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

Burild, Anders; Lauridsen, Charlotte; Faqir, Nasrin; Sommer, Helle Mølgaard; Jakobsen, Jette

Published in:
Journal of Nutritional Science

Link to article, DOI:
10.1017/jns.2015.28

Publication date:
2016

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

Citation (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Vitamin D3 and 25-hydroxyvitamin D3 in pork and their relationship to vitamin D status in pigs

Anders Burild1, Charlotte Lauridsen2, Nasrin Faqir3, Helle M. Sommer3 and Jette Jakobsen1*

1National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark
2Department of Animal Science, Aarhus University, Blichers Alle 20, 8830 Tjele, Denmark
3Department of Applied Mathematics and Computer Science, Technical University of Denmark, 2800 Lyngby, Denmark

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


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). 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
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 D\(_3\) stored depends on the fat content of the given tissue\(^3,17\). However, information regarding the difference between the distribution of oral 25(OH)D\(_3\) and oral vitamin D\(_3\) stored in the body is sparse. Since 25(OH)D\(_3\) is more polar than vitamin D\(_3\) and the affinity of 25(OH)D\(_3\) for vitamin D-binding protein is more than 500 times stronger than that of vitamin D\(_3\), more vitamin D\(_3\) 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 D\(_3\) and 25(OH)D\(_3\) in pork meat (muscle, adipose and liver tissue) from pigs that were fed different doses of vitamin D\(_3\) or 25(OH)D\(_3\), and to assess whether serum 25(OH)D\(_3\) is a biomarker for the tissue concentration of vitamin D\(_3\) and 25(OH)D\(_3\).

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 x Danish Yorkshire) and Duroc; body weight 156 (\(\text{SE} = 7.1\)) 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 \(\mu\)g/kg feed) of one of the two different vitamin D sources: vitamin D\(_3\) and 25(OH)D\(_3\). A subpopulation of four animals from each of the eight dietary treatments, except three from the group receiving 50 \(\mu\)g vitamin D\(_3\) and 25(OH)D\(_3\), and to assess whether serum 25(OH)D\(_3\) is a biomarker for the tissue concentration of vitamin D\(_3\) and 25(OH)D\(_3\).

The tissue samples were analysed by a previous published method using HPLC\(^5,19\). In short, the internal standard of vitamin D\(_2\) and 25(OH)D\(_2\) 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 D\(_3\) and 25(OH)D\(_3\) above 1 \(\mu\)g/kg showed a between-day precision of \(\pm 5-6\) and \(\pm 5-1\) %, respectively. For samples with contents below 1 \(\mu\)g/kg the between-day precision was \(\pm 0-06\) \(\mu\)g/kg for both compounds.

Serum was analysed for 25(OH)D\(_3\) 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 \(\leq 5-7\) % 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 \(\mu\)g/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 \(\mu\)g/kg feed.

Statistics

The effects of vitamin D\(_3\) form (vitamin D\(_3\), 25(OH)D\(_3\)) and level in feed (5, 20, 35, 50 \(\mu\)g/kg) on the content of 25(OH)D\(_3\) and vitamin D\(_3\) in tissues were analysed by the regression model:

\[
y_{ijk} = \beta_0 + \beta_1 \text{form}_y + \beta_2 \text{feeding level}_i + \beta_3 (\text{feeding level} \times \text{form})_{ik},
\]

where \(y\) is the response variable for eight different types of measurements \((i = 1, 2, \ldots, 8)\) of vitamin D, i.e. the combination of measured vitamin D\(_3\) or 25(OH)D\(_3\) in four different meat cuts: adipose tissue, white muscle tissue, red muscle tissue, and liver tissue. All samples were stored in plastic bags at \(–20^\circ\text{C}\) until analysis. Before analysis each sample was slowly thawed and homogenised for 2 min (1094 Homogenizer; Tecator).

Analysis of 25-hydroxyvitamin D\(_3\) and vitamin D\(_3\) in tissue and 25-hydroxyvitamin D\(_3\) in serum and tissues

The tissue samples were analysed by a previous published method using HPLC\(^5,19\). In short, the internal standard of vitamin D\(_2\) and 25(OH)D\(_2\) 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 D\(_3\) and 25(OH)D\(_3\) above 1 \(\mu\)g/kg showed a between-day precision of \(\leq 5-6\) and \(\leq 5-1\) %, respectively. For samples with contents below 1 \(\mu\)g/kg the between-day precision was \(\leq 0-06\) \(\mu\)g/kg for both compounds.

Serum was analysed for 25(OH)D\(_3\) 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 \(\leq 5-7\) % 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 \(\mu\)g/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 \(\mu\)g/kg feed.

Statistics

The effects of vitamin D\(_3\) form (vitamin D\(_3\), 25(OH)D\(_3\)) and level in feed (5, 20, 35, 50 \(\mu\)g/kg) on the content of 25(OH)D\(_3\) and vitamin D\(_3\) in tissues were analysed by the regression model:

\[
y_{ijk} = \beta_0 + \beta_1 \text{form}_y + \beta_2 \text{feeding level}_i + \beta_3 (\text{feeding level} \times \text{form})_{ik},
\]

where \(y\) is the response variable for eight different types of measurements \((i = 1, 2, \ldots, 8)\) of vitamin D, i.e. the combination of measured vitamin D\(_3\) or 25(OH)D\(_3\) in four different meat cuts: adipose tissue, white muscle tissue, red muscle tissue, and liver tissue. All samples were stored in plastic bags at \(–20^\circ\text{C}\) until analysis. Before analysis each sample was slowly thawed and homogenised for 2 min (1094 Homogenizer; Tecator).
liver. The index $\gamma$ (1, 2) is used for the two forms of vitamin D, vitamin D$_3$ and 25(OH)D$_3$, in the feed. For each type of response variable $j$ ($\beta$), $\beta$ refers to the intercept, $\beta_1$ is a categorical parameter, and $\beta_2$ and $\beta_3$ are regressor parameters.

$\beta_0$, $\beta_1$, and $\beta_2$ represent the cut-offs and $\beta_3$, $\beta_4$, and $\beta_5$ represent the slopes of the regression lines for feeding level of vitamin D$_3$ and 25(OH)D$_3$, 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 ($\beta_1$) were significantly different from each other, and to determine whether the slopes ($\beta_2$) were significantly different from each other.

The associations between serum 25(OH)D$_3$, dietary vitamin D form, and vitamin D$_3$ and 25(OH)D$_3$ 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.$^{23}$ 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 D$_3$ and 25(OH)D$_3$ in pig feed on the

<table>
<thead>
<tr>
<th>Vitamin D$_3$ in feed (µg/kg feed)…</th>
<th>5</th>
<th>20</th>
<th>35</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D$_3$ (ng/ml)$^{*}$</td>
<td>8.7</td>
<td>1.5</td>
<td>12.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D$_3$ (µg/kg)$^{+}$†</td>
<td>2.08</td>
<td>0.53</td>
<td>2.78</td>
<td>0.13</td>
</tr>
<tr>
<td>25(OH)D$_3$ (µg/kg)$^{*}$</td>
<td>0.86</td>
<td>0.10</td>
<td>1.03</td>
<td>0.15</td>
</tr>
<tr>
<td>White muscle tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D$_3$ (µg/kg)$^{+}$†</td>
<td>0.20</td>
<td>0.04</td>
<td>0.46</td>
<td>0.05</td>
</tr>
<tr>
<td>25(OH)D$_3$ (µg/kg)$^{*}$</td>
<td>0.37</td>
<td>0.04</td>
<td>0.53</td>
<td>0.02</td>
</tr>
<tr>
<td>Red muscle tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D$_3$ (µg/kg)$^{+}$†</td>
<td>0.49</td>
<td>0.15</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>25(OH)D$_3$ (µg/kg)$^{*}$</td>
<td>0.54</td>
<td>0.18</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D$_3$ (µg/kg)$^{+}$†</td>
<td>0.68</td>
<td>0.07</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>25(OH)D$_3$ (µg/kg)$^{*}$</td>
<td>1.33</td>
<td>0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A, not applicable.

* Increasing doses of vitamin D$_3$ in the feed increased the content of vitamin D$_3$ and 25(OH)D$_3$ ($P < 0.001$).

† The vitamin D$_3$ content was significantly higher ($P < 0.001$) for pigs fed vitamin D$_3$ compared with 25(OH)D$_3$ in the feed (data shown in Table 2).

**Discussion**

In the present study, we compared the effects of different levels of 25(OH)D$_3$ and vitamin D$_3$ in pig feed on the
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 (2,18). 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 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 (18); 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 vs. 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). Hölter et al. (18) 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 3–7–4–4–4 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.
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. 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.

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.

References