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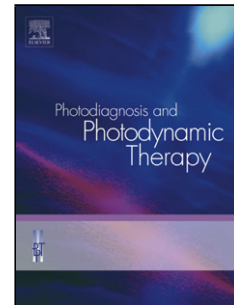
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- The fluorescence signal produced by the Protoporphyrin IX (PpIX) build up was measured as a function of time.
- Fluorescence signal increased linearly with time during the occlusive treatment phase.
- Faster build up was noticed for lesion located on the face region.

A Quantitative Study of In Vivo Protoporphyrin IX Fluorescence Build Up During Occlusive Treatment Phases

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Abstract

Background: Topical photodynamic therapy (PDT) is a non-invasive light based therapy used to treat non-melanoma skin cancer (NMSC) and dysplasia. During PDT, the light sensitive molecule protoporphyrin IX (PpIX) is activated, resulting in the production of singlet oxygen, which subsequently leads to cell death. PpIX is metabolised from a topically applied pro-drug and the strong fluorescence signal associated with PpIX can be utilised as an indicator of the amount of PpIX present within the tumour tissue. In this work we measure the build up PpIX during the occlusive treatment phase and investigate how the PpIX production rate is affected by different lesion and patient characteristics.

Methods: Fluorescence measurements were used to investigate the build up of PpIX within the tumour tissue during the 3 hour long occlusive treatment prior to irradiation. The study included *in vivo* measurements of 38 lesions from 38 individual patients. Actinic keratosis (AK) and basal cell carcinoma (BCC) were the lesion types included in this study. The resulting data from the study was analysed using generalised linear mixed effects models.

Results: It was found that the surface fluorescence signal linearly increased with occlusive treatment time. The predictive models suggest that there is a significant difference in PpIX production between lesion location, however no significant difference is demonstrated between different lesion types, gender and skin type.

Conclusions The study extends and supports previous knowledge of PpIX production during the occlusive treatment phase.

1 Introduction

Topical photodynamic therapy (PDT) relies on the interaction between light, molecular oxygen and a photosensitive chemical to produce singlet oxygen and other reactive oxygen species (ROS), leading to tissue injury and death of targeted cells [1]. PDT is a well established method for treating superficial skin lesions such as basal cell carcinoma (BCC), Bowen's disease (BD) and actinic keratosis (AK) [2]. During topical photodynamic therapy (PDT), the prodrug aminolevulinic acid (ALA) or methyl aminolevulinate (MAL) is applied topically to the skin. After the application of the prodrug the lesion is covered with an occlusive dressing which blocks out any ambient light. During this occlusive treatment (with a typical duration of three hours) the prodrug diffuses through the skin and is converted to the photosensitive molecule protoporphyrin IX (PpIX) in the tumour cells [3]. PpIX is a naturally occurring molecule which

is produced in the mitochondria as part of the heme biosynthesis. When precursors to PpIX such as ALA or MAL are added to the tumour cells, rate limiting steps are bypassed and an excess amount of PpIX is selectively accumulated in the tumour cells.

Even though PDT is a well established treatment method, there is still a desire to improve the response rate. One suggested reason for a reduced response rate is the insufficient accumulation of PpIX within the lesional tissue [4]. Typically the occlusive treatment phase (OTP) is three hours long, but improved results with a longer OTP have been reported [5]. The work presented here provides a study of the production of PpIX during the OTP to determine the rate at which PpIX builds up within the tumour tissue during the OTP where different lesion and patient characteristics are compared.

Many skin properties change when tissue is removed from the body. Thus it is important to consider *in vivo* data when investigating and representing skin dynamics during light based therapies such as PDT. Clinical studies are vital for development of the PDT treatment as well as determination of skin properties to allow accurate theoretical simulations. In PDT the *in vivo* data acquisition is mostly limited to surface measurements, due to the non-invasive nature of the treatment. One measurable quantity is the characteristic fluorescence signal associated with the PpIX molecule, where the level of measured fluorescence is used as an indicator of the amount of PpIX present within the skin tissue [6]. It is important to be aware of the limitations of the penetration of light through tissue which means that fluorescence from depth will be attenuated to some extent when measured at the skin surface. In this study fluorescence observations were used to measure the production of PpIX during the OCP.

2 Materials and Methods

2.1 Patients

38 patients (14 men and 24 women) between 43 and 92 years old with BCC (27 lesions) or AK (11 lesions) were included. Skin types I - IV was examined (I: 11, II: 20, III: 5 and IV: 2). Only one lesion per patient was included to reduce measurement correlations. The study took place between October and December 2014 at the skin department of Amaral Carvalho Hospital (Jaú, Brazil) and all patients underwent routine PDT after measurements were taken. The study was approved by the University of St Andrews Teaching and Research Ethics Committee (Ref.: PA11325) as well as the Review Board on Ethics in Research from the Amaral Carvalho Hospital Foundation (Ref: CEPFCHAC 140/11). Written consent was obtained from all patients before participating in the clinical study.

2.2 Photosensitiser

The pro-drug that was used contained 20% MAL (PDTPharma LTDA., Cravinhos-SP, Brazil) which was converted to ALA that subsequently induced the production of PpIX within the mitochondria. Both ALA and PpIX are naturally occurring in human cells and are main components in heme production. Exogenous addition of ALA leads to bypassing of rate limiting steps and accumulation of PpIX in tumour cells [3].

2.3 Fluorescence device

The fluorescence measurements were recorded using an optical biopsy system (OBS) illustrated in figure 1. The OBS houses a blue, 405 nm laser diode (approximately 1mW output), which is coupled to a fibre probe (diameter of 600 μm). The probe was used both for the excitation light as well as collecting the resulting fluorescence. A 455 nm long-pass filter and a fibre splitter are included in the OBS to allow the excitation light and collected signal to be separated and the

backscattered light from the excitation to be eliminated from the recorded spectra. The collected light was recorded using a compact grating spectrometer (Ocean Optics, Inc.) interfaced with a PC.

2.4 Study design

Appropriate lesions were examined and selected for the study by a qualified dermatologist. All lesions were photographed, measured and biopsied to ensure correct identification. Before the prodrug was added to the lesion, a curettage was applied to remove the surface crust to improve penetration of the prodrug. The biopsy that was removed during the curettage was sent away for analysis. After the cream application (containing MAL), the lesion was covered with a PVC plastic film, aluminium foil and a compress, which was secured with surgical tape. The occlusive dressing was applied for a total of three hours. During this OTP the fluorescence signal was recorded at regular time intervals. At each measurement point the dressing was removed, any excess cream was removed and the fluorescence signal was recorded from the lesion. Five measurements from approximately the same area were recorded before the cream and dressing was reapplied. This was repeated every 20 min for a total of 3 hours. When required, additional cream was added to the lesion to ensure that there was always an excess amount of cream on the lesion. The fluorescence signal was also recorded prior to cream application to establish the autofluorescence from each patient/lesion. Test measurements were carried out (not reported) which showed that the presence of MAL did not affect the penetration of light. MAL does not emit any fluorescence light that would interfere with the measurements of the PpIX fluorescence.

The probe was placed in light contact with and perpendicular to the skin. The measurements were performed in a darkened environment and the probe was repositioned at approximately the same point of the lesion to record repeated measurements. By using lesion landmarks it

was possible to measure the fluorescence from approximately the same area throughout the OTP. Most patients with AK only received one treatment, and for this reason only data from this session was included in this work although some patients with BCC received a second treatment. For all measurements analysed, the autofluorescence spectrum measured prior to prodrug application was subtracted. From each resulting measurement the peak value of the fluorescence measurement (635 nm) was obtained (figure 3) and subsequently used to analyse the PpIX production as a function of time.

Following the occlusive treatment the lesion was treated using a therapeutic light source for 20 min [7]. The measurements were performed under such conditions that it was assumed to not affect the build up of PpIX within the tumour lesion.

2.5 Statistical analysis

Our aim was to generate a statistical model that accurately predicts the fluorescence signal as it changes with time for different patient characteristics such as skin type and lesion types. When measuring a longitudinal data set, correlation between the measured data points is present. For this reason the data points cannot be assumed to be independent and simple linear regression is not appropriate. In addition the spread of the data was proven to not be normal nor homogenous, which indicates that generalised statistical models are required.

A common and convenient way of modelling variation between individuals within a data set is by adopting random effects. The random effects represent the variation not explained by the covariates (fixed effects). The difference in, for example, genetic make up associated with different individuals results in different PpIX production rates. This effect is not considered within the fixed effects model and by including the random effects associated with the individual and explaining the associated noise, the information gained from the estimated model can be applied to the wider population and not only the small subset of patients included in the study.

A mixed effects model considers the correlation between repeated measurements as well as the longitudinal correlations [8]. This makes generalised mixed effects models (GLMMs) the most appropriate framework to adopt in the presented study. All statistical analysis was carried out in R [9].

3 Results

Figure 2 shows a patient with multiple BCC lesions located on the shoulder/back with typical characteristics for the study. The size of the lesions were approximately 2 cm (left) and 1.5 cm (right). Only one of the lesions (right) was included within the study (magnified figure 2) to reduce correlations within the analysis. The characteristic fluorescence signal generated from the PpIX within the lesion tissue is shown in figure 3. In the figure both the characteristics peaks at 635 nm and 705 nm are shown. As discussed above, the work presented here focuses on the peak at 635 nm. I.e. the fluorescence value at the peak at 635 nm was used as the indicator of the PpIX distribution within the lesion.

An initial analysis showed that the data collected here gives the linear trend that has previously been suggested [10, 11]. For this reason the adopted model was assumed to follow a positive linear trend. Since all data points were background subtracted, the y-intercept was forced to the origin. The skewness of the residuals in combination with the requirement of a positive continuous model indicates a Gamma distribution of the observations, where the variance increases quadratically with expectations [8, 12].

To consider the correlations as well as random effects, a GLMM was adopted using the 'glmmPQL' function in the 'MASS' package in R. The explanatory variables that were investigated were: gender, location of the lesion, skin type and type of lesion. During the first treatment session the results show that there was no significant difference associated with gender, skin type or lesion type. However, the GLMM showed that there was a significant difference

($p < 0.05$) in lesion location. Lesions located on the face/head area shows a faster increase of PpIX production during the occlusive treatment face compared to other lesion locations. Figure 4 shows the linear trends associated with the lesion locations.

4 Discussion

Our results suggested that the strength of the fluorescence signal is affected by the location of the lesion supporting previous findings [13, 14]. Other characteristics such as lesion type, gender and skin type did not show any significant difference. The effect of gender and skin type could be explained by the fact that these should not have an effect on the lesion itself. A more significant difference between lesion type (when comparing AK and BCC) was expected both due to the potential anatomical and physiological variation between lesion types as well as previous published results [10]. In addition the difference in diffusion barrier between different individuals (affected by both lesion type and location of the lesion) will influence the penetration of the prodrug and thereby the distribution of the PpIX. However, no significant difference was found within the data obtained within this study.

Since the light source in the OBS is blue, the penetration of this light is as previously mentioned, limited. This means that the whole depth of the lesion might not be measured successfully. PpIX from depths beyond the measuring range will therefore not contribute to this study. To remove this limitation further investigations are encouraged. This would include measurements of biopsied sample sections as well as using alternative deeper penetrating light sources to measure the fluorescence.

The linear trend was found to be an appropriate fit for our data and is, as previously mentioned, supported by previous studies [10, 11]. There is no way of determining the trend after the three hours of occlusive treatment. It could be argued that the linear trend would flatten out if the occlusive treatment was extended due to the saturation of the PpIX. To measure this,

the subjects would have to stay in the clinic for a longer period of time and the PDT treatment would not be able to be performed according to the regulated protocol. In a previous study, where the fluorescence was studied in healthy human skin, no significance was observed when ALA was applied for 1 hours or 6 hours. This shows that the differences between NMSC and healthy skin modifies the PpIX production kinetics [15].

The results show a large variation within patients as well as between patients. This could be explained by the non-uniform nature of tumour tissue. Even though the same area was targeted for the repeated measurements, non-uniformities on small size-scales can lead to large variations in the fluorescence signal [16].

Due to the slight variation in surface preparation (degree of curettage) between institutes a study which considers this would be appropriate. Including several institutes would strengthen the results and increase the number of patients included in the study.

The statistical framework that was developed for this study should be considered for other clinical studies. By considering the correlation, more appropriate results and conclusions can be drawn from clinical data. The collected data extends the previous knowledge where the frequency of measurements was increased as well as the comparison between more explanatory variables, has to our knowledge, not previously been undertaken.

5 Conclusion

The PpIX fluorescence signal from patients presented with superficial skin lesions (BCC and AK) was measured at a regular time interval during a three hour long OTP. It was found that the PpIX increased linearly during the first three hours of the occlusive phase where results indicate a larger overall amount of PpIX produced in lesions located on the face compared to all other body location. Even though the results show a large variability both intra- and inter-subject, the linear trend supports previously published data. This study extends and supports previous

knowledge of PpIX production during the occlusive treatment phase.

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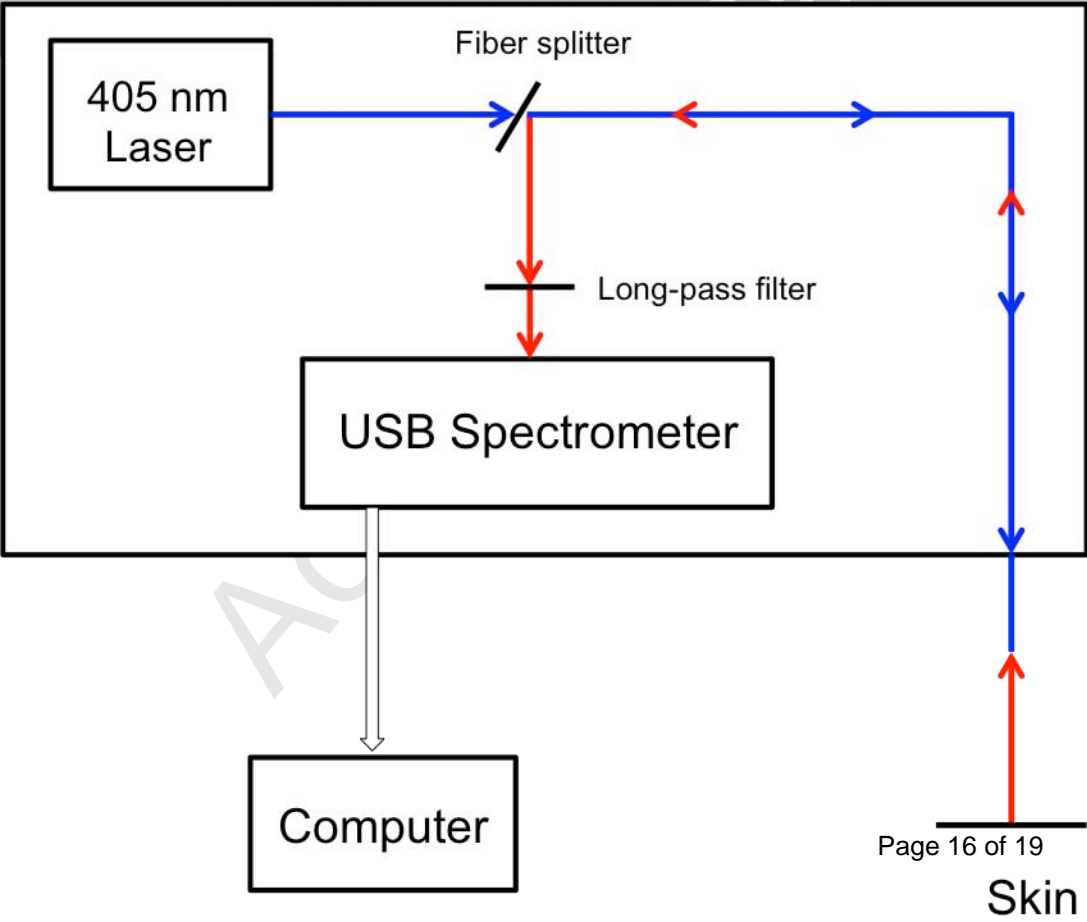
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Figure 1: Schematics of the optical biopsy system (OBS) where all components, except for the laptop computer are eclipsed within a box to prevent any stray light effecting the measurements.

Figure 2: Image of typical lesions included within the study. Two superficial BCCs are displayed in the image. Only the lesion to the right (magnified) was included within the study.

Figure 3: Figure showing a typical fluorescence signal (arbitrary units (a.u.)) observed from one of the patients included within the study. The fluorescence signal is generated when the PpIX is exposed to blue, 405 nm, light. The fluorescence spectrum displays two characteristic peaks at 635 nm and 705 nm. In the analysis presented here, the focus lies within the peak at 635 nm.

Figure 4: Generalized Linear Mixed Effects Model (first session).Figure showing the linear trend between the fluorescence signal (arbitrary units, a.u.) and time (minutes) for the first treatment. The predicted model was generated using a Generalised Linear Mixed Effects Model (GLMM) where the results demonstrate a significant difference ($p < 0.05$) between lesions located on the face (dashed) compared to other body sites (solid). The fluorescence was shown to be higher for lesions located on the face (dashed). Repeated measurements were averages to reduce clutter on the graph. However all repeated (five) measurements were used to fit the model. The black circles represents lesions located on other body sites other than the face and the black crosses corresponds to observations from lesions on the face. The confidence intervals (dotted for lesion located on the face and dash-dot for lesion) are included for the different situations (Note overlap between confidence intervals between the two sub-groups).



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