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# Pigment genes not skin pigmentation affect UVB-induced vitamin D†

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Skin pigmentation is believed to contribute to the generally low serum 25-hydroxyvitamin D (25(OH)D) concentrations observed in darker-skinned persons. The influence of measured skin pigmentation on UVB-induced 25(OH)D increase was investigated together with 9 demographic and 13 genetic parameters (pigment SNPs). Forty participants representing a wide range in measured skin pigmentation were exposed to identical UVB doses on identical body areas over nine weeks with weekly measurements of serum 25(OH)D. This study took place in Denmark during winter, a period with negligible ambient UVB, so variation in 25(OH)D synthesis was not influenced by latitude, season, sun and clothing habits. The increase in 25(OH)D concentration displayed considerable variation (range: 2.9 to 139 nmol $L^{-1}$). Constitutive and facultative skin pigmentation exerted separate influence on the variation of the UVB-induced linear 25(OH)D increase. However, this influence was statistically non-significant in the presence of separate significant pigment SNPs. The variation in the 25(OH)D increase in the combined linear model was not explained by measured skin pigmentation but by sex, height, age and seven SNPs located in the *ASIP, MTAP, MIR196A29* and Solute Carrier Family genes. This linear model including individual intercepts and the 10 parameters influencing the slope explained 77.4% of the variation. This study confirmed the influence of sex, age and height on 25(OH)D increase and found that pigment genes provided a better relation to UVB-induced 25(OH)D increase compared to the actual measured skin pigmentation. Therefore, only investigating skin pigmentation obscures other causal parameters for low 25(OH)D.

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## 1. Introduction

The short wavelengths in terrestrial sunlight (UV type B, UVB) are absorbed by the previtamin $D_3$ precursor 7-dehydrocholesterol (7-DHC) in human skin leading to the photo-conversion of 7-DHC to previtamin $D_3$. This is the initial process of a pathway ultimately leading to the formation of the bioactive form of vitamin D, $1,25(OH)_2D_3$. Melanin (pigment) in skin absorbs UVB and is thought to reduce the photo-conversion of 7-DHC.[1] Darker-skinned immigrant populations residing in countries located at high latitudes have been reported more frequently with deficient (<25 nmol $l^{-1}$) serum 25-hydroxyvitamin D (25(OH)D) compared to the native and light-skinned inhabitants.[2–13] Moreover, several intervention studies have demonstrated significantly lower 25(OH)D response to artificial UV in darker-skinned persons.[14–17] Consequently, it is widely believed that skin pigmentation has an important influence on UVB-induced conversion of 7-DHC to previtamin $D_3$. However, studies on darker-skinned populations in their native countries, with more intense sunlight, revealed a high incidence of inadequate 25(OH)D levels too.[6] Furthermore, other intervention studies have failed to demonstrate an influence of skin pigmentation on serum 25(OH)D response to UVB.[18–22] Consistent with this, we have previously found no influence of measured skin pigmentation in a short-term UVB study during winter, when melanin is predominantly located in the deepest layer of the epidermis, stratum basale.[23] After summer, the upper epidermis contains more melanin, which might result in a pronounced reduction in converted 7-DHC.[24] In our previous work, the UVB-induced 25(OH)D synthesis was found to be influenced by their inborn (constitutive) skin pigmentation and not their sun-induced and UVB-induced (facultative) skin pigmentation.[25] This could suggest a possible, underlying genetic background. As this previous study represented a narrow range of skin pigmentation, it was not optimally designed to

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clarify the influence of skin pigmentation on 25(OH)D synthesis.

In the present study, the main purpose was to investigate the influence of skin pigmentation on the UVB-induced 25(OH)D response after long-term and identical UVB doses on summer pigmented skin. Furthermore, the influence of demographic parameters and genetic parameters (pigment SNPs) on the UVB-induced 25(OH)D response was also investigated. The study took place during autumn/winter, a season where ambient UVB in Denmark does not induce a significant 25(OH)D increase.[26–28]

## 2. Results

### 2.1. Compliance

Forty out of the 43 participants included, all residents in Denmark, completed the study, as three participants dropped out for personal reasons. All completing participants had a 100% treatment and 25(OH)D sampling compliance.

### 2.2. Demographic characteristics

Demographic data are shown in Table 1. Individual facultative and constitutive skin pigmentation were objectively measured as pigment protection factor (PPF) assessed with a skin reflectance meter (measuring range of 1 to 25). Individual facultative PPF ranged from 5.3 to 19 and individual constitutive PPF ranged from 2.4 to 21. Thus, both measures represented a wide range. Facultative PPF was significantly ($P = 1.7 \times 10^{-8}$) higher (*i.e.* darker) than constitutive PPF. Constitutive PPF ($P = 0.059$) and facultative PPF ($P = 0.81$) did not change significantly after the non-erythemogenic UVB treatment with UV6 lamps (F85/100W, broadband UVB: 290–365 nm).

### 2.3. Individual variation in the UVB-induced 25(OH)D response

At study-end, the mean increase in 25(OH)D was 51 nmol $l^{-1}$ with a range of 2.9 to 139 nmol $l^{-1}$ (Table 1) and thus displayed considerable individual variation despite the identical UVB exposure. The individual variation in 25(OH)D at first and final sample for all participants is shown in Fig. 1. The mean 25(OH)D start level was 67 nmol $l^{-1}$ (range 10–120 nmol $l^{-1}$) and the mean 25(OH)D end level was 118 nmol $l^{-1}$ (range 73–216 nmol $l^{-1}$, Table 1). The increase in 25(OH)D over time was best described by a linear model including individual intercepts (*i.e.* measured 25(OH)D start level) and was individually significant in 38 out of 40 participants. The slope of the 25(OH)D increase was not influence by 25(OH)D start level ($P = 0.20$) The mean weekly 25(OH)D levels and UVB doses are shown in Table S1.† The course of the UVB-induced 25(OH)D increase over time is shown Fig. 2.

Table 1 Demographic data

| Parameters | *N*/mean ± SD (range) |
|---|---|
| Participants | 40 |
| Sex (women/men) | 21/19 |
| Age, years | 42 ± 8.9 (22–62) |
| Weight, kg | 76 ± 11 (50–100) |
| Height, cm | 171 ± 9.0 (153–188) |
| BMI, kg $m^{-2}$ | 26 ± 3.2 (21–36) |
| Body surface area, $m^2$ | 1.9 ± 0.18 (1.5–2.3) |
| Fatty fish meals per week[a] | 2.0 ± 1.7 (0–7.2) |
| Constitutive PPF[b] | 6.0 ± 3.7 (2.4–21) |
| Facultative PPF[b] | 8.0 ± 2.6 (5.3–19) |
| 25(OH)D start level, nmol $l^{-1}$ | 67 ± 27 (10–120) |
| 25(OH)D end level, nmol $l^{-1}$ | 118 ± 32 (73–216) |
| Absolute increase in 25(OH)D, nmol $l^{-1}$ | 51 ± 26 (2.9–139) |

[a] Maximally 14 meals per week. [b] Pigment protection factor (PPF) is an objective measurement of skin pigmentation with a measurement range of 1–25.

### 2.4. Explainable part of the variation in the 25(OH)D increase

A general linear model (GLM) with common slope and common intercept explained 22.5% (Table 2) of the total observed individual variation. When the common intercept was substituted with individual intercepts (*i.e.* measured 25(OH)D start levels), the explainable individual variation of the 25(OH)D increase was improved to 62.9% (Table 2). The maximal explainable variation was assessed with the GLM including individual slopes as well as individual intercepts,

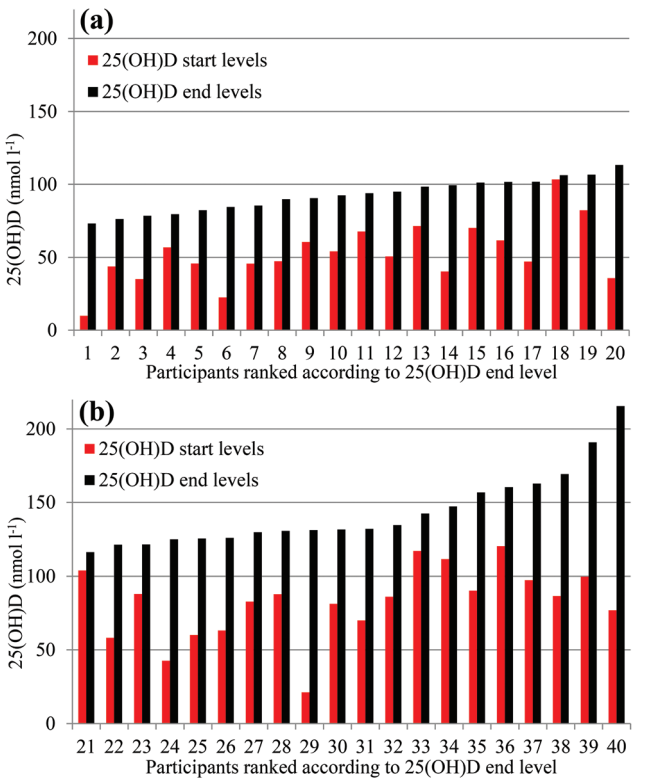


Fig. 1 Individual 25(OH)D start levels (red bars) and end levels (black bars) after nine weeks of UVB treatment in (a) the 20 participants with lowest 25(OH)D end levels and (b) the 20 participants with highest 25(OH)D end levels. Participant numbers are shown on the *x*-axis. Each participant is represented on the *x*-axis and ranked according to their 25(OH)D end levels.

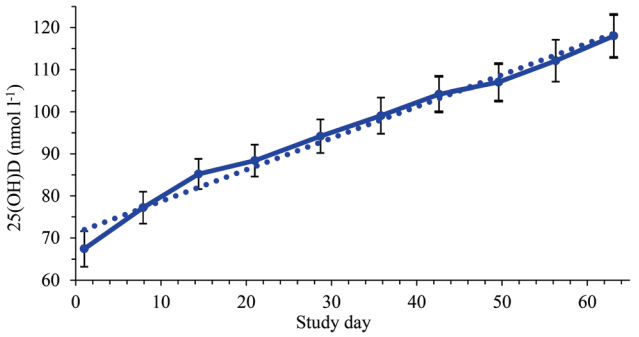


**Fig. 2** Mean 25(OH)D levels (*y*-axis, blue curve) ± 1 standard error of the mean (SEM) over time in study days (*x*-axis). The blue dotted line represents a linear fit line. All participants received identical UVB doses over nine weeks corresponding to a total of 26.2 kJ $m^{-2}$ (56 SED). Significant (*P*-value < 0.05) increase in 25(OH)D occurred after first week.

**Table 2** The explainable part of the variation in UVB-induced increase of 25(OH)D assessed by general linear models

| Intercept | Slope | $R^2$ | *P*-value |
|---|---|---|---|
| Common | Common | 0.225 | $8.4 \times 10^{-24}$ |
| Individual | Common | 0.629 | $2.7 \times 10^{-86}$ |
| Individual | Individual | 0.919 | $3.5 \times 10^{-170}$ |

The measured 25(OH)D start level was used as individual intercept.

increasing the explainable variation to 91.9% (Table 2). Thus, the individual variation of the slope constituted 29.0% (the difference between 91.9% and 62.9%) of the total observed variation. The background for the variation of the slope was assessed by investigating the influence of different parameters on the slope in the GLM including individual intercepts. This analysis was investigated in three steps. In Step 1 and Step 2, demographic and pigment SNPs were selected for a combined analysis in Step 3 (Fig. 3).

### 2.5. Step 1: Selection of demographic parameters for combined analysis

Nine demographic parameters were investigated separately for their influence on the slope (Table S2 in the ESI†). We have previously found a strong influence of height on 25(OH)D increase.[25] Apart from height, body mass index (BMI), body surface area (BSA) and weight was also investigated as separate parameters.

Eight parameters (facultative PPF, constitutive PPF, sex, age, height, BMI, BSA and intake of fatty fish) had separate, significant influence on the variation of the slope (Table S2†). Facultative PPF explained 2.6% (65.5%–62.9%, Table S2†) and constitutive PPF explained 1.9% (64.8%–62.9%, Table S2†) of the variation in the 25(OH)D increase as separate parameters without considering interaction.

Height was the strongest influential ($R^2$-value) parameter of the three parameters, BMI, BSA and height and was selected for further analysis. Height, facultative PPF, constitutive PPF, sex, age, and intake of fatty fish were deployed in a combined GLM. After stepwise backward elimination according to *P*-value (<0.05) and power (>0.750) was performed. Facultative PPF, sex, age, height and intake of fatty fish remained to influence the slope of the 25(OH)D increase significantly ($P = 7.6 \times 10^{-101}$, $R^2 = 0.709$, Table 3, Step 1). These five demographic parameters were selected for further analysis in Step 3.

### 2.6. Step 2: Selection of pigment SNPs (genetics) for combined analysis

Thirty-one single nucleotide polymorphisms (SNPs) located in genes with possible influence on pigmentation were analysed in all participants (Table S3, ESI†). After retaining at least five participants in each SNP allele subgroup and excluding SNPs with no allele dose effect or dominant allele effect, 13 SNPs displayed separate significant influence on the variation of the slope (Table S3, ESI†). These 13 SNPs were deployed in a combined GLM for stepwise backward elimination according to *P*-value (<0.05) and power (>0.750). In the combined GLM, the individual influence of the seven SNPs (rs4911414, rs4911442, rs28777, rs16891982, rs6475555, rs12896399 and rs11614913) on the slope remained significant ($P = 1.2 \times 10^{-111}$, $R^2 = 0.738$, Table 3, Step 2). These seven SNPs were located in the Agouti signalling protein gene (*ASIP*, rs4911414 and rs4911442), Solute Carrier Family 24, member 4 gene (*SLC24A4*, rs12896399), Solute Carrier Family 45, member 2 gene (*SCL45A2*, rs28777 and rs16891982) and *MIR196A29* gene (rs11614913) and were selected for further analysis in Step 3 (Table 3).

### 2.7. Step 3: Combined influence of demographic and pigment SNPs on the 25(OH)D increase

In Step 3, the influence of the five significant demographic parameters (Table 3, Step 1) and the seven significant pigment SNPs parameters (Table 3, Step 2) on the slope of the 25(OH)D increase was investigated in a combined analysis. All selected parameters were deployed in a combined GLM for backward and stepwise elimination according to *P*-value (<0.05) and power (>0.750). Subsequently, the GLM included 10 parameters; sex, height, age, rs4911414, rs4911442, rs6475555, rs28777, rs16891982, rs12896399 and rs11614913. In the combined GLM, the individual influence of these 10 parameters on the slope remained significant ($P = 5.2 \times 10^{-116}$, $R^2 = 0.774$) and the model explained 77.4% of the variation.

### 2.8. Relations between pigment SNPs (genetics) and skin pigmentation

Facultative PPF and constitutive PPF exerted separate influence on the slope of the 25(OH)D increase. But, this influence was not significant in the presence of pigment SNPs. Thus, the pigment SNPs provided an improved explanation of the variation in the UVB-induced 25(OH)D response compared to the objectively skin pigmentation measures. Therefore, the relation between the seven SNPs included in the final GLM and PPF was investigated. Out of the seven final SNPs, three SNPs (rs28777, rs16891982 and rs11614913) had separate sig-

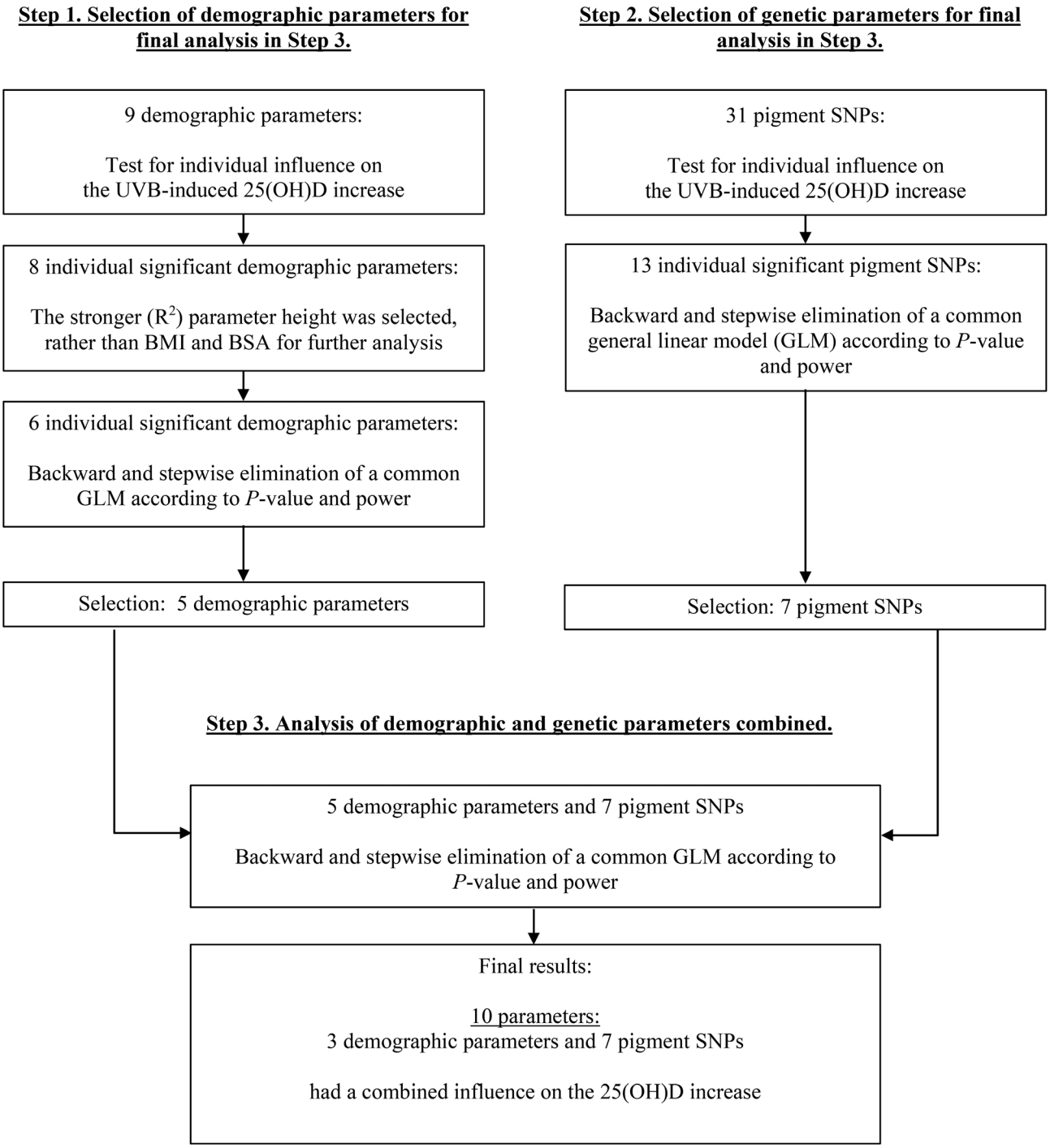


**Fig. 3** Flowchart of the analysis process.

nificant influence ($P < 0.05$, Table S4 ESI†) on facultative and constitutive PPF. In combined analysis only rs28777 remained to influence the variation of facultative PPF ($P = 7.2 \times 10^{-8}$) and constitutive PPF ($P = 7.5 \times 10^{-9}$). Rs28777 explained 58.9% and 63.6% of the variation in facultative PPF and constitutive PPF, respectively. The relation between PPF and all investigated SNPs included in the final GLM as well as the remaining investigated SNPs are shown in Table S4.†

## 3. Discussion

In early autumn, the skin pigmentation of the body areas, exposed to sun during summer, is usually maximal.[29–31] The epidermal turnover time for healthy persons is between 40–56 days.[32,33] Therefore, pigmentation is most likely located throughout the upper layers of the epidermis during the study period. This study was therefore designed to maximize the

 

Table 3 Parameters influencing the variation in the 25(OH)D increase

| Parameter | *P*-Value | Power | Range/category | Weekly change (95% CI) in nmol $l^{-1}$ |
|---|---|---|---|---|
| **Step 1. Selection of demographic parameters** | | | | |
| Age | $2.9 \times 10^{-7}$ | 0.999 | | |
| Height | $4.7 \times 10^{-7}$ | 0.999 | | |
| Facultative PPF | $1.5 \times 10^{-5}$ | 0.992 | | |
| Fatty fish meals per week | $9.6 \times 10^{-5}$ | 0.976 | | |
| Sex | $5.4 \times 10^{-4}$ | 0.797 | | |
| **Step 2. Selection of pigment SNPs** | | | | |
| rs4911414, *ASIP* | $8.6 \times 10^{-13}$ | 1.000 | | |
| rs12896399, *SCL24A4* | $4.9 \times 10^{-11}$ | 1.000 | | |
| rs6475555, *MTAP* | $2.8 \times 10^{-6}$ | 0.997 | | |
| rs4911442, *ASIP* | $4.8 \times 10^{-4}$ | 0.940 | | |
| rs11614913, *MIR196A29* | $5.4 \times 10^{-4}$ | 0.935 | | |
| rs16891982, *SCL45A2* | $7.6 \times 10^{-4}$ | 0.923 | | |
| Rs28777, *SCL45A2* | $5.4 \times 10^{-3}$ | 0.797 | | |
| **Step 3. Analysis of demographic and pigment SNPs combined** | | | | |
| Age, years | $1.2 \times 10^{-12}$ | 1.000 | 22–62 | −5.6 (−7.3 to −4.2) |
| rs4911414, *ASIP* | $1.5 \times 10^{-11}$ | 1.000 | TT + GT/GG[a] | −2.4 (−3.1 to −1.8) |
| rs4911442, *ASIP* | $1.8 \times 10^{-6}$ | 0.998 | AG/AA[a] | 2.0 (1.2–2.8) |
| rs12896399, *SCL24A4* | $4.3 \times 10^{-5}$ | 0.985 | TT + GT/GG[a] | 1.6 (0.83–2.3) |
| Height, cm | $2.6 \times 10^{-4}$ | 0.957 | 153–188 | 4.4 (2.0–6.6) |
| rs6475555, *MTAP* | $3.1 \times 10^{-4}$ | 0.953 | AA + AG/GG[a] | 1.2 (0.56–1.9) |
| rs16891982, *SCL45A2* | $4.1 \times 10^{-4}$ | 0.945 | CC/GC + GG[a] | 3.5 (1.6–5.5) |
| Sex | $1.1 \times 10^{-3}$ | 0.905 | Female/male[a] | 1.8 (0.72–2.9) |
| Rs28777, *SCL45A2* | $1.8 \times 10^{-3}$ | 0.945 | CC/CA/AA[a] | −5.2 (−7.2 to −3.1)<br>−2.1 (−3.0 to −1.3) |
| rs11614913, *MIR196A29* | $1.9 \times 10^{-3}$ | 0.878 | TT + CT/CC[a] | −1.1 (−1.9 to −0.42) |
| Mean change in common course | | | | 5.3 (4.6–5.9) |

In Step 1 separate significant demographic parameters (Table S1, ESI) that combined remained to display significant influence on the slope of the 25(OH)D increase in a general linear model (GLM) including individual intercepts (measured) after backward and stepwise elimination according to *P*-value (<0.05) and power (>0.750) were selected for further analysis. In Step 2, separate significant pigment SNPs (Table S3, ESI) were selected in a similar way as in Step 1. In Step 3, the selected five demographic (Step 1) and seven pigment SNPs (Step 2) were deployed in a combined GLM. After backward and stepwise elimination according to *P*-value (<0.05) and power (>0.750), the final GLM included 10 parameters with significant influence on the slope ($R^2 = 0.774$, $P = 5.2 \times 10^{-116}$, Power = 1.000). $R^2$ is squared correlation coefficient. Power is the probability of confirming the given result in a new material with similar size and uncertainties as this material. [a] Represents the parameter category used as reference for estimating the influence of the other categories.

potential influence of skin pigmentation. Even though we took care to avoid erythema, the cumulative UVB dose was relatively high and similar to what an individual may be expected to receive during the summer months in Denmark.[34]

By conducting this study in one centre (same latitude) and during a season with negligible ambient UVB-induced 25(OH)D[26–28] this study was minimally influenced by ambient UVB exposure and variations in this. By using a UV cabinet, the UVB exposure doses as well as the exposed body areas was identical.

The influence of measured skin pigmentation (facultative and constitutive PPF) was investigated rather than the subjective assessment of Fitzpatrick Skin Type. It is preferable to use variables with individual values instead of categorical variables when possible. In addition, PPF is stronger related to UVR sensitivity than Fitzpatrick Skin Type and[35] PPF is significantly related to the content of melanin in epidermal biopsies.[36] The range of both facultative and constitutive PFF largely covered the PPF measuring range.

Both facultative PPF and constitutive PPF displayed separate influence on the 25(OH)D synthesis and explained a minor part of the variation. However, in the final combined analysis, the influence of both facultative and constitutive PPF on the slope of the 25(OH)D increase was superseded by pigment SNPs. Genes have information of the ability to tan and the constitutive and inborn skin pigmentation. However, facultative skin pigmentation is also a product of lifelong and recent solar exposure. This information is not encoded in the genes.[37] Apparently, the pigment SNPs also have an effect on 25(OH)D synthesis that seems to be different from that of the actual content of melanin in skin. Actually, one of the influential SNPs were located in the MATP gene related to eye colour. Rs28777 was strongly correlated to facultative PPF ($R^2 = 0.589$) and explained 58.9% of the variation in facultative PPF. However, rs28777 did not have an equally strong influence on the variation in the 25(OH)D increase. The GLM without rs28777 had an $R^2$-value that was 0.005 (0.5%) lower compared to the final GLM with rs28777 (Step 3 final GLM with and without rs28777: $R^2 = 0.774$ and $R^2 = 0.769$, respectively). In reality, several genes associated to the pigmentation pathway also have other functions.[38] In this context, it is interesting that the conclusion of this study would have indicated an influence of skin pigmentation, confirming the findings by several other previous studies, if we had not chosen to

broaden this investigation with pigment SNPs.[14–17] Thus, according to our findings focus on skin pigmentation alone may obscure the finding of other causal parameters.

After long-term exposure to relatively high UVB doses the individual variation in 25(OH)D end levels were wide and corresponded to a difference of 3-fold between the lowest and the highest end level (73 nmol $l^{-1}$ and 216 nmol $l^{-1}$). Likewise, the 25(OH)D increase ranged from 2.9 nmol $l^{-1}$ to 139 nmol $l^{-1}$. However, the mean 25(OH)D start level was relatively high as the study started in early autumn. This may be reflected in the limited increase in especially two participants (participant no. 18 in Fig. 1, 2.9 nmol $l^{-1}$ and no. 21, 12.4 nmol $l^{-1}$). This could suggest that these participants nearly had reached an upper maximal 25(OH)D level at study start limiting their response to UVB. Interestingly, these two participants did not by far have high-end 25(OH)D levels at study end compared to the other participants, indicating a substantial variation in the upper maximal 25(OH)D level as well. This is consistent with our previous findings.[25]

The GLM including measured 25(OH)D start levels explained 62.9% of the variation in the UVB-induced 25(OH)D increase. The variation of the slope was responsible for an additional 29.0% of the total variation. The parameters, age, height, sex and seven pigment SNPs combined explained 14.5% out of the possible 29% of the variation. Thus, an additional 14.5% of the variation due to the slope remains to be explained by the influence of parameters presently not investigated. In total, the final GLM explained 77.4% out of the variation.

Height was positively correlated to 25(OH)D increase and age was negatively correlated. Both parameters have previously been shown to influence 25(OH)D levels.[39,40] Sex also influenced the slope of the 25(OH)D increase and may be related to the phenomenon that women generally have a higher percentage of body fat[41] where excess 25(OH)D may to some extent be stored.[42] BMI did not in the combined model influence the variation of the 25(OH)D increase significantly. As the high-end and low-end of the BMI categories were not represented in this study group, this could be an explanation for this.

Human pigmentation is a polygenic trait. The SNP polymorphism investigated presently are located in genes that previously have been associated with pigmentation.[43–47] As we investigated only a modest selection of SNPs, it is most likely that additional genetic parameters influencing the slope of the 25(OH)D increase through other mechanisms remain to be identified.

It could be argued that genetic parameters can be determined with higher precision than skin pigmentation (PPF) measured with a reflectance-meter. To maximize the accuracy of the assessment method, PPF at each location was based on measurements performed at three different time points. Facultative PPF was based on average measurements at five different sites. More importantly, a precise and complete selection of the pigment SNPs could not be investigated either, as the polygenetic background for inborn skin pigmentation is not yet entirely resolved. Only a selection of SNPs located in genes with possible influence on skin pigmentation based on literature was available for analysis.

The UVB-induced 25(OH)D increase over time was best described by a linear model after non-linear and increasing UVB doses. This indicates a non-linear dose–response relationship in accordance with the findings by others.[48–50]

There were no significant changes in facultative PPF during study course after UV irradiation with UV6 tubes. Constitutive skin was not exposed to UV and constitutive PPF did not change. Normally, a decrease in facultative PPF during winter would be expected in Denmark.[51,52] But, it is possible that the artificial UV radiation may have prevented this during the study.

We intentionally presented raw *P*-values as this enables the reader to correct for multiple testing according to their own preferences. The most frequent used Bonferroni method is a simple but conservative method to correct for multiple testing. Our results allow correction for at least 27 tests (rs28777: $P = 1.8 \times 10^{-3}$) while six demographic and 13 pigment SNPs (a total of 19 parameters) were tested for combined influence. Thus, such a correction would not have changed the final results.

The LC-MS/MS method used in this study, could not distinguish the 25-hydroxy-3-epi3-vitamin $D_3$ (3-*epi*-25(OH)D) form from 25(OH)D and may therefore potentially overestimate the 25(OH)D levels.[53] In a large Swedish study, 3-*epi*-25(OH)D was detected in 7.7% of the participants with a mean concentration of 8.3 nmol $l^{-1}$ and a detection rate inversely associated with age.[54] The mean relative contribution of 3-*epi*-25(OH)D of total 25(OH)D was 11.8%. Therefore, the impact of 3-*epi*-25(OH)D is most likely low.

In conclusion, polymorphisms in pigment SNPs were better related to UVB-induced 25(OH)D increase than actual measured skin pigmentation. Therefore, only investigating skin pigmentation may obscure other causal parameters for low 25(OH)D.

# 4. Materials and methods

## 4.1. Study design

This single-centre, open and non-blinded clinical trial was conducted at Bispebjerg Hospital, University of Copenhagen, Denmark (55.7°N) from 1st October to 22st December in 2010. During this period the mean ambient UVR (UVA and UVB) was 2.2 SED per day and the mean outdoor temperature was 2.6° Celsius. The solar exposed body areas are therefore limited to faces and sometimes hands. Between October and in the middle of April, ambient UVB do not induce significant increase in 25(OH)D as a decline in 25(OH)D occurs instead.[26–28] For this reason, a non-irradiated (*i.e.* placebo) group was not included.

## 4.2. Participants

Forty-three healthy participants took part in this study. They represented a wide range of skin pigmentation (PPF) and had

 

ancestors originating from a number of countries (Denmark, Faroe Islands, United Kingdom, Pakistan, India, Palestine, Turkey, Afghanistan, Ukraine, Russia, Morocco, Yugoslavia, the Philippines, Gambia, Sri Lanka and Lebanon). All participants were residents in Denmark. The UVB-induced increase in 25(OH)D in 22 of these participants have previously been described.[25]

The inclusion criteria were: (1) age 18–70 and (2) Danish residents. The exclusion criteria were (1) supplementary vitamin D intake exceeding 10 μg per day one month prior to study start; (2) use of supplementary vitamin D during study period; (3) sun holiday south of latitude 45°N one month prior to or during the study period; (4) use of solarium one month prior to or during the study period; (5) chronic disease; (6) skin disease; (7) intake of cholesterol-lowering or photosensitising medication; (8) pregnancy; (9) drug addiction; (10) psychiatric disorder; (11) physical disabilities.

The number of daily consumed fatty fish meals was recorded in a questionnaire. Vitamin D fortified food is not available in Denmark.

Written, informed consent was obtained from all participants. Study protocol (H-2-2010-097) was approved by the Committees for Biomedical Research Ethics for the Capital Region in Denmark and completed in accordance with the Declaration of Helsinki.

### 4.3. Skin pigmentation

Objective measurement of skin pigmentation (pigment protection factor, PPF) was performed non-invasively with a skin reflectance meter (UV-Optimize Scientific, Chromo-light, Vedbaek, Denmark).[55] PPF is a measure of melanin in the skin as the content of eumelanin and pheomelanin in epidermal biopsies is significantly and linearly related to measured PPF.[35,35,36,36,36,55,56,56] The PPF number corresponds to the number of SED that is needed to elicit a just perceptible erythema *i.e.* minimal erythema dose (MED).[57] One SED is defined as 10 mJ $cm^{-2}$ at 298 nm according to the CIE erythema action spectrum and will elicit a just perceptible erythema in the most sensitive person in a group of persons with very fair skin.[58]

There is a highly significant correlation between clinical measured MED and PPF.[59] The measuring range of PPF is 1 to 25, meaning that a UVB dose between 1 standard erythema dose (SED) and 25 SED is needed to elicit a just perceptible erythema. To limit the influence of possible measured outlier values arising from improper measurement technique, the median value of the three consecutive PPF measurements were used. In the following, PPF is therefore used as a measure of skin pigmentation and referred to as such. The constitutive PPF was measured on the buttocks, a body area not normally exposed to UVB. The facultative PPF was assessed as a mean of measurements on the chest, midriff, back of shoulder, and the medial and lateral sides of the arm. These are body areas influenced by prior lifelong and recent solar exposure.[37] Both constitutive PPF and facultative PPF were measured at study start, after $4\frac{1}{2}$ weeks, and at study end and did not change significantly ($P > 0.05$) during the study. Therefore, mean values of the three time-points for facultative and constitutive PPF were used for further investigation to maximise measurement accuracy.

### 4.4. Intervention/UVR treatment

UVR cabinets (Waldmann, Willingen-Schwenningen, Germany) with 26 UV6 tubes were used. The light spectrum covered the vitamin D action spectrum (Fig. S1 in ESI†). Ninety % (295–365 nm) of the previtamin $D_3$ weighed emission spectrum of the UV6 lamp is present in daylight during a summer day in Denmark. During UV irradiation, the participants wore a UV protective helmet covering head/face and underwear covering buttocks. Mosteller's formula was used to calculate the UV exposed BSA,[60] Thus, approximately 80% of the participants' body area was exposed.[61]

All participants received identical UVB doses gradually increasing over nine weeks from October to December. UVB treatments, each of 0.94 kJ $m^{-2}$ (2 SEDs), were administered bi-weekly during the first two weeks. In the following five weeks, the frequency was increased to three sessions per week. During the last two weeks, 3 weekly sessions, each of 1.4 kJ $m^{-2}$ (3 SEDs), were administered. The total UVB dose during the nine weeks was 26 kJ $m^{-2}$ (56 SEDs). Irradiation time was determined and regulated by measuring UV intensity with a Sola-Hazard spectroradiometer (Solatell, Cornwall, UK) at study start, after five weeks and at the end of the exposure period. The lowest facultative PPF measured on summer pigmented skin in this group of participants was 5.3, meaning that a dose of 5.3 SED is needed to cause a just perceptible erythema in this particular participant. As the dose per session was maximally 3 SED and as only facultative skin was irradiated, the UVB irradiation did not cause erythema in any participants. Ambient UVB radiation and solar-exposed body areas are negligible at this time of the year.[26–28] Long-term UVB treatment in non-erythemogenic doses do not increase skin pigmentation (PPF).[25]

### 4.5. Blood analysis

**4.5.1. 25(OH)D.** Serum 25(OH)D was used as a parameter of vitamin D status and analysed on a liquid chromatography tandem mass spectrometer (LC-MS/MS).[18,26] To minimise analysis variance, at least triplet analyses (technical replicates) were performed and all 25(OH)D samples from the same participant were analysed in one batch. The total relative standard deviation (SD) varied between 4.9% at 20 nmol $l^{-1}$ and 14.1% at 222 nmol $l^{-1}$ reflecting experimental variability. Serum 25(OH)D was measured at study start, and from this point weekly (400 samples) and two days after the last UVB treatment since 25(OH)D production is sustained for around two to three days after UVB exposure.[62]

**4.5.2. Pigment SNP genotyping.** The DNA purification method is described by others.[25] Thirty-one SNPs were genotyped. Detailed method is described in the ESI.† The SNPs were placed in pigmentation-associated loci. SNPs with genotype subgroups containing less than five participants were

merged with other allele-sharing subgroups (*e.g.* genotype AA with AG and not with GG). Heterozygote subgroups with less than five participants were merged with an allele-sharing subgroup displaying non-significant ($P > 0.05$) difference in influence on the slope of the 25(OH)D increase. SNPs with no allele dose effect (an allele that is associated with the absolute outcome measure) or dominant allele effect on 25(OH)D were excluded. For SNPs with dominant allele effect (an allele that will produce a certain phenotype, even in the presence of other alleles), subgroups with no significant differences in influence on the slope of the 25(OH)D increase were merged according to allele sharing.

### 4.6. Statistics

Individual data were tested with the Kolmogorov–Smirnov test to assess whether the data were normally distributed. The residuals of the 25(OH)D measurements, constitutive PPF and facultative PPF were normally distributed. The parameters, age, weight, height, BMI, BSA, intake of fatty fish, were normally distributed. Comparisons of continuous and normally distributed parameters at different time points were tested with paired Students *t*-test. Linear regression (general linear models, GLMs) analysis was used for assessing relations between SNPs and PPF. All tests were 2-tailed.

To describe the increase in 25(OH)D over time, the following models were investigated: linear, inverse, quadratic, cubic, power, sigmoid and exponential. The derivate function was defined as the average daily change in 25(OH)D between two sample time-points (Δ25(OH)D per day). The linear model was determined to provide the best suitable model based on: (1) the $R^2$ value for each model, (2) the accordance between the investigations of the 25(OH)D increase over time (individual- and group-based) and (3) the accordance between the investigations of the 25(OH)D increase over time the derivate function.

The inter-individual variations in the linear increase of 25 (OH)D were explored by comparing GLMs with: (1) common intercept and common slope, (2) individual intercepts (*i.e.* measured) and common slope and (3) individual intercepts and individual slope (*i.e.* an individual constant). The separate influence of the investigated parameters on the variation in the slope of the 25(OH)D increase was examined in a GLM including individual intercepts. The influence of separate significant parameters was not independent and therefore subsequently investigated by a stepwise backward elimination of a combined GLM according to *P*-value (<0.05) and power (>0.750).[63]

Data were statistically analysed using SPSS 24.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant.

**4.6.1. Sample size calculation.** In our previous study an influence of measured constitutive skin pigmentation (PPF) was found.[26] The range of constitutive PPF was 2.0–6.0 (span: 4) and resulted in a lower 25(OH)D increase corresponding to 3.2 nmol $l^{-1}$ per week. The expected difference over nine weeks is therefore calculated to be 28.8 nmol $l^{-1}$. To detect an additional difference of 28.8 nmol $l^{-1}$ with a standard deviation (SD) of 29 nmol $l^{-1}$ for high-end 25(OH)D samples, a significance level of 5% and a power of 80%, additional 17 participants were required. Due to the relatively long study period, 21 extra participants were included allowing five drop-outs. Sample size calculation was performed using the program: "*Power and Sample Size Calculation version 3.1.2*" available online on http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize.

**4.6.2. Hypothesis.** We hypothesised that skin pigmentation (measured as PPF) and/or polymorphisms in pigment genes influence UVB-induced 25(OH)D increase significantly in combination with other influential parameters.

## Conflicts of interest

None of the authors reported any conflict of interest related to this study.

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## References

1 P. Meredith and J. Riesz, Radiative relaxation quantum yields for synthetic eumelanin, *Photochem. Photobiol.*, 2004, **79**, 211–216.
2 C. Brot, P. Vestergaard, N. Kolthoff, J. Gram, A. P. Hermann and O. H. Sorensen, Vitamin D status and its adequacy in healthy Danish perimenopausal women: relationships to dietary intake, sun exposure and serum parathyroid hormone, *Br. J. Nutr.*, 2001, **86**(Suppl 1), S97–S103.
3 R. Andersen, C. Molgaard, L. T. Skovgaard, C. Brot, K. D. Cashman, J. Jakobsen, C. Lamberg-Allardt and L. Ovesen, Pakistani immigrant children and adults in Denmark have severely low vitamin D status, *Eur. J. Clin. Nutr.*, 2008, **62**, 625–634.
4 M. Z. Erkal, J. Wilde, Y. Bilgin, A. Akinci, E. Demir, R. H. Bodeker, M. Mann, R. G. Bretzel, H. Stracke and M. F. Holick, High prevalence of vitamin D deficiency, secondary hyperparathyroidism and generalized bone pain in Turkish immigrants in Germany: identification of risk factors, *Osteoporosis Int.*, 2006, **17**, 1133–1140.
5 P. Lips, Worldwide status of vitamin D nutrition, *J. Steroid Biochem. Mol. Biol.*, 2010, **121**, 297–300.
6 A. Prentice, G. R. Goldberg and I. Schoenmakers, Vitamin D across the lifecycle: physiology and biomarkers, *Am. J. Clin. Nutr.*, 2008, **88**, 500S–506S.

 

7 L. Ovesen, R. Andersen and J. Jakobsen, Geographical differences in vitamin D status, with particular reference to European countries, *Proc. Nutr. Soc.*, 2003, **62**, 813–821.
8 P. Lips and R. T. de Jongh, Vitamin D deficiency in immigrants, *Bone Rep.*, 2018, **9**, 37–41.
9 A. R. Eggemoen, K. V. Knutsen, I. Dalen and A. K. Jenum, Vitamin D status in recently arrived immigrants from Africa and Asia: a cross-sectional study from Norway of children, adolescents and adults, *BMJ Open*, 2013, **3**, 1–8.
10 S. A. Jadoon, A. Ahmed and M. A. Alam, Vitamin D Deficiency In Pakistan: Tip Of Iceberg, *J. Ayub. Med. Coll. Abbottabad*, 2018, **30**, 78–80.
11 R. Mandlik, N. Kajale, V. Ekbote, V. Patwardhan, V. Khadilkar, S. Chiplonkar and A. Khadilkar, Determinants of Vitamin D Status in Indian School-children, *Indian J. Endocrinol. Metab.*, 2018, **22**, 244–248.
12 R. E. Man, L. J. Li, C. Y. Cheng, T. Y. Wong, E. Lamoureux and C. Sabanayagam, Prevalence and Determinants of Suboptimal Vitamin D Levels in a Multiethnic Asian Population, *Nutrients*, 2017, **9**, 1–12.
13 R. Bachhel, N. R. Singh and J. S. Sidhu, Prevalence of vitamin D deficiency in north-west Punjab population: A cross-sectional study, *Int. J. Appl. Basic Med. Res.*, 2015, **5**, 7–11.
14 L. A. Armas, S. Dowell, M. Akhter, S. Duthuluru, C. Huerter, B. W. Hollis, R. Lund and R. P. Heaney, Ultraviolet-B radiation increases serum 25-hydroxyvitamin D levels: the effect of UVB dose and skin color, *J. Am. Acad. Dermatol.*, 2007, **57**, 588–593.
15 T. C. Chen, F. Chimeh, Z. Lu, J. Mathieu, K. S. Person, A. Zhang, N. Kohn, S. Martinello, R. Berkowitz and M. F. Holick, Factors that influence the cutaneous synthesis and dietary sources of vitamin D, *Arch. Biochem. Biophys.*, 2007, **460**, 213–217.
16 T. L. Clemens, J. S. Adams, S. L. Henderson and M. F. Holick, Increased skin pigment reduces the capacity of skin to synthesise vitamin D3, *Lancet*, 1982, **1**, 74–76.
17 L. Y. Matsuoka, J. Wortsman, J. G. Haddad, P. Kolm and B. W. Hollis, Racial pigmentation and the cutaneous synthesis of vitamin D, *Arch. Dermatol.*, 1991, **127**, 536–538.
18 M. K. Bogh, A. V. Schmedes, P. A. Philipsen, E. Thieden and H. C. Wulf, Vitamin D production after UVB exposure depends on baseline vitamin D and total cholesterol but not on skin pigmentation, *J. Invest. Dermatol.*, 2010, **26**, 546–553.
19 W. F. Brazerol, A. J. McPhee, F. Mimouni, B. L. Specker and R. C. Tsang, Serial ultraviolet B exposure and serum 25 hydroxyvitamin D response in young adult American blacks and whites: no racial differences, *J. Am. Coll. Nutr.*, 1988, **7**, 111–118.
20 M. F. Holick, J. A. MacLaughlin and S. H. Doppelt, Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator, *Science*, 1981, **211**, 590–593.
21 C. W. Lo, P. W. Paris and M. F. Holick, Indian and Pakistani immigrants have the same capacity as Caucasians to produce vitamin D in response to ultraviolet irradiation, *Am. J. Clin. Nutr.*, 1986, **44**, 683–685.
22 T. C. Stamp, Factors in human vitamin D nutrition and in the production and cure of classical rickets, *Proc. Nutr. Soc.*, 1975, **34**, 119–130.
23 D. Fajuyigbe, S. M. Lwin, B. L. Diffey, R. Baker, D. J. Tobin, R. P. E. Sarkany and A. R. Young, Melanin distribution in human epidermis affords localized protection against DNA photodamage and concurs with skin cancer incidence difference in extreme phototypes, *FASEB J.*, 2018, 1–7.
24 L. O. Bjorn, Vitamin D synthesis may be independent of skin pigmentation only with UV of short wavelength, *J. Invest. Dermatol.*, 2010, **130**, 2848–2850.
25 P. Datta, P. A. Philipsen, P. Olsen, B. Petersen, P. Johansen, N. Morling and H. C. Wulf, Major inter-personal variation in the increase and maximal level of 25-hydroxy vitamin D induced by UVB, *Photochem. Photobiol. Sci.*, 2016, **15**, 536–545.
26 P. Datta, M. K. Bogh, P. Olsen, P. Eriksen, A. V. Schmedes, M. M. Grage, P. A. Philipsen and H. C. Wulf, Increase in serum 25-hydroxyvitamin-D(3) in humans after solar exposure under natural conditions compared to artificial UVB exposure of hands and face, *Photochem. Photobiol. Sci.*, 2012, **11**, 1817–1824.
27 P. Datta, P. A. Philipsen, P. Olsen, M. K. Bogh, P. Johansen, A. V. Schmedes, N. Morling and H. C. Wulf, The half-life of 25(OH)D after UVB exposure depends on gender and vitamin D receptor polymorphism but mainly on the start level, *Photochem. Photobiol. Sci.*, 2017, **16**, 985–995.
28 A. R. Webb, L. Kline and M. F. Holick, Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin, *J. Clin. Endocrinol. Metab.*, 1988, **67**, 373–378.
29 C. Galzote, R. Estanislao, M. O. Suero, A. Khaiat, M. I. Mangubat, R. Moideen, H. Tagami and X. Wang, Characterization of facial skin of various Asian populations through visual and non-invasive instrumental evaluations: influence of seasons, *Skin Res. Technol.*, 2014, **20**, 453–462.
30 K. Togsverd-Bo, P. A. Philipsen, M. Haedersdal and H. C. O. Wulf, Skin autofluorescence reflects individual seasonal UV exposure, skin photodamage and skin cancer development in organ transplant recipients, *J. Photochem. Photobiol., B*, 2018, **178**, 577–583.
31 C. M. Sawicki, M. I. Van Rompay, L. E. Au, C. M. Gordon and J. M. Sacheck, Sun-Exposed Skin Color Is Associated with Changes in Serum 25-Hydroxyvitamin D in Racially/Ethnically Diverse Children, *J. Nutr.*, 2016, **146**, 751–757.
32 H. Iizuka, Epidermal turnover time, *J. Dermatol. Sci.*, 1994, **8**, 215–217.
33 K. M. Halprin, Epidermal "turnover time"–a re-examination, *Br. J. Dermatol.*, 1972, **86**, 14–19.
34 E. Thieden, P. A. Philipsen and H. C. Wulf, Ultraviolet radiation exposure pattern in winter compared with summer based on time-stamped personal dosimeter readings, *Br. J. Dermatol.*, 2006, **154**, 133–138.

35 H. C. Wulf, P. A. Philipsen and M. H. Ravnbak, Minimal erythema dose and minimal melanogenesis dose relate better to objectively measured skin type than to Fitzpatricks skin type, *Photodermatol., Photoimmunol. Photomed.*, 2010, **26**, 280–284.

36 B. Kongshoj, A. Thorleifsson and H. C. Wulf, Pheomelanin and eumelanin in human skin determined by high-performance liquid chromatography and its relation to in vivo reflectance measurements, *Photodermatol., Photoimmunol. Photomed.*, 2006, **22**, 141–147.

37 G. Bieliauskiene, P. A. Philipsen and H. C. Wulf, Phototype reproducibility and relation to objectively measured skin sensitivity is best when burn and tan reactivity to sun are answered separately, *Photodermatol., Photoimmunol. Photomed.*, 2018, **4**, 233–236.

38 L. Yi and S. Kaler, ATP7A trafficking and mechanisms underlying the distal motor neuropathy induced by mutations in ATP7A, *Ann. N. Y. Acad. Sci.*, 2014, **1314**, 49–54.

39 J. MacLaughlin and M. F. Holick, Aging decreases the capacity of human skin to produce vitamin D3, *J. Clin. Invest.*, 1985, **76**, 1536–1538.

40 K. Pazaitou-Panayiotou, P. D. Papapetrou, A. Chrisoulidou, S. Konstantinidou, E. Doumala, E. Georgiou, V. Panagiotou, E. Sotiriadou, E. Mavroudi and M. Apostolaki-Christopoulou, Height, whole Body Surface Area, gender, working outdoors, and sunbathing in previous summer are important determinants of serum 25-hydroxyvitamin D levels, *Exp. Clin. Endocrinol. Diabetes*, 2012, **120**, 14–22.

41 B. F. Palmer and D. J. Clegg, The sexual dimorphism of obesity, *Mol. Cell. Endocrinol.*, 2015, **402**, 113–119.

42 R. P. Heaney, R. R. Recker, J. Grote, R. L. Horst and L. A. Armas, Vitamin D(3) is more potent than vitamin D(2) in humans, *J. Clin. Endocrinol. Metab.*, 2011, **96**, E447–E452.

43 S. I. Candille, D. M. Absher, S. Beleza, M. Bauchet, B. McEvoy, N. A. Garrison, J. Z. Li, R. M. Myers, G. S. Barsh, H. Tang and M. D. Shriver, Genome-wide association studies of quantitatively measured skin, hair, and eye pigmentation in four European populations, *PLoS One*, 2012, **7**, 1–12.

44 H. Nan, P. Kraft, D. J. Hunter and J. Han, Genetic variants in pigmentation genes, pigmentary phenotypes, and risk of skin cancer in Caucasians, *Int. J. Cancer*, 2009, **125**, 909–917.

45 R. A. Sturm, R. D. Teasdale and N. F. Box, Human pigmentation genes: identification, structure and consequences of polymorphic variation, *Gene*, 2001, **277**, 49–62.

46 P. Sulem, D. F. Gudbjartsson, S. N. Stacey, A. Helgason, T. Rafnar, K. P. Magnusson, A. Manolescu, A. Karason, A. Palsson, G. Thorleifsson, M. Jakobsdottir, S. Steinberg, S. Palsson, F. Jonasson, B. Sigurgeirsson, K. Thorisdottir, R. Ragnarsson, K. R. Benediktsdottir, K. K. Aben, L. A. Kiemeney, J. H. Olafsson, J. Gulcher, A. Kong, U. Thorsteinsdottir and K. Stefansson, Genetic determinants of hair, eye and skin pigmentation in Europeans, *Nat. Genet.*, 2007, **39**, 1443–1452.

47 P. Sulem, D. F. Gudbjartsson, S. N. Stacey, A. Helgason, T. Rafnar, M. Jakobsdottir, S. Steinberg, S. A. Gudjonsson, A. Palsson, G. Thorleifsson, S. Palsson, B. Sigurgeirsson, K. Thorisdottir, R. Ragnarsson, K. R. Benediktsdottir, K. K. Aben, S. H. Vermeulen, A. M. Goldstein, M. A. Tucker, L. A. Kiemeney, J. H. Olafsson, J. Gulcher, A. Kong, U. Thorsteinsdottir and K. Stefansson, Two newly identified genetic determinants of pigmentation in Europeans, *Nat. Genet.*, 2008, **40**, 835–837.

48 O. A. Hakim, K. Hart, P. McCabe, J. Berry, R. Francesca, L. E. Rhodes, N. Spyrou, A. Alfuraih and S. Lanham-New, Vitamin D production in UK Caucasian and South Asian women following UVR exposure, *J. Steroid Biochem. Mol. Biol.*, 2016, **164**, 223–229.

49 R. McKenzie, R. Scragg, B. Liley, P. Johnston, J. Wishart, A. Stewart and R. Prematunga, Serum 25-hydroxyvitamin-D responses to multiple UV exposures from solaria: inferences for exposure to sunlight, *Photochem. Photobiol. Sci.*, 2012, **11**, 1174–1185.

50 W. J. Olds, A. R. McKinley, M. R. Moore and M. G. Kimlin, In vitro model of vitamin D3 (cholecalciferol) synthesis by UV radiation: dose-response relationships, *J. Photochem. Photobiol., B*, 2008, **93**, 88–93.

51 J. Lock-Andersen, N. D. Knudstorp and H. C. Wulf, Facultative skin pigmentation in caucasians: an objective biological indicator of lifetime exposure to ultraviolet radiation?, *Br. J. Dermatol.*, 1998, **138**, 826–832.

52 J. Lock-Andersen and H. C. Wulf, Seasonal variation of skin pigmentation, *Acta Derm.-Venereol.*, 1997, **77**, 219–221.

53 D. Bailey, K. Veljkovic, M. Yazdanpanah and K. Adeli, Analytical measurement and clinical relevance of vitamin D(3) C3-epimer, *Clin. Biochem.*, 2013, **46**, 190–196.

54 C. Karefylakis, P. Pettersson-Pablo, S. Sarnblad, E. Rask, M. Bitar, A. Magnuson and C. G. Eriksson, Vitamin D C3 epimer in a mid-Swedish region-Analytical measurement and epidemiology, *Clin. Chim. Acta*, 2018, **478**, 182–187.

55 H. C. Wulf, Method and an apparatus for determining an individual's ability to stand exposure to ultraviolet radiation, America Patent and Trademark Office, *US Patent* 4882598, 1986.

56 H. C. Wulf, *Method and apparatus for determining an individual's ability to stand exposure to UV*, 1986, pp. 1–32.

57 J. Lock-Andersen, F. de Fine Olivarius, M. Haedersdal, T. Poulsen and H. C. Wulf, Minimal erythema dose in UV-shielded and UV-exposed skin predicted by skin reflectance measured pigmentation, *Skin Res. Technol.*, 1999, **5**, 88–95.

58 B. L. Diffey, C. T. Jansen, F. Urbach and H. C. Wulf, The standard erythema dose: a new photobiological concept, *Photodermatol., Photoimmunol. Photomed.*, 1997, **13**, 64–66.

59 M. Henriksen, R. Na, M. S. Agren and H. C. Wulf, Minimal erythema dose after multiple UV exposures depends on pre-exposure skin pigmentation, *Photodermatol., Photoimmunol. Photomed.*, 2004, **20**, 163–169.

 

60 R. D. Mosteller, Simplified calculation of body-surface area, *N. Engl. J. Med.*, 1987, **317**, 1098–1098.
61 A. Augustsson, U. Stierner, I. Rosdahl and M. Suurkula, Regional distribution of melanocytic naevi in relation to sun exposure, and site-specific counts predicting total number of naevi, *Acta Derm.-Venereol.*, 1992, **72**, 123–127.
62 M. F. Holick, J. A. MacLaughlin, M. B. Clark, S. A. Holick, J. T. Potts Jr., R. R. Anderson, I. H. Blank, J. A. Parrish and P. Elias, Photosynthesis of previtamin D3 in human skin and the physiologic consequences, *Science*, 1980, **210**, 203–205.
63 P. Armitage, G. Berry and J. N. S. Matthews, *Statistical methods in medical research*, 4th edn, 2001, pp. 312–377.