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

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Abstract

Calcium (Ca) and phosphorus (P) homeostasis in pigs are intricately regulated by key factors, including vitamin D and fibroblast growth factor 23 (FGF23). This research (1st study) investigated the cross-tissue expression of candidate genes involved in vitamin D metabolism and FGF23 signaling, including cytochromes (CYP) mediating biosynthesis, activation and degradation and receptors, in pigs exposed to a conventional and a divergent P diet. Expression patterns revealed non-renal tissues and cells actively contributing to vitamin D synthesis based on site-specific requirements. A low P diet elevated serum calcitriol level and *CYP24A1* expression in the small intestine, indicating local vitamin D signaling suppression. A high P diet increased *CYP27B1* expression in bone, promoting local vitamin D synthesis. Ubiquitous expression of fibroblast growth factor receptor 1-4 (*FGFR1-4*) and tissue-specific expression of klotho (*KL)* in FGF23 signaling were observed. Skeletal *FGF23* remained unaffected by dietary P, but *FGFR4* and *KL* exhibited increased expression in bone with high P supply, suggesting a regulatory role in mineralization balance. Specific non-renal tissue responses influence vitamin D metabolism and P homeostasis, highlighting considerations for optimal P supply in pig nutrition.

In current pig husbandry systems, vitamin D status is predominantly reliant on dietary supply. This research (2nd study) explored the potential benefits of endogenous vitamin D production through daily UVB exposure in growing pigs. Transcriptomic profiling of the liver revealed differential gene expressions associated with vitamin D metabolism, particularly the significant upregulation of *CYP2R1*. Serum analysis showed elevated calcidiol concentrations in response to UVB exposure, indicating enhanced vitamin D status. The overall study suggests that UVB exposure could complement dietary vitamin D supply in pig husbandry, offering opportunities for functional food development and improved animal welfare.

In pig husbandry, the dietary use of vitamin D_3 and 25(OH)D_3 as sources of active vitamin D has diverse effects beyond the primary target organs. A comprehensive literature review (3rd study) comparing the impact of vitamin D_3 and 25(OH)D_3 on pig physiology, including reproductive capacities, growth performance, immunity, and bone development, revealed nuanced differences. Maternal intake of 25(OH)D_3 positively influenced piglet growth, immune markers, and bone mineralization, surpassing the effects of vitamin D_3. These findings emphasize the importance of selecting the appropriate form of vitamin D for optimal utilization efficiency, nutritional benefits, and therapeutic potency in pigs.

Zusammenfassung

Die Kalzium (Ca)- und Phosphor (P)-Homöostase bei Schweinen wird durch Schlüsselfaktoren wie Vitamin D und Fibroblastenwachstumsfaktor 23 (FGF23) auf komplexe Weise reguliert. In dieser Studie wurde die gewebeübergreifende Expression wesentlicher Gene im Zusammenhang mit dem Vitamin-D-Stoffwechsel und der FGF23-Signalübertragung bei Schweinen untersucht, die einer konventionellen und einer abweichenden P-Diät unterzogen wurden. Expressionsmuster zeigten, dass nicht-renale Gewebe und Zellen aktiv zur Vitamin-D-Synthese beitragen, basierend auf spezifischen Anforderungen des Standorts. Eine Diät mit niedrigem P-Gehalt erhöhte die Serum-Calcitriol-Konzentration und *CYP24A1*-Expression im Dünndarm, was auf eine Unterdrückung der lokalen Vitamin-D-Signalübertragung hindeutet. Eine Ernährung mit hohem P-Gehalt erhöhte die *CYP27B1*-Expression in den Knochen, was die lokale Vitamin-D-Synthese fördert. Es wurde eine allgegenwärtige Expression von *FGFR1-4* und eine gewebespezifische Expression von *KL* bei der FGF23-Signalübertragung beobachtet. *FGF23* im Skelett blieb von der P-Diät unbeeinflusst, aber *FGFR4* und *KL* zeigten eine erhöhte Expression im Knochen bei hoher P-Zufuhr, was auf eine regulierende Rolle im Mineralisierungsgleichgewicht hindeutet. Spezifische nicht-renale Gewebereaktionen beeinflussen den Vitamin-D-Stoffwechsel und die P-Homöostase, was auf Überlegungen zur optimalen P-Versorgung in der Schweineernährung hinweist.

In den derzeitigen Haltungssystemen für Schweine hängt der Vitamin-D-Status überwiegend von der Ernährung ab. Diese Studie untersuchte die potenziellen Vorteile einer endogenen Vitamin-D-Produktion durch tägliche UVB-Exposition bei wachsenden Schweinen. Die Transkriptom-Profilierung der Leber ergab eine unterschiedliche Genexpression im Zusammenhang mit dem Vitamin-D-Stoffwechsel, insbesondere die deutliche Hochregulierung von *CYP2R1*. Die Serumanalyse zeigte erhöhte Calcidiol-Konzentrationen als Reaktion auf die UVB-Exposition, was auf einen verbesserten Vitamin-D-Status hinweist. Die Studie legt insgesamt nahe, dass die UVB-Exposition die Vitamin-D-Versorgung in der Schweinehaltung ergänzen könnte, was Möglichkeiten für die Entwicklung funktioneller Lebensmittel und eine Verbesserung des Tierschutzes bietet.

In der Schweinehaltung hat die diätetische Verwendung von Vitamin D_3 und 25(OH)D_3 als Quellen für aktives Vitamin D vielfältige Auswirkungen über die primären Zielorgane hinaus. Eine umfassende Literaturübersicht, in der die Auswirkungen von Vitamin D_3 und 25(OH)D_3 auf die Physiologie von Schweinen, einschließlich Fortpflanzungsfähigkeit,

Wachstumsleistung, Immunität und Knochenentwicklung, verglichen wurden, ergab nuancierte Unterschiede. Die mütterliche Zufuhr von 25(OH)D_3 wirkte sich positiv auf das Ferkelwachstum, die Immunmarker und die Knochenmineralisierung aus und übertraf die Wirkung von Vitamin D_3. Diese Ergebnisse unterstreichen, wie wichtig es ist, die geeignete Form von Vitamin D auszuwählen, um eine optimale Verwertungseffizienz, ernährungsphysiologische Vorteile und therapeutische Wirksamkeit in der Schweine zu erzielen.

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Abbreviations

$1,25(OH)_2D_3$ – Calcitriol

$25(OH)D_3$ – Calcidiol

ATP – Adenosine triphosphate

Ca – Calcium

CaBPs – Ca^{2+} binding proteins

CaSRs – Ca^{2+} sensing receptors

CYP24A1 – Vitamin D 24 hydroxylase

CYP27B1 – 25-hydroxyvitamin D 1-alpha-hydroxylase

CYP2R1 – Vitamin D 25 hydroxylase

DBP – Vitamin D binding protein

DHCR7 – 7-Dehydrocholesterol reductase

DLG – German agricultural society

ECM – Extracellular matrix

FGF23 – Fibroblast growth factor 23

FGFR – Fibroblast growth factor receptor

GH – Growth hormone

HOXA10 – Homeobox protein A10

IFN-γ – Interferon γ

Ig – Immunoglobulin

IGF-1 – Insulin-like growth factor 1

IGFBP-3 – Insulin-like growth factor binding protein 3

IL – Interleukin

IP_3 – Inositol triphosphate

LW – Live weight

M-CSF – Macrophage-colony stimulating factor

MED – Minimal erythemal dose

MSCs – Mesenchymal stromal cells

Na^+/P – Sodium-dependent phosphorus cotransporter

NaPT2a – Sodium-dependent phosphorus cotransporter 2a

NaPT2b – Sodium-dependent phosphorus cotransporter 2b

NCX1 – Na^+/Ca^{2+} exchanger

NRC – National research council

OPG – Osteoprotegerin

P – Phosphorus

PBMCs – Peripheral blood mononuclear cells

PTH – Parathyroid hormone

PTHR1 – Parathyroid hormone receptor 1

RANKL – Receptor activator of nuclear factor-kappa B ligand

RXR – Retinoid x receptor

SED – Standard erythemal dose

STTD – Standardized total tract digestible

Th – Helper T-cell

TRPV – Transient receptor potential vanilloid

UV – Ultraviolet radiation

VDR – Vitamin D receptor

VDRE – Vitamin D response element

Vitamin D_2 – Ergocalciferol

Vitamin D_3 – Cholecalciferol

1. State of the art

1.1. Phosphorus

Phosphorus (P) is an essential macro element for all living organisms and crucial for the growth and development of animals. It is the second most abundant element in an animal's body after calcium, with 85% found in bones and teeth, 14% in the soft tissues (cell membrane phospholipids, carbohydrates), and 1% in extracellular fluids as a cofactor in numerous enzyme systems involved in carbohydrate, lipid, and protein metabolism (National Research Council, 1989). P contributes to the metabolic potential of cells by forming high-energy phosphate (PO_4^{3-}) compounds. Without an adequate P supply, an animal will suffer from P deficiency, followed by a decline in the serum P concentration. However, estimating the exact amount of P for the individuals is a difficult task. For this purpose, two methods are usually utilized: the empirical and factorial approaches, with the factorial approach being randomly used (Agricultural Research Council. Technical Committee on the Nutrient Requirements of Farm, 1967; Guéguen & Perez, 1981; Jongbloed *et al.*, 1999b; National Research Council, 2012). The requirement of P is determined by several factors, including the animal (physiological condition, level of production, and type of production), the feeding strategy (quantity of feed provided), the diet (main ingredients used, composition and binding form of the chemical, and a large number of interactions), the environment (health condition, temperature, maintenance, conditions of the housing, goal of production), and the criterion used (minimal or safe level of P, assessment method, or response criteria). The requirement of P for maintenance purposes is assessed by the losses of P through feces and urine (Jongbloed & Kemme, 2002). Standardized total tract digestible (STTD)-P content in feed is expressed as a percentage of NRC requirement estimates. NRC (2012) estimates the requirement for young pigs of 5 to 7 kg and 7 to 11 kg to be 0.45% and 0.40% STTD-P, respectively (National Research Council, 2012). The P requirements of the nursery pigs seem to be greater than the National Research Council (NRC) recommendations (Vier *et al.*, 2017; Wu *et al.*, 2018). According to Jongbloed *et al.* (1999a), the amount of P for the maintenance of the pigs could be equal to 7 mg/kg live weight (LW). A daily maintenance requirement of 10 mg P/kg LW was adopted by the German Agricultural Society (DLG) (Kamphues *et al.*, 2017). For breeding and production purposes, P should be incorporated at a higher rate than for maintenance. The consequences of P insufficiency are manifold, but in all cases, they impair the animal's physical well-being and economic performance. As a result of the deficiency, the

PO_4^{3-} level in the blood plasma falls, leading to Ca^{2+} and P withdrawal from the bones, and they develop rickets or osteomalacia due to the weakened skeletons (Johnson, 2007). As a result of osteomalacia, the newly formed or remodeled bone is not mineralized, leading to an excess of non-mineralized bone matrix (Karpman *et al.*, 2007). Additionally, P deficiency affects the appetite, growth performances, and feed efficiency of swine (Crenshaw, 2000; National Research Council, 2012). However, high dietary P is also detrimental to swine health resulting in reduced performance, impaired bone development, lower feed intake, and lower growth (Oster *et al.*, 2018; Reinhart & Mahan, 1986). Therefore, dietary P should be provided in an adequate amount.

The pig is considered a major source of P excretion from agricultural systems, which has a significant impact on the environment. Using novel approaches to P management, economic and environmental sustainability may be balanced by reducing emissions through system-wide transformations or incremental processes (Kebreab, 2013; Oster *et al.*, 2018). The approaches include information and measures aimed at reducing P excretion to ensure animal welfare and the environment (Oster *et al.*, 2018).

1.1.1. Importance of phosphorus

P is involved in many physiological processes along with the mineralization of bone and teeth. Most of the P in our bodies is devoted to maintaining and supporting the skeleton, where it is co-precipitated with Ca^{2+} as hydroxyapatite. As well as providing support, the skeleton also stores Ca^{2+} and P for the rest of the body. P also plays a crucial role in regulating the body's acid-base balance, forming cell structures, and controlling cell signals. The genetic "memory unit" of all living things is DNA, which contains P. P is also part of an RNA molecule that reads DNA genetic code and builds proteins and other compounds essential for growth, development, and genetic transfer. The structure of both DNA and RNA are linked together by P bonds (Erdman Jr *et al.*, 2012). P is also an essential component of adenosine triphosphate (ATP). ATP is considered the storage and source of energy of the cells and it supplies easily releasable energy in the bond between the second and third PO_4^{3-} groups (Dunn & Grider, 2020). The bond between the second and third PO_4^{3-} groups in ATP gives it releasable energy, making it the "energy currency" of cells. ATP hydrolysis not only provides energy but also plays an important role in signaling and DNA/RNA synthesis. Multiple catabolic mechanisms are utilized in the synthesis of ATP, including cellular respiration, beta-oxidation, and ketosis (National Research Council, 1989). P is also crucial for maintaining osmotic pressure, buffer

capacity, and acid-base balance (National Research Council, 1989; Soetan *et al.*, 2010). This compound plays directly or indirectly a crucial role in all major physiological functions. Phosphorylation of proteins is an essential step in controlling metabolism, cell proliferation, apoptosis, subcellular trafficking, inflammation via cell signaling, gene expression, differentiation, and other important biochemical processes (Ardito *et al.*, 2017).

1.1.2. Homeostasis of phosphorus

P is vital for the proper functioning of the cells, tissues, and body homeostasis. The homeostasis of P is defined as the maintenance of the serum P in the body through the coordinated actions of the specific organs, hormones, and transcriptomes. The balance is crucial for the body to maintain the skeletal integrity and other important biological functions.

1.1.2.1. Regulatory organs involved in phosphorus homeostasis

P homeostasis is mediated through the complex interactions among the gut, kidney, and bone (Figueres *et al.*, 2021). The complex interactions involve intestinal absorption, skeletal resorption, urinary reabsorption and excretion through urine, and exchange of P between extracellular tissues and bone storage pools (Marks *et al.*, 2010; Penido & Alon, 2012). In the event of P intake, these organs interact with each other and respond to the alteration in the concentration of the serum P via complicated processes utilizing P transporters, sensors, and circulating hormones (Figueres *et al.*, 2021).

In detail, bones are constantly remodeled by processes of bone deposition and resorption, and maintaining this balance is crucial to regulating its shape, size, and integrity. Bone remodeling plays a key role in determining plasma P concentration to maintain the P homeostasis, as excessive resorption of bone will elevate the plasma P levels, while increased mineralization lowers them (Berndt *et al.*, 2005).

Intestinal P absorption studies have resulted in variable outcomes, partly due to nutritional status, alteration in gut transit, species differences, and the impacts of studying whole organisms rather than isolated segments of the intestine (Carpenter, 2015). The absorption of P mainly occurs in the small intestine (Jongbloed, 1987) and the absorption is transpired by at least two distinct mechanisms: passive diffusion driven by paracellular P transport, and active diffusion driven by sodium-dependent P (Na^+/P) co-transporters (Carpenter, 2015; Gallant & Vorland, 2021; Sabbagh *et al.*, 2011). Paracellular P transport mainly depends on the electromagnetic gradients through an epithelial layer, with actual paracellular movement facilitated by tight junction complexes created by the interactivity of the complementary

adhesive proteins of adjacent cells. Recent studies have demonstrated that these tight junctions are regulated by signal transduction pathways, actively interact with the cytoskeleton, and have a specific affinity for specific ions (Balda *et al.*, 1993; Benais-Pont *et al.*, 2003; Gonzalez-Mariscal *et al.*, 2003; Sabbagh *et al.*, 2011; Will *et al.*, 2008). Occludins and claudins, two major components of tight junctions, appear to be vital for ion specificity and is illustrated by the findings that mutation in claudin 16 (key protein within the tight junctions of the gut) results in hypermagnesia with hypercalciuria (Balda *et al.*, 1993; Gonzalez-Mariscal *et al.*, 2003). Despite the recent advances in understanding the cellular regulation of passive transport, there is no evidence that P is specifically associated with specific tight junction proteins (Sabbagh *et al.*, 2011). However, there is a controversy regarding the proportion of the intestinal absorption of P via Na^+-dependent and independent mechanisms. The transport of P into intestinal and other cells is predominantly mediated by Na^+/P cotransporters (Lederer & Miyamoto, 2012). Both type II (NaPT2) and III Na^+-dependent P transporters (PT1 and PT2) are expressed in the intestines (Forster *et al.*, 2013). So far, NaPT2b encoded by the *SLC34A2* (Forster *et al.*, 2013), a member of the SLC34 solute carrier family, has been identified in intestinal brush border membranes as a major (approximately 50%) Na^+/P cotransporter that transports Na^+ and P at a ratio of 3:1 across the membrane (Wagner *et al.*, 2014). In the study of Wubuli *et al*., the expression of *SLC34A2* was found to be lower than the expression of *SLC34A3*, suggesting that the role of SLC34A2 in the intestine of pigs is less pronounced compared with other animal species (Wubuli *et al.*, 2019). Inversely, type III Na^+ dependent P transporter and other unknown mechanisms play a minor role in intestinal P uptake (Carpenter, 2015). The Na^+ gradient, maintained by Na^+-potassium ATPase, provides energy for the electrochemical uphill process (Pirahanchi *et al.*, 2022). P taken up into intestinal cells by this mechanism is transported from the apical pole to the basolateral pole, probably through restricted channels such as microtubules. The process by which P leaves the enterocyte and enters the circulation is yet poorly understood. Currently, the broadly distributed SLC20 carrier family, PT1 and PT2 is found to be widely expressed in the intestine (Marks *et al.*, 2010), and PT2 primarily in the ileum. PT1 and PT2 favor the conveyance of the monovalent P species (HPO_4^-), and regulate a Na:P (2:1) stoichiometry. The role of these transporters in adaptive responses to the conveyance of intestinal P may be broader than previously recognized. In rats, PT1 expression is slowly upregulated in response to P deprivation, whereas PT2 and NaPT2b expression increases within 24 hours (Candeal *et al.*,

2017). A recent study in rats suggests that a major part of the adaptive response to intestinal P transport likely occurs through as yet unknown transporters or transport pathways (Candeal *et al.*, 2017).

The kidney is considered the major regulator of P homeostasis due to its reabsorptive capacity. Renal excretion of P is determined by the balance between glomerular filtration and tubular reabsorption. In healthy conditions, 80-90% of P that is filtered is reabsorbed, while the remaining 10-20% is excreted in the urine (Choi, 2008). Renal tubular reabsorption of P proceeds primarily in the proximal tubule at the apical brush border membrane through the action of Na^+ gradients (Na^+/P cotransport) (Murer *et al.*, 2000). It has been shown that the majority of hormonal and metabolic factors influencing renal tubular P reabsorption, including changes in the amount of dietary P and parathyroid hormone, modulate the expression of Na^+/P cotransporter protein type II in the proximal tubular membrane (Berndt & Knox, 1992; Kempson, 1996) belonging to the SLC20A2, SLC34A1 and SLC34A3 families (Murer & Biber, 2010). The abundance of these Na^+/P-cotransporters at the apical membrane is adjusted by hormones/phosphatonins and metabolic factors to balance the renal P reabsorption corresponding to the demand of the body. P reabsorption is mainly regulated via hormones by altering SLC34A1 (NaPT2a) abundance through modulating interaction between NaPT2a and post synaptic density (PDZ)-protein NHERF1 (Murer & Biber, 2010). A PDZ (soluble cytoplasmic adapter protein) is characterized by the presence of a PDZ domain, which is a distinct structural motif consisting of 80-90 amino acids and acts as scaffolds for the assembly of multiprotein signaling complexes using highly conserved molecules (Romero *et al.*, 2011).

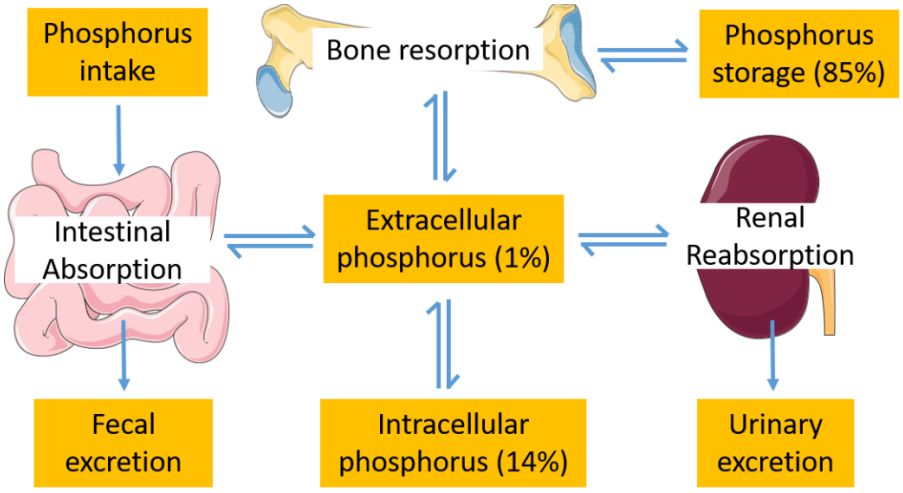

Figure 1. A schematic illustration of the coordinated actions of intestine, bone, and kidney in maintaining P homeostasis. Adapted from the study of Brown *et al*. (Brown & Razzaque, 2018).

1.1.2.2. Endocrine control of phosphorus homeostasis

Hormones play a critical role in maintaining P homeostasis. There are three different hormones associated with homeostasis. These include parathyroid hormone (PTH), vitamin D [1,25$(OH)_2D_3$], and FGF23. The homeostasis of P is achieved through the coordinated action of these hormones.

Physiologically, the parathyroid glands serve as calciostats (the mechanism by which parathyroid hormone maintains blood Ca^{2+} levels), and therefore the production of PTH by the parathyroid glands is tightly regulated at the transcriptional and post-transcriptional levels by the extracellular Ca^{2+} concentration (Kemper *et al.*, 1974). In addition, it is also involved in regulating the serum P levels via its action in multiple organs, which in turn increases the secretion of PTH, possibly by lowering extracellular Ca^{2+} and increasing PTH mRNA stability (Moallem *et al.*, 1998). In response to high serum P levels, the hormone regulates P homeostasis through two opposing effects. It lowers the serum P by reducing renal reabsorption and elevates the level by either direct stimulation of bone turnover for the P release or by indirect stimulation of the intestinal absorption of P through its triggering impact on the activation of vitamin D_3 via 1-α hydroxylation in the kidney (Torres & De Brauwere, 2011). PTH activates a number of intracellular second messengers, including Gs-dependent cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP_3), phospholipase C, free Ca^{2+}, and diacylglycerol through binding to PTH/PTHrP receptor type 1 (PTHR1) in renal and bone cells (Potts, 2005). Specifically, cAMP and phospholipase C initiate the internalization of Na^+/P cotransporters NaPT2a and NaPT2c on the renal proximal tubule of the kidney and decline in the renal reabsorption of P. PTHRs are predominantly found in the osteoblasts of the bone, and not present in osteoclast, that initiates the anabolic activity of PTH to enhance bone formation (Torres & De Brauwere, 2011). PTH is also involved in stimulating receptor activator of nuclear factor-kappa B ligand (RANKL) in osteoblast to elevate the osteoclast number, resorption of bone, and consequently P release (Potts, 2005).

The active form of vitamin D, calcitriol/1,25$(OH)_2D_3$, is one of the most important hormones in regulating P homeostasis. The activation of vitamin D is triggered by a low serum P level in the blood. In response to low serum P level, calcitriol is transported from the kidney to the intestine to enhance the absorption of P and Ca^{2+} by increasing the expression of the Na^+/P cotransporter (NaPT2b), leading to an increase in serum P levels (DeLuca, 1986; Takeyama *et al.*, 1999). Calcitriol also promotes renal reabsorption of P by upregulating the expression of

NaPT2a after sensing the low serum P level in the blood (Torres & De Brauwere, 2011). However, the positive effect of calcitriol on intestinal absorption and renal reabsorption is compensated by the stimulation of FGF23. The increased serum P [mediated by $1,25(OH)_2D_3$] stimulates the FGF23 hormone secretion from bone to lower the serum P status (Trummer *et al.*, 2019). Vitamin D is also implicated in the suppression of the production of PTH by directly inhibiting the expression of its gene and, indirectly, by enhancing the expression of Ca-sensing receptor (CaR) and parathyroid cell sensitivity to extracellular Ca^{2+} (Carrillo-López *et al.*, 2009). Vitamin D thus has the potency to raise or lower the serum P levels, based on its balanced impacts on the parathyroid glands, bones, and intestines (Torres & De Brauwere, 2011).

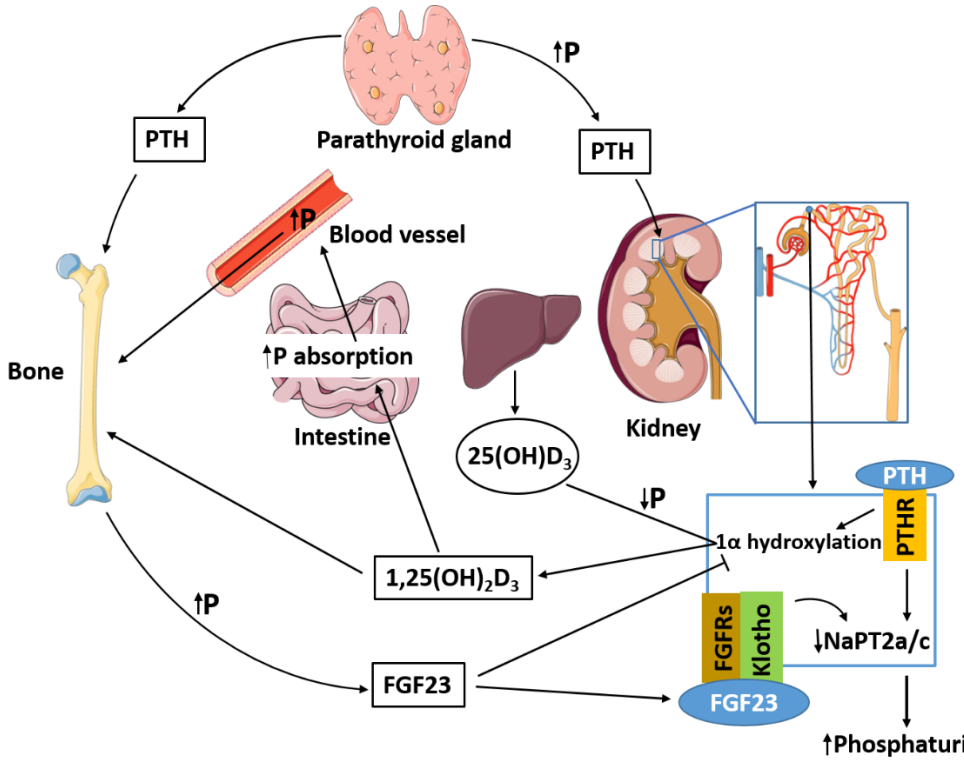

Figure 2. A schematic illustration of the hormonal regulation of P homeostasis in response to low (↓) and high (↑) serum P mediated by the major players (PTH-Vitamin D-FGF23 axis) in the P regulatory network and their functional interactions (Blau & Collins, 2015). FGF23 = fibroblast growth factor 23, FGFRs = fibroblast growth factor receptors, PTH = parathyroid hormone, PTHR = parathyroid hormone receptor, NaPT = sodium phosphate cotransporter.

The discovery of FGF23, a novel bone-derived hormone secreted by osteoblasts, osteocytes, and osteoclasts which inhibits renal P reabsorption and calcitriol production, has revealed primary modulatory pathways and novel system biology insights into bone mineralization, vitamin D metabolism, parathyroid function, and renal P processing (Naveh-Many *et al.*, 2008). The physiological action of FGF23 is mediated by FGFRs and KL (a co-receptor that increases its binding affinity of FGF23 to FGFRs) (Richter & Faul, 2018). In response to high serum P levels, this hormone reduces the level by two mechanisms: it downregulates the expression

of NaPT2a and NaPT2c, which lowers the renal P reabsorption, and suppresses the production of calcitriol by inhibiting 1α-hydroxylation and increasing its catabolizing enzyme 24,25-hydroxylase in the kidney to elevate the excretion of the excess urinary P. Thus, the enhanced loss of urinary P and declined renal tubular synthesis of $1,25(OH)_2D_3$ lead to a return to normal serum P level (Clarke, 2011). As a result of the reduction in calcitriol synthesis, intestinal NaPT2b expression and P uptake are also reduced. FGF23 is also directly involved in downregulating the mRNA expression (via the increased activity of parathyroid 1α hydroxylase) and the secretion of PTH (Torres & De Brauwere, 2011). However, the mechanism of action of FGF23 on PTH is still not fully understood.

1.2. Vitamin D

Fat soluble hormone, vitamin D is crucial for maintaining bone health through intestinal absorption and serum regulation of Ca^{2+} and P in the body. However, a recent investigation suggests that the function of vitamin D is not only bone mineralization but also involved in maintaining other important functions of the body to ensure optimal health (Konowalchuk *et al.*, 2013; Weber *et al.*, 2014). Ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) are the most important sources of vitamin D. Both forms of vitamin D are consumed through diets or supplements to avoid vitamin D deficiency. Vitamin D_2 is of plant origin whereas cholecalciferol is animal origin. Vitamin D_2 is produced by some plants (e.g., fungi, yeast) from provitamin D_2 in the presence of ultraviolet radiation (UVB) derived from the sunlight (Boushey *et al.*, 2001). Vitamin D_3 can also be obtained in the body following the sunlight exposure of the animals (Alexander *et al.*, 2017). Vitamin D_2 differs from vitamin D_3 by the presence of a double bond between C22 and C23 and the existence of a methyl group at C24. In commercial farming, vitamin D_3 is preferable to vitamin D_2 because of its potency to elevate the serum concentration of vitamin D in the body (Grundmann *et al.*, 2023). Compared to equimolar D_2, D_3 is approximately 87% more effective at raising and maintaining serum 25(OH)D levels and produces 2- to 3-fold greater storage of vitamin D in humans (Heaney *et al.*, 2011).

1.2.1. Metabolism of vitamin D via dietary intake and UVB exposure

Vitamin D_3 is mainly provided as a dietary supplement to the pigs. However, its dosage is important to ensure optimal serum concentration and maximum health benefits of vitamin D. In commercial pig farming, the dosage of cholecalciferol is limited by law to 2000 IU/kg feed (European Commission, 2017). Another source of cholecalciferol is sunlight exposure. Sunlight

is a component of the electromagnetic spectrum emitted by the sun, specifically infrared, visible, and ultraviolet light (UV). Radio waves, micro waves, X-rays, and γ radiations are also parts of this spectrum. However, a small portion of the electromagnetic spectrum is covered by UV radiation. Any particular region of the UV spectrum is characterized by its wavelength. UV radiation has a wavelength range of 400 to 100 nm (Blaustein & Searle, 2013). Even in the UV portion of the spectrum, the biological effects of the radiation vary greatly in wavelength, hence the UV spectrum is further separated into three regions. The idea of dividing the UV spectrum into spectral zones was first proposed during the Copenhagen conference of the Second International Congress on Light in August 1932. It was suggested that three spectral zones be defined based on the wavelengths: UVA (400-315 nm), UVB (315-280 nm), and UVC (280-100 nm). Photo biologists further defined three spectral regions considering a bit different wavelength: UVA (400-320 nm), UVB (320-290 nm), and UVC (290-200 nm) (Bero & Abukassem; Diffey, 2002). In pigs, sunlight-emitted UVB photons (290-315 nm) are absorbed by 7-dehydrocholesterol reductase (DHCR7) in the skin to convert into previtamin D_3 (Holick, 2016). The previtamin D_3 is rapidly converted to vitamin D_3 within the plasma membrane. However, vitamin D_3 is an inactive form of vitamin D that requires hydroxylations to become active. Upon ingestion as supplements or synthesis by the skin, vitamin D_3 enters the circulation and is hydroxylated to form 25(OH)D_3/calcidiol in the liver by vitamin D-25-hydroxylase (CYP2R1). To deliver vitamin D throughout the body via circulation, the transporter protein GC/DBP (vitamin D binding protein) is also specifically formed in the liver (Hurwitz & Cooke, 2003). Moreover, the liver is not only involved in vitamin D activation and transportation, but also involved in many processes that support vitamin D storage, digestion, metabolism, immunity, and other important functions (Kalra *et al.*, 2018). After conversion, 25(OH)D_3 is further transported to the kidney via DBP and hydroxylated to form 1,25(OH)$_2D_3$/calcitriol (the active form of vitamin D_3) via 25-hydroxyvitamin D 1-alpha-hydroxylase (CYP27B1). Once activated, it is transported to the target organs via DBP to perform its function.

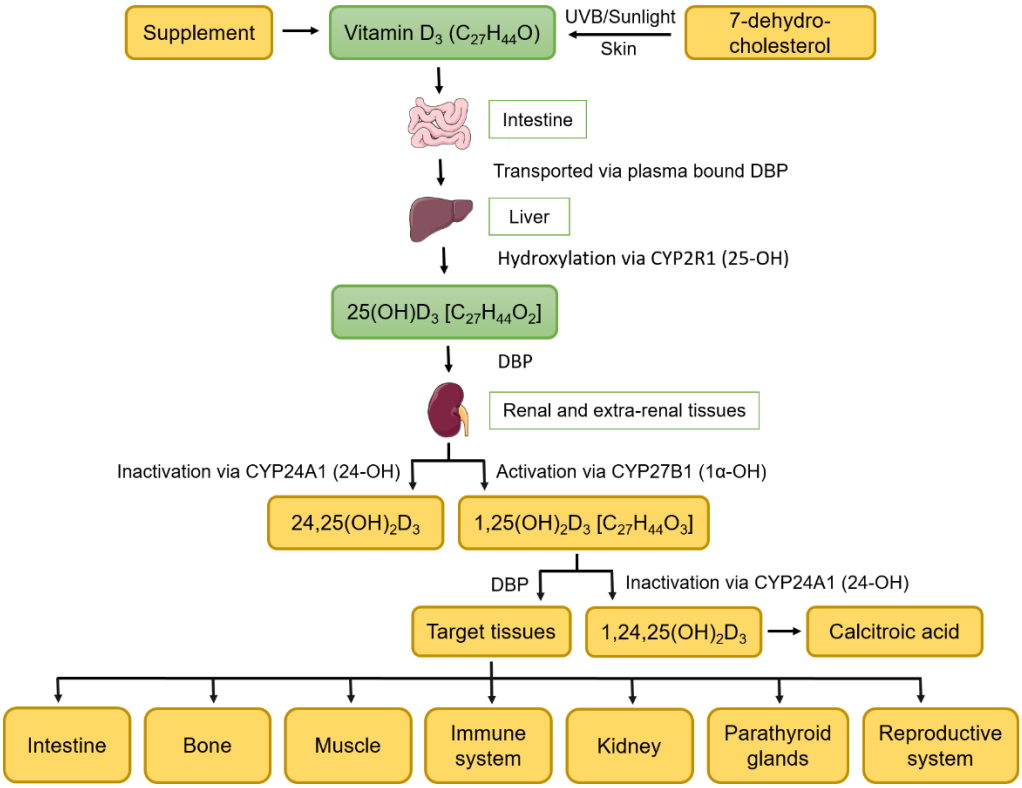

Figure 3. An illustration of the endogenous synthesis of active [1,25(OH)$_2$D$_3$] and inactive [1,24,25(OH)$_2$D$_3$] forms of vitamin D$_3$ from supplemental or skin sources (Hasan *et al.*, 2023a). DBP = vitamin D binding protein.

According to Hasan *et al.*, vitamin D can also be locally activated by different parts of the body depending on their specific requirements, including the kidneys and liver (Hasan *et al.*, 2022). However, an excess amount of vitamin D$_3$ is excreted through the kidney following deactivation by CYP24A1 (24-hydroxylase). CYP24A1 is the major enzyme that breaks down both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. 25(OH)D$_3$ is the most reliable form of vitamin D for assessing the vitamin D status in the body due to its good correlation with vitamin D supply and longer half-life (10 days for pigs) than other forms (Flohr *et al.*, 2014; Holick, 2009; Watson *et al.*, 2014).

In commercial farming, pigs do not have access to sunlight exposure. Therefore, it faces difficulties in reaching the full potential of vitamin D. This is because di*et al*.one does not supply the body with enough vitamin D (Alexander *et al.*, 2017; Kolp *et al.*, 2017). To overcome this limitation, researchers are using artificial UVB light to simulate sunlight exposure for pigs in commercial facilities. There is evidence that artificial UVB exposure elevates serum 25(OH)D$_3$ levels in pigs in a similar manner to sunlight exposure. However, we have to be careful about the overexposure of UVB radiation. Because overexposure to UVB is linked to negative consequences on the body (Kolp *et al.*, 2017; Salian *et al.*, 2021). Therefore, exposing

the animals to a specific dose of UVB is crucial to ensure the optimum level of vitamin D synthesis and corresponding positive outcomes in the body. Thus, to evaluate the proper dosage of UVB light, a minimal erythema dose (MED) is determined to avoid sunburns and other negative consequences for the body. MED is defined as the lowest dosage of UVB required to produce just perceptible erythema 24 hours after a single UVB exposure (Heckman *et al.*, 2013). However, the use of this method is limited due to its subjectivity and dependence upon different variables involving skin pigmentation and exposure sites. Therefore, standard erythemal dose (SED) is used as a standardized measurement of UV radiation that causes erythemogenesis and independent of the skin type (Diffey *et al.*, 1997). SED corresponds to an erythemal radiant exposure of 100 J m^{-2} (Salvadori *et al.*, 2019). The dosage of SED is crucial for optimal vitamin D synthesis. The pigs exposed to UVB at 1 SED are found to have the highest levels of vitamin D_3 in their bodies (serum, skin, lean meat, subcutaneous fat, and liver), compared to those exposed to UVB at 0.3 and 0.7 SED (Barnkob *et al.*, 2019). According to some researchers, the animals exposed to natural or artificial UVB at 1 SED produce more vitamin D_3 and 25(OH)D_3 in the body than those receiving vitamin D_3 at 2000 IU/kg feed (Burild *et al.*, 2016; Jakobsen *et al.*, 2007; Kruczynska, 2019).

1.2.2. Forms of vitamin D

There are four different forms of vitamin D: vitamin D_2, vitamin D_3, 25(OH)D_3 and 1,25$(OH)_2D_3$. Vitamin D_2 is the type of vitamin D that is derived from plant sources or dietary supplements from irradiated fungi. Vitamin D_3 is the most common form of vitamin D, obtained through skin exposure to ultraviolet radiation (UVB), and animal sources or dietary supplements (Clarke, 2019). The relative efficacy of these two forms of vitamin D in raising serum levels of total 25(OH)D is still debated. However, several studies have demonstrated that the serum level of 25(OH)D_3 is elevated more efficiently with vitamin D_3 than vitamin D_2 (Nasim *et al.*, 2019; Trang *et al.*, 1998; Tripkovic *et al.*, 2012). The plasma half-life of vitamin D_2 is shorter, and it has a lower affinity for the vitamin D binding protein, the vitamin D hydroxylase, and the vitamin D receptor in the liver (Mistretta *et al.*, 2008). Therefore, vitamin D_3 is mainly provided as a dietary supplement to the pigs on commercial farms. It is usually administered at 2000 IU/kg feed and also the maximum allowed by regulation in Europe (Barnkob *et al.*, 2019). Like vitamin D_3, 25(OH)D_3 is also allowed up to 2000 IU/kg feed (European Food Safety Authority, 2009). So far, the active form of vitamin D_3, 1,25$(OH)_2D_3$ is rarely used as a supplement to the pigs. In recent years, there has been debate about the most effective form

of vitamin D between vitamin D_3 and $25(OH)D_3$ to achieve optimal vitamin D status and maximum health benefits for the body (Burild *et al.*, 2016; Hines *et al.*, 2013).

1.2.3. Mechanisms of actions of vitamin D

As we already know, $1,25(OH)_2D_3$ is the active form of vitamin D. However, the activation or genomic action of $1,25(OH)_2D_3$ depends on the binding with vitamin D receptor/retinoid X receptor (VDR/RXR) heterodynamic complex to specific DNA sequences (Christakos *et al.*, 2016). Once $1,25(OH)_2D_3$ enters the nucleus, it attaches to the VDR and activates another nuclear receptor RXR. The formulated VDR/RXR complex then binds to vitamin D response elements (VDREs) in the presence of $1,25(OH)_2D_3$, initiating a cascade of cellular interactions that modulate gene transcription. It has been found that thousands of VDREs are located throughout the genome and the activation of VDRs by 1,25-dihydroxyvitamin D may result in the regulation of multiple genes by direct or indirect mechanisms (Pike & Meyer, 2012). The main regulators of renal 1α-hydroxylase [CYP27B1, the enzyme that produces $1,25(OH)_2D$] are PTH, FGF23, Ca^{2+}, and PO_4^{3-}. Unlike the kidney, extra renal 1α-hydroxylase is regulated by cytokines (Bikle, 2021).

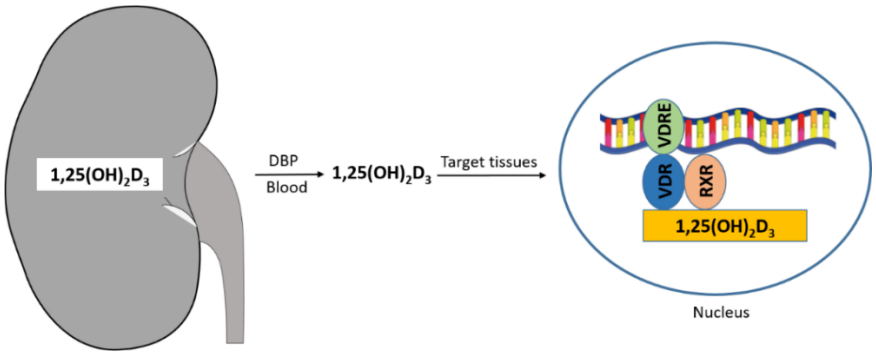

Figure 4. An illustration of the mechanisms of actions of vitamin D on the target tissues of the body after activation [$1,25(OH)_2D_3$] in the kidney. DBP = vitamin D binding protein, VDR = vitamin D receptor, RXR = retinoid x receptor, VDRE = vitamin D response element.

1.2.4. Effects of vitamin D

1.2.4.1. Effects on mineralization

The secosteroid hormone vitamin D [$1,25(OH)_2D_3$] plays a crucial role in the maintenance, growth, development, and mineralization of the skeleton. Since normal growth and mineralization of the skeleton depends on the availability of the optimum level of serum Ca^{2+} and P. The major function of $1,25(OH)_2D_3$ is to regulate the homeostasis of the serum levels of Ca^{2+} and P to maintain proper cellular function and promote bone mineralization (Holick, 1996). The deficiency of these minerals due to a shortage of vitamin D in the body leads to rickets, osteomalacia, and osteoporosis (De Martinis *et al.*, 2021; Uhl, 2018).

Bone is a composite tissue containing 60% inorganic component (hydroxyapatite), organic component (30%), specialized cells, and water (10%) (Bourne *et al.*, 2021; Feng, 2009). The formation of bone involves two steps and starts with deposition and mineralization of the extracellular matrix (ECM) or osteoid. Approximately 90% of the ECM is composed of type I collagen, with the rest made up of non-collagenous proteins, growth factors, and glycoproteins. Ca^{2+} hydroxyapatite [$Ca_{10}(PO_4)_6(OH_2)$] is the principal mineral component of bone, which is composed of inorganic $PO4^{3-}$ or P and Ca^{2+}. Osteoblasts are derived from mesenchymal stromal cells (MSCs) for the formation of bone tissue. It contains a large Golgi apparatus, secretory vesicles, rough endoplasmic reticulum, and mitochondria as a result of the large number of ECM proteins they synthesize and export (Bourne *et al.*, 2021; Dudley & Spiro, 1961). $1,25(OH)_2D_3$ stimulates bone mineralization by directly acting on the osteoblasts, osteocytes, and osteoclasts (Chen *et al.*, 2016; van Driel & van Leeuwen, 2023; Woeckel *et al.*, 2010; Woeckel *et al.*, 2013). It regulates the mineralization process via regulation of transcription of the gene, differentiation, and mineralization of the osteoblasts, and production of the matrix vesicle. Some collagenous proteins, such as osteocalcin and osteopontin, as well as bone sialoprotein BSP1, are affected by it. In the bone matrix, osteocalcin and osteopontin bind Ca^{2+} and hydroxyapatite to facilitate their deposition. Bone matrix organization is also supported by sialoproteins (Atkins *et al.*, 2007). Vitamin D stimulates osteocytes to produce FGF23, a phosphatonin that inhibits PO_4^{3-} reabsorption, regulates the hydroxylation of $1,25(OH)_2D_3$ in the kidney, and decreases the production of PTH. Additionally, vitamin D is critical for osteoclast differentiation and function. Preosteoblasts are stimulated by this hormone to secrete macrophage-colony stimulating factor (M-CSF), which inhibits preosteoclast apoptosis and promotes their proliferation. RANKL released by preosteoblasts binds to RANK found on the membrane of preosteoclasts leading to the activation of the differentiation pathway and activation of osteoclasts to form a mature cell. RANKL may bind with osteoprotegerin protein (OPG) from osteoblasts and impair its binding to RANK, inhibiting osteoclast maturation pathways. Vitamin D upregulates the expression of RANKL in osteoblasts, inhibiting the expression of OPG and stimulating osteoclastogenesis (Kogawa *et al.*, 2010).

The indirect effect of $1,25(OH)_2D_3$ is proceeded by the regulation of Ca^{2+} and P metabolism of the body. It plays a key role in intestinal P and Ca^{2+} absorption and renal reabsorption to balance the serum mineral status for the proper mineralization of the bone. In the

gastrointestinal and renal tracts, Ca^{2+} is transported passively via concentration gradients and actively via ATP-dependent transcellular transport (Hoenderop *et al.*, 2005). The latter is crucial when the Ca^{2+} supply is insufficient and is largely governed by $1,25(OH)_2D_3$. The transcellular transport of Ca^{2+} consists of three steps: I) Ca^{2+} influx into the epithelial cells, mediated by transient receptor potential vanilloid (TRPV) channels and initiated by steep electrochemical gradients through the apical membranes II) Transport of Ca^{2+} bound to Ca^{2+} binding proteins (CaBPs) in the cytosol and III) Energy intensive process mediated extrusion of Ca^{2+} through the basolateral membrane into the extracellular fluid (Bouillon *et al.*, 2008; Leunissen *et al.*, 2013; Peng *et al.*, 2018). However, both intestines and kidneys differ in terms of the site of active and passive Ca^{2+} transport. In the kidney (95% filtered Ca^{2+} is reabsorbed through the renal tubules), Ca^{2+} reabsorption is fine-tuned by $1,25(OH)_2D$ in the distal nephron following an abundance of combined reabsorption of Ca^{2+} and Na^+ in the proximal segment of the nephron. 60% of the filtered Ca^{2+} is passively reabsorbed in the proximal tubules and 15% of Ca^{2+} is reabsorbed via paracellular diffusion through the action of paracellin-1 (claudin-16). As Ca^{2+} levels change in the thick ascending limb, Ca^{2+} sensing receptors (CaSRs) inhibit Ca^{2+} reabsorption independent of PTH and $1,25(OH)_2D_3$ (Jeon, 2008). In the intestine, active Ca^{2+} absorption is largely regulated by $1,25(OH)_2D_3$ in the duodenum, and passive Ca^{2+} absorption in the jejunum. Molecular players involved in Ca^{2+} transport in the intestine and kidney differ as well: TRPV6 is prevalent in the duodenum, whereas TRPV5 is the major apical Ca^{2+} channel inside the kidney; In the intestine, only CaBP-9k (cytosolic Ca^{2+} transporter, molecular mass, 9 kDa) is present, whereas both CaBP-9k and CaBP-28k (molecular mass, 28 kDa) are found in the kidney; Ca^{2+} extrusion in the intestine is regulated by plasma membrane Ca^{2+} ATPase, while in the kidney it is regulated by Na^+/Ca^{2+} exchanger (NCX1) and PMCA1b (Bouillon *et al.*, 2008).

1.2.4.2. Effects besides mineralization

Vitamin D is well known for the mineralization of the skeleton via intestinal absorption of Ca^{2+} and P. It also plays a crucial role in growth (Flohr *et al.*, 2014; Hines *et al.*, 2013), reproduction (Upadhaya *et al.*, 2021; Weber *et al.*, 2014), immunity (Konowalchuk *et al.*, 2013; Zhang *et al.*, 2021), antioxidative (Rey *et al.*, 2020; Yang *et al.*, 2019) status and other biologically important functions of the body (Flohr *et al.*, 2014). However, the forms (vitamin D_3/$25(OH)D_3$) and doses of vitamin D play an important role in maintaining the non-mineralizing effects on the body. The legislation limits the vitamin D supply to 1000-2000 IU/kg diet, which corresponds to 25-50 µg/kg of feed (Efsa Panel on Additives Products or Substances used in Animal Feed, 2012),

while 25(OH)D_3 in conjunction with vitamin D_3 is allowed up to 50 µg/kg of feed (European Food Safety Authority, 2009).

There are some contradictory findings regarding the impact of vitamin D on the growth performance of the pigs. According to some researchers, there is no direct effect of any form of vitamin D on the body weight or weight gain of the pigs (Thayer *et al.*, 2019; Witschi *et al.*, 2011). However, some studies suggest that supplementing sows with 25(OH)D_3 enhances the growth performance of their progeny (Upadhaya *et al.*, 2021; Zhang *et al.*, 2019; Zhao *et al.*, 2022). 25(OH)D_3 also shows a significant impact on the muscle development (primary muscle fiber number, total muscle fiber number, and the expression of the genes linked to muscle development) of the pigs (Hines *et al.*, 2013; Thayer *et al.*, 2019). Some researchers have hypothesized that improved growth performance might be due to an increase in growth hormone (GH)/insulin-like growth factor (IGF)-1 activity, which is associated with the progression of bone, skeletal muscle, and general body size (Upadhaya *et al.*, 2022). However, it is still unclear how vitamin D interacts with the GH/IGF-1 system (Brameld *et al.*, 1996; Upadhaya *et al.*, 2022). In humans, it has been reported that vitamin D levels affect the hepatic release of IGF-1 and insulin-like growth factor binding protein 3 (IGFBP-3) and expression of IGF-1 receptors across different tissues of the body (Ameri *et al.*, 2013; Matilainen *et al.*, 2005). Vitamin D metabolism and GH are interconnected: on the one hand, the supplementation of vitamin D elevates the level of IGF-1 (Wei *et al.*, 1998), and on the other hand, IGF-1 is involved in stimulating the activity of the 1α-hydroxylase which in turn controls the synthesis of the active form of vitamin D/calcitriol in the kidney (Henry, 2011). Furthermore, GH itself stimulates the synthesis of 1,25$(OH)_2D_3$ (Marcus *et al.*, 1990). Both GH and IGF-1 are also predicted to elevate the action of CYP27A1, which is involved in the 25-hydroxylation of vitamin D in hepatic cells (Araya *et al.*, 2003).

The research on the potential effect of vitamin D on the reproductive performance of the animal is ongoing. A growing body of evidence indicates that vitamin D_3 can also act on non-classical tissues (Hasan *et al.*, 2022), including reproductive organs (Lerchbaum, 2012). In females, physiological processes within the ovaries, uterus, and placentas are influenced by vitamin D_3 (Muscogiuri *et al.*, 2017; Shahrokhi *et al.*, 2016). The expression of homeobox protein A10 (HOXA10) is upregulated during implantation in human endometrium, which is necessary for normal development. 1,25$(OH)_2D_3$ is shown to regulate key target genes (HOXA10) implicated in implantation (Du *et al.*, 2005). Mutant mice lacking the VDR gene

exhibited hypoplastic uteri and impaired folliculogenesis, while mutant mice lacking the VDR gene had low sperm production, reduced motility, and histologically abnormal testicle formation (Kinuta *et al.*, 2000). Several lines of evidence suggest that vitamin D plays an important role in pig fertility. However, the efficacy of two forms of vitamin D on the reproductive performance of pigs is different. According to Upadhaya *et al*. (2021), supplementing 25(OH)D_3 to sows can increase the survivability and body condition score of the piglets. Although preweaning mortality was not reported, feeding 25(OH)D_3 increased the number of fetuses in the litter by 2.5 when compared to vitamin D_3 (Coffey *et al.*, 2012). As compared to vitamin D_3 supplementation, sows fed 25(OH)D_3 weaned 1.17 more piglets, and the treatment also improved bone density, strength, and ash content in piglets (Zhou *et al.*, 2017). The number of mummified fetuses in the sow farrowing group is reported to have decreased significantly from 0.5 to 0.1, while their birth weight increased from 1.18 to 1.44 kg (Zhang *et al.*, 2017). However, some studies reported no effect of vitamin D on the reproductive performance (litter size, survivability) of the pigs except live birth per sow (Lauridsen *et al.*, 2010; Thayer *et al.*, 2019; Upadhaya *et al.*, 2022) demanding further investigation.

Recently, vitamin D has gained a lot of attention due to its role in the body's immune function. To date, most studies have examined vitamin D's effects on human immunity, but few have examined its effects on porcine immunity. VDR signaling has been discovered in several immune cells, including macrophages, monocytes, natural killer cells, dendritic cells, and T and B lymphocytes (Baeke *et al.*, 2010; Chishimba *et al.*, 2010; Jeffery *et al.*, 2009; Lin *et al.*, 2017). According to Jackson *et al*., vitamin D has powerful effects on cytokine production and immune function (O'Brien & Jackson, 2012). Vitamin D is also involved in the maintenance of intestinal homeostasis through its effects on autophagy (Wu *et al.*, 2015) and the composition of the intestinal microbiome (Waterhouse *et al.*, 2019), as well as in the regulation of mucosal barrier function by upregulating the expression of tight junction and adherent junction proteins and inhibiting epithelial cell apoptosis (Del Pinto *et al.*, 2017). However, the forms of vitamin D differ in their ability to maintain the body's immune function. In vitro, the active form of vitamin D, 1,25(OH)$_2D_3$ has been reported to suppress the immune response by inhibiting the activity of T helper/inducer cells (Th cells) that activates peripheral blood mononuclear cells to suppress the production of immunoglobulins, which is associated with a decrease in the inflammatory cytokine interleukin-2 (IL-2) (Zhao *et al.*, 2014). 1,25(OH)$_2D_3$ also

induces the synthesis of interleukin-4 (IL-4) in Th2 cells (improve the potency of the macrophages and monocytes for killing the bacteria) (Mahon *et al.*, 2003), increases the activity of $CD4^+$, $CD25^+$ T regulatory cell (crucial for the inhibition of inflammation) and suppresses the inflammatory Th1 secreted cytokines (IL-2, IFN-γ, IL-17, IL-21) (Jeffery *et al.*, 2009). However, recent studies have demonstrated that $25(OH)D_3$ has also a positive impact on pig health. It has been reported that $25(OH)D_3$ improves humoral immunity, innate immunity, and gut immunity in pigs (Zhang *et al.*, 2022). The concentrations of IgG, IgA, and IgM in serum (Meuter *et al.*, 2016; Zhang *et al.*, 2022) and IgG in milk (Zhang *et al.*, 2019) were found to be significantly elevated in response to $25(OH)D_3$ treatment. The dietary supplementation of $25(OH)D_3$ was positively associated with anti-inflammatory responses, resulting in an increase in leukocyte counts per milliliter of blood, individual granulocyte and lymphocyte subpopulations, improving their survivability under basal conditions and also enhancing their phagocytic capacity (Konowalchuk *et al.*, 2013).

1.2.5. Target genes of vitamin D

The biological actions of $1,25(OH)_2D_3$ are mediated through specific alterations in gene expression proceeded by a VDR. In response to direct interaction with $1,25(OH)_2D_3$, VDR quickly binds to regulatory regions of the target genes to nucleate the formation of large protein complexes crucial for the transcriptional alterations directed by the receptor. Most target cells respond to these actions by expressing networks of target genes, whose functional activities coordinate to orchestrate specific biological responses. Depending on the tissue, these responses can be complex, ranging from those essential for mineral metabolism homeostatic to focal responses that control cell growth, differentiation, and functional activity, including those in the immune system, skin, pancreas, bone, and many others (Pike & Meyer, 2012). In most cases, vitamin D target genes have been studied in humans, whereas the studies on pigs are limited. Numerous studies have shown that vitamin D can affect up to 900-1000 genes (Carlberg, 2019; Nurminen *et al.*, 2019b; Wang *et al.*, 2005). A human acute monocytic leukemia cell line, THP-1 (Tsuchiya *et al.*, 1980) has long been used as a model system to investigate the vitamin D-mediated physiological processes in innate immunity research. In the study of Nurminen *et al*. (2019a), transcriptomic analysis of THP-1 cells revealed 951 vitamin D target genes, of which a total of 273 overlapped with the previously reanalyzed RNA-seq dataset (Neme *et al.*, 2017; Seuter *et al.*, 2016). Based on the later dataset, 189 (69%) out of 273 genes were assigned as primary vitamin D targets. Among 189

genes, the top 5 genes with the highest fold changes (fold change>40) were: *CD14* (encoding a toll-like receptor coreceptor, mediate the innate immune response against viruses and bacterial lipopolysaccharide) (Zanoni & Granucci, 2013), *ORM1* (encoding key acute phase plasma protein, involved in lipid homeostasis and protein quality control) (Han *et al.*, 2010), *CAMP* (universal regulator of cellular function), *FBP1* (encoding a glucose metabolizing enzyme, involved in glucose metabolism) (Ghanem *et al.*, 2017), and *CYP26B1* (encoding an enzyme metabolizing retinoids, involved in retinoic acid metabolism) (Isoherranen & Zhong, 2019). Identification of the underlying biological processes is the most important factor to consider when analyzing lists of hundreds of vitamin D target genes. A gene ontology analysis is often used to assess whether a list of target genes is statistically significant in enriching a predefined list of terms, which include (i) the molecular function (the molecular activity of the gene), (ii) the biological process (the role a gene plays in the cell or physiological context), and (iii) the cellular component (the site where the product of the gene functions in the cell). The authors performed an analysis to observe the biological functions of those listed genes (951, 273, and 126 genes). The top 5 enriched biological pathways were neutrophil activation, inflammatory response, neutrophil degranulation, negative regulation of T cell proliferation, and positive regulation of cytokine production (Nurminen *et al.*, 2019a) indicating the modulation of innate immunity in response to vitamin D-mediated stimulation of human monocyte (Nurminen *et al.*, 2019a). The enrichment of these biological pathways is in agreement with the known key function of vitamin D in monocytes (Prietl *et al.*, 2013). In the study of Hanel & Carlberg (2022), due to the high vitamin D responsiveness of monocytes in PBMCs, the gene ontology analysis of 662 vitamin D target genes in this tissue (stimulated with vitamin D) reveals similar immune functions, including neutrophil degranulation, inflammation, cytokine-mediated signaling pathways, extracellular matrix organization, and positive regulation of angiogenesis.

1.3. Aims of the thesis

The secosteroid hormone, vitamin D is crucial for the optimal health status and performance of the 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 is 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 phosphorus and artificial UVB exposure in pigs. Therefore,

I. In the first study, 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 phosphorus 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 D_3 and 25(OH)D_3 and at evaluating the efficacy of appropriate dietary supplementation in improving reproductive capacities, growth performance, immunity, and bone development in pigs.

2. Summary of the studies and personal contributions

2.1. 1st study

The key theme of this dissertation is to identify the molecular mechanisms of vitamin D metabolism and phosphorus utilization to maintain mineral homeostasis for the improvement of the existing animal breeding program. Phosphorus and vitamin D play an important role in animal growth and reproduction. Additionally, gene expression analysis is a crucial step in predicting the continuous phenotypes for optimal animal production and animal breeding. This study was designed to observe the effect of the divergent dietary P on the expression of genes associated with vitamin D metabolism and FGF23 signaling to maintain vitamin D and P homeostasis and ultimately mineral homeostasis. The tissue-wide distribution of vitamin D metabolism genes from the first study revealed the ability of the non-renal tissues to synthesize active vitamin D according to their needs. As a result of the divergent dietary phosphorus intake, variations in serum vitamin D status and expressions of vitamin D metabolism genes divulged the intrinsic mechanism of the body to maintain phosphorus homeostasis through alterations of vitamin D level and associated transcripts [Metabolites (2022): 12(8), 729].

The following is my contribution to the publication summarized in this dissertation:

- Tissue processing (partial)
- Gene expression profiling by performing RT-qPCR
- Analyzing and visualizing all gene expression data
- Data interpretation and discussions
- Writing the manuscript

2.2. 2nd study

In commercial pig farming, the traditional method of confinement limits the access of pigs to sunlight exposure containing UVB radiation and the ability of the endogenous production of vitamin D_3. The pigs have to rely solely on the dietary source to fulfill the requirement of vitamin D in the body, which could be detrimental to the optimal growth, production, and reproduction efficiency of the animals. Therefore, this study aimed to evaluate the impact of artificial UVB exposure on the hepatic transcriptome and vitamin D metabolism in pigs. UVB-induced increased serum vitamin D levels and hepatic molecular changes towards positive vitamin D status suggest that dietary source of vitamin D could be complemented by endogenous calcidiol synthesis induced by artificial UVB irradiation, paving the way for long-term improvement of mineral utilization and confirming the possibility of producing biofortified animal products for human consumption [The Journal of Steroid Biochemistry and Molecular Biology (2023): 106428].

The following is my contribution to the publication summarized in this dissertation:

- Tissue sampling and processing
- RNA extraction, quantity, and integrity checks
- Gene expression profiling by performing RT-qPCR
- Next-generation sequencing (NGS) data analysis and visualization
- Interpretation of the results and discussions
- Writing the manuscript

2.3. 3rd study

The local synthesis of vitamin D (as observed in the 1st study) confirms the previous fact that the function of vitamin D is not only to maintain the skeleton development but also to perform other crucial functions of the body. Observations from the 2nd study demonstrated the ability of the UVB exposure to elevate the serum concentration of 25(OH)D_3 in pigs. Therefore, this study was conducted to reveal the mineralizing and non-mineralizing effects of these two different sources of active vitamin D and their comparative efficacies in improving the overall performance of the pigs. The study showed that dietary intake of 25(OH)D_3 demonstrates limited improvement in the reproductive capacities of the pigs demanding further investigations. The maternal supply of 25(OH)D_3 significantly improved the growth performance of the piglets compared to sows fed vitamin D_3-enriched feeds. Serum markers of innate and humoral immunity were also found to be significantly improved following the dietary intake of 25(OH)D_3. Pigs receiving basal diets low in calcium and phosphorus demonstrated significant improvement in bone mineralization following the dietary ingestion of 25(OH)D_3 in contrast to vitamin D_3. In summary, 25(OH)D_3 could be a promising and potential alternative to vitamin D_3 in promoting growth, reproduction, immunity, and bone development in pigs [British Journal of Nutrition (2023): 1-10].

The following is my contribution to the publication summarized in this dissertation:

- Conceptualization
- Systematic search and article selection following the PRISMA flow diagram
- Analyzing and combining the results of the selected articles
- Results interpretation and discussions
- Writing the manuscript

3. Lists of publications

3.1. Publication 1

Tissue-wide expression of genes related to vitamin D metabolism and FGF23 signaling following variable phosphorus intake in pigs

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Conceptualization: M.O., H.R., D.-C.F. and K.W.; methodology: M.H., M.O., H.R., S.P. and E.M.; formal analysis: M.H., M.O. and H.R.; investigation: M.H., M.O. and H.R.; resources: S.P., E.M., P.W. and K.W.; data curation: M.H., M.O. and H.R.; writing—original draft preparation: M.H.; writing—review and editing: M.H., M.O., H.R., S.P., E.M., P.W., D.-C.F. and K.W.; visualization: M.H.; supervision: M.O., H.R., D.-C.F. and K.W.; project administration: K.W.; funding acquisition: P.W., D.-C.F. and K.W.

Abstract

Calcium (Ca) and phosphorus (P) homeostasis is maintained by several regulators, including vitamin D and fibroblast growth factor 23 (FGF23), and their tissue-specific activation and signaling cascades. In this study, the tissue-wide expression of key genes linked to vitamin D metabolism (*CYP2R1*, *CYP27A1*, *CYP27B1*, *CYP24A1*, *GC*, *VDR*) and FGF23 signaling (*FGF23*, *FGFR1-4*, *KL*) were 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. Expression patterns revealed that non-renal tissues and cells (NRTC) express genes to form active vitamin D [1,25$(OH)_2D_3$] according to site-specific requirements. A low P diet resulted in higher serum calcitriol and increased *CYP24A1* expression in the small intestine, indicating local suppression of vitamin D signaling. A high P diet prompted increased mRNA abundances of *CYP27B1* for local vitamin D synthesis, specifically in bone. For FGF23 signaling, analyses revealed ubiquitous expression of *FGFR1-4*, whereas *KL* was expressed in a tissue-specific manner. Dietary P supply did not affect skeletal *FGF23*; however, *FGFR4* and *KL* showed increased expression in bone at high P supply, suggesting regulation to balance mineralization. Specific NRTC responses influence vitamin D metabolism and P homeostasis, which should be considered for a thrifty but healthy P supply.

3.2. Publication 2

Exposure to artificial ultraviolet-B light mediates alterations on the hepatic transcriptome and vitamin D metabolism in pigs

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Conceptualization: EM, KW; Data curation: MH, HR, MO, NT; Formal analysis: MH, HR, MO, NT; Funding acquisition: EM, DCF, KW; Investigation: MH, HR, MO; Methodology: MH, NT; Resources: NT, SP; Supervision: DCF, KW; Visualization: MH, HR; Roles/Writing - original draft: MH; and Writing - review & editing: MH, HR, MO, NT, SP, EM, DCF, KW.

Abstract

In the currently prevailing pig husbandry systems, the vitamin D status is almost exclusively dependent on dietary supply. Additional endogenous vitamin D production after exposure to ultraviolet-B (UVB) light might allow the animals to utilize minerals in a more efficient manner, as well as enable the production of functional vitamin D-enriched meat for human consumption. In this study, growing pigs (n = 16) were subjected to a control group or to a daily narrowband UVB exposure of 1 standard erythema dose (SED) for a period of 9 weeks until slaughter at a body weight of 105 kg. Transcriptomic profiling of liver with emphasis on the associated effects on vitamin D metabolism due to UVB exposure were evaluated via RNA sequencing. Serum was analyzed for vitamin D status and health parameters such as minerals and biochemical markers. The serum concentration of calcidiol, but not calcitriol, was significantly elevated in response to UVB exposure after 17 days on trial. No effects of UVB exposure were observed on growth performance and blood test results. At slaughter, the RNA sequencing analyses following daily UVB exposure revealed 703 differentially expressed genes (DEGs) in liver tissue (adjusted p-value < 0.01). Results showed that molecular pathways for vitamin D synthesis (*CYP2R1*) rather than cholesterol synthesis (*DHCR7*) were preferentially initiated in liver. Gene enrichment (p < 0.05) was observed for reduced cholesterol/steroid biosynthesis, SNARE interactions in vesicular transport, and CDC42 signaling. Taken together, dietary vitamin D supply can be complemented via endogenous production after UVB exposure in pig husbandry, which could be considered in the development of functional foods.

3.3. Publication 3

Efficacy of dietary vitamin D_3 and 25(OH)D_3 on reproductive capacities, growth performance, immunity, and bone development in pigs

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Conceptualisation: M. H., M. O., H. R., K. W. and D-C. F.; systematic search and articles selection: M. H.; first draft: M. H.; revision: M. H., M. O., H. R., K. W. and D-C. F.

Abstract

Vitamin D_3 (Vit D_3) and 25(OH)D_3 are used as dietary sources of active vitamin D [1,25$(OH)_2D_3$] in pig husbandry. Although acting primarily on intestine, kidney, and bone, their use in pig nutrition has shown a wide range of effects also in peripheral tissues. However, there is an ambiguity in the existing literature about whether the effects of Vit D_3 and 25(OH)D_3 differ in attributing the molecular and phenotypic outcomes in pigs. We searched Web of Science and PubMed databases concerning the efficacy of Vit D_3 in comparison with 25(OH)D_3 on pig physiology, i.e., reproductive capacities, growth performance, immunity, and bone development. Dietary intake of Vit D_3 or 25(OH)D_3 did not influence the reproductive capacity of sows. Unlike Vit D_3, the maternal intake of 25(OH)D_3 significantly improved the growth performance of piglets, which might be attributed to maternally induced micronutrient efficiency. Consequently, even in the absence of maternal vitamin D supplementation, 25(OH)D_3-fed offspring also demonstrated better growth than the offspring received Vit D_3. Moreover, a similar superior impact of 25(OH)D_3 was seen with respect to serum markers of innate and humoral immunity. Last but not least, supplements containing 25(OH)D_3 were found to be more effective than Vit D_3 to improve bone mineralization and formation, especially in pigs receiving basal diets low in calcium and phosphorus. The insights are of particular value in determining the principal dietary source of vitamin D to achieve its optimum utilization efficiency, nutritional benefits, and therapeutic potency and to further improve animal welfare across different management types.

4. General discussions and conclusions

The supplementation of minerals, as well as the body's ability to maintain homeostasis, is crucial for the successful breeding program and management systems of livestock/domesticated animals. Minerals play a vital role in the normal health, growth, production, and reproduction of animals along with structural, physiological, catalytic, and regulatory functions of the body (National Research Council, 2012; McDowell, 1992; Suttle, 2022), as well as serving as components of proteins, enzymes, and enzymatic factors in a wide variety of biochemical processes (Fleet *et al.*, 2011; McDowell, 1992) in the body. Although minerals are essential, deficiencies and overloading are both harmful to the animals. It is therefore essential to maintain their optimum concentrations in the body through the balance among uptake, transport, metabolism, and storage processes. P is an essential macro mineral and providing sufficient amounts of P to livestock is vital because it contributes to the skeletal development, growth, and productivity of the animals. In the livestock industry, its efficiency of utilization is below 40%, which is detrimental to optimal animal performance along with the concerns of environmental issues due to the excretion of unused P through urine and feces (Kebreab *et al.*, 2012). Though there are recommendations for dietary feed supply there is still a debate about whether these are adequate, especially in the background that these recommended might be outdated and do not consider differences in demands of various breeds, developmental or production stages, or even individual needs. Vitamin D is one of the most critical hormones in the body to maintain mineral homeostasis, especially serum P and Ca balance and bone metabolism. In recent years, studies have revealed that vitamin D has a broader range of functions than previously thought. Given that, the overall aim of the dissertation was to determine the molecular mechanisms of vitamin D metabolism in response to low and high dietary P and UVB exposure. The identification of the superior form of vitamin D was also part of the study to improve the growth, productivity, reproductive capacity, and immune status of the animals.

4.1. Vitamin D metabolism and P utilization in response to divergent dietary P

Understanding animal physiology in the context of sustainable agriculture and animal husbandry remains a major task in the breeding and production of livestock, especially in response to the demands of a changing and diversified consumer market. The physiological processes of animals are governed by the expression of many genes working in concert with

each other. Therefore, knowledge of the expression of genes is crucial to improving animal performance, especially in pigs. In recent years, extensive research has been conducted to determine how genes are expressed and how they are regulated by biological and environmental factors (e.g., genetic determinants, nutritional factors, and animal management) (Cassar-Malek *et al.*, 2008). P is an essential macro element for all living organisms and its serum levels are regulated by a complex set of processes involving the intestine, bone, and kidneys with the help of hormones vitamin D, FGF23, and PTH. The function of these hormones is also regulated by many genes. Therefore, the first aim of this study was to determine the expression patterns of the candidate genes related to vitamin D metabolism and FGF23 signaling in pigs following the ingestion of the basal diets. The second goal was to determine the alterations in the expression of the candidate genes in response to divergent dietary phosphorus intake (low vs high). This study uncovered some of the following findings:

4.1.1. Non-renal synthesis of vitamin D

It is already established that the activation of vitamin D is mediated by the liver and kidney following the actions of specific hydroxylating enzymes. The function of vitamin D is mediated through its binding to its receptor VDR, a nuclear receptor ligand-dependent transcription factor. However, the findings of this study suggest that the conversion of 25(OH)D_3 also occurs in several non-renal tissues and cells (e.g., lung, aorta, bone, stomach, small intestine, large intestine) in the body (Hasan *et al.*, 2022) which are in agreement with the previous studies (Hewison *et al.*, 2007; Pike & Meyer, 2020). The basis for this idea originates from the fact that vitamin D activating enzyme encoding genes (*CYP2R1*, *CYP27B1*) and *VDR* are also expressed in these tissues along with the liver and kidney (Hasan *et al.*, 2022). It has been proposed that renal endocrine calcitriol regulates mineral metabolism primarily, whereas local NRTC production of this hormone is central to the control of numerous biological activities that are not calcemic in these cell types. The hypothesis is further supported by the finding that the expression of *CYP27B1* in NRTC is regulated differently from that of the kidney (Bikle & Christakos, 2020). While the expression of *CYP27B1* in the kidney is strongly modulated by PTH, calcitriol and phosphaturic FGF23, none of these mineralotropic hormones are active in NRTCs, where inflammatory mediators such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), lipopolysaccharides (LPS) and probably others predominate (Hewison, 2011). According to Bikle (2009), the non-classical actions of 1,25(OH)$_2$$D_3$ can be divided into

three categories: regulation of hormone secretion, regulation of immune function, and regulation of cellular proliferation and differentiation. The impact of this hormone may extend beyond these categories. The immune-modulatory effect of vitamin D is derived from two observations: the presence of VDR within proliferating immune cells, and immune cells' ability to metabolize vitamin D (Chun *et al.*, 2014). According to Umar *et al*., locally produced vitamin D affects innate and adaptive immune systems in an intracrine, autocrine, or paracrine manner (Umar *et al.*, 2018). Further studies are required to identify the unknown functions of vitamin D in NRTC. A knock-out study of the specific candidate gene (*CYP27B1*) could be a good approach to determine the specific function of locally produced active vitamin D. A future study could also investigate whether the activity of locally produced calcitriol depends on or is influenced by circulating calcitriol levels. In addition, it is essential to determine whether circulating 25(OH)D_3 concentrations have a differential effect on calcitriol production in kidneys and NRTCs.

4.1.2. Potential candidate genes for the improvement of vitamin D status and successful animal breeding

Vitamin D plays an important role in the growth, reproduction, and overall productive performance of the animals. It is therefore crucial to maintain the optimum vitamin D status in pigs. As a result of insufficient dietary supplementation and lack of sunlight exposure, vitamin D deficiency is very common in pigs reared for commercial purposes (Alexander *et al.*, 2017). While research in this field is ongoing, some potential candidate genes have been identified from this study, which could be considered to improve vitamin D metabolism and serum vitamin D status. Here are some candidate genes and considerations:

VDR: The VDR gene encodes for the vitamin D receptor, which plays a key role in mediating the biological effects of vitamin D. A tissue-wide distribution of the gene in this study indicates that all organs require vitamin D for maintaining the normal function of the body. However, polymorphisms of this gene may have been associated with different pathological and non-pathological conditions of the body. The divergent dietary P used in this study did not alter the expression of this gene in any tissues including the kidney but changed the serum vitamin D status indicating no effect of variable P on the mutation of this gene. A polymorphism in this gene may have different effects on different tissues. For example, in one study, overexpression of muscular VDR stimulated muscle hypertrophy through enhanced protein synthesis, translation efficiency, and ribosomal expansion (Bass *et al.*, 2020) and

downregulation of this gene may do the opposite. Thus, any mutation of this gene could potentially reduce the growth and productive performance of the pigs. Therefore, the mutation of this gene should be thoroughly investigated to select the animals for the breeding purpose.

CYP2R1 and *CYP27B1*: These genes encode the enzymes involved in the synthesis of the active form of vitamin D. The expression of both hydroxylating enzyme-encoding genes was similar to that of VDR in this study, indicating that almost all organs in the body can synthesize vitamin D. Therefore, mutations of these genes may influence the efficiency of vitamin D synthesis and ultimately, animal productivity. Surprisingly, in our study neither *CYP2R1* in the liver nor *CYP27B1* in the kidney was upregulated in response to low or high dietary P. Though, the serum concertation of vitamin D increased significantly in response to low dietary P. Vitamin D is essential for the absorption of Ca and P in the intestines. Any mutation or downregulation that impairs the function of CYP2R1 and CYP27B1 may lead to decreased levels of active vitamin D, affecting calcium and phosphorus homeostasis (Norlin & Wikvall, 2023). This, in turn, can impact the health and development of bones and teeth in animals. Calcium is critical for muscle function, including the muscles involved in reproduction genes (Lee, 2010; Uchida & Bohr, 1969). Breeding success may be influenced by the availability of Ca and the proper functioning of the reproductive organs, which can be affected by mutations in these two. Vitamin D also plays an important role in the regulation of the immune system (Aranow, 2011). Animals with mutations may have altered immune responses, potentially impacting their overall health and resistance to diseases. This could have indirect effects on their ability to reproduce successfully. The physiological functions of vitamin D are beyond bone health, including cell growth, neuromuscular function, and immune response. Mutations in *CYP2R1* may affect these processes, influencing the overall health and growth of animals.

CYP24A1: This gene encodes for the enzyme involved in the catabolism of vitamin D. In this study, the expression of this gene was upregulated in response to low dietary P in the small intestine and kidney. The expression could be implicated in the regulation of vitamin D synthesis and its homeostasis triggered by low serum P. Abnormal upregulation of *CYP24A1* causes vitamin D insufficiency and associated complications in the body (Li *et al.*, 2019; Wang *et al.*, 2016). The loss of function following the abnormal mutation of this gene can also lead to hyperphosphatemia and hypercalcemia in the body (Gigante *et al.*, 2016). This could severely impact the growth, productivity, and reproductive performance of the pigs.

GC: The *GC* gene codes for the vitamin D-binding protein, which transports vitamin D in the bloodstream. Genetic variations in GC can affect circulating levels of vitamin D. In this study, a high level of *GC* expression was observed in the liver and also in the kidney, indicating the ability of these two organs to transport vitamin D throughout the body. However, the expression of this gene remained unaltered in response to variable dietary P, indicating its expression is independent of serum vitamin D status. In the only human case of biallelic mutation of the *DBP*/*GC* gene, serum DBP levels were undetectable, but $1,25(OH)_2D$ was nearly undetectable or detectable in tissue with no effects on Ca or PTH levels (Bikle & Schwartz, 2019; Henderson *et al.*, 2019). Until now, there has been no conclusive evidence that mutations of DBP cause serum vitamin D insufficiency or animal performance.

It is important to conduct thorough genomic and functional studies to validate the associations between these candidate genes and the desired traits. Additionally, the genetic architecture of these traits can be complex, involving multiple genes and interactions. Genetic selection in animal breeding programs should consider a combination of traits related to vitamin D metabolism, reproductive success, and overall performance. Ongoing advancements in genomics and molecular biology will likely contribute to the identification of additional candidate genes in the future.

4.1.3. Side-effects on metabolic health

- **Role of FGF23 on metabolism**

FGF23 is a hormone primarily known for its role in P metabolism and vitamin D homeostasis. It is mainly produced by osteoblasts and osteocytes, which are found in bone tissue. FGF23 regulates P levels by inhibiting phosphate reabsorption in the kidneys and reducing the production of active vitamin D, which, in turn, affects Ca and P metabolism. In our study, a gene encoding bone-derived hormone *FGF23* was expressed in bone and liver. Surprisingly, the genes encoding the receptors of FGF23 (*FGFR1*, *FGFR2*, *FGFR3,* and *FGFR4*) demonstrated ubiquitous expression of the body indicating the tissue-wide capacity for FGF23 signal transduction and regulation of P metabolism. While its primary functions are related to mineral homeostasis, there is emerging evidence suggesting potential connections between FGF23 and metabolic processes, including insulin sensitivity. Therefore, special attention should be paid to the mutation of this gene during the selection of the pigs for breeding purposes. The effects of FGF23 on insulin resistance and metabolisms are described below:

Insulin resistance: Some studies have suggested a potential link between elevated FGF23 levels and insulin resistance. Insulin resistance is a condition in which the body's cells become less responsive to the effects of insulin, leading to impaired glucose uptake. Elevated FGF23 levels have been associated with insulin resistance in conditions such as diabetes, chronic kidney disease (CKD), and obesity in mice and humans (Bär *et al.*, 2018; Hanks *et al.*, 2015).

Glucose metabolism: In addition to regulating blood phosphate levels, FGF23 also influences glucose metabolism. According to researchers, ablation of the FGF23 gene in mice results in hypoglycemia and an increase in peripheral insulin sensitivity (Hesse *et al.*, 2007; Shimada *et al.*, 2004). Experimental studies have shown that FGF23 can impair insulin signaling in various tissues, including adipose tissue and skeletal muscle. Interference with insulin signaling pathways could adversely affect glucose metabolism (Nakashima *et al.*, 2018). In pigs, the disturbance in glucose metabolism could adversely affect the productive performance of the pigs.

Adipose tissue and energy metabolism: FGF23 receptors are present in adipose tissue, and FGF23 may influence the function of the adipocytes (Afsar *et al.*, 2023). Adipose tissue plays a crucial role in energy metabolism and insulin sensitivity (Smith & Kahn, 2016). The interaction between FGF23 and adipose tissue could have implications for the metabolic health of the pigs.

Association with metabolic disorders: Elevated FGF23 levels have been observed in conditions associated with metabolic dysfunction, such as obesity and type 2 diabetes (Hanks *et al.*, 2015). The exact mechanisms by which FGF23 influences metabolic processes are not fully understood, and research in this area is ongoing. It is important to note that while there is evidence suggesting a potential association between FGF23 and metabolic processes, the exact mechanisms and causality are still being investigated, especially in pigs.

- **Role of FGF23 on osteoimmunology**

While the primary focus of FGF23 is on mineral homeostasis (especially serum P balance), there is emerging research suggesting its potential involvement in osteoimmunology, which is the study of the interactions between the skeletal and immune systems. The skeletal system, particularly bone, is increasingly recognized as an active participant in immune responses. Since the information on osteoimmunology is crucial for the optimal health status of the animal, there is a potential relationship between animal breeding as well. The mutation of

FGF23 should be considered carefully before selecting the animal for breeding purposes. Here are some potential roles of FGF23 in osteoimmunology:

Inflammation and immune regulation: FGF23 has been associated with inflammation in various conditions, and inflammation plays a crucial role in osteoimmunology (Francis & David, 2016). According to Francis *et al*., inflammation increases the expression and stabilization of hypoxia-inducible factor-1α (HIF-1α) and this transcriptionally active HIF-1α/HIF-1β heterodimers translocate into the nucleus and bind hypoxia-reactive elements (HRE) to upregulate the expression of FGF23 (Francis & David, 2016). The inflammatory condition of the body may influence bone remodeling, and FGF23 could be a mediator in this process.

Osteoclastogenesis and bone resorption: FGF23 may influence osteoclastogenesis (Sirikul *et al.*, 2022), the process by which bone-resorbing cells (osteoclasts) are formed. Changes in osteoclast activity may affect bone remodeling and impact the immune microenvironment within the bone which could be detrimental to the growth and overall performance of the animals.

Interaction with immune cells: There is evidence suggesting that FGF23 receptors are expressed on immune cells, including monocytes and macrophages, and associated with the immune function of the body (Fitzpatrick *et al.*, 2018; Rossini *et al.*, 2005). This raises the possibility that FGF23 may directly influence the function of immune cells within the bone microenvironment.

Impact on bone marrow: FGF23 may influence the bone marrow microenvironment, which is a critical component of the immune system (Simic & Babitt, 2021). Changes in the bone marrow can affect the production and function of immune cells. It is important to note that while there is a growing body of research suggesting a potential link between FGF23 and osteoimmunology, the exact mechanisms and implications of this relationship are not fully understood. Further research is needed to elucidate the specific roles of FGF23 in immune regulation within the bone microenvironment and its relevance to various diseases that involve both mineral metabolism and the immune system in pigs.

4.2. Vitamin D metabolism and P utilization in response to ultraviolet radiation

Vitamin D deficiency is very common among animals, especially in pigs. There are two different sources of vitamin D in the body, one is a dietary source and another one is a natural source. A natural source of vitamin D production is the sunlight exposure of the animals

containing UVB radiation. In commercial pig framing, pigs usually do not have access to sunlight. Therefore, they are greatly deprived of the natural source of vitamin D production. This study aimed to evaluate the effectiveness of the artificial UVB light on vitamin D synthesis in the body and associated transcriptomic effects in the liver to improve the serum vitamin D and obtain the accompanying benefits e.g., improved growth, immunity, and reproduction status and also to reduce the utter reluctance of the pigs on the dietary sources of vitamin D. The outcome of this study could help to divulge the following issues:

4.2.1. Molecular mechanisms of vitamin D metabolism and P utilization in response to artificial UVB light exposure

As we already know, sun exposure in animals contributes to the formation of vitamin D in the body through the conversion of 7-dehydrocholesterol in the skin into previtamin D_3, which in turn isomerizes to vitamin D_3. The synthesized vitamin D_3 is then hydroxylated in the liver and kidney to become active. Our study suggests that artificial UVB light might also be able to initiate the same molecular mechanism of vitamin D synthesis in the body as sunlight exposure. Artificial UVB exposure to the animal also increases serum vitamin D levels similar to sunlight as seen in our study, which is also supported by other researchers (Neill *et al.*, 2023; Osmancevic *et al.*, 2015). The gene expression pattern observed in response to UVB exposure reveals intriguing insights into the regulation of vitamin D metabolism. It was expected that the serum status of P would also elevate in response to the increased serum status of vitamin D. However, the serum concentration of P remained unaltered. This might be due to the balance in the expression of the genes linked to P transport (specific *SLC* gene superfamily) to maintain its homeostasis (Wubuli *et al.*, 2019). The study focused on various key genes involved in vitamin D synthesis, transport, and receptor activity. Notably, the elevated expression of 25-hydroxylase *CYP2R1* correlated with increased serum calcidiol levels, indicating an active role in the conversion of cholecalciferol to calcidiol in response to artificial UVB light (Cheng *et al.*, 2003). Given the role of CYP27A1 in both bile acid and cholesterol metabolism, the downregulation of this gene suggests a potential link between UVB exposure and reduced fatty acid synthesis (Dubrac *et al.*, 2005; Li *et al.*, 2007), opening the possibility of implementing artificial UVB exposure on the weight loss program in the animal industry. However, it requires further investigation to make a concrete conclusion. The gene encoding the vitamin D binding protein, *GC*, showed unchanged expression despite elevated serum calcidiol levels suggesting efficient transportation of calcidiol in circulation, supported by

recent findings in pigs subjected to a low P diet (Hasan *et al.*, 2022). Expression patterns of *CYP27B1* (responsible for 1α-hydroxylase activity) remained unaffected by UVB exposure, indicating no significant impact on hepatic calcitriol production (Brunette *et al.*, 1978; Hasan *et al.*, 2022; Meyer & Pike, 2020). The unaltered expression of *CYP24A1* (Hasan *et al.*, 2022), *VDR*, and specific *RXRs* indicates that the expression pattern of these genes is independent of serum vitamin D status, especially in the liver in response to artificial UVB light. Specific vitamin D target genes also demonstrated significant alteration, indicating their important contributions to vitamin D metabolism. Overall, the gene expression pattern provides valuable insights into the complex regulatory network of vitamin D metabolism in response to UVB exposure, shedding light on the potential connections of UVB with lipid metabolism and the transcriptional control of vitamin D target genes in porcine tissues.

4.2.2. Vitamin D biofortification of meat using UVB light for human consumption

Deficiency of vitamin D is a major health concern among the populations worldwide, especially in Europe and Northern latitudes where sunlight exposure is limited. Therefore, there is a clear need for innovative vitamin D-enhancement strategies that are based on natural food sources and reflect the diversity of dietary patterns and fortification policies in the population (Black *et al.*, 2012; Wilson *et al.*, 2017). Biofortification refers to the process of enhancing the nutritional quality of the food using specific nutrients. Meat is one of the few food sources that naturally contain vitamin D, which makes it an ideal candidate for biofortification. Additionally, meat contains the 25(OH)D metabolite, which has been shown to have a faster absorption and to raise serum 25(OH)D more effectively than other vitamin D metabolites (Duffy *et al.*, 2019) in the body. The outcomes of our study indicated the improved serum status of vitamin D in response to artificial UVB light, which could pave the way for the biofortification of animal meat with vitamin D. Neill *et al*. (2023), also observed similar outcomes and suggested that as an additional food-based strategy to help consumers achieve vitamin D intake recommendations without changing their habitual diet, pork biofortification may complement traditional fortification measures to reduce the rate of hypovitaminosis D and adverse resulting health outcomes. He also stressed the importance of the dietary source of vitamin D along with UVB exposure of the animals. Nevertheless, our study suggests that artificial sources of UVB could replace the dietary source of vitamin D. The increased expression pattern of the genes linked to vitamin D metabolism indicates that the liver can

also be a good source of vitamin D. Since, the liver is the main the organ where 25(OH)D (used to measure the serum status of vitamin D) is primarily produced from vitamin D_2 or D_3 following 25-hydroxylation. The future study should consider these important things before performing biofortification of the meat: I) duration and intensity of the UVB exposure, II) the selection of the animals, III) environmental considerations, and IV) targeted consumers and their acceptance towards the biofortification strategy.

4.2.3. Improvement of the farming practices

Leveraging the knowledge of artificial UVB-induced synthesis of vitamin D can significantly enhance pig farming practices. Here are practical ways in which this knowledge can be applied to improve the overall well-being, health, and productivity of pigs:

Supplemental UVB lighting systems: Implementing supplemental UVB lighting systems in enclosed environments can provide a controlled and consistent source of UVB radiation. This ensures that pigs receive adequate exposure for vitamin D synthesis, even in areas with limited natural sunlight. Even in outdoor systems, where pigs have access to natural sunlight, supplemental UVB lighting can be beneficial during cloudy days or in shaded areas where exposure may be limited. According to researchers, in contrast to sunlight, which may vary daily and seasonally, UVB exposure via artificial lights ensures a more controlled and consistent environment (Burild *et al.*, 2015; Larson-Meyer *et al.*, 2017) and increased vitamin D status in pigs may also improve animal welfare irrespective of human health implications (Neill *et al.*, 2023). Animal behavior may also affect vitamin D synthesis, and controlled artificial UVB exposure can overcome this limitation, ensuring the rational distribution of vitamin D in the body regardless of animal behavior.

Optimal light intensity and duration: The synthesis of vitamin D via UVB requires the skin to be exposed at the wavelength of 290-315 nm (Bouillon *et al.*, 2010) and a given UVB dosage (erythemal dose) (Diffey, 1994). Therefore, careful consideration should be given to the intensity and duration of UVB exposure. Monitoring and adjusting these factors based on the specific needs of the breeds, age groups, and environmental conditions are crucial for maximizing the health benefits of the pigs without causing harm. Moreover, the outcomes of this experiment also demonstrate the importance of the lighting system on the health status of the pigs.

Enclosure design and layout: When designing pig enclosures, incorporate features that facilitate exposure to artificial UVB light. This may include strategically placing UVB lights,

creating areas where pigs can bask, and ensuring that the lighting system covers the entire enclosure effectively.

UVB exposure during critical life stages: Providing UVB radiation during critical life stages, such as gestation and lactation for sows, and growth phases for piglets. Especially, piglets are usually born with very low levels of vitamin D and feed is the only source of this vitamin (Jakobsen *et al.*, 2020). Ensuring optimal vitamin D levels during these periods contributes to better reproductive success, bone development, and overall health (Heyden & Wimalawansa, 2018) of the pigs.

Integration with feeding practices: Complementing UVB exposure with proper nutrition, including feed that supports overall health and provides essential nutrients might be a good practice in commercial pig farming. The synergy of UVB-induced vitamin D synthesis and a balanced diet could optimize overall pig health and performance and reduce the risk of vitamin D deficiency (Hasan *et al.*, 2023b). However, it requires further investigation to conclude.

Monitoring vitamin D levels: Monitoring the vitamin D levels in pigs regularly through blood tests or other non-invasive methods might help assess the effectiveness of UVB exposure and allow for adjustments to lighting conditions or dietary supplementation as needed (Holick, 2009).

Regulatory compliance: Ensuring compliance with relevant regulations and guidelines regarding UVB exposure for animals might be a good idea to maximize the benefits following UVB-induced production of vitamin D. This includes adherence to the recommended light intensity levels, duration of exposure, and animal welfare standards (Schutkowski *et al.*, 2013).

By combining artificial UVB exposure with other aspects of pig management, such as nutrition, housing design, and health monitoring; farmers can enhance the overall health, well-being, and productivity of their pig herds. Regular veterinary consultation and ongoing assessment of the effectiveness of UVB exposure practices could be key components of successful implementation.

Moreover, in recent times, organic farming has gained popularity among farmers and consumers day by day due to its eco-friendliness and animal welfare practices. The objective of organic animal husbandry is to provide livestock with a comfortable and stress-free environment following their natural needs to emphasize the use of biodegradable and certified organic inputs from the environment for animal nutrition, health, housing, and

breeding, and avoid synthetic inputs like drugs, feed additives, or genetically engineered breeding materials (Ahsan Kabir, 2019). The outcome of this experiment may prove the importance of sunlight exposure for pigs (outdoor farming system) as well as the potentiality of practicing organic farming and less dependency on drugs for vitamin D synthesis in the body. The future study should focus more on the overall health status of the animals in response to artificial UVB light exposure.

4.3. Vitamin D_3 and 25(OH)D_3: skeletal and non-skeletal effects on the body

As we already know, the two major dietary sources of active vitamin D are vitamin D_3 and 25(OH)D_3. Both forms of vitamin D play a significant role in maintaining or improving animal performance. Vitamin D_3 is the most commonly used form of vitamin D on both commercial and non-commercial pig farms. However, vitamin D insufficiency is still a major problem in the majority of these facilities. It is difficult for farmers to determine which dietary form of vitamin D would be the best to improve serum vitamin D levels and maximize its corresponding benefits. The 1st study implied the non-renal/local production of vitamin D raising some unanswered questions regarding its non-skeletal effects. In the 2nd study, the UVB demonstrated a significant increase in the serum concentrations of 25(OH)D_3 but no effect on 1,25$(OH)_2D_3$. Given this, it is imperative to determine the diverse functions of both forms of vitamin D. So, the goals of this study were to figure out the skeletal and non-skeletal effects of these two forms of vitamin D and compare their efficacies in the body. Vitamin D plays a very important role in improving the reproductive capacities of the pigs. Pigs fed either form of vitamin D do not show a significant difference in overall reproductive performance (Thayer *et al.*, 2019; Upadhaya *et al.*, 2022). According to some researchers, the survival rate of the piglets from sows fed 25(OH)D_3 is significantly higher than that of piglets from sows fed vitamin D_3 (Coffey *et al.*, 2012; Upadhaya *et al.*, 2021; Zhou *et al.*, 2016) which could be an important consideration in improving the animal breeding practices. In contrast to vitamin D_3, the progeny from the sows fed a diet containing 25(OH)D_3 shows improved weight gain and body condition scores (Upadhaya *et al.*, 2022; Weber *et al.*, 2014; Zhang *et al.*, 2021). The supplementation of 25(OH)D_3 directly to the offspring can also boost their growth performance (Zhang *et al.*, 2022; Zhao *et al.*, 2022). In addition to increasing muscle fibers, 25(OH)D_3 also improves muscle cell proliferation and differentiation (Hines *et al.*, 2013; Thayer *et al.*, 2019).

Vitamin D plays a vital role in improving the immune status of the body. The immune function of vitamin D is currently under intense investigation due to its promising effect against some bacterial and viral infections (Djukic *et al.*, 2015; F Gunville *et al.*, 2013). However, it is crucial to determine the best dietary source of active vitamin D to improve the maximal immune status of the body to maintain the optimal health of the animals. In contrast to vitamin D_3, dietary intake of 25(OH)D_3 significantly enhances the body's immune status. It particularly boosts humoral immunity by increasing serum immunoglobulin levels and the phagocytic capacity of macrophages in piglets, enabling a more effective response to health challenges (Meuter *et al.*, 2016; Zhang *et al.*, 2021). Additionally, 25(OH)D_3 positively influences inflammatory cytokine levels to maintain immune homeostasis in the body (Ghaseminejad-Raeini *et al.*, 2023; Zhang *et al.*, 2019; Zhang *et al.*, 2021). It surpasses vitamin D_3 in modulating systemic and mucosal antimicrobial responses, improving leucocyte numbers, survival, and phagocytic abilities (Jones *et al.*, 2023; Konowalchuk *et al.*, 2013). Above all, in contrast to vitamin D_3, 25(OH)D_3 also significantly improves gut immunity by altering the gut microbiota, promoting metabolic processes crucial for intestinal health (Belkaid & Hand, 2014; Konowalchuk *et al.*, 2013).

Proper bone development is crucial for the overall health, growth, reproductive success, and longevity of the breeding animals. Stronger bone is also essential for supporting the body weight and physiological demands of reproduction. The mineral homeostasis of the body greatly depends on the proper growth of the bone (Office of the Surgeon General, 2004; Shane *et al.*, 2013). The majority of the studies demonstrate that vitamin D_3 and 25(OH)D_3 show similar impacts on skeleton development (von Rosenberg *et al.*, 2016; Witschi *et al.*, 2011; Zhang *et al.*, 2022) in normal conditions of pigs. However, the efficacy of vitamin D_3 and 25(OH)D_3 vary significantly in pigs fed a diet containing low Ca and P. 25(OH)D_3 performs better than vitamin D_3 in improving the serum mineral status e.g., Ca, BALP, and OC to ensure the proper mineralization of bone in pigs experiencing Ca and P deficiency due to low dietary Ca and P intake (Zhang *et al.*, 2022; Zhao *et al.*, 2022). So, it is quite evident from the above discussion that 25(OH)D_3 might be preferred over vitamin D_3 in improving growth; reproduction; immune status; and bone mineralization in pigs fed a low Ca-P diet. The outcomes of this study could contribute to valuable insights into animal breeding and genetics, potentially informing future breeding strategies and supplementation practices.

4.4. Conclusions and outlooks

In summary, the supplementation of minerals and the careful management of their homeostasis are pivotal aspects of successful livestock breeding programs. Minerals play multifaceted roles in the health, growth, reproduction, and overall physiological functions of animals. The balance between mineral uptake, transport, metabolism, and storage processes is essential to avoid deficiencies or overloading, both of which can harm animals. This dissertation focused on the importance of P and vitamin D in the context of pig farming. Essential macro mineral, P and secosteroid hormone vitamin D contribute to the skeletal development, growth, and productivity. However, their inefficient utilizations in the livestock industry pose challenges for optimal animal performance and raises environmental concerns. The discussion explored the ongoing debate on the optimal dietary phosphorus levels for animals, considering health, welfare, and environmental factors.

The investigation of the molecular mechanisms of vitamin D metabolism in response to low and high dietary P reveals that

- genes of vitamin D synthesis are not only expressed in the kidney but are active in various tissues outside the liver and kidney, shedding light on possible new functions of locally produced active vitamin D.
- candidate genes, such as *VDR*, *CYP2R1*, *CYP27B1*, *CYP24A1*, and *GC*, were highlighted as potential targets for improving vitamin D metabolism, reproductive success, and overall animal performance.

Considering the involvement of the FGF-system in mineral balance it is found that

- *FGFR* transcripts are ubiquitously distributed, suggesting tissue-wide signal transduction via FGF23.

Overall, analysis of the tissue-wide transcript expression reveals

- tissue-specific and diet-dependent expression patterns of genes encoding vitamin D and FGF23 metabolism enzymes and receptors.

There is a need for further studies to examine the effects of diets on health and tissue integrity under specific conditions and in selected tissues at the level of protein expression and enzyme activation.

The dissertation further explores the effects of UVB exposure on vitamin D synthesis and associated transcriptomic changes in the liver. The artificial UVB-induced synthesis of vitamin

D is discussed in the context of improving vitamin D metabolism and its potential benefits for growth, immunity, and reproduction in pigs. The overall findings reveal that

- daily exposure of the pigs to artificial UVB light significantly raises the serum level of calcidiol enhancing the vitamin D status in pigs.
- hepatic gene expression pattern validates systemic impacts of artificial UVB light on vitamin D metabolism and molecular pathways in the liver indicating the preference for endogenous vitamin D synthesis in the liver over cholesterol formation.
- the potential biofortification of animal products with vitamin D using UVB light is highlighted as a promising strategy to address vitamin D deficiency in human populations.
- UVB exposure could replace or complement dietary sources of vitamin D, with the liver identified as a potential source for vitamin D production.

Finally, the dissertation emphasizes the importance of implementing UVB exposure practices thoughtfully and integrating them with other aspects of pig management, such as nutrition, housing design, and health monitoring. The adaptability of UVB exposure practices to seasonal changes and outdoor farming systems was discussed, along with the need for ongoing research, innovation, and compliance with regulatory standards. The future study should explore the impact of UVB exposure on the metabolic health marker of the pigs considering the body weight, carcass composition, and serum indicators. Further research should also focus on the long-term effects of UVB light on mineral utilization and biofortified animal production. Investigating the molecular mechanism in gene expression linked to vitamin D metabolism and cholesterol pathways can unveil the interplay between UVB exposure, lipid metabolism, and cholesterol synthesis in pigs.

Determining the non-skeletal effects and the ideal form of vitamin D in performing these actions is also part of this dissertation. Dietary supplementations of vitamin D_3 and 25(OH)D_3 result in similar reproductive outcomes in sows, except for the survivability of the piglets. Piglets born to sows fed 25(OH)D_3 have a higher survival rate than those born to sows fed vitamin D_3. The growth performance of piglets and sows fed 25(OH)D_3 is significantly better than that of those fed vitamin D_3. 25(OH)D_3 is more effective than vitamin D_3 in enhancing both innate and humoral immunity. Animal welfare and resource efficiency can be balanced through improved bone mineralization in pigs fed diets containing low Ca and P by adding

25(OH)D_3 to the diet. Therefore, 25(OH)D_3 has great potential to improve growth, reproduction, immunity, and bone development in pigs as an alternative to vitamin D_3.

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.

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5. Appendix

Manuscript I

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.

Tissue-wide expression of genes related to vitamin D metabolism and FGF23 signaling following variable phosphorus intake in pigs

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Abstract

Calcium (Ca) and phosphorus (P) homeostasis is maintained by several regulators, including vitamin D and fibroblast growth factor 23 (FGF23), and their tissue-specific activation and signaling cascades. In this study, the tissue-wide expression of key genes linked to vitamin D metabolism (*CYP2R1*, *CYP27A1*, *CYP27B1*, *CYP24A1*, *GC*, *VDR*) and FGF23 signaling (*FGF23*, *FGFR1-4*, *KL*) were 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. Expression patterns revealed that non-renal tissues and cells (NRTC) express genes to form active vitamin D [1,25$(OH)_2D_3$] according to site-specific requirements. A low P diet resulted in higher serum calcitriol and increased *CYP24A1* expression in the small intestine, indicating local suppression of vitamin D signaling. A high P diet prompted increased mRNA abundances of *CYP27B1* for local vitamin D synthesis, specifically in bone. For FGF23 signaling, analyses revealed ubiquitous expression of *FGFR1-4*, whereas *KL* was expressed in a tissue-specific manner. Dietary P supply did not affect skeletal *FGF23*; however, *FGFR4* and *KL* showed increased expression in bone at high P supply, suggesting regulation to balance mineralization. Specific NRTC responses influence vitamin D metabolism and P homeostasis, which should be considered for a thrifty but healthy P supply.

5.1. Introduction

Maintaining calcium (Ca) and phosphorus (P) homeostasis is crucial for all mammals, including pigs, to enable proper bone metabolism, growth processes, and cellular functions. Due to physiological turn-over, the organism excretes Ca and P, which must be continuously replaced by a diet that meets mineral requirements (Flachowsky, 2006). However, the availability of minerals in the organism is strictly regulated in mammals, with the kidneys, liver, bones, and intestines specifically interacting in a complex manner to meet the metabolic demand at each stage of development (Berndt & Kumar, 2009).

Key regulators of mineral homeostasis include vitamin D and fibroblast growth factor 23 (FGF23). In particular, the secosteroid hormone calcitriol [$1,25(OH)_2D_3$], the most potent natural metabolite of vitamin D, acts on numerous tissues for both calcemic and non-calcemic effects, although intestinal Ca and P absorption, bone metabolism, and renal mineral reabsorption are the primary subjects of its regulation (Berndt & Kumar, 2009; Chande & Bergwitz, 2018). Calcitriol is formed from precursors by sequential hydroxylation. This involves the conversion of cholecalciferol via CYP2R1 and CYP27A1 in the liver and the subsequent conversion of calcidiol via CYP27B1 in the kidneys (Table 1). Moreover, calcitriol levels are also subject to CYP24A1-mediated degradation (Jones *et al.*, 2014). An additional autocrine regulation is known to be present at peripheral sites in non-renal tissues and cells (NRTC) such as the lungs, skin, or parathyroid glands (Hewison *et al.*, 2007). However, the eventual secretion of non-renal calcitriol into the blood circulation and transport via vitamin D binding protein (DBP, encoded by *GC*) or its autocrine action via the vitamin D receptor (VDR) is still unclear (Pike & Meyer, 2020). In conventionally farmed pigs, enteral intake of precursors ensures the subsequent formation of calcitriol (Duffy *et al.*, 2018) although there are also approaches using the UV-B light regimen (Barnkob *et al.*, 2016). Vitamin D metabolism is tightly interlinked with fibroblast growth factor 23 (FGF23) signaling. In mammals, FGF23 is a hormone produced primarily by osteoblasts and osteocytes (Mirams *et al.*, 2004), leading to a decrease in both systemic and non-renal calcitriol production through suppression of *CYP27B1* and induction of *CYP24A1* (Bacchetta *et al.*, 2013; Shimada *et al.*, 2004). In kidney, FGF23 reduces P reabsorption and increases urinary P excretion by down-regulating the expression of sodium-dependent phosphate co-transporters in the proximal tubule (Gattineni *et al.*, 2009). The physiological function of FGF23 is mediated by its interaction with protein-encoding FGF receptors (FGFR1–4) and αKlotho (KL), which serves as a transmembrane or

soluble co-receptor (Hu *et al.*, 2019; Kurosu *et al.*, 2006). In addition to its control of mineral homeostasis, FGF23 also exerts KL-independent autocrine and paracrine effects on cytokine secretion, such as in the liver (Singh *et al.*, 2016).

Knowledge of the regulation of mineral homeostasis in mammals is currently reaching to a new level. The evidence continues to grow for the direct actions of vitamin D and FGF23 and their interactions, with peripheral tissues triggering a variety of non-calcemic functions, including immunity and cell development, as reviewed elsewhere (Gil *et al.*, 2018; Vervloet, 2019). In fact, FGF23 is evolving from a biomarker of disturbed P balance to a pathogenic factor (Staude *et al.*, 2013). For pigs, recent dietary intervention studies established the conclusive pattern of endocrine control following variable dietary P supplies (Gerlinger *et al.*, 2021). Transcriptome profiles in respective target tissues such as the kidneys and jejunum (Wubuli *et al.*, 2020) and parathyroid glands (Oster *et al.*, 2021) showed endogenous responses in maintaining mineral homeostasis, which consistently highlighted the vitamin D system and associated regulatory pathways. Notably, the assessment of effective mechanisms of mineral homeostasis in pigs is usually based on single measurements that include blood levels of P, Ca, and calcidiol, and therefore do not allow comprehensive conclusions on tissue-level specificities. The expression of all currently annotated sodium-dependent phosphate co-transporters in pigs showed a tissue-specific pattern, with adaptive responses to low dietary P intake compared with high dietary P intake, i.e., increased *SLC34A1* and *SLC34A3* abundances in kidney cortex and reduced *SLC20A2* abundances in the jejunum (Wubuli *et al.*, 2019).

Table 1. Studied genes involved in vitamin D metabolism and FGF23 signaling.

Gene	Ensembl ID (v. 102)	Description	Function
CYP2R1	ENSSSCG00000013389	Cytochrome P450, family 2, R1	Hydroxylation (25-OH) of cholecalciferol (liver) * (Strushkevich *et al.*, 2008)
CYP27A1	ENSSSCG00000016199	Cytochrome P450 family 27, A1	Hydroxylation (25-OH) of cholecalciferol (liver) * (Hosseinpour *et al.*, 2003)
CYP27B1	ENSSSCG00000028637	Cytochrome P450 family 27, B1	Hydroxylation (1α-OH) of calcidiol (kidney)* (Ohyama & Yamasaki, 2004)
CYP24A1	ENSSSCG00000007486	Cytochrome P450 family 24, A1	Hydroxylation (24-OH) of calcidiol and calcitriol (kidney) * (Ohyama & Yamasaki, 2004)
VDR	ENSSSCG00000020864	Vitamin D receptor	Transcription factor (Omdahl *et al.*, 2002)
GC	ENSSSCG00000027609	Vitamin D binding protein	Binding of calcitriol (Daiger *et al.*, 1975)
FGF23	ENSSSCG00000052449	Fibroblast growth factor 23	Regulator of P homeostasis (Haussler *et al.*, 2012)
FGFR1	ENSSSCG00000015815	Fibroblast growth factor receptor 1	Receptor of FGF23 and other FGFs (Erben & Andrukhova, 2017)
FGFR2	ENSSSCG00000010698	Fibroblast growth factor receptor 2	Receptor of FGF23 and other FGFs (Erben & Andrukhova, 2017)

FGFR3	ENSSSCG00000030827	Fibroblast growth factor receptor 3	Receptor of FGF23 and other FGFs (Erben & Andrukhova, 2017)
FGFR4	ENSSSCG00000014047	Fibroblast growth factor receptor 4	Receptor of FGF23 and other FGFs (Erben & Andrukhova, 2017)
KL	ENSSSCG00000009347	Klotho	Co-receptor of FGF23 (Choi *et al.*, 2014)

* site of hydroxylation; *CYP2R1*, Cytochrome P450 Family 2 Subfamily R Member 1; *CYP27A1*, Cytochrome P450 Family 27 Subfamily A Member 1; *CYP27B1,* Cytochrome P450 Family 27 Subfamily B Member 1; *CYP24A1*, Cytochrome P450 Family 24 Subfamily A Member 1.

Expanding on these findings (Wubuli *et al.*, 2019), the present study examines the tissue-specific expression of genes involved in vitamin D metabolism and FGF23 signaling following a conventional standard diet (trial 1) and a P-divergent diet (trial 2). The mRNA expression of a set of genes encoding components of vitamin D metabolism and signaling pathways, as well as FGF23-signaling pathways (Table 1), was investigated in a number of tissues of systemic mineral homeostasis (kidney, bone, various intestinal segments), as well as in additional peripheral tissues (lung, liver, aorta), to gain basic insight into the potential abilities of these tissues to participate in the production, transduction, and elimination of vitamin D metabolites and in the response to FGF23 and its (co)receptors at the very basal level of transcription.

5.2. Results

The study was conducted in pigs, a valuable biomedical model that shares many similarities with humans in its physiology, and at the same time is a target species for improving phosphorus efficiency for more animal and environmentally friendly husbandry (Oster *et al.*, 2018; Schook *et al.*, 2005). Therefore, animals fed a standard conventional diet (experiment 1) or variable P diets (experiment 2) were used to quantify the abundance of transcripts of genes encoding components of vitamin D metabolism and signaling pathways, as well as FGF23-signaling pathways in a variety of tissues, which was complemented by measuring serum calcitriol concentrations.

5.2.1. Tissue-Specific Expression of Genes Linked to Vitamin D Metabolism and FGF23 Signaling under Conventional Standard Dietary P Intake

The serum calcitriol concentration in pigs fed the conventional diet was 384.5 ± 82.5 pmol/L (mean ± SD) at about 180 days of age. The expression of 12 key genes involved in the synthesis, transport, and metabolism of vitamin D and FGF23 signaling on 14 different tissues is summarized in Figure 1. The hydroxylating enzymes encoding *CYP2R1, CYP27A1,* and *CYP27B1* showed a tissue-wide expression across the investigated panel, whereas the expression of *CYP24A1* was exclusively detectable in the kidneys, stomach, and duodenum. Similarly, *GC*

profiles revealed a tissue-specific distribution with its highest transcript abundance in the liver. Moreover, *GC* was found to be expressed in the kidney cortex, kidney medulla, stomach, duodenum and to a lower extent in the aorta. *VDR* was present throughout the tested tissues, but with considerably lower expression in the liver and the aorta. Among the genes related to FGF23 signaling, the expression of *FGF23* and *KL* was shown to be restricted to specific tissues such as kidneys, lung, bone, and the distal intestine, whereas FGF receptors were detectable in all tissues studied. 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 the other analyzed tissues. Interestingly, *FGFR2* showed a very similar tissue-specific pattern, with slightly less variation between tissues. In contrast, *FGFR3* profiling revealed an almost constitutive expression in all tissues examined. The highest expression of *FGFR4* was found in the liver; however, considerable amounts were also found in the kidneys, lungs and the intestine. Finally, a high expression of *KL* was noticed in the kidney cortex, kidney medulla, and lung. In general, it was found that almost all genes for vitamin D metabolism and FGF23 signaling were found to be expressed in a considerable and similar extent in the kidney cortex and medulla.

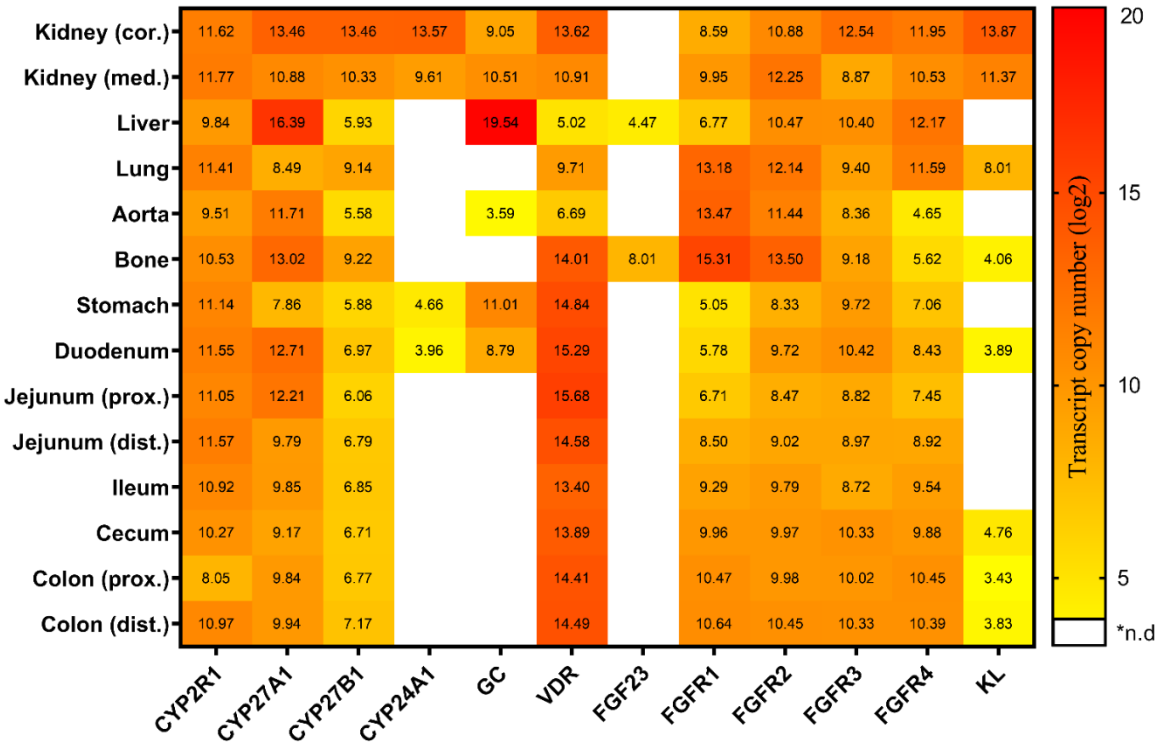

Figure 5. Heatmap illustrating the tissue-specific expression of the key genes linked to vitamin D metabolism and FGF23 signaling in pigs fed on a conventional standard diet (trial 1). Transcript copy numbers of 12 genes were evaluated in 14 tissues by RT-qPCR. The copy numbers were displayed as log2 values and were indicated by a color scale from yellow to red. *n.d, not detectable; cor, cortex; med, medulla; prox, proximal; dist, distal.

5.2.2. Changes in the Expression of Genes Linked to Vitamin D Metabolism and FGF23 Signaling as a Result of Divergent Dietary P Intake

The effectiveness of dietary treatment in stimulating regulatory circuits to maintain P homeostasis was demonstrated by analysis of serum calcitriol levels. Following the divergent P diets, calcitriol levels were 751.1 ± 112.4 pmol/L (mean ± SD) on a high-P diet, and 1079.3 ± 134.0 pmol/L on a low P diet, indicating a significant difference (p = 0.0047) at 120 days of life. The mRNA expression of a number of the key genes responded in a tissue-specific manner to variable dietary P intake (Table S1, Figure 2). Specifically, the expression of *CYP2R1* significantly differed in proximal jejunum (L > H; FC = 1.38). Another gene encoding one of the 25-hydroxylating enzymes, *CYP27A1*, illustrated a considerable variation in mRNA expression in the kidney cortex (L > H; FC = 2.06) and proximal colon (L > H; FC = 2.39). Furthermore, the vitamin D activating enzyme (1α-hydroxylation) encoding gene *CYP27B1* showed a significant variation in mRNA expression in bone (H > L; FC = 2.39). *CYP24A1*, which encodes for calcitriol 24-hydroxylase and therefore calcitriol elimination, demonstrated the highest response to the P-divergent diet particularly in duodenum (L > H; FC = 34.78), distal jejunum (L > H; FC = 106.52), and ileum (L > H; FC = 16.22). Additionally, a significant change in the mRNA abundance of *CYP24A1* was detected in kidney cortex (H > L; FC = 2.79). *FGF23* exhibited a considerable change in mRNA abundance in liver (L > H; FC = 5.21). Among the *FGF23* receptors, *FGFR4* showed significant differences in mRNA abundances in bone (H > L; FC = 2.45) and distal jejunum (H > L; FC = 1.62). Expression of co-receptor *KL* differed significantly in bone (H > L; FC = 10.16) between the two dietary groups. Among the tissues studied, the expression pattern of bone showed adaptive responses of both vitamin D metabolism and FGF23 signaling after varying dietary P intake.

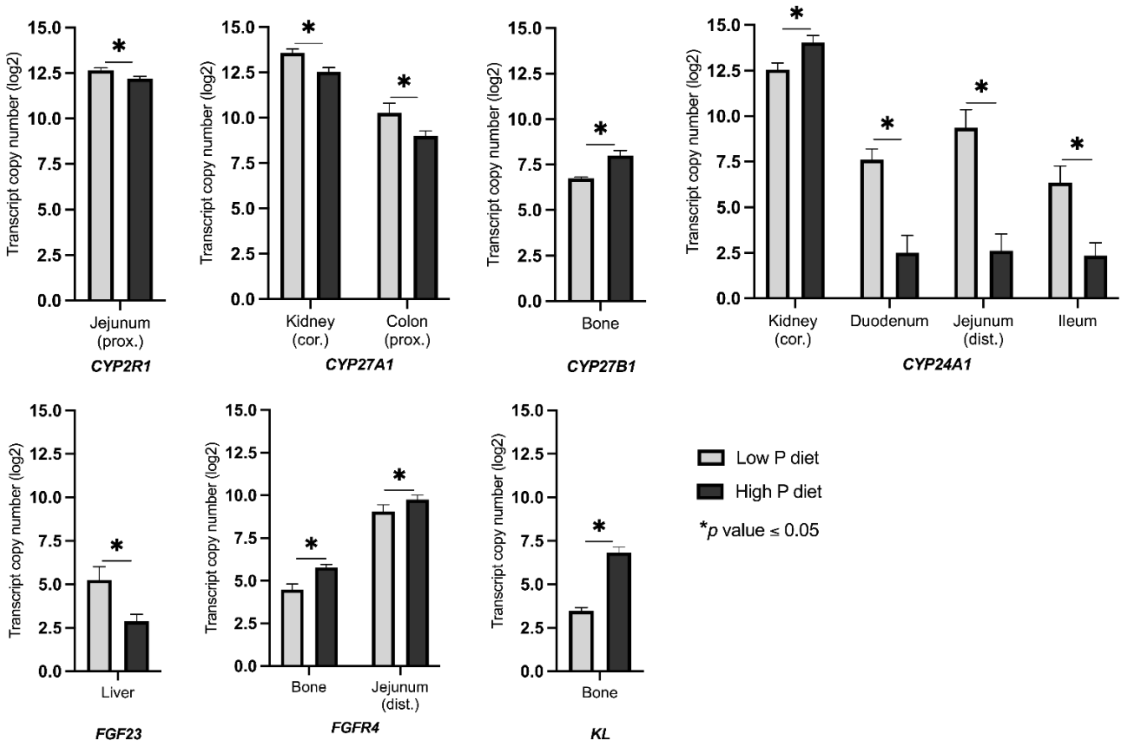

Figure 6. Transcript abundance (copy number) of differentially expressed genes related to vitamin D metabolism and FGF23 signaling in pigs receiving diets with divergent P levels from weaning until 120 days of life (trial 2). Asterisks indicate statistical significance between dietary groups (* $p \leq 0.05$). cor, cortex; prox, proximal; dist, distal.

5.3. Discussion

In monogastric vertebrates, mineral homeostasis is maintained by the tight regulation of intestinal absorption, osseous mobilization, and renal excretion rates involving a number of known and yet to be identified regulators, transporters, and endocrine and paracrine signals. In addition, the complex interplay to maintain mineral homeostasis includes meeting the specific requirements of peripheral NRTC. This involves pathways of mineral metabolism, as well as non-calcemic biological functions of the vitamin D system and FGF23 signaling.

5.3.1. Status Quo and Reactivity of the Vitamin D System to Maintain Mineral Homeostasis

In pigs, expression of the hydroxylases *CYP2R1*, *CYP27A1*, and *CYP27B1* was evident in all tissues examined, which matches previous findings in humans and rodents [5,33,34]. However, in pigs, the mRNA abundance of *CYP2R1* is higher in the kidney cortex and medulla than in the liver, which is in contrast to other animal species (Cheng *et al.*, 2003; Elkhwanky *et al.*, 2020). Indeed, CYP2R1 has been shown to work in close physical proximity to 1α-hydroxylase, which may explain the presence of *CYP2R1* transcripts in the kidney as the main site of calcitriol production (Zhu & DeLuca, 2012). For *CYP27A1*, the highest mRNA abundance

was found in the liver of pigs and other species such as mice (Cheng *et al.*, 2003). While CYP27A1 shows some activity in 25-hydroxylation, it plays an important role in 27-hydroxylation of cholesterol and bile acid synthesis, i.e., processes that are clearly attributed to the liver (Sawada *et al.*, 2000; Zhu & DeLuca, 2012). Interestingly, CYP27A1 has been further proposed to be involved in 25-hydroxylation of vitamin D_3 also in kidney (Postlind & Wikvall, 1988). CYP27B1 represents the gatekeeper for the renal activation of systemically acting calcitriol and accordingly it is highly abundant in the renal cortex of pigs. In fact, several modulators including PTH regulate *CYP27B1* expression in kidney, whereas in NRTC, its mRNA abundance may be independent of PTH action (Meyer *et al.*, 2019a). The mRNA abundance of *CYP27B1* in the bone of pigs was substantial, which is in agreement with findings in human and mouse osteocytes and osteoclasts (Atkins *et al.*, 2007; Chanakul *et al.*, 2013). Moreover, *CYP27B1* showed a relatively high mRNA abundance in lung tissue samples. Here, it is assumed that lung epithelial cells and macrophages depend on calcitriol, which triggers immune effects against microbial and viral infections via antimicrobial peptides and cytokines, as well as by initiating immune signaling cascades (L Bishop *et al.*, 2021). Taken together, the tissue-wide distribution of *CYP2R1*, *CYP27A1* and *CYP27B1* in pigs suggests that not only liver and kidney but also peripheral tissues might have the ability to produce calcidiol and calcitriol, which is in agreement with the previous findings in human and mice (Bikle, 2010; Dusso *et al.*, 1994). However, the extent and eventual secretion of calcitriol into the circulation or its autocrine effect on gene expression are still unclear. It has been argued that calcitriol of non-renal origin elicits a variety of effects that are both connected to and independent of mineral homeostasis, including effects on the immune system (L Bishop *et al.*, 2021) and control of cell growth and differentiation processes (Pike & Meyer, 2020). Calcitriol levels are further modulated via a catabolic enzyme encoded by *CYP24A1*. In pigs, *CYP24A1* showed high expression in the renal cortex and exhibited low mRNA abundances in the stomach and duodenum. Despite the fact that the renal CYP24A1 enzyme helps to balance systemic calcidiol and calcitriol levels, its expression in peripheral tissues is likely to precisely adjust cellular hormone levels in a negative feedback loop (Petkovich & Jones, 2011). Furthermore, the transport of vitamin D metabolites via the bloodstream is facilitated up to 90% by vitamin D binding proteins (DBP, encoded by *GC*) with different binding affinities (calcidiol > calcitriol > cholecalciferol) (Vilaça & Lazaretti-Castro, 2018). In pigs, *GC* demonstrated the highest mRNA abundance in the liver, which is also the production site of this protein and corresponds to physiologic conditions in

humans (Feldman *et al.*, 2008; Schiødt, 2008). Consistent with the results, *GC* showed low mRNA abundances in a number of tissues in mice, including the kidneys and intestine (Cooke *et al.*, 1991). Interestingly, the expression of *GC* in tissues other than the liver has been associated with binding of fatty acids, chemotaxis, binding of endotoxins and impact of T cell response (Delanghe *et al.*, 2015). In pigs, the vitamin D receptor (*VDR*) showed high mRNA abundances in all analyzed tissues. Its ubiquitous expression highlights the fact that vitamin D plays a key role in every single tissue in mammals. Indeed, VDR exerts vitamin-D-dependent and -independent effects in a tissue-specific manner and controls a large number of target genes via vitamin D response elements (VDRE) in the respective promotor region (Wang *et al.*, 2005). The high mRNA abundance of *VDR* in the small intestine, bone and kidney demonstrates its involvement in balancing mineral absorption, retention and re-absorption in extracellular fluids (Hendy *et al.*, 2006; Xue & Fleet, 2009). Furthermore, novel actions of the calcitriol-VDR complex have been described, e.g., on cell differentiation and proliferation, immune system functions and intracellular signaling cascades (Bikle & Christakos, 2020; Colotta *et al.*, 2017; Samuel & Sitrin, 2008).

Indeed, the results demonstrated the effect of divergent dietary P supply on serum calcitriol, with higher levels in L animals compared with H animals. This matches previous findings in growing pigs, but also other species (Gerlinger *et al.*, 2021; Tanaka & Deluca, 1973). Interestingly, the dietary responses were less pronounced at the major tissue sites known for endocrine synthesis, as the gene expression for *CYP2R1* and *CYP27A1* in the liver as well as for *CYP27B1* in the kidneys did not differ between the experimental groups. In fact, *CYP27B1* showed decreased mRNA abundances in the kidneys of pigs after both low P diets (Wubuli *et al.*, 2020) and diets with reduced P and Ca levels (Oster *et al.*, 2018) compared with control animals. However, the present pig trial showed a marked increase in *CYP24A1* expression in the kidney, suggesting calcitriol elimination in H animals which contributes to the prevention of hypercalcemia and hyperphosphatemia. Therefore, 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.*, 2019b). Thus, calcitriol and FGF23 induce renal *CYP24A1* and suppress renal *CYP27B1*, whereas opposite effects have been reported for PTH (Kaufmann *et al.*, 2015; Meyer *et al.*, 2017). It is conceivable that the feedback loops of calcitriol were masked by PTH and FGF23, which in sum favored *CYP24A1* expression in H animals compared with L animals with unchanged renal *CYP27B1* mRNA abundances. In

addition, some of the intestinal sections and kidney responded to dietary P supply, as shown by profiles of *CYP2R1* in the proximal jejunum and *CYP27A1* in the proximal colon and renal cortex. Moreover, H animals showed increased *CYP27B1* mRNA abundance in bone. Results suggest a physiological demand for local calcitriol synthesis as a bone-specific response of H animals to compensate for lower endocrine calcitriol concentrations in serum compared with L animals. Reduced osteoclastogenesis has been shown previously in mutant mice lacking *CYP27B1* expression in chondrocytes (Naja *et al.*, 2009). Conversely, a transgene strain overexpressing *CYP27B1* in chondrocytes exhibited lowered bone volume and the trabecular number (Naja *et al.*, 2009). This supports previous results on physiology in pigs that showed that microstructural bone characteristics were found to plateau with increasing dietary P supply (Gerlinger *et al.*, 2021). In the small intestine, results revealed regulatory mechanisms to reduce calcitriol levels in L animals compared to H animals. The inducible character of intestinal *CYP24A1* after calcidiol injection (Shinki *et al.*, 1992) suggests that high *CYP24A1* mRNA abundances in duodenum, jejunum, and ileum might help to precisely control cellular calcitriol levels. Specifically, systemic calcitriol levels can directly affect intestinal mineral absorption processes, e.g., by regulating *SLC34A3* and *TRPV6* expression (Christakos *et al.*, 2014; Wubuli *et al.*, 2019), which are counterbalanced at the local level by calcitriol elimination to prevent hypercalcemia in L animals. Indeed, activity of CYP24A1 has been shown to be targeted via negative feedback loops of calcidiol and calcitriol (Christensen *et al.*, 2013). In the context of respective endocrine serum levels, control of anabolic and catabolic enzymes related to calcitriol and vitamin D metabolism is important for maintaining serum P and Ca levels and tissue integrity. Regarding the transport of vitamin D metabolites, the *GC* mRNA abundances were unaltered by variable dietary P supply in porcine NRTC. The results match current reports in humans in which high dietary vitamin D levels had no effect on GC protein abundances (Björkhem-Bergman *et al.*, 2018). Although GC expression has been reported to be dependent on TGFβ, IL-6, and glucocorticoids (Guha *et al.*, 1995), the associated physiological states of sexual maturation and inflammation are not present in pigs at 120 days of age.

5.3.2. Systemic and Autocrine Regulations of FGF23 Signaling

In pigs, FGF23 expression is highly tissue-specific and primarily restricted to bone and also to liver. It has been shown that the liver has the capacity to express FGF23 in mice (Kolek *et al.*, 2005), especially in an inflammatory state (Daryadel *et al.*, 2021; Onal *et al.*, 2018). Numerous

studies have shown that the kidney is the main target organ of FGF23, with effects on regulation of P reabsorption triggered by activation of the mitogen-activated protein kinase (MAPK) cascade (Farrow *et al.*, 2009). Moreover, FGF23 has been associated with an increasing number of side effects in other tissues (Erben, 2018). Similarly to kidney, binding between FGF23 and the FGFR-KL receptor complex is also required in NTRC to mediate downstream effects, although KL-independent cascades have been reported (Saito *et al.*, 1998). 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 its involvement in osteoblast proliferation and bone formation (Jacob *et al.*, 2006; Yu *et al.*, 2003). Moreover, functions of these two FGF receptors are conceivable in the aorta as well as in the lung, where the pig analysis also revealed high abundances of *FGFR1* mRNA (Hubert *et al.*, 2018; Ruiz-Camp & Morty, 2015). Interestingly, *FGFR1* transcripts were also detected in the intestine, where ascending mRNA abundances along the gastrointestinal sections were observed. An effect of FGF23 on the intestine is attributed to indirect effects via lowering of calcitriol levels and, consequently, P absorption (Takashi & Fukumoto, 2018). Although *FGFR3* and *FGFR4* show high mRNA abundances in the renal cortex, they are thought to make little or no contribution to renal FGF23 effects (Gattineni *et al.*, 2009; Liu *et al.*, 2008). However, other ligands such as FGF19 and FGF21 have been shown to interact with FGFR1-4 in a tissue-specific manner to regulate, e.g., fat metabolism and bile acid synthesis (Kuro-o, 2008). The important co-receptor *KL* appeared to be expressed in a relatively tissue-specific manner with the highest mRNA abundances in both the kidney cortex and the medulla (Andrukhova *et al.*, 2012; Urakawa *et al.*, 2006). This supports 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). Furthermore, the subtle *KL* expression in intestinal sections such as the duodenum and colon points to functions of FGFs in an autocrine manner (Gavaldà-Navarro *et al.*, 2018). In addition to the membrane-bound form of KL, its soluble form released into the circulation is known to trigger both FGF23-mediated and FGF23-independent responses in NRTC (Dalton *et al.*, 2017; Hu *et al.*, 2019). Besides mediating mineral metabolism, the occurrence of soluble KL is currently associated with cell-protective functions, such as the inhibition of apoptosis, senescence, and oxidative stress [72,87,88], i.e., molecular themes that are also attributed to membrane-bound KL in

the lung and alveolar cells, which showed relatively high *KL* mRNA abundances in pigs (Ravikumar *et al.*, 2014; Richter & Faul, 2018).

Transcriptional responses due to variable dietary P supply showed unaltered mRNA abundances of the *FGFR* and *KL* in porcine kidneys. Furthermore, *FGF23* expression in the bones of pigs was unaltered between dietary groups. However, it has been reported elsewhere that a low dietary P supply decreases FGF23 protein secretion and therefore a lowered renal P excretion (Ferrari *et al.*, 2005). Interestingly, L animals showed higher *FGF23* mRNA abundances in liver tissue than H animals. It is conceivable that results represent adaptive responses as the liver has been shown previously to express *FGF23* following Jak1/Stat3-induced local inflammation (Daryadel *et al.*, 2021). In mice, hepatic *FGF23* expression was accompanied by increased levels of serum calcitriol, decreased PTH, and unaltered levels of FGF23 derived from bone (Daryadel *et al.*, 2021), revealing another potential mechanism for the tissue-wide response to variable dietary P supply. Importantly, both the intact protein (iFGF23) and the c-terminal cleavage product (cFGF23), which lacks phosphaturic activity, can be secreted (Goetz *et al.*, 2010). Due to the latter, 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). In this context, FGF23 has been demonstrated to induce the production of inflammatory cytokines such as TNF-α and IL-6 in the liver, highlighting FGFR4 as a therapeutic target (Singh *et al.*, 2016). Regarding FGF23 signal transduction, *FGFR4* and *KL* in bone showed significant diet-dependent transcriptional effects with increased mRNA abundance in H animals compared with L animals. As FGFR4 and KL are key regulators of osteogenesis affecting the differentiation and function of osteoblasts (Cool *et al.*, 2004; Kawaguchi *et al.*, 2000), transcriptional responses might be attributed to balance bone mineralization in H animals as a part of potential autocrine regulatory circuits (Komaba *et al.*, 2017).

5.4. Materials and methods

5.4.1. Animals and Diets

Animal trials used for this study were approved by the Scientific Committee of the Research Institute for Farm Animal Biology (FBN). The experimental setup was licensed and endorsed by the ethics committee of the federal state of Mecklenburg-Western Pomerania, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei). It was registered under the license LALLF M-V/TSD/7221.3-1-053-15 (16 Dec 2015). The sample set used for this study

has been introduced previously (Wubuli *et al.*, 2019). In trial 1, five German Landrace fattening pigs (two females, three castrates; purebred animals of the species Sus scrofa domesticus) were fed ad libitum a complete standard diet according to the current recommendation (Flachowsky, 2006). The animals reached an average body weight of 118.4 ± 1.7 kg at slaughter (6 months of age). In trial 2, a total of ten crossbred pigs (German Landrace × Large White × Pietrain; crossbred animals representing typical slaughter pigs) of three litters were supplemented with P-divergent diets from weaning (28th day of life) until they were slaughtered (4 months of age) at an average body weight of 90.7 ± 4.6 kg (L) and 99.8 ± 12.3 kg (H) (mean ± SD). Among them, five piglets (three males, two females) were fed low (L) phosphorus (P) diets and five piglets (three males, two females) were fed high (H) P diets (Wubuli *et al.*, 2019). At the juvenile ages used, no differences were found between male, female and castrated animals with regard to P homeostasis. In the grower period (28th–70th day of life), the fed dietary P and Ca levels were 5.2 and 9.8 g/kg (L), and 7.8 and 9.1 g/kg (H). The finisher (71st–120th day of life) diet had 4.1 and 6.5 g/kg (L), and 7.0 and 6.7 g/kg (H) of P and Ca (Table S2). Neither phytase nor other phosphatases were included in the diet. The pigs were given ad libitum access to pelleted feed and water.

5.4.2. Tissue and Serum Sampling

The tissue sampling has been described previously (Wubuli *et al.*, 2019). In the slaughterhouse at FBN, the pigs were anesthetized using electrical stunning and sacrificed by exsanguination. A total number of 14 tissues (trial 1) and 11 tissues (trial 2) were sampled according to Table 2. Prior scraping, the mucosa was washed with ice-cold saline solution (0.9% NaCl) to remove any residual digesta.

Table 2. Tissue samples collected for the pig trials, as previously stated (Wubuli *et al.*, 2019).

Tissue Labeling	Description	Trial
Kidney cortex	Cortex of left kidney	1, 2
Kidney medulla	Medulla of left kidney	1, 2
Liver	*Lobulus spigelii*	1, 2
Lung	Lower tip of the left lung lobe	1
Aorta	Aorta, descending thoracic aorta	1
Bone	Calvarial bone along the sagittal suture	1, 2
Stomach	Fundus mucosa	1
Duodenum	Mucosa 30–40 cm distal of pylorus	1, 2
Jejunum (prox.)	Mucosa 2 m distal of pylorus	1, 2
Jejunum (dist.)	Mucosa 2 m proximal of the ileocecal junction	1, 2
Ileum	Mucosa 20 cm proximal of the ileocecal junction	1, 2
Caecum	Mucosa	1, 2

Colon (prox.)	Mucosa 50–60 cm distal of cecolic junction	1, 2
Colon (dist.)	Mucosa 50–60 cm proximal of rectum	1, 2

prox, proximal; dist, distal.

The samples were prepared by cutting them into pieces and freezing in liquid nitrogen immediately. Additionally, trunk blood was collected, clotted for about 30 min, and centrifuged (3500× *g*; 10 min) to prepare serum. All samples were kept frozen at −80°C until downstream analysis.

5.4.3. RNA Isolation and cDNA Synthesis

The total RNA was extracted from all tissue samples using TRI reagent according to the user's guidelines (Sigma-Aldrich, Taufkirchen, Germany), and treated with DNase1 to ensure the removal of any residuals of genomic DNA. The RNA samples were purified using the column-based NucleoSpin RNA II-kit (Macherey-Nagel, Düren, Germany). The final concentration of the purified RNA was measured using the NanoDrop 2000 Spectrophotometer. The absence of genomic DNA in total RNA samples was checked by PCR amplification with beta-actin (*ACTB*)-specific primers (Table 3). First-strand cDNA was synthesized from total RNA using random primers (Promega, Fitchburg, WI, USA) and oligo d(T) primers in the presence of SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The absence of genomic DNA contamination was checked following PCR amplification of porcine *ACTB* with intron-spanning primers.

Table 3. Primer sequences, annealing temperatures and resulted fragment sizes.

Sl. No.	Genes	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	AT * (°C)	FS ** (bp)
1	*CYP2R1*	TTGCTTCAGCGATTTCACTTG	TGTGCATTTTCAGCGTCTTTC	60	123
2	*CYP27A1*	CAAGTACCCAGTACGGAACGAC	AGCATCCGCTGGTTCAGAG	60	132
3	*CYP27B1*	CCATCAGCCACTGTTCTATCC	TCCCTTGAAGTGGCATAGTGAC	60	179
4	*CYP24A1*	GGAATTGTATGCGTCTGTGAC	CATCTGATTCTCAGGCAGTACAC	60	154
5	*GC*	AAGTTGCCCACAAACAAAGATG	TCAGGGTTGGCTCAAGTATTTTAC	60	130
6	*VDR*	CTTCTGTGACCCTGGACCTG	GCACTTGACTTCAGCAGCAC	60	157
7	*FGF23*	CAGGCTTCGTGGTCATAACAG	CTGACGAGGAAGCGGTAGTG	60	172
8	*FGFR1*	GACTCCTAACCCCACCTTGC	GTGTAGTTGCCCTTGTCGGA	60	141
9	*FGFR2*	CCTCACAGAGACCCACCTTC	GTTCGAGAGGCTGACTGAGG	60	212
10	*FGFR3*	TCATAGGCGTGGCTGAGAAG	CACCACCAGGATGAAGAGGAG	60	187
11	*FGFR4*	AGAGTACCTTGACCTCCGCT	CTCATGGCTGAAGACCGAGT	60	213
12	*KL*	ACTGGCTGAGGTCCAAGTACG	GGAGCTGTGCGATCATTAAATG	60	199
13	*RPL32* ***	AGCCCAAGATCGTCAAAAAG	TGTTGCTCCCATAACCAATG	60	165
14	*ACTB*	GAGAAGCTCTGCTACGTCGC	CCTGATGTCCACGTCGCACT	60	231

***** Annealing temperature, ** Fragment size at cDNA level, *** Housekeeping gene.

5.4.4. Quantitative Real-Time PCR

The primers of all target genes (Table 3) were designed using the sequence information from the Ensembl database (https://www.ensembl.org; accessed on 18 January 2021) and the NCBI primer blast online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast; accessed on 18 January 2021). The performance of primers and an initial evaluation of expected amplification conditions were assessed by PCR using SupraTherm Taq Polymerase (GeneCraft, Lüdinghausen, Germany) with standard cycling conditions (initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at corresponding annealing temperature for 30 s, and extension at 72 °C for 60 s with a final extension at 72 °C for 5 min). For the preparation of the initial standards for the standard curve, the respective amplificates were visualized after electrophoresis on an agarose gel, purified with magnetic beads (Beckmann Coulter, Krefeld, Germany) and measured with the NanoDrop 2000. The expression levels of target genes and *RPL32* (housekeeping gene) were quantified using quantitative real-time qPCR. The transcript copy numbers were measured in duplicate using the LightCycler 480 SYBR Green I master mix (Roche, Mannheim, Germany) according to the user's guidelines. In detail, the reaction mix contained the following: 6 µL of SYBR Green Master I mix, 0.6 µL of each primer (10 µM), 2.8 µL of nuclease-free water, and 2 µL of cDNA. Using the LightCycler480 system (Roche), the PCR amplification program was set as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 25 s. Finally, a melting curve analysis was performed to evaluate the amplified products. The transcript copy numbers of each sample were revealed based on the standard curve method, which utilizes the cycle threshold values of serial dilutions (10^7–10^0 copies) to the corresponding standard.

5.4.5. Serum Measurement of Calcitriol

The serum concentration of calcitriol [$1,25(OH)_2D_3$] was measured in duplicate for all samples of trial 1 and trial 2 using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (AC-62F1, Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany) according to the manufacturer's protocol.

5.4.6. Data Analyses

The data analyses of this experiment were performed using the open-sourced R software (v4.1.1; R foundation for statistical computing, Vienna, Austria). For gene expression analysis, the transcript copy numbers were factorially normalized based upon the expression of the

housekeeping gene *RPL32* and transformed log2. To be considered for further analysis, the mean log2 copy number of duplicates had to be above 2.5 for at least 50% of the samples in each tissue. The tissue-specific transcript copy numbers (mean value) were visualized by a heatmap using GraphPad Prism v9.2.0 (GraphPad software, San Diego, CA, USA). For trial 2, a linear model (R package stats v4.1.1) was used to compare the gene expression and serum calcitriol concentrations between dietary groups. Sex was used as fixed effect. Differences at $p \leq 0.05$ were considered statistically significant. Fold changes (FC) have been calculated based on mean expression values between the two dietary groups.

5.5. Conclusions

The ubiquitous distribution of *CYP2R1*, *CYP27A1*, and *CYP27B1* in pigs suggest a tissue-wide capacity for systemic and local calcidiol and calcitriol synthesis. In contrast, *CYP24A1* expression and thus calcitriol clearance appeared to be site-specific and occur at normal P supply essentially only in kidney. Nevertheless, the significant response of intestinal *CYP24A1* due to variable P diets demonstrated the crucial role of autocrine mechanisms to balance local calcitriol actions. The tissue-wide expression of *VDR* under-lines the multifactorial impact of the vitamin D system with an emphasis on intestine. Regarding the involvement of the FGF-system in mineral balance, the ubiquitous distribution of *FGFR*s implies tissue-wide capacity for signal transduction mediated by FGF23 of bone origin. The significant increase in expression of skeletal *FGFR4* and *KL* following high dietary P supply point to autocrine circuits to regulate bone mineralization. The tis-sue-wide contributions to the maintenance of vitamin D metabolism and mineral homeostasis reflect complex endogenous mechanisms through specific gene expression patterns and are essential for monitoring the dietary impact on health and tissue integrity.

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Manuscript II

Hasan M, Reyer H, Oster M, Trakooljul N, Ponsuksilli S, Magowan E, Fischer D-C, Wimmers K (2023) 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: 106428.

Exposure to artificial ultraviolet-B light mediates alterations on the hepatic transcriptome and vitamin D metabolism in pigs

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Abstract

In the currently prevailing pig husbandry systems, the vitamin D status is almost exclusively dependent on dietary supply. Additional endogenous vitamin D production after exposure to ultraviolet-B (UVB) light might allow the animals to utilize minerals in a more efficient manner, as well as enable the production of functional vitamin D-enriched meat for human consumption. In this study, growing pigs (n = 16) were subjected to a control group or to a daily narrowband UVB exposure of 1 standard erythema dose (SED) for a period of 9 weeks until slaughter at a body weight of 105 kg. Transcriptomic profiling of liver with emphasis on the associated effects on vitamin D metabolism due to UVB exposure were evaluated via RNA sequencing. Serum was analyzed for vitamin D status and health parameters such as minerals and biochemical markers. The serum concentration of calcidiol, but not calcitriol, was significantly elevated in response to UVB exposure after 17 days on trial. No effects of UVB exposure were observed on growth performance and blood test results. At slaughter, the RNA sequencing analyses following daily UVB exposure revealed 703 differentially expressed genes (DEGs) in liver tissue (adjusted *p*-value < 0.01). Results showed that molecular pathways for vitamin D synthesis (*CYP2R1*) rather than cholesterol synthesis (*DHCR7*) were preferentially initiated in liver. Gene enrichment ($p < 0.05$) was observed for reduced cholesterol/steroid biosynthesis, SNARE interactions in vesicular transport, and CDC42 signaling. Taken together, dietary vitamin D supply can be complemented via endogenous production after UVB exposure in pig husbandry, which could be considered in the development of functional foods.

5.6. Introduction

Vitamin D plays a critical role in bone development by maintaining calcium-phosphate homeostasis (Fleet, 2017; Gerlinger *et al.*, 2021) and has a wide range of important biological functions in the body, including metabolism, growth, immunity, and the antioxidative status (F Holick, 2011; Hasan *et al.*, 2022a). In pigs, vitamin D forms can be directly obtained from the dietary sources or produced in the body following the skin exposure to solar ultraviolet-B (UVB) (Alexander *et al.*, 2017; Burild *et al.*, 2015; Larson-Meyer *et al.*, 2017; Neill *et al.*, 2023). During exposure to solar light, UVB (290-315 nm) is absorbed in the skin to convert endogenously produced provitamin D_3 (7-dehydrocholesterol) to previtamin D_3 (Holick *et al.*, 2007). Subsequently, the previtamin D_3 is rapidly converted to vitamin D_3 (cholecalciferol). Once cholecalciferol is produced in the skin or obtained from the diet, it enters the circulation and undergoes 25-hydroxylation via vitamin D 25-hydroxylase (encoded by *CYP2R1*) to form 25(OH)D_3, also known as calcidiol, in liver. The liver is also the specific site where the vitamin D binding protein (encoded by *GC*) is produced to distribute calcidiol throughout the body via circulation (Hurwitz & Cooke, 2003). Calcidiol is further hydroxylated (1α-hydroxylation) in kidney via 25-hydroxyvitamin D 1-α-hydroxylase (encoded by *CYP27B1*) to form 1,25$(OH)_2D_3$, also known as calcitriol, for activation (Bikle, 2021; Hasan *et al.*, 2022a). There is a body of evidence that calcitriol can affect the expression of more than 900 genes in a tissue-specific manner (Carlberg, 2019; Nurminen *et al.*, 2019; Wang *et al.*, 2005). Recent studies suggest that vitamin D metabolism is not limited to the kidney and liver, since various tissues express genes encoding the corresponding hydroxylases to locally provide vitamin D according to specific requirements (Hasan *et al.*, 2022b; Hasan *et al.*, 2022a).

In pigs, exposure to sunlight or artificial UVB has been demonstrated to elevate the serum level of calcidiol in circulation (Kolp *et al.*, 2017; Larson-Meyer *et al.*, 2017). Exposure to sunlight for one hour daily at midday (41.3114°N, 105.5911°W; 2,184 m) increased the serum concentrations of calcidiol in pigs by up to 200% (Alexander *et al.*, 2017). Also, a short-term application of 6 minutes daily has been shown to be effective (Alexander *et al.*, 2017; Burild *et al.*, 2015; Larson-Meyer *et al.*, 2017; Neill *et al.*, 2023). Recent evidence suggests that direct UVB exposure of pigs can also elevate cholecalciferol and calcidiol levels in muscle and adipose tissue, which may enable the fortification in functional meat products for human consumption (Larson-Meyer *et al.*, 2017; Maurya *et al.*, 2020; Neill *et al.*, 2023).

The total radiation received can be represented in standard erythemal dose units (SED), with 1 SED equaling 100 J/m^2 erythemally effective radiation (Salvadori *et al.*, 2019). This standard unit is a fixed measure and independent of the skin type and species. At the latitude of 55 degrees north (55°N), 1 SED is equal to 10 minutes of sun exposure at the zenith in summertime (Bogh *et al.*, 2012). Natural or artificial UVB exposure of animals with 1 SED results in higher cholecalciferol and calcidiol levels in the blood compared to animals relying on dietary vitamin D_3 at 2000 IU/kg feed (Burild *et al.*, 2016; Jakobsen *et al.*, 2007). According to Barnkob *et al.*, UVB exposure of pigs at 1 SED results in higher amounts of vitamin D_3 levels in various body compartments such as serum, skin, subcutaneous fat, lean meat, and liver compared to exposure at 0.3 and 0.7 SED (Barnkob *et al.*, 2019). Apart from this, a 7-second exposure of pigs to UVB light can produce 0.5 µg of vitamin D_3 per cm^2 of skin (Barnkob *et al.*, 2016). Under the conditions that pigs might be increasingly exposed to sunlight e.g., in housing systems with outdoor access, the question arises how this will affect the endogenous vitamin D metabolism. Various consequences on management and feeding of pig herds are conceivable. This could comprise appropriate modifications of the nutritional mineral and vitamin D supply or additional value-adding opportunities through the fortification of vitamin D in meat.

We hypothesized that UVB exposure contributes to the increase in systemic levels of vitamin D_3 metabolites and might impact on zoo-technical performance parameters secondary to the direct effects on cholecalciferol synthesis in the skin. Thus, this is the first study to provide a holistic transcriptome profile of liver tissue to investigate hepatic responses to UVB exposure in pigs compared to untreated controls. This approach enabled the uncovering of effector molecules and genes involved in hepatic vitamin D_3 metabolism.

5.7. Materials and methods

5.7.1. Animals and diets

The entire experiment was performed at the Agri-Food and Bioscience Institute (AFBI), UK, received approval from the Ethical Review Body (Project License Number PPL2751) for animal welfare, and was conducted in accordance with the Animals Scientific Procedures Act 1986. DanBred Duroc boars (n = 16) were assigned to either UVB exposure (UVB, n = 8) or no UVB exposure (control, n = 8). UVB exposure started at 14 weeks of age (average body weight (mean ± SD): 46.88 ± 3.44 kg) and continued for 9 weeks until slaughter. The pigs were exposed to UVB with a narrow band lamp (0.11 mW/cm^2; Koninklijke Philips N.V., Amsterdam, The

Netherlands) for 30 seconds daily, which corresponds to 1 SED equivalent to 10 minutes of natural daylight at 55°N. All pigs had *ad libitum* access to basal diets (Supplementary Table 1) and drinking water. Body weight, average daily weight gain, and feed conversion were recorded.

5.7.2. Serum and tissue sampling

Blood samples were taken from the jugular vein on days 2 and 17 and were allowed to clot for about 30 minutes prior to centrifugation (2200 x g; 15 min at 4°C). Serum was aliquoted and stored at -80° until analysis. At an average age of 150.9 days, 16 pigs were anesthetized using gas stunning and sacrificed by exsanguination at the Karro Food Group (Cookstown, UK) slaughter facility. The back fat thickness and cold carcass weight of the pigs were determined at slaughter and used in combination with the live weight to calculate the kill out percentage. For each individual, two grams of liver sample were collected from the *lobus Spigeli*, cut into pieces and frozen in liquid nitrogen immediately prior to long-term storage at -80°C.

5.7.3. Analysis of serum

The serum concentrations of albumin, alkaline phosphatase, calcium, glucose, inorganic phosphorus, total protein, and total cholesterol was measured by means of the Fuji DriChem 4000i system (FujiFilm, Minato, Japan). The amounts of calcidiol and calcitriol were determined in duplicate for all samples using enzyme-linked immunosorbent assays (ELISA; calcidiol: EIA-5396, DRG instruments GmbH, Marburg, Germany; calcitriol: AC-62F1, Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany) according to the manufacturer's protocol.

5.7.4. Isolation of RNA, library preparation and RNA sequencing

Total RNA was extracted from liver samples using TRI reagent (Sigma-Aldrich, Taufkirchen, Germany) and treated with DNaseI for the removal of potential genomic DNA residues as previously described (Omotoso *et al.*, 2021). The isolated RNA was purified using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) and the concentration was measured using a NanoDrop 2000 spectrophotometer. Polymerase chain reaction amplification of RNA with beta-actin (ACTB)-specific primers (forward primer: 5'-GAGAAGCTCTGCTACGTCGC-3'; reverse primer: 5'-CCTGATGTCCACGTCGCACT-3') was performed to warrant the complete absence of genomic DNA in total RNA content. The quality of liver RNA extracts was assessed using a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany), giving RNA integrity numbers of 7.0 to 8.8 (average 8.1), which allowed further processing of the samples. Stranded mRNA

libraries were constructed (Illumina, San Diego, CA, USA) from purified total RNAs, and their quality was confirmed using an Agilent DNA-1000 chip kit (Bioanalyzer 2100). The libraries were sequenced for single reads of 101bp using an Illumina HiSeq2500 equipment.

5.7.5. Sequencing data processing and differential gene expression analysis

Raw reads were quality verified and preprocessed with FastQC v.0.12.0 and Trim Galore v.0.6.5, with low quality reads (mean Q score 20) and adapters removed. The HISAT2 (v.2.2.1) (Pertea *et al.*, 2016) and HTSeq (v.2.0.3) (Anders *et al.*, 2015) tools were used to map high-quality reads to the reference Sscrofa11.1 (Ensembl Release 109) including 35,670 genes. The mixOmics (v.6.24.0) R package (Rohart *et al.*, 2017) was used to perform preliminary data visualization in a principal component analysis of gene expression patterns following a variance stabilizing transformation of count data. In addition, overall transcriptional profiles were compared between groups using a distance matrix-based approach implemented in the vegan (v.2.6-2) R package. The R package DESeq2 v1.40.1 was used to identify differentially expressed genes (DEGs) between UVB and control (Love *et al.*, 2014). The significance threshold for detecting DEGs between diet groups was set at a Benjamini-Hochberg adjusted *p*-value of 0.01. To examine significant biological changes between two groups (UVB vs control), the lists of DEGs (n = 703) were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [release 106, accessed 23/05/2023, reference organism is pig (*Sus scrofa*)] using the g: Profiler (v.e109_eg56_p17_1d3191d) web server (Raudvere *et al.*, 2019). In addition, ingenuity pathway analysis (IPA) were conducted on the list of DEGs using IPA database (QIAGEN, Redwood City, CA, USA) (Krämer *et al.*, 2014). Pathways were considered significant at a Benjamini-Hochberg-adjusted *p*-value less than or equal to 0.05. In addition, genes involved in the synthesis, activation, transport and inactivation of vitamin D were considered specifically, as well as those previously described as primary vitamin D target genes (Nurminen *et al.*, 2019).

5.7.6. Data analyses

The data analysis of this experiment was carried out using the open sourced R software (v4.2.2; R foundation for statistical computing, Vienna, Austria). Statistical analyses of zootechnical parameters and serum measurements were performed with a linear model including the two experimental groups (UVB and control) as fixed effect and slaughter order as covariate. The graphs were prepared using GraphPad Prism v9.2.0 (GraphPad software, San Diego, CA, USA).

5.8. Results

5.8.1. Effects of UVB exposure on growth performance and carcass traits

The animals examined in this study had a uniform birth weight and comparable weights at the start of UVB treatment at 14 weeks of age. When compared to controls, the UVB exposure showed no significant ($p > 0.05$) impact on the growth performance of the pigs including body weight, average daily weight gain, backfat thickness, and feed conversion ratio (Figure 1). No significant effect of UVB exposure was observed on the carcass characteristics comprising cold weight and kill out percentage.

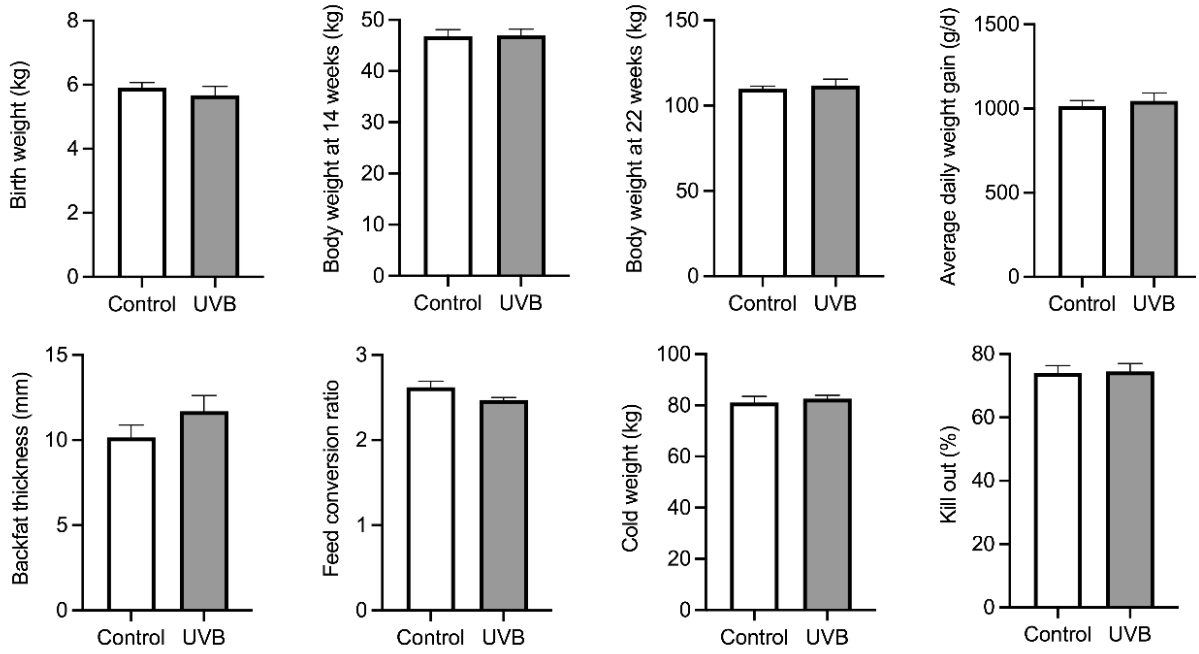

Figure 7. Comparison of growth performance and carcass traits in pigs subjected to a control group (control; white bar) and UVB exposure (UVB; grey bar).

5.8.2. Effects of UVB exposure on serum parameters

The UVB exposure demonstrated no significant effect on the serum parameters related to liver integrity, renal function, and mineral homeostasis (Table 1). The serum level of calcidiol was significantly increased following a daily UVB exposure for 17 days compared to the unexposed control group ($p = 0.01$). The activity of alkaline phosphatase in serum tended to increase at the initial phase after exposure to UVB for 2 days compared to controls ($p = 0.06$).

Table 4. The serum concentration of parameters related to liver integrity, renal function, and mineral homeostasis of control and UVB-exposed pigs (mean ± SE). Differeneces with p-values ≤ 0.05 were considered significant.

Traits	Control	UVB	*p*-value
Initial serum analytics (trial day 2)			
Calcidiol [25(OH)D_3] (ng/ml)	14.77±0.85	19.55±2.94	0.17
Calcitriol [1,25$(OH)_2D_3$] (pmol/l)	1111.26±117.35	1291.74±125.93	0.30
Albumin (g/dl)	3.67±0.13	3.42±0.31	0.49
Alkaline phosphatase activity (U/l)	278.17±63.02	453.50±73.42	0.06
Calcium (mg/dl)	11.10±0.26	11.23±0.32	0.76
Creatinine (mg/dl)	1.10±0.22	1.40±0.26	0.37
Glucose (mg/dl)	115.33±8.45	117.17±4.43	0.86
Inorganic phosphorus (mg/dl)	9.32±0.39	10.00±0.51	0.34
Total cholesterol (mg/dl)	88.17±8.40	77.50±4.99	0.26
Total protein (g/dl)	5.90±0.17	5.68±0.27	0.53
Serum analytics during the study (trial day 17)			
Calcidiol [25(OH)D_3] (ng/ml)	15.65±1.20	22.02±1.41	0.01
Calcitriol [1,25$(OH)_2D_3$] (pmol/l)	561.89±25.91	498.93±58.55	0.36
Albumin (g/dl)	3.79±0.18	3.78±0.19	0.96
Alkaline phosphatase activity (U/l)	197.38±20.34	211.38±10.70	0.53
Calcium (mg/dl)	11.00±0.28	11.28±0.36	0.55
Creatinine (mg/dl)	0.83±0.08	0.85±0.05	0.76
Glucose (mg/dl)	112.38±6.28	116.13±4.46	0.64
Inorganic phosphorus (mg/dl)	10.00±0.46	10.80±0.35	0.20
Total cholesterol (mg/dl)	73.75±3.09	72.25±4.13	0.78
Total protein (g/dl)	6.20±0.18	5.91±0.18	0.29

5.8.3. Effects of UVB exposure on hepatic gene expression profiles

The gene expression profiles in liver were assessed after 8 weeks of UVB exposure. The average number of reads per sample was 15,876,602 and the mean alignment rate was 89%. The principal component analysis based on gene expression data showed a distinct separation between samples exposed to control and UVB conditions (Figure 2). Principal component 1 (PC1) explained 20 percent of the variance and principal component 2 (PC2) explained 14 percent of the variance in the transcriptomic profiles. Based on the analysis of variance considering the first two PCs, the UVB exposure had a significant effect on the overall transcriptional profiles (p = 0.004). The differential expression analysis for the comparison between the control and UVB groups resulted in 703 DEGs (adjusted $p \leq 0.01$) (Supplementary Table 2).

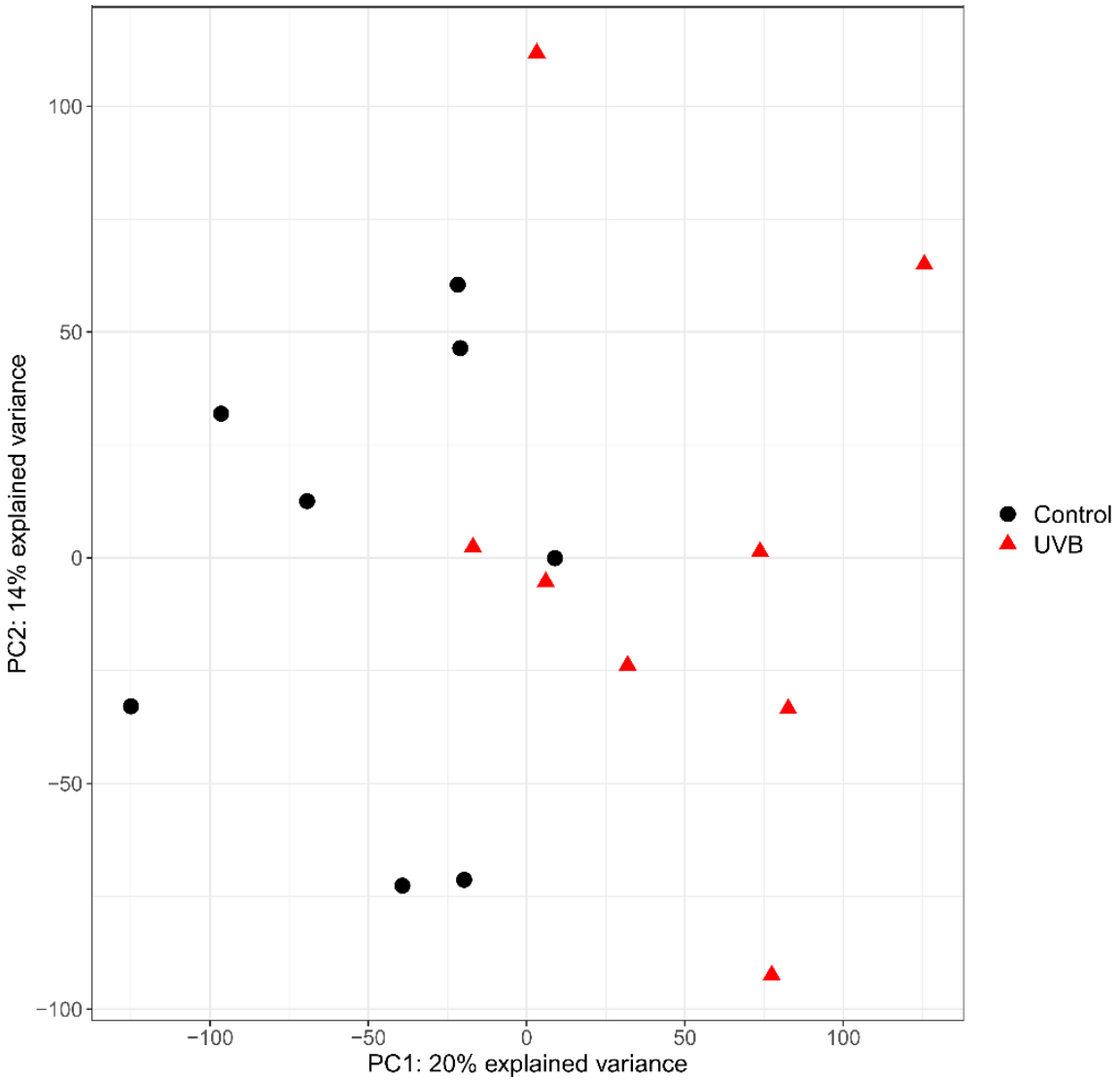

Figure 8. Principal component analysis of the liver expression profiles of control and UVB-exposed pigs.

5.8.4. Pathway enrichment analysis

The DEGs were subjected to biological pathway analysis via IPA and KEGG databases to determine their enrichment in metabolic pathways following the UVB exposure of the pigs (Table 2). IPA demonstrated a significant enrichment of the superpathway of cholesterol biosynthesis and cholesterol biosynthesis I, II, and III. The DEGs contributing to the enrichment of these pathways were significantly downregulated (UVB < control) in UVB-exposed animals compared to the controls, resulting in a predicted inactivation of the pathways (Supplementary Table 3). The CDC42 signaling pathway was also found to be significantly enriched. Additionally, KEGG analysis revealed a significant enrichment of steroid biosynthesis and SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) interactions in the vesicular transport pathway (Supplementary Table 4).

Table 5. Representation of significant pathways derived from Ingenuity Pathway Analysis (IPA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using DEGs from hepatic expression profiles obtained from control and UVB-exposed pigs. Pathway enrichment with an adjusted p-value ≤ 0.05 was considered significant.

Pathway	Number of genes in the pathway	Adjusted *p*-value	Involved genes
IPA			
Superpathway of Cholesterol Biosynthesis	26	0.008	*ACAT2*, *DHCR7*, *EBP*, *FDPS*, *LSS*, *MVD*, *NSDHL*, *TM7SF2*
Cholesterol Biosynthesis I	13	0.026	*DHCR7*, *EBP*, *LSS*, *NSDHL*, *TM7SF2*
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	13	0.026	*DHCR7*, *EBP*, *LSS*, *NSDHL*, *TM7SF2*
Cholesterol Biosynthesis III (via Desmosterol)	13	0.026	*DHCR7*, *EBP*, *LSS*, *NSDHL*, *TM7SF2*
CDC42 Signaling	139	0.026	*ACTR2*, *APC*, *CD247*, *CDC42BPA*, *CLIP1*, *EXOC7*, *ITGA1*, *ITGA2*, *ITGA4*, *ITGAV*, *ITGB1*, *PAK2*, *PPP1R12A*, *PRKCI*, *RASA1*, *TNK2*, *TRBC1*
KEGG			
Steroid biosynthesis	15	0.019	*DHCR7*, *EBP*, *LSS*, *NSDHL*, *TM7SF2*
SNARE interactions in vesicular transport	24	0.024	*STX2*, *STX3*, *STX5*, *STX7*, *STX17*, *SNAP23*

5.8.4.1. Expression profiles of vitamin D metabolism genes

Using the RNA sequencing data, the impact of UVB exposure was further assessed regarding the expression of genes responsible for the synthesis, activation, transport, and inactivation of vitamin D metabolites in liver (Table 3). The expression of *CYP2R1* was significantly upregulated in UVB-exposed pigs compared to controls (adjusted $p \leq 0.05$). The overall expression level of *CYP2R1* was moderate. At the same time, transcript levels of *CYP27A1* were significantly reduced in UVB-exposed pigs compared to controls (adjusted $p \leq 0.05$). The expression of *CYP27B1* was unaffected between the experimental groups. Moreover, the expression of the vitamin D receptor encoded by *VDR* and its heterodimer partners *RXRA*, *RXRB,* and *RXRG* remained unaltered following UVB exposure. Compared to controls, UVB illustrated no significant impact on the expression of genes linked to transport (*GC*) and inactivation (*CYP24A1*). The expression of *DHCR7* was significantly reduced in pigs exposed to UVB compared with controls (adjusted $p \leq 0.05$).

Table 6. Expression of genes linked to vitamin D metabolism in liver of control and UVB-exposed pigs. Gene expression differences with djusted p-values < 0.05 were considered significant.

Gene	Function	Base mean expression	Fold change (UVB vs control)	Adjusted *p*-value
CYP2R1	Hydroxylation (25-OH) of cholecalciferol	31.53	1.82	0.033
CYP27A1	Hydroxylation (25-OH) of cholecalciferol	10681.15	-1.67	0.041
CYP27B1	Hydroxylation (1α-OH) of calcidiol	9.50	-1.24	0.814
CYP24A1	Hydroxylation (24-OH) of calcidiol and calcitriol	0.66	-3.60	0.596
DHCR7	Formation of cholesterol	2624.63	-2.73	<0.01
GC	Binding of calcidiol	37745.80	-1.07	0.948
RXRA	Nuclear receptor	1938.26	-1.26	0.613
RXRB	Nuclear receptor	208.46	-1.24	0.174
RXRG	Nuclear receptor	2.44	1.12	0.978
VDR	Transcription factor	3.69	1.11	0.978

5.8.4.2. Expression profiles of vitamin D target genes

The list of 703 DEGs was aligned with a set of 189 validated primary vitamin D target genes [13] to analyze their transcriptional alteration in the liver in response to UVB exposure (Supplementary Table 5). In total, 12 transcripts were found to be significantly differentially expressed in UVB-exposed pigs compared to controls (adjusted $p \leq 0.05$) (Table 4).

Table 7. Primary vitamin D target genes found to be significantly differentially expressed in the liver of control and UVB-exposed pigs. The full list of the target genes according to Nurminen *et al*. (Nurminen *et al.*, 2019) is provided in Supplementary Table 5. Differences in gene expression with adjusted *p*-values < 0.05 were considered significant.

Gene	Function	Base mean expression	Fold change (UVB vs control)	Adjusted *p*-value
MARCKS	Regulation of developmental processes	300.93	2.11	0.002
OCEL1	Undefined	329.05	-1.72	0.004
LTC4S	Mediation of inflammation	1126.27	-2.30	0.009
SHE	Signal transduction	54.27	1.71	0.014
G0S2	Promotes apoptosis by binding to BCL2	268.76	-2.41	0.015
LAMB3	Cell adhesion, migration, and differentiation	107.38	-2.25	0.016
FXYD6	Affects the activity of Na, K-ATPase	394.03	-1.57	0.017
SLC35A4	Sugar transporter	143.71	-1.82	0.018
NRIP1	Modulates the function of transcription factors	66.09	1.88	0.022
PKP2	Desmosome formation and function	161.90	1.56	0.023
ITSN1	Cytoplasmic membrane-associated protein	158.07	1.40	0.048
SDS	Metabolizing serine and glycine	3210.57	-5.87	0.049

5.9. Discussions

Our study demonstrated that UVB exposure shows no significant impact on body weight, average daily weight gain, carcass weight, and yield percentage of the pigs. This is consistent

with previous findings where different dosages of UVB (0 SED, 0.7 SED, 1 SED) as well as timings of daily UVB exposure (2 min, 6 min) showed non-significant effects on performance data including body weight (Larson-Meyer *et al.*, 2017; Neill *et al.*, 2023). In the current study, the backfat thickness, a key carcass composition trait (Gozalo-Marcilla *et al.*, 2021; Reyer *et al.*, 2017) in breeding programs, was unaltered between experimental pig groups. This is in agreement with previous results where exposure of the pigs to natural sunlight at solar noon for 2 weeks (1 hour daily) during the summer solstice and fall equinox showed no significant impact on the backfat thickness weight (Larson-Meyer *et al.*, 2017; Neill *et al.*, 2023). However, adipose tissue exhibited higher levels of vitamin D_3 and calcidiol compared to liver and lean meat (Barnkob *et al.*, 2016), suggesting that a number of tissues are involved in maintaining body stores according to specific local demands (Hasan *et al.*, 2022a). Interestingly, a radiation regimen of 1 kJ/m^2 with 65% UVB and 35% UVA exposure of overweight mice significantly reduced body weight and weight gain when challenged with a high-fat diet (Fleury *et al.*, 2017). This phenomenon was also confirmed in other studies on mice (Allemann *et al.*, 2020; Ferguson *et al.*, 2019; Geldenhuys *et al.*, 2014). Since pig husbandry is also a subject of economic interests, the outcome might be considered in terms of improving metabolic health in proliferative sows.

The serum concentration of calcidiol is used to assess the vitamin D status in humans (Holick, 2009). In this study, the applied UVB exposure showed to be effective on serum calcidiol levels, but not on serum calcitriol levels, suggesting a short- to medium-term capacity for endogenous synthesis of calcidiol. This is in agreement with previous findings on sows with their piglets (Jakobsen *et al.*, 2020), where calcidiol levels were increased following a daily UVB exposure of 6 h with 0.7 and 1.0 SED at day 12 and 24 compared to controls. Interestingly, 0.7 and 1 SED of UVB showed similar effects on the serum concentration of calcidiol, indicating that an upper limit for calcidiol concentration from endogenous synthesis exists. In addition, daily narrowband UVB exposure of 2 min and 6 min for 10 weeks was recently reported to elevate the vitamin D status of slaughtered pigs (Alexander *et al.*, 2017; Burild *et al.*, 2015; Larson-Meyer *et al.*, 2017; Neill *et al.*, 2023). Therefore, 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. However, there is not yet a comprehensive, scientifically validated recommendation for serum calcidiol levels in pigs to ensure bone health and efficient mineral utilization. In addition to presumed genetic factors (Nan *et al.*,

2020; Reyer *et al.*, 2019) generalizable conclusions are further confounded by the tissue-specific requirement for vitamin D metabolites (Hasan *et al.*, 2022a). The UVB exposure showed no significant impact on the serum levels of albumin, total protein, total cholesterol, alkaline phosphatase, calcium, inorganic phosphorus, creatinine, and glucose at experimental days 2 and 17. This suggests that the applied UVB irradiation has no adverse effect on liver integrity and renal function. This is in agreement with previous reports, where serum alkaline phosphatase and minerals were unaltered due to UVB exposure in pigs (Kolp *et al.*, 2017).

The UVB exposure has been evaluated for transcripts encoding genes related to vitamin D hydroxylation in liver. The higher expression of 25-hydroxylase *CYP2R1* compared to controls corresponds to the elevated serum calcidiol levels (Cheng *et al.*, 2003). However, another enzyme known to exhibit 25-hydroxylase activity, i.e., to convert cholecalciferol to calcidiol, *CYP27A1*, was lowered in expression following UVB exposure compared with controls. Since CYP27A1 is a bifunctional enzyme known to participate in both bile acid and vitamin D metabolism (Jones *et al.*, 2014), its expression patterns may indicate the involvement of UVB exposure in lipid metabolism. Despite known species specificities, there is evidence for yet unknown enzymes exhibiting 25-hydroxylation activity (Zhu *et al.*, 2013). The expression of *GC*, which encodes the vitamin D binding protein with the highest affinity to calcidiol (Daiger *et al.*, 1975), was unaltered between the experimental groups. Given the elevated serum concentration of calcidiol, results reflect the high transportation capacity via GC in the circulation (Cooke & Haddad, 1989). The expression pattern is consistent with recent reports, where a low phosphorus diet significantly upregulated the expression of *CYP2R1* in the jejunum of pigs compared to controls, whereas no effect was observed in the expression of *GC* in liver (Hasan *et al.*, 2022a). Moreover, the expression of *CYP27B1*, encoding the 1α-hydroxylase, was unaffected by UVB exposure indicating no significant effects on hepatic calcitriol production. For the kidney, as the main production site of systemic calcitriol levels, an unaffected *CYP27B1* expression can also be assumed due to the unchanged serum calcitriol levels (Brunette *et al.*, 1978; Hasan *et al.*, 2022a; Meyer & Pike, 2020). However, *CYP27B1* is known to be sensitive to low dietary P intake in lactating sows with increased renal expression (Wubuli *et al.*, 2020). Hepatic expression of *CYP24A1*, known for its 24-hydroxylase activity that induces renal excretion of vitamin D metabolites, was barely detectable in liver tissue and unaltered by UVB exposure. Consistently, *CYP24A1* was not expressed in porcine liver as reported previously (Hasan *et al.*, 2022a). For the vitamin D receptor encoded by *VDR* and its

heterodimer partners *RXRA*, *RXRB,* and *RXRG* (Barsony & Prufer, 2002), the UVB exposure illustrated no significant impact on their expression. Nevertheless, the analysis revealed that 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). Although calcitriol has the highest affinity for VDR, calcidiol also activates VDR at certain serum concentrations with an affinity 100-200 times lower than calcitriol (de Brito Galvao *et al.*, 2013; Dusso & Tokumoto, 2011). However, comprehensive lists of vitamin D target genes in porcine tissues are still missing.

The pathway analysis consistently showed the inhibition of the cholesterol/steroid hormone biosynthesis pathway in the liver of UVB-exposed pigs. In fact, the molecular pathways that contribute to the biosynthesis of vitamin D and cholesterol have considerable overlap. Thus, both the serum calcidiol levels and the synthesis of steroid hormones such as vitamin D from 7-dehydrocholesterol indicate that the findings in hepatic gene expression are most probably due to the increased production of calcidiol (Miller & Bose, 2011; Yang *et al.*, 2021). Specifically, *DHCR7* seems to act as a key regulator controlling the balance between cholesterol biosynthesis and vitamin D biosynthesis (Warren *et al.*, 2021). Vitamin D is thought to be involved in reducing cholesterol biosynthesis by decreasing the activity of DHCR7 and affecting cholesterol levels in both, cells and plasma (Warren *et al.*, 2021). Moreover, the current study found that not only *DHCR7* but also other genes encoding cholesterol-synthesizing enzymes were altered, thereby limiting cholesterol biosynthesis. Interestingly, several studies are available that show the relationship between vitamin D status and fat metabolism in different contexts (Du *et al.*, 2023; Quach *et al.*, 2018; Yin *et al.*, 2012). Especially genes encoding fatty acid synthase (FASN), acetyl-CoA carboxylase alpha (ACACA), and genes related to fatty acid oxidation, e.g., acetyl-CoA oxidase (ACOX1), were also shown to be affected by UVB exposure in the current study. Further molecular studies on the liver are needed to clarify the effects of vitamin D metabolites in general and the role of additional UVB-induced calcidiol production in particular on lipid metabolism. The pathway analysis further demonstrated the significant enrichment of the SNARE interactions in vesicular transport pathway. SNARE is a highly conserved protein superfamily that facilitates the membrane fusion and transport of molecules within cells (Jahn & Scheller, 2006). Certain

SNARE proteins have been shown to facilitate the transport of cholesterol in an *in vitro* rodent model and thus influence steroidogenesis (Lin *et al.*, 2016). Accordingly, the enrichment of this pathway appears to be a consequence of UVB-induced shifts in the biosynthesis from cholesterol precursors to vitamin D. The biological pathway analysis illustrated significant enrichment of the CDC42 signaling. CDC42 is considered as a member of the RhoGTPase family of intracellular molecular switches involved in several signaling pathways crucial for actomyosin (actin-myosin complex) organization within the cytoskeleton (Melendez *et al.*, 2011). It is likely that these are secondary effects due to the shift in cholesterol-vitamin D balance (Chadda *et al.*, 2007; Nofer *et al.*, 2003).

5.10. Conclusions

Daily UVB exposure significantly increased serum calcidiol levels and thus improved vitamin D status in pigs. In addition, the hepatic gene expression pattern confirms systemic effects of artificial UVB light on vitamin D metabolism. Molecular signaling pathways in liver suggested that endogenous vitamin D synthesis had priority over cholesterol formation. Hence, vitamin D obtained from mainly dietary sources can be complemented via endogenous calcidiol synthesis induced by UVB exposure. This paves the way for long-term improvements of mineral utilization and confirms the possibility of producing biofortified animal products for human consumption.

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Manuscript III

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, 1-10.

Efficacy of dietary vitamin D_3 and 25(OH)D_3 on reproductive capacities, growth performance, immunity, and bone development in pigs

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Abstract

Vitamin D_3 (Vit D_3) and 25(OH)D_3 are used as dietary sources of active vitamin D [1,25$(OH)_2D_3$] in pig husbandry. Although acting primarily on intestine, kidney, and bone, their use in pig nutrition has shown a wide range of effects also in peripheral tissues. However, there is an ambiguity in the existing literature about whether the effects of Vit D_3 and 25(OH)D_3 differ in attributing the molecular and phenotypic outcomes in pigs. We searched Web of Science and PubMed databases concerning the efficacy of Vit D_3 in comparison with 25(OH)D_3 on pig physiology, i.e., reproductive capacities, growth performance, immunity, and bone development. Dietary intake of Vit D_3 or 25(OH)D_3 did not influence the reproductive capacity of sows. Unlike Vit D_3, the maternal intake of 25(OH)D_3 significantly improved the growth performance of piglets, which might be attributed to maternally induced micronutrient efficiency. Consequently, even in the absence of maternal vitamin D supplementation, 25(OH)D_3-fed offspring also demonstrated better growth than the offspring received Vit D_3. Moreover, a similar superior impact of 25(OH)D_3 was seen with respect to serum markers of innate and humoral immunity. Last but not least, supplements containing 25(OH)D_3 were found to be more effective than Vit D_3 to improve bone mineralization and formation, especially in pigs receiving basal diets low in calcium and phosphorus. The insights are of particular value in determining the principal dietary source of vitamin D to achieve its optimum utilization efficiency, nutritional benefits, and therapeutic potency and to further improve animal welfare across different management types.

5.11. Introduction

Vitamin D_3, cholecalciferol (Vit D_3), and 25-hydroxycholecalciferol [$25(OH)D_3$] are the two major dietary forms to supply the organism with vitamin D (Vit D). In recent years, $25(OH)D_3$ is being studied as an alternative to Vit D_3, as it is more bioavailable, efficiently absorbed, bypasses hepatic metabolism, and is three to five times more potent than Vit D_3 (Quesada Gómez & Bouillon, 2018). Nevertheless, both dietary forms of Vit D are biologically inactive and require two (Vit D_3) and one sequential hydroxylation reaction [$25(OH)D_3$] for activation (Figure 1). After ingestion or dermal synthesis, Vit D_3 is transported via Vit D binding protein (DBP) and hydroxylated via Vit D 25-hydroxylase (encoded by *CYP2R1*) in liver to form $25(OH)D_3$ and via 25-hydroxyvitamin D 1-alpha-hydroxylase (encoded by *CYP27B1*) in kidney to $1,25(OH)_2D_3$ [the active form of Vit D]. The excess amount of $1,25(OH)_2D_3$ is subject to renal elimination following CYP24A1-mediated hydroxylation (Quach, 2016). Nevertheless, both synthesis and elimination also occur in non-renal tissues (i.e., intestine) (Hasan *et al.*, 2022). After activation, $1,25(OH)_2D_3$ migrates via DBP to various target tissues such as intestine, bone, muscle, immune system, kidney, parathyroid glands and reproductive system to attribute its functions.

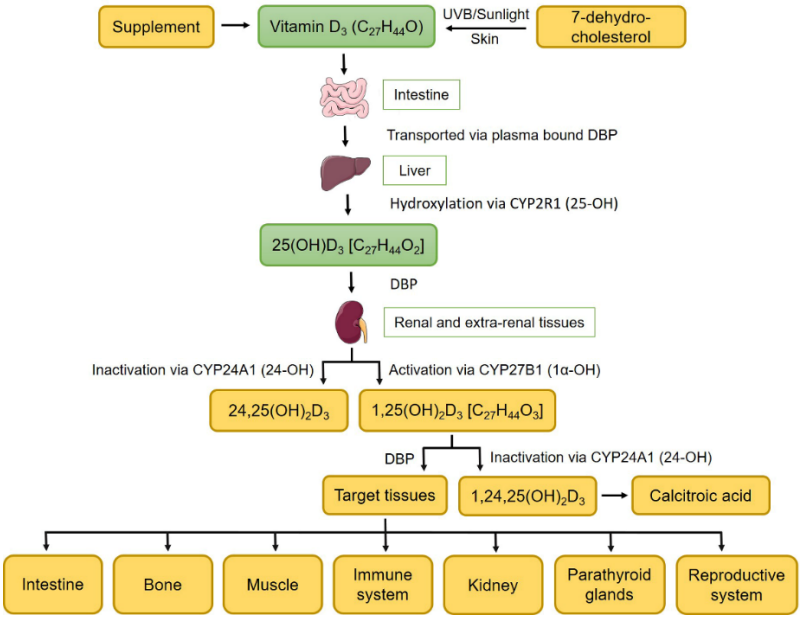

Figure 9. Schematic illustration of the endogenous synthesis of active [$1,25(OH)_2D_3$] and inactive [$24,25(OH)_2D_3$ or $1,24,25(OH)_2D_3$] forms of vitamin D from supplemented or dermally synthesized vitamin D_3.

The mediation of the biological functions of Vit D depends on its successful binding with the intracellular Vit D receptor (VDR, ligand-dependent transcription regulatory molecule) forming the VDR-1,25$(OH)_2D_3$ complex. This complex interaction initiates the formation of two autonomous protein interaction surfaces on the VDR; one of them modulates interplay with the retinoid X receptor (RXR) for DNA binding and the other recruits co-regulators to control gene expression. After dimerization of VDR-1,25$(OH)_2D_3$ with RXR, the heterodimer translocates to the nucleus, binds to the Vit D responsive element (VDRE), which in turn regulates the expression of numerous genes (>900), and implements the specific function of Vit D in particular tissues (Kongsbak *et al.*, 2013; Pike *et al.*, 2012).

The secosteroid hormone Vit D is critical for the maintenance of serum calcium (Ca) and phosphorus (P) homeostasis for optimum bone development, as it modulates the active uptake of minerals through the intestine (Holick, 1996; Sutton & MacDonald, 2003). The tissue-wide expression of VDR and other genes involving Vit D metabolism underscores the fact that the function of Vit D is not limited to osteogenesis or serum mineral balance (Bouillon *et al.*, 2019; Hasan *et al.*, 2022). In fact, an array of tissues are capable to express relevant genes encoding hydroxylation enzymes for calcitriol synthesis and elimination in mammals (Hewison *et al.*, 2007; Pike *et al.*, 2012). Thus, it turns out to be equally important for growth (Flohr *et al.*, 2014; Hines *et al.*, 2013; Wilborn *et al.*, 2004; Zhang *et al.*, 2022), immunity (Konowalchuk *et al.*, 2013; Zhang *et al.*, 2021), oxidative status (Rey *et al.*, 2020; Yang *et al.*, 2019), reproductive capacity (Upadhaya *et al.*, 2021; Weber *et al.*, 2014), and progeny performance (Upadhaya *et al.*, 2022). In pig feeding, legislation limits the Vit D supply to 1000–2000 IU/kg diet, which corresponds to 25–50 µg/kg feed (FEEDAP, 2012), while 25$(OH)D_3$ in combination with Vit D_3 is allowed up to doses of 50 µg/kg feed (European Food Safety, 2009). However, the most potent form of Vit D in terms of nutritional benefits remains unclear, largely due to divergent functional demands of tissues and cell types (Hasan *et al.*, 2022; Hewison *et al.*, 2007). Given the background, this review summarizes the available literature for molecular and phenotypic outcomes of Vit D_3 in comparison with 25$(OH)D_3$ supplements in terms of reproductive capacities, growth performance, immunity, and bone development in pigs.

5.12. Search strategies and selection of articles

We conducted a systematic query in Web of Science and PubMed to retrieve all the articles dealing with Vit D_3 and 25(OH)D_3 in pigs published from 1st January 2000 to 23rd May 2022. The search strategy included appropriate MeSH terms (supplementary material S1) 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 (supplementary material S2). Of these, a total of 35 articles were selected for review, paying attention to relevance, overlap, and experimental design (supplementary material S3).

5.13. Comparative performance of vitamin D_3 and 25(OH)D_3 on the reproduction and growth

Recent investigations suggest that the dietary supply of both Vit D_3 and 25(OH)D_3 plays an important role in fertility and maturation. The objective of this section was to identify arguments for the more effective form of Vit D in the diet to improve sow reproductive capacities and growth performance of the piglets (Table 1). Parts of the reported findings could be attributed to adaptive responses resulting from maternal nutritional programming, i.e., long-term consequences for growth, function, and structure of various tissues and cell types, and thus for health and welfare (Fleming *et al.*, 2018).

Thayer *et al*. (Thayer *et al.*, 2019) studied the effects of feeding Vit D_3 and 25(OH)D_3 on sow reproductive capacity, muscle fibre morphometry, and subsequent growth performance of piglets. For this purpose, 69 sows were randomly allocated to one of three dietary groups comprising (i) Vit D_3 as control [37.5 µg/kg], (ii) Vit D_3 and 25(OH)D_3 at low levels [DL, 12.5 µg/kg Vit D_3 + 25 µg/kg 25(OH)D_3], or (iii) Vit D_3 and 25(OH)D_3 at high levels [DH, 37.5 µg/kg Vit D_3 + 50 µg/kg 25(OH)D_3]. On the other hand, the piglets (n = 216) from these 69 sows were treated similarly to their mothers from birth until day 59 of life. No significant effect was observed on the reproductive performance of sows regardless of the dietary Vit D regimens. At the time of birth, piglets from DH-fed sows showed a significant increase in the number of primary muscle fibres in contrast to piglets from control sows at birth. However, this effect was no longer present when weaning piglets were investigated. During the nursery period, there was no effect of dietary Vit D sources on the growth performance, except for feed

efficiency from day 28 to 59 and day 0 to 59. During this period, piglets from DH-fed sows showed a significant increase in feed efficiency (FE), i.e., the body weight gain to feed intake ratio, in contrast to piglets from sows assigned to DL. Overall, dietary intake of 25(OH)D_3 resulted in a significant increase in the number of primary muscle fibres at birth, but the total number of muscle fibres did not improve at birth or weaning.

Upadhaya *et al*. (Upadhaya *et al.*, 2022; Upadhaya *et al.*, 2021) conducted two different studies to compare the effects of maternal Vit D_3 and 25(OH)D_3 supplementations on sow reproduction and offspring growth performance. In one study (Upadhaya *et al.*, 2022), 48 multiparous sows were provided a basal diet containing Vit D_3 (CON, 50 µg/kg Vit D_3) or 25(OH)D_3 [TRT, CON + 50 µg/kg 25(OH)D_3] depending on their body weight and expected farrowing date. At weaning, 80 piglets each from CON and TRT sows were fed diets containing 62.5 µg/kg Vit D_3 (weaning diet) and 43.75 µg/kg Vit D_3 (growing-finishing diet) with or without 25(OH)D_3 [50 µg/kg] for 140 days. Unlike Vit D_3, the maternal intake of 25(OH)D_3 significantly improved the average daily weight gain (ADWG) and FE of the piglets at the early stage of the nursery period. However, in the later phase of the nursery period, maternal ingestion of either source of Vit D demonstrated a similar effect on the growth performance of the piglets. In this period, the piglets alone supplemented with 25(OH)D_3 showed significant improvement in ADWG, FE, and average daily feed intake (ADFI). Moreover, in contrast to Vit D_3, 25(OH)D_3 significantly increased the water-holding capacity and reduced pork drip loss. Feeding 25(OH)D_3 to sows and their progenies had a significant impact on the expression of candidate genes associated with muscle formation. After dietary supplementation to sows and their offspring (post-weaning diet) with 25(OH)D_3, the up-regulation of myogenic markers *MYOD1* (myogenic differentiation 1), *MYF5* (myogenic factor 5) and down-regulation of *MSTN* (myostatin) were observed, suggesting the importance of 25(OH)D_3 for muscle development. MYOD1 and MYF5 are involved in the regulation of myoblast proliferation and differentiation and critically determine the survival of muscle progenitor cells as well (Alway *et al.*, 2011; Megeney & Rudnicki, 2011). On the contrary, MSTN is the negative regulator of muscle development (Elkina *et al.*, 2011). In the second study (Upadhaya *et al.*, 2021), a total of 48 multiparous sows received either a basal diet fortified with Vit D_3 (control, 50 µg/kg) or a

control diet containing 25(OH)D_3 [TRT, 50 µg/kg] based on their body weight and expected farrowing date. The sows fed 25(OH)D_3 demonstrated a significant increase in body weight gain and body condition score during the suckling period of the piglets. Unlike Vit D_3, piglets from sows fed 25(OH)D_3 also had significantly higher survival, ADWG, and weaning weight than piglets from sows fed the control diet. These results indicate that dietary supplementation with 25(OH)D_3 significantly enhanced growth performance in both sows and their offspring.

To compare the two dietary forms of Vit D in terms of reproductive performance, Weber *et al*. (Weber *et al.*, 2014) performed their study into two parts. Firstly, 227 primi- and multiparous sows received a basal diet containing Vit D_3 (114 sows, 50 µg/kg) and 25(OH)D_3 [113 sows, 50 µg/kg] from mating to day 110 of gestation. 25(OH)D_3-supplemented sows exhibited a significant positive impact on total litter weight and total weaning weight of the offspring compared to sows that received Vit D_3. The intrauterine development of the embryos was also positively correlated with the maternal serum levels of 25(OH)D_3. For the second experiment, 39 sows received basal diets fortified with Vit D_3 (DL, 5 µg/kg; DN, 50 µg/kg), and 25(OH)D_3 [DH, 50 µg/kg] from the day of the mating and the treatments were continued for 4 reproductive cycles. Following the intake, the two dietary sources of Vit D exhibited similar impacts on sow reproductive performance. However, a significant increase in weight gain between birth and weaning was observed in the offspring of the DN-fed sows, in contrast to the offspring of DL- and DH-fed sows. In summary, the authors concluded that sows fed 25(OH)D_3 showed a significant improvement in the birth weight of their offspring. But sows provided with either form of Vit D for more than one reproductive cycle may not show any noticeable impact on the growth performance of their progeny.

Witschi *et al*. (Witschi *et al.*, 2011) conducted a comparative study in 39 primi- and multiparous sows randomly assigned to Vit D_3 (DL, 5 µg/kg or DN, 50 µg/kg) and 25(OH)D_3 [HD, 50 µg/kg] from the day of mating to day 21 of lactation. Similar to other studies, the serum level of 25(OH)D_3 increased significantly in pigs after the administration of 25(OH)D_3 compared with Vit D_3. DL led to a decrease in ADFI and a tendency for a decrease in body weight and body weight gain compared to the other groups. Additional supplementation of

HD had no significant effect. So, maternal diets supplemented with either form of Vit D (50 µg/kg) demonstrated a similar impact on the growth performance of their offspring.

Zhang *et al*. (Zhang *et al.*, 2019) compared Vit D_3 (50 µg/kg) and 25(OH)D_3 [50 µg/kg] to determine their relative effects on the reproductive capacity of 24 sows and the growth performance of their offspring from day 107 of gestation to day 21 of lactation. No significant effect of Vit D treatments on sow reproductive performance was observed. However, in contrast to Vit D_3, offspring from 25(OH)D_3-fed sows showed a significant increase in litter weight, total litter weight, and piglet weight gain during the lactation period. Unlike Vit D_3, ADWG and total weight gain also tended to improve in piglets from 25(OH)D_3-supplemented sows. Overall, the dietary supplementation of 25(OH)D_3 in pregnant sows significantly improved piglet growth performance (5.3% increase in weight gain on average).

Hines *et al*. (Hines *et al.*, 2013) studied the effects of maternal supplementation of Vit D_3 or 25(OH)D_3 on piglet growth and muscle development. They provided 40 sows with a control diet containing Vit D_3 (62.5 µg/kg) and an experimental diet fortified with 25(OH)D_3 [DH, 12.5 µg/kg Vit D_3 + 50 µg/kg 25(OH)D_3] beginning 43 days before artificial insemination through day 90 of gestation. Both diets contained 12.5 µg/kg of Vit D_3 to avoid possible Vit D deficiency. In comparison with Vit D_3, foetuses from 25(OH)D_3-fed dams showed a significant increase in the number of *longissimus dorsi* muscle fibres by 9.3%. However, there were no significant effects of the maternal diet on the cross-sectional area of the muscles in piglets. Piglets from 25(OH)D_3-fed sows had more Pax7+ myoblast (72 and 96 hours of post-plating) in the *longissimus dorsi* muscle than piglets from sows fed Vit D_3. Foetuses from 25(OH)D_3-fed sows also had increased total myoblast proliferative capacity. Thus, a maternal diet containing 25(OH)D_3 exhibited a significant positive impact on foetal muscle development.

In contrast to the aforementioned studies, there are very few studies that have investigated the growth performance after supplementing two dietary sources of Vit D directly to piglets rather than to pregnant sows. In the study of Zhang *et al*. (Zhang *et al.*, 2022), 144 piglets were randomly assigned to three different dietary treatments with a follow-up period of 28 days, namely a normal Ca-P (PC), low Ca-P (NC) diet supplemented with 62.5 µg/kg Vit D_3; and low Ca-P diet supplemented with 25(OH)D_3 [NC + 25D, 50 µg/kg 25(OH)D_3]. The experiment was

conducted for 28 days and consisted of phase 1 (days 0-14) and phase 2 (days 15-28). PC diet contained 0.81% Ca, 0.60% total P and 0.72% Ca, 0.53% total P, and NC diet contained 0.56% Ca, 0.47% total P, and 0.45% Ca, 0.39% total P in phase 1 and phase 2, respectively. Compared to NC, the body weight (day 28) and ADWG (days 15-28 and days 1-28) of the weaned piglets increased significantly after their dietary ingestion of NC + 25D. Overall, the piglets fed 25(OH)D_3 showed noticeable growth performance even when no dietary source of Vit D was provided to their mothers.

Corresponding to the study of Zhang *et al*., Zhao *et al*. also conducted feeding trials only in piglets to evaluate growth efficiencies (Zhao *et al.*, 2022). They supplemented a total of 240 weaned piglets (21 days of age, initial body weight about 6 kg) with a positive control diet (PSC, 50 µg/kg Vit D_3), negative control diet (NC, -0.15% P and -0.25% Ca of PSC), phytase (Phy diet, NC + 37.5 µg/kg phytase), and 25(OH)D_3 [HyD, NC + 50 µg/kg 25(OH)D_3; Phy + HyD, NC + 37.5 µg/kg Phy + 50 µg/kg HyD]. The experiment was divided into two phases (6-10 kg and 10-20 kg). Regardless of the body weight, HyD supplements showed no significant effect in ADFI or FE of the weaned piglets in contrast to NC. However, ADWG of piglets (10-20 kg) increased noticeably after dietary intake of HyD compared to NC. In essence, piglets from Vit D-deprived sows demonstrated noticeable improvement in their growth after dietary ingestion of 25(OH)D_3 without altering their feed intake or feed efficiency. However, data on body composition traits have not been reported in this study.

Thus, most studies show that sows fed either form of Vit D have a similar impact on their reproductive ability. However, the survivability of piglets from sows fed 25(OH)D_3 is significantly better than piglets from sows fed Vit D_3 (Coffey *et al.*, 2012; Zhou *et al.*, 2016). Unlike Vit D_3, feeding 25(OH)D_3 to sows helps to noticeably increase offspring growth performance by improving weaning weight, ADWG, total body weight gain, and body condition scores. In the absence of maternal supply of Vit D sources, supplementation of 25(OH)D_3 to the offspring alone can also significantly boost their growth performance. 25(OH)D_3 also outperforms Vit D_3 in increasing the number of muscle fibres and improving the proliferation and differentiation ability of muscle cells.

Table 8. Overview of the comparative studies between vitamin D3 (Vit D3) and 25(OH)D3 supplementation, indicating the study designs, dosages of supplements, underlying conditions, and significant results of the experiments with regard to sow reproductive capacity and growth performance of their progeny. Only supplemented diets (control and treatment) and the conditions that led to noticeable outcomes are listed.

Authors	n	DTP[§] (days)	Doses of vitamin D (µg/kg)		Conditions	Outcomes linked to reproduction and growth performance*	
			Vit D_3[¶]	25(OH)D_3		Vit D_3>25(OH)D_3	25(OH)D_3>Vit D_3
Thayer *et al*. (Thayer *et al.*, 2019)	69 sows 216 piglets	ET[a] (S[b]) 0-59 (P[c])	37.5	37.5 (Vit D_3) + 50	MNDE[d]	-	↑Primary muscle fibre number
Upadhaya *et al*. (Upadhaya *et al.*, 2022)	160 piglets	1-140	50	50	PDE[e]	↑*MSTN*[g]	↑ADWG[h] (days 1-42, 99-140, 1-140), ↑FE[i] (days 1-42), ↑ADFI[j] (days 1-140), ↑*MYF5*[k]
	48 sows		50	50	MDE[f]	↑*MSTN*	↑ADWG, ↑FE (days 1-42); ↑*MYOD*[l], ↑*MYF5*
Upadhaya *et al*. (Upadhaya *et al.*, 2021)	48 sows	-	50	50	-	-	↑Survivability, ↑Weaning weight, ↑ADWG (P); ↑Body condition score, ↑Body weight gain (S)
Weber *et al*. (Weber *et al.*, 2014)	227 sows	ET	50	50	MDE	-	↑Total litter weight, ↑Birth weight
	39 sows	ET	50	50	MDE	↑Weight gain (P)	-
Witschi *et al*. (Witschi *et al.*, 2011)	39 sows	ET	50	50	MDE	-	-
Zhang *et al*. (Zhang *et al.*, 2019)	24 sows	ET	50	50	MDE	-	↑Litter weight, ↑Piglet weight (day 21 of lactation); ↑Total litter weight gain
Hines *et al*. (Hines *et al.*, 2013)	40 sows	ET	62.5	12.5 (Vit D_3)+ 50	MDE	-	↑Total muscle fibre number; ↑Pax7+ Myoblast (cell culture, 72 h and 96 h)
Zhang *et al*. (Zhang *et al.*, 2022)	144 piglets	14/28	62.5	50	-	-	↑ADWG (days 15-28, 1-28), ↑Body weight (day 28)
Zhao *et al*. (Zhao *et al.*, 2022)	240 piglets	-	50	50	-	-	↑ADWG (10-20 kg)

*Asterisk indicates significant outcomes (p<0.05); [§]DTP, dietary treatment period; [¶]1 µg = 40 IU Vit D_3; [a]ET, explained on text; [b]S, sows; [c]P, piglets; [d]MNDE, maternal-nursery diet effect on piglets; [e]PDE, piglet diet effect; [f]MDE, maternal diet effect on piglets; [g]*MSTN*, myostatin; [h]ADWG, average daily weight gain; [i]FE, feed efficiency; [j]ADFI, average daily feed intake; [k]*MYF5*, myogenic factor 5; [l]*MYOD*, myogenic differentiation.

5.14. Comparative performance of vitamin D_3 and 25(OH)D_3 on immunity

In recent years, a great deal of research has been conducted to investigate the immunoregulatory functions of Vit D. This section aimed to discuss the efficacy of Vit D_3 and 25(OH)D_3 to promote immunity in pigs (Table 2).

In the study of Zhang *et al*. (Zhang *et al.*, 2022), 25(OH)D_3 was more effective than Vit D_3 in fortifying the body's immune system when receiving a low Ca-P diet. In contrast to NC, dietary supplementation of 25(OH)D_3 [NC + 25D] reduced the incidence of streptococcal infections by significantly increasing the serum concentrations of immunoglobulin A (IgA) and G (IgG) in piglets at weaning. Since these two types of immunoglobulins (Igs) are involved in inhibiting streptococcal and other bacterial infections (Charles *et al.*, 2001; Norrby-Teglund *et al.*, 1996). Furthermore, in pigs receiving the 25(OH)D_3 supplement, the serum concentration of IgM was enhanced at day 28, while one of the complement components (C3) was reduced at day 14. Compared to NC, diets containing 25(OH)D_3 led to a significant rise in the serum concentration of catalase (CAT) at day 28 and this might be taken as a hint towards an improved antioxidant capacity. CAT is regarded as a first-line defence antioxidant enzyme that is fundamental and vital to the overall defensive mechanisms in biological systems (Ighodaro & Akinloye, 2018). However, NC and NC + 25D demonstrated similar effects on the serum status of other oxidative enzymes like super oxidase dismutase (SOD), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px). In contrast to Vit D_3, the intestinal concentration of short-chain fatty acids (SCFA) significantly increased following the dietary intake of NC + 25D. For example, the abundance of Lachnospiraceae, which plays a crucial role in maintaining gut health, increased. The principal function of Lachnospiraceae is to break down the complex polysaccharide into short-chain fatty acids (SCFAs), including acetate, butyrate, and propionate (Martin-Gallausiaux *et al.*, 2021). SCFAs are involved in boosting immunity and reducing inflammatory reactions in the gut and other organs by i.e., acetyl-CoA synthesis, suppression of histone deacetylase (HDAC), signalling via G protein-coupled receptors (GPR), and metabolic integration (Kim, 2021). Thus, dietary inclusion of 25(OH)D_3 resulted in a

significant improvement in humoral as well as innate immunity; and gut immunity of the weaned piglets.

In another study, Zhang *et al*. (Zhang *et al.*, 2019) compared the immune competence of sows supplemented with Vit D_3 and 25(OH)D_3. Sows fed either source of Vit D demonstrated a similar effect on the level of Igs (IgA, IgG, and IgM) in colostrum. However, diet containing 25(OH)D_3 significantly increased IgG levels in milk on day 21 of lactation. The expression of genes concerning fatty acid metabolisms, i.g., *ACCa* (Acetyl-CoA carboxylase α) and *FAS* (Fatty-acid synthase) increased considerably in the mammary gland in response to 25(OH)D_3 enriched diets. Lipid metabolism is directly involved in the regulation of the immune system through the activation of M1 and M2 macrophages (Batista-Gonzalez *et al.*, 2020). The piglets from 25(OH)D_3-fed sows demonstrated a significant elevation in the concentration of butyrate in the cecal digesta compared to piglets from sows fed Vit D_3. The elevated concentration of butyrate following the breakdown of dietary fibres indicates improvement in bacterial metabolism (Lallès *et al.*, 2016; Louis *et al.*, 2007) which plays a crucial role in adaptive immune response via two specific pathways: firstly, it acts directly on monocyte-derived dendritic cells (DC) (Millard *et al.*, 2002; Nastasi *et al.*, 2015; Nastasi *et al.*, 2017), and secondly through its action on T lymphocytes. So, in contrast to Vit D_3, the dietary inclusion of 25(OH)D_3 during lactation period led to a significant increase in the concentration of milk IgG; and butyrate concentration in the cecal digesta of suckling piglets due to improved bacterial metabolism in the gut.

Konowalchuk *et al*. (Konowalchuk *et al.*, 2013) compared Vit D_3 and 25(OH)D_3 to evaluate the immune capacity by offering three different dietary treatments to 149 piglets weighing 5-7 kg for 14 or 21 days. The dietary supplements contained a baseline diet mixed with Vit D_3 (NC, 37.5 µg/kg), which served as a control diet. The second regimen included the control diet mixed with an additional 50 µg/kg of Vit D_3 (PC, NC + 50 µg/kg Vit D_3). The third one consisted of the control diet mixed with 25(OH)D_3 [HyD, NC + 50 µg/kg 25(OH)D_3]. Leucocytes are involved in both innate and humoral immune responses and play a critical role in fighting against infections and defence against foreign elements (Tigner *et al.*, 2020). The authors observed that supplementation of 25(OH)D_3 resulted in a significant increase in total blood

leucocyte (granulocytes, lymphocytes) counts in piglets, accompanied by a parallel increase in serum 25(OH)D_3. Compared to NC, piglets fed HyD demonstrated a significant improvement in the viability of total blood leukocytes and monocytes. The HyD-fed group also showed a significant increase in the viability of bronchoalveolar leukocytes compared to PC. However, unlike Vit D_3, 25(OH)D_3 did not only increase the number and viability of leucocytes but also significantly improved their phagocytic capacity. Overall, dietary supplementation of 25(OH)D_3 compared to Vit D_3 showed a significant positive influence on systemic blood and peripheral bronchoalveolar mucosal compartments, resulting in an increase in leukocyte count as well as the survival and phagocytic ability of the discrete leucocyte populations.

In the study of Upadhaya *et al*. (Upadhaya *et al.*, 2022), considerably lower serum levels of interleukin-6 (IL-6) at day 42 and higher serum levels of interleukin-1 (IL-1) at day 140 were observed in growing pigs supplemented with 25(OH)D_3 compared to Vit D_3. IL-6 is an anti-inflammatory cytokine that contributes to host defence via stimulation of the acute phase response, haematopoiesis, and immune responses (Tanaka *et al.*, 2014). IL-1 is a strong inflammatory cytokine involved in a wide range of immunological responses. It is predominantly produced by macrophages during defensive reactions to protect the body from infection and disease through inflammation and innate and adaptive immune responses (Fields *et al.*, 2019). The authors were unable to explain the underlying reason for this up- or down-regulation of the pro- and anti-inflammatory cytokines. According to Tanaka *et al*., the fluctuation of inflammatory cytokines is due to the maintenance of immune homeostasis (Alhassan Mohammed *et al.*, 2017). In conclusion, the supplementation of 25(OH)D_3 exhibited a more positive impact than Vit D_3 on pig health.

Meuter *et al*. (Meuter *et al.*, 2016) compared two different dietary sources of Vit D to observe their effects on humoral immunity by measuring the serum concentration of IgG and lysozyme. For this purpose, 160 post-weaned piglets (weaned at 21 days of age) were divided into two groups matched for maternal origin and body weight. Each group included 16 pens with 5 animals each and received the same diet, differing only in the source and doses of Vit D used. The piglets received either Vit D_3 (50 µg/kg) or 25(OH)D_3 [50 µg/kg] for 48 days. The serum concentration of 25(OH)D_3 increased considerably after dietary supplementation of

25(OH)D_3 compared to Vit D_3. The increased concentration of 25(OH)D_3 was associated with the positive modulation of parameters related to the humoral immune systems. In contrast to Vit D_3, dietary supplementation of 25(OH)D_3 led to a significant increase in the serum concentration of IgG and lysozyme. So, unlike Vit D_3, the dietary supplementation of 25(OH)D_3 significantly improved humoral immunity and strengthened the immune response of the piglets without compromising their health.

Thus, unlike Vit D_3, the dietary intake of 25(OH)D_3 significantly improves immune status of the body. In particular, the improvement of humoral immunity is reflected in the increased concentration of serum immunoglobulins and phagocytic capacity of the macrophages in piglets, which allows the animals to respond more effectively to potential health challenges. 25(OH)D_3 also positively affects the serum concentration of inflammatory cytokines to maintain immune homeostasis. Moreover, 25(OH)D_3 is superior over Vit D_3 in modulating the systemic and mucosal antimicrobial responses, suggested by increased numbers of leucocytes and their survival and phagocytic ability in blood and bronchoalveolar compartments. Compared to Vit D_3, the significant expression of genes related to fatty acid metabolism due to dietary intake of 25(OH)D_3 is also noteworthy. Fatty acids positively affect immune cell functions through a variety of complex mechanisms by improving phagocytosis, T-cell signalling, and antigen presentation capability (Calder, 2008). In contrast to Vit D_3, the role of 25(OH)D_3 in improving gut immunity is also noteworthy. Dietary supplementation of 25(OH)D_3 alters the gut microbiota and thus might promote specific metabolic processes, which plays a crucial role in maintaining intestinal health (Belkaid & Hand, 2014).

Table 9. Overview of the comparative studies between vitamin D3 (Vit D3) and 25(OH)D3, demonstrating the study designs, dietary doses, underlying conditions, and significant outputs of the experiments corresponding to immunity. Only the dietary doses (control and treatment) and the conditions that led to noticeable outcomes are listed.

Authors	n	DTP[§] (days)	Doses of vitamin D (µg/kg)		Conditions	Outcomes linked to immunity*	
			Vit D_3[¶]	25(OH)D_3		Vit D_3>25(OH)D_3	25(OH)D_3>Vit D_3
Zhang *et al*. (Zhang *et al.*, 2022)	144 piglets	1-28	62.5	50	Day 14 (low Ca-P)	↑C3[d] (serum)	-
					Day 28 (low Ca-P)	-	↑IgG[f], ↑IgA[g], ↑IgM[h], ↑CAT[i] (serum)
					-	-	↑SCFA[j] (feces)
Zhang *et al*. (Zhang *et al.*, 2019)	24 sows	ET[a]	50	50	-	-	↑*ACCA*[k], ↑*FAS*[l]; ↑IgG (milk), ↑Butyrate (cecum)
Konowalchuk *et al*. (Konowalchuk *et al.*, 2013)	149 piglets	14/21	50	50	Serum	-	↑TLC[m], ↑GLC[n], ↑LPC[o] (day 14); ↑TPLC[p]
					BA[b]	-	↑PLC[q] (day 21)
					Cell cultures	-	↑TMNC[r], TLC, TBLC[s] (viable; 24 h)
Upadhaya *et al*. (Upadhaya *et al.*, 2022)	48 sows	ET	50	50	-	-	-
	160 piglets	1-140	62.5	50	Day 42	↑IL-6[e] (serum)	-
			43.75	50	Day 140	-	↑IL-1[t] (serum)
Meuter *et al*. (Meuter *et al.*, 2016)	160 piglets	48	50	50	21 days (PW[c])	-	↑IgG, ↑Lysozyme (serum)
					48 days (PW)	-	↑IgG, ↑Lysozyme (serum)

*Asterisk indicates significant outcomes (p<0.05); [§]DTP, dietary treatment period; [¶]1 µg = 40 IU Vit D_3; [a]ET, explained on text; [b]BA, bronchoalveolar; [c]PW, post-weaning; [d]C3, complement component 3; [e]IL-6, interleukin 6; [f]IgG, immunoglobulin G; [g]IgA, immunoglobulin A; [h]IgM, immunoglobulin M; [i]CAT, catalase; [j]SCFA, short-chain fatty acids; [k]*ACCA, acetyl-CoA carboxylase* α; [l]*FAS*, *fatty-acid synthase*; [m]TLC, total leucocyte count; [n]GLC, granulocytes; [o]LPC, lymphocytes; [p]TPLC, total phagocytic leucocytes; [q]PLC, phagocytic leucocytes; [r]TMNC, total monocytes; [s]TBLC, total bronchoalveolar leucocytes; [t]IL-1, Interleukin 1.

5.15. Comparative performance of vitamin D_3 and 25(OH)D_3 on bone development

Vit D regulates bone and mineral metabolism. This section reviews the available pig studies highlighting the efficacy of Vit D_3 and 25(OH)D_3 on mineral status and bone development (Table 3).

Zhang *et al*. (Zhang *et al.*, 2022) compared the efficacy of Vit D_3 and 25(OH)D_3 in improving serum mineral status. In contrast to Vit D_3 (NC), the dietary inclusion of 25(OH)D_3 [NC + 25D] significantly normalized the serum levels of Ca, bone-specific alkaline phosphatase (BALP), and osteocalcin (OC) in low Ca-P fed pigs depending on the duration of the dietary supplementation. The increased serum concentration of Ca indicates the compensatory efforts of 25(OH)D_3 to maintain Ca-P homeostasis in pigs fed with low Ca-P diets. Serum level of BALP (synthesized by osteoblasts) positively correlates with bone formation (Konukoglu, 2019). OC is also considered as a serum marker of osteoblastic bone formation which acts in the bone matrix to regulate bone mineralization via resorption (Zoch *et al.*, 2016). Unlike OC, the supplementation of piglets with both forms of Vit D exhibited a similar impact on the serum status of bone resorption markers like tartrate-resistant acid phosphatase (TRAP) and pyridinoline (PYD). TRAPs perform bone resorption by catalysing the hydrolysis of various phosphates and anhydrides in an acidic environment (Oddie *et al.*, 2000) and PYD is synthesized by the reaction at the side chain of the collagen residues during trimerization in skeletal development (Kline *et al.*, 2017). Overall, 25(OH)D_3-enriched diets proved to be more effective in improving bone integrity in low Ca-P-fed pigs compared with Vit D_3, as indicated by increased serum bone formation biomarkers to balance bone mineralization and its homeostasis.

Similar to the study of Zhang *et al*. (Zhang *et al.*, 2022), Zhao *et al*. (Zhao *et al.*, 2022) also demonstrated the superiority of dietary 25(OH)D_3 over Vit D_3 in restoring bone mineral status in pigs supplemented with low Ca-P. Unlike Vit D_3 (NC), the serum level of Ca increased significantly in piglets (10-20 kg) after dietary ingestion of 25(OH)D_3 [HyD]. Serum Ca needs to be regulated in narrow physiological ranges and serves as an important marker of bone turnover and osteoblast functions (Åkesson *et al.*, 1998; Kuo & Chen, 2017). Its elevation in

piglets fed low Ca-P indicates the body's compensatory effort to balance the level of Ca and P to achieve optimal mineralization. However, no major dietary effect on the serum status of Ca was observed in 6-10 kg piglets. Dietary supplementation of Vit D had also no discernible effect on the serum levels of P. This could be due to the body's ability to maintain serum P levels in pigs fed low P (Gerlinger *et al.*, 2021; Hasan *et al.*, 2022). Thus, the supplementation of dietary 25(OH)D_3 led to a significant impact on increasing the serum Ca to improve the mineral homeostasis in pigs receiving low Ca-P diet.

Doherty *et al*. (O'Doherty *et al.*, 2010) performed a similar experiment, but with a different dosage of 25(OH)D_3 compared to the aforementioned studies. For the mineral balance study, 24 finishing boars (13 weeks of age) with an initial live weight of 42 kg were used and fed the following diets containing low P (T1, 50 µg/kg Vit D_3), T1 + Phytase (T2, 50 µg/kg Vit D_3 + 750 IU/kg phytase), T1 + 25(OH)D_3 [T3, 25 µg/kg Vit D_3 + 25 µg/kg 25(OH)D_3], or T1 + Phytase + 25(OH)D_3 [T4, 25 µg/kg Vit D_3 + 750 IU/kg Phytase + 25 µg/kg 25(OH)D_3] for 66 days. Accordingly, in contrast to Vit D_3 (T1), dietary inclusion of 25(OH)D_3 [T3] significantly improved Ca digestibility, and retention in low Ca-P fed pigs, suggesting the same remedial endeavour as reported in the above studies to balance mineral homeostasis. Ca digestibility and P retention are closely associated with bone mineralization and growth performance (Lautrou *et al.*, 2021). However, the dietary intervention did not affect P metabolism, serum mineral (Ca, P) status, bone ash, and bone strength.

Weber *et al*. (Weber *et al.*, 2014) compared the effectiveness of Vit D_3 and 25(OH)D_3 to determine the plasma mineral status following the supplementation of sows with basal diets containing Vit D_3 and 25(OH)D_3. Dietary intake of Vit D_3 and 25(OH)D_3 did not consistently affect the plasma status of Ca, P, and OC associated with skeletal development and resorption throughout the reproduction cycle. However, the concentration of CrossLaps (β-CTx) increased significantly before parturition in both treatments and was the highest at the end of lactation. This observation suggests the mobilization of Ca from the sow's skeleton to the developing foetuses and piglets for their optimum bone development (Bonde *et al.*, 1995). Thus, in healthy pigs, Vit D_3 and 25(OH)D_3 resulted in similar impacts on the concentrations of bone mineralization markers.

In the study of Witschi *et al*. (Witschi *et al.*, 2011), 39 sows (13 in each treatment) were assigned to Vit D_3 and 25(OH)D_3 supplementation from the day of mating until day 21 of lactation. Rosenberg *et al*. (von Rosenberg *et al.*, 2016) used six weeks old piglets (n = 40) supplemented with Vit D_3 (control, 50 µg/kg) and 25(OH)D_3 [50 or 250, or 500 µg/kg] for 42 days. Witschi *et al*. and Rosenberg *et al*. reported no significant difference in ash%, weight, length, mineral content, and mineral density of bone in response to the dietary intakes of Vit D_3 and 25(OH)D_3. However, low dietary intake of Vit D_3 (200 IU) was associated with reduced bone-breaking strength, cortical bone mineral content, and density at the tibial midshaft of the piglets (Witschi *et al.*, 2011). Thus, both forms of Vit D implied a similar effect on bone formation in healthy pigs.

In summary, 25(OH)D_3 outperforms Vit D_3 to improve the serum mineral status associated with bone mineralization in pigs fed low Ca-P diet. Unlike Vit D_3, the serum concentrations of Ca, BALP, and OC increase significantly in pigs receiving basal diets containing 25(OH)D_3 and low Ca-P. Therefore, the upregulation of serum markers associated with bone turnover indicates a noticeable positive influence of 25(OH)D_3 to balance mineral homeostasis in pigs experiencing Ca-P deficiency. The comparative efficacy of Vit D_3 and 25(OH)D_3 on mineralization in healthy pigs appears to be similar.

Table 10. Overview of the comparative studies between vitamin D3 (Vit D3) and 25(OH)D3, demonstrating the study designs, dietary doses, underlying conditions, and significant outputs of the experiments corresponding to skeletal development. Only the dietary doses (control and treatment) and the conditions that led to noticeable outcomes are listed.

Authors	n	DTP[§] (days)	Doses of vitamin D (µg/kg)		Conditions	Outcomes linked to bone development*	
			Vit D_3[¶]	25(OH)D_3		Vit D_3>25(OH)D_3	25(OH)D_3>Vit D_3
Zhang *et al*. (Zhang *et al.*, 2022)	144 piglets	14/28	62.5	50	14 days	-	↑Ca (serum)
					28 days	-	↑Ca, ↑BALP[e], ↑OC[f] (serum)
Zhao *et al*. (Zhao *et al.*, 2022)	240 sows	-	50	50	6-10 kg	-	-
					10-20 kg	-	↑Ca (serum)
Doherty *et al*. (O'Doherty *et al.*, 2010)	24 boars	66	50	25 (Vit D_3) + 25	-	↑Faecal Ca[d]	↑Ca digestibility, ↑Ca retention
Weber *et al*. (Weber *et al.*, 2014)	227 sows	-	50	50	IN[b]	↑CrossLaps (plasma)	-
					PI[c]	-	↑CrossLaps (plasma)
Witschi *et al*. (Witschi *et al.*, 2011)	39 sows	ET[a]	50	50	-	-	-
Rosenberg *et al*. (von Rosenberg *et al.*, 2016)	48 piglets	42	50	50	-	-	-

*Asterisk indicates significant outcomes (p<0.05); [§]DTP, dietary treatment period; [¶]1 µg = 40 IU Vit D_3; [a]ET, explained on text; [b]IN, insemination; [c]PI, post-insemination; [d]Ca, calcium; [e]BALP, bone-specific alkaline phosphatase; [f]OC, osteocalcin.

5.16. Implications for further research

Vitamin D metabolism is attributed to endogenous adaptive mechanisms for maintaining mineral homeostasis which is critical for animal health. In particular, potential long-term consequences due to nutritional strategies in the early life stages need to be addressed as summarized in the concept of nutritional programming, thereby inducing resource-efficient phenotypes. However, evaluation must also consider a range of effects in peripheral tissues to account for unintended metabolic effects (Figure 1). With regard to alternative housing conditions of pigs, such as exposure to natural sunlight, dietary Vit D requirements and the physiological synergy with endogenous syntheses need to be investigated. Identifying the complex genetic architecture of the Vit D system is key to improving mineral efficiency and animal health aspects as a basis for developing new breeding criteria.

5.17. Conclusions

In sows, dietary supplementation of Vit D_3 and 25(OH)D_3 results in similar reproductive outcomes. The growth performance of piglets and sows fed 25(OH)D_3 is significantly better than those fed Vit D_3. The superiority of 25(OH)D_3 over Vit D_3 is evident in enhancing innate and humoral immunity. Improved bone mineralization in pigs supplemented with 25(OH)D_3 on diets low in calcium and phosphorus provide an option to balance animal welfare and resource efficiency. Thus, 25(OH)D_3 is a promising and potential alternative to Vit D_3 for promoting growth, immunity, and bone development in pigs.

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5.19. Curriculum vitae

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5.20. Declaration of Independence

By signing, I hereby certify that this thesis has been written independently and no other sources or aids have been used other than those indicated by me. I have marked those passages as taken verbatim and in terms of content from the sources that I have used.

Rostock, 11 September 2024 ----------

Maruf Hasan