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A Clinical Trial to Determine the Impact of Tumor Size, Histological Subtype, and Vitamin D Status on the Therapeutic Response of Basal Cell Carcinoma to Photodynamic Therapy.

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  Supp 1: Supplementary Methods 1. 3D photographic analysis, with examples and calibration curve.
  Supp 2: Supplementary Methods 2. Remote clinical trial data management (Medocity online platform).
  Supp 3: Supplementary Table S1.   Compendium of 3D analyses of all BCC lesions in the clinical trial.
  Supp 4: Supplementary Figure S1. Flow chart of patient enrollment.
  Supp 5: Supplementary Figure S2. Flow chart of available BCC lesions.
  Supp 6: Supplementary Table S2.   Comparison of BCC histologic subtype, depth, height (3DAvHt).
  Supp 7: Supplementary Table S3.   Initial size of aggressive versus banal BCC tumors.
  Supp 8: Supplementary Table S4.   PDT responsiveness of BCC to Vitamin D versus tumor thickness.
  Supp9: Supplementary Table S5.   Effect of biopsy upon BCC tumor clearance.
  Supp10: Supplementary Table S6.   VDR alleles and BCC tumor clearance.

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53 **ABSTRACT**   (248 words; 250 max)

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55 Photodynamic therapy (PDT) with topical aminolevulinic acid (ALA) can be effective for select basal cell

56 carcinoma (BCC) lesions. However, the histological depth and subtype of tumors that respond to PDT

57 remain uncertain. Here, we report a clinical trial of high-dose oral Vitamin D (VD), used as a PDT

58 neoadjuvant for BCC. In this multi-institutional, intra-patient, randomized trial, 35 patients (9 with Gorlin

59 Syndrome) received three PDT sessions (20% ALA; 417 nm blue light) preceded by oral VD, placebo, or

60 no pretreatment. Tumors (122 BC) were monitored using 3D photography and computer-assisted

61 volumetric analysis. Values for absolute volume (3DAbsVol) and average height (3DAvHt) were

62 calculated and used to quantify tumor response kinetics. From histological sections, 3DAvHt was found

63 to correlate with actual tumor depth, although 3DAvHt is only ~10-20% of the latter. Importantly, 3DAvHt

64 measurements revealed a distinct depth threshold that predicts PDT responsiveness. Of 122 tumors

65 analyzed, 70% cleared after PDT; remaining tumors were micronodular or other aggressive histologic

66 subtypes. To evaluate VD's effects upon treatment response kinetics after PDT, only 40% of original

67 lesions were available for analysis. By stratifying remaining tumors by 3DAvHt, we found 65% of thin

68 tumors to be VD-responsive, whereas only 28% of thick tumors responded to VD. Overall, PDT was

69 effective for the majority of BCC lesions in our study. Tumors most likely to respond can be predicted

70 histologically and by noninvasive 3D morphometry. For PDT-appropriate BCC lesions, neoadjuvant oral

71 Vitamin D represents a safe and beneficial way to accelerate tumor resolution.

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78 **CLINICAL TRANSLATION STATEMENT**   (144/150 words)

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81 For photodynamic therapy (PDT) of basal cell carcinoma (BCC), a clinical challenge is deciding which

82 tumors to treat since the penetration depth of visible light into the skin is limited. In this clinical trial,

83 noninvasive 3D photography and computer analysis were used to determine the height and volume of

84 BCC tumors and to correlate these calculated parameters with tumor clearance after PDT, with or without

85 the use of oral vitamin D3 (VD) as a neoadjuvant. Two very practical findings emerged. First, tumor

86 height (3DAvHt) was found to correlate with BCC tumor depth and to predict therapeutic response;

87 tumors below a height threshold of 0.13 mm (~1.5 - 2 mm histological depth) were highly likely to

88 respond. Second, adding VD as a neoadjuvant prior to PDT of appropriate BCC tumors (superficial and

89 thin nodular subtypes) is a safe and effective way to boost PDT efficacy.

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93 **INTRODUCTION** (567 words)

94   Basal cell carcinomas (BCC), skin tumors which typically arise after decades of chronic sun

95 exposure, is the most common of all human cancers *(1,2)*. Although the current standard of care for BCC

96 is surgical excision, post-surgical scars can be very problematic, especially in cosmetically sensitive

97 areas and in patients with a high tumor burden. Examples of the latter are organ transplant recipients on

98 immunosuppressive drugs, and patients with Gorlin syndrome (a heritable condition in which patients

99 develop dozens-to-hundreds of BCC tumors beginning in adolescence)*(3,4)*. In Gorlin patients, multiple

100 surgeries over a lifetime are often associated with disfigurement, functional compromise, and even the

101 development of major depression with a high risk of suicide *(5)*.

102   Photodynamic therapy (PDT) is a non-scarring, noninvasive treatment modality for cancer in

103 which tumor-selective accumulation of a photosensitizer (PS), either 5-aminolevulinic acid (ALA) or its

104 methyl ester, is converted into protoporphyrin IX (PpIX) within mitochondria which is then activated using

105 strong visible light *(6-8)*. Although PDT can be used for different cancers at various locations in the body

106 (wherever laser light can be delivered using fiber optics). PDT is in fact most commonly used for skin

107 neoplasia because of the relative ease of shining a conventional light source (blue or red lamp or laser)

108 onto the skin *(9)*. Most European countries, Canada, and Australia have approved the use of PDT for

109 BCC treatment. PDT is known to work quite well for superficial BCC (sBCC), with 5-year clearance rates

110 (CR) between 71%-88% at 5 years post-treatment *(10,11)* but it is less reliable for nodular BCC (nBCC)

111 in which the CR can be considerably lower *(12)*. In general, a barrier to the more widespread use of PDT

112 for BCC is the fact that histological and tumor-size parameters associated with a good BCC response are

113 not yet sufficiently well-defined. In the United States, the FDA has not approved the use of PDT for

114 treating BCC.

115   To try to improve the overall response of nonmelanoma skin cancer to PDT, we have worked for

116 many years to identify agents that can be combined with PDT (as neoadjuvants) to improve lesion

117 clearance *(13)*. As one example, high-dose Vitamin D (VD) was shown to be a safe and effective

118 neoadjuvant with PDT that improves the resolution of cutaneous squamous cell carcinoma (SCC) and

119 actinic keratoses (pre-SCC) in murine models *(14)*, and more recently in human patients *(15,16)*. In the

120 case of BCC, pretreatment with VD increases ALA-mediated PpIX accumulation in a *ptch1* mutant

4

121   mouse model of BCC *(17,18)*. However, *ptch1*/p53 BCC mice are not a perfect model of human disease,

122   and therefore the question of how size and histological subtype of BCC influence PDT response in

123   patients has remained unanswered. Therefore, we designed a clinical study in patients with BCC to

124   systematically address the following questions: (1) How does the initial size of the BCC correlate with the

125   number of PDT treatments needed to clear it? Can a size threshold for responsiveness be defined? (2)

126   What is the relationship between tumor response after PDT and BCC histological subtype? (3) Does oral

127   VD, administered as a pretreatment prior to PDT, tend to accelerate or otherwise affect the rate of BCC

128   tumor shrinkage? For this study we developed a new high-precision 3D photographic method to

129   accurately monitor changes in the size of individual BCC tumors. This non-invasive analytic technique is

130   described in detail in the report, along with the results of the clinical trial.

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MATERIALS AND METHODS   (774 words)

Clinical trial design

This clinical trial was registered at ClinicalTrials.gov (NCT03483441) and conducted at two different sites, Cleveland Clinic (Cleveland, Ohio) and US Dermatology Partners (Phoenix, Arizona) under related but separate IRB approvals. For inclusion, all patients were required to have a minimum number of biopsy-proven BCC tumors (2 or 3, depending upon the trial location), and up to 10 lesions per patient could be studied. At Cleveland Clinic, patients with Gorlin syndrome (BCNS; clinically defined in *(3)*) were recruited. Two histologically proven BCC were required for enrollment; any lesion that persisted after 3 rounds of PDT was biopsied. At the Arizona site, primarily chronically sun-damaged patients were recruited; 3 biopsy-proven BCC tumors were required. Exclusion criteria included pregnancy, history of renal disease or porphyria, and treatment with Vismodegib or VD supplementation within the past month.

The primary aim of the clinical study was to test whether high dose oral VD (cholecalciferol; D3), when used in combination with PDT, will hasten the kinetic clearance rate of BCC tumors. The intrapatient comparative study design featured five study visits and three different VD pretreatment conditions (**Fig. 1**). Each patient served as his/her own control for evaluation of VD effects, and overall Vitamin D status was determined at each visit by measuring levels of serum 25-hydroxy vitamin D3 (calcidiol; 25-OH D3). Some blood samples were also used to obtain leukocytes for VD receptor (VDR) gene analysis. The correct content of active VD ingredient (D3) in study drug capsules was verified by laboratory testing (Heartland Assays, Ames, Iowa). The order of administration of agents (either high-dose D3 or placebo capsules) prior to PDT was randomized 1:1 at Visits 2 and 3 by our research pharmacy, as follows. One PDT session was preceded by high dose neoadjuvant D3, 10,000 international units [IU] daily, taken for either 5 or 14 days, depending upon whether the patient's initial serum 25-OH D3 level was normal or low, respectively. The other PDT session had no D3 pretreatment beforehand. Prior to the third PDT session (Visit 4), patients took D3 2,000 IU daily to achieve a normal serum 25-OH D3 level. Final assessments of lesion clearance were performed at 6 months (Visit 5). Tumors were photographed at every study visit using the 3D camera described below.

PDT treatments were administered as follows. A solution of 20% ALA (Levulan Kerastick™, Sun Pharmaceuticals) was applied to each tumor and a 5 mm rim of surrounding skin, covered with an occlusive

160   dressing, and 4 hours later illuminated with blue light (Blu-U lamp, 417 nm; 20 J/cm^2 ; Sun/DUSA) as

161   described *(19)*. Pain relief was provided using ice-cold cloths as needed. Patients were sent home with

162   instructions to avoid direct sun exposure for 48 h.
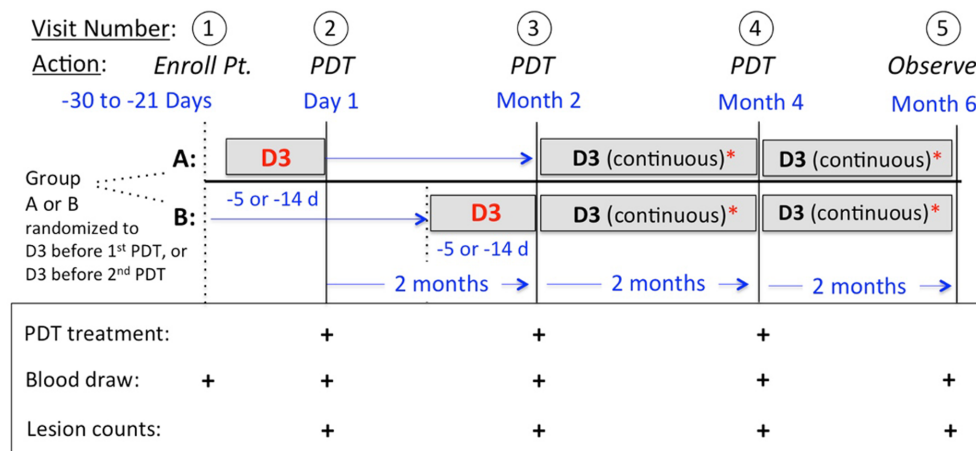
163

164   **Figure 1. Clinical trial protocol.** Patients received 10,000 units of high-dose oral cholecalciferol ("D3") prior to
165   only one of the first two PDT treatments. For those visits, the delivery of the neoadjuvant (either D3, or a placebo
166   capsule) was randomized as follows. Patients in Group A received D3 prior to PDT at Visit 2, whereas patients in
167   Group B received D3 prior to PDT at Visit 3. All patients then received D3 supplementation (2,000 units daily) prior
168   to their PDT at Visit 4. Any lesion not resolved at Visit 5 was biopsied.

169

170   Evaluation of BCC tumor volume and thickness using 3-D photographic quantitative analysis

171   Using a 3D camera (LifeViz Micro) and software (Dermapix) from Quantificare, Inc., pairs of digital

172   images of individual tumors were taken. These were processed to create 3-D mathematical reconstructions

173   from which a variety of tumor dimensions could be calculated. The most useful parameters for us were

174   *absolute volume* (3DAbsVol) and *average height* (3DAvHt). For example, 3DAbsVol was plotted against

175   time as a visual representation of tumor clearance kinetics. These methods are described in detail in

176   **Supplementary Methods 1** *(Supp1).*

177

178   Evaluation of BCC histologic subtype

179   For each biopsied lesion, the histological diagnosis was determined by a board-certified

180   dermatopathologist at the respective institutions. To analyze depth of BCC lesions, extra slides were recut

181   from the original paraffin blocks, stained with hematoxylin and eosin (H&E), and the maximum depth

182   (distance from epidermis to deepest aspect of tumor nests) was analyzed using QuPath software *(20).*

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184 <u>Evaluation of Vitamin D levels and Vitamin D receptor (VDR) status</u>

185   For all patients (including those from Arizona), serum 25-OH D3 analyses were performed in the

186 clinical pathology department at Cleveland Clinic. For 24 patients, DNA was extracted from blood

187 leukocytes to test genetic alleles of the VDR gene as previously described *(21)*.

188

189 <u>Study coordination and data management at multiple study sites</u>

190   To facilitate the secure and efficient transfer of patient information and data between the Cleveland

191 and Arizona sites, we collaborated with Medocity, Inc. (Parsippany, NJ), a company that develops online

192 tools for sharing of medical information. Medocity's customized clinical trial management website allowed

193 us to remotely share study patients' data (recruitment status, 3-D clinical photos, biopsy reports, and lab

194 results) in a safe and protected digital environment. Details about the Medocity platform are provided in

195 **Supplementary Methods 2** (*Supp2).*

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197 **RESULTS** (1,624 words)

198   Raw data from the clinical trial are provided in **Table 1** and **in Supplementary Table S1** (Supp3).

199 As described in Methods, patients were recruited at two different clinical sites, comprising 14 patients at

TABLE 1. Patient demographics, Vitamin D serum levels, and BCC tumor counts

A	B	C	D	E					F			G	H	I
		Age range (Years)	Gorlins	Serum Vitamin D level (ng/mL)					Yes/No answers to VD questions:			Total BCC eligible for 3D analysis	BCC that resolved with PDT	VD-responsive BCC / BCC available for analysis
Patient ID	Sex			Visit 1	Visit 2 (PDT1)	Visit 3 (PDT2)	Visit 4 (PDT3)	Visit 5	#1	#2	#3			
CLEVELAND STUDY PATIENTS														
1	F	66-70	yes	29.0	24.1	21.2	30.0	30.7	no	no	Y	6	4	3 of 4
2	F	76-80	--	30.2	29.5	32.4	37.8	35.3	Y	Y	Y	3	3	1 of 1
3	F	56-60	yes	17.6	25.6	24.7	26.6	27.8	Y	Y	Y	6	6	3 of 3
4	M	51-55	--	33.0	29.5	44.1	52.8	43.9	Y	Y	Y	0	n/a	(scar/keloids)
5	F	66-70	yes	55.8	47.9	37.2	32.4	41.7	Y	no	no	10	4	3 of 7
6	M	61-65	--	27.8	26.2	32.1	44.3	42.9	Y	Y	Y	5	4	2 of 2
7	M	66-70	--	38.0	49.3	61.2	60.0	45.1	Y	Y	Y	3	3	1 of 3
8	M	41-45	yes	14.6	21.5	35.5	19.3	20.8	Y	Y	Y	4	3	0 of 1
9	M	56-60	yes	14.2	24.2	18.5	28.1	29.4	Y	Y	Y	10	5	5 of 7
10	F	21-25	yes	33.8	32.5	40.9	43.9	48.8	Y	Y	Y	4	4	(all gone by V3)
12	M	66-70	--	15.4	33.3	26.3	29.5	32.7	Y	Y	Y	10	10	1 of 5
13	F	26-30	yes	13.5	31.4	34.1	31.0	36.9	no	Y	Y	0	n/a	(scar/keloids)
14	M	61-65	yes	25.1	30.4	23.0	31.8	32.3	Y	Y	Y	10	7	5 of 9
15	M	66-70	yes	18.2	22.9	45.6	48.4	nd	Y	Y	Y	10	1	2 of 9
ARIZONA STUDY PATIENTS														
26	M	61-65	--	26.5	35.2	30.5	37.9	50.1	Y	Y	Y	0	0	n/a
27	M	71-75	--	30.9	27.0	32.7	32.8	nd	Y	Y	Y	0	0	n/a
28	M	71-75	--	47.5	nd	33.6	37.3	45.5	n/a	no	no	1	1	n/a
29	M	61-65	--	40.9	40.2	31.5	43.7	44.6	no	no	Y	0	0	n/a
31	M	71-75	--	37.4	38.3	39.5	37.7	42.7	Y	Y	Y	1	1	n/a
33	M	61-65	--	29.0	33.9	23.5	29.0	32.9	Y	Y	Y	0	0	n/a
34	M	46-50	--	30.0	23.1	30.6	40.0	31.5	Y	Y	Y	1	1	n/a
35	F	56-60	--	95.5*	53.9	55.1	59.4	77.8	Y	no	no	2	2	n/a
36	M	71-75	--	31.6	41.2	34.2	34.5	34.3	Y	Y	Y	3	3	1 of 2
38	M	75-80	--	29.0	26.1	nd	35.3	32.5	n/a	n/a	Y	3	3	1 of 3
39	M	46-50	--	53.1	66.9	58.8	48.8	53.4	Y	Y	no	2	1	n/a
41	M	66-70	--	37.7	nd	nd	53.3	nd	n/a	n/a	Y	5	2	1 of 5
42	F	41-45	--	66.8	nd	78.5	nd	63.9	n/a	n/a	no	3	3	1 of 1
43	M	31-35	--	21.3	23.9	nd	23.8	26.5	n/a	Y	Y	2	1	1 of 1
44	F	51-55	--	--	48.4	nd	49.1	nd	n/a	n/a	n/a	0	0	n/a
45	M	46-50	--	46.1	41.3	nd	43.6	37.1	n/a	n/a	no	6	4	1 of 2
46	M	51-55	--	107.2*	59.0	nd	71.9	87.4	n/a	n/a	no	3	3	(gone by V3)
47	M	61-65	--	27.6	32.9	26.3	nd	nd	no	no	n/a	1	1	1 of 2
48	M	51-55	--	39.5	nd	32.6	37.9	44.3	n/a	n/a	Y	0	0	n/a
49	M	66-70	--	48.1	nd	45.7	nd	39.3	n/a	no	no	3	3	0 of 1
50	F	61-65	--	21.5	nd	27.1	30.6	32.4	n/a	n/a	Y	5	4	1 of 4
35 patients total (25 M, 10 F)				Patients evaluable for the particular VD question:					24	27	32	122 BCC		

Footnotes (KEY to each column):

A   Patient ID = study identification number.

B   Sex = male (M) or female (F).

C   Age at time of enrollment.

D   Gorlins = patient known to have Gorlins syndrome.

E   Serum VD levels = Blood levels of 25-hydroxy-vitamin D (25-OH D3) were measured at each study visit. *Color code is as follows:*

      Red font: Serum VD value abnormally low (< 31 ng/mL)

      Green box: Randomization assignment; patient received high dose VD (10,000 IU) in the week prior to visit.

      Yellow box: Randomization assignment; patient received placebo capsules in the week prior to visit.

F   Observations about serum VD levels are posed as answers to the following questions (Yes/No; or n/a if data not available)

      Question 1: Is the serum VD level observed after High-dose VD supplementation (at either V2 or V3) higher than that seen after Placebo?

      Question 2: Is the serum VD level observed after High-dose VD supplementation (at either V2 or V3) higher than that seen at Visit 1?

      Question 3: Is the serum VD level after medium-dose VD supplementation (at Visits V4 and V5) higher than that observed at Visit 1?

G   Evaluable BCC = number of BCC tumors that had data from all five study visits, usable for 3D analysis in time course studies.

H   PDT-responsive BCC; number of tumors that comletely cleared after 1, 2, or 3 PDT treatments

I   VD-responsive BCC = Number of tumors showing an increased response after high-dose VD, amongst tumors eligible for VD analysis.

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201   the Cleveland site (including 9 with Gorlin syndrome) and 22 patients with at the Arizona location. Of 36

202   total subjects enrolled, 35 completed the entire protocol; see enrollment flow chart in **Supplementary**

203   **Figure S1** (Supp4). Patients were generally older adults (median age, 62 years) with Fitzpatrick skin

204   types I – II and male gender (70%), except for two female Gorlin patients in their mid-twenties. **Table 1**

205   lists the demographic data and lesion counts for all patients. A total of 211 lesions were monitored during

206   the trial. Amongst these, 89 were later excluded due to a subsequent non-BCC diagnosis (SCC;

207   fibrosis/scar) or technical issues that precluded a complete analysis at all five study visits; see lesion

208   flowchart in **Supplementary Figure S2** (Supp 5). Thus, 122 lesions were ultimately available for a

209   complete analysis during the entire study time-course. All data for those 122 tumors and the original 211

210   lesions is provided in **Supplementary Table S1** (Supp3).

211   **<u>Approach and overall results from analysis of changes in BCC tumor size after PDT</u>**

212   As described in Methods, three-dimensional images (mathematical reconstructions) of tumors

213   from dual high-resolution photographs were used to determine 3DAbsVol and 3DAvHt for each tumor,

214   relative to the horizontal surface plane of the skin. Data were plotted, and kinetic rates of tumor

215   shrinkage were assessed from slopes of the volume-versus-time curve. Examples of typical 3D

216   photographic data are shown in **Figure 2**. Here, we first summarize the overall results. The large majority

217   of BCC lesions resolved after PDT. Specifically, 41% of tumors resolved by Visit 3, 58% by Visit 4, and

218   70% by Visit 5, leaving 30% of the tumors classified as PDT-resistant. In general, most BCC that

219   resolved after PDT were small, relatively thin tumors (**Fig. 2A and 2B**). Most were superficial BCC (**Fig.**

220   **2A**), but also a significant number were nodular BCC (**Fig. 2B**). Other tumors responded initially to PDT

221   (shrinking after one or more treatments) but then grew back (**Fig. 2C** and **2D**). As an important general

222   result, the data show that height really matters. Thus, the height (3D AvHt) of PDT-responsive lesions

223   was typically less than for PDT-resistant lesions, probably reflecting a relationship to depth of

224   photosensitizer and light penetration. However, exceptions to the apparent inverse correlation between

225   tumor height and PDT responsiveness are observed for PDT-resistant tumors with aggressive

226   histological subtypes, such as the one illustrated in **Fig 2E** which showed no inhibition in tumor growth

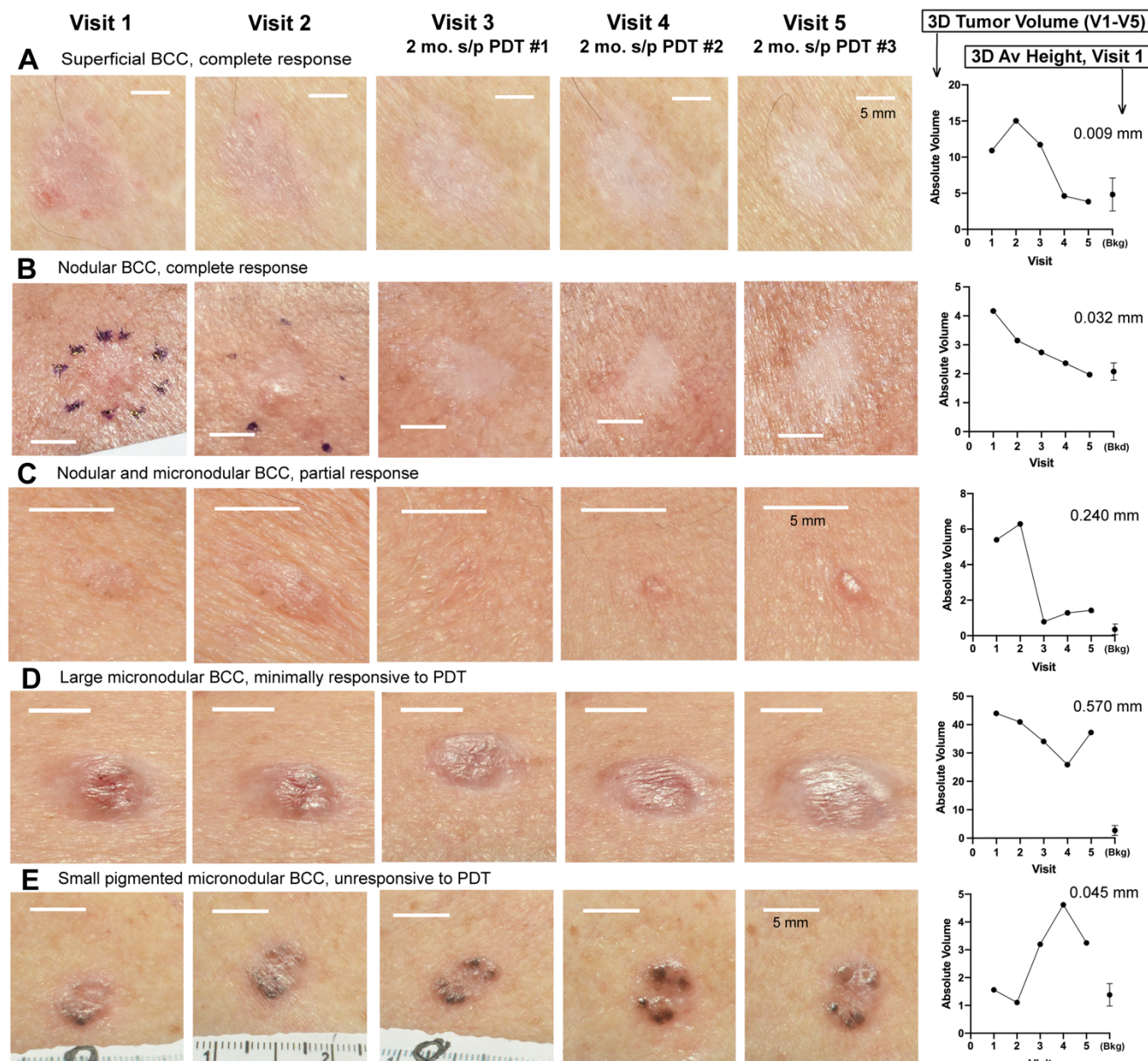227   rate after PDT, despite being relatively thin.

10

Figure 2. 3D reconstructed images of BCC tumors photographed at each of five study visits, to show typical appearances and corresponding changes in absolute tumor volume for each lesion calculated using the DermaPix software. Absolute Volume (in mm^3) of each tumor, over the time course of 5 study visits, along with the average background value (Bkg), is shown in the graphs. The 3D average height (in mm) of each tumor at Visit 1 is indicated at the upper right of the graphs. Scale bars, 5 mm.

Influence of BCC tumor size upon responsiveness to PDT

To more precisely define the influence of tumor volume and thickness upon therapeutic response, we

focused on two size parameters, *absolute volume* (3DAbsVol) and *3D average height* (3DAvHt). The

time course graphs constructed using 3DAbsVol are compiled in **Supplementary Table S1**. Evaluable

tumors were grouped into 4 categories, based upon their ultimate clearance status at Visit 5; see **Fig. 3**.
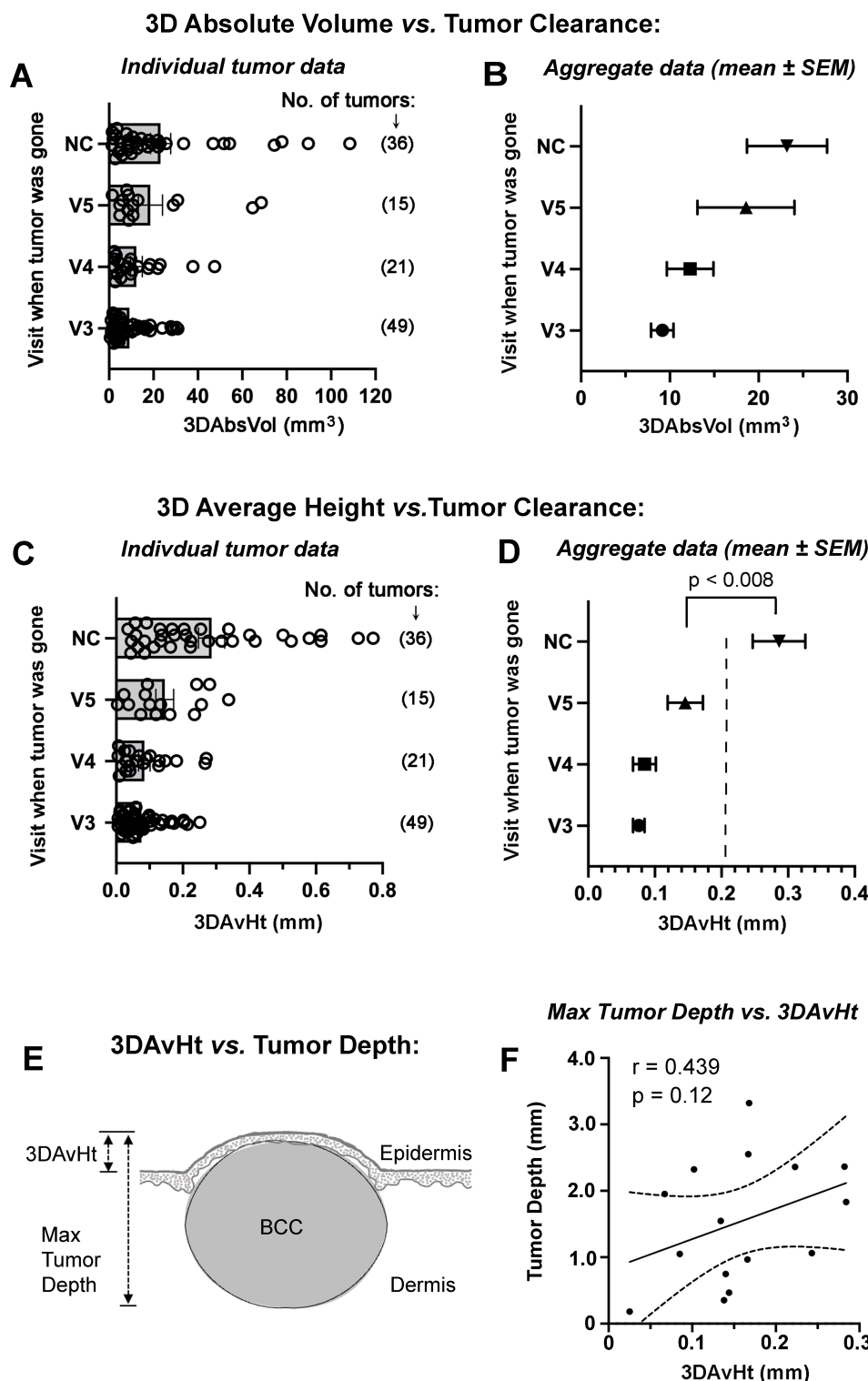
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Fig. 3. Correlation study between calculated size parameters from 3D analysis of BCC tumors, *versus* tumor clearance as indicated by disappearance of tumor at Visits V3, V4, or V5 (after 1, 2, or 3 PDT treatments, respectively). *NC*, tumors that failed to clear after 3 PDT treatments. (**A, B**), *3D Absolute Volume*, shown as data for individual tumors or in aggregate. (**C, D**) *3D Average height*, shown as data for individual tumors or in aggregate. *Dotted line* in panel *D* is apparent threshold of 3DAvHt. Beyond this threshold, BCC are unlikely to respond to PDT. The p-value from Student t-test is shown. (**E**) Schematic diagram showing the definitions of 3DAvHt and histological tumor depth. (**F**) Plot to show the modest correlation between 3DAvHt and histologic depth for a subset of tumors that were biopsied at the same visit; maximum depth of tumor nests was measured from digital images of H&E stained histological sections; depths are in millimeters.

249   Tumors that cleared completely on clinical exam were grouped by the visit of first disappearance (either

250   V3, V4, or V5). Tumors that persisted at V5 were labelled as *not cleared* ("NC"). Considering absolute

251   volume, a negative relationship between 3DAbsVol and number of PDT treatments to clear the tumor

252   was observed, as expected (**Fig. 3A, B**). However, 3DAbsVol did not provide the ability to unequivocally

253   distinguish between cleared versus persistent tumors. 3DAvHt, on the other hand, revealed large

254   differences between tumors that cleared after 1 - 3 PDT treatments and those that failed to clear (**Fig.**

255   **3C, D**). For 3DAvHt, differences between PDT-responsive and PDT-resistant groups were statistically

256   significant. Interestingly, a 3DAvHt value of ~0.2 mm appears to represent an operational threshold; i.e.,

257   tumors with values > 0.2 are unlikely to respond to PDT, whereas those below the threshold have a high

258   likelihood of response (**Fig. 3D**, dotted line).

259   To understand how 3DAvHt might relate to the true intradermal depth of the tumor (since 3DAvHt

260   is a calculated height and may or may not be related to actual histological depth of the tumor; see **Fig.**

261   **3E**), we performed the following analysis. For BCC tumors that were photographed immediately prior to

262   biopsy at V5, the 3DAvHt was compared to the depth of tumor nests observed in the corresponding H&E

263   stained specimen. A roughly linear correlation between 3DAvHt and maximum depth of tumor was

264   observed (**Fig. 3F**). Although 3DAvHt values were only ~10-20% of the actual tumor depth, a positive

265   correlation between the two parameters was seen, even when tumors were stratified by histological

266   subtype (**Supplementary Table S2**). Thus, we conclude that 3DAvHt represents a surrogate measure of

267   dermal depth for these BCC tumors.

268   **Influence of BCC histologic subtype upon responsiveness to PDT**

269   From an analysis of 36 tumors diagnosed by pre-treatment biopsy, we examined the relationship between

270   histological subtype and number of treatments required to achieve tumor clearance. Amongst these lesions,

271   100% of superficial BCCs cleared completely after 1 - 3 PDT sessions (**Fig. 4A**). For nodular BCC, many

272   failed to respond, yet a significant number (61%) did clear (**Fig. 4B**). For tumors that were biopsied because

273   they failed to clear (**Fig. 4C**), histological subtypes (e.g. micronodular; infiltrative) different from the usual

274   superficial and nodular variants (**Supplementary Table S2**) were predominantly found. The numerical

275   distribution ratios for tumors amongst the various histological subtypes, was 1: 6: 12: 3: 3: 1 for Superficial:

13

276 Nodular: Micronodular: Infiltrative: Trichoepithelial: Adenoid in PDT-resistant tumors (**Supplementary**

277 **Table S2**), versus 16 : 9 for sBCC : nBCC (with no rare histologic subtypes) amongst PDT-responsive

278 tumors (**Table 1**, Arizona patients). To ask whether there was any additional way to demonstrate that rare

279 BCC subtypes have more aggressive biological behavior, we hypothesized that repeated PDT treatments

280 tend to select for smaller, more aggressive tumors. This is because a PDT dose that would kill a typical

281 BCC fails to kill the aggressive tumor which continues to proliferate after PDT, despite being relatively thin

282 and accessible to drug and light. To test this idea, two groups of PDT-resistant BCC (10 nodular *versus* 15

283 aggressive histological subtypes) were compared to determine their initial sizes at Visit 1; see

284 **Supplementary Table S3 (Supp7**). For these tumors, the mean 3DAbsVol was 3-fold less for aggressive

285 subtypes compared to nodular BCC, and the mean 3D AvHt was 2-fold smaller. This supports the notion

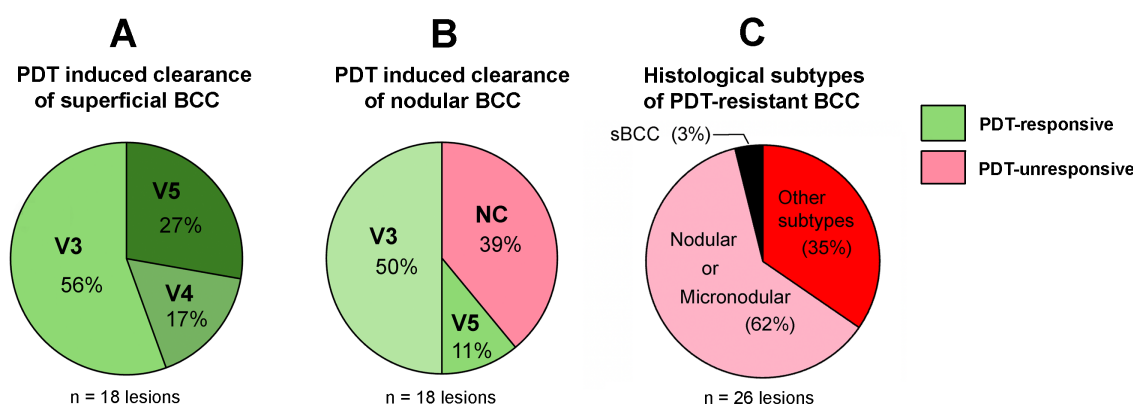286 that BCC with aggressive histologic subtypes can resist PDT even when relatively small in size.

287

288 **Fig. 4**. Percentage of tumors that cleared at visits V3, V4, or V5 (*green color*), or failed to respond (*red color*), within
289 categories of superficial (**A**) or nodular (**B**) BCC. Amongst PDT-resistant lesions biopsied at V5 to diagnose the
290 histology of the resistant tumor (**C**), 97% were relatively aggressive BCC subtypes and included a mixture of
291 nodular and micronodular, as well as other rare subtypes including adenoid, infiltrative, and trichoepitheliomatous
292 variants.
293

294 **Influence of Vitamin D upon BCC responsiveness to PDT**

295 One of the first observations in Table 1 is that baseline vitamin D status (25-O-D3 levels) were

296 different in the Cleveland and Arizona cohorts, i.e., 71% of Cleveland patients compared to 40% of

297 Arizona patients were Vitamin D deficient at Visit 1. This might be due to a relative lack of sunlight in

298 Cleveland vs. Phoenix, or to the fact that 65% of the Cleveland patients had Gorlin syndrome; the latter

299 is known to be associated with low serum VD levels, for unknown reasons *(22)*. Regardless, baseline 25-

300 OH-D3 levels did not seem to have any discernible effect upon PDT outcomes reported in our study.

14

301   In the trial, each patient took either a high-dose oral VD capsule, placebo capsule, or low-dose

302   VD capsule prior to each study visit as described in **Fig. 1**. To assess the effects on serum VD levels,

303   blood was sampled at each visit. As shown in **Table 1**, ~80% of patients, 25-OH D3 serum levels were

304   higher after taking high-dose VD, compared to the prior visit (at which no VD was taken). At their initial

305   study visit, 70% of the Cleveland patients and 40% of the Arizona patients were Vitamin D deficient per

306   our hospital's standard criteria. Those percentages dropped to 21% and 14%, respectively, by the end of

307   the trial, indicating excellent compliance by our participants when taking their assigned study drugs.

308   To assess our primary endpoint, we examined the kinetics of tumor regression after PDT with or

309   without high-dose VD pretreatment. A positive VD response was defined as a situation in which

310   neoadjuvant VD combined with PDT caused a faster rate of tumor shrinkage (downward sloping line

311   segment labeled "a" in **Fig. 5A** or **5B**, for example), when compared to the PDT session preceded only

312   by placebo (line segment labelled "b"). Conversely, an absence of VD response was determined if the

313   slope of "a" was the same or greater than the slope of "b" (**Figs. 5 C, D**). A major challenge here was the

314   requirement of having a visible tumor present at 4 study visits to perform a reliable VD kinetic analysis; in

315   fact, a majority of BCCs in the study disappeared before Visit 4. In addition, many tumors from the

316   Arizona dataset cleared more rapidly than we initially expected due to inflammatory effects from prior

317   biopsy (see Discussion). In total only 49 tumors in 11 patients were suitable for kinetic analysis. Amongst

318   the 11 patients, a majority of their tumors responded favorably to VD in 6 of 11 patients (**Supplementary**

319   **Table S1**, column G). At first glance, this is not very convincing for a meaningful VD effect. However,

320   realizing that a significant proportion of the 49 lesions were thick, non-PDT-responsive tumors that were

321   unlikely to yield any observable effect even with VD present, we performed a stratified analysis; tumors

322   were grouped into "thick" versus "thin" tumors based upon a 3DAvHt threshold (similar to **Fig. 3D**).

323   Results shown in **Supplementary Table S4** (Supp8) indicated that for thick tumors, only 28% were

324   responsive to VD compared to 72% that were nonresponsive. In contrast for thin tumors, 65% were

325   responsive to VD. This provides reasonable evidence that neoadjuvant VD may indeed be beneficial as

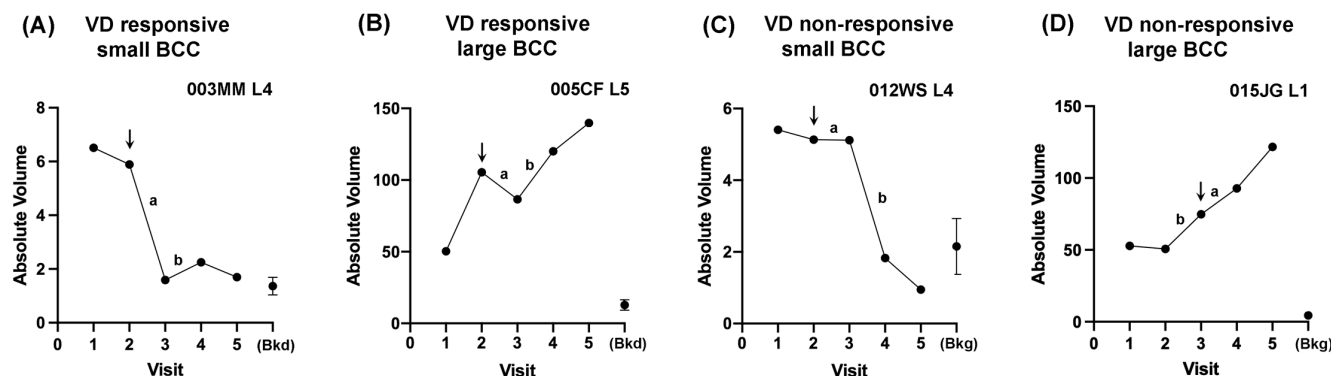326   an accelerant of PDT-induced clearance in thin BCC tumors.

Fig. 5. Examples of the changes in BCC tumor volume kinetics used to define Vitamin D-induced PDT responsiveness. *Arrows*, study visit at which patients received neoadjuvant high-dose VD prior to PDT. The slopes of the line segments labelled "a" (occurring after high-dose VD) or "b" (occurring after placebo) were compared; if segment "a" was smaller (more negative) than segment "b", then the tumor was scored as VD-responsive.

DISCUSSION: (1,932 words)

Adoption of PDT as an accepted treatment for BCC has been slow in the United States, due to continuing uncertainty about which tumors are likely to respond to PDT versus those likely to persist. In this clinical trial, we systematically examined 122 BCC tumors in 35 patients to ask how (1) tumor size, (2) histologic subtype, and (3) pretreatment with high-dose vitamin D might influence the therapeutic response to PDT. For each question, we report new findings and insights.

In terms of size, we show that tumor volumes and average heights calculated by 3D photographic morphometry can define a size threshold for PDT efficacy which discriminates between PDT-responsive and PDT-resistant tumors (**Fig. 3D**). Because this noninvasive technique provides a surrogate measure of actual tumor size, predicts therapeutic responsiveness, and is relatively easily to perform, it should be helpful in dermatology clinics to identify which BCC are appropriate candidates for PDT. The calculated parameter, 3DAvHt, is roughly correlated with the depth of BCC tumor nests below the surface, even while representing only ~10-20% of the actual tumor depth (**Fig. 3E**). The threshold value (3DAvHt = ~0.15 – 0.20 mm) (**Fig. 3D**) corresponds to an approximate tumor depth of 1.5 mm (**Fig. 3F**), which matches quite closely to results from a clinical study by Mosterd in 2008 that comparing PDT outcomes to BCC histological tumor depth *(23)*. In that study, PDT responses of 78 BCC tumors with a wide range of thickness values (0.30–3.10 mm) were categorized into two groups with widely different outcomes,

16

351   i.e., tumors < 1.3 mm thick had a risk of treatment failure of 15.5%, whereas tumors > 1.3 mm thick had a

352   risk of treatment failure of 42.2% % (P = 0.09). Their 1.3 mm depth threshold *(23)* and our estimated 1.5

353   mm threshold are very similar. Importantly, these values are consistent with European guidelines

354   recommending that PDT be performed only for BCC < 2 mm in depth *(24,25)*. Our new 3D photographic

355   method represents an objective, noninvasive way of judging whether a BCC tumor falls within these

356   established clinical guidelines.

357   The recommendation of 2 mm as a depth threshold originated with a pioneering study by Morton

358   of 53 BCC tumors which demonstrated that thickness significantly affected PDT response, with no

359   response in 4 patients whose tumors exceeded 2 mm in thickness *(26)*. Subsequently, most PDT clinical

360   trials have focused on patients with BCC tumors < 2 mm thick, primarily superficial BCC but some with

361   nodular BCC as well *(27)*. As a result, many clinicians who perform PDT have learned to visually

362   estimate tumor depth with a surprising degree of accuracy; but the ability to correctly predict the

363   histological BCC subtype is relatively poor, i.e., only 72% in a recent large study *(28)*. Surface diameter

364   can be indicative of BCC depth, but only for large tumors. In 235 biopsies of relatively large nodular BCC

365   (mean surface diameter 13.0 ± 8 mm), Takenouchi et al. found that horizontal surface diameter

366   correlated with and was the strongest predictor of tumor depth, followed by histologic subtype *(29)*. Our

367   new 3D morphometry technique permits a relative depth evaluation of any BCC tumor, whether large or

368   small, which could be quite helpful for PDT screening purposes. That said, any BCC tumor that is large

369   or has clinically suspicious features should be histologically evaluated before PDT.

370   An important and fascinating question is how 3DAvHt is able to predict PDT responses when

371   most of the tumor lies below the surface. Here, we need to consider the concept of "phodynamic

372   priming", in which only a portion of a tumor needs to be damaged by PDT to trigger local events

373   (expression of DAMPs, alteration of tumor stroma, stimulation of innate immune cells) that eventually

374   lead to activation of systemic anti-tumor immunity *(30-33)*. Light penetration into the skin is controlled by

375   the laws of physics, and therefore photodynamic efficiency of light at various depths within the skin can

376   be modeled and predicted *(34)*. Combining this idea with the concept of photodynamic priming, we

377   postulate that 3DAvHt (a parameter proportionately related to histological tumor depth) is useful because

378   it correlates with the light penetration depth required to successfully induce photodynamic priming.

17

379 The above discussion about tumor thickness should not obscure the fact that BCC histological

380 subtype is an overriding determinant of PDT responsiveness. Four BCC growth patterns are thought to

381 have prognostic significance post-treatment: superficial, nodular, micronodular, and infiltrative *(35)*. In a

382 study of BCC skin biopsies by Welsch et al. *(36)*, superficial (23%) and nodular (59%) subtypes were

383 most prevalent, while micronodular (13%) and infiltrative (5%) subtypes were less common. This

384 compares well with our data in which the distribution of subtypes was superficial (31%), nodular (44%),

385 micronodular (15%), infiltrative (5%), and other (7%). The rank order of mean tumor depths in the Welsch

386 study *(36)* was reported as micronodular (2.01 mm) > infiltrative (1.82 mm) > nodular (1.68 mm) >

387 superficial (0.71 mm), very similar to the rank order in our patients: micronodular (1.26 mm) = infiltrative

388 (1.56 mm) > nodular (0.77 mm) > superficial (0.13 mm). Micronodular and infiltrative subtypes are often

389 reported as the thickest tumors and the most likely to recur, and indeed these were the deepest and most

390 frequent histologic subtypes in our dataset (**Supplemental Table S2, Graph A**).

391 Now, let us consider how BCC histological subtype affects response to PDT. The most extensive

392 studies have been done with sBCC, and show tumor clearance rates of 95%-100% between 3 months

393 and 1 year *(12,37,38)*, falling to 76%-88% at 5 years *(10,11)*. Overall results with nBCC are somewhat

394 worse *(39,40)*. Thus, in terms of pre-selection of BCC lesions for PDT, the best responses will be

395 achieved with sBCC tumors but excellent results can also be obtained for nBCC if tumors with 3DAvHt <

396 0.15 mm are selected. On the other hand, micronodular, infiltrative, adenoid, or trichoepitheliomatous

397 BCC are best treated with surgery.

398 In our study, we compared results from BCC that were biopsy proven versus those that had not

399 undergone biopsy. Amongst 70 biopsy-proven lesions in the Arizona patient cohort, 53% showed no

400 evidence of clinical disease by Visit 2 (before any PDT), whereas patients without any pre-enrollment

401 biopsy showed no lesion disappearance at Visit 2; see **Supplementary Table S5** (Supp 9). This finding

402 suggests the possibility of an anti-tumor immune response triggered after surgical biopsy. Previous

403 studies have shown that over 20% of tumors undergoing Mohs surgery reveal no remaining tumor on

404 intraoperative examination *(41)*. Conversely, in patients presenting with no clinically apparent BCC tumor

405 after biopsy, residual tumor may still be found in over 80 % of cases *(42)*. To ask whether rates of tumor

406 clearance after PDT were faster in patients with a pre-enrollment biopsy, the percentages of tumor

18

407 clearance in two groups (biopsy versus no biopsy) were compared (**Supplementary Table S5**).

408 However, no evidence for acceleration in tumor clearance after PDT at Visits 3, 4, and 5 in the biopsy

409 group was found, suggesting that inflammatory or immunological effects that promote tumor clearance

410 post-surgery must be relatively short-lived.

411 The third question addressed by this trial was whether Vitamin D modifies the therapeutic effects

412 of PDT. The scientific rationale for this idea is strong. Preclinical work in mice with SCC *(14,18)* and

413 recent clinical trials in patients with squamous pre-cancers demonstrated that neoadjuvant VD has a

414 significant positive effect upon PDT response *(15,16,43)*. Originally, we attributed this to the fact that VD

415 promotes selective accumulation of PpIX within tumor cells, driving the improved therapeutic response

416 *(14,18)*. However, recent evidence in a mouse model of UV-induced squamous precancer revealed

417 significant involvement of the immune system, with striking increases in neutrophils, macrophages, and

418 T-cells into neoplastic lesions within the first few days after PDT *(44)*; this immunocyte recruitment was

419 greatly enhanced by pretreatment with VD *(45)*. We anticipate that similar mechanisms are at play in

420 BCC, but this remains to be investigated. However, results of the current clinical trial demonstrate that

421 neoadjuvant VD does indeed hasten lesion shrinkage rates for sBCC, and for nBCC tumors thin enough

422 to respond to PDT (although VD does not convert highly PDT-resistant tumors into PDT-sensitive ones).

423 Given the excellent safety profile of vitamin D3, there appears to be a clear potential benefit and no

424 downside of using oral VD together with PDT for BCC.

425 Most effects of VD are mediated by the Vitamin D receptor (VDR), which can be affected by

426 single-nucleotide polymorphisms (SNPs) within the VDR gene. In a previous study, we had demonstrated

427 a strong association between two of these SNPs (Fok1 and Poly-A) alleles and the development of

428 actinic keratoses and squamous cell carcinoma, and also between the SNPs and 25-OH-D3 serum

429 levels *(21)*. Specifically, the presence of Poly-A homozygous short alleles (SS) and Fok1 homozygous

430 dominant alleles (FF) were associated with high levels of serum 25-OH-D3 *(21)*. In the current work on

431 BCC, we were intrigued to find a possible involvement of the Poly-A (SS) and Fok1 (FF) alleles in tumor

432 clearance. Patients with 100% tumor clearance in **Supplementary Table S6** (Supp 10) featured primarily

433 a Poly-A (SS) or a Fok1 (FF) genotype, whereas the heterozygous variants had a less complete

434   therapeutic response. This observation, while still preliminary due to the small sample size, is

435   nonetheless interesting because VDR proteins encoded by the Poly-A (SS) and/or Fok1 (FF) gene

436   variants are thought to have a higher-than-average functional activity in cell-based reporter assays

437   designed to measure transcriptional activity of the VDR (discussed in *(21)*). Exactly how different levels

438   of VDR expression might influence BCC growth and involution rates after PDT remains an open question

439   but it may involve effects on the immune system as well as on the tumor itself.

440   This clinical trial had several limitations. First, the number of patients available for analysis of

441   vitamin D effects was limited, because for comparative kinetics it was necessary to have a visible BCC

442   tumor to observe at 4 or more consecutive visits. During trial planning, we had not anticipated how well

443   PDT would work; 58% of BCC lesions disappeared by Visit 4, leaving only 42% of lesions to analyze.

444   Another limitation was the strong effect of pre-enrollment biopsy upon BCC tumor behavior, such that

445   ~21% of lesions were already gone by Visit 2 (although the effect is compatible with previous studies,

446   see above). A third issue was that the trial design, as a practical necessity, did not require all lesions to

447   be biopsy-proven. Consequently, of the 211 lesions chosen for monitoring, ~20% were clinically

448   misdiagnosed and later proved to be either scars, keloids, or SCC.

449   In summary, the following conclusions can be drawn from this study: *First*, our discovery of a

450   noninvasive way to measure tumor depth, which reflects actual histological depth and predicts tumor

451   responsiveness to PDT, provides a new tool to help reduce uncertainty when choosing which BCC

452   lesions to treat with PDT. *Second*, our data confirm that proper histological categorization is critical to

453   PDT response. Thin nodular BCC tumors respond quite well, but aggressive subtypes (micronodular,

454   infiltrative, adenoid, or trichoepitheliomatous) do not respond to PDT and are best treated with surgery.

455   The combination of histological assessment to exclude aggressive BCC subtypes, combined with 3D

456   photographic screening for appropriate thickness (3DAvHt < 0.2 mm), should make it possible to

457   personalize PDT treatment and maximize favorable results. *Third,* addition of neoadjuvant oral VD is a

458   safe and effective technique to augment PDT-induced therapeutic responses. *Finally,* it is our hope that

459   this report will stimulate optimism about the prospects of using PDT for basal cell carcinoma by making

460   physicians aware that good results are achievable when tumor thickness and subtype are taken into

461   account.

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