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Highlights

- Differences in the metabolic routes of dietary-supplied vs. skin-produced vitamin D
- UV exposure was associated with higher levels of vitamin D_3 in plasma and tissues
- 3-epi-25-hydroxyvitamin D_3 increased in response to UV exposure
- exposure to UV light affects nitric oxide concentrations independently of vitamin D
- UV exposure reduces cytokine release in blood cells independently of vitamin D

UV light exposure vs. vitamin D supplementation: a comparison of health benefits and vitamin D metabolism in a pig model

Julia Kühn[1*], Corinna Brandsch[1], Anja C. Bailer[1,2], Mikis Kiourtzidis[1,2], Frank Hirche[1], Chia-Yu Chen[3,4,5,6], Lajos Markó[3,4,5,6], Theda U. P. Bartolomaeus[3,4,5,6], Ulrike Löber[3,4,5,6], Samira Michel[1], Monika Wensch-Dorendorf[1], Sofia K. Forslund-Startceva[3,4,5,6,7], Gabriele I. Stangl[1,2]

[1] Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

[2] Competence Cluster for Nutrition and Cardiovascular Health (nutriCARD), Halle-Jena-Leipzig, Germany

[3] Experimental and Clinical Research Center, Max Delbrück Center for Molecular Medicine and Charité-Universitätsmedizin Berlin, Berlin, Germany

[4] Charité-Universitätsmedizin Berlin, Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

[5] Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany

[6] DZHK (German Centre for Cardiovascular Research), Berlin, Germany

[7] Structural and Computational Biology Unit, EMBL, Heidelberg, Germany

[*] Corresponding author: Martin Luther University Halle-Wittenberg, Von-Danckelmann-Platz 2, 06120 Halle (Saale), Germany, phone: +49 345 5522724; E-mail address: julia.kuehn@landw.uni-halle.de

Running Title: UV light exposure vs. vitamin D supplementation

Abstract

There is limited data on the effect of UV light exposure versus orally ingested vitamin D_3 on vitamin D metabolism and health. A 4-week study with 16 pigs (as a model for human physiology) was conducted. The pigs were either supplemented with 20 µg/d vitamin D_3 or exposed to UV light for 19 min/d to standardise plasma 25-hydroxyvitamin D_3 levels. Important differences were higher levels of stored vitamin D_3 in skin and subcutaneous fat, higher plasma concentrations of 3-epi-25-hydroxyvitamin D_3 and increases of cutaneous lumisterol$_3$ in UV-exposed pigs compared to supplemented pigs. UV light exposure compared to vitamin D_3 supplementation resulted in lower hepatic cholesterol, higher circulating plasma nitrite, a marker of the blood pressure-lowering nitric oxide, and a reduction in the release of pro- and anti-inflammatory cytokines from stimulated peripheral blood mononuclear cells. However, plasma metabolome and stool microbiome analyses did not reveal any differences between the two groups. To conclude, the current data show important health relevant differences between oral vitamin D_3 supplementation and UV light exposure. The findings may also partly explain the different vitamin D effects on health parameters obtained from association and intervention studies.

Keywords (max 6)

Vitamin D_3, lumisterol$_3$, 3-epi-25-hydroxyvitamin D_3, cardiovascular risk factors, immune response, microbiome

1 Introduction

Vitamin D plays an essential role in regulating calcium homeostasis and bone health. In recent years, there has been growing interest in the potential non-skeletal health benefits of vitamin D, such as on the immune and cardiovascular system or the intestinal microbiome. Thus, several observational and interventional studies were conducted to investigate the role of vitamin D in disease prevention and therapy. Interestingly, observational studies often found associations between low plasma levels of 25-hydroxyvitamin D (25(OH)D) and increased disease risk such as hypertension [1–3], while intervention studies using vitamin D supplements do not seem to lower this risk [4–6].

Vitamin D is known as the "sunshine vitamin" as it is produced in the skin in response to ultraviolet (UV) light exposure, but it can enter the body by oral routes through vitamin D-containing foods or supplements as well. With some exceptions, endogenous vitamin D seems to contribute predominantly to the vitamin D supply in humans [7]. This means that the primary source of vitamin D in individuals included in observational studies comes from UV exposure, while oral vitamin D is the exclusive source of vitamin D in intervention studies. It has been assumed for a long time that orally consumed vitamin D is physiologically equivalent to vitamin D synthesized in the skin, but there are differences in the transport and metabolic fate of dietary and skin-derived vitamin D [8]. Moreover, it is a well-known fact that large quantities of orally ingested vitamin D can have toxic effects, whereas overdosing through endogenously produced vitamin D is prevented by degradation of excess metabolites in the skin.

Apart from potential differences in the metabolism of oral and endogenously produced vitamin D, sunlight or UV light can also affect health outcomes independent of vitamin D, such as lowering blood pressure or modulating the immune cells [9,10]. It is therefore likely that epidemiological data investigating the association between vitamin D and health

outcomes might be biased by UV light exposure. Consequently, it can be assumed that the observed correlation between blood pressure and vitamin D in association studies [1,3,11] is attributable to sunlight or UV light. It may also explain the absent effects of oral vitamin D on blood pressure in intervention studies [4,12,13]. Whether the conflicting results on vitamin D and health parameters between association and intervention studies are due to vitamin D-independent effects of UV light or differences in the efficacy to improve vitamin D status is largely unknown.

The vitamin D status is usually assessed by the measure of 25(OH)D in plasma or serum and does not provide any information on the origin of vitamin D - endogenously synthesized or orally supplied. There is currently no biomarker that allows distinguishing UV light-produced from oral vitamin D. It is further unknown whether UV light affects vitamin D metabolism differently than oral vitamin D.

Due to the need for data on the effects of UV light compared to oral vitamin D, the current study was conducted to investigate the metabolic routes and health implications of dietary and UV light-produced vitamin D in a pig model. The obtained data may help explain the contradictory data from observational and interventional studies on vitamin D and health. To this end, pigs received either dietary vitamin D or were exposed to UV light, and vitamin D metabolism, blood pressure regulators, immunological parameters, microbial stool composition, and several plasma metabolites were assessed. To avoid divergences in the effects between these two interventions that are simply caused by differences in the improvement of vitamin D status, we first conducted a pre-study to identify the UV exposure time per day that leads to similar 25(OH)D levels as the intake of 20 µg/d vitamin D.

2 Experimental section

To investigate possible differences in the metabolic fate and health effects of oral versus endogenously produced vitamin D, two studies (a pre-study to find the UV exposure time that

is equivalent to oral vitamin D intake and the main study) using pigs as a model were conducted. The study protocol and the animal husbandry were approved by the local council of Saxony-Anhalt (Landesverwaltungsamt, Halle (Saale), Germany; approval number: 42502-2-1527 MLU). Care and handling of the pigs were in accordance with the German animal welfare regulations (Tierschutzgesetz, version of 18 May 2006 and last revised on 1 January 2019 and Tierschutz-Versuchstierverordnung, version of 1 August 2013 and last revised on 8 September 2015). The studies are described in accordance with the ARRIVE guidelines.

The pre- and the main study were performed using female weaned piglets [Piétraine x (Large White x Landrace)] with an initial age of 6 weeks at baseline and a mean body weight of 12.2 ± 1.73 kg. The studies were conducted in an animal facility that was controlled for temperature and lighting, with a UV-free lighting program from 06:00 am to 06:00 pm and a colour temperature of 4,000 K and 80 lux. All pigs had free access to pellet feed and water from nipple drinkers. The individual pen compartments in the facility consisted of concrete slatted floor and plastic floor grids. Before starting the pre-study and the main study, the pigs were vitamin D depleted for two weeks by feeding a vitamin D free pre-starter diet (supplemental table 1). During the experimental periods, the pigs were daily treated with either 20 µg orally supplemented vitamin D_3 dissolved in 2 ml soybean oil or UV light (complemented by 2 ml of vitamin D-free soybean oil as sham treatment). The soybean oil was administered with an oral dispenser. The basal diet that was fed during the experimental periods contained no analytically detectable vitamin D_3. For the UV treatment, UV lamps (Exo Terra Reptile UVB 200, Reptilienkosmos, Viersen, Germany) were mounted 95 cm above the floor. The mean emitted UVB intensity (measured on the shoulder height of the pigs) was 17.7 µW/cm^2. The UV spectrum of the lamps is shown in supplemental figure 1. An automated timer was used to control the on/off switch of the UV lamps.

2.1 Experimental design of the pre-study – Identification of the suitable UV exposure

The pre-study was conducted to ascertain the UV exposure time, which leads to the same plasma 25(OH)D levels in pigs as feeding 20 µg vitamin D per day. The standardization of 25(OH)D levels was intended to avoid differences in vitamin D metabolism and health parameters between the two groups caused by differences in vitamin D status. Therefore, a total of 20 pigs were randomly assigned to 6 groups (3 to 4 pigs per group) and were either fed 20 µg/d vitamin D_3, or daily exposed to UV light for 1 min (1.1 mJ/cm^2), 5 min (5.3 mJ/cm^2), 10 min (10.6 mJ/cm^2), 20 min (21.2 mJ/cm^2) and 30 min (31.9 mJ/cm^2). The animals were housed in groups of 10 pigs per pen and coloured ear tags identified the corresponding intervention group. For the daily UV exposure procedure, the pigs were separated group-wise and brought to a cabin that was equipped with UV lamps. Blood was drawn weekly to monitor the 25(OH)D_3 levels of all pigs over a period of four weeks. To this end, blood was collected in heparinized monovettes (Sarstedt, Nümbrecht, Germany) and then centrifuged at 2,000 g for 10 min to obtain plasma. Analyses were conducted to reveal if there is a linear or quadratic relationship between the 25(OH)D_3 levels in the pigs and the UV exposure time.

2.2 Experimental design of the main study - Investigating physiological differences between oral vitamin D and UV exposure

The main study was conducted to compare the tissue levels of vitamin D metabolites and health parameters in pigs treated with oral vitamin D_3 or UV light. The UV radiation conditions for the main intervention study were deduced from the pre-study. A total of 16 pigs with an initial age of four weeks and a median body weight of 7.45 kg (7.10-8.13 kg) were randomly divided in two groups and housed in two identical pen compartments (8.32 m^2).

One group was supplemented daily with 20 µg vitamin D_3 and the other group was daily exposed to UV light for 19 min (20,2 mJ/cm^2) to produce endogenous vitamin D_3. To this end, the compartment of the UV-exposed group was equipped with four UV lamps. The experimental period lasted four weeks.

During the intervention period, the pigs were weighted weekly. Blood samples were also taken weekly by venepuncture from the *vena jugularis.* Blood collected in heparinized monovettes (Sarstedt) was then centrifuged at 2,000 g for 10 min to obtain plasma. Five pigs per group were randomly selected to isolate peripheral blood mononuclear cells (PBMC) at baseline (week 0) and at the end of the intervention period (week 4) to analyse cytokine expression. Stool samples were collected at week 0 and week 4 to analyse microbial composition, bile acids and short-chain fatty acids. The stool samples were collected from the individuals during defecation, stored immediately at -20°C and transferred to -80°C on the next day. At the end of the study, the pigs were food deprived for 12 h, anesthetized by an intramuscular injection of xylazine (Serumwerk, Bernburg, Germany) plus ketamine (Ursotamin, Serumwerk) and a continuous isoflurane/oxygen gas flow before being euthanized by an intracardiac injection of pentobarbital sodium (Release, WDT, Garbsen, Germany) to gather tissue samples. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further analyses.

2.3 Isolation and treatment of the PBMC

Peripheral blood mononuclear cells (PBMC) were obtained from the blood of 10 randomly selected pigs (five per group) at baseline and after four weeks of intervention to analyse the expression of cytokines. The heparinized blood was layered on Histopaque®-1077 (Sigma-Aldrich, Taufkirchen, Germany) to isolate the PBMC by density gradient centrifugation, according to the manufacturer's protocol [14]. The resulting cell pellet was re-suspended in RPMI 1640 medium (Life Technologies, Darmstadt, Germany), supplemented with 5% foetal

bovine serum. For the stimulation, PBMC were seeded in 24-multiwell plates (about 4 x 10^6 cells per well) and incubated with lipopolysaccharide (LPS, 1 µg/ml RPMI 1640 medium) for 20 h at 37°C and 5% CO_2. The cells were harvested by centrifugation at 900 g for 5 min. The supernatant was centrifuged a second time (13,000 g at 4°C) to remove cell debris and was stored at -20°C until analyses of the secreted cytokines. PeqGOLD TriFast™ (Peqlab, Erlangen, Germany) was added to the cell pellets and the tubes were stored at -80°C until analysis of mRNA abundance of cytokines by using PCR and analysis of the protein amount by using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Schwerte, Germany).

2.4 Analysis of vitamin D metabolites

The concentrations of 25(OH)D_3, vitamin D_3, 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) and lumisterol$_3$ were analysed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Before the identification and quantification of these vitamin D metabolites, the plasma samples were spiked with deuterated internal standards (Sigma-Aldrich). The plasma was then hydrolysed as described elsewhere [15] and transferred to extraction columns (Extrelut NT1, Merck KGaA, Darmstadt, Deutschland). The metabolites were extracted with *n*-hexane/*tert*-butyl methyl ether and the dried eluates were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione [15]. The plasma samples were injected to the HPLC-MS/MS (1260 Series, Agilent Technologies; QTRAP 5500, Sciex, Darmstadt, Germany) that was equipped with a Poroshell column (EC-C18, 50 x 4.6 mm^2, 2.7 µm). For the chromatographic separation, the column temperature was set to 40°C and the mobile phases consisted of (A) acetonitrile and (B) a mixture of acetonitrile/water (1/1, v/v) with 5 mM ammonium formate and 0.1% formic acid. The following gradient was used: 0.0 min, 0% A, 600 µl/min; 2.1 min, 0% A, 600 µl/min; 4.0 min, 23.5% A, 600 µl/min; 6.0 min, 40% A, 600 µl/min; 8.0 min, 60% A, 600 µl/min; 10.0 min, 80% A, 600 µl/min; 11.0 min, 100% A,

600 µl/min; 16.0 min, 100% A, 600 µl/min; 18.0 min, 100% A, 1000 µl/min; 20.0 min, 100% A, 1000 µl/min; 21.0 min, 0% A, 1000 µl/min; 24.0 min, 0% A, 800 µl/min, 25.0 min, 0% A, 600 µl/min. Mass spectrometric conditions, aspects of lumisterol analysis and quantification procedures are described elsewhere [16,17].

The concentration of 3-epi-25(OH)D_3 was assessed in plasma using the MassChrom® kit for HPLC-MS/MS with atmospheric pressure chemical ionisation (Chromsystems, Gräfelfing, Germany). In plasma samples, the limit of quantifications (LOQ) were: vitamin D_3, 0.26 nmol/l; 24,25$(OH)_2D_3$, 1.44 nmol/l; 3-epi-25(OH)D_3, 2.5 nmol/l, lumisterol$_3$, 4 nmol/l.

Tissue samples for the determination of vitamin D_3, 25(OH)D_3 and lumisterol$_3$ were prepared as describe previously [15,18]. The chromatographic separation was performed on a Hypersil ODS C18 column (5 µm, 2.0 x 150 mm^2, VDS Optilab, Berlin, Germany) [19]. The LOQ for 25(OH)D_3 in tissue samples was 7 ng/g.

Precision of the HPLC-MS/MS methods was assessed using the MassCheck® controls (Chromsystems) and pooled liver samples. The coefficients of variation for intra- and inter-day precision were all lower than 10%.

The concentration of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) in the plasma was analysed by a commercial enzyme-linked immunoassay (Immunodiagnostic Systems, Frankfurt am Main, Germany) according to the manufacturer's protocol. The intra-day precision was 5.0%.

2.5 Analysis of the relative mRNA abundance

The relative mRNA abundance of genes involved in vitamin D metabolism and tissue distribution as well as immune response and blood pressure regulation was quantified by real-time RT-PCR in different tissues and PBMC. Total RNA was isolated with the peqGOLD TriFast™ according to the kit's manual. The concentration of total RNA in the sample was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc.) and the

integrity of the RNA was confirmed by agarose gel electrophoresis. The synthesis of cDNA was performed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Subsequently, real-time RT-PCR was carried out as described elsewhere [20] using the Rotorgene 6000 system and the Rotorgene software version 1.7 (Corbett Research, Mortlake, Australia). The relative mRNA abundance was calculated by the method of Pfaffl [21] using two appropriate reference genes. The primers used for the analyses are summarized in table 1.

2.6 Analysis of minerals, lipids, nitrite, and folate

The concentrations of calcium, phosphorus, triglycerides and cholesterol were quantified in the plasma of pigs with test kits from DiaSys (Diagnostic Systems GmbH, Holzheim, Germany). The concentration of cholesterol in bile and liver was determined with the same test kit from DiaSys. Prior to quantification, lipid extracts of bile and liver were prepared as previously described [15,22]. The concentration of nitrite in the plasma, used as a biomarker of nitric oxide production, was determined by a colorimetric assay from Sigma-Aldrich. All procedures were in accordance with the manufacturer's protocols. The intra-day precisions were as follows: calcium, 4.4%; phosphorus, 13%; triglycerides, 10%; cholesterol, 5.6%; nitrite, 7.9%. The quantification of folate species in plasma was conducted by Bevital (Bergen, Norway) using LC-MS.

2.7 Analysis of the microbiome

The microbiome composition of faeces and duodenal chyme was analysed. To this end, total DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Freiburg, Germany) and as described in details elsewhere [23]. 16S sequencing was performed by LGC Genomics (Berlin, Germany) using the Klindworth V3-V4 primers [24] and an Illumina MiSeq sequencer.

LotuS 1.62 [25] was employed to process the 16S rRNA amplicons from both stool and chyme microbiomes, utilizing the SILVA [26], Greengenes [27], and HITdb databases, thereby generating tables depicting microbiome abundance at all taxonomic levels. Subsequently, the abundance tables underwent rarefaction using RTK [28], and alpha diversities were calculated by the same tool.

2.8 Analysis of endothelin-1 in the renal artery

The concentration of endothelin-1 was determined in tissue homogenates of the porcine renal artery, using a commercial enzyme-linked immunosorbent assay (Cusabio, Texas, USA). The preparation of tissue homogenates and the assay procedure were following the manufacturer's protocol. The protein concentration of the tissue homogenates was assayed by the Bradford method [29].

2.10 Analysis of cytokine concentration

The concentrations of tumor necrosis factor alpha (TNF-alpha), interleukin 6 (IL6) and interleukin 10 (IL10) were determined in the supernatant of the PBMC after stimulation by use of enzyme-linked immunoassays (Bio-Techne GmbH, Wiesbaden, Germany). All procedures were in accordance with the manufacturer's protocol. Intra-day precisions were 2.4% for TNF-alpha, 4.2% for IL6, and 3.9% for IL10. The determined cytokine concentrations were related to the protein amount of the corresponding cells.

2.10 Analysis of bile acids and short-chain fatty acids (SCFA)

The concentration of bile acids was determined in bile and freeze-dried faeces samples by MS-Omics (Vedbaek, Denmark) using LC-MS [30]. The analysis of SCFA in faecal water samples was carried out with gas chromatography-mass spectrometry by MS-Omics. For the

faecal water extraction, faeces samples were mixed with phosphate-buffered saline (1/3, w/v), vortexed and centrifuged at 16,000 g for 30 min. The supernatant was filtered through 0.2 µm centrifuge filters at 15,000 g for 2 min. The faecal water was stored at -20°C and shipped to MS-Omics on dry ice.

2.11 Metabolomic analysis in plasma

Metabolomic analysis of plasma samples was carried out by MS-Omics (Vedbaek) using a Thermo Scientific Vanquish LC coupled to Thermo Q Exactive HF MS. An electrospray ionization interface was used as ionization source. Analysis was performed in negative and positive ionization mode. Peak areas were extracted using Compound Discoverer 2.0 (Thermo Scientific). Identification of compounds was performed at four annotation levels; level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; level 2a: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm); level 2b: identification by accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm).

2.12 Statistical analyses

Data from plasma and faeces samples with repeated measurements were analysed using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Prior to the *mixed-model procedure* (*PROC MIX*), the data were tested for normal distribution or log-normal distribution. The treatment (group), time of intervention and their interaction (group x time) were considered as fixed effects and the animals were considered random. For significant interaction effects, the Tukey-Kramer post-hoc was conducted. For all data from tissue

samples (no repeated measurements) and plasma 3-epi-25(OH)D_3, the two groups were compared by the non-parametric Mann-Whitney *U* test (GraphPad Prism version 9; GraphPad Software, Boston, MA, USA). Values that were below the LOQ were included as LOQ/2 in the statistical analyses, if more than 25% of the data within one group were above the LOQ. Otherwise, no statistical analysis was conducted for this parameter.

For statistical analysis of microbiome, Wilcoxon rank-sum and Spearman correlation tests were carried out using the base R environment. Multivariate analysis, employing the Adonis test based on distance matrices, was conducted using the Vegan [31] R package. Univariate tests were executed utilizing the glmmTMB [32] package, where the abundance of each microbe was fitted through the interaction term of plasma vitamin D concentration and treatment, with the subject included as a random factor. A significance threshold for q-values, adjusted via the Benjamini-Hochberg method, was set at 0.05. Heatmaps were generated using the gplots package. Finally, the repeated measurement correlation test was performed with the Rmcorr [33] package.

For the statistical analysis of metabolome data, the log2 values of both groups were subjected to a student's t-test with Benjamini-Hochberg correction.

Significant differences were assumed for $P < 0.05$. If not otherwise stated, data are presented as median and interquartile ranges.

3 Results

3.1 Determining the UV exposure time in the pre-study

The pre-study aimed to assess the daily UV exposure time which leads to similar plasma 25(OH)D levels as an oral administration of 20 µg/d vitamin D_3 (figure 1). At baseline, the 25(OH)D_3 plasma concentration (given as mean ± standard deviation) of all pigs was 10.6 ± 2.3 nmol/l. The highest increase of plasma 25(OH)D_3 was observed within the first

week of treatment in all groups (figure 1). After week 1, orally supplemented pigs showed plasma 25(OH)D_3 concentrations that corresponded to those of groups exposed to UV light for 20min/d and 30 min/d, respectively. After two weeks of treatment, orally supplemented pigs had plasma levels of 25(OH)D_3 similar to the groups exposed to UV light for 10 min/d and 20 min/d, respectively (figure 1). To determine what daily UV exposure time over the entire treatment period of 4 weeks represents best the 25(OH)D_3 concentrations obtained by dietary vitamin D supply, a model comparison analysis was conducted. Based on the Akaike information criterion values and F-test, a linear association between UV exposure time and plasma 25(OH)D_3 was found in week 1, 2, 3 and 4 of the experiment. By using the weekly assessed regression equations, the daily UV exposure time that resulted in plasma 25(OH)D_3 levels similar to those of daily supplemented pigs was calculated. Based on this calculation, a UV exposure time of 19 min/d was considered to be sufficient to produce 25(OH)D_3 plasma levels similar to those analysed in the group fed 20 µg/d vitamin D_3 (supplemental figure 2). This UV exposure time was used for the main study.

3.2 Main Study

3.2.1 Vitamin D metabolism in pigs treated with oral vitamin D_3 or UV light

All pigs were vitamin D deficient at week 0. The plasma concentration of 25(OH)D_3 (measured as the sum of non-epimerized 25(OH)D_3 and 3-epimerized 25(OH)D_3) increased during the study in both groups ($P < 0.001$, figure 2a) with the highest rise within the first week of intervention. As intended, plasma 25(OH)D_3 concentrations were not different between both groups throughout the experimental period (figure 2b). Interestingly, the concentration of 3-epi-25(OH)D_3 increased in response to UV light exposure, but remained below the LOQ (2.5 nmol/l) in the supplemented group during the whole study (figure 2b). The concentration of plasma vitamin D_3 was below the LOQ (0.26 nmol/l) at baseline and

strongly increased within the first week of vitamin D supply in both groups (figure 2c). Interestingly, the treatment affected the plasma concentration of vitamin D_3, whereby the UV-exposed group had higher levels than the supplemented group (figure 2b, $P < 0.05$). The most marked difference between the two groups was found at the end of the study. The concentration of 3-epi-25(OH)D_3 increased in response to UV light exposure, but remained below the LOQ in the supplemented group during the whole study. However, the plasma concentrations of 24,25$(OH)_2D_3$ (figure 2d) and 1,25$(OH)_2D_3$ (figure 3a) increased during the experimental period ($P < 0.001$), but were not affected by the treatment. The plasma concentration of parathyroid hormone (PTH) did not change with the treatment and time (figure 3b). Lumisterol$_3$, a photoproduct of the UV irradiation, was not quantifiable in the plasma of pigs (LOQ: 4 nmol/l).

Vitamin D metabolites were then quantified in the skin of the dorsal back, where the highest UV light exposure and vitamin D synthesis was expected. Remarkably, the quantities of vitamin D_3 and lumisterol$_3$ were nearly 50 times higher in the UV-exposed group than in the supplemented group ($P < 0.001$, figure 4). Additionally, higher quantities of vitamin D_3 were also seen in kidney ($P = 0.010$), subcutaneous fat ($P < 0.001$), and bile ($P < 0.05$) of UV-exposed that in the supplemented pigs (figure 5). However, analysis of faecal vitamin D_3 revealed lower values in the UV-exposed group compared to the supplemented group ($P < 0.001$, figure 5). There were no significant differences in the quantities of vitamin D_3 in liver, visceral fat, trapezius muscle and intestinal mucosa (figure 5). The concentration of 25(OH)D_3 in liver (dietary vitamin D: 2.57 ng/g [1.99-4.35 ng/g], UV exposure: 2.54 ng/g [2.11-2.71 ng/g]) and kidney (dietary vitamin D: 2.98 ng/g [1.43-3.70 ng/g], UV exposure: 2.63 ng/g [2.19-4.53 ng/g]) were not different. The tissue concentrations of 25(OH)D_3 were below the LOQ (7 ng/g) in subcutaneous fat, visceral fat, muscle, intestinal mucosa, bile and faeces.

Next, the mRNA abundance of cytochrome P450 (CYP) genes that encode the porcine-specific vitamin D hydroxylase *CYP2D25*, the mitochondrial *CYP27A1* and the 1α-hydroxylase *CYP27B1* was assessed (figure 6). Dorsal skin of UV-exposed pigs showed a higher mRNA abundance of *CYP2D25* ($P < 0.05$) and a trend toward higher mRNA abundance of *CYP27A1* ($P < 0.1$) than that of supplemented pigs, whereas in liver, intestinal mucosa and kidney the mRNA abundance of the vitamin D hydroxylases did not differ.

For intestinal and hepatic lipid transporter, the only difference in mRNA abundance was observed for the hepatic sterol transporter NPC1 like intracellular cholesterol transporter 1 (*NPC1L1*) which was higher in the UV-exposed group than in the supplemented group ($P < 0.05$, table 2). In contrast, intestinal *NPC1L1*, and ATP binding cassette subfamily G member 5 (*ABCG5*), CD36 molecule (*CD36*) and scavenger receptor class B member 1 (*SCARB1*) in the intestinal mucosa and liver did not differ between the two groups (table 2).

3.2.2 Concentration of minerals, lipids, and folate

The plasma concentrations of calcium and phosphorus, cholesterol, triglycerides (table 3) and folate (supplemental table 2) did not differ between the two groups. Interestingly, the hepatic cholesterol level was moderately lower in UV-exposed pigs than in supplemented pigs ($P < 0.1$), although biliary cholesterol was not different between the two groups (table 3).

3.2.3 Microbiome composition, bile acids and short-chain fatty acids

In the examination of the stool microbiome, no statistically significant differences (Wilcoxon rank-sum test $P \geq 0.05$) in alpha diversities (including richness, evenness, Shannon diversity, and Chao1 index) were discerned neither among various treatments nor across distinct time points. Employing multivariate analysis via the Adonis test, with the time variable representing variations in vitamin D concentration and pig growth, it was found that the time

variable significantly influenced microbial composition ($P < 0.05$), while the treatment factor did not exhibit any significant impact. Although significant alterations in microbial populations were observed across different time points, indicative of fluctuations in vitamin D levels, our investigation did not yield substantive evidence linking the sources of vitamin D to discernible effects on the stool microbiome, whether through multivariate or univariate assessments. On the other hand, when scrutinizing the chyme microbiome, no statistically significant variations in alpha diversities (encompassing richness, evenness, Shannon diversity, and the Chao1 index) were detected among treatments. Employing a multivariate analysis (Adonis test) at the species level, the treatment factor did not exert a discernible influence on microbial composition. Likewise, no evidence was found to suggest that the sources of vitamin D significantly impacted the chyme microbiome, whether through multivariate or univariate analytical approaches.

Correlation analyses conducted between the stool and chyme microbiomes have unveiled a multitude of statistically significant correlations existing at the species, genus, and family taxonomic levels (supplemental figure 3-5). This evidence underscores a robust and close association between the stool and chyme microbiome. Furthermore, when analysing the correlation between the stool microbiome and plasma metabolite concentrations, a positive correlation was identified between the abundance of *Mailhella massiliensis* and creatine (supplemental figure 6).

Additionally, bile acids in faeces and bile, as well as short-chain fatty acids in faeces did not differ significantly between the two groups of pigs (supplemental table 3-5).

3.2.4 Regulators of blood pressure

The concentration of plasma nitrite, a marker of nitric oxide production, decreased during the study period ($P < 0.001$), whereby the decrease was less pronounced in UV-exposed pigs (figure 7), indicating a vasodilatative response to UV radiation. However, the mRNA

abundance of nitric oxide synthase 2 (*NOS2*) and nitric oxide synthase 3 (*NOS3*) in the renal artery, skin and subcutaneous fat was not differently affected by the treatments (figure 7). To investigate factors involved in vasoconstriction, the relative mRNA abundance of endothelin-1 (*EDN1*), endothelin receptor type A (*EDNR*A) and endothelin receptor type B (*EDNR*B) was analysed in the renal artery. A trend toward lower *EDN1* mRNA abundance was observed in pigs exposed to UV light compared to vitamin D_3-supplemented pigs ($P < 0.1$), whereas the protein expression of endothelin-1 and the relative mRNA abundance of *EDRNA* and *EDNRB* did not differ between the two groups (figure 8).

3.2.5 Cytokine expression in PBMC

PBMC isolated from both groups and stimulated with LPS showed a lower relative mRNA abundance and protein release of TNF-alpha and IL10 in pigs exposed to UV light compared to pigs supplemented with vitamin D_3 ($P < 0.05$, table 4). No differences between the groups were observed for the mRNA abundance and the concentration of released IL6 (table 4).

3.2.6 Plasma metabolome

An untargeted metabolome analysis was conducted in plasma samples of UV-exposed pigs and pigs with dietary vitamin D supplementation and 153 compounds with an annotation level 1, 2a or 2b were identified (supplemental table 6). Four compounds were increased in UV light-exposed pigs with a high significant P value < 0.001: hexadecanamide, trans-anethole, 1-dodecyl-2-pyrrolidinone and (2E,4E)-N-(2-methylpropyl)deca-2,4-dienamide. Two compounds were increased in the UV-exposed group with a significant P value < 0.05: 4-methylumbelliferone hydrate and monobutyl phthalate. However, none of these metabolites could be assigned a physiological function.

4 Discussion

The current study compared the effects of UV light exposure versus vitamin D_3 supplementation on vitamin D metabolites, and factors indicating health or disease risk. The most marked findings of this study were differences in the profile and tissue distribution of vitamin D_3 metabolites, factors associated with blood pressure regulation, and the immune response. Many other factors with relevance for health, including plasma lipids, gut microbiome, short-chain fatty acids, folic acid status, and other metabolites, did not show differences between the two treatments. An important finding of this study was that UV light-exposed pigs had more vitamin D_3 in plasma, skin, kidney, and subcutaneous fat tissue than supplemented pigs, although the plasma 25(OH)D_3 levels were similar in both groups. We assume that the higher vitamin D_3 stores in skin, and subcutaneous fat, which constitute a large part of the whole body fat [34,35], of UV-exposed pigs are physiologically important as they may contribute to counteract declining plasma 25(OH)D_3 levels in times of insufficient vitamin D_3 synthesis. The importance of vitamin D stores in adipose tissue has already been described in a human study that found high vitamin D stores associated with a smaller decline in 25(OH)D during winter periods [36]. It is important to note that the increase of cutaneous vitamin D_3 in UV-exposed pigs was accompanied by a higher mRNA expression of *CYP2D25*, a vital vitamin D_3 hydroxylase. An upregulated cutaneous expression of vitamin D hydroxylases after UV treatment has also been found by others [37,38], and is probably a response to higher substrate availability. However, recent research has uncovered alternative pathways of vitamin D_3 metabolism, particularly involving the enzyme CYP11A1 [39], which has been upregulated in human skin samples exposed to UV light [40]. It must therefore be assumed that UV light-exposed pigs in the current study had produced some alternative vitamin D_3 metabolites that exhibit biological activities that differ from the classical vitamin D metabolites. Interestingly, UV light primarily increases vitamin D_3 in the subcutaneous fat rather than in visceral fat, which is probably because vitamin D is stored mainly close to the

site of production. The current data might also confirm previous findings demonstrating that circulating 25(OH)D_3 does not necessarily reflect the tissue stores of vitamin D_3 in the body [41].

When UV radiation penetrates the skin, 7-dehydrocholesterol is converted to pre-vitamin D_3, which undergoes a temperature-dependent isomerization to vitamin D_3. Pre-vitamin D_3 can also isomerize to photochemical by-products of UV light exposure such as lumisterol$_3$ [42]. Remarkably high quantities of lumisterol$_3$ is what we found in the skin of UV light-exposed pigs in contrast to supplemented pigs. We assume that this finding is also very important with respect to the fact that lumisterol$_3$ has been identified as a molecule with beneficial effects in keratinocytes such as proliferation and antioxidative responses [43].

In addition, we found another quite important difference in plasma 3-epi-25(OH)D_3, that largely increased in UV-exposed pigs over the time. To date, little is known about the precise function and health implication of C3-epimerized vitamin D forms, and also the gene encoding for the epimerization enzyme has not yet been identified [44]. It is suggested that 3-epi-1,25(OH)$_2D_3$, which has been shown to be synthesized in the endoplasmatic reticulum of hepatocytes, bone cells and keratinocytes [45], can stimulate the expression of vitamin D regulated genes by binding to and activating the vitamin D receptor, but less effective than the non-epimeric calcitriol [45]. Data on external factors that stimulate 3-epi-25(OH)D_3 formation are scare. There are only two studies that identified higher epimeric vitamin D levels in oral supplemented compared to UV light-exposed mice [46], whereas in another study an increase of 3-epi-25(OH)D_3 was observed in mice exposed to LED lamps emitting wavelengths in the range of 275-900 nm compared to mice kept in the dark [47]. The current study has contributed to identify UV light exposure as an environmental factor that stimulates the production of 3-epi-25(OH)D_3.

One of the most apparent differences between dietary vitamin D supply and endogenous synthesis is the way vitamin D enters the body. The uptake of vitamin D from food sources is a complex process that involves both passive diffusion and active transport mechanisms that are facilitated by intestinal lipid transporters, such as NPC1L1, located at the apical site of the enterocyte [16,18]. The expression of these lipid transporters was measured, in order to test if the expression is modified by oral vitamin D_3 intake, but observed no differences between the supplemented and the UV light-exposed pigs. However, NPC1L1 is a sterol transporter that is not only located in the gut, but also in the liver, where it mediates the hepatic re-uptake of cholesterol to prevent excessive biliary loss [48]. Telford et al. observed an increase of hepatic NPC1L1 in response to tissue cholesterol depletion in pigs [49]. Interestingly, our study found moderately lower hepatic cholesterol levels and a higher mRNA abundance of hepatic *NPC1L1* in UV light-exposed pigs that in supplemented pigs. We hypothesize that the upregulation of hepatic *NPC1L1* was caused by the lower levels of liver cholesterol and that UV-exposed pigs might benefit from an enhanced biliary re-uptake of vitamin D_3 as a secondary effect.

The role of vitamin D and UV light in blood pressure regulation is still a topic of scientific debate [10,50]. Vitamin D has been shown to affect the production of nitric oxide, the most potent endothelial vasodilator, and is therefore notably involved in the modulation of vascular tone and resistance [51]. Due to its very short lifespan, measuring nitric oxide in biological samples is challenging, and nitrite has become an essential surrogate biomarker for nitric oxide production [52,53]. The higher plasma levels of nitrite observed in the UV-exposed pigs are probably indicative of a higher nitric oxide release from the skin into the circulation, although the gene expression of *NOS2* and *NOS3* was not changed by the UV exposure. This phenomenon has already been described in the context of UV light exposure [54,55]. An antagonist of nitric oxide is endothelin-1, the most potent vasoconstrictor that mediates its effects through the activation of endothelin receptors on vascular smooth muscle cells [56].

Although it is suggested that both vitamin D and UV light can stimulate endothelin expression and/or response *in vitro* and *in vivo* [57–60], we found a moderate down-regulation of endothelin-1 in UV-exposed pigs in comparison to supplemented pigs. On the other hand, nitric oxide has been described to be capable of decreasing circulating endothelin-1 levels [61–63], which may explain the reduced expression of *EDN1* in the renal artery of UV-exposed pigs. However, a limitation of the study is that blood pressure was not directly measured in pigs due to their high stress level induced by restraining.

Vitamin D and UV light are known to influence immune response. In the present study, UV light exposure was associated with lower expression and release of the proinflammatory TNF-alpha and the anti-inflammatory IL10 in stimulated PBMC in comparison to the vitamin D_3 supplementation. This finding fits well with the commonly known immune suppressive impact of UV radiation [9,64], and data from humans exposed to artificial or natural UV light whose isolated and *ex vivo* stimulated PBMC showed a reduced capacity to secrete the cytokines TNF-alpha and IL10 [65,66]. In the present study, the reduced release of TNF-alpha and IL10 might indicate an overall suppression or attenuation of the innate immune response, which might be beneficial in controlling inflammation but could also impair the ability to fight infections effectively.

When comparing both sources of vitamin D supply, one should keep in mind that UV light exposure is associated with numerous systemic effects that arise independently of vitamin D [40,67,68]. To better point out health outcomes which are derived from vitamin D and are not the consequence of UV exposure, it would be helpful to identify sensitive and reliable biomarkers of UV exposure. Unfortunately, the untargeted plasma metabolome analysis in the current study did not provide promising metabolites.

To summarize, the current study demonstrates some health relevant differences between oral vitamin D_3 supplementation and UV light exposure. UV light exposure in contrast to vitamin D_3 supplementation resulted in higher levels of stored vitamin D_3 in skin and subcutaneous fat, which might counteract a decline in vitamin D levels in times of insufficient vitamin D synthesis. UV light exposure further contributed to an increase in the production of the physiologically active lumisterol$_3$ and 3-epi-25(OH)D_3. And finally, it was shown that UV light exposure in contrast to supplemented vitamin D_3 can modify regulators of blood pressure and the immune response. These differences are important with respect to recommendations concerning the best option to improve people's vitamin D status and may also explain discrepancies in the results from observational studies and intervention studies.

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Author contributions

JK, ACB and GIS designed the study; JK and ACB conducted the study; JK, CB, ACB, MK, FH, C-YC, LM, TUPB, UL and SM conducted the analyses; JK, CB, C-YC, MW-D analysed the data; SKF-S supervised microbiome analysis, JK and GIS wrote the initial draft of the manuscript, JK and CB prepared tables and figures. All authors read and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available on reasonable request from the corresponding author. Bacterial 16S rRNA reads were deposited in the NCBI Sequence Read Archive (SRA) database (BioProject ID: PRJNA1062174).

Conflict of interest

The authors declare that they have no competing financial interests.

Figure legends

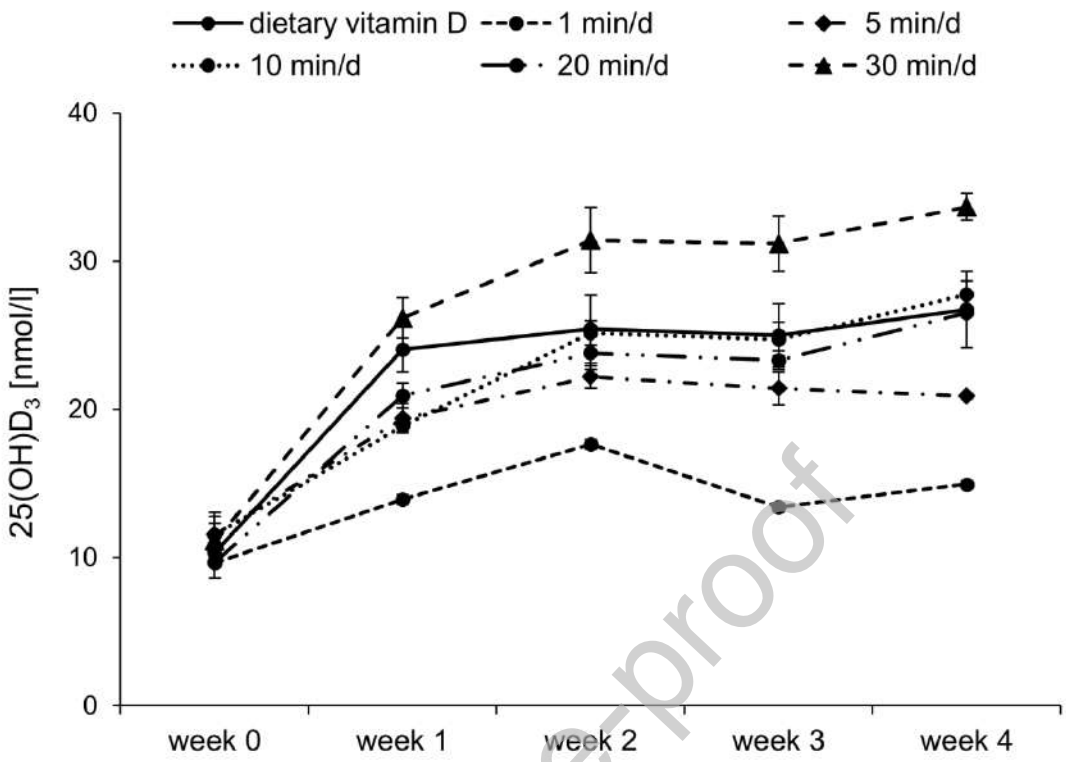

Figure 1: Plasma concentration of 25-hydroxyvitamin D_3 ($25(OH)D_3$) in pigs with a daily UV exposure of 0 min (dietary vitamin D), 1 min, 5 min, 10 min, 20 min or 30 min. Pigs exposed to UV light did not receive oral vitamin D. Data are reported as mean values ± standard deviation.

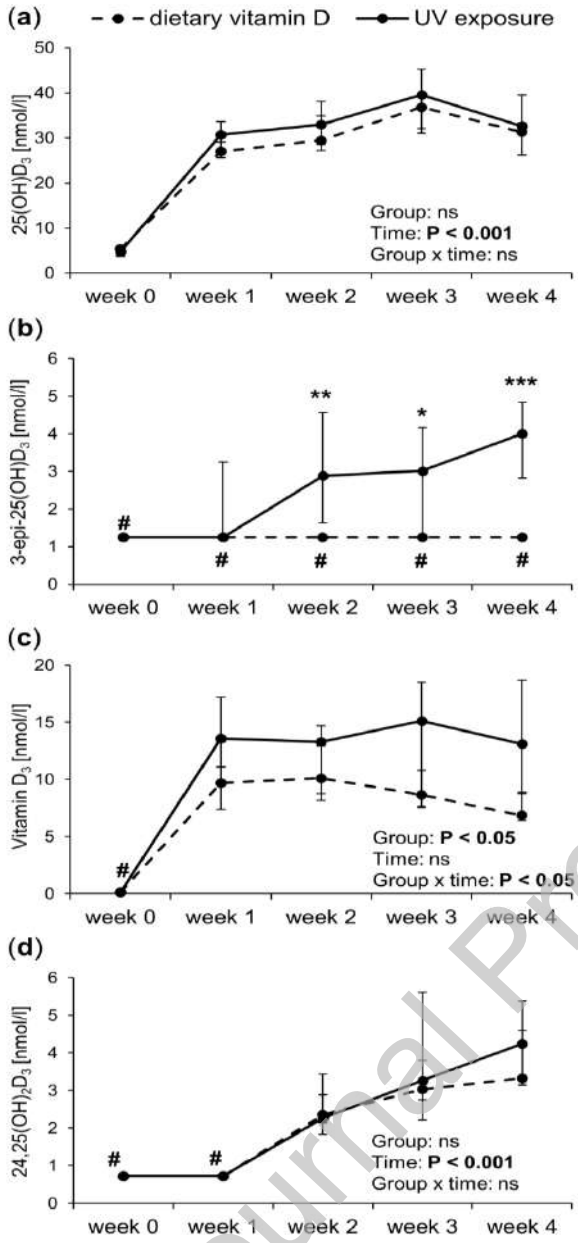

Figure 2: Plasma vitamin D metabolite concentration in pigs over four weeks of intervention. (a) 25-hydroxyvitamin D_3 ($25(OH)D_3$, reflecting the sum of 3-epimerized and non-epimerized $25(OH)D_3$), (b) 3-epi-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$), (c) Vitamin D_3, (d) 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$). Data are reported as median and interquartile range (n = 8). * Significant difference between the groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, (b) Mann-Whitey *U* test, (c) Tukey-Kramer test). # Median values below the limit of quantification (3-epi-$25(OH)D_3$: 2.5 nmol/l, vitamin D_3: 0.26 nmol/l, $24,25(OH)_2D_3$: 1.44 nmol/l).

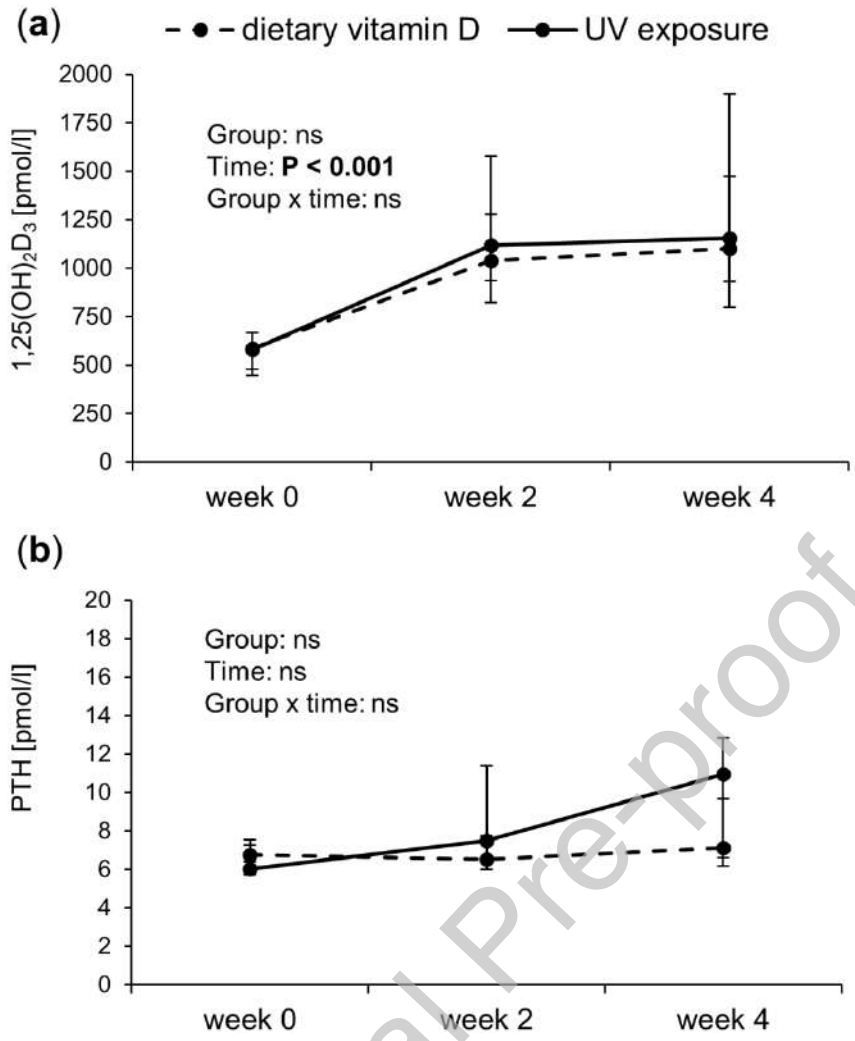

Figure 3: Plasma concentration of (a) 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and (b) parathyroid hormone (PTH) in pigs that were supplemented with 20 µg/d vitamin D_3 or treated with UV light for 19 min/d during four weeks of intervention. Data are reported as median and interquartile range (n = 8). Not significant (ns).

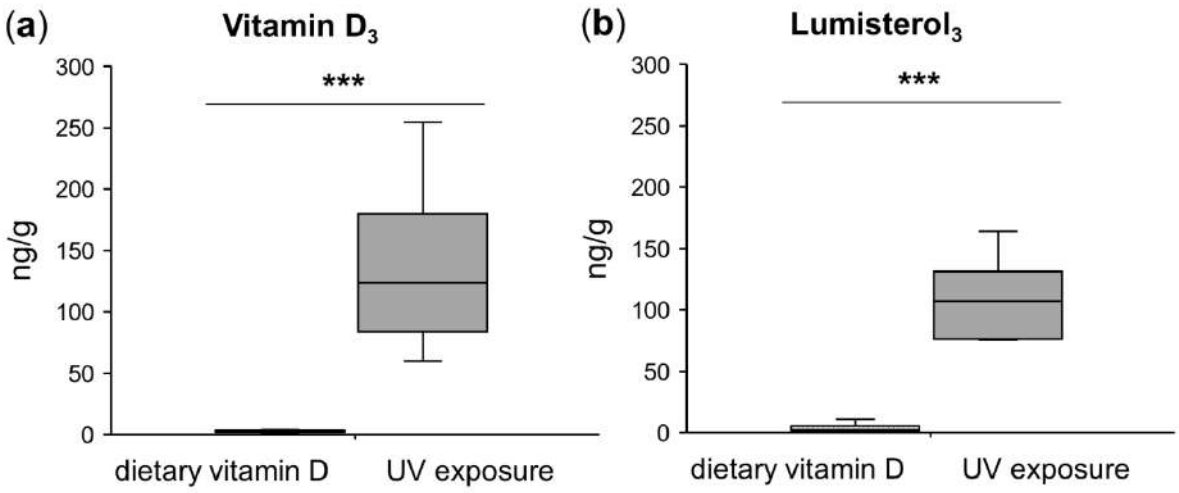

Figure 4: Concentration of (a) vitamin D_3 and (b) lumisterol$_3$ in the skin of pigs that were supplemented with 20 µg/d vitamin D_3 or treated with UV light for 19 min/d after four weeks of intervention. (n = 8) *** Significant difference between the groups (Mann-Whitney *U* test, P < 0.001).

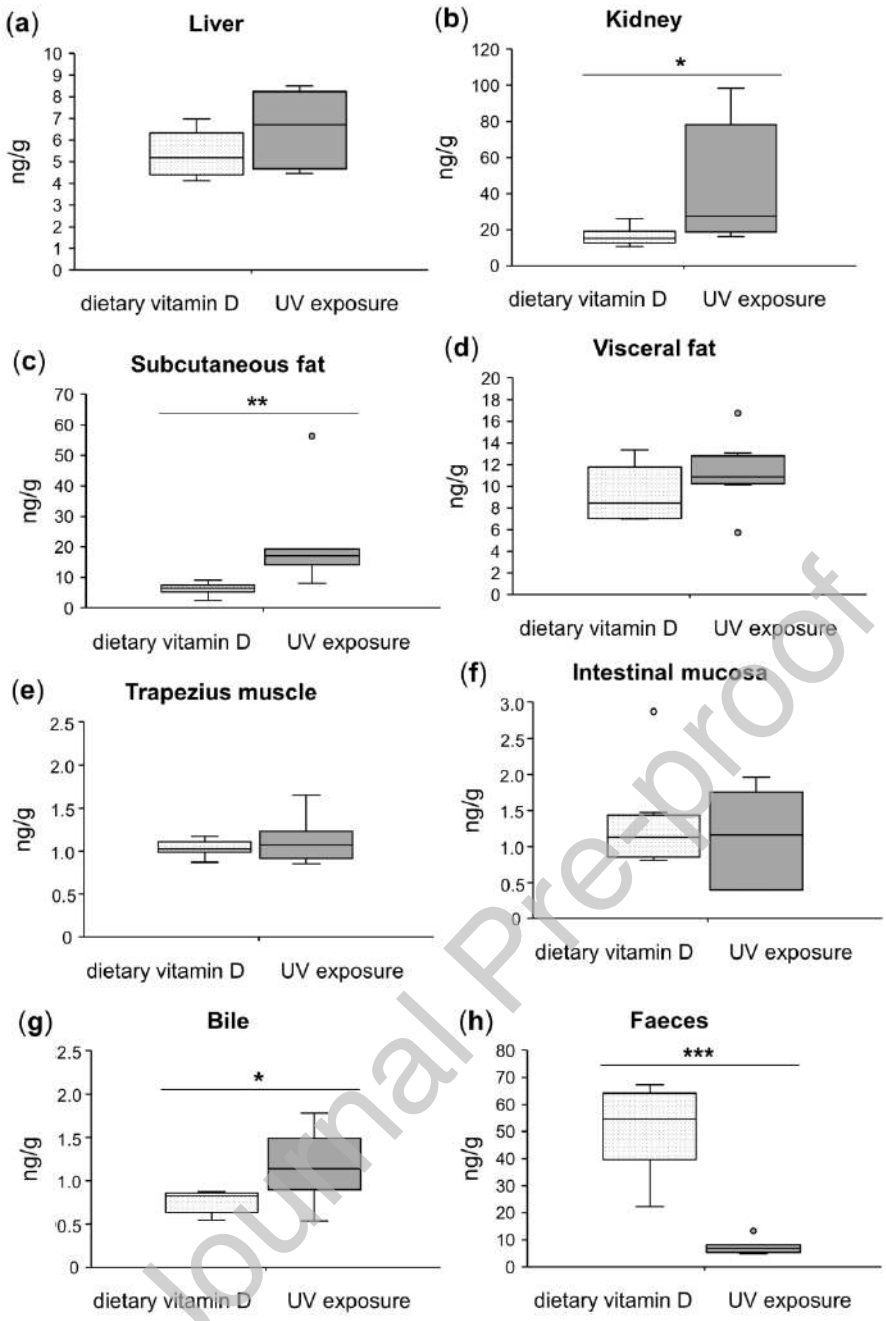

Figure 5: Concentration of vitamin D_3 in samples of pigs that were supplemented with 20 µg/d vitamin D_3 or treated with UV light for 19 min/d after four weeks of intervention. (a) liver, (b) kidney, (c) subcutaneous fat, (d) visceral fat, (e) trapezius muscle, (f) intestinal mucosa, (g) bile, (h) faeces. (n = 8) * Significant difference between the groups (Mann-Whitney *U* test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

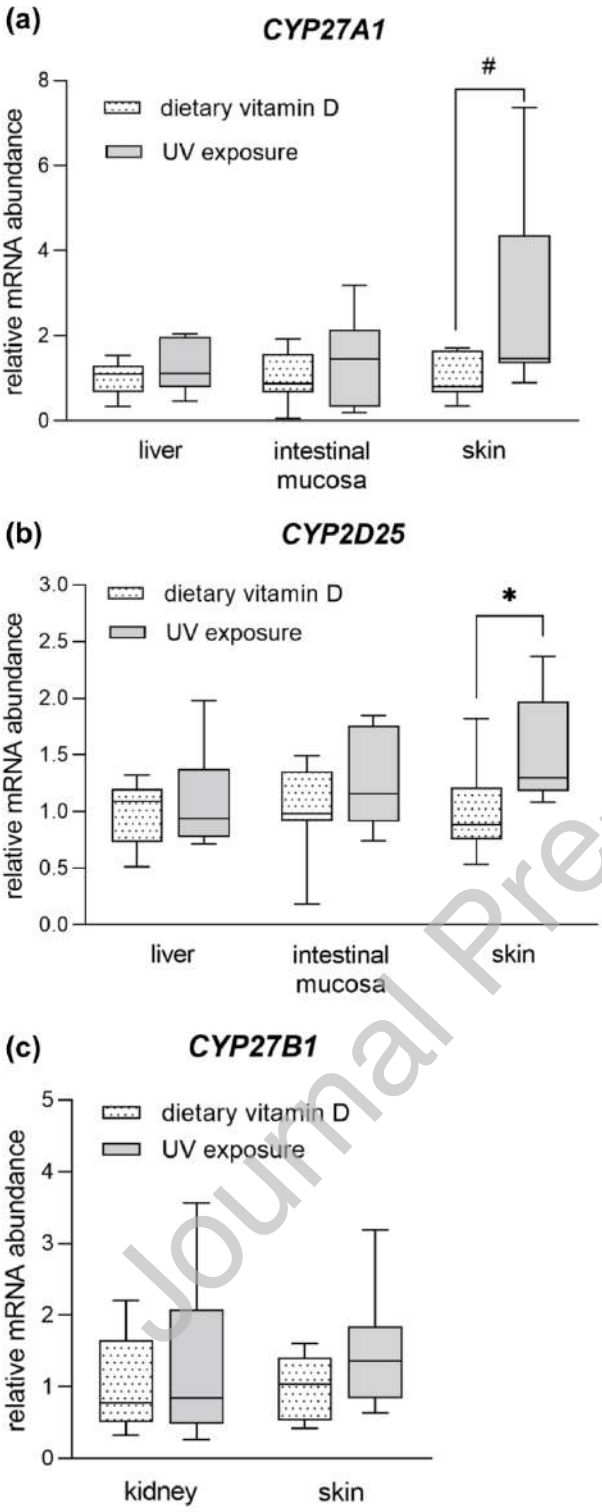

Figure 6: Relative mRNA abundance of vitamin D hydroxylases in tissues of pigs that were supplemented with 20 µg/d vitamin D_3 or treated with UV light for 19 min/d after four weeks of intervention. (a) Cytochrome P450 family 27 subfamily A member 1 (*CYP27A1*), (b) cytochrome P450 family 2 subfamily D member 25 (*CYP2D25*), (c)

cytochrome P450 family 27 subfamily B member 1 (*CYP27B1*). Reference genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein S9 (*RPS9*), (n = 8). * Significant difference between the groups (Mann-Whitney *U* test, $P < 0.05$). # Trend toward significant difference between the groups (Mann-Whitney *U* test, $P < 0.1$).

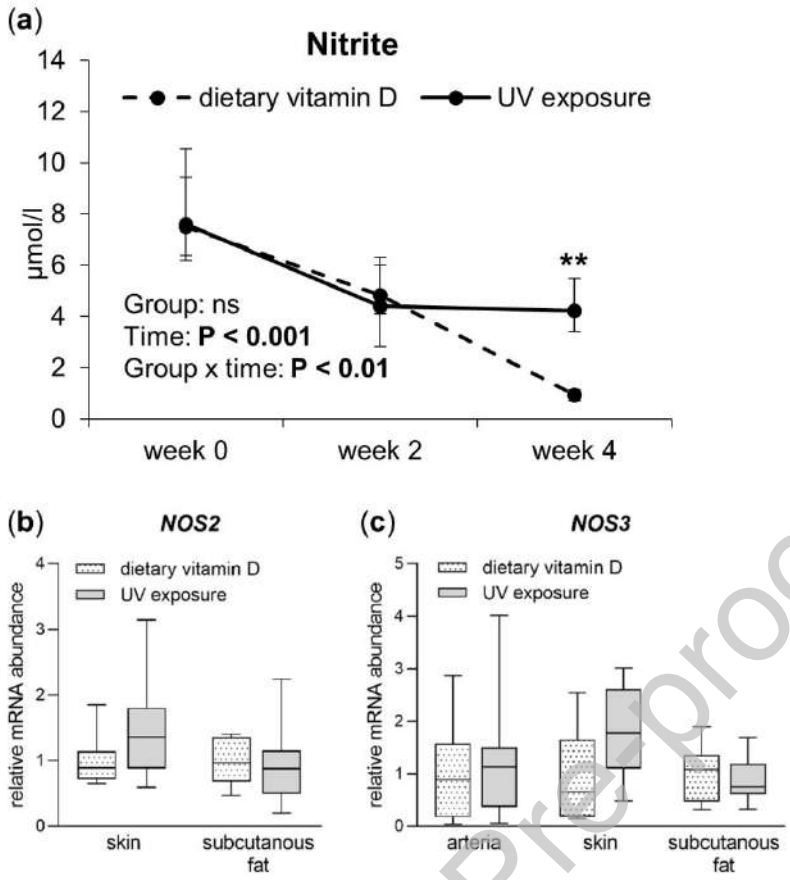

Figure 7: Plasma nitrite concentration and relative mRNA abundance of nitric oxide synthases in pigs that were supplemented with 20 µg/d vitamin D_3 or treated with UV light for 19 min/d. (a) Plasma nitrite reflects the concentration of nitric oxide. Data are reported as median and interquartile range. (b) Inducible nitric oxide synthase (*NOS2*), (c) endothelial nitric oxide synthase (*NOS3*). Data of (b) and (c) were measured after four weeks of treatment. (n = 8). ** Significant difference between the groups ($P < 0.01$, Tukey-Kramer test). Not significant (ns).

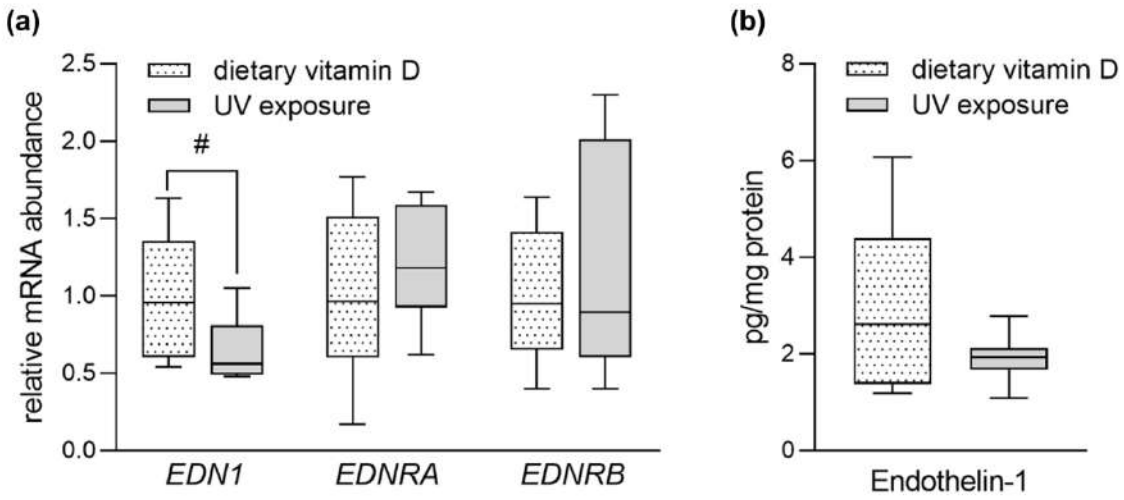

Figure 8: Expression of endothelin-1 and endothelin receptors in the renal artery of pigs that were supplemented with 20 µg/d vitamin D_3 or treated with UV light for 19 min/d after four weeks of intervention. (a) relative mRNA abundance of endothelin-1 (*EDN1*) and endothelin receptor A (*EDNRA*) and B (*EDNRB*), (b) Protein expression of endothelin-1. (n = 8) # Trend toward significant difference between the groups (Mann-Whitney *U* test, P = 0.1).

Table 1: Primer of target and reference genes used for the analysis of mRNA abundance in tissues and PBMC of pigs.

Gene	Obtained from	Product size [bp]
ABCG5	Eurofins Genomics	282
ACTB [1]	Eurofins Genomics	204
CD36	Eurofins Genomics	103
CYP2D25	Sigma-Aldrich[2]	104
CYP27A1	Sigma-Aldrich[2]	107
CYP27B1	Sigma-Aldrich[2]	170
EDN1	Sigma-Aldrich[2]	145
EDNRA	Sigma-Aldrich[2]	149
EDNRB	Sigma-Aldrich[2]	189
GAPDH [1]	Eurofins Genomics	446
IL6	Sigma-Aldrich[2]	125
IL10	Eurofins Genomics	446
NOS2	Sigma-Aldrich[2]	191
NOS3	Sigma-Aldrich[2]	174
NPC1L1	Eurofins Genomics	201
RPS9 [1]	Eurofins Genomics	327
SCARB1	Eurofins Genomics	171
TNF	Sigma-Aldrich[2]	144

[1] Reference gene. [2] Pre-designed primer pair. ATP binding cassette subfamily G member 5 (*ABCG5*), actin beta (*ACTB*), CD36 molecule (*CD36*), cytochrome P450 family 2 subfamily D member 25 (*CYP2D25*), cytochrome P450 family 27 subfamily A member 1 (*CYP27A1*), cytochrome P450 family 27 subfamily B member 1 (*CYP27B1*), endothelin 1 (*EDN1*),

endothelin receptor type A (*EDNRA*), endothelin receptor type B (*EDNRB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), interleukin 6 (*IL6*), interleukin 10 (*IL10*), nitric oxide synthase 2 (*NOS2*), nitric oxide synthase 3 (*NOS3*), NPC1 like intracellular cholesterol transporter 1 (*NPC1L1*), ribosomal protein S9 (*RPS9*), scavenger receptor class B member 1 (SCARB1), tumor necrosis factor (*TNF*).

Table 2: Relative mRNA abundance of transporters expressed in small intestine mucosa and liver of pigs after four weeks of intervention.

	Dietary vitamin D_3		UV exposure		P value
	Median	IQR	Median	IQR	
Intestinal mucosa					
ABCG5	0.84	0.60-1.22	0.78	0.64-0.87	Ns
CD36	0.93	0.78-1.20	1.63	0.87-2.51	< 0.1
NPC1L1	0.87	0.49-1.25	1.54	0.58-2.07	Ns
SCARB1	0.94	0.45-1.53	2.05	0.51-3.05	Ns
Liver					
ABCG5	1.06	0.63-1.24	1.16	0.82-1.25	Ns
NPC1L1	1.07	0.46-1.44	2.01	1.36-2.66	**< 0.05**
SCARB1	1.00	0.74-1.30	1.44	0.82-1.77	Ns

Reference genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein S9 (*RPS9*). ATP binding cassette subfamily G member 5 (*ABCG5*), CD36 molecule (*CD36*), interquartile range (IQR), not significant (ns), NPC1 like intracellular cholesterol transporter 1 (*NPC1L1*), scavenger receptor class B member 1 (*SCARB1*). Groups were compared by the Mann-Whitney *U* test (n = 8).

Table 3: Concentrations of minerals and lipids in pigs during four weeks of intervention.

	Dietary vitamin D_3		UV exposure		P values		
	Median	IQR	Median	IQR	Group	Time	Group x time
Plasma calcium (mmol/l)							
Week 0	2.55	2.47-2.67	2.57	2.49-2.82			
Week 2	2.66	2.39-2.75	2.76	2.65-2.82	Ns	Ns	Ns
Week 4	2.54	2.34-2.73	2.67	2.45-2.85			
Plasma phosphorus (mmol/l)							
Week 0	2.76	2.46-2.96	2.96	2.52-3.45			
Week 2	3.24	3.00-3.60	3.32	3.13-3.55	Ns	**P < 0.001**	Ns
Week 4	4.03	3.84-4.31	4.20	3.97-4.49			
Plasma cholesterol (mmol/l)							
Week 0	2.17	2.08-2.29	2.22	1.91-2.38			
Week 2	2.54	2.26-2.88	2.75	2.36-3.40	Ns	**P < 0.01**	Ns
Week 4	2.77	2.63-2.88	2.57	2.45-3.02			
Plasma triglycerides (mmol/l)							
Week 0	0.42	0.35-0.56	0.42	0.29-0.57			
Week 2	0.51	0.42-0.61	0.50	0.43-0.58	Ns	**P < 0.01**	Ns
Week 4	0.57	0.34-0.90	0.56	0.51-0.93			
Hepatic cholesterol (mg/g)							
Week 4	2.56	2.28-2.73	2.31	1.79-2.41		Ns[1]	
Biliary cholesterol (mg/g)							
Week 4	3.96	3.12-4.46	3.32	2.99-3.63		Ns[1]	

Interquartile range (IQR), not significant (ns). Plasma concentrations with repeated measures were statistically analysed by the *mixed-model procedure*. [1] Tissue concentrations without repeated measures were analysed by the Mann-Whitney *U* test (n = 8).

Table 4: Cytokine expression in stimulated peripheral blood mononuclear cells (PBMC) of pigs after four weeks of intervention.

	Dietary vitamin D_3		UV exposure		P value
	Median	IQR	Median	IQR	
Relative mRNA abundance[1]					
IL6	1.05	0.61-1.38	0.49	0.13-1.26	Ns
IL10	1.03	0.80-1.19	0.46	0.38-0.79	< **0.05**
TNF	0.99	0.91-1.10	0.42	0.30-0.67	< **0.05**
Cytokine concentration [pg/mg protein]					
IL6	283	184-482	226	81-355	Ns
IL10	140	121-404	83	65-100	< **0.05**
TNF-alpha	3236	2261-3892	863	457-1276	< **0.05**

[1] Reference genes: actin beta (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Interleukin 6 (*IL6*), interleukin 10 (*IL10*), interquartile range (IQR), not significant (ns), tumor necrosis factor (*TNF*). Groups were compared by the Mann-Whitney *U* test (n = 5).