Oxygen saturation and perfusion changes during dermatological methyl-aminolevulinate photodynamic therapy

Short title: Oxygen changes during photodynamic therapy

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Abstract

Background: Methyl-aminolevulinate photodynamic therapy (MAL-PDT) is a successful topical treatment for a number of (pre)cancerous dermatological conditions. In combination, light of the appropriate wavelength, the photosensitiser, protoporphyrin IX (PpIX) and tissue oxygen result in the production of singlet oxygen and reactive oxygen species inducing cell death. This study investigates real-time changes in localised tissue blood oxygen saturation and perfusion in conjunction with PpIX fluorescence monitoring for the first time during dermatological MAL-PDT.

Methods: Oxygen saturation, perfusion and PpIX fluorescence were monitored non-invasively utilising optical reflectance spectroscopy, laser Doppler perfusion imaging and a fluorescence imaging system respectively. Patients attending for standard dermatological MAL-PDT were recruited to this ethically approved study and monitored prior to, during and after light irradiation.

Results: Significant reductions in mean blood oxygen saturation ($P<0.005$) and PpIX fluorescence ($P<0.001$) were observed within the first minute of irradiation (4.75 Jcm$^{-2}$), whilst in contrast perfusion was observed to significantly increase ($P<0.01$) during treatment. The changes in oxygen saturation and PpIX fluorescence were positively correlated during the initial phase of treatment ($r^2=0.766$).

Conclusion: Rapid reductions in the localised blood oxygen saturation have been observed to occur clinically for the first time within the initial minutes of light irradiation and positively correlate with the concurrent PpIX
photobleaching. Furthermore perfusion increases suggesting that the microvasculature compensates for the PDT induced oxygen depletion.

Keywords: Dermatology; Fluorescence diagnosis (FD); Methyl-aminolevulinate (MAL); Oxygen saturation, Photodynamic therapy (PDT)
Introduction

Photodynamic therapy (PDT) utilises a photosensitiser, light of an appropriate wavelength and tissue oxygen to selectively ablate (pre)malignant tissue (Henderson & Dougherty, 1992; Peng et al, 1997). These three critical components, when available in a plentiful supply, result in the production of singlet oxygen and other reactive oxygen species (ROS) which lead to cellular damage and ultimately cell death via apoptotic and necrotic pathways. The absence of any one of the three components prevents the formation of ROS and thus tissue ablation.

Methyl-aminolevulinate (MAL) PDT employs a topical prodrug to accumulate the endogenous photosensitiser, protoporphyrin IX (PpIX), preferentially within tumour cells and this treatment has found a particular niche in the treatment of (pre)cancerous dermatological indications including actinic keratosis (AK), Bowen's disease (BD) and superficial basal cell carcinoma (sBCC). PpIX (the penultimate molecule in the haem biosynthesis pathway) selectively accumulates within (pre)cancerous lesions/cells due to their disrupted stratum corneum, upregulation of the haem biosynthesis pathway and alterations in the expression of the porphobilinogen deaminase and ferrochelatase enzymes within the pathway.

Activation of the accumulated PpIX with red light (635 nm) results in the localised production of ROS and singlet oxygen, providing sufficient tissue oxygen is present. The critical importance of a plentiful oxygen supply during PDT has long been recognised with studies demonstrating that tumours with
pre-existing hypoxia or tumours that develop hypoxia during PDT respond with limited efficacy to PDT 8-10.

Monitoring tissue oxygenation during and after PDT therefore has the potential to enhance our understanding of the basic physiological mechanisms involved during light irradiation. Additionally, monitoring oxygen during PDT may enable the efficacy of the PDT treatment to be predicted as the cytotoxic effect of PDT is partly dependent on the availability of oxygen 11,12.

Many studies have utilised a variety of different techniques to monitor oxygen saturation (or the partial pressure of oxygen, \( pO_2 \)) in the tissue before, during and after the light irradiation phase of PDT. All but one of these studies have been in animal models. These studies have highlighted several general conclusions: Significant changes in oxygenation were observed to occur during and after PDT, with markedly different responses noted for the different photosensitisers employed 8,9,13-15. A rapid decline in \( pO_2 \) immediately on the initiation of light treatment was also noted in a number of studies 10,14,16,17. This rapid depletion in oxygen is attributed to the photochemical consumption of oxygen and damage to the microvasculature reducing the capacity of the circulation to replenish the tissue with oxygen 13,18.

A steep decline in \( pO_2 \) was noted immediately after commencing light treatment in mouse and rat models treated with PpIX mediated-PDT where aminolaevulinic acid (ALA) was utilised as the topical pro-drug 14,19, although other studies have demonstrated large increases in tumour oxygenation during ALA-PDT 15. The only clinical observations with ALA-PDT monitored eight
nodular BCC lesions, indicating 5-20% reductions in haemoglobin saturation, which were highly dependent on the fibre position. To date we are unaware of any clinical MAL-PDT studies which have considered $pO_2$.

Furthermore insights into the physiological changes induced by PDT can be gained by monitoring the alteration in perfusion. To date perfusion alterations during PpIX mediated PDT have not demonstrated unified alterations with fluence and fluence rate influencing the perfusion alterations. Increased perfusion within BCC lesions was observed following ALA-PDT in one study, although alternative clinical studies have indicated no immediate perfusion alterations during either ALA or MAL-PDT and significant reductions in perfusion during high fluence ALA-PDT (100 Jcm\(^{-2}\)) have been observed in pre-clinical and clinical studies. To date we are unaware of any clinical MAL-PDT studies which have employed a standardised irradiation protocol.

This investigation therefore monitored, for the first time, mean blood oxygen saturation, perfusion and PpIX fluorescence within three histologically distinct dermatological lesion types (AK, BD and sBCC) during clinical MAL-PDT treatment. Changes in the localised tissue blood oxygen concentration and perfusion were monitored during treatment and common trends between the three distinct lesion types treated were identified. The relationship between changes in tissue oxygen saturation and changes in PpIX fluorescence during light irradiation were also considered.
Materials and Methods

Clinical data

Patients attending the PDT service at the Dermatology Department, Royal Cornwall Hospital, Truro, UK were provided with verbal and written information about this ethically approved non-invasive monitoring study (Plymouth and Cornwall Research Ethics Committee) prior to providing written consent. Fifty-five patients (20 males and 35 females; mean age 70 +/- 10 years) were recruited with a range of licensed indications (24 AK, 14 BD and 17 sBCC) localised at both acral and non-acral sites (22 acral and 33 non-acral). The average surface area of the lesion was 35 mm$^2$ +/- 9 mm$^2$. BD and sBCC were biopsy proven prior to treatment, whilst AK lesions were referred at the Consultant Dermatologist’s discretion. The clinical nurse specialists treated all lesions as per the current National Institute of Clinical Excellence (NICE, 2006) guidelines for MAL-PDT. Following appropriate lesion preparation, MAL was applied for three hours at approximately 1 mm thickness with a 5 mm border around the lesion. After the three hour application time any excess MAL was removed and the area was then irradiated with red light (Aktilite®, Galderma, UK; 635 nm; 37 Jcm$^{-2}$; 80 mWcm$^{-2}$).

Fluorescence imaging: The lesions were monitored with a previously validated non-invasive fluorescence imaging system (Dyaderm, Biocam, Germany) following the previously derived standard operating procedure to ensure reproducible images were acquired$^{24}$. Each lesion was imaged at four distinct time points during treatment; (1) prior to the application of MAL, (2) after the
three hour application of MAL, (3) during light irradiation either after 1 minute (4.75 Jcm\(^{-2}\), 10 patients - 5 males and 5 females, mean age 75 +/- 4 years; 3 AK, 3 BD and 4 sBCC) or at the half way stage (18.5 Jcm\(^{-2}\)) during treatment (45 patients (based on previous studies where statistical significance was demonstrated at 90 % power at \(P<0.001\)) - 15 males and 30 females; mean age 69 +/- 11 years; 12 AK, 7 BD and 6 sBCC)) and (4) following the completion of light treatment (37 Jcm\(^{-2}\)). The acquisition of each image took a maximum of 60 seconds.

**Oxygen saturation** monitoring: In addition to the fluorescence imaging, the mean blood oxygen saturation (\(S_{\text{mbO}_2}\) - percentage of haemoglobin carrying oxygen, averaged across all the microvessels in the volume of skin studied) of the tissue was monitored prior to, during (10 patients (5 males and 5 females, mean age 75 +/- 4 years; 3 AK, 3 BD and 4 sBCC ) at 4.75 Jcm\(^{-2}\) and 25 patients (8 males and 17 females; mean age 69 +/- 11 years; 12 AK, 7 BD and 6 sBCC) at 18.5 Jcm\(^{-2}\)) and at the end of light irradiation. The \(S_{\text{mbO}_2}\) was calculated by spectroscopic quantification of the two chromophores oxyhaemoglobin (\(\text{HbO}_2\)) and deoxyhaemoglobin (\(\text{Hb}\)), using a previously validated ‘in house’ optical reflectance spectroscopy (ORS) instrumentation (School of Physics, University of Exeter, UK). Light from a stabilised quartz tungsten halogen white light source was delivered to the skin via a fibre optic probe. Backscattered light was collected by six concentric fibres set 250 µm from the source enabling depths of 200 µm to be monitored which approximated to the depth of the lesions being investigated. Backscattered spectra ranging from 470-1120 nm were recorded in 0.05 seconds through a grating
spectrometer and charge-coupled device camera and processed with custom written Windows-based software. The concentrations of HbO$_2$ and Hb were derived from the modified Beer-Lambert law using a four component multi-linear regression algorithm on a narrow spectral range (500-600 nm) where a linear relationship between scattering of blood and wavelength was assumed. To obtain spectra from the blood volume alone, an initial reference spectrum from the surrounding interstitium of the lesion was acquired by applying sufficient pressure on the probe to occlude the microvessels $^{26,28}$. The mean blood oxygen saturation was derived from the measured concentrations of HbO$_2$ and Hb and defined as oxygen saturation (%) = \([HbO_2] \times 100 / ([HbO_2] + [Hb])\). This oxygen saturation is the mean blood oxygen saturation across the arterioles capillaries and venules in the cutaneous microcirculation under investigation. For each time point the oxygen saturation within the tissue was monitored for 60 seconds. Consistent probe placement within the lesion limited the effect of the heterogeneous microvasculature. Reproducibility studies of probe placement on normal skin produced a coefficient of variance (CV) of less than 7% (data not shown).

*Laser Doppler Perfusion Imaging*: The skin microcirculation was studied with a well established technique, laser Doppler fluximetry, which has previously been employed to monitor dermatological ALA-PDT $^{21,29}$. A Periscan PIM II perfusion imager (Perimed, Sweden) instrument was utilised to monitor perfusion within normal skin and the (pre)cancerous lesion treated with MAL-PDT. A single wavelength of low power (1 mW) laser light (670 nm) was directed onto the surface of the skin and the entire lesion scanned. Light backscattered from the
tissue was collected by the photodetector, light scattered from moving objects (e.g. red blood cells) underwent frequency changes, or Doppler shift, whereas light backscattered from stationary objects remained unaltered. The magnitude and frequency distribution of the Doppler shift is a measure of the number and velocity of blood cells in the volume of tissue under investigation. The perfusion in the skin is quantified by the term flux (in arbitrary units) which is the product of the mean moving red blood cell velocity and the number of moving red blood cells. Following the scan, perfusion was monitored at a number of discrete points using the LDPIWin software (Perimed, Sweden) and the average perfusion of the lesion and normal skin was calculated, reducing the signal noise and the effects of spatial variations in the tissue. Patients (20 patients – 7 males and 13 females; average age 69 years +/- 8.3 years; 9 AK, 4 BD and 7 sBCC) were monitored prior to light treatment, during light irradiation (at the half way stage) and following the completion of treatment for a maximum of 90 seconds. To ensure the continuity of the system a calibration box was scanned before and after every patient and no significant variation was observed ($P=0.526$; data not shown).

**Temperature:** Skin temperature was monitored with a digital thermometer (Fluke Ltd, UK) during the acquisition of oxygen saturation and perfusion measurements.

**Data analysis**
Fluorescence images (BMP – to ensure no data was lost by condensing the image) from patients were exported into NIH ImageJ software (http://rsb.info.nih.gov/ij/) and analyzed from the same point within the lesion.

The normality of the data was analysed with the Kolmogorov-Smirnov test. Normally distributed data were either analysed with the unpaired t-test or ANOVA analysis for between group comparisons or the paired t-test or repeated measures ANOVA for intra patient data.

Least squares regression analysis and Spearman Rank analysis was utilised to determine the relationship between changes in oxygen saturation and PpIX concentration during treatment.
**Results**

*Oxygen saturation*

In the twenty-five patients whose mean blood oxygen saturation was measured after the delivery of 0, 18.5 and 37 Jcm$^{-2}$ of light, a significant reduction ($P<0.005$) in oxygen saturation during light treatment was observed. Oxygen saturation was significantly lower ($P<0.005$) both during and at the end of irradiation compared to the pre-irradiation value (Figure 1). No statistically significant difference was noted between the final oxygen saturation value and that recorded at the half-way stage ($P=0.165$).

A significant decrease ($P<0.005$) in lesion oxygen saturation was also observed for the ten patients monitored after 0, 4.75 and 37 Jcm$^{-2}$ of light delivery (Figure 2). The two latter measurements were found to be significantly lower ($P<0.005$) than the initial oxygen saturation recorded (Figure 2). In contrast the oxygen saturation recorded at 4.75 and 37 Jcm$^{-2}$ were noted to be similar ($P=0.314$; Figure 2).

Significant reductions ($P<0.005$) in oxygen saturation during light irradiation were noted irrespective of the histological lesion type, anatomical location of the lesion (8 acral versus 17 non-acral skin) and patient gender (8 males and 17 females).

The initial oxygen saturation values recorded within these lesions were in the same range as previously determined oxygen saturation values for normal skin in identical anatomical locations (data not shown).
The average decrease in oxygen saturation within the first minute ($4.75 \text{ Jcm}^{-2}$; 10 patients) was noted to be similar ($P=0.845$, Figure 3) to the average decrease in oxygen saturation after the first half of light treatment ($18.5 \text{ Jcm}^{-2}$; 25 patients) although significantly more data would be required to demonstrate whether the changes observed within the first minute were similar to the changes in oxygen saturation in the first half of light irradiation.

**Perfusion**

Perfusion within lesions was noted to be significantly greater ($P<0.01$; Figure 1) than perfusion within distal normal skin both before and after light irradiation. During the first half of light irradiation perfusion within the lesion increased significantly ($P<0.001$) and perfusion then remained significantly greater at the cessation of treatment ($P<0.01$). In contrast perfusion at the half-way stage was similar to the perfusion at the cessation of treatment ($P=0.160$). The perfusion within distal normal skin remained unaltered ($P=0.42$).

The change in perfusion during the first half of light treatment was significantly greater ($P<0.01$) than the changes during the second half of the irradiation.

**Temperature**

The localised skin temperature was observed to fluctuate during MAL-PDT with 25% of patients undergoing significant increases in temperature (up to $+2^\circ \text{C}$, $P<0.05$, data not shown). However when the raw data was analysed as a whole no significant difference was noted in skin temperature during irradiation ($P=0.18$; Figure 1). 

**Fluorescence**

During light irradiation PpIX fluorescence was observed to significantly decrease \( (P<0.001; \text{Figure 1}) \). The decrease in PpIX fluorescence during light irradiation was significantly greater during the first half of irradiation \( (P<0.001) \) than during the second half of treatment, however during the second half of the irradiation period the decrease in PpIX fluorescence still remained statistically significant \( (P<0.01) \). A weak positive correlation was noted between total PpIX fluorescence prior to irradiation and PpIX photobleaching \( (r^2=0.55, \ P<0.01) \); as previously demonstrated \(^{31}\).

Potential correlations between the oxygen saturation and PpIX fluorescence data were investigated. No correlations were observed between the initial oxygen saturation within the tissue and the level of PpIX photobleaching that occurred during irradiation \( (r^2=0.1, \ P=0.335; \text{data not shown}) \) and the total change in oxygen saturation and the total level of PpIX photobleaching during light irradiation \( (r^2=0.122, \ P=0.169; \text{data not shown}) \). Stronger correlations were observed between the changes in oxygen and PpIX photobleaching observed during the first half \( (r^2=0.281, \ P<0.05; \text{Figure 4a}) \) and the second half of irradiation \( (r^2=0.312, \ P<0.05; \text{Figure 4b}) \) respectively. The strongest correlation was observed between changes in oxygen saturation and fluorescence recorded at the end of the first minute of irradiation \( (r^2=0.766, \ P<0.001; \text{Figure 4c}) \). These relationships were confirmed with the Spearman Rank analysis.
Discussion

This investigation measured for the first time the changes in oxygen saturation and perfusion at various stages during real-time clinical dermatological MAL-PDT in a range of licensed indications. The oxygen saturation within lesions was monitored prior to the application of light, after the delivery of either 4.75 Jcm$^2$ or 18.5 Jcm$^2$ light and after the completion of irradiation (37 Jcm$^2$). A wide variety of responses were noted in different lesions, but the general trend observed demonstrated significant reductions in the localised mean blood oxygen saturation after the delivery of 18.5 Jcm$^2$ (Figure 1) and 4.75 Jcm$^2$ (Figure 2) of light respectively. Furthermore the change in oxygen saturation observed during the first minute of irradiation was similar to the decrease observed in the first half of irradiation (Figure 3). Significantly more patients (over 1000 in each group) would be required to determine if this was a true result. The significant reduction in oxygen saturation was observed during light irradiation irrespective of the histological lesion type receiving the MAL-PDT treatment, patient gender and lesion location. Perfusion was also observed to significantly alter during treatment with a significant increase in perfusion occurring after the onset of irradiation (Figure 1).

These data provide evidence in man that the effects are similar to those previously observed in animals, that is that rapid reductions in the $pO_2$ are observed immediately after the initiation of light treatment during PDT $^{14,17,20,32}$. Figure 2 suggests that the $pO_2$ is significantly depleted within the initial 4.75 Jcm$^2$ of light with this form of PDT and then remains at a similar level for the remainder of treatment. This suggests that it is the very early stages of
irradiation where the maximal photochemical reactions take place, as previously suggested by our PpIX photobleaching study\textsuperscript{33}. The reduction in oxygen saturation during MAL-PDT is proposed to occur primarily as a result of oxygen consumption during the photochemical reactions within cells and to a lesser extent, damage to the localised microvasculature may also be involved\textsuperscript{13}. Mean blood oxygen saturation can only provide a measure of the oxygen content of the blood in the lesion during irradiation and is not a direct measure of tissue oxygenation. A fall in mean blood oxygen saturation can be induced by a decrease in arterial inflow as well as an increase in oxygen consumption\textsuperscript{34}. However, when considered in conjunction with the perfusion data presented here, which demonstrated the vasodilatory effect of irradiation it is reasonable to attribute this fall in oxygen saturation to an increase in oxygen consumption during light irradiation.

Several studies have investigated perfusion alterations following light irradiation during ALA-PDT. Increased perfusion following light irradiation (as observed here in Figure1) was previously noted following the light irradiation stage of ALA-PDT treatment of BCC (where a light dose of 60 Jcm\textsuperscript{-2} was delivered at 100 mWcm\textsuperscript{-2})\textsuperscript{21} and during PDT with other photosensitisers in animal models\textsuperscript{35,36}. However, another study indicated no significant perfusion alterations within 18 BCC lesions pre and post ALA-PDT treatment although this study utilised a wide range of fluence rates (20-300 mWcm\textsuperscript{-2}) and total light dose delivered (30-100 Jcm\textsuperscript{-2}) and did not investigate the effect of these parameters on perfusion\textsuperscript{29}. In contrast our study utilised a standardised irradiation protocol (37 Jcm\textsuperscript{-2}, 80 mWcm\textsuperscript{-2}), ensuring a more robust investigation of perfusion.
alterations during MAL-PDT treatment. Furthermore this study highlighted the importance of monitoring during light irradiation to ensure a more complete insight into the physiological changes of PDT treatment; one BCC within this study underwent no significant difference in perfusion pre and post irradiation, but significant changes were observed at the half way stage. Our findings have suggested that perfusion does not solely increase or decrease during MAL-PDT, rather perfusion initially increases before beginning to decrease, this corresponds with previous research suggesting that the concept of unidirectional perfusion changes during PDT was misconceived \(^8\).

The initial lesional mean blood oxygen saturation recorded was compared with previously determined mean values for normal skin in an identical location to the lesion and was demonstrated to fall within a similar range. This indicates that despite the disorganised microvasculature and the greater percentage of the tissue occupied by blood vessels in the (pre)cancerous lesions monitored \(^{37-39}\), the levels of oxygen within lesional and normal microvasculature are similar. This suggests a limited hypoxic environment exists within these lesion types, potentially enhancing the efficacy of MAL-PDT.

The changes in PpIX fluorescence were monitored for the first time during MAL-PDT in conjunction with the ORS data utilising a well established, validated, non-invasive fluorescence imaging technique \(^{24}\). Figure 1 demonstrates that both PpIX photobleaching and oxygen consumption were greater in the first half of light treatment when compared to the latter stages of treatment, with significant decreases occurring in both the PpIX level and oxygen saturation during the first half of treatment. The significant decrease in PpIX fluorescence
is attributed to the destruction of ground state PpIX by singlet oxygen forming the photoproduct, photoporphyrin $^{1,4}$. In contrast to oxygen saturation, PpIX fluorescence continued to significantly decrease in the second half of light treatment (Figure 1) and this may have been via an oxygen independent mechanism.

Intricate relationships have previously been demonstrated between the oxygen concentration and PpIX photobleaching in tumour tissue $^{40}$. The relationships between oxygen saturation and PpIX fluorescence during MAL-PDT were therefore investigated further. These data suggested a positive correlation between the change in PpIX fluorescence and changes in oxygen saturation within the first minute of light irradiation (Figure 4c), although this relationship requires confirmation and further investigation in a much larger patient cohort. Weaker correlations were observed for the changes in PpIX fluorescence and oxygen saturation during the first and second half of light irradiation and total changes in oxygen saturation and PpIX fluorescence. The initial oxygen saturation of the lesion did not significantly relate to the level of PpIX photobleaching either. The relationships between oxygen saturation and PpIX fluorescence were also investigated with the Spearman rank correlation and the same conclusions were reached, indicating that despite some outliers the data were not significantly influenced by these.

When considered concurrently, the oxygen saturation data, perfusion data and PpIX fluorescence changes suggest that on the initiation of light irradiation, singlet oxygen/other ROS are rapidly produced via photochemical reactions. Hence the pool of available oxygen and ground state PpIX are reduced, via
photochemical consumption and singlet oxygen destruction respectively. The rapid oxygen consumption would result in significant reductions in the partial pressure of oxygen and therefore potentially the development of a hypoxic environment. This may initiate hypoxia induced vasodilation leading initially to increased perfusion as observed to replenish the depleted oxygen supply. These data also demonstrate that the oxygen saturation remains low for the remainder of treatment, therefore suggesting that the increased perfusion either does not significantly replenish the partial pressure of oxygen in the tissue or it continues to be consumed by photochemical reactions as the light delivery is continued. It can be concluded however that in the latter stages of treatment oxygen consumption is reduced as no significant alterations in this parameter were observed at the monitoring points investigated during the second half of treatment. This probably occurs as a result of the significant reduction in PpIX availability (due to its photochemical destruction/photobleaching). As a result the perfusion in the tissue being treated may potentially decrease from this point onwards.

The rate of photobleaching is thought to be dependent on the light fluence (maintained in this study at 37 Jcm$^{-2}$), the photosensitiser concentration prior to light irradiation ($r^2=0.55$, $P<0.01$; data not shown) and the $pO_2$ within the tissues under investigation. Whilst only relatively weak correlations were observed between changes in PpIX fluorescence and oxygen saturation during light irradiation this can perhaps be explained by the phased photobleaching that is observed during light treatment. PpIX photobleaching has been demonstrated to follow a double exponential decay pre-clinically and recently we have
observed a similar relationship clinically during MAL-PDT \(^{33}\). The greatest correlation existed in the initial minute of light irradiation, which appears to be when the majority of oxygen is consumed (in fact changes in tissue oxygen saturation within the first minute (4.75 Jcm\(^{-2}\), 10 patients) were not dissimilar to the changes that occurred in the whole first half (18.5 Jcm\(^{-2}\), 25 patients) of light irradiation), suggesting that the majority of PpIX should be photobleached in the early stages of light irradiation (as observed). The weaker correlations observed between PpIX photobleaching and oxygen saturation for the first half, second half and total light dose could be explained by oxygen-independent photobleaching occurring when the local environment is more hypoxic \(^{41}\) or an increase in arterial inflow due to the vasodilatory effect of irradiation. Furthermore our clinical data demonstrated significant interpatient variations as a result of numerous external factors, which may potentially alter the capacity for photochemical reactions and therefore the relationship between PpIX photobleaching and oxygen saturation.

Monitoring the oxygen saturation of lesions at more time points during light irradiation would have been preferable however this study aimed to monitor standard clinical treatments without significant intervention. Due to the problems of cross-talk with PDT irradiating at 635 nm and laser Doppler perfusion imaging 670 nm, acquisition of measurements required the PDT light source to be off and was therefore limited to three occasions, with only one reading acquired during the light irradiation procedure. It is important to note that for more accurate monitoring of PpIX photobleaching, oxygen saturation and perfusion during light irradiation in the clinical setting a technique enabling
continuous monitoring throughout treatment needs to be developed such as has been employed with other photosensitisers pre-clinically. Until such time as a technique is developed the temporary pausing of the light irradiation protocol for a short time (maximum of three minutes within this study) remains the only way of gaining an insight into clinical PpIX photobleaching kinetics. By pausing the light a fractionated protocol was employed with a short dark phase, which has previously noted to enhance the level of cellular damage/death during ALA-PDT. Whilst pausing the light to monitor during treatment may not be ideal, the photobleaching of PpIX appeared to respond as previously demonstrated in pre-clinical models. This observation increased our confidence in the validity of the data despite having to temporarily pause the light delivery.

Furthermore it would have been preferential to monitor both the oxygen saturation and perfusion within all of the patients. However with clinical time constraints this was not feasible and therefore this study was limited by the number of patients in each group. We have demonstrated however that both the ORS and LDPI devices can be successfully utilised in a standard clinical setting to aid dermatological MAL-PDT investigation/monitoring. Future work should involve the monitoring of a larger number of patients, to confirm the observations within this cohort of patients and to extend the study to investigate conclusively whether lesion type and lesion location alters changes in oxygen saturation and perfusion. However it should be noted that the results recorded here, fit both with our previous work and the work of other groups in animal models, increasing our confidence in the data despite limited numbers.
The different techniques employed within this study probe slightly different depths of the skin, which need to be considered in conjunction with our findings. Both the fluorescence images and the ORS provide detailed information at the epidermis and dermal-epidermal interface respectively. The LDPI however, utilises a longer wavelength of light and therefore probes the skin at deeper depths investigating the flow of the superficial plexus within the dermis. Whilst it would be preferable to use techniques that all investigate the same depth this is not currently possible with the technology available. The deeper depth of the LDPI may explain why limited correlations were observed between changes in perfusion and changes in fluorescence during treatment.

The heterogeneity of the microvasculature may reduce the reliability of the data as only one area within the lesion was monitored. However our data showed that within a lesion oxygenation varied by only 10% and our techniques of reproducible probe placement ensured the same region of microvasculature was monitored. There remains the possibility that the values obtained may not truly reflect the overall actual oxygen saturation of the lesion, as previous probe placement at different sites within the lesion was observed to significantly alter the oxygen saturation \(^{20}\). However, in light of the consistent trends observed when these data were analysed by fluence delivered, lesion type, lesion location and patient gender, we consider these observations to be robust and the trends valid.

Overall these findings demonstrated for the first time a rapid decline in oxygen saturation occurring during dermatological MAL-PDT following the initiation of irradiation and this finding is related to the PpIX photobleaching that is also
observed to occur over this time period. Furthermore this study demonstrated an increase in perfusion within lesions during light irradiation, which suggests that as oxygen is consumed perfusion increases to replenish depleted tissue oxygen stores. These findings suggest that treatment enhancement could investigate improving the oxygen saturation of the tissue either prior to or following the initial minute of light irradiation to increase ROS production and ultimately treatment efficacy.
References

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Figure legends

Figure 1: Graphical representation of the relative oxygen saturation (n=25), PpIX fluorescence (n=45), perfusion (n=20) and temperature (n=45) as a function of the light dose delivered. The error bars represent the standard deviation of data and ∆ and ** represents statistical significance at $P<0.005$ and $P<0.001$ respectively from the pre-irradiation value. * represents significance at the $P<0.01$ level between the post-irradiation value and the value at the half way point of irradiation.

Figure 2: Bar chart representing the oxygen saturation as a function of the light dose delivered for the ten patients monitored pre-light application, after 1 minute and immediately after the completion of treatment (n=10). The error bars represent the standard deviation and ∆ represents statistical significance at $P<0.005$ from the pre-irradiation value.

Figure 3: Bar chart representing the mean change in oxygen saturation during the first minute (4.75 Jcm$^{-2}$) and the first half of light treatment (18.5 Jcm$^{-2}$). The error bars represent the standard deviation of the data.

Figure 4: Linear regression graphs of the change in oxygen saturation and PpIX fluorescence that occurred A) during the first half of irradiation (n=25), B) during the second half of irradiation (n=25) and C) during the first minute of irradiation (n=10). The linear regression coefficient is noted.
Figure 1
Figure 2
Figure 3
A

$PpIX$ fluorescence during the first half of light irradiation (%)

Percentage change in oxygen saturation during the first half of light irradiation (%)

$r^2 = 0.281 (P < 0.05)$

B

$PpIX$ fluorescence during the second half of light irradiation (%)

Percentage change in oxygen saturation during the second half of light treatment (%)

$r^2 = 0.312 (P < 0.05)$
Percentage change in PpIX fluorescence during the first minute of light treatment (%)

Percentage change in oxygen saturation during the first minute of light treatment (%)

$r^2 = 0.766; (P < 0.001)$

Figure 4