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Published in:
Journal of Nutritional Science

Link to article, DOI:
[10.1017/jns.2015.28](https://doi.org/10.1017/jns.2015.28)

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Burild, A., Lauridsen, C., Faqir, N., Sommer, H. M., & Jakobsen, J. (2016). Vitamin D3 and 25-hydroxyvitamin D3 in pork and their relationship to vitamin D status in pigs. *Journal of Nutritional Science*, 5, Article e3. <https://doi.org/10.1017/jns.2015.28>

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RESEARCH ARTICLE

Vitamin D₃ and 25-hydroxyvitamin D₃ in pork and their relationship to vitamin D status in pigs

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(Received 3 June 2015 – Final revision received 18 September 2015 – Accepted 05 October 2015)

Journal of Nutritional Science (2016), vol. 5, e3, page 1 of 6

doi:10.1017/jns.2015.28

Abstract

The content of vitamin D in pork produced in conventional systems depends on the vitamin D concentration in the pig feed. Both vitamin D₃ and 25-hydroxyvitamin D₃ (25(OH)D₃) are essential sources of dietary vitamin D; however, bioavailability assessed by serum 25(OH)D₃ concentration is reported to be different between the two sources. Furthermore, the relationship between serum 25(OH)D₃ level and the tissue content of vitamin D₃ and 25(OH)D₃ is unknown. The objective of this study was to investigate the potential of increasing the content of vitamin D in different pig tissues by increasing the levels of vitamin D₃ and 25(OH)D₃ in the pig feed for 49 d before slaughter. Concurrently, the 25(OH)D₃ level in serum was investigated as a biomarker to assess the content of vitamin D₃ and 25(OH)D₃ in pig tissues. Adipose tissue, white and red muscle, the liver and serum were sampled from pigs fed feed containing either vitamin D₃ or 25(OH)D₃ at 5, 20, 35 or 50 µg/kg feed for 7 weeks before slaughter. The tissue 25(OH)D₃ level was significantly higher in the pigs fed 25(OH)D₃ compared with those fed vitamin D₃, while the tissue vitamin D₃ level was higher in the pigs fed vitamin D₃ compared with those fed 25(OH)D₃. The content of 25(OH)D₃ in the different tissues fully correlated with the serum 25(OH)D₃ level, whereas the correlation between the tissue content of vitamin D₃ and serum 25(OH)D₃ was dependent on the source of the ingested vitamin D₃.

Key words: Vitamin D₃; 25-Hydroxyvitamin D₃; Biofortification; Bioavailability

Vitamin D belongs to the group of lipophilic vitamins and accumulates in the adipose tissue of rats, pigs and humans^(1–3). Serum or plasma 25-hydroxyvitamin D is considered the best biomarker of vitamin D status⁽⁴⁾, but its correlation with the tissue concentration of vitamin D is unknown⁽⁵⁾.

Vitamin D exists in two major forms: vitamin D₂ and vitamin D₃. Vitamin D₃ is synthesised in the skin after UV exposure⁽⁶⁾ and is naturally found as vitamin D₃ and 25-hydroxyvitamin D₃ (25(OH)D₃) in products of animal origin, e.g. meat and eggs^(7,8). Within the European Union, vitamin D₃ is the main vitamin D source in animal feed, but recently, 25(OH)D₃ has been approved for supplementary use in pigs and hens. The

maximum permitted level of vitamin D sources is 50 µg/kg pig feed^(9,10).

Comparing the serum and plasma 25(OH)D₃ levels after the administration of either of the two sources of vitamin D₃, oral 25(OH)D₃ has been found to be more potent than oral vitamin D₃, although the data are inconsistent. In humans, oral 25(OH)D₃ is 2- to 5-fold more potent than oral vitamin D₃^(11,12); and in pigs, oral 25(OH)D₃ is 1- to 3-fold more potent than oral vitamin D₃^(2,13–15).

For humans 25(OH)D₃ is a significant compound for the dietary intake of vitamin D⁽¹⁶⁾. However, the vitamin D source, either vitamin D₃ or 25(OH)D₃, used in pig feed

Abbreviation: 25(OH)D₃, 25-hydroxyvitamin D₃.

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determines distribution of the two vitamin D metabolites in the fat in slaughter pigs fed the same feed throughout their lives⁽²⁾. Moreover, the amount of vitamin D₃ stored depends on the fat content of the given tissue^(2,17). However, information regarding the difference between the distribution of oral 25(OH)D₃ and oral vitamin D₃ stored in the body is sparse. Since 25(OH)D₃ is more polar than vitamin D₃ and the affinity of 25(OH)D₃ for vitamin D-binding protein is more than 500 times stronger than that of vitamin D₃, more vitamin D₃ is assumed to be present in its free form, which will allow it to diffuse directly into adjacent tissues⁽¹⁸⁾.

The aims of this study were to investigate the concentrations of vitamin D₃ and 25(OH)D₃ in pork meat (muscle, adipose and liver tissue) from pigs that were fed different doses of vitamin D₃ or 25(OH)D₃, and to assess whether serum 25(OH)D₃ is a biomarker for the tissue concentration of vitamin D₃ and 25(OH)D₃.

Materials and methods

Animal experiment

Details of the animal study including diet composition can be obtained in Lauridsen *et al.*⁽¹³⁾. In brief, 160 prepubertal gilts ((Danish Landrace × Danish Yorkshire) and Duroc; body weight 156 (SE = 7.1) kg) were included in the main pig study investigating the nutritional benefits of vitamin D for reproducing female pigs, with special emphasis on bioefficiency of the two vitamin D sources in terms of bioavailability, early reproduction, bone status markers, and transfer of vitamin D to the progeny⁽¹³⁾. The animals were randomly assigned to dietary treatments containing four concentrations (5, 20, 35 and 50 µg/kg feed) of one of the two different vitamin D sources: vitamin D₃ and 25(OH)D₃. A subpopulation of four animals from each of the eight dietary treatments, except three from the group receiving 50 µg vitamin D₃ and five from the group receiving 35 µg 25(OH)D₃/kg (total *n* = 32 pigs, body weight = 186 kg), was randomly selected from the population of 160 gilts (body weight 186 (SE = 7.2) kg at slaughter). The basal diet consisted of barley (75 %), soyabean meal (8 %), wheat bran (5 %), green grass (5 %), molasses (3 %), animal fat (2 %), and vitamins and minerals (2 %). The analysed concentrations in the diets were very close to the formulated amounts⁽¹³⁾. The study was carried out at Aarhus University, Foulum, Denmark, and the pigs were housed indoors with an artificial lighting (no UVB) regimen 12 h per d. The pigs were killed after 49 d on the experimental diets.

Sampling

Blood samples obtained at slaughter from the *vena jugularis* were collected in Vacutainer tubes containing no additives and processed to serum, which was immediately stored at −80°C until analysis. After the carcasses were eviscerated, samples of the liver, *longissimus dorsi* (loin), and of the *psosas major* (red muscle tissue) were obtained. Adipose tissue and muscle tissue (white muscle tissue) were carefully dissected from the loin. All samples were stored in plastic bags at

−20°C until analysis. Before analysis each sample was slowly thawed and homogenised for 2 min (1094 Homogenizer; Tecator).

Analysis of 25-hydroxyvitamin D₃ and vitamin D₃ in tissue and 25-hydroxyvitamin D₃ in serum and tissues

The tissue samples were analysed by a previous published method using HPLC^(2,19). In short, the internal standard of vitamin D₂ and 25(OH)D₂ were added to the test sample. The samples were saponified, liquid/liquid extracted, cleaned-up in a solid-phase step, followed by a preparative normal-phase HPLC step. For the final separation, detection and quantification reversed-phase chromatography coupled to a UV detector and a diode array detector (DAD) was used. The analyses were performed accredited according to ISO17025⁽²⁰⁾, and quality control included participation in proficiency testing (FAPAS; www.fapas.com). Duplicate analyses were used to assess precision, which for samples with contents of vitamin D₃ and 25(OH)D₃ above 1 µg/kg showed a between-day precision of ≤5.6 and ≤5.1 %, respectively. For samples with contents below 1 µg/kg the between-day precision was ≤0.06 µg/kg for both compounds.

Serum was analysed for 25(OH)D₃ by HPLC equipped with a DAD and a UV detector for detection and quantification as described in detail elsewhere⁽²¹⁾, showing a between-day precision of ≤5.7 % from duplicate analyses. Participation in the Vitamin D External Quality Assessment Scheme (DEQAS; Charing Cross Hospital, London, UK) ensured trueness of the results. Samples of liver and red muscles were analysed for the groups fed 5 or 50 µg/kg feed only, due to economic resources.

Analysis of fat content in muscle tissue

The content of fat in the muscle tissue was determined by the gravimetric method by a modified Schmid–Bondzynski–Ratslaff (SBR) method⁽²²⁾. In short, the sample was boiled with hydrochloric acid followed by the addition of ethanol and extraction of the lipids with diethyl ether–petroleum ether (1:1, v/v). After evaporation of the solvent, the fat was weighed. Due to a limited amount of sample of the red muscle, fat content was only determined on samples from pigs receiving 20 or 35 µg/kg feed.

Statistics

The effects of vitamin D₃ form (vitamin D₃, 25(OH)D₃) and level in feed (5, 20, 35, 50 µg/kg) on the content of 25(OH)D₃ and vitamin D₃ in tissues were analysed by the regression model:

$$y_{ijk} = \beta_{0,i} + \beta_1 \text{form}_{ij} + \beta_2 \text{feeding level}_{ik} + \beta_3 (\text{feeding level} \times \text{form})_{ijk}, \quad (1)$$

where 'y' is the response variable for eight different types of measurements (*i* = 1, 2, ..., 8) of vitamin D, i.e. the combination of measured vitamin D₃ or 25(OH)D₃ in four different meat cuts: adipose tissue, white muscle tissue, red muscle tissue, and



liver. The index j ($= 1, 2$) is used for the two forms of vitamin D, vitamin D₃ and 25(OH)D₃, in the feed. For each type of response variable (i) β_0 refers to the intercept, β_{1j} is a categorical parameter, and β_2 and β_{3j} are regressor parameters.

β_0 and β_{1j} represent the cut offs and β_2 and β_{3j} represent the slopes of the regression lines for feeding level of vitamin D₃ and 25(OH)D₃, respectively. The two regression lines for each response variable were analysed simultaneously. This way not only the power of the test was increased by increased df, the simultaneously estimation also served the purpose of being able to determine whether the two cut-offs (β_{1j}) were significantly different from each other, and to determine whether the slopes (β_{3j}) were significantly different from each other.

The associations between serum 25(OH)D₃, dietary vitamin D form, and vitamin D₃ and 25(OH)D₃ in tissues were investigated by a similar type of model as shown above (equation 1), except that the explanatory variable 'feeding level' was replaced by 'serum'.

In the dataset one outlier was detected using the methods described by Cook & Weisberg^(2,3). Results in the tables and figures are given as means with their standard errors. All data were analysed using proc glm, SAS version 9.3 (SAS Institute), and a significant level of $\alpha = 5\%$ was used as cut-off value for the P values. For plotting, the program Prism 5 for Windows (GraphPad Software) was used.

Ethics statement

The experiment complied with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study.

Results

All results (means with their standard errors) of the content of vitamin D₃ and 25(OH)D₃ in meat cuts, i.e. adipose tissue,

white muscle tissue, red muscle tissue and liver, and serum 25(OH)D₃ are shown in Tables 1 and 2. Overall, the content of 25(OH)D₃ in serum was between 8.7 and 67.1 ng/ml. In the tissues the content of 25(OH)D₃ was between 0.37 and 11.9 $\mu\text{g}/\text{kg}$, while the content of vitamin D₃ was between 0.10 and 8.41 $\mu\text{g}/\text{kg}$.

The content of fat in red muscle tissue and in white muscle tissues was 3.7 (SEM 1.2) % ($n = 32$) and 2.1 (SEM 0.7) % ($n = 16$), respectively.

Overall, for all eight types of response variables the increasing doses of either vitamin D₃ or 25(OH)D₃ in feed increased the content of 25(OH)D₃ and vitamin D₃ in all tissues (β_2 : $P < 0.001$). The vitamin D₃ content in all analysed tissues was significantly (β_3 : $P < 0.001$) higher for pigs fed vitamin D₃, whereas the tissue content of 25(OH)D₃ was significantly higher (β_3 : $P < 0.002$) in all tissues when 25(OH)D₃ was provided in the feed. 'Dose 0' is a theoretical result for vitamin D in tissues if concentration of vitamin D in the feed is zero. The parameter β_1 was not significant for any of the analyses meaning that for 'dose 0' the content of vitamin D₃ and 25(OH)D₃ in all tissues was the same for the two regression lines (Figs 1(a)–(d)). The baseline values for the two groups are thus the same.

As shown in Figs 2(a) and (b), the content of vitamin D₃ in adipose and white muscle tissues was linearly correlated with serum 25(OH)D₃ (β_2 : $P < 0.001$). Furthermore, the concentration was dependent on the dietary vitamin D₃ form, as the interaction term was significant (β_3 : $P < 0.001$).

The content of 25(OH)D₃ in adipose and white muscle tissues (Figs 2(c) and (d)) was also linearly correlated with serum 25(OH)D₃ (β_2 : $P < 0.001$). The correlation coefficient was, however, independent of the dietary vitamin D₃ form (β_3 : $P > 0.72$).

Discussion

In the present study, we compared the effects of different levels of 25(OH)D₃ and vitamin D₃ in pig feed on the

Table 1. Serum level and content of vitamin D₃ and 25-hydroxyvitamin D₃ (25(OH)D₃) in adipose tissue (subcutaneous fat from loin), white muscle tissue (lean meat from loin), red muscle tissue (chain muscle) and in the liver following feeding for 49 d with 5, 20, 35 and 50 μg vitamin D₃/kg feed (n 4, 4, 4, 3, respectively)
(Mean values with their standard errors)

Vitamin D ₃ in feed ($\mu\text{g}/\text{kg}$ feed)...	5		20		35		50	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum								
25(OH)D ₃ (ng/ml)*	8.7	1.5	12.2	1.5	21.7	1.3	27.2	1.7
Adipose tissue								
Vitamin D ₃ ($\mu\text{g}/\text{kg}$)*†	2.08	0.53	2.78	0.13	5.45	0.62	7.59	0.39
25(OH)D ₃ ($\mu\text{g}/\text{kg}$)*	0.86	0.10	1.03	0.15	1.29	0.15	2.03	0.10
White muscle tissue								
Vitamin D ₃ ($\mu\text{g}/\text{kg}$)*†	0.20	0.04	0.46	0.05	0.69	0.06	1.18	0.10
25(OH)D ₃ ($\mu\text{g}/\text{kg}$)*	0.37	0.04	0.53	0.02	0.66	0.04	1.07	0.19
Red muscle tissue								
Vitamin D ₃ ($\mu\text{g}/\text{kg}$)*†	0.49	0.15	N/A		N/A		2.50	0.26
25(OH)D ₃ ($\mu\text{g}/\text{kg}$)*	0.54	0.18	N/A		N/A		1.81	0.24
Liver								
Vitamin D ₃ ($\mu\text{g}/\text{kg}$)*†	0.68	0.07	N/A		N/A		8.41	1.36
25(OH)D ₃ ($\mu\text{g}/\text{kg}$)*	1.33	0.05	N/A		N/A		4.52	0.36

N/A, not applicable.

* Increasing doses of vitamin D₃ in the feed increased the content of vitamin D₃ and 25(OH)D₃ ($P < 0.001$).

† The vitamin D₃ content was significantly higher ($P < 0.001$) for pigs fed vitamin D₃ compared with 25(OH)D₃ in the feed (data shown in Table 2).



Table 2. Serum level and content of vitamin D₃ and 25-hydroxyvitamin D₃ (25(OH)D₃) in adipose tissue (subcutaneous fat from loin), white muscle tissue (lean meat from loin), red muscle tissue (chain muscle) and in the liver following feeding for 49 d with 5, 20, 35 and 50 µg of 25(OH)D₃/kg of feed (n 4, 4, 5, 4, respectively)
(Mean values with their standard errors)

25(OH)D ₃ in feed (µg/kg feed)...	5		20		35		50	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum								
25(OH)D ₃ (ng/ml)*	11.4	1.8	31.1	2.8	49.2	6.1	67.1	9.4
Adipose tissue								
Vitamin D ₃ (µg/kg)*†	1.51	0.28	2.71	0.54	3.37	1.05	3.25	0.59
25(OH)D ₃ (µg/kg)*	0.95	0.17	1.42	0.16	3.09	0.53	4.15	0.83
White muscle tissue								
Vitamin D ₃ (µg/kg)*†	0.10	0.03	0.12	0.02	0.46	0.28	0.17	0.01
25(OH)D ₃ (µg/kg)*	0.41	0.05	1.14	0.09	1.85	0.25	2.81	0.32
Red muscle tissue								
Vitamin D ₃ (µg/kg)*†	0.34	0.04	N/A		N/A		0.45	0.08
25(OH)D ₃ (µg/kg)*	0.67	0.07	N/A		N/A		4.02	0.68
Liver								
Vitamin D ₃ (µg/kg)*†	0.20	0.02	N/A		N/A		0.40	0.07
25(OH)D ₃ (µg/kg)*	2.74	0.31	N/A		N/A		11.9	1.96

N/A, not applicable.

* Increasing doses of 25(OH)D₃ in the feed increased the content of vitamin D₃ and 25(OH)D₃ ($P < 0.001$).

† The 25(OH)D₃ content was significantly higher ($P < 0.002$) for pigs fed 25(OH)D₃ compared with vitamin D₃ in the feed (data shown in Table 1).

concentrations of both 25(OH)D₃ and vitamin D₃ in different pig tissues. Adipose, liver and muscle tissues were chosen for the analysis as representative of the major tissues in the pig that are consumed by humans.

Jakobsen *et al.*⁽²⁾ found that vitamin D₃ and 25(OH)D₃ levels depend on the fat content. In the present study we found a higher content of vitamin D in adipose tissue than in muscle tissue. We presume that the difference between red muscle tissue compared with white muscle tissue is due to the difference in fat content.

Other studies in pigs have investigated a single feeding level of each of the two vitamin D metabolites on their tissue contents^(2,15). Comparing absolute levels obtained in different studies has to be done with caution, due to differences in study design, experimental animals and analytical methods.

The pigs fed 50 µg vitamin D₃/kg feed had vitamin D₃ levels of 7.59 µg/kg in adipose tissue (subcutaneous fat) and 1.18 µg/kg in white muscle tissue (lean meat). These levels are in the same range as pigs fed 24 µg vitamin D₃/kg feed for 70 d resulting in 7.47 µg vitamin D₃/kg in adipose tissue and 1.11 µg vitamin D₃/kg in white muscle tissue, respectively⁽²⁾. The corresponding concentrations of 25(OH)D₃ in subcutaneous fat and lean meat we found to be at 2.03 and 1.07 µg/kg, respectively, were also similar to previously found⁽²⁾. Although the amount of vitamin D₃ in the feed of the two studies was different, 50 µg/kg feed and 24 µg/kg feed, the levels of vitamin D₃ and 25(OH)D₃ in the tissues were similar. Another study also provided 50 µg vitamin D₃/kg feed to slaughter pigs, and reported no tissue content of 25(OH)D₃⁽¹⁵⁾; however, the method used showed a limit of quantification of 5 µg/kg, thus excluding quantification at the levels that we report.

The pigs (females) in the present study provided with 20 µg of 25(OH)D₃/kg feed had similar levels of 25(OH)D₃ in adipose tissue and lean meat as slaughter pigs (both sexes) fed 20 µg of 25(OH)D₃/kg feed⁽²⁾. In contrast, the corresponding level of vitamin D₃ was five times higher in the

pigs in the present study: 2.71 *v.* 0.57 µg/kg. This discrepancy may be due to differences in study design. In the present study, all pigs were fed vitamin D₃ up to inclusion in the study, while the pigs were divided into the different feeding groups after weaning⁽²⁾. Höller *et al.*⁽¹⁵⁾ reported a content of 5.7 µg of 25(OH)D₃/kg lean meat of slaughter pigs fed 50 µg of 25(OH)D₃/kg feed for 119 d. This value is 50–100 % higher than we found in white and red muscle tissue.

Interestingly, the vitamin D level found in the present study was relatively low compared with the vitamin D content determined in a study of Göttingen minipigs⁽²⁴⁾. The vitamin D₃ levels in the adipose tissues of the minipigs were 98 µg vitamin D₃/kg and 67 µg of 25(OH)D₃/kg, i.e. more than 10 times as high as found in the present study (7.59 and 2.03 µg/kg, respectively). The minipigs were fed 3.7–4.4 µg vitamin D₃/kg body weight (approximately 200 µg/kg feed) for 5 weeks, which is about four times higher than the highest level in the present study. However, it can be speculated that the content of fat in the different breeds affects the concentration of vitamin D, e.g. the Göttingen minipig is a breed with small stores of fat, which will result in a higher concentration in the fat if the same amount of vitamin D is stored.

Our study showed that increased vitamin D₃ levels in feed increase the contents of vitamin D₃ and 25(OH)D₃ in pork meat, and increased 25(OH)D₃ levels in feed increase the content of 25(OH)D₃ in pork. These findings demonstrate that increasing the levels of vitamin D in pig feed has the potential to increase the dietary intake of vitamin D from biofortified pork; however, the potential has a limitation due to the maximum level of vitamin D allowed in feed^(9,10).

Furthermore, we demonstrated that the adipose and white muscle tissue content of 25(OH)D₃ could be predicted from the serum 25(OH)D₃ level independently of the ingested form of vitamin D₃. The content of vitamin D₃ in these tissues was also related to the serum 25(OH)D₃ level, but the correlation depended on the dietary source of vitamin D₃.

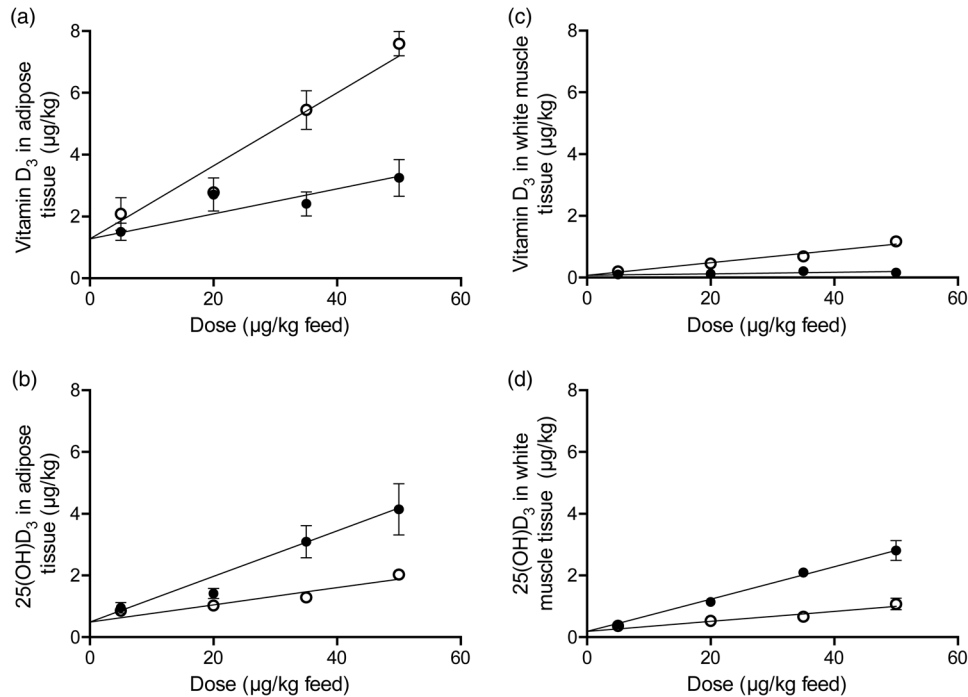


Fig. 1. Vitamin D₃ (○) (a and c) and 25-hydroxyvitamin D₃ (25(OH)D₃; ●) (b and d) in adipose tissue and white muscle tissue plotted against content of vitamin D₃ or 25(OH)D₃ in feed. Values are means, with standard errors represented by vertical bars.

Extrapolation from pig data has estimated the level of vitamin D stores in humans⁽²⁵⁾. However, the content of vitamin D in human adipose tissues is generally higher (32–45 µg vitamin D₃/kg)^(25–27) than in adipose tissues from pigs found in the

present study at 7.6 µg vitamin D₃/kg. However, content of 200 µg vitamin D₃/kg in adipose tissue in Göttingen minipigs has been shown following daily exposure to UV light similar to 10–20 min in the midday sun at 55°N during summer⁽²⁴⁾.

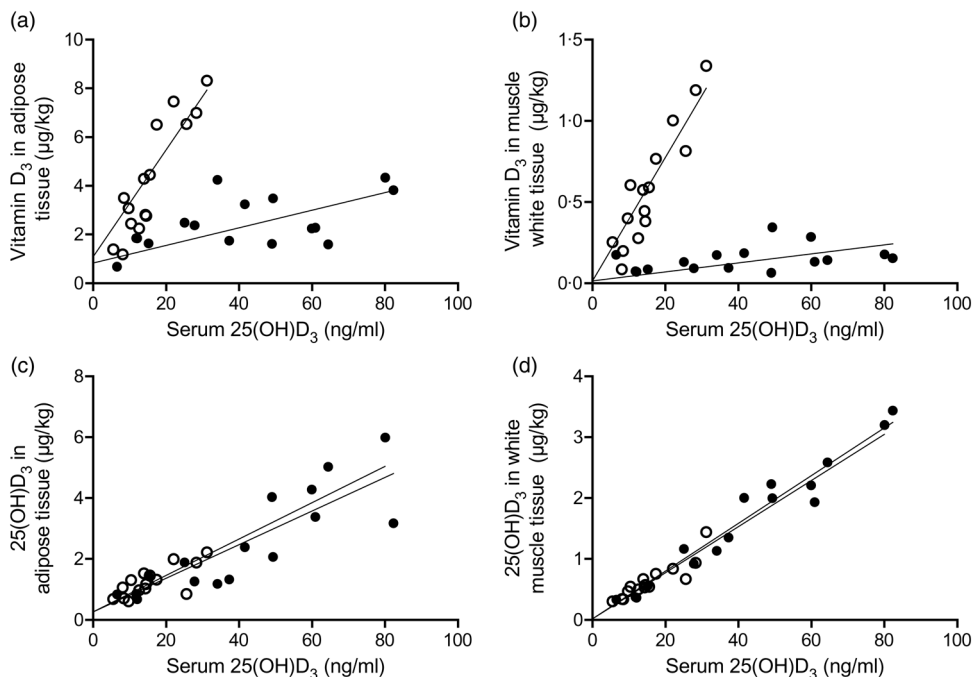


Fig. 2. Content of vitamin D₃ in adipose and white muscle tissues *v.* serum 25-hydroxyvitamin D₃ (25(OH)D₃) (a and b) were linearly correlated ($P < 0.001$), and concentration dependent on the dietary vitamin D₃ form ($P < 0.001$). Content of 25(OH)D₃ in adipose and muscle tissues *v.* serum 25(OH)D₃ (c and d) for pigs fed either vitamin D₃ or 25(OH)D₃ were linearly correlated ($P < 0.001$), but concentration independent on the dietary vitamin D₃ form ($P > 0.72$). (○), Vitamin D₃ in feed; (●), 25(OH)D₃ in feed.



Conclusion

This study showed that the concentration and distribution of vitamin D₃ metabolites (vitamin D₃ and 25(OH)D₃) in tissues depend on the ingested form of vitamin D. Furthermore, this study showed that serum 25(OH)D₃ in pigs is a poor biomarker for the tissue vitamin D₃ content if the dietary vitamin D source contains both vitamin D₃ and 25(OH)D₃.

Acknowledgements

This nutritional subproject received funding from Danish Ministry of Science.

C. L. was responsible for the design of the pig trial and performed all sample collections. J. J. was responsible for the design of this nutritional related subproject and performed the chemical analyses. A. B., J. J., N. F. and H. M. S. designed the statistical test, and N. F. performed the statistical analysis. A. B. wrote the draft paper. All authors approved the final version to be published.

There were no conflicts of interest.

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