



University of Dundee

Fluorescence and thermal imaging of non-melanoma skin cancers before and during photodynamic therapy

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5 educational conferences. SI has received funding from Galderma UK Ltd and Biofrontera for attendance at
6 conferences.
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14 Photodynamic therapy, non-melanoma skin cancer, fluorescence, temperature
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19 **Summary**

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21 Background:

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23 Photodynamic therapy (PDT) has been shown to be less effective on the extremities. Protoporphyrin-IX
24 (PpIX) fluorescence and skin surface temperature are variables that have been implicated in the differences in
25 efficacy with body site, but objective studies have not been undertaken.
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29 Objectives:

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31 To further investigate observations from our previous study that temperature and fluorescence during pro-
32 drug incubation are correlated, through a prospective objective investigation of the relationships between
33 fluorescence and skin surface temperature before and during PDT and relationships with body site and
34 efficacy.
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38 Methods:

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40 Eighteen patients with Bowen's disease or basal cell carcinoma, who had been referred for PDT, were
41 recruited to this study. PpIX fluorescence and thermal measurements were recorded at intervals during the
42 pro-drug incubation and irradiation phases of PDT. Pain immediately after irradiation, and outcome at 3- and
43 12-months were recorded.
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48 Results:

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50 Temperature and PpIX fluorescence were higher on the trunk than lower leg immediately before treatment
51 (median temperature 32.7°C vs. 27.8°C, $p < 0.05$ and median fluorescence 16.5 vs. 6.7, $p < 0.05$). Higher pain
52 levels were reported during PDT on the extremities (median 5.7 vs. 2.2, $p < 0.05$). Clearance rates at 12-
53 months were 80%.
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58 Conclusions:

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4 The study supports a correlation between temperature and PpIX fluorescence during PDT, providing robust
5 objective data to support our previous hypothesis and observations. The higher pain levels, lower PpIX
6 fluorescence on the lower leg, and the high efficacy rates at all body sites irrespective of temperature and
7 fluorescence indicates that relationships between PDT treatment conditions and parameters is likely to be
8 multifactorial.
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15 **Introduction**

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17 Photodynamic therapy (PDT) is widely used to treat superficial basal cell carcinoma (BCC), Bowen's disease
18 (BD) and actinic keratosis[1]. Whilst PDT is at least as effective as standard comparators and high levels of
19 efficacy can be achieved, evidence indicates that PDT is less effective when used on the extremities, e.g. the
20 arm and lower leg, when compared with outcomes achieved when treating lesions on the head and neck or
21 trunk[2]. It has also been shown that the conversion of ALA or MAL to PpIX is reduced at acral sites which
22 may contribute to this reduction in efficacy [3,4].
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28 Our group previously hypothesised that temperature differences between body sites may play a role in the
29 rate of cutaneous ALA or MAL to PpIX conversion[5]. Using objectively measured fluorescence data and
30 temperature data derived from the literature, we proposed that, after a 3h photosensitiser pro-drug incubation
31 period, the higher fluorescence measurements recorded at sites on the head, neck and trunk and the lower
32 fluorescence levels at leg sites, could possibly be attributed to higher surface temperatures at the former sites
33 [5]. However, this study did not objectively measure surface temperature, nor did it investigate possible
34 associations with PDT efficacy.
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40 As summarised by Mordon[6], the dependence of PpIX production on temperature has been investigated,
41 although again no direct link has been shown between increasing temperature during PDT and efficacy when
42 PDT is used clinically to treat BD and BCC. 'Thermally-modulated' PDT has however been investigated by
43 Willey *et al.* for the treatment of AK on the extremities[7]. In their study, they heated up the arms or legs with
44 a heating pad during a 1-hour incubation period. During treatment, sites were air cooled for comfort, which
45 has independently been shown to result in lower efficacy[8]. Although high (90%) clearance rates were
46 reported, there was no control group, and the results were compared to those of a previous study[9].
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52 Juzeniene *et al.* reported in normal mouse skin that low temperatures had a negative impact on PpIX
53 formation[10]. PDT was not performed in this study though the authors speculated that thermal modulation
54 of PpIX concentration during incubation could potentially improve clinical outcomes. It has been shown that
55 'thermal PDT' can lead to greater reactive oxygen species generation and cell apoptosis[11]. It is clear that
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4 thermal modulation during incubation alters the dynamics of PDT, and it may have the potential to improve
5 treatment outcomes at body sites where PDT is less effective.
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8 Thermal imaging has been used widely for imaging skin surface temperature[12]. Skin temperature has often
9 been measured either by conduction (thermocouple) or radiation (infrared thermometer, thermal camera).
10 The use of a thermal camera has benefits over other methods: it is non-contact, and allows for analysis over a
11 field-of-view, as opposed to point measurements. With respect to PDT, to the best of our knowledge, there
12 are no reports of using thermal cameras to measure skin surface temperatures during treatment.
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17 A comprehensive review of pain in PDT was published by Ibbotson *et al.*[13]. While the mechanisms of
18 PDT-induced pain are not well understood, there is increasing, albeit sometimes contradictory evidence of
19 factors affecting pain in clinical studies. Attili *et al.* reported a correlation between fluorescence and pain in a
20 patient group consisting of those with BD, BCC and actinic keratosis (AK) lesions, although the authors of
21 this study also noted that previous studies reported conflicting results[14]. In addition, there is no consensus
22 agreement on the relationship between pain and body site. Nissen *et al.* reported that increasing the PpIX
23 concentration on the hands by increasing the incubation period of the prodrug did not increase efficacy, and
24 indeed a positive correlation between pre-treatment fluorescence and pain was found.
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31 The present study aims to further examine the hypothesis of Kulyk *et al.*, through investigating possible
32 relationships between PpIX fluorescence and skin surface temperature, and discussing these within the
33 context of PDT efficacy outcomes when using PDT to treat BD and BCC at different body sites.
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39 **Methods**

40 Aims:

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42 The primary aim of the study was to investigate the relationships between body site temperature and
43 fluorescence prior to and during PDT. Secondary objectives included assessing relationships of temperature
44 and fluorescence and body site with clinical outcome at 12 months and pain.
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48 Imaging systems:

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50 The fluorescence imaging system is described in detail by Kulyk *et al* [5,15]. Briefly, a 3D printed casing
51 houses a Lumenera LM075 M CCD camera, sixteen 405 nm LEDs and a longpass dichroic filter. The LEDs
52 are used to stimulate fluorescence and the dichroic filters out this excitation light, such that the stimulated
53 fluorescence may be imaged by the camera. Thermal images of the skin were recorded using a thermal camera
54 (FLIR T420bx, FLIR, USA). Images were taken normal to the skin at approximately 20 cm distance.
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59 Study design:

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4 This pilot study was sponsored by the University of Dundee and approved by the West of Scotland Research
5 Ethics Committee (17/WS/0055) and registered on clinicaltrials.gov (NCT03167762). Patients who were
6 referred to the photodynamic therapy clinic at Ninewells Hospital & Medical School between July 2017 and
7 July 2020 for treatment of BD or BCC were screened for the study and recruited if eligible and written
8 informed consent was obtained. Inclusion and exclusion criteria are presented in Table 1. Due to the field of
9 view restrictions of the camera, maximum lesion diameter was limited to < 2 cm.

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15 A formal power calculation was not carried out for this study as this is a pilot study, and patient numbers
16 were selected based on how many we anticipated we would be able to recruit.

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19 A standard PDT protocol, approved for use in clinical practice was used and initial surface preparation of the
20 lesion was undertaken using standard practice with a disposable ring curette. A reference frame[5] was taped
21 to the skin and used to align camera measurements. Baseline fluorescence and thermal images were taken of
22 the lesion, and the photosensitiser prodrug (Metvix®, Galderma UK) was then applied and occluded
23 (Tegaderm™ film and Mepore™ occlusive dressings used). During the three-hour cream incubation period,
24 images were taken every 30 minutes. This was achieved by removal of the dressing and excess cream, and the
25 reference frame was fixed on the skin. Fluorescence and thermal images were recorded in sequence and the
26 Metvix® cream (topped up as required) and the dressing were reapplied. Starting at t=0 minutes, fluorescence
27 and thermal images of the lesion were taken at 30-minute intervals for the duration of the three-hour
28 incubation period, prior to final removal of the dressing and any cream residue.

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36 During irradiation, 75 J cm⁻² (Aktilite CL128, Galderma UK) was delivered to the lesion. From the start of
37 irradiation at time points t=1, 2, 4 minutes, halfway (8.5 minutes; 37.5 J cm⁻²) and at the end of treatment (17
38 minutes; 75 J cm⁻²) fluorescence and thermal images were recorded as before. In order to undertake these
39 measurements during the irradiation, PDT had to be briefly paused but the duration of these pauses was not
40 normally for longer than one minute. At no time before or during PDT were body sites artificially heated or
41 cooled.

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46 After irradiation, the pain score was assessed by participants completing a visual analogue scale (VAS, 1-10
47 cm), and clearance rates were assessed during follow-up visits at 3 and 12 months, based on clinical
48 assessment using a scale of: clear (no evidence of any remaining active disease visually or on palpation),
49 partial, and no response.

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53 Data analysis:

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56 Fluorescence images were analysed in a custom MATLAB script (MATLAB 2016, Mathworks) derived from
57 Kulyk *et al.* One image was analysed per time point. Images were aligned using the fluorescent contour on the
58 template and the baseline signal subtracted, measured at the start of the incubation phase. Each pixel in the
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4 black and white image represents a white value between 0 and 255 – by averaging these pixel values over a
5 region of interest an arbitrary value of mean fluorescence may be obtained for the area of the lesion. Hence,
6 for each image, mean fluorescence is recorded over a region of interest. Gain was set on the camera to avoid
7 saturation of fluorescence signal. As the gain is kept constant and baseline signal subtracted for each patient,
8 these fluorescence values are comparable in this study.
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12 Thermal images were imported into ResearchIR (FLIR, USA). Similarly, a region of interest is setup around
13 the inner edges of the template, and the mean temperature for each image was recorded.
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17 Statistical analyses (descriptive statistics, unpaired t-test, simple linear regression) were carried out using
18 GraphPad Prism (Prism 9, GraphPad Software, USA). In all cases, statistical significance is represented by:
19 *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001
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25 **Results**

26 Patient population:

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29 Whilst the only additional study-specific procedures during this study were the fluorescence and thermal
30 measurements, this did mean that overall treatment times were increased and as a consequence, several
31 patients did not wish to participate in the study. Due to difficulty in recruitment, compounded by the need to
32 halt recruitment during the COVID-19 pandemic, we were only able to recruit 18 of the 20 planned
33 participants. Datasets from two of these participants were deemed unusable due to equipment failure,
34 resulting in 16 usable datasets for analysis. Of those 16 (9 female and 7 male) participants, 11 had lesions
35 located on the trunk (9 BCC and 2 BD) and 5 were located on the lower leg (2 BCC and 3 BD). Median age
36 of the participants was 75.5 (range 45 - 80) years. Two patients did not complete 12-month follow-up, and
37 one patient did not provide a pain score.
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44 Fluorescence and temperature:

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47 Examples of fluorescence and thermal images recorded during the study are shown in Figure 1. Fluorescence
48 and temperature measurements for each patient are shown in Figure 2. Both peak fluorescence and mean
49 lesion surface temperature at 180 minutes were independently modelled against body site (Figure 3) and both
50 were found to be significantly higher on the trunk than the lower leg (p<0.05; 95%CI 2.73-19.65, t(14)=2.84
51 and p<0.001; 95%CI 2.59-5.98, t(14)=5.43 respectively).
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56 Fluorescence intensity at 180 minutes (peak fluorescence, immediately before treatment) was modelled as a
57 function of mean surface temperature of the lesion at the same timepoint (Figure 4), and fluorescence was
58 seen to increase with increasing surface temperature and a linear relationship within the bounds of the
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4 measured values fitted ($R^2=0.4$; $\beta=2.25$, 95%CI 0.67-3.83; $p<0.01$; $F(1,14)=9.34$). Separating the data into
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6 body site however, linear trends of increasing fluorescence with temperature are observed but are not
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8 significant ($R^2=0.07$; $\beta=1.58$, 95%CI $-2.78-5.94$; $p=0.43$; $F(1,9)=0.67$ and $R^2=0.46$; $\beta=0.83$, 95%CI $-1.32-$
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10 3.96 ; $p=0.21$; $F(1,3)=2.53$ for trunk and lower leg respectively).

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12 There is a sharp rise in temperature at both body sites within the first four minutes of light irradiation. The
13
14 change in temperature between the start of light irradiation and the maximum temperature recorded during
15
16 treatment was modelled independently against both peak fluorescence and body site, however no significant
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18 relationship was found for either ($R^2=0.03$; $\beta=-0.82$, 95%CI $-3.72-2.07$; $p=0.552$; $F(1,14)=0.37$ and $p=0.244$;
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20 95%CI $-3.09-0.85$, $t(14)=1.22$).

21 Pain:

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23 Immediately after irradiation, pain was reported as being higher on the lower leg than on the trunk although
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25 there was a lot of crossover (Figure 5b, $p<0.05$; 95%CI $-5.15--0.33$, $t(13)=2.45$). There was no significant
26
27 relationship found between either fluorescence ($R^2=0.00$; $\beta=0.01$, 95%CI $-0.17-0.18$; $p=0.912$; $F(1,13)=0.01$)
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29 or temperature ($R^2=0.07$; $\beta=-0.28$, 95%CI $-0.86-0.31$; $p=0.325$; $F(1,13)=1.05$) at 180 minutes and reported
30
31 pain levels. Maximum temperature recorded during PDT was not an indicator of pain ($R^2=0.07$; $\beta=-0.32$,
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33 95%CI $-1.02-0.38$; $p=0.344$; $F(1,13)=0.96$).

34 Efficacy:

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36 This study was not powered to determine efficacy however from the available data there were no significant
37
38 relationships between clearance rates and peak fluorescence ($p=0.95$; 95%CI $-13.02-12.34$, $t(12)=0.06$),
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40 maximum temperature ($p=0.53$; 95%CI $-2.65-4.91$, $t(12)=0.65$) or maximum change in temperature ($p=0.84$;
41
42 95%CI $-2.76-2.27$, $t(12)=0.21$). At 12-month follow-up, 78% (7/9) and 80% (4/5) of lesions on the trunk
43
44 and leg were clear respectively (Figure 5a).

45 Other parameters:

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47 While 100% of BD lesions were clear at 12 months compared to 67% of BCCs, there were no significant
48
49 relationships between lesion type and fluorescence ($p=0.6$; 95%CI $-13.11-7.91$, $t(14)=0.53$), temperature
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51 ($p=0.06$; 95%CI $-5.11-0.14$, $t(14)=2.03$) at 180 minutes, or maximum temperature during PDT ($p=0.1$;
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53 95%CI $-3.82-0.39$, $t(14)=1.75$).

54 Discussion

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58 The relationship between fluorescence and skin surface temperature of the body site was proposed by Kulyk
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60 *et al*, which inspired this study in order to objectively investigate this hypothesis. With this study we also
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4 hoped to produce data that would inform the optimal parameters for a future interventional study to
5 investigate modifying temperature of the extremities during PDT and observing impact on treatment efficacy.
6 Even with the restricted sample size in this study, there are several relationships noted here that would be of
7 interest to investigate further.
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11 Fluorescence, and therefore conversion of ~~ALA-MAL~~ to PpIX, is higher in lesions where the skin surface
12 temperature is higher, which tended to be on the trunk rather than the lower leg (Figure 2). This confirms the
13 hypothesis laid out in the previous study by Kulyk *et al.* Within body site there was a trend, though not
14 statistically significant, of increasing fluorescence with a corresponding increase in temperature (Figure 4). As
15 body sites were not artificially heated during this study it is possible that such a relationship may be revealed
16 in more detail with a greater range of temperatures at each body site.
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22 Fluorescence increased gradually over the three-hour incubation period at both body sites, with fluorescence
23 increasing at a higher rate on the trunk than the lower leg. After PDT was complete, both sites were
24 photobleached down to an equal measure of fluorescence.
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28 During the three-hour incubation period the temperature of the skin remained relatively stable after an initial
29 decrease in temperature during the first hour of incubation, particularly at the lower leg site. It is thought that
30 this may be due to the patient resting on arrival at the clinic and the natural reduction in heart rate and
31 decrease in blood flow to the extremities. However, during PDT the temperature increased greatly within the
32 first four minutes at both body sites. The sharp increase in temperature may be due to the inflammatory
33 reaction initiated during PDT and increased blood flow to the area. Though not well demarcated, the thermal
34 imaging indicated that most of the increased temperature occurred at the lesion site, with surrounding healthy
35 tissue being less affected.
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42 There is a suggestion that the lower leg experienced a greater maximum change in temperature through pre-
43 treatment to post-treatment, despite having overall lower fluorescence intensity, though this was not found to
44 be statistically significant. It was thought that a greater change in temperature might be observed in lesions
45 with a higher fluorescence measurement, and thus higher concentrations of PpIX leading to higher rates of
46 photodynamic effect, however there may be some rate-limiting effects at higher temperatures that limit the
47 increase of temperature at the site.
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52 It was observed in this study that lesions which had the greatest maximal change in temperature during
53 treatment fared no better or worse than those associated with a smaller temperature change, however this
54 does not preclude the possibility of interventional heating of the skin having a significant effect. Rather what
55 we have shown is that, though present and significant, natural changes in the physiology between body sites
56 concerning PpIX fluorescence and skin surface temperature are unlikely to impact efficacy when PDT is
57 delivered using a red light dose of 75 J cm^{-2} .
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4 Although the fluorescence at the start of PDT was approximately three times higher on the trunk than the
5 lower leg, this did not result in higher efficacy, with clearance rates practically equal at trunk and lower leg
6 sites. This parity in efficacy is contrary to current literature and is possibly an artefact of the small study
7 population.
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11 There is another compelling explanation for the efficacy rates shown in this study, however. As noted in the
12 methods section, we used 75 J cm⁻² of red light during PDT, which is twice as much as that in the licensed
13 protocol, which uses radiant exposure of 37 J cm⁻². However, our recently published data showed PDT
14 clearance rates of 90% at 12-month follow-up when using 75 J cm⁻² as opposed to a regimen using 37 J cm⁻²
15 (68% clearance)[16]. In both studies and in standard clinical practice, if a lesion is not clear at three-month
16 follow-up, PDT is repeated. It is therefore possible that any differences in efficacy normally observed
17 between lesions treated with PDT at different body sites were negated by delivering a higher radiant
18 exposure.
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25 Additionally, it has been suggested that PpIX is still present deeper in the tissue even after apparent
26 photobleaching. Apparent photobleaching in this case would be determined by stimulating red (~635 nm)
27 fluorescence with blue (~405 nm) light, as in the present study. This will stimulate largely superficial
28 fluorescence and reveal little about the PpIX concentration deeper into the lesion. Even in this study
29 photobleaching was still occurring up to the end of the PDT, indicating the presence of superficial PpIX.
30 These results strengthen the case for delivering a higher radiant exposure during PDT than is currently
31 recommended.
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37 It is yet to be confirmed whether heating up the skin prior to treatment would either increase fluorescence
38 (and hence PpIX uptake) or increase adverse effects or PDT efficacy. A study by Nissen *et al.* investigated
39 whether increased PpIX uptake at acral sites on the hands led to better treatment outcomes for AKs treated
40 with PDT[17]. Their study showed increased fluorescence when curettage or increased incubation time was
41 applied, although no increase in efficacy was seen. In their study they also noted an increase in pain with an
42 increase in fluorescence. Other studies[14,18], have shown a positive correlation between fluorescence and
43 pain, though notably there were no analyses of pain and body site. Each of these studies runs counter to our
44 results as overall we found no relationship between fluorescence and pain.
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51 Although the sample size in this study was smaller than intended, the significant results obtained are
52 encouraging and support future studies to investigate whether heating extremity sites during PDT may
53 increase outcomes, particularly efficacy. Notably 12-month clearance rates in this study were high, possibly
54 ~~due to the delivery of a higher radiant exposure than the current standard for PDT~~, however the relatively
55 small number of patients in this study, particularly those with lesions on the lower leg, make extrapolating
56 conclusions on this particular aspect difficult.
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4 Importantly, the results of this study facilitate a more knowledgeable design of future studies. Patient
5 engagement with the study may be improved by making the study design less intrusive or inconvenient,
6 therefore increasing recruitment. Now that it is known how temperature and fluorescence behave during the
7 incubation phase of PDT, a more targeted, larger study could investigate the irradiation phase more closely.
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11 This study lends evidence to the suggestions by Kulyk *et al.* that temperature and PpIX-induced fluorescence
12 are correlated and based on these data, further interventional studies would be of benefit to deepen our
13 understanding of the interplay of fluorescence and temperature, and how these parameters might impact on
14 PDT adverse effects and efficacy.
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22
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27 methods and analysis.
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34 **CRedit Statement**

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36 **Paul O'Mahoney:** Conceptualisation, Methodology, Software, Validation, Formal Analysis, Investigation,
37 Writing – Original Draft, Visualisation, Funding acquisition. **Ifor Samuel:** Conceptualization, Resources,
38 Writing - Review & Editing. **Ewan Eadie:** Conceptualization, Methodology, Writing - Review & Editing,
39 Supervision. **Sally Ibbotson:** Conceptualization, Methodology, Writing - Review & Editing, Supervision,
40 Project Administration.
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20 Figure Legends

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24 Figure 1. a) Fluorescence images at all time points in incubation and irradiation phases. White signal in the
25 images represents PpIX fluorescence, and the PpIX concentration (arbitrary) is indicated in the white
26 box in the lower right corner of each image. b) Thermal images at time points 0- and 180-minutes
27 during incubation phase, and 17 minutes in irradiation phase. The region of interest (ROI, black
28 dotted and dashed line) can be seen within the rectangular cut-out section of the template. The mean
29 temperature of the ROI is indicated in the white box in the lower right corner of each image. The
30 range of the temperature scale on the right-hand side of each image is automatically adjusted. An
31 indicative colour temperature scale is shown on the right-hand side. All temperatures are measured in
32 degrees Celsius.
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40 Figure 2. Time course measurements of a) fluorescence and b) skin surface temperature. Initial temperature
41 decrease measured on the lower leg in b) is thought to be the result of the patient resting on arrival at
42 the clinic and the natural reduction in heart rate and decrease in blood flow to the extremities. Solid
43 lines show the mean values, and grey shaded areas represent one standard deviation either side of the
44 mean.
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49 Figure 3. a) Fluorescence and b) temperature measured immediately prior to irradiation were significantly
50 higher on the trunk than on the lower leg.
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53 Figure 4. Fluorescence and temperature measured immediately prior to irradiation are positively correlated
54 when considering the data set as a whole (dashed line). When separated into body site, there are non-
55 significant positive trends in each subset (solid lines).
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59 Figure 5. a) Clearance rates at 12 months were comparable between body sites. b) Pain reported immediately
60 after irradiation was significantly higher on the lower leg than on the trunk.
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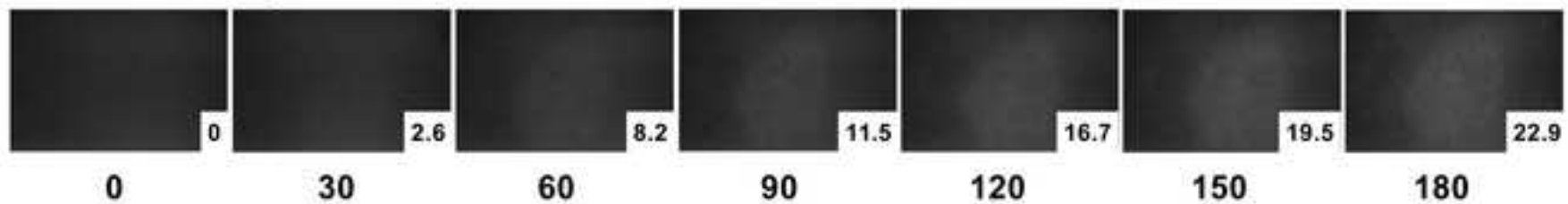
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4 **Tables**
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8 Table 1: Inclusion and exclusion criteria
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Inclusion criteria	Exclusion criteria
Patients referred for PDT for superficial BCC or Bowen's disease (one or two lesions and diagnosed either clinically or histologically and untreated or having had no treatment for 4 months or longer)	Patients skin lesions have had previous treatment in the last 4 months
Adult males and females, >18 years only	Unable to give informed consent
Capable of giving informed consent	Known allergy to Metvix®
Able to understand and adhere to protocol requirements	Known to have a light sensitive disorder
	Pregnant, breastfeeding or planning to conceive

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a) Incubation phase**Irradiation phase**