somewhat less than the average, it was sufficient for treating the tumor extensions. As experience with conventional methods indicates, insufficient treatment to the safety margins theoretically increases the risk of recurrence. With FDPS, it is possible to investigate the margins for photobleaching and administer repeat illuminations as insufficient photobleaching is detected.

It was not possible to explain all PDT failures in this cohort with FDPS. A group of lesions showed incomplete response or recurrence even though substantial photobleaching was observed both at the tumor center and the safety margins. This was to be expected; as experiences with conventional methods indicate, sufficient and correct treatment does not always guarantee clinical success. FDPS, however when incorporated into clinical practice can help us compensate for preventable errors described in previous paragraphs. The frequency of these preventable errors are not known, but their presence in such a small cohort of patients suggest that these occur not very infrequently.

**5. CONCLUSION**

Reflectance (DPS) and fluorescence (FDPS) spectroscopy provide insight into the working mechanism of PDT, by providing data about oxygen related parameters and the photosensitizer. By incorporating FDPS into clinic it might be possible to detect technical problems, such as insufficient photosensitizer concentration or lack of photobleaching in the target tissues. Furthermore FDPS can be used to investigate the treatment surface/volume for sub-optimally treated areas. By developing protocols to compensate for these preventable errors the clinical success of PDT can be improved.
6. REFERENCES


