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Final report

PhD project of
P-Campus Graduate School

Molecular mechanisms of vitamin D metabolism and phosphorus utilization to maintain mineral homeostasis

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1 Summary and conclusions

Calcium (Ca) and phosphorus (P) homeostasis in pigs are intricately regulated by key factors, including vitamin D and fibroblast growth factor 23 (FGF23). In this research (1st study), the cross-tissue expression of genes involved in vitamin D metabolism and FGF23 signaling in pigs fed conventional and divergent P diets was investigated. The results emphasized the non-renal synthesis of vitamin D in the body. Low P diets increased serum calcitriol and *CYP24A1* expression in the small intestine, suggesting vitamin D signal suppression, while high P increased *CYP27B1* expression in bone, indicating active vitamin D production. Upregulation of *FGFR4* and *KL* in bone following high P diets suggested the body's efforts to balance mineralization, highlighting the importance of optimal P supply in pig nutrition.

Dietary cholecalciferol is the primary source of vitamin D in pigs. This research (2nd study) explored the potential benefits of endogenous vitamin D production through daily UVB exposure in growing pigs. UVB exposure elevated serum calcidiol and increased hepatic expression of *CYP2R1*, indicating an elevated vitamin D status. The overall outcomes suggested that UVB exposure could complement dietary vitamin D supply in pig husbandry, offering opportunities for functional food development and improved animal welfare.

Vitamin D3 and its storage form, 25(OH)D3, have diverse effects in pigs. A comprehensive literature review (3rd study) comparing the impact of vitamin D3 and 25(OH)D3 on pig physiology revealed nuanced differences. Maternal intake of 25(OH)D3 positively influenced piglet growth, immune markers, and bone mineralization, surpassing the effects of vitamin D3. These findings emphasize the importance of selecting the appropriate form of vitamin D for optimal utilization efficiency, nutritional benefits, and therapeutic potency.

In summary, the dissertation provides valuable insights into the intricate relationship between minerals, vitamin D, and gene expression in pigs, offering practical implications for optimizing farming practices, improving animal health, and addressing nutritional challenges in both animal and human populations. The findings contribute to the evolving landscape of sustainable agriculture and animal husbandry practices

2 Introduction and objectives of PhD project

Phosphorus (P) is an essential mineral for all living cells and is vital for animal growth and development. It is the second most abundant element in animals after calcium (Ca^{2+}), with 85% found in bones and teeth, 14% in soft tissues (cell membrane phospholipid, carbohydrate), and 1% in the extracellular fluid as part of various enzymes (Lewis et al., 2006). P is involved in many physiological processes, including the mineralization of bones and teeth. It plays a crucial role in nucleic acid synthesis, ATP synthesis, fatty acid balance, cell structure, and cell signaling (Serna & Bergwitz, 2020). P homeostasis is a complex process involving interactions among the intestine, kidneys, and bones. It encompasses intestinal absorption, bone resorption, urinary excretion, and the transfer of phosphorus between extracellular tissues and bone deposition sites. Bone remodeling is crucial in maintaining plasma P concentrations; high absorption increases plasma P levels, whereas high mineralization decreases plasma P levels (Berndt et al., 2005; Penido & Alon, 2012). Transport of P to the intestine and other

cells is mediated by Na+/P cotransporters. Na+-dependent P transporters types II (NaPT2) and III (PT1 and PT2) are expressed in the intestine, and the SLC34A2-encoded NaPT2b is the major Na+/P cotransporter (about 50%) (Gallant & Vorland, 2021; Sabbagh et al., 2011). Due to its reabsorptive capacity, the kidney is considered the main regulator of phosphorus homeostasis. Hormonal and metabolic factors influencing P reabsorption in the tubules, including changes in P from the diet and parathyroid hormone, alter the expression of type II Na+/P cotransporters in the proximal tubule membrane. Hormones play an essential role in maintaining P homeostasis; three hormones are involved: parathyroid hormone (PTH), vitamin D [1,25(OH)2D3], and fibroblast growth factor 23 (FGF23). In response to high serum P concentrations, PTH regulates P homeostasis through two opposing actions. It lowers serum P by reducing renal reabsorption and elevates the level by either direct stimulation of bone turnover for P release or indirect stimulation of the intestinal absorption of P through its triggering impact on the activation of vitamin D3 via 1-α hydroxylation in the kidney (Torres & De Brauwere, 2011). On the other hand, vitamin D is activated by low serum P concentrations in the blood and transports calcitriol from the kidneys to the intestine to improve P absorption for the elevation of the serum P level (Del Valle et al., 2011). However, the positive effects of calcitriol on intestinal absorption and renal reabsorption are off-set by the stimulation of FGF23. Vitamin D, a fat-soluble hormone, is essential for maintaining bone health and other bodily functions. However, this hormone must be activated to function. Following dietary intake or sunlight exposure, vitamin D3 is transported to the liver, where it is hydroxylated (25-OH) via vitamin D-25-hydroxylase (CYP2R1) to form 25(OH)D3 (Norlin & Wikvall, 2023). The transport protein GC/DBP (vitamin D-binding protein) is specifically produced in the liver, involved not only in the activation and transport of vitamin D but also in many processes that support vitamin D storage, digestion, metabolism, immunity, and other essential functions (Hurwitz & Cooke, 2003). After conversion, 25(OH)D3 is further transported to the kidney via DBP and hydroxylated via 25-hydroxyvitamin D 1-α-hydroxylase (CYP27B1) to form 1,25(OH)2D3. Similar to sunlight, there is evidence that artificial UVB exposure increases serum 25(OH)D3 levels in pigs. Therefore, it is important to expose animals to specific amounts of UVB to ensure optimal levels of vitamin D synthesis and corresponding positive outcomes in the body. The main function of vitamin D is to regulate the homeostasis of serum Ca2+ and P concentrations to maintain proper cellular function and promote bone mineralization (Fleet, 2017). However, the form and dosage of vitamin D (vitamin D3/25(OH)D3) play an important role in maintaining non-mineralizing effects in the body. Along with the candidate genes, the target genes of vitamin D have also been studied in humans, but research in pigs is limited. However, the exact function of vitamin D target genes in the body is still unclear. According to Nurminen et al., the top five functions of the target genes of vitamin D are neutrophil activation, inflammatory response, neutrophil degranulation, control of T cell proliferation, and cytokine production (Nurminen et al., 2019).

Objectives of the PhD

The secosteroid hormone, vitamin D, is crucial for the optimal health status and performance of animals. The homeostatic mechanisms of vitamin D metabolism, FGF23 signaling, and phosphorus utilization in response to divergent dietary P are mostly unknown. Vitamin D synthesis and its metabolism in response to artificial UVB light are also very little studied in pigs. So, the overall aim of the thesis was to determine the molecular mechanisms of vitamin D metabolism and P utilization in response to variable dietary P and artificial UVB exposure in pigs. Therefore,

I. In the first manuscript, the tissue-specific expression of genes related to the biosynthesis, inactivation, degradation, and signaling mediation of vitamin D and FGF23, and their changes in response to basal and aberrant P uptake, were investigated to elucidate their role in P utilization for mineral homeostasis in the body.

II. The second study was performed to assess the impact of artificial UVB light exposure on the alterations of the hepatic transcriptome and vitamin D metabolism in pigs.

III. The third part is a literature review aimed at elaborating on the described skeletal and non-skeletal effects of vitamin D3 and 25(OH)D3 and at evaluating the efficacy of appropriate dietary supplementation in improving reproductive capacities, growth performance, immunity, and bone development in pigs.

3 Material and methods

1st manuscript:

"This study involved animal trials approved by the Scientific Committee of the Research Institute for Farm Animal Biology (FBN) and was licensed and endorsed by the ethics committee of the federal state of Mecklenburg-Western Pomerania, Germany. In this study, the tissue-wide expression of key genes linked to vitamin D metabolism (CYP2R1, CYP27A1, CYP27B1, CYP24A1, GC, VDR) and FGF23 signalling (FGF23, FGFR1-4, KL) was investigated in pigs fed conventional (trial 1) and divergent P diets (trial 2). The tissue set comprised kidney, liver, bone, lung, aorta, and gastrointestinal tract sections. The sample set used for the study included five German Landrace fattening pigs and ten crossbred pigs. The pigs were given ad libitum access to pelleted feed and water. Total RNA was extracted from all tissue samples and purified using the NucleoSpin RNA II-kit. Quantitative real-time PCR was performed using SupraTherm Taq Polymerase with standard cycling conditions. The expression levels of target genes and RPL32 (housekeeping gene) were quantified using quantitative real-time qPCR.
Serum measurement of calcitriol was performed in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Data analyses were performed using open-sourced R software, with transcript copy numbers factorially normalized based upon the expression of the housekeeping gene RPL32 and transformed log2. A linear model was used to compare gene expression and serum calcitriol concentrations between dietary groups. Sex was used as a fixed effect, and differences at $p \leq 0.05$ were considered statistically significant. Fold changes (FC) were calculated based on mean expression values between the two dietary groups.

2nd manuscript:

"The study involved DanBred Duroc boars exposed to UVB radiation for 9 weeks until slaughter. The pigs were given basal diets and drinking water, and their body weight, daily weight gain, and feed conversion were recorded. Blood samples were collected from the jugular vein on days 2 and 17, and the serum concentrations of albumin, alkaline phosphatase, calcium, glucose, inorganic phosphorus, total protein, and total cholesterol were measured. Total RNA was extracted from liver samples and purified using the NucleoSpin RNA II kit. Stranded mRNA libraries were constructed and sequenced for single reads of 101 bp using Illumina HiSeq2500 equipment. The sequencing data processing and differential gene expression analysis were performed using FastQC v.0.12.0 and Trim Galore v.0.6.5, with low-quality reads and adapters removed. The HISAT2 and HTSeq tools were used to map high-

quality reads to the reference Sscrofa11.1 and identify differentially expressed genes (DEGs) between UVB and control groups.
The study used open-sourced R software to analyse the data, including a linear model with the two experimental groups (UVB and control) as fixed effects and slaughter order as a covariate. The graphs were prepared using GraphPad Prism v9.2.0. The results of the study provide valuable insights into the effects of UVB exposure on pig health and the role of vitamin D in cellular processes.

3rd manuscript:
"We conducted a systematic query in Web of Science and PubMed to retrieve all the articles dealing with vitamin D3 and 25(OH)D3 in pigs published from 1st January 2000 to 23rd May 2022. The search strategy included appropriate MeSH terms without any language restriction. In total, 454 articles from the Web of Science and 241 articles from PubMed were identified using the appropriate search query. Of these, a total of 35 articles were selected for review, paying attention to relevance, overlap, and experimental design.

4 Results
1st manuscript:
The study examined the tissue-specific expression of 12 key genes related to vitamin D metabolism and FGF23 signalling in pigs fed a conventional standard diet. The serum calcitriol concentration in pigs was 384.5 ± 82.5 pmol/L at 180 days of age. The hydroxylating enzymes encoding CYP2R1, CYP27A1, and CYP27B1 showed tissue-wide expression across the investigated panel, while the expression of CYP24A1 was exclusively detectable in the kidneys, stomach, and duodenum. GC profiles revealed a tissue-specific distribution with its highest transcript abundance in the liver. VDR was present throughout the tested tissues, but with significantly lower expression in the liver and the aorta. Among the genes related to FGF23 signalling, the expression of FGF23 and KL was shown to be restricted to specific tissues such as kidneys, lung, bone, and the distal intestine. The expression of the FGF23 transcript was limited to bone and liver. FGFR1 expression was the highest in bone and showed considerable variation in transcript abundance in other analysed tissues. The effectiveness of dietary treatment in stimulating regulatory circuits to maintain P homeostasis was demonstrated by the analysis of serum calcitriol levels.

2nd manuscript:
The study examined the effects of UVB exposure on pig growth performance and carcass traits. The animals had uniform birth weights at 14 weeks of age, and UVB exposure had no significant effect on these factors. However, UVB exposure had a significant effect on the overall transcriptional profiles of 703 DEGs in the liver after 8 weeks of exposure. The study also assessed the expression profiles of genes responsible for the synthesis, activation, transport, and inactivation of vitamin D metabolites in the liver. CYP2R1 expression was significantly upregulated in UVB-exposed pigs, while CYP27A1 and DHCR7 expression were reduced. The study found that 12 genes were significantly differentially expressed in UVB-exposed pigs compared to controls. These findings suggest that UVB exposure may have a negative impact on pig growth and carcass traits.

3rd manuscript:

Vitamin D3 and 25(OH)D3 are two major dietary sources of active vitamin D, which play a significant role in maintaining and improving animal performance. Vitamin D3 is the most commonly used form on pig farms, but insufficiency remains a major problem. This study aimed to determine the skeletal and non-skeletal effects of these two forms of vitamin D and compare their efficacies in the body.

Vitamin D plays a vital role in improving the reproductive capacities of pigs, with piglets fed 25(OH)D3 showing significantly higher survival rates than those fed vitamin D3. Supplementation of 25(OH)D3 directly to offspring can also boost growth performance. It also improves muscle fibres, muscle cell proliferation, and differentiation.

Vitamin D plays a vital role in improving the immune status of the body, with 25(OH)D3 significantly enhancing the body's immune status. It boosts humoral immunity, modulates systemic and mucosal antimicrobial responses, and improves gut immunity by altering the gut microbiota.

Proper bone development is crucial for overall health, growth, reproductive success, and longevity of breeding animals. The efficacy of vitamin D3 and 25(OH)D3 varies significantly in pigs fed a diet containing low Ca and P. 25(OH)D3 performs better than vitamin D3 in improving serum mineral status, suggesting that 25(OH)D3 might be preferred over vitamin D3 in improving growth, reproduction, immune status, and bone mineralization in pigs fed a low Ca-P diet.

5 Discussion

The expression of hydroxylases *CYP2R1*, *CYP27A1*, and *CYP27B1* in pigs is evident in all examined tissues, consistent with previous findings in humans and rodents (Elkhwanky et al., 2020; Hewison et al., 2007). However, *CYP2R1* exhibits higher expression in the kidney cortex and medulla than in the liver, suggesting that not only the liver and kidney but also peripheral tissues may possess the ability to produce calcidiol and calcitriol.

Calcitriol levels are further modulated by a catabolic enzyme encoded by CYP24A1, which shows high expression in the renal cortex and low mRNA abundances in the stomach and duodenum. Vitamin D binding proteins (DBP) with different binding affinities play a crucial role in balancing mineral absorption, retention, and re-absorption in extracellular fluids (Bouillon et al., 2020).

The results highlight the effect of divergent dietary P supply on serum calcitriol, with higher levels in low P-fed animals compared to high P-fed animals. The renal expression pattern *of* CYP27B1 and *CYP24A1* represents the result of reciprocal effects, likely mediated by calcitriol, FGF23, and PTH in the kidneys (Meyer et al., 2019).

Calcitriol and FGF23 induce renal *CYP24A1* and suppress renal *CYP27B1*, while opposite effects have been reported for PTH. It is possible that the feedback loops of calcitriol were masked by PTH and FGF23, which collectively favoured CYP24A1 expression in H animals compared with L animals, with unchanged renal *CYP27B1* mRNA abundances.

In conclusion, the tissue-wide distribution of *CYP2R1*, *CYP27A1*, and *CYP27B1* in pigs suggests that not only the liver and kidney but also peripheral tissues may have the ability to produce calcidiol and calcitriol.

FGF23 expression in pigs is highly tissue-specific, primarily restricted to bone and liver. The liver's capacity to express FGF23 in mice, especially in an inflammatory state (Daryadel et al., 2021; Onal et

al., 2018), contrasts with the kidney as the main target organ of FGF23. The effects on the regulation of P reabsorption are triggered by the activation of the mitogen-activated protein kinase (MAPK) cascade. FGF23 has been associated with an increasing number of side effects in other tissues. The ubiquitously expressed *FGFR1-4* receptors in pigs indicate a tissue-wide capacity for FGF23 signal transduction. The highest mRNA abundances for *FGFR1* and *FGFR2* were found in bone, suggesting their involvement in osteoblast proliferation and bone formation. *FGFR3* and *FGFR4* show high mRNA abundances in the renal cortex but are thought to make little or no contribution to renal FGF23 effects. The important co-receptor *KL* appears to be expressed in a relatively tissue-specific manner, supporting the assumption that the kidney acts as the primary target site of FGF23 in pigs, the same as in other mammals, to maintain P homeostasis (Agoro et al., 2020). FGF23 is currently being discussed as a biomarker for pathophysiological implications in nephrology and cardiology, although its reliable translation into clinical utility is still pending (Komaba & Fukagawa, 2021).

The study found that UVB exposure did not significantly impacted body weight, average daily weight gain, carcass weight, and yield percentage in pigs. This is consistent with previous findings where different dosages of UVB and timings of daily exposure showed non-significant effects on performance data (Larson-Meyer et al., 2017; Neill et al., 2023). However, adipose tissue exhibited higher levels of vitamin D3 and calcidiol compared to liver and lean meat. The use of UVB lamps is an effective means of improving vitamin D status at various life stages for pigs, showing efficacy in addition to dietary vitamin D supplementation.
The serum concentration of calcidiol was found to be effective on UVB exposure, but not on serum calcitriol levels, suggesting a short- to medium-term capacity for endogenous synthesis of calcidiol. UVB exposure showed no significant impact on serum levels of albumin, total protein, total cholesterol, alkaline phosphatase, calcium, inorganic phosphorus, creatinine, and glucose at experimental days 2 and 17. The expression of GC, which encodes the vitamin D binding protein with the highest affinity to calcidiol, was unaltered between the experimental groups.
For the vitamin D receptor encoded by VDR and its heterodimer partners RXRA, RXRB, and RXRG, the UVB exposure illustrated no significant impact on their expression. However, a number of known vitamin D target genes were differentially expressed between the experimental groups. The higher serum calcidiol levels might be attributed to the observed transcriptional effects, as an improved serum status of calcidiol was associated with several hundred genes in human white blood cells (Hossein-Nezhad et al., 2013).

In sows, dietary supplementation of vitamin D3 and 25(OH)D3 results in similar reproductive outcomes. The growth performance of piglets and sows fed 25(OH)D3 is significantly better than those fed vitamin D3. The superiority of 25(OH)D3 over vitamin D3 is evident in enhancing innate and humoral immunity. Improved bone mineralization in pigs supplemented with 25(OH)D3 on diets low in calcium and phosphorus provide an option to balance animal welfare and resource efficiency. Thus, 25(OH)D3 is a promising and potential alternative to vitamin D3 for promoting growth, immunity, and bone development in pigs.

In summary, the dissertation sheds light on the complex interaction among minerals, vitamin D, and gene expression in pigs, with practical implications for improving farming practices, improving animal

health, and tackling nutritional challenges in both animal and human populations. Findings in this study contribute to the development of sustainable agricultural and animal husbandry.

6 References

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Attachment

Publications

1. **Hasan, M.**, Oster, M., Reyer, H., Ponsuksili, S., Murani, E., Wolf, P., Fischer D-C., Wimmers, K. (2022). Tissue-wide expression of genes related to vitamin D metabolism and FGF23 signaling following variable phosphorus intake in pigs. Metabolites, 12(8), 729.

2. **Hasan M.**, Reyer H., Oster M., Trakooljul N., Ponsuksilli S., Magowan E., Fischer D-C., Wimmers K. (2024) Exposure to artificial ultraviolet-B light mediates alterations on the hepatic transcriptome and vitamin D metabolism in pigs. The Journal of Steroid Biochemistry and Molecular Biology, 236, 106428.

3. **Hasan, M.**, Oster, M., Reyer, H., Wimmers, K., Fischer, D. C. (2023). Efficacy of dietary vitamin D_3 and 25(OH)D_3 on reproductive capacities, growth performance, immunity and bone development in pigs. British Journal of Nutrition, 130, 1298-1307.

Presentations

1. **Hasan M.**, Oster M., Reyer H., Ponsuksili S., Murani E., Fischer D-C., Wimmers K. Tissue-wide expression of genes related to vitamin D metabolism and FGF23 signaling following variable phosphorus intake in pigs. Annual conference of the German Society for Animal Production and the Society for Animal Sciences (DGfZ/GfT-Gemeinschaftstagung), Kiel, Germany; 2022.

2. **Hasan M.**, Oster M., Reyer H., Ponsuksili S., Murani E., Fischer D-C., Wimmers K. Tissue-wide expression of genes related to vitamin D metabolism and FGF23 signaling following variable phosphorus intake in pigs. P campus symposium at The Leibniz ScienceCampus Phosphorus Research Rostock, Rostock, Germany; 2022.

3. **Hasan M.**, Oster M., Reyer H., Ponsuksili S., Murani E., Fischer D-C., Wimmers K. Tissue-wide expression of genes related to vitamin D metabolism and FGF23 signaling following variable phosphorus intake in pigs. Annual PhD day at The Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany; 2022.

4. **Hasan M.**, Reyer H., Oster M., Trakooljul N., Ponsuksilli S., Magowan E., Fischer D-C., Wimmers K. Molecular determinants of vitamin D metabolism for improved phosphorus efficiency in pigs. Annual conference of the German Society for Animal Production and the Society for Animal Sciences (DGfZ/GfT-Gemeinschaftstagung), Wittenberg, Germany; 2023.

5. **Hasan M.**, Reyer H., Oster M., Trakooljul N., Ponsuksilli S., Magowan E., Fischer D-C., Wimmers K. Molecular determinants of vitamin D metabolism for improved phosphorus efficiency in pigs. P campus symposium at The Leibniz ScienceCampus Phosphorus Research Rostock, Rostock, Germany; 2023.

6. **Hasan M.**, Reyer H., Oster M., Trakooljul N., Ponsuksilli S., Magowan E., Fischer D-C., Wimmers K. Molecular determinants of vitamin D metabolism for improved phosphorus efficiency in pigs. Annual PhD day at The Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany; 2023.