Vitamin D metabolism in growing pigs: Influence of UVB irradiation and dietary vitamin D supply on calcium homeostasis, its regulation and bone metabolism

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Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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2017

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Summary

The aim of this study was to prove if pigs are able to synthesise vitamin D in the skin and to investigate the influence of UVB irradiation on vitD status and Ca homeostasis of growing pigs. Thirty-two eleven-week-old pigs were kept without access to sunlight and divided into four groups receiving the following treatment in a 2x2-factorial-design: a) UVB irradiation or not and b) vitD in feed or not. Blood, urine and faeces were sampled every third week. In serum, vitD metabolites, Ca, P, Mg and bone markers were analysed. Digestibility of Ca, P and Mg as well as urinary excretion of these minerals were analysed. After 14 weeks, the animals were slaughtered and samples of skin, intestines, kidneys and bones were taken for further analyses. Irradiated animals showed higher levels of 7-DHC and tachysterol in the skin, higher levels of 25-OH-D and 1,25-$(OH)_2$-D in the serum and higher Ca net flux rates were determined in Ussing chambers. In contrast, the expression of genes involved in Ca transport in the intestines and kidneys was not altered. Similarly, the digestibility of Ca and P as well as the urinary excretion were not affected. With respect to the metatarsus, no differences in mineral contents and bone mineral density were found between groups. Some subclinical signs of beginning vitD insufficiency were observed in the group without access to vitD (represented by higher expression of 1α-hydroxylase in the kidney and increased PTH in serum).

Keywords: bone mineral content, peripheral quantitative computed tomography, skin, Ussing chamber

Zusammenfassung

Ziel der Studie war es, zu beweisen, dass Schweine Vitamin D in der Haut produzieren können sowie den Einfluss von UVB Bestrahlung auf den VitD Status und die Ca Homöostase von wachsenden Schweinen zu untersuchen. Dazu wurden 32 Schweine ab dem Alter von 11 Wochen ohne Zugang zu Sonnenlicht in 4 Gruppen aufgeteilt und unter folgenden Bedingungen gehalten: a) UVB Bestrahlung oder nicht und b) VitD im Futter oder nicht. Alle 3 Wochen wurden Blut-, Urin- und Kotproben genommen. Im Serum wurde der Gehalt der VitD-Metaboliten, Ca, P, Mg und der Knochenmarker bestimmt. Ebenso wurde die Verdaulichkeit und die renale Ausscheidung von Ca, P und Mg bestimmt. Nach 14 Wochen wurden die Tiere geschlachtet und Proben der Haut, der Därme, der Niere und der Knochen entnommen. Bestrahlte Tiere zeigten einen höheren Gehalt von 7-DHC und Tachysterol in der Haut und höhere Konzentrationen von 25-OH-D und 1,25-$(OH)_2$-D im Serum. Mittels Ussingkammer wurden bei bestrahlten Tiere höhere Ca-Netto-Fluxe gemessen. Im Gegensatz dazu war die Expression der Gene, welche am aktiven Ca-Transport in Darm und Niere beteiligt sind, nicht beeinflusst. Ebenso war die Verdaulichkeit und die renale Ausscheidung von Ca und P nicht beeinflusst. Im Metatarsus wurden zwischen den Gruppen keine Unterschiede im Mineralgehalt und Knochendichte gefunden. Die Gruppe ohne Zugang zu VitD zeigte subklinishce Anzeichen einer VitD-Insuffizienz (höhere Expression der 1α-Hydroxylase in der Niere, erhöhtes PTH im Serum).

Schlüsselwörter: Mineralgehalt der Knochen, peripheral quantitative computed tomography, Haut, Ussing-Kammer

Manuskript

Diese Arbeit wurde im Journal of Animal Physiology and Animal Nutrition zur Publikation publiziert:
J Anim Physiol Anim Nutr. 2017; 101 Suppl1:79-94. DOI: 10.1111/jpn.12707

Vitamin D metabolism in growing pigs:

Influence of UVB irradiation and dietary vitamin D supply on calcium homeostasis, its regulation and bone metabolism

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SUMMARY

The aim of this study was to prove if pigs are able to synthesise vitamin D (vitD) in the skin and to investigate the influence of ultraviolet irradiation (UVB) on vitD status and calcium (Ca) homeostasis of growing pigs. Thirty-two eleven-week-old pigs were kept without access to sunlight and divided into four groups receiving the following treatment in a 2x2-factorial-design: a) UVB irradiation or not and b) vitD in feed or not. Blood, urine and faeces were sampled every third week. In serum, vitD metabolites, Ca, phosphorus (P), magnesium (Mg) and bone markers were analysed. Digestibility of Ca, P and Mg as well as urinary excretion of these minerals were analysed. After 14 weeks, the animals were slaughtered and samples of skin, intestines, kidneys and bones (metatarsus) were taken for further analyses: sterols of vitD-synthesis in the skin, Ca flux rates in the intestines, expression of genes involved in Ca transport in the intestines and kidneys, bone mineral density (BMD) with the aid of peripheral quantitative computer tomography and bone mineral content by ashing the metatarsus. Irradiated animals showed higher levels of 7-dehydrocholesterol and tachysterol in the skin, higher levels of 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol in the serum and higher Ca net flux rates were determined in Ussing chambers. In contrast, the expression of genes involved in Ca transport in the intestines and kidneys was not altered. Similarly, the digestibility of Ca and P as well as the urinary excretion were not affected. With respect to the metatarsus, no differences in mineral contents and BMD were found between groups. At the end of the study some subclinical signs of beginning vitD "insufficiency" were observed in the group without access to vitD (represented by higher expression of 1α-hydroxylase in the kidney and increased parathyroid hormone in serum).

Key words: bone mineral content, intestinal absorption of macro minerals, parathyroid hormone, skin, urinary excretion of macro minerals, Ussing chamber

INTRODUCTION

Vitamin D (vitD, here representing vitD_2, ergocalciferol, and vitD_3, cholecalciferol) requirements of humans and animals can be met either by supply from dietary sources or by cutaneous synthesis of vitD_3. The synthesis of vitD in the skin was demonstrated for many species such as human (Holick et al., 1980, Adams et al., 1982), rat (Holick et al., 1979), cattle (Hymoller and Jensen, 2010), sheep, goat (Kohler et al., 2013, Kovacs et al., 2015), guinea pig (Watson et al., 2014), rabbit (Emerson et al., 2014) and recently for pigs (Burild et al., 2015) and hens (Kuehn et al., 2015). Ultraviolet B irradiation (UVB, 290 – 315 nm) changes 7-dehydrocholesterol (7-DHC, provitamin D_3) in the skin to previtamin D_3 (previtD_3) which is instable and converts heat-dependent (faster at warmer temperatures) into vitD_3. Under further irradiation, previtD_3 is changed into lumisterol and tachysterol (Webb and Holick, 1988). However, different factors influence the vitD status: either the UVB irradiation reaching (latitude, season, time, clothes, sun blocker – humans) or penetrating (pigmentation) the skin as well as the 7-DHC-content in the skin which is age-dependent (Holick, 1995). Because the livestock husbandry systems restrict the access to sunlight for farm animals, they are mainly dependent on dietary vitD sources (Dittmer and Thompson, 2011).

Vitamin D is bound to vitamin-D-binding-protein (DBP; Haddad et al., 1993) and transported to the liver where it is transformed to 25-hydroxy-vitamin D (25-OH-D, Calcidiol). This is the main metabolite in the blood and the most suitable to evaluate the vitD status (Adams et al., 1982, Watson et al., 2014), because of its good correlation with vitD supply and its long half-life time (10 days for swine; Flohr et al., 2014). Transferred to different tissues (primarily fat, muscle and liver) it can be stored in the body (Heaney et al., 2009, Arnold et al., 2015). In the kidneys, 25-OH-D is metabolised to 1,25-dihydroxy-vitamin D (1,25-$(OH)_2$-D, Calcitriol), the most active metabolite, by the 1α-hydroxylase (CYP27B1). This enzyme is stimulated in the kidney by parathyroid hormone (PTH) and inhibited by calcium (Ca), phosphorus (P) and 1,25-$(OH)_2$-D (Lips, 2006, Dittmer and Thompson, 2011, Bikle, 2012). Both metabolites (25-OH-D and 1,25-$(OH)_2$-D) are degraded by 24-hydroxylase (CYP24A1) in the kidney and skin (Holick, 2007), stimulated by high serum levels of themselves and P and inhibited by PTH (Dittmer and Thompson, 2011). The main function of 1,25-$(OH)_2$-D is the maintenance of Ca homeostasis, although it is also involved in cell differentiation and immunomodulatory functions (Dittmer and Thompson, 2011).

In the intestines and kidney, 1,25-$(OH)_2$-D is responsible for active Ca absorption and reabsorption, respectively. Binding to the cellular vitamin-D-receptor (VDR) it stimulates the

expression of different proteins involved in Ca transport (Bronner, 2003). In bone, 1,25-$(OH)_2$-D mobilises Ca to maintain the Ca concentration in the blood (Dittmer and Thompson, 2011). This effect depends on PTH (stimulated by low Ca in the blood; Lips and van Schoor, 2011).
In humans, two levels of lack of vitD are defined as vitD deficiency and "insufficiency": vitD deficiency means 25-OH-D levels below 12.5 nmol/l and is accompanied by hypocalcaemia and rickets. Levels of 25-OH-D between 13 and 50 nmol/l indicate a vitD "insufficiency" and are accompanied with increase of PTH, bone loss and risk of fractures (Chapuy et al., 1997, Need, 2006), but no clinical signs can be observed.
The aim of this study was to show the ability of pigs to produce vitD in the skin, the influence of the vitD source on vitD status in blood and its effect on intestinal Ca absorption, renal Ca excretion and bone metabolism in growing pigs. The hypothesis was that pigs are able to synthesise enough vitD in the skin under the influence of UVB irradiation with all the respective consequences for their vitD blood levels and associated parameters.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the official veterinary authority of the canton of Zurich (Switzerland, No. 162/2012.) and were in accordance with the animal welfare law of Switzerland.

Animals and treatments

In this trial, 32 Swiss Large White fattening pigs that originated all from the same breeding farm were used during a 14-week trial (age 11 weeks until 6 months). They were housed under controlled conditions (21 to 19 °C, 12:12-h light-dark cycle by artificial light, no sunlight) on concrete floor and straw bedding as lying area. Animals were fed individually restrictively meeting all nutritional requirements (Stoll et al., 2004) and water for drinking was available *ad libitum*. All animals were weighed weekly.
The animals were randomly assigned to four groups (4 gilts and 4 barrows in each group) and the following treatments were applied in a multifactorial 2x2 design: UV irradiation with a minimum of 23 µW/cm^2 for 75 to 105 min a week (15 to 21 min daily, 5 days per week) applied in the morning. An UV-lamp (Ultra-Vitalux 300-230 E27, OSRAM GmbH, Munich, Germany) was used as UV-source and irradiation was daily measured with a UVB-Radiometer (Solarmeter 6.2, Solartech, Glenside, Pennsylvania, USA, response range of 280 – 320 nm) on pigs back level in the centre between two lamps. Diet variant D was a standard mixture for pigs (UFA 331-3, UFA AG, Herzogenbuchsee, Switzerland, Table 1) with 1,000 IU vitD/kg feed,

variant NO only differed in vitD-content (no vitD supplemented). Group UV+/D+ received UV irradiation and was fed with variant D, groups UV-/D+ and UV-/D- were fed with variant D and NO respectively and did not receive UV irradiation, group UV+/D- received UV irradiation and was fed with variant NO.

Collection and analysis of blood, urine and faeces samples

In weeks 0, 3, 6, 9 and 13, urine, blood and faeces samples were collected.

Blood samples were taken before morning feeding from the jugular vein (S-Monovette® 9ml Z, Sarstedt, Nümbrecht, Germany and Neopoint® disposable needle 18Gx1½", Servoprax GmbH, Wesel, Germany). The blood was centrifuged (3000*g*, 20 min) within 30 min after sampling and the serum was stored at -20 °C and -80 °C until analyses for 25-OH-D, 1,25-$(OH)_2$-D, Ca, P, magnesium (Mg), PTH, alkaline phosphatase (AP), osteocalcin (OC) and serum crosslaps

Table 1: Composition of the used feed mixtures (complete diet)

	Variant D		**Variant NO**	
	Manufacturers specifications	analysed	Manufacturers specifications	analysed
Crude ash, g/kg	40	41.5	40	47.2
Crude protein, g/kg	150	152	150	156
Crude fat, g/kg	35	38.0	35	36.8
Crude fiber, g/kg	35	38.6	35	37.4
Digestible Energy, MJ/kg	13.6		13.6	
Lysin, g/kg	10		10	
Methionin, g/kg	6		6	
Calcium, g/kg	6	6.91	6	7.34
Phosphor, g/kg	4	3.99	4	4.23
Magnesium, g/kg	No declaration	1.75	No declaration	1.75
Sodium, g/kg	1.5		1.5	
Vitamin A, IU/kg	8,000		8,000	
Vitamin D_3, IU/kg	1,000	912	0	< 100*
Vitamin E, IU/kg	40		40	
Iron, mg/kg	65		65	
Copper, mg/kg	10		10	
Zinc, mg/kg	65		65	
Manganese, mg/kg	20		20	
Iodine, mg/kg	1.05		1.05	
Selenium, mg/kg	0.25		0.25	
Natuphos 5000 L, mg/kg	100		100	
Phytase units, FTU/kg†	500		500	

* detection limit: 100 IU/kg

† 500 FTU/kg feed replace 0.8 g available P/kg feed (Schmid, 2011)

(SCL) by commercial available test kits as in previous studies (ALP IFCC for AP, CALC CPC for Ca, PHOS for P, MG for Mg from Diatools AG, Villmergen, Switzerland, Osteocalcin EIA Kit, MDSS GmbH, Hannover, Germany for OC, 25-Hydroxy Vitamin D RIA for 25-OH-D, 1,25-Dihydroxy Vitamin D RIA for 1,25-$(OH)_2$-D and Serum Crosslaps ELISA for SCL all from Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany, Porcine Intact PTH ELISA Kit, Immunotopics Inc., San Clemente, USA for PTH, Buehler et al., 2010, Schmid, 2011, Kovacs et al., 2015). Colorimetric measurements for Ca, P and Mg determination were done with an autoanalyzer (Cobas Mira Roche-autoanalyzer, F. Hoffman-La Roche Ltd., Basel, Switzerland). The bone turnover ratio was calculated (SCL : OC*1000, following called SCL:OC, Kovacs et al., 2015) to characterise the bone remodeling.

First spontaneous urine in the morning was sampled, cooled at 5 °C and pH was measured (827 pH Lab, Metrohm AG, Herisau, Switzerland) within 90 min. Afterwards, the urine was frozen and stored by -20 °C until analyses for dissolved Ca, P and Mg using the same tests as in serum after hydrolysis in hydrochloric acid. Creatinin (Crea) was analysed using a commercial available test kit (CREA JAFFE, Diatools AG, Villmergen, Switzerland, Kovacs et al., 2015). Results of Ca, P and Mg were related to Crea.

Faeces samples were taken from the rectum during 5 days, pooled per animal and stored at -20 °C. Acid detergent fiber (ADF), acid detergent lignin (ADL) and hydrochlorid acid-insoluble ash (HCl-insoluble ash) were analysed by van Soest et al. (1991) and proximate analysis. In crude ash Ca, P and Mg were analysed after hydrolysis in hydrochloric acid using the same tests as in serum.

Collection and Analyses of Bone Samples

Bone mineral density (BMD) and cortical bone thickness (CBT) of the left metatarsus were measured by peripheral quantitative computed tomography (pQCT, XCT 960A bone scanner, Stratec Medizinaltechnik, Pforzheim, Germany) in week 0 before starting the experiment and after slaughter. Measurements were done in the diaphysis (50% of the length, cortical and total BMD, CBT, cortical mode 2, threshold for cortical bone >640 mg/cm^3) and distal metaphysis (10% of the length, trabecular and total BMD, peel mode 2, threshold for trabecular bone >710 mg/cm^3) and parameters were calculated by automated computation (Liesegang et al., 2005). For the first measurement, animals were anesthetised intravenously (V. auricularis) with ketamine 15 mg, azaperone 2 mg and butorphanol 0.2 mg per kg bodyweight. After slaughtering the left metatarsus was collected, prepared from tissue and stored at -20 °C until the second measurement was performed.

After analyses by pQCT, all bones were ashed (dried at 105 ºC for 118 hours, ashed at 600 ºC for 128 hours and dissolved in 250 ml of 12% HCl) and contents of Ca, Mg and P were analysed as in faeces.

Collection and analysis of skin samples

At slaughtering, skin samples from the forehead, neck and back (all clipped in the evening before) were collected, frozen with liquid nitrogen and stored at -80 °C. They were prepared as described by Morris (1999) with small modifications and analysed for 7-DHC, previtD_3, vitD_3, tachysterol and lumisterol by high performance liquid chromatography (Kovacs et al., 2015).

Collection and analysis of intestine and kidney samples

Samples from duodenum (behind the minor duodenal papilla), jejunum (middle), caecum (apex, ventral *haustra*) and kidney (cross section) were collected. Parts of duodenum and jejunum were used for determination of active Ca fluxes (nmol/h/cm^2, J_{sm} = sero-mucosal, J_{ms} = mucosa-serosal) by a modified Ussing-Chamber experiment (Sidler-Lauff et al., 2010). Net Ca flux was calculated from the mean unidirectional flux rates ($J_{net} = J_{ms} - J_{sm}$).

From the remaining intestine samples, the mucosal layer was mechanically removed and like the kidney frozen in liquid nitrogen and stored at -80 °C. Expression of VDR, transient receptor potential vanilloid 6 (TRPV6), Calbindin-D9k (Calb-D9k) and the basal CaATPase (PMCa1b) on mRNA level was measured in the intestines. In the kidney, expression of VDR, Calb-D28k, 1α-hydroxylase and 24-hydroxylase was measured.

For RNA isolation 20mg of tissue was homogenized in 500µl TRI Reagent (TRI Reagent® Solution, Ambion, Rotkreuz, Switzerland) using a handheld homogenizer (Polytron PT 1200E, Kinematica, Lucerne, Switzerland). After 5 min of incubation at room temperature (RT) 50µl of 1-Bromo-3-Chloropropane were added followed by another 5 – 15 min of incubation at RT. Samples were then centrifuged for 15 min at 12,000*g* at 4 ºC and the aqueous phase was transferred into a fresh tube for further processing. After adding 250µl isopropanol the sample was vortexed for 10s and incubated at RT for 5 – 10 min followed by centrifugation with 12,000*g* for 8 min at 4 – 25 ºC. Once the supernatant was discarded, 500µl of 75% ethanol were added and the samples were centrifuged with 7,500*g* for 5 min at 4 – 25 °C. The ethanol was then removed, the RNA pellet briefly air dried and finally dissolved in 200µl RNAse-free water. Concentrations were measured by spectrophotometry using NanoDrop. Previously measured RNA samples served as a template for reverse transcription by using a commercial kit (QuantiTect Revese Transcription Kit, Qiagen, Hombrechtikon, Switzerland). All steps were performed according to the manufacturer's instructions using 1,000ng RNA in a reaction volume of 20µl.

For quantification of RNA expression specific primers and probes (Table 2) were purchased.
For quantification of TRPV6, Calb-D9k, PMCA1b and β-actin as housekeeping-gene, reaction mixtures (20µl) contained TaqMan™ Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), 50pM specific primers, 25pM specific probe and an amount of 50ng reverse transcribed RNA. PCR products were amplified (50 °C, 2 min, 95 °C, 10 min, 40 cycles of 95 °C, 15 s and 60 °C, 1 min) and analysed on a real-time PCR cycler (CFX96™, Bio-Rad, Munich, Germany). Absolute copy numbers were determined using calibration curves generated with cloned PCR fragment standards as described elsewhere (Wilkens et al., 2009).
For quantification of VDR, Calb-D28k, 1α-hydroxylase, 24-hydroxylase and β-Actin (for VDR) and GAPDH as housekeeping genes, reaction mixtures (20µl) contained TaqMan™ Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), 1µM specific primers, 0.75µM specific probe and an amount of 50ng reverse transcribed RNA. PCR products were amplified (95 °C, 2 s, 45 cycles of 95 °C, 3 s and 60 °C, 30s except for 24-hydroxylase: 50 °C, 2 min, 95 °C, 10 min, 40 cycles of 95 °C, 15 s and 60 °C, 1 min) and analysed on a 7500 Fast Real Time PCR System (ABI 7500Fast Sequence Detection System, Life Technologies, Waltham, USA). CT-values were determined with a threshold of 0.1.
Efficiency of the different PCR assays tested in advance for dilutions of intestinal or renal cDNA and cloned standard ranged from 90 to 120%. Specificity of the amplicons of TRPV6, Calb-D9k and PMCa1b was verified using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Parallel PCR assays for each gene were performed with cDNA samples, plasmid standards and a no-template control containing water. Each series of experiments was carried out twice.

Table 2: Primers and probes used for TaqMan™ assays (5′→ 3′)

Gene	Sense and antisense primers	Probes
β-actin*	ctcgatcatgaagtgcgacgt gtgatctccttctgcatcctgtc	HEX-atcaggaaggacctctacgccaacacgg-BHQ1
GAPDH†	gtcaccagggctgcttttaa atctcgctcctggaagatgg	FAM-aagttccacggcacagtcaa-BHQ-1
TRPV6*	ctgcccttcatgtacagcgtc gtcccccagcccaaactt	6FAM-ccgctctgctcatgctcaacctcctta-BBQ
Calb-D9k*	ccaggacaccaaaatgagtgc gggttctcggacctttcagtaaa	6FAM-tggggaattcagcctgaatcagttgct-BBQ
PMCA1b*	tctaaaagaagctggtcatggaacac tcttgattctggcttttctaaccct	6FAM-ctcttccacatcctctgctaactcctcct-BBQ
Calb-D28k†	cagaatcccacctgcaatca tcaagttctgaagctcctttcc	FAM-ctggcttcatttcgacgctga-BHQ-1
CYP27b1†	cactggtcactctgtgtcact cgcttgccaaagccaaagg	FAM-acttcaagggaccctgccca-BHQ-1

*TIB MOLBIOL, Berlin, Germany
† Mycrosynth AG, Balgach, Switzerland

Collection and analysis of feed samples

Samples of both feed variants were taken during the whole experiment, pooled over time and analysed for contents of $vitD_2$ and $vitD_3$ by an external laboratory (Eurofins Scientific AG, Schönenwerd, Switzerland, EN 12821:2009-08). The $vitD_2$ content in both feed variants D and NO was under the detection limit of 100 IU/kg. The $vitD_3$ content of feed variant NO was also under the detection limit while in feed variant D it was 912 IU/kg (Table 1).

Acid detergent fiber, ADL, HCl-insoluble ash, Ca, P and Mg of feed variants D and NO were analysed as in faeces and used for calculation of apparent digestibility of Ca, P and Mg (determined with HCl-insoluble ash, Stangl, 2014).

$$\text{apparent digestibility (\%)} = 100 - \left(\frac{Indicator\ in\ feed}{Indicator\ in\ faeces} x \frac{Mineral\ in\ faeces}{Mineral\ in\ feed}\right) x100$$

Statistical analyses

Statistical analyses were performed with Systat® (Version 11 for Windows, Systat Software Inc., San Jose, California, USA). All parameters were tested for normal distribution by Shapiro-Wilk test. Effects of irradiation (UV), feed (D) and time were tested by 2way ANOVA. Results of not normal distributed variabels were verified with non-parametric tests (Kruskal-Wallis between groups, Wilcoxon sign ranks test within groups). Correlations were tested by t-test. The level of significance was set to $p<0.05$ for all analyses.

RESULTS

Bodyweight

Bodyweight did not differ significantly between the groups at any time but increased weekly in all groups due to normal growth from 26.6±1.90 kg before starting up to 108.2±5.33 kg at slaughtering (mean daily weight gain of 0.899±0.055 kg).

Vitamin D metabolites and PTH in the serum

Mean serum concentrations of 25-OH-D and 1,25-$(OH)_2$-D both increased in irradiated groups (UV+/D+, UV+/D-, $p<0.001$ for both, Figure 1) compared to unirradiated groups (UV-/D+, UV-/D-). The mean serum concentration of 25-OH-D revealed higher levels in groups fed with vitD (UV-/D+, UV+/D+, $p=0.003$).

Mean serum 25-OH-D concentration increased in irradiated groups (UV+/D+, UV+/D-) from week 0 until week 13 and 9, respectively. In group UV-/D- 25-OH-D decreased between week 0 and 6. In group UV-/D+ an increase was observed from week 6 until week 13.

Mean serum concentration of 1,25-$(OH)_2$-D decreased from week 3 until the end in unirradiated groups (UV-/D-, UV-/D+) while in irradiated groups (UV+/D-, UV+/D+) the levels decreased only after week 6 and 9 respectively.

For PTH no influence of irradiation (p=0.265) but a trend for vitD in feed (higher levels in groups fed without vitD, p=0.077) was found. Over all groups a decrease between week 0 and week 3 and an increase after week 6 was observed (p<0.001, Figure 2).

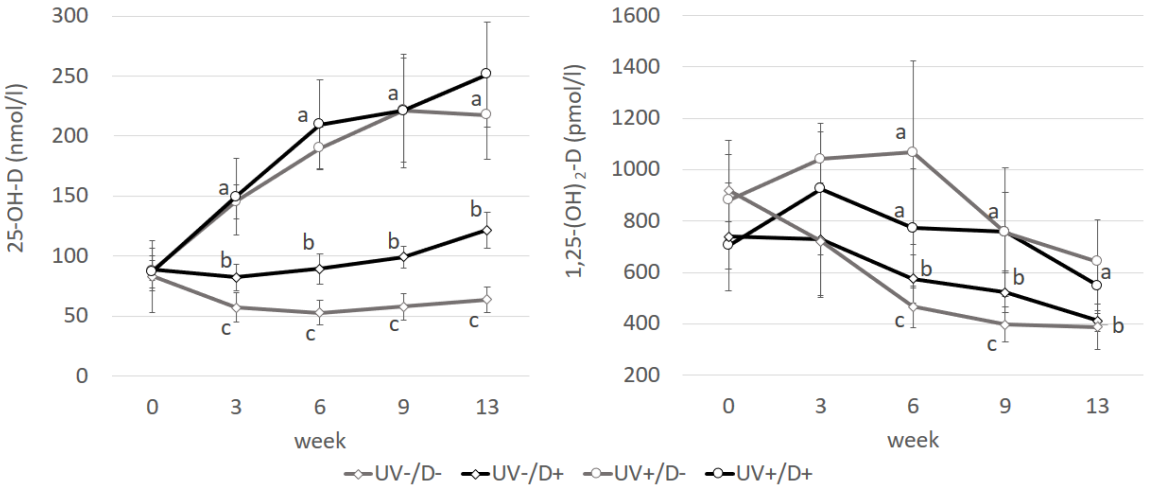

Figure 1: Mean 25-OH-D and 1,25-$(OH)_2$-D concentrations in serum in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respective equivalents exposed to irradiation (UV+/D- and UV+/D+). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Groups within one time point with different letters differ significantly (p<0.05). Mean±SD

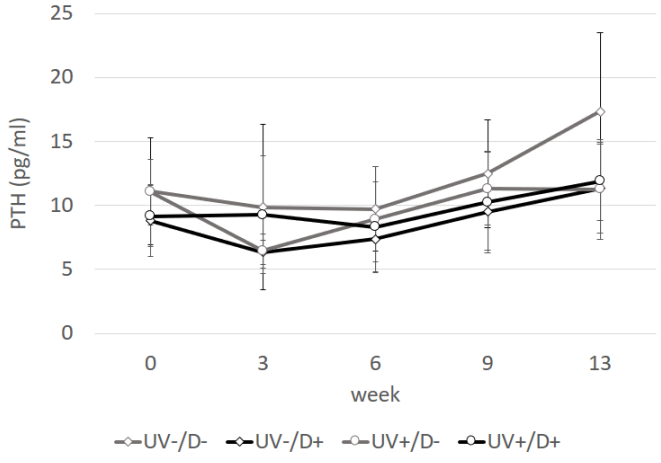

Figure 2: Mean PTH concentration in serum in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respective equivalents exposed to irradiation (UV+/D- and UV+/D+). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Groups within one time point with different letters differ significantly (p<0.05). Mean±SD

Minerals and bone markers in serum

The mean concentrations of Ca, P and Mg in the serum did not differ between groups but time effects could be observed (Table 3). For bone markers (SCL, OC, AP, SCL:OC) no influence of irradiation or vitD in feed was observed except an interaction-effect for OC in week 3 (Table 4). Differences between the time points were detectable for all parameters. Mean SCL concentration and therefore SCL:OC increased over time after week 3. Mean serum OC concentrations increased between week 0 and 3 and declined between week 6 and 9, while mean serum AP concentration declined continuously after week 3. No correlations with each other or with 25-OH-D were found.

Table 3: Serum concentrations of minerals in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D+). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Kruskal-Wallis test was used for data that were not normally distributed. Not significant: n.s., not applied: n.a.. Mean± SD

	UV-/D-	UV-/D+	UV+/D-	UV+/D+	2way ANOVA			Kruskal-Wallis
					UV	D	UV x D	
Calcium, mmol/l								
Week 0	2.57±0.33	2.59±0.47	2.54±0.40	2.66±0.39	n.s.	n.s.	n.s.	
Week 3	2.67±0.29[x]	2.68±0.47	2.69±0.34	2.68±0.36	n.s.	n.s.	n.s.	
Week 6	2.56±0.36[x]	2.58±0.35	2.61±0.32	2.60±0.41	n.s.	n.s.	n.s.	
Week 9	2.50±0.33	2.66±0.30	2.69±0.15	2.61±0.33	n.a.	n.a.	n.a.	n.s.
Week 13	2.53±0.31	2.61±0.42	2.72±0.25	2.58±0.37	n.a.	n.a.	n.a.	n.s.
p-value time	0.039	n.s.	n.s.	n.s.				
Phosphorus, mmol/l								
Week 0	2.04±0.21	2.20±0.31	2.05±0.20	2.06±0.26	n.s.	n.s.	n.s.	
Week 3	2.13±0.21	2.16±0.16	2.07±0.22	2.12±0.30	n.s.	n.s.	n.s.	
Week 6	2.20±0.25	2.10±0.23	2.14±0.24	2.14±0.24	n.s.	n.s.	n.s.	
Week 9	2.22±0.13	2.26±0.20	2.19±0.14	2.23±0.22	n.s.	n.s.	n.s.	
Week 13	2.22±0.05	2.22±0.12	2.16±0.09	2.28±0.20	n.s.	n.s.	n.s.	
p-value time	n.s.	n.s.	n.s.	n.s.				
Magnesium, mmol/l								
Week 0	0.72±0.06	0.72±0.09	0.73±0.08	0.73±0.12	n.s.	n.s.	n.s.	
Week 3	0.77±0.03[x]	0.76±0.10	0.74±0.06	0.74±0.07	n.s.	n.s.	n.s.	
Week 6	0.70±0.09	0.71±0.11	0.71±0.08	0.72±0.11	n.s.	n.s.	n.s.	
Week 9	0.78±0.06[x]	0.73±0.10	0.77±0.05	0.74±0.07	n.s.	n.s.	n.s.	
Week 13	0.80±0.06	0.76±0.08	0.78±0.03	0.77±0.06	n.s.	n.s.	n.s.	
p-value time	0.001	0.066	0.023	n.s.				

[x] mean values within a column with a superscript significantly differ ($p<0.05$) from the time point 3 weeks before

Table 4: Mean serum concentration of bone markers in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D+). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Kruskal-Wallis test was used for data that were not normally distributed. Not significant: n.s., not applied: n.a.. Mean± SD

	UV-/D-	UV-/D+	UV+/D-	UV+/D+	2way ANOVA			Kruskal-Wallis
					UV	D	UVxD	
Serum Crosslaps, ng/ml								
Week 0	0.296±0.08	0.291±0.07	0.269±0.06	0.264±0.08	n.s.	n.s.	n.s.	
Week 3	0.381±0.10^{x}	0.385±0.06^{x}	0.346±0.08	0.311±0.07	p = 0.062	n.s.	n.s.	
Week 6	0.472±0.10^{x}	0.462±0.10^{x}	0.420±0.08^{xy}	0.419±0.14^{x}	n.s.	n.s.	n.s.	
Week 9	0.502±0.09^{y}	0.527±0.12^{x}	0.460±0.10^{y}	0.474±0.14^{x}	n.a.	n.a.	n.a.	n.s.
Week 13	0.682±0.12^{x}	0.756±0.04^{x}	0.580±0.13^{x}	0.685±0.16^{x}	p = 0.062	p = 0.055	n.s.	
p-value time	<0.001	<0.001	<0.001	<0.001				
Osteocalcin, ng/ml								
Week 0	219±45.1	219±53.0	187±8.96	233±65.1	n.s.	n.s.	n.s.	
Week 3	267±24.0^{ax}	236±37.1^{ab}	228±35.3^{bx}	273±78.6^{ab}	n.s.	n.s.	p = 0.040	
Week 6	267±35.0^{y}	281±65.9^{y}	232±42.3	271±53.4	n.s.	n.s.	n.s.	
Week 9	239±50.5^{y}	273±66.6	217±28.2	232±42.3	p = 0.084	n.s.	n.s.	
Week 13	233±28.8^{y}	249±32.3	208±28.6	247±59.9	n.s.	n.s.	n.s.	
p-value time	0.003	0.010	0.037	n.s.				
Bone turnover ratio (SCL : OC * 1000)								
Week 0	1.38±0.36	1.51±0.25	1.41±0.31	1.21±0.53	n.s.	n.s.	n.s.	
Week 3	1.46±0.43	1.57±0.37	1.55±0.42	1.30±0.41	n.s.	n.s.	n.s.	
Week 6	1.80±0.43^{xy}	1.68±0.34^{y}	1.90±0.60^{xy}	1.56±0.44	n.s.	n.s.	n.s.	
Week 9	2.19±0.63^{y}	1.99±0.53^{y}	2.18±0.70^{y}	2.12±0.77^{xy}	n.s.	n.s.	n.s.	
Week 13	3.01±0.80^{xy}	2.96±0.85^{xy}	2.85±0.83^{xy}	2.85±0.67^{xy}	n.s.	n.s.	n.s.	
p-value time	<0.001	<0.001	<0.001	<0.001				
Alkaline Phosphatase, U/l								
Week 0	261±100.5	223±67.8	241±89.9	215±84.8	n.s.	n.s.	n.s.	
Week 3	275±65.0	251±79.0^{x}	244±58.8	216±27.5	n.s.	n.s.	n.s.	
Week 6	236±82.6^{x}	213±68.0^{x}	212±57.3^{x}	200±44.3	n.s.	n.s.	n.s.	
Week 9	219±68.1^{y}	197±71.1^{y}	188±51.5^{xy}	182±43.0^{y}	n.s.	n.s.	n.s.	
Week 13	200±58.1^{xy}	185±65.8^{y}	171±43.0^{y}	170±36.4^{y}	n.a.	n.a.	n.a.	n.s.
p-value time	0.004	<0.001	<0.001	0.013				

^{ab} mean values within a row with different superscripts differ significantly ($p<0.05$)

^{xy} mean values within a column with a superscript significantly differ ($p<0.05$) from the time point 3 weeks (^{x}) or 6 weeks (^{y}) before

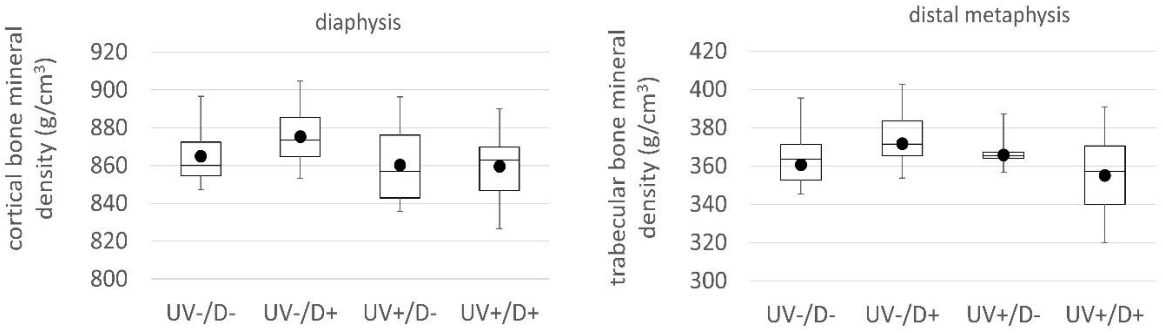

Figure 3: Bone mineral density of left metatarsus in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D+) after slaughtering in week 13. Bone mineral densities were determined with pQCT(peripheral quantitative computer tomography) at diaphysis (50% of length) and distal metaphysis (10% of length).

Urine

The mean concentrations of Ca, P and Mg in urine were not influenced neither by irradiation nor vitD in feed. Excretion of Ca increased from week 0 to week 3 and decreased afterwards every three weeks ($p<0.001$, 1.5±2.20 mmol/mmol Crea in week 0, 2.2±1.8 mmol/mmol Crea in week 3, 0.2±0.08 mmol/mmol Crea in week 13). After week 6 excretion of P increased until week 13 ($p<0.001$, 0.1±0.02 mmol/mmol Crea in week 6, 0.4±0.29 mmol/mmol Crea in week 13) while excretion of Mg decreased until week 9 ($p<0.001$, 1.5±0.72 mmol/mmol Crea in week 6, 1.0±0.40 mmol/mmol Crea in week 9).

Bone mineral densities and bone mineral contents

At the beginning of the experiment no differences were found between the groups, neither in BMD nor CBT. Parameters of BMD as well as CBT increased over time, with trabecular BMD (metaphysis) as the only exception ($p=0.260$ between week 0 and week 13).

Analysed mineral contents of Ca, P and Mg in bones (Table 5) as well as the parameters of BMD in week 13 were not influenced neither by irradiation nor vitD in feed (Figure 3). Correlations between the content of Ca and P and the serum level of 25-OH-D were found (Table 5).

Vitamin D metabolites in the skin

Mean 7-DHC concentration in the skin of the forehead was higher in irradiated (UV+/D+, UV+/D-) compared to unirradiated groups (UV-/D-, UV-/D+, Table 6). Mean concentration of previtD_3 was not influenced neither by irradiation nor vitD in feed. Due to missing data for vitD_3 and tachysterol only a two-factorial ANOVA was performed for every single localisation (leaving repeated measurements as factor). For vitD_3 in the skin no influence of irradiation or vitD in feed was observed. The concentration of tachysterol in the skin of the back was higher in irradiated groups (UV+/D+, UV+/D-) compared to unirradiated groups (UV-/D+, UV-/D-).

Table 5: Mean macromineral contents of ashed bones in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D+) and their correlation with 25-OH-D in serum in week 13. Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Not significant: n.s., Mean± SD

	UV-/D-	UV-/D+	UV+/D-	UV+/D+	2way ANOVA			Correlation with 25-OH-D in serum	
					UV	D	UV x D	r	p-value
Calcium, % DM	14.2±0.707	14.2±1.119	14.2±1.116	13.27±0.953	n.s.	n.s.	n.s.	-0.380	0.035
Phosphorus, % DM	5.58±0.270	5.77±0.209	5.54±0.279	5.43±0.0365	p=0.077	n.s.	n.s.	-0.423	0.018
Magnesium, % DM	0.469±0.052	0.469±0.052	0.476±0.023	0.459±0.059	n.s.	n.s.	n.s.	-0.208	n.s.

Table 6: Content of sterols in the skin of pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D+). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Kruskal-Wallis test was used for data that were not normally distributed. Not significant: n.s., not applied: n.a.. Mean± SD

	UV-/D-	UV-/D+	UV+/D-	UV+/D+	2way ANAOVA			Kruskal-Wallis
					UV	D	UV x D	
7-DHC µg/g*								
Forehead	13.5±2.26[a]	11.8±3.33[a]	17.6±1.10[b]	17.5±4.93[ab]	p = 0.000	n.s.	n.s.	
Neck	27.0±8.25	22.2±4.81	24.7±7.04	26.7±5.40	n.s.	n.s.	n.s.	
Back	25.4±4.93	21.6±5.19	24.1±9.60	21.6±4.84	n.s.	n.s.	n.s.	
Previtamin D3 µg/g*								
Forehead	7.49±1.70	6.93±2.20	6.62±0.87	6.79±2.20	n.a.	n.a.	n.a.	n.s.
Neck	6.08±2.34	6.51±1.20	5.79±1.59	7.39±0.74	n.s.	n.s.	n.s.	
Back	6.07±2.03	5.66±1.19	5.69±1.24	5.52±0.73	n.s.	n.s.	n.s.	
Vitamin D3 µg/g*								
Forehead	0.688±0.350	0.571±0.360	1.004±0.126	0.683±0.052	n.s.	p = 0.093	n.s.	
Neck	0.480±0.105	0.550±0.303	0.734±0.377	0.857±0.576	p = 0.074	n.s.	n.s.	
Back	1.345±0.986	1.165±0.273	1.972±1.540	2.389±1.027	n.a.	n.a.	n.a.	n.s.
Tachysterol µg/g*								
Forehead	0.117±0.160	0.106±0.062	0.827±0.425	0.105±0.052	n.a.	n.a.	n.a.	p = 0.077
Neck	0.113±0.035	0.151±0.050	0.165±0.079	0.209±0.197	n.s.	n.s.	n.s.	
Back	0.104±0.046[a]	0.179±0.125[a]	1.720±1.236[b]	0.931±0.969[ab]	p = 0.003	n.s.	n.s.	

* wet tissue

[ab] mean values within a row with different superscripts differ significantly ($p<0.05$)

Lumisterol was found just in very few samples, therefore, no statistical analyses were performed.

Ussing chamber experiment

In both duodenum and jejunum Ca net absorption was found (Table 7), which was positively correlated with 25-OH-D level in serum (r=0.466 and p=0.011 for duodenum and r=0.550 and p=0.002 for jejunum). For 1,25-$(OH)_2$-D only a trend was observed (p=0.061 and p=0.055 respectively). Ultraviolet B irradiation enhanced muco-serosal Ca fluxes and therefore the net absorption of Ca in both duodenum and jejunum. Sero-mucosal Ca fluxes were not influenced neither by UVB irradiation nor vitD content in feed. In unirradiated groups, the Ca net absorption was higher in duodenum compared to the jejunum (p=0.037). This effect was not seen in the irradiated groups.

Expression of mRNA in the kidney and the intestines

Expression of 1α-hydroxylase was negatively influenced by vitD in feed and UVB irradiation (Table 8) and correlated with 25-OH-D level in serum (r=0.494, p=0.006). Expression of 24-hydroxylase was higher in group UV+/D- compared to group UV-/D+. Expression of VDR was not influenced neither by irradiation nor vitD in feed but trends were observed in the small intestines and kidney. Expression of Calb-D28k in kidney was influenced by irradiation.

Expression of VDR, TRPV6, Calb-D9k and PMCa1b in the intestine was influenced only by localisation with decreasing levels in lower intestines and did not correlate with serum levels of 25-OH-D or 1,25-$(OH)_2$-D.

Digestibility

Digestibility of Ca decreased with time (Table 9) but no influence of UVB irradiation or vitD in feed was observed. Digestibility of Ca did not correlate with serum levels of 25-OH-D or 1,25-$(OH)_2$-D at any time point nor with the results of Ussing chamber experiment (p=0.634 for duodenum and p=0.511 for jejunum) in week 13.

Digestibility of P was influenced by UVB irradiation only in week 13 resulting in higher digestibility in group UV+/D- than UV-/D-. No other effects were observed. Mean digestibility of Mg was increased in groups fed with vitD (UV-/D+, UV+/D+) and showed no time effect (Table 9).

Table 7: Ca fluxes across the mucosa of duodenum and jejunum (Ussing-chamber experiment) in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D-). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Kruskal-Wallis test was used for data that were not normally distributed. Not significant: n.s., not applied: n.a.. Mean± SD

	UV-/D-	UV-/D+	UV+/D-	UV+/D+	2way ANOVA			Kruskal-Wallis
					UV	D	UV x D	
Duodenum, nmol/h/cm^2								
mucoserosal	41.1±12.6^a	41.1±15.7^a	53.2±10.6^{ab}	65.4±23.0^b	p = 0.004	n.s.	n.s.	
seromucosal	15.1±8.6	14.6±7.0	19.3±8.9	28.8±24.0	n.a.	n.a.	n.a.	n.s.
net	25.9±7.4^a	26.4±5.4^a	34.0±10.3^{ab}	36.3±6.7^b	p = 0.004	n.s.	n.s.	
Jejunum, nmol/h/cm^2								
mucoserosal	37.8±10.3^a	47.5±5.4^b	59.3±19.6^b	58.1±24.4^b	p = 0.014	n.s.	n.s.	
seromucosal	18.7±5.6	23.6±11.0	26.8±12.5	20.9±3.9	n.s.	n.s.	n.s.	
net	18.9±6.2^a	19.9±8.3^a	27.9±5.1^b	34.7±14.5^b	p = 0.002	n.s.	n.s.	

^{ab} mean values within a row with different superscripts differ significantly ($p<0.05$)

DISCUSSION

Vitamin D synthesis in the skin

Under continuing UV irradiation the metabolites lumisterol and in a lower amount tachysterol are formed from previtD_3 (Holick et al., 1981) preventing a vitD intoxication. In our study a higher content of tachysterol than lumisterol was found. The reason for this remains unclear but proportion of synthesis depends on the applied wavelength (Webb and Holick, 1988), absorption capacity in the skin and transfer of the products from skin by DBP which has highest affinity for vitD (Haddad et al., 1993). An incubation for 30 min at body temperature – comparable with the delay between irradiation and slaughter in our study – previtD_3 isomerises to vitD_3 but does not change the ratio between lumisterol and tachysterol (Obi-Tabot et al., 2000), but Tuckey et al. (2014) showed metabolism of lumisterol by CYP11A1 (Cytochrome P450scc, catalyzes the synthesis of pregnenolone from cholesterol) within 90 min of incubation. Also a different equilibrium in pig skin can explain the higher amount of tachysterol than lumisterol in our study.

In line with Takada et al. (1981), high UVB doses lead to an increase of 7-DHC in the skin. The explanation seems to be a decreased activity of the 7-DHC-reductase in keratinocytes induced by high vitD and 25-OH-D concentrations in serum (Zou and Porter, 2015) with the goal to provide all the 7-DHC in skin for the vitD synthesis as a positive feedback regulation. Although the used UVB-dose in our study was lower compared to the dose used by Takada et al. (1981), it was enough to enhance the 7-DHC-content (in forehead) or at least prevent a decrease in the skin as a long term effect (other localisations).

VitaminD status in the pigs

The 2x2-factorial design of the study resulted in three different levels of vitD status (concentration of 25-OH-D in serum: UV+/D+ and UV+/D- > UV-/D+ > UV-/D-). Arnold et al. (2015) also found an increase in 25-OH-D levels in both summer and outdoor pig herds (for finisher: 61.4±3 nmol/l in January, in June for confined pigs 70±3.3 nmol/l and for outdoor housed pigs 214±8.2 nmol/l). In minipigs, Burild et al. (2015) demonstrated an increase of vitD and 25-OH-D levels in blood after daily exposure to UV irradiation (study 1: from 12.5-20 nmol/l before start to over 300 nmol/l after 120 days) and a decrease after stopping the exposition to UV light (study 2: from 22.5 – 37.5 nmol/l at start up to 100 – 200 nmol/l after 49 days of irradiation to 8.7 – 12.5 nmol/l after 67 days without irradiation or oral supply).

Table 8: RNA expression of VDR (vitamin D receptor), TRPV6 (transient receptor potential vanilloid type 6), Calb-D9k (calbindin D_{9k}), PMCA1b (plasma membrane calcium ATPase type 1b), CYP24A1 (24-hydroxylase), CYP27B1 (1α-hydroxylase) and Calb-D28k (calbindin D_{28k}) normalized to either β-actin (intestinal segments) or GAPDH (glyceraldehyde 3-phosphate dehydrogenase, kidney) in pigs fed with a diet adequate in vitamin D and irradiated (UV+/D+), kept on a vitamin D-deficient diet without (UV-/D-) and combined with irradiation (UV+/D-) in relation to results from pigs kept on a diet adequate in vitamin D without any further treatment (UV-/D+, determined as "1"). Statistical analysis was done using 2way ANOVA for the factors D und UV followed by Tukey's multiple comparisons test. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for data that were not normally distributed. Not significant: n.s. Not applied: n.a. Mean±SEM

	UV-/D+	UV+/D+	UV-/D-	UV+/D-	2way ANOVA			Kruskal-Wallis
					UV	D	D x UV	
Duodenum								
VDR	1.00 ± 0.23	1.22 ± 0.22	2.26 ± 0.57	1.19 ± 0.27	n.s.	p = 0.089	p = 0.076	
TRPV6	1.00 ± 0.12	1.14 ± 0.21	1.23 ± 0.14	0.95 ± 0.15	n.s.	n.s.	n.s.	.
Calb-D9k	1.00 ± 0.20	1.02 ± 0.28	1.25 ± 0.28	0.89 ± 0.23	n.a.	n.a.	n.a.	n.s.
PMCA1b	1.00 ± 0.18	0.98 ± 0.25	1.49 ± 0.25	1.41 ± 0.36	n.a.	n.a.	n.a.	n.s.
Jejunum								
VDR	1.00 ± 0.20	0.88 ± 0.24	1.81 ± 0.32	1.10 ± 0.28	n.s.	p = 0.062	n.s.	
TRPV6	1.00 ± 0.50	0.44 ± 0.15	0.59 ± 0.25	0.37 ± 0.11	n.a.	n.a.	n.a.	n.s.
Calb-D9k	1.00 ± 0.29	0.65 ± 0.43	0.83 ± 0.28	0.82 ± 0.40	n.a.	n.a.	n.a.	n.s.
PMCA1b	1.00 ± 0.29	0.50 ± 0.19	1.22 ± 0.34	1.04 ± 0.21	n.a.	n.a.	n.a.	n.s.
Caecum								
VDR	1.00 ± 0.20	1.74 ± 0.30	1.23 ± 0.20	1.04 ± 0.13	n.a.	n.a.	n.a.	n.s.
TRPV6	1.00 ± 0.44	1.65 ± 0.43	1.71 ± 0.77	1.16 ± 0.41	n.a.	n.a.	n.a.	n.s.
Calb-D9k	1.00 ± 0.46	1.18 ± 0.38	0.73 ± 0.32	0.48 ± 0.15	n.a.	n.a.	n.a.	n.s.
PMCA1b	1.00 ± 0.19	1.17 ± 0.16	1.25 ± 0.27	0.91 ± 0.15	n.a.	n.a.	n.a.	n.s.
Kidney								
VDR	1.00 ± 0.13	1.17 ± 0.13	1.28 ± 0.23	0.81 ± 0.18	n.s.	n.s.	p = 0.075	
CYP24A1	1.00 ± 0.23^{a}	2.53 ± 0.67^{ab}	2.31 ± 0.68^{ab}	6.65 ± 1.78^{b}	n.a.	n.a.	n.a.	p = 0.004
CYP27B1	1.00 ± 0.17^{a}	0.73 ± 0.14^{a}	2.11 ± 0.41^{b}	1.28 ± 0.21^{ab}	p = 0.043	p = 0.004	n.s.	
Calb-D28k	1.00 ± 0.17	0.84 ± 0.20	1.46 ± 0.16	2.69 ± 1.09	p = 0.032	n.s.	n.s.	

^{ab} mean values within a row with different superscripts differ significantly ($p<0.05$)

Irradiated groups had a 25-OH-D level in serum comparable or higher than outdoor housed herds in June (Arnold et al., 2015). The high availability of 25-OH-D resulted in higher 1,25-$(OH)_2$-D levels in serum than in unirradiated groups (Vieth, 1990). The activity of the renal 1α-hydroxylase is strongly regulated by serum PTH, Ca and P (Holick, 2007), which were not altered in our study (except PTH in week 13).

Levels of 25-OH-D in group UV+/D- increased at the beginning but stagnated in the last three weeks. A possible explanation can be the higher level of 24-hydroxylase compared to group UV-/D+ resulting in a higher degradation of 25-OH-D and 1,25-$(OH)_2$-D. An insufficient vitD synthesis in the skin by UVB seemed to be unlikely, since an elevated 24-hydroxylase and a increased active Ca-absorption were observed. In addition, the used UVB dose was supposed to produce over 9,600 IU vitD per week at the end which would cover the demand of a 100-kg pig of 3,500 – 14,000 IU per week (Kamphues et al., 2004, Kamphues et al., 2014, Stoll et al., 2004). An irradiance of 20μW/cm^2 for 100 min a week leads to a radiant exposure of 1,200 J/m^2 a week which is equivalent to 0.96 MED for unshaved pig skin (Kawagishi et al., 1998). In humans 1 MED enables the production of 10,000 IU vitD (Holick, 1995).

Recommendations for vitD in feed for pigs range from 200 IU/kg DM (Kamphues et al., 2014) to 400 IU/kg feed (Stoll et al., 2004) for fattening pigs, while a 100 kg pig has an estimated intake of 3 kg feed/day. Group UV-/D+ had vitD only from feed. Content of vitD in feed was over the Swiss official recommendation of 400 IU/kg (Stoll et al., 2004) therefore group UV-/D+ could cover its demand easily what is also confirmed by 25-OH-D levels in serum which were higher than in other studies (Arnold et al., 2015).

Study group UV-/D- showed higher levels of 25-OH-D compared to pigs in other studies (Burild et al., 2015). In addition, no clinical signs of vitD deficiency like rickets or lameness were evident although body stores are known to cover a lack for only a week (Heaney et al., 2009). A beginning vitD "insufficiency" in group UV-/D- is indicated by an upregulation of 1α-hydroxylase in the kidney, resulting in a similar level of 1,25-$(OH)_2$-D as in group UV-/D+, which is necessary for holding Ca level in blood. Another possible explanation can be a lower activity of 24-hydroxylase. It would have been interesting to observe these animals over a longer period.

Table 9: Digestibility of Ca, P and Mg (calculated with HCl-insoluble ash as indicator) in pigs fed with a vitamin-D deficient diet (UV-/D-) or a diet adequate in vitamin D (UV-/D+) and their respectives exposed to irradiation (UV+/D- and UV+/D+). Statistical analysis was done using a 2way ANOVA for the factors D and UV followed by Tukey's multiple comparisons test. Kruskal-Wallis test was used for data that were not normally distributed. Not significant: n.s., not applied: n.a.. Mean± SD

	UV-/D-	UV-/D+	UV+/D-	UV+/D+	2way ANOVA			Kruskal-Wallis
					UV	D	UV x D	
Calcium, %								
Week 0	65.1±8.9	73.4±7.3	63.4±8.0	65.4±7.5	n.s.	n.s.	n.s.	
Week 3	68.7±6.8	70.9±7.8	66.0±5.7	67.3±10.5	n.s.	n.s.	n.s.	
Week 6	67.1±2.0	69.6±1.1	67.3±5.7	63.9±9.9	n.s.	n.s.	n.s.	
Week 9	57.0±3.5^{x}	64.9±6.7	61.5±4.4^{x}	59.6±11.0	n.s.	n.s.	p = 0.078	
Week 13	46.1±10.5^{x}	60.8±6.3^{x}	56.8±3.2	57.2±10.7	n.s.	n.s.	n.s.	
p-value time	0.000	0.002	0.014	0.042				
Phosphorus, %								
Week 0	61.3±9.0	68.6±6.2	62.a6±7.2	61.9±8.0	n.s.	n.s.	n.s.	
Week 3	65.4±4.6	69.0±3.4	64.3±6.6	63.6±1.7	n.s.	n.s.	n.s.	
Week 6	63.8±7.0	67.4±3.9	65.7±4.7	60.9±6.7	n.s.	n.s.	n.s.	
Week 9	62.5±3.8	65.4±4.9	61.4±5.8	61.3±7.1	n.s.	n.s.	n.s.	
Week 13	58.5±5.9^{a}	61.4±6.3^{abx}	65.4±4.1^{b}	62.9±2.6^{ab}	p = 0.032	n.s.	n.s.	
p-value time	n.s.	0.021	n.s.	n.s.				
Magnesium, %								
Week 0	14.9±19.8^{a}	41.9±12.7^{b}	24.1±15.3^{a}	27.6±6.9^{a}	n.a.	n.a.	n.a.	p = 0.028
Week 3	16.5±12.87^{a}	40.5±1.6^{b}	17.2±17.0^{a}	31.3±6.1^{c}	n.s.	p = 0.001	n.s.	
Week 6	14.6±21.4^{a}	45.0±4.6^{b}	18.3±14.7^{a}	30.0±9.8^{a}	n.s.	p = 0.002	n.s.	
Week 9	14.7±11.8^{a}	41.1±7.0^{b}	13.7±13.8^{a}	32.5±6.1^{c}	n.s.	p < 0.001	n.s.	
Week 13	12.6±18.1^{a}	37.3±15.8^{b}	29.2±6.9^{b}	35.1±8.0^{b}	n.a.	n.a.	n.a.	p = 0.004
p-value time	n.s.	n.s.	p = 0.066	n.s.				

^{abc} mean values within a row with different superscript differ significantly ($p<0.05$)

^{x} mean values within a column with superscript differ significantly ($p<0.05$) to the time point 3 weeks before

Vitamin D is known to have other functions than Ca homeostasis. For example, higher levels of 25-OH-D in serum are associated with lower risk of cancer. The local production of 1,25-$(OH)_2$-D induces apoptosis in malignant cells as a paracrine effect (Holick, 2007). Also a decreased risk of auto-immune diseases such as diabetes type 1 and multiple sclerosis is known (Lips, 2006, Holick, 2007), while vitD deficiency is also associated with recurrent infections (Dusso et al., 2005). These effects were not included in this study but should not be disregarded for evaluating the optimal vitD status and vitD supply respectively.

Influence on intestinal absorption

Although the results of Ussing chamber experiment showed clearly the effect of 25-OH-D in serum on active Ca absorption, neither the expression of involved genes (TRPV6 for apical Ca entrance, CalbD9k for transcellular Ca transport, PMCa1b for basal Ca excretion) nor the calculation of digestibility of Ca confirmed this. No correlations of 25-OH-D and 1,25-$(OH)_2$-D concentration in serum with the expression of involved genes were detectable. Numerous studies demonstrated a vitD dependent regulation of the envolved transcellular pathways by vitD (Bronner, 2003, Hoenderop et al., 2005, Schroeder and Breves, 2006, Diaz de Barboza et al., 2015). Most studies used a Ca deficient diet to investigate the intestinal Ca absorption. The diet in this study was sufficient in Ca, therefore it can be argued that the paracellular pathway played a more crucial role (Bronner and Pansu, 1999). For mice and rats the paracellular pathway can ensure the Ca level in the serum when Ca content of the diet is high enough, contrary to the situation in growing pigs (Schlumbohm and Harmeyer, 2004, Schroeder and Breves, 2006). In addition, an influence of 1,25-$(OH)_2$-D on paracellular Ca absorption by solvent-drag was found for duodenum in female rats (Tudpor et al., 2008) by crosstalk between transcellular and paracelluar pathways (Wasserman, 2004, Diaz de Barboza et al., 2015) and non-genomic action of 1,25-$(OH)_2$-D was found to stimulate the vesiculare Ca transport to the basal membrane (Dusso et al., 2005, Bikle, 2012). Besides, other currently unknown involved pathways might intervene in the absorption of Ca as well. In $TRPV6^{D541A/D541A}$ knock-in mice (impairing the TRPV6 function) active Ca net absorption was high enough to ensure normal serum Ca levels, indicating the presence of alternative Ca channels such as TRPV5 or Cav1.3 or paracellular pathway (Diaz de Barboza et al., 2015). In addition, at the basolateral membrane a Na/Ca-exchanger responsible for 20% of basal Ca excretion and a 4.1R protein modulating the PMCa1b-activity (Diaz de Barboza et al., 2015) are known.

Digestibility of Mg was increased by vitD in feed indicating a higher absorption in the intestines and also found in other studies (Pointillart et al., 1995) but dependence on vitD is controversly discussed (Hardwick et al., 1991, Lameris et al., 2015). In our study, Mg digestibility was

influenced by vitD content in feed but did not correlate with 25-OH-D or 1,25-$(OH)_2$-D in serum, indicating that presence of vitD in the intestinal lumen changes conditions and therefore influences the absorption of Mg like it is known by intestinal acidity (Thongon et al., 2014). Therefore, the apparent digestibility in the groups fed with vitD (UV-/D+ and UV+/D+) are high but known in growing pigs (around 30-40%; Pointillart et al., 1995, Kammlott et al., 2005 Matsui, 2007).

Influence to the bone metabolism

The reported contents of Ca and P as well as the Ca:P ratio are within the range of results found in bones of other studies with pigs (Ursprung, 2002, Liesegang et al., 2005, Schmid, 2011).

If Ca requirements are not covered by intestinal absorption due to deficiency in dietary Ca or a lack of vitD, Ca is mobilised from the bone leading to rickets in growing individuals (Dittmer and Thompson, 2011). Demineralisation due to mobilisation of Ca will weaken the bone. This bone weakness will be compensated by the up-building of more bone tissue, which will consequently be poorly mineralised. In pQCT these changes can be demonstrated by a higher CBT and decreased cortical BMD. When mineralisation subsequently takes place, BMD will increase (Dittmer et al., 2011). On the other side, 1,25-$(OH)_2$-D is known to inhibit bone mineralisation (St-Arnaud et al., 2000, Sun et al., 2016). Sun et al. (2016) found that subcutaneous injections of a physiologically-high-dose of 1,25-$(OH)_2$-D (250 ng/kg body weight, releasing a maximal physiologic response in normal rats) over 3 weeks in newborn rats led to decreased trabecular bone volume measured in histologically sections of the tibial metaphysis. In contrast, piglets were reported to show serum levels of 25-OH-D up to 900 nmol/l without any changes in content of Ca and P of the bones (von Rosenberg et al., 2016). These pigs were fed 500µg 25-OH-D/kg feed for 42 days (corresponding to 20,000 IU vitD/kg feed; Lauridsen et al., 2010). The effect of 1,25-$(OH)_2$-D on bone (stimulation or inhibition of bone resorption and mineralisation) depends on dose and PTH level in blood (Sun et al., 2016). Reported values for 25-OH-D with negative effects on bone metabolism (reduced mineral contents) are rare and vary highly. Wimsatt et al. (1998) reported on pot-bellied pigs with clinical signs of hypervitaminosis D (weakness, polyuria, polydipsia, hypercalcemia), low serum PTH of 4 pmol/l and high 25-OH-D levels over 320 nmol/l. These pigs were fed more than 40,000 IU vitD/kg feed for 18 days. In contrast, Chakraborty et al. (2015) described a woman with 25-OH-D level of 1,060 nmol/l without any clinical signs.

In our study, no differences in the measured parameters were found between groups (pQCT, parameters in bones and blood). This indicates a sufficient vitD status for physiological bone

mineralisation by the time in all pigs and goes along with the fact that no clinical signs of rickets could be observed.

Increase in SCL reflects the increasing bone mass in growing individuals while decrease in AP and OC are founded in a lower activity of osteoblasts and therefore slower increase of bone mass over time. Increase in SCL and decrease in OC both increase the SCL:OC which is known in growing individuals (Liesegang et al., 2010).

Influence on urinary excretion

Active Ca reabsorption in the kidney takes places in the distal tubule nephron by the same mechanisms as in the intestines. Expression of TRPV5, Calb-D28k and NCX1 is stimulated by 1,25-$(OH)_2$-D and PTH (van Abel et al., 2005, Boros et al., 2009). Therefore, it is not surprising that no influence on the expression of Calb-D28k or mean Ca concentration in urine was observed.

Special attention should be paid to the decreasing Ca excretion via urine. Considering the measured feed intake of 2.7 kg/day, the mean Ca content in feed of 7.13 g/kg (Table 1) and the mean apparent digestibility of Ca of 55.2% in week 13 (Table 9), the animals had a mean Ca absorption of 10.6 g Ca/day in week 13. With an estimated urine production of 17 ml/kg bodyweight/day (Kahn et Line, 2010; 1.8 l urine/day at a mean bodyweight of 108 kg) urinary Ca excretion ranged from 0.22 to 0.34 g/day (on average 0.3 g/day). Consequently, Ca retention of the pigs was 10.3 g/day in week 13 (97.2% of the absorbed Ca). High Ca retention is known in pigs. Nieto et al. (2012) described a Ca retention of 98% of the apparently absorbed Ca in Iberian pigs and an urinary Ca excretion between 0.23 and 0.29 g/day. At the end of our study, Ca retention was nearly twice of the Ca retention of 5.5 g/day in week 0 (94.7% of absorbed Ca; calculated with feed intake of 1.2 kg/day and apparent digestibility of Ca of 66.8%). This can be based on the increasing bone mass, which requires more Ca for bone formation.

CONCLUSIONS

Irradiated groups showed a clear increase in 25-OH-D serum levels compared to the animals supplemented with vitD in feed. This allows the conclusion that pigs are able to synthesize vitD in the skin. Within the irradiated groups (UV+/D-, UV+/D+) no difference in the vitD status was observed, allowing the conclusion that no addition in the feed is needed when adequate exposure to sunlight is ensured. Although no effect of 25-OH-D on Ca homeostasis (Ca digestibility, bone mineralisation, Ca level in serum) between the irradiated groups and group UV-/D+ was observed, other functions than the Ca homeostasis may be affected, e.g. immune

response. It is put into question whether current reference values for 25-OH-D in serum reflect the optimal vitD status in pigs and therefore, whether current recommendations for vitD in feed are high enough to receive the optimal 25-OH-D level in serum. In the same way, the adequate UVB dose is not known at this time and further studies over longer periods and with different UVB doses would be informative and might lead to recommendations for UVB doses. Specially to monitor the situation in breeding animals would be of great relevance.

ACKNOWLEDGEMENTS

The authors thank Ines Mittner, Susanne von Waldow and Reto Mühlemann for their technical assistance. The laboratory work was partly performed using the logistics of the Centre for Clinical Studies at the Vetsuisse Faculty of the University of Zurich.

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Danksagung

An dieser Stelle möchte ich allen herzlich danken, welche zum Gelingen dieser Arbeit beigetragen haben und mit deren Unterstützung ich jederzeit rechnen konnte:

Prof. Dr. med. vet. A. Liesegang für das Bereitstellen des Themas, die fachliche und praktische Unterstützung, die gewissenhafte Durchsicht der Arbeit und die gute Zusammenarbeit.

PD Dr. med. vet. habil. M. Wilkens und ihren Mitarbeitern für die Analyse der Proben, ihre Inputs und fachliche Unterstützung.

Ines Mittner, Susanne von Waldow und **Reto Mühlemann** für die umfangreichen Laborarbeiten sowie ihre unermüdliche Geduld mit uns Doktoranden.

Den Tierpflegern **Holger Born** und **Joanna** für die lustigen Kaffeepausen und den Metzgern **Paul Müller** und **Peter Bänziger** für die Unterstützung bei der Schlachtung.

Der Abteilung für Schweinemedizin (im Besonderen **Dr. med. vet. Esther Bürgi, Patricia Hirsiger, Bettina Jenny**) für die Beantwortung aller Fragen und die tatkräftige Mithilfe bei der ersten Beprobung aller Tiere.

Besonderen Dank geht an meinen Mitdoktoranden **Lukas Küffer** und meine Mitdoktorandinnen **Monika Bieri, Vivien Rampling** und **Malin Nemeth** für ihre Mithilfe bei der Versorgung meiner Tiere und der Probenentnahme, die allzeitige Hilfsbereitschaft und die gute Stimmung im Team.