ANALYSIS OF FIBROBLAST GROWTH FACTOR-23 (FGF23) FUNCTIONS ON MINERAL BONE HOMEOSTASIS IN 3-MONTH-OLD VITAMIN D RECEPTOR-ABLATED MICE

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THESIS
To obtain the dignity of MAGISTRA MEDICINAE VETERINARIAE

by
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**Abbreviations**

ALB    albumin
ALP    alkaline phosphatase activity
AST    aspartate aminotransferase activity
ATP    adenosine triphosphate
B.Ar/T.Ar  bone area/trabecular area
B.Pm/T.Ar  bone perimeter/tissue area
BILT   serum bilirubin
BMD    bone mineral density
BS/TV  bone surface/tissue volume
BW     body weight
CA     total calcium
Ca²⁺   ionized blood calcium
CAU    urine calcium
CHOL   cholesterol
CKD    chronic kidney disease
CKL    creatine kinase activity
Cl-U   urine chloride
CL     serum chloride
CRE    creatinine
CRU    urine creatinine
ELISA  enzyme linked immunoabsorbant assay
Fgf23³⁻/⁰⁺[VDRΔ]  Fgf23/ vitamin D receptor compound mutants
Fgf23⁺⁻/⁰⁺[VDRΔ]  heterozygote compound mutants
Fgf23  fibroblast growth factor 23
FV3mo  Fgf23 and vitamin D receptor ablation in 3-month-old mice
IU     international units
K-U    urine potassium
K      serum potassium
K⁺     blood potassium
MAPK  mitogen-activated protein kinase
MMA  methylmethacrylate
N.Tb/B.Ar  trabecular number/bone area
N.Tb/T.Ar  trabecular number/tissue area
Na-U  urine sodium
NA  serum sodium
Na+  blood sodium
NaHCO₃  sodium hydrogen carbonate
p  probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true
PHOS  total phosphate
PHOU  urine phosphate
PKA  adenylyl cyclase protein kinase A
PKC  phospholipase C-protein kinase C
pQCT  peripheral quantitative computed tomography
PTH  parathyroid hormone
PTH1R  PTH/PTHrP type 1 receptor
PTHrP  parathyroid hormone-related protein
RD  rescue diet
s. c.  subcutaneous
SEM  standard error of the mean
Tb.Ar  trabecular area
Tb.N  trabecular number
Tb.Sp  trabecular separation
Tb.Th  trabecular thickness
Tb.Wi  trabecular width
TRIGL  triglycerides
UREL  serum urea
VDR  vitamin D receptor
VDR⁺/Δ  heterozygous VDR-ablated mice
VDR⁻/⁻  vitamin D receptor-ablated mutants
vs.  

**WT**  

**XCT**
1. Introduction

The calcium and phosphate balance is of crucial physiological importance for the maintenance of mineral homeostasis. Constant level of these two electrolytes is preserved in the organism by several essential molecular mechanisms, alterations of which underlie numerous pathophysiological disorders (GUTIERREZ et al. 2008; COVIC et al. 2009; PEACOCK 2010; FOLEY 2009; LEVIN et al. 2007). This balance is controlled by a wide range of hormones keeping the extracellular calcium and phosphate concentration at a constant level.

Calcium is an electrolyte involved in a great variety of functions, such as intracellular regulation of muscle contraction, synaptic transmission and control of cellular membrane excitability. It plays a part in the blood clotting process and has an essential role in regulation of bone mineralization (PEACOCK 2010; CLAPHAM 2007; BRONNER 2009). Under physiological conditions calcium is bound to a variety of proteins and anions in the extra- and intracellular space (CLAPHAM 2007).

Since diet is the only source of calcium, dietary calcium content is essential for the body. With high calcium intake it is adsorbed via passive paracellular diffusion in the jejunum and ileum. However with low calcium intake, active transcellular calcium transport in the duodenum, controlled by the active form of vitamin D (1, 25(OH)₂D₃), predominates. Furthermore both passive and active processes for calcium resorption are active in the large intestine. Importantly, serum calcium balance is also tightly controlled through renal filtration by an integrated hormone-controlled system that involves 1, 25(OH)₂D₃ and parathyroid hormone (PTH). Serum calcium levels are also affected by retention and mobilisation in bone and excretion via the sweat glands (PEACOCK 2010; CLAPHAM 2007; BRONNER 2009).

In contrast, phosphate is a fundamental part of a variety of important compounds of e. g. the cellular energy metabolism, nucleic acids, phospholipids and the phosphate buffer system. The regulation of serum phosphate takes mainly place at the proximal tubular system in the
kidneys in order to balance the intestinal phosphate absorption. The phosphate transport through the proximal tubular epithelial cells is a sodium dependent co-transport along the sodium gradient towards the lower intracellular concentration (GAASBEEK et al. 2005; RENKEMA et al. 2008; RAZZAQUE and LANSKE 2007).

 Secreted PTH is released from the parathyroid glands in response to a low extracellular calcium concentration respectively high phosphate levels. PTH binds to its receptor and activates multiple signalling pathways leading to restoration of serum phosphate and calcium levels, including an increase of calcium concentration through bone degradation and stimulation of the distal tubular renal resorption at the same time as inhibition of renal resorption of bicarbonate and phosphate (RENKEMA et al. 2008; RAZZAQUE and LANSKE 2007). PTH mainly functions through its PTH/PTHrP receptor (PTH1R) by activating adenylyl cyclase - protein kinase A (PKA) and phospholipase C - protein kinase C (PKC) signalling pathways (ABOU-SAMRA et al. 1992).

In bone PTH has a dual role regarding bone metabolism. In studies demonstrating the constant in vivo PTH infusion in mice, the catabolic effect of PTH was observed resulting in bone loss. Constant PTH shifts gene expression patterns in osteoblasts in order to engage in the process of bone resorption by regulating osteoclasts (LEE and PARTRIDGE 2009). Intermittent injections of PTH, however, cause an anabolic response in bone, leading to an increased bone mineral density (BMD) (LI et al. 2007). Although the classical catabolic effect is well studied, the anabolic role of PTH in bone still requires further investigations.

In the kidney, the primary organ for regulation of ion homeostasis, PTH stimulates calcium resorption by activating specific ion calcium channels in the distal tubules of the nephron. It is also involved in the regulation of the phosphate excretion by regulating sodium-coupled cotransporters in the proximal tubule. In addition, PTH enhances intestinal calcium and phosphate absorption indirectly by inducing the conversion of 25-hydroxyvitamine D to 1, 25(OH)dihydroxycholecalciferol (vitamin D hormone) in the kidneys (LEE and PARTRIDGE 2009; HEALY et al. 2005).
Vitamin D is a cholesterol derivative, assimilated from nutritional origin or synthesized in liver and skin. It is then transformed in the kidneys into its active form, a process that is regulated by extracellular calcium and phosphate levels, by hormones, and by the end product itself. The vitamin D hormone stimulates the enteral absorption and renal resorption of calcium by active ATP-dependent transport beside a constant vitamin D-independent concentration gradient-dependent transport.

Vitamin D hormone acts through binding to the vitamin D receptor (VDR), which has been detected in nearly every human tissue. This demonstrates its wide spectrum of activities besides the role in bone homeostasis. Vitamin D is known to inhibit PTH-induced bone resorption (reviewed in TATSUO et al. 2003; BOUILLON et al. 2008).

It was demonstrated in earlier studies that mice with a non-functioning VDR (VDR\(\Delta/\Delta\)) show a wide spectrum of changes in growth, bone formation, as well as secondary hyperparathyroidism and alopecia on a normal mouse diet after weaning compared to their wild type (WT) littermates (ERBEN et al. 2002).

VDR\(\Delta/\Delta\) mice on a normal mouse diet show normal development until weaning at the age of 3 weeks; thereafter they show reduced body weight throughout their life (BOUILLON et al 2008; ERBEN et al. 2002). During the rapid growth phase, secondary hyperparathyroidism was observed accompanied by hypocalcaemia and high serum vitamin D hormone levels. Moreover, 10-week-old mice showed histological signs of rickets (ERBEN et al. 2002). At about 6-8 weeks the animals started to show progressive alopecia, up to almost complete hair loss at about 4 months of age. A diet of high calcium, phosphorus, and lactose (rescue diet) normalized blood calcium and serum PTH levels in these mice (ERBEN et al. 2002).

Another central calcium- and phosphate-regulating hormone is fibroblast growth factor 23 (Fgf23). Fgf23 is the last member of the FGF family, identified by WHITE et al. 2000. FGFs are polypeptides, with a variety of biological activities in multiple metabolic and developmental processes (SHIMADA et al. 2004).
*Fgf23* is synthesized predominantly by osteocytes and is an important regulator of the vitamin D hormone metabolism and phosphate homeostasis. A high *Fgf23* blood level leads to hypophosphatemia, reduced serum vitamin D hormone concentration and consequent hyperparathyroidism (COZZOLINO and MAZZAFERRO 2010). Other studies have found *Fgf23* acting on the parathyroid glands to decrease PTH expression (SHIMADA et al. 2004; GALITZER et al. 2010). A decrease in the *Fgf23* blood level causes hyperphosphatemia, increased vitamin D blood hormone concentration and PTH suppression (COZZOLINO and MAZZAFERRO 2010). Recent studies have shown a link between high *Fgf23* blood level and progression of chronic kidney disease (CKD) and higher mortality rates in haemodialysis patients; but the clinical relevance of high *Fgf23* levels still remains unknown (GUTIERREZ et al. 2008; GALITZER et al. 2010; VAN HUSEN et al. 2010).

To gain further understanding of *Fgf23*, its way of acting and its function, studies with *Fgf23* knock-out mice have been made. Four-week-old *Fgf23* knock-out mice developed a significant increase in serum phosphate, with no significant changes in serum PTH. They showed impaired bone mineralization, ectopic calcifications and organ atrophy associated with high vitamin D hormone serum levels. The mutant mice remained significant smaller in size than wild-type controls. The maximum age was reached with 13 weeks (RAZZAAQUE et al. 2005; HESSE et al. 2007).

The exact mechanisms of *Fgf23* action and regulation of its production is not fully understood at present (HESSE et al. 2007). Experiments with *Fgf23*+/−/VDRΔ/Δ compound mutant mice, in which the vitamin D signalling pathway was disrupted, suggested that the alterations in mineral and carbohydrate metabolism, as well as the premature aging present in *Fgf23*−/− mice, depend on an intact signalling through the VDR (HESSE et al. 2007). The latter study suggested that the main physiological function of *Fgf23* is its suppressive action on renal 1α-hydroxylase activity, which converts 25-dihydroxycholecalciferol into its bioactive form 1, 25-dihydroxycholecalciferol (vitamin D hormone) (HESSE et al. 2007). However, these experiments were all performed in fast-growing 4- to 6-week-old *Fgf23*−/− mice (RAZZAAQUE et al. 2005; HESSE et al. 2007).
In 9-month-old \( Fgf23^{-/-}/VDR^{\Delta/\Delta} \) compound mutant mice organ atrophy, lung emphysema, and ectopic tissue or vascular calcifications were absent in compound mutants, as compared to wild type and \( VDR^{\Delta/\Delta} \) mice (STREICHER et al. 2012). Due to the short life span in \( Fgf23^{-/-} \) mice, phenotypic analyses in older \( Fgf23^{-/-} \) mice are not possible. Additionally, body weight, pancreatic beta cell volume, insulin secretory capacity, insulin tolerance, glucose tolerance, epididymal and retroperitoneal fat mass as well as serum triglycerides and cholesterol did not show differences between vitamin D receptor and compound mutants. These data suggest \( Fgf23 \) has no molecular vitamin D-independent role in aging or energy metabolism in mice (HESSE et al. 2007).

To be able to bind to its receptor \( Fgf23 \) requires Klotho, a transmembrane protein acting as a co-receptor of \( Fgf \) receptor 1c (FGFR1c) (KUROSU et al. 2006). Klotho was first identified as a gene, associated with the premature aging-like phenotypes characterized again by a short lifespan (KUROSU et al. 2005). Beside the membrane form, a soluble form has been detected in blood, urine and cerebrospinal fluid, described to have an independent hormone activity (KUROSU et al. 2005).

Klotho ablated mice showed similar phenotypic changes compared to \( Fgf23 \) knock-outs, characterized through shortened life span, growth retardation, ectopic calcification and muscle and skin atrophy as well as increased vitamin D hormone level (HESSE et al. 2007; ANOUR et al. 2012). The membrane form has been found to play a key role in the regulation of calcium homeostasis, and leading to release of PTH (IMURA et al. 2007).

Aim of this study was to analyse mineral and bone homeostasis in 3-month-old wild type (WT), vitamin D hormone receptor-ablated (VDR\(^{\Delta/\Delta}\)) and \( Fgf23^{-/-}/VDR^{\Delta/\Delta} \) compound mutant mice in comparison to recent studies with 4-week-old \( Fgf23^{-/-} \) mutants and 9-month-old WT, \( VDR^{\Delta/\Delta} \), and \( Fgf23^{-/-}/VDR^{\Delta/\Delta} \) mice (HESSE et al. 2007; STREICHER et al. 2012). The study is based on the hypothesis, that 3-month-old \( Fgf23^{-/-}/VDR^{\Delta/\Delta} \) compound mutant mice will represent the same signs of secondary hyperparathyroidism compared to \( VDR^{\Delta/\Delta} \) similar to the observations made in the former study with 9-month-old mutants (unpublished data).
2. Materials and methods

2.1. Animals

All animal procedures were approved by the Ethical Committees of the University of Veterinary Medicine Vienna. Heterozygous VDR$^{+/\Delta}$ (ERBEN et al. 2002) were mated with heterozygous $Fg/23^{+/\Delta}$ (SITARA et al. 2004) (Lexicon Genetics, Mutant Mouse Regional Resource Centers, University of California, Davis, CA, USA) mutant mice to generate double heterozygous animals. VDR$^{+/\Delta}/Fg/23^{+/\Delta}$ mutant mice on C57BL/6 background were interbred to generate WT, VDR$^{\Delta\Delta}$ and compound $Fg/23^{\Delta\Delta}$/$VDR^{\Delta\Delta}$ mutant mice. Genotyping of the mice was performed by multiplex PCR using genomic DNA extracted from tail as described (HESSE et al. 2007). The mice were kept at 24°C with a 12 hour/12 hour light/dark cycle, and were allowed free access to tap water and rescue diet (RD) (Ssniff, Soest, Germany) containing 2.0% calcium, 1.25% phosphorus, 20% lactose, and 600 IU vitamin D per kilogram from 16 days of age on. It has been shown that this diet normalizes mineral homeostasis in VDR ablated mice (PANDA et al. 2004; AMLING et al. 1999; LI et al. 1998).

Urine was collected in metabolism cages before necropsy.

To measure the bone formation rate by histomorphometric analysis, animals were injected (s.c.) with calcein (20 mg/kg BW in 1.4% NaHCO$_3$) 4 and 2 days in advance of necropsy.

2.2. Necropsy

At necropsy, the mice were first anaesthetized with an intraperitoneal injection of ketamine/xyazine (67/7 mg/kg). To collect blood for examination of serum parameters, the animals were exsanguinated from the abdominal Vena cava. Body weight, heart weight and pancreas weight were taken at necropsy.
2.3. Serum and urine biochemistry

Ionized calcium (Ca²⁺), sodium (Na⁺) and potassium (K⁺) were analyzed by the AVL 9180 electrolyt analyzer (Roche Diagnostics, Mannheim, Germany).

Serum albumin (ALB), total bilirubin (BILT), alkaline phosphatase activity (ALP), aspartate aminotransferase activity (AST), total calcium (CA), phosphorus (PHOS), creatinine kinase activity (CKL), creatinine (CRE), urea (UREL), triglycerides (TRIGL), cholesterol (CHOL), total potassium (K), total sodium (NA) and chloride (CL) as well as urine calcium (CAU), phosphate (PHOU), potassium (K-U), sodium (NA-U), chloride (CL-U) and creatinine (CRU) were analysed by the cobas c 111 ISE analyser (Roche Diagnostics, Mannheim, Germany).

Quantification of PTH was performed using human PTH enzyme linked immunosorbant assay (ELISA, Immutopics, San Clemente, California, USA).

2.4. Bone mineral density and histomorphometry

Bone mineral density (BMD) of the tibial metaphysis and shaft were analyzed by peripheral quantitative computed tomography (pQCT) using a XCT Research M+ pQCT machine (Stratec Medizintechnik).

Cancellous bone histomorphometry in the distal femoral metaphysis as well as cortical bone histomorphometry in the femoral shaft have been performed using OsteoMeasure 3.0 (OsteoMetrics) and AxioVision 4.6 (C. Zeiss) software.

After routine dehydration steps without decalcification, the bone samples were embedded in methylmethacrylate (MMA). A modified MMA embedding suitable for histomorphometry, histochemistry and immunohistochemistry was used (ERBEN 1997). After the embedding procedure the bone samples were trimmed and cut into 3 μm thick sections. They were
covered with Fluoromount to visualize the incorporated calcein. Other sections were stained for mineralisation of bone trabeculae with von Kossa/McNeal staining procedure.

2.5. Organ histology

For organ histology kidney, spleen, liver and lung were fixed in 4% paraformaldehyd (PFA) over night. They were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin according to standard procedures.

2.6. Statistical analyses

Statistics were performed by using PRISM 5.0 (Graph Pad Software, La Jolla, CA). The data were analyzed by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. \( P \leq 0.05 \) was considered significant and all values are means ± standard error of the mean (SEM) of 6 to 8 animals in each group.
3. **Results**

3.1. Changes in phenotype

All animals included in the present study were 3 months old. According to an earlier study (ERBEN et al. 2002) VDR\(^{Δ/Δ}\) mutants demonstrate severely progressing alopecia starting from the age of 6 weeks. Three-month-old \(Fgf23^{-/-}/VDR^{Δ/Δ}\) compound mutants also showed progressive alopecia. This phenomenon can be explained by VDR ablation (Fig. 1). Neither body weight nor heart weight, heart weight/body weight ratio and pancreatic weight showed significant differences in \(Fgf23^{-/-}/VDR^{Δ/Δ}\) compound mutants as compared to the VDR\(^{Δ/Δ}\) and WT animals. This was true for both the female and male animals (Fig. 2). These data indicate that at an age of 3 months a lack of \(Fgf23\) does not cause any gross phenotypic changes in the VDR ablated mice.

![FIG. 1. Comparison of the 3 genotypes at an age of 3 months. WT, VDR\(^{Δ/Δ}\) and \(Fgf23^{-/-}/VDR^{Δ/Δ}\) (from left to right)](image-url)
FIG. 2. Comparison of body weight, heart weight, heart/body rate and pancreatic weight, given as g and %, in male and female WT, VDRΔ/Δ and Fgf23+/-/VDR Δ/Δ; all on RD.
Each data column represents the mean ± SEM of six to eight mice each group.
3.2. Histological organ analysis

To monitor histopathological changes in the inner organs, kidney, spleen, liver and lung were fixed in 4% paraformaldehyde (PFA) overnight and embedded in paraffin. Subsequently, organs were sectioned at 5 μm and stained with hematoxylin and eosin according to standard procedures. Microscopic evaluation of the stained sections were performed for 6 sections/organ/animal. In each section 4 representative images were recorded. The morphological appearance of the stained organ sections was compared between the animal groups.

No morphological abnormalities in lung, spleen, liver and kidneys were observed in Fgf23^−/−/VDR^Δ/Δ compound mutants compared to the VDR^Δ/Δ and WT animals (Figs. 3, 4).

**FIG. 3.** H&E stained paraffin sections of lung and spleen of WT, VDR^Δ/Δ and Fgf23^−/−/VDR^Δ/Δ mutants.
3.3 Biochemical parameters of organ metabolism and function

To monitor serum parameters of organ metabolism and function serum triglycerides (TRIGL), cholesterol (CHOL), albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase activity (AST), creatine kinase activity (CKL), bilirubin (BILT), creatinine (CREA), and urea levels (UREL) serum levels were measured.

In the present study, no significant difference in serum TRIGL in Fgf23−/−/VDRΔ/Δ compound mutants was observed compared to the VDRΔ/Δ and WT animals. This was true for both male and female animals (Fig. 5). Cholesterol showed a significantly lower level in male VDRΔ/Δ mutant mice (1.58 ± 0.07 mmol/L, n=6) and Fgf23−/−/VDRΔ/Δ compound mutants (1.56 ± 0.07 mmol/L, n=8) compared to the WT (1.88 ± 0.07 mM, n=6) animals. A similar trend (statistically not significant) was observed in the female animals, with Fgf23−/−/VDRΔ/Δ (1.28 ± 0.08 mmol/L, n=6), VDRΔ/Δ (1.25 ± 0.10 mmol/L, n=6) and WT (1.54 ± 0.08 mmol/L, n=7)
Serum ALB, ALP, AST, and serum creatine kinase activity (CKL) did not differ between the groups (Fig. 6 and Fig. 7).

Comparable values were also observed for serum creatinine (CREA) and urea levels (UREL), parameters for renal function (JOHNSON et al. 2012). Both showed no significant difference between the groups (Fig. 7).

Bilirubin (BILT) is the ultimate breakdown product of haeme and serves as a diagnostic marker of liver and blood disorders (FEVERY 2008). In this study, a significant increase of serum bilirubin in both, male Fgf23<sup>-/-</sup>/VDR<sup>Δ/Δ</sup> compound mutants (1.93 ± 0.13 μmol/L, n=8) and VDR<sup>Δ/Δ</sup> ablated mice (1.93 ± 0.16 μmol/L, n=6), compared to WT animals (1.28 ± 0.06 μmol/L, n=7), was found. No significant differences, however, were observed between the
Fig. 6. Comparison of ALB, ALP and AST, given as g/L or U/L in male and female WT, VDRΔ/Δ and Fgf23/-/VDRΔ/Δ, all on RD.
Each data column represents the mean ± SEM of six to eight mice each group.

Fgf23/-/VDRΔ/Δ compound mutants and VDRΔ/Δ ablated mice. In the female groups no significant changes in the serum bilirubin level occurred (1.61 ± 0.13 μmol/L, n=7 in WT; 1.76 ± 0.20 μmol/L, n=5 in VDRΔ/Δ; 1.83 ± 0.13 μmol/L, n=6 in Fgf23/-/VDRΔ/Δ) (Fig. 7).

These data indicate that at 3 months of age Fgf23/-/VDRΔ/Δ compound mutants have no changes in organ function as compared to the VDR ablated mice.
FIG. 7. Comparison of BILT, CKL, CREA, UREL, given as mmol/L or U/L in male and female WT, VDR⁻/⁻ and Fgf23⁻/⁻/VDR⁻/⁻, all on RD.

Each data column represents the mean ± SEM of five to eight mice in each group.
*p < 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.
3.4. Evaluation of the mineral homeostasis in blood

Ionized calcium (Fig. 8) was significantly decreased in blood of both, male and female Fgf23⁻/⁻/VDRΔΔ compound mutants compared to VDRΔΔ ablated mice and WT. No significant difference in ionized calcium was found between Fgf23⁻/⁻/VDRΔΔ compound mutants and VDRΔΔ ablated mice. The blood levels of ionized calcium were as following: WT (1.3 ± 0.02 mM, n=6 in male and 1.3 ± 0.02 mM, n=8 in female), VDRΔΔ (1.24 ± 0.04 mM, n=6 in male and 1.28 ± 0.04, n=5 in female) and Fgf23⁻/⁻/VDRΔΔ (1.22 ± 0.02 mM, n=8 in male and 1.14 ± 0.05, n=5 in female).

The blood potassium and sodium showed no significant changes between the different genotypes for both, male and female animal groups (Fig. 8).

**FIG. 8.** Comparison of ionized Ca²⁺, K⁺ and Na⁺ given as mM in male and female WT, VDRΔΔ and Fgf23⁻/⁻/VDRΔΔ compound mutants, all on RD.
Each data column represents the mean ± SEM of five to eight mice each group.
*p<0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.
The total serum calcium was significantly reduced in VDR$^{Δ/Δ}$ mutant mice and $Fgf23^{-/-}/VDR^{Δ/Δ}$ compound mutants versus WT in male animals. In female mice a significant decrease in serum calcium was only found in $Fgf23^{-/-}/VDR^{Δ/Δ}$ compound mutants compared to the WT animals. The mean calcium values in WT were $2.22 \pm 0.05 \text{ mmol/L}$, $n=6$ in male and $2.29 \pm 0.04 \text{ mmol/L}$, $n=7$ in female, in $VDR^{Δ/Δ}$ $2.09 \pm 0.03 \text{ mmol/L}$, $n=6$ in male and $2.18 \pm 0.04 \text{ mmol/L}$, $n=6$ in female and in $Fgf23^{-/-}/VDR^{Δ/Δ}$ $2.07 \pm 0.04 \text{ mmol/L}$, $n=8$ in male and $2.10 \pm 0.03 \text{ mmol/L}$, $n=6$ in female (Fig. 9).

Serum potassium, sodium and chloride levels were not significantly different between the genotypes for both male and female groups (Fig. 9).

The serum phosphate level was significantly increased in male $Fgf23^{-/-}/VDR^{Δ/Δ}$ compound mutants compared to VDR$^{Δ/Δ}$ mutants ($3.17 \pm 0.20 \text{ mmol/L}$, $n=6$ in WT; $2.49 \pm 0.18 \text{ mmol/L}$, $n=6$ in VDR$^{Δ/Δ}$ and $3.64 \pm 0.28 \text{ mmol/L}$, $n=8$ in $Fgf23^{-/-}/VDR^{Δ/Δ}$). In the female $Fgf23^{-/-}/VDR^{Δ/Δ}$ compound mutants serum phosphate was not significantly increased in comparison to VDR$^{Δ/Δ}$ mutants and WT animals ($3.39 \pm 0.25 \text{ mmol/L}$, $n=7$ in WT; $3.44 \pm 0.39 \text{ mmol/L}$, $n=6$ in VDR$^{Δ/Δ}$ and $3.95 \pm 0.27 \text{ mmol/L}$, $n=6$ in $Fgf23^{-/-}/VDR^{Δ/Δ}$) (Fig.9).
**FIG. 9.** Comparison of total serum Ca\(^{2+}\), K\(^{+}\) and Na\(^{+}\), Cl and P, given as mmol/L in male and female WT, VDR\(^{\Delta/\Delta}\) mutant mice and Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants, all on RD. Each data column represents the mean ± SEM of six to eight mice in each group.

* p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

# p< 0.05 vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.
Interestingly, the serum level of the parathyroid hormone (PTH) was significantly increased in male and female Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants compared to VDR\(^{\Delta/\Delta}\) mutants and WT mice. No significant difference in serum PTH level was detected between the VDR\(^{\Delta/\Delta}\) mutants and WT mice. The PTH serum values were as following for male: 38.70 ± 21.56 pg/ml (n=6) in WT, 573.80 ± 255.40 pg/ml (n=6) in VDR\(^{\Delta/\Delta}\) and 1914.0 ± 395.0 pg/ml (n=8) in Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\). The PTH serum values were as follows for female: 13.60 ± 4.0 pg/ml (n=8) in WT, 125.5 ± 46.94 pg/ml (n=6) in VDR\(^{\Delta/\Delta}\) and 1131 ± 401.3 pg/ml (n=6) in Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) (Fig. 10).

These data indicate that at 3 months of age a lack of Fgf23 signalling leads to hypocalcaemia and hyperphosphatemia associated with elevated serum PTH level in the VDR ablated mice. These findings indicate the presence of secondary hyperparathyroidism in Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants.

**FIG. 10.** Comparison of PTH, given as pg/ml in male and female WT, VDR\(^{\Delta/\Delta}\) mutant mice and Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants, all on RD. Each data column represents the mean ± SEM of six to eight mice each group.

* * p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.
# # p< 0.05 vs. Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants, one-way ANOVA followed by Student-Newman-Keuls test.
3.5. Evaluation of mineral excretion in urine

A significantly higher urine Ca/CREA rate was only observed in female Fgf23-/-/VDRΔΔ compound animals (4.69 ± 0.47 mM/mM, n=6) compared to the WT animals (2.35 ± 0.59 mM/mM, n=7) (Fig. 11). No difference for urine Ca/CREA values was detected between VDRΔΔ (3.48 ± 0.86 mM/mM, n=5) and WT. Although the corresponding values of the male mice were increased as well, this did not reach significance (male: 0.24 ± 0.09 mM/mM, n=5 in WT; 1.01 ± 0.74 mM/mM, n=5 in VDRΔΔ and 1.81 ± 0.71 mM/mM, n=7 in Fgf23-/-/VDRΔΔ).

Additionally, K/CREA (61.81 ± 5.58 mM/mM, n=6 in WT, 64.04 ± 7.90 mM/mM, n=6 in VDRΔΔ and 82.52 ± 4.16 mM/mM, n=7 in Fgf23-/-/VDRΔΔ), Na/CREA (50.17 ±9.26 mM/mM, n=6 in WT, 106.6 ± 53.09 mM/mM, n=5 in VDRΔΔ and 52.86 ± 5.88 mM/mM, n=8 in Fgf23-/-/VDRΔΔ) and Cl/CREA (78.52 ± 5.15 mM/mM, n= 5 in WT, 113.0 ± 13.03 mM/mM, n=5 in VDRΔΔ and 98.55 ± 8.54 mM/mM, n=8 in Fgf23-/-/VDRΔΔ) showed no significant changes between the genotypes in the male mice (Fig. 11).

Moreover, in the female mice K/CREA and Na/CREA showed significantly higher values in VDRΔΔ (68.39 ± 4.91 mM/mM, n=6 for potassium and 78.03 ± 13.88 mM/mM, n= 5 for sodium) and Fgf23-/-/VDRΔΔ (72.57 ± 5.48 mM/mM, n=6 for potassium and 98.65 ± 14.29 mM/mM, n=5 for sodium) both compared to WT mice (45.40 ± 2.43 mM/mM, n=8 for potassium and 38.02 ± 5.21 mM/mM, n=7 for sodium). However, Cl/CREA values were significantly higher only in Fgf23-/-/VDRΔΔ compound mutants compared to WT (60.55 ± 5.70 mM/mM, n=8 in WT, 76.61 ± 8.20 mM/mM, n=5 in VDRΔΔ and 85.77 ± 4.53 mM/mM, n=6 in Fgf23-/-/VDRΔΔ).

Based on the knowledge about the Fgf23 phosphaturic action (Razzaque et al. 2007) P/CREA was determined in the 3-month-old animals as well. Male Fgf23-/-/VDRΔΔ compound mutants showed a significantly higher urinary P/CREA value compared to both WT and VDRΔΔ mutant mice. In females, VDRΔΔ and Fgf23-/-/VDRΔΔ exhibited a significant
increase compared to WT, no difference was seen between the two mutant groups (Fig. 11). The values of urinary P/CREA in male animals were: 25.83 ± 4.07 mM/mM, n=6 in WT, 22.01 ± 1.99 mM/mM, n=6 in VDR^{Δ/Δ} and 35.57 ± 2.30 mM/mM, n=8 in Fgf23^{-/-}/VDR^{Δ/Δ}. The values of urinary P/CREA in female animals were: 17.61 ± 1.77 mM/mM, n=8 in WT, 35.79± 2.53 mM/mM, n=6 in VDR^{Δ/Δ} and 41.26 ± 3.56 mM/mM, n=6 in Fgf23^{-/-}/VDR^{Δ/Δ}.

These data indicate that at 3 months of age a lack of Fgf23 signalling leads to increased urinary excretion of calcium and phosphate in VDR ablated mice.
FIG. 11. Comparison of the relation of urine calcium, potassium, sodium, chloride and phosphate in to creatinine, given as mM/mM in male and female WT, VDRΔ/Δ mutant mice and Fgf23−/−/VDRΔ/Δ compound mutants, all on RD. Each data column represents the mean ± SEM of five to eight mice in each group.

* p< 0.05 vs. WT, # p< 0.05 vs. Fgf23−/−/VDRΔ/Δ mutants, one-way ANOVA followed by Student-Newman-Keuls test.
3.6. Bone mineral density and bone histomorphometry

Bones were analysed by pQCT and histomorphometry.

The total bone mineral density (BMD) of the femoral metaphysis and shaft showed no significant changes between the groups (Figs. 12, 13). The cortical/subcortical BMD of the femoral metaphysis was significantly lower in VDR$^{\Delta/\Delta}$ mutants compared to WT mice as well as to Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant mice, with 779.9 ± 22.74 mg/cm$^3$, n=6 in WT; 711.0 ± 17.91 mg/cm$^3$, n=6 in VDR$^{\Delta/\Delta}$ and 760.0 ± 5.36 mg/cm$^3$, n=8 in Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$ (Fig.12).

For the trabecular BMD in the femoral metaphysis, no significant differences were detected between the groups (Fig. 12). The periosteal perimeter and the total cross-sectional area also did not differ between the groups in both the metaphysis and the shaft (Figs. 12, 13).

The cortical thickness was significantly higher in Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutants (0.23 ± 0.024 mm in the metaphysis and 0.26 ± 0.011 mm in shaft, n=8) compared to VDR$^{\Delta/\Delta}$ mutant mice (0.16 ± 0.007 mm in metaphysis and 0.23 ± 0.007 mm in shaft, n=6). No significant difference was detected between the Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant mice and WT, and between the VDR$^{\Delta/\Delta}$ and WT mice (0.20 ± 0.015 mm in metaphysis and 0.25 ± 0.008 mm in shaft, n=6) in metaphysis and shaft (Figs. 12, 13).

The results for the endocortical perimeter in the metaphysis showed a significant increase in VDR$^{\Delta/\Delta}$ mutants compared to WT mice. No significant difference was found between VDR$^{\Delta/\Delta}$ and Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutant mice (5.13 ± 0.10 mm, n=6 in WT, 5.88 ± 0.19 mm, n=6 in VDR$^{\Delta/\Delta}$ and 5.46 ± 0.13 mm, n=8 in Fgf23$^{-/-}$/VDR$^{\Delta/\Delta}$) (Figs. 12, 13). No significant changes in the endocortical perimeter of the shaft were detected between the groups (Figs. 12, 13).
VDR\textsuperscript{Δ/Δ} mutants had higher trabecular area in the metaphysis than the WT group and the Fgf23\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} mutants (1.60 ± 0.07 mm\textsuperscript{3}, n=6 in WT, 2.16 ± 0.14 mm\textsuperscript{3}, n=6 in VDR\textsuperscript{Δ/Δ} and 1.72 ± 0.09 mm\textsuperscript{3}, n=8 in Fgf23\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ}) (Fig. 12).

**FIG. 12. Femoral metaphysis** Comparison of total BMD, cortical/subcortical BMD (mg/cm\textsuperscript{3}), trabecular BMD, cortical thickness, endocortical perimeter, periostal perimeter, trabecular area, and total cross-sectional area of the metaphysis in male WT, VDR\textsuperscript{Δ/Δ} and Fgf23\textsuperscript{-/-}/VDR\textsuperscript{Δ/Δ} mice, all on RD. Each data column represents the mean ± SEM of six to eight mice per group. * p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test. ** p< 0.05 vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.
No significant changes in shaft cortical BMD and cortical area were found between the groups (Fig. 13).

Quantitative bone histomorphometric analysis was performed using the Osteomeasure® image analysis system. The analysis of calcein double layers in the femur bone provided information about the mineralizing surface, mineral apposition rate and the bone formation rate. In our 3-month-old animals we detected no significant changes in all mentioned values (Fig. 14).
**FIG. 14.** Von Kossa/McNeil stained sections of femora. **a.** WT, **b.** VDR\(^{Δ/Δ}\) mutant mouse, **c.** Fgf23\(^{-/-}\)/VDR\(^{Δ/Δ}\) compound mutant. Magnification: x 10.

And comparison of mineralizing surface, mineralizing apposition rate and bone formation rate in WT, VDR\(^{Δ/Δ}\) mutant mice and Fgf23\(^{-/-}\)/VDR\(^{Δ/Δ}\) compound mutants, given as %, μm/d or μm\(^3\)/μm\(^3\)/d, all on RD. Each data column represents the mean ± SEM of six to eight mice each group.
Static bone histomorphometry showed a significant increase in number of trabecular/bone area (N.Tb/ B.Ar) in the VDR$^{\Delta/\Delta}$ mutants compared to the WT and $Fg23^{-/-}$/VDR$^{\Delta/\Delta}$ animals with $135.7 \pm 29.16$, n=6 in WT, $241.8 \pm 22.72$, n=6 in VDR$^{\Delta/\Delta}$ and $164.1 \pm 21.28$, n=7 in $Fg23^{-/-}$/VDR$^{\Delta/\Delta}$ (Fig. 15).

The results for trabecular width (Tb.Wi) showed a significant decrease in VDR$^{\Delta/\Delta}$ mutants compared to WT, but no significant difference between $Fg23^{-/-}$/VDR$^{\Delta/\Delta}$ compound mutants and the VDR$^{\Delta/\Delta}$ mutants or WT (Fig. 15).

Bone area (B.Ar/T.Ar), bone perimeter (B.Pm/T.Ar), number of trabecular/tissue area (N.Tb/T.Ar), trabecular area (Tb.Ar), trabecular number (Tb.N) and trabecular separation (Tb.Sp) did not differ between the groups (Fig. 15).
FIG. 15 Comparison of bone area/tissue area, bone perimeter/tissue area, number of trabecula/tissue area, number of trabecular/bone area, trabecular area, number of trabecular, trabecular width, trabecular separation in male WT, VDR$^{Δ/Δ}$ mutant mice and Fgf23$^{−/−}$/VDR$^{Δ/Δ}$ compound mutants, all on RD. Each data column represents the mean ± SEM of six to eight mice in each group.

* p < 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

# p < 0.05 vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.
4. Discussion

This study was designed in order to close the gap between former studies analyzing WT, \( Fgf23^{-/-} \), \( \text{VDR}^{\Delta\Delta} \) and \( Fgf23^{-/-}/\text{VDR}^{\Delta\Delta} \) mice at an age of 4 weeks (HESSE et al. 2007) and WT, \( \text{VDR}^{\Delta\Delta} \) and \( Fgf23^{-/-}/\text{VDR}^{\Delta\Delta} \) mice at an age of 9 months (STREICHER et al. 2012). In addition, we aimed to elucidate a possible role of \( Fgf23 \) signalling on biochemical parameters and bone metabolism in mature VDR-ablated animals at an age of 3 months.

Ablation of \( Fgf23 \) in mice results in complex morphological and histological changes including growth retardation (Fig. 16), hypogonadism, emphysema, vascular calcification, extensive soft tissue calcification as well as atrophy of the intestinal villi, thymus and spleen (RAZZAQUE and LANSKE 2007; HESSE et al. 2007; SITARA et al. 2004; SHIMADA et al. 2004). In addition, hypoglycaemia and profoundly increased peripheral insulin sensitivity have been found (HESSE et al. 2007). These changes are accompanied with a shortened life span of up to 13 weeks only (RAZZAQUE et al. 2005; HESSE et al. 2007; SHIMADA et al. 2004).
Due to the lacking suppressive effect of $Fgf23$ on renal 1α-hydroxylase activity, $Fgf23^{-/-}$ mice produce excessive amounts of 1, 25(OH)$_2$D$_3$ leading to hypervitaminosis D, hypercalcaemia and hyperphosphataemia (24). The same is true for Klotho-ablated mice, because Klotho is an obligate co-receptor for $Fgf23$ function (RAZZAQUE and LANSKE 2007; HESSE et al. 2007; KUROSU et al. 2006; ANOUR et al. 2012; IMURA et al. 2007). In order to examine vitamin D independent effects of $Fgf23$ deficiency on bone metabolism in skeletally mature mice, the animals with a non-functioning vitamin D receptor (VDR$^{\Delta/\Delta}$) were crossed with $Fgf23^{-/-}$ mice obtaining double mutants ($Fgf23^{-/-}$/VDR$^{\Delta/\Delta}$), and were examined at the ages of 4 weeks and 9 months (HESSE et al. 2007; STREICHER et al. 2012).

Ablation of vitamin D signalling by a non-functioning VDR (VDR$^{\Delta/\Delta}$) and feeding with rescue diet has been shown to reverse the changes seen in $Fgf23^{-/-}$ knock-out mice (HESSE et al. 2007; STREICHER et al. 2012). The comparison between data obtained in $Fgf23^{-/-}$/VDR$^{\Delta/\Delta}$ double mutants with data from VDR$^{\Delta/\Delta}$ single mutants should reveal the influences of lacking $Fgf23$ on different organs and their biochemical functions. In addition, comparing VDR$^{\Delta/\Delta}$ mutants with WT animals does show the influence of a 1, 25(OH)$_2$D$_3$ hypovitaminosis.

As expected from previous studies using mice at an age of 4 weeks or 9 months, body weight of 3-month-old mice in the present study was identical in all examined animal groups including single and double mutants (see Figs. 2 and 16). There were no statistically significant differences between the animal groups regarding organ weight and histological analysis, indicating that $Fgf23$ has no direct impact on heart, spleen or pancreas. This confirms previous data that also indicated no effect of lacking $Fgf23$ on organ morphology such as spleen, pancreas as well as the body fat content of the mutant mice at 4 weeks and 9 months (HESSE et al. 2007; STREICHER et al. 2012).

In addition to histological analysis of organs, we examined various blood parameters. In 3-month-old $Fgf23^{-/-}$/VDR$^{\Delta/\Delta}$ double mutant mice no difference in liver function occurred compared to single VDR$^{\Delta/\Delta}$ mutant animals. The same finding has been reported from $Fgf23^{-/-}$
compound mutants at 9 months of age (STREICHER et al. 2012). As in 4-week-old
$Fgf23^{-/-}/VDR^{\Delta/\Delta}$ double and VDR$^{\Delta/\Delta}$ single mutants comparable values were obtained (HESSE et al. 2007), it can be concluded that $Fgf23$ does not seem to influence liver function in any way.

To examine whether $Fgf23$ influences fat metabolism, triglycerides and cholesterol were measured in the present study. The triglyceride serum level decrease observed in 6-week-old $Fgf23$ ablated mice by SHIMADA et al. (2004) stands in contrast to the present results, which showed no significant difference between $Fgf23^{-/-}/VDR^{\Delta/\Delta}$ double mutants and VDR$^{\Delta/\Delta}$ ablated mice. These findings indicate unchanged energy balance in compound mutants in the present study, which has also been observed in 9-month-old compound mutants (STREICHER et al. 2012).

Interestingly, the comparison between VDR-ablated mice and WT animals revealed a significant decrease in cholesterol and an increase of bilirubin content in serum of VDR mutant mice. These changes, however, were only found in male animals. The reasons for these changes are unknown and may require further investigation, as other examined liver parameters remain unchanged, indicating no general liver impairment.

To investigate the influence of $Fgf23$ on the mineral homeostasis, electrolytes were measured in blood and urine. The serum content of potassium and sodium as well as chloride was found to be unchanged in $Fgf23^{-/-}/VDR^{\Delta/\Delta}$ double mutants compared to VDR$^{\Delta/\Delta}$ ablated mice, indicating a normal mineral metabolism in these mice. This was underlined by similar values for urine calcium, sodium, potassium and chloride in male $Fgf23^{-/-}/VDR^{\Delta/\Delta}$ double mutant relative to VDR mutant mice.

In females, however, VDR ablation in both $Fgf23^{-/-}/VDR^{\Delta/\Delta}$ double mutants and VDR$^{\Delta/\Delta}$ mice led to a significantly increased urinary sodium and potassium excretion. The reason for these gender specific changes of the mineral metabolism remains unknown, but might be explained by an influence of estrogen on the electrolyte homeostasis (SLADEK and SONPONPUN 2008; TREMBLAY et al. 2012; CARRILLO-LOPEZ et al. 2009).
Ablation of VDR results in a significant hypocalcaemia in mice on a normal mouse diet (STREICHER et al. 2012). The rescue diet was able to compensate the impaired uptake of calcium in studies using 4-week- and 9-month-old mutant mice (HESSE et al. 2007; STREICHER et al. 2012). In the present study a significant decrease of total serum calcium and ionized calcium was only observed between $Fgf23^{-/-}/VDR^\Delta\Delta$ mutants and wild types. In contrast to the results found in 9-month-old animal, no significant decrease was observed in 3-month-old mice between $Fgf23^{-/-}/VDR^\Delta\Delta$ and $VDR^\Delta\Delta$ mutants.

$Fgf23$ is known as phosphaturic hormone (LIU et al. 2006), influencing the phosphate content in blood and urinary excretion. However, in the present study the serum phosphate increase became significant only in $Fgf23^{-/-}/VDR^\Delta\Delta$ male mice compared to $VDR^\Delta\Delta$ mutants. This significant increase was also observed in 9-month-old compound mutants (STREICHER et al. 2012). Interestingly, the significant increase of urinary phosphate excretion in male $Fgf23^{-/-}/VDR^\Delta\Delta$ mutant mice in comparison to both, $VDR^\Delta\Delta$ mutants and wild type animals, found in the present study, has not been observed in 9-month-old animals (STREICHER et al. 2012). This might be due to a kind of renal adaption in response to lacking $Fgf23$ during lifetime. Again this observation was not made in the female mice in the present study. The gender specific reaction remains unknown.

In the study using 4-week-old mutants, an increased secretion of PTH was observed in $VDR^\Delta\Delta$ and $Fgf23^{-/-}/VDR^\Delta\Delta$ double mutants compared to the WT mice. However, there was no significant difference between $VDR^\Delta\Delta$ ablated and $Fgf23^{-/-}/VDR^\Delta\Delta$ compound mutants (HESSE et al 2007). Three-month-old animals, investigated in the present study, showed a marked increase in serum PTH levels between $VDR^\Delta\Delta$ ablated mice and $Fgf23^{-/-}/VDR^\Delta\Delta$ compound mutants. The same difference in serum PTH level was also observed in 9-month-old $Fgf23^{-/-}/VDR^\Delta\Delta$ compound mutants as compared to $VDR^\Delta\Delta$ ablated mice, indicating the presence of a secondary hyperparathyroidism (ANDRUKHOVA et al. 2011). As shown in rats, the parathyroid gland is a target organ for $Fgf23$ that decreases PTH secretion through the MAPK pathway (BEN-DOV et al. 2007). Therefore, the absence of $Fgf23$ in $Fgf23^{-/-}/VDR^\Delta\Delta$ double mutant mice may trigger an increased secretion of this hormone. The high serum PTH, which is also known as a phosphaturic hormone (SITARA et al. 2004), may also
contribute to the higher urinary phosphate excretion that we found in $\text{Fgf23}^{-/-}/\text{VDR}^{\Delta/\Delta}$ mutant mice. To understand the exact intercrossing action of PTH and $\text{Fgf} 23$ on the phosphate homeostasis, further studies will have to be made.

The lack of vitamin D signaling, studied by comparing $\text{VDR}^{\Delta/\Delta}$ mutants with WT mice, did not affect the serum concentrations of all examined electrolytes except a significant reduced content of calcium in male animals. In females the serum calcium content was reduced as well, but this effect did not reach statistical significance. In previous studies using 4-week- or 9-month-old mice no significant changes were observed (HESSE et al. 2007; STREICHER et al. 2012) indicating the benefit of the rescue diet used in all experiments.

The total bone mineral density (BMD) was not influenced by the absence of $\text{Fgf23}$. Significant increases in $\text{Fgf23}^{-/-}/\text{VDR}^{\Delta/\Delta}$ double mutants relative to $\text{VDR}^{\Delta/\Delta}$ mice became evident for cortical to subcortical BMD in the metaphysis, paralleled by a significant increase in cortical thickness and a reduction of the trabecular area.

In contrast to these results, VDR ablation lead to opposite changes in bones affecting mainly the cortical part of bone and increasing the trabecular area and the number of trabecular per bone area found in the metaphysis as compared to WT. In 4-week-old $\text{VDR}^{\Delta/\Delta}$ mutants the reduction of cortical parts of the bone is more prominent (HESSE et al. 2007). These data may indicate that VDR ablation affects the bone composition age dependently and can be more readily compensated by the rescue diet at older ages.

In conclusion, the study shows that a lack of $\text{Fgf23}$ did not induce organ morphological and histological changes in mice with a non-functioning VDR. However, male and female $\text{Fgf23}^{-/-}/\text{VDR}^{\Delta/\Delta}$ double mutants showed profoundly increased serum concentrations of parathyroid hormone, together with increased phosphate levels in serum and urine of male mutants. These alterations, however, were gender specific, possibly depending on the influence of estrogen in $\text{Fgf23}$ signalling. The bone composition showed a shift to a more dense cortical part paralleled by a reduction of the trabecular portion, an effect which may be explained by higher serum phosphate levels in compound mutants. The obtained results demonstrate that 3-
month-old \( Fgf23^{-/-}/VDR^{\Delta/\Delta} \) double mutants show signs of secondary hyperparathyroidism. It is currently unclear what drives the development of secondary hyperparathyroidism in compound mutants, and why bone in compound mutant mice does not seem to be negatively influenced by profoundly increased circulating parathyroid hormone.
5. **Summary**

It is still uncertain, if the bone-derived hormone fibroblast growth factor-23 (Fgf23) has further physiological functions besides the well-known phosphaturic action of and regulation of the vitamin-D production. Hypervitaminosis D may mediate many of the pathophysiological consequences of Fgf23 deficiency. In fact, it has been shown that ablation of vitamin D signaling in Fgf23-/ mice through expressing a non-functioning VDR rescues the premature aging like phenotype on a rescue diet. This Fgf23-/VDRΔ/Δ compound mutants together with VDRΔ/Δ and WT litters used in the present study are the excellent tools to improve understanding of the direct, vitamin D independent, physiological role of Fgf23. The present study closes the evidence gap between former studies with 4-week-old and 9-month-old WT, VDRΔ/Δ and Fgf23Δ/Δ/VDRΔ/Δ compound mutants. We hypothesize that adult 3-month-old animals would represent similar outline in biochemical parameters, mineral homeostasis, bone mineral histomorphometry and mineral density to that observed in 9-month-old Fgf23Δ/Δ/VDRΔ/Δ animals as compared to the VDRΔ/Δ animals and WT. All animals were fed on rescue diet been shown to normalize calcium homeostasis in mice with a non-functioning VDR.

The results of the present study demonstrate that similarly to the 9-month-old Fgf23Δ/Δ/VDRΔ/Δ compound mutants in 3-month-old animals we found no changes in the organ morphology and organ histology as compared to the VDRΔ/Δ mutants and WT mice. There were no statistically significant differences between the animal groups regarding organ weight and histological analysis, indicating that Fgf23 has no direct impact on heart, spleen or pancreas.

We determine similar event in the mineral homeostasis of blood and urine between 9-month-old and 3-month-old Fgf23Δ/Δ/VDRΔ/Δ compound mutants as compare to the VDRΔ/Δ mutants and WT animals. Moreover, marked elevation in the serum parathyroid hormone observed in 3-month-old Fgf23Δ/Δ/VDRΔ/Δ compound mutants was accompanied by enhanced serum and
urine phosphate levels as well as urine calcium level and decreased serum calcium level, the observation also made for 9-month-old Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants. These alterations, however, were gender specific, possibly depending on the influence of estrogen in Fgf23 signaling. The exact explanation for PTH elevation in old Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) compound mutants and the mechanisms for possible regulation of PTH expression by Fgf23 are still missing.

The total bone mineral density of the femoral metaphysis and shaft showed no significant changes between the 3-month-old experimental groups. Quantitative histomorphometrical bone analysis of the femur examined in the present study revealed no difference in mineralizing surface, mineral apposition rate and the bone formation rate in 3-month-old animal groups. Static bone histomorphometry showed a significant increase in number of trabecular/bone area in the VDR\(^{\Delta/\Delta}\) mutants compared to the WT and Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) animals. The bone composition showed a shift to a more dense cortical part paralleled by a reduction of the trabecular portion, an effect which may be explained by higher serum phosphate levels in compound mutants.

In conclusion, the obtained results demonstrate that 3-month-old Fgf23\(^{-/-}\)/VDR\(^{\Delta/\Delta}\) double mutants show signs of secondary hyperparathyroidism, manifested in 9-month-old animals. It is currently unclear what drives the development of secondary hyperparathyroidism in compound mutants, and why bone in compound mutant mice does not seem to be negatively influenced by profoundly increased circulating parathyroid hormone.
6. **Acknowledgements**

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7. References


8. List of figures

FIG. 1. Comparison of the 3 genotypes at the age of 3 months. WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$ (from left to right)

FIG. 2. Comparison of body weight, heart weight, heart/body rate and pancreatic weight, given as g and %, in male and female WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$, all on RD. Each data column represents the mean ± SEM of six to eight mice each group.

FIG. 3. H&E stained paraffin sections of lung and spleen of WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$ mutants.

FIG. 4. H&E stained paraffin sections of kidney and liver of WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$ mutants.

FIG. 5. Comparison of triglyceride and cholesterol serum levels, given as mmol/L, in male and female WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$, all on RD. Each data column represents the mean ± SEM of six to eight mice each group. * p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

FIG. 6. Comparison of ALB, ALP and AST, given as g/L or U/L, in male and female WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$, all on RD. Each data column represents the mean ± SEM of six to eight mice each group. Each data column represents the mean ± SEM of six to eight mice each group.

FIG. 7. Comparison of BILT, CKL, CREA, UREL, given as mmol/L or U/L, in male and female WT, VDR$^{\Delta\Delta}$ and Fgf23$^{\Delta}$/VDR$^{\Delta\Delta}$, all on RD. Each data column represents the mean ± SEM of five to eight mice in each group. * p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.
FIG. 8. Comparison of ionized Ca$^{2+}$, K$^{+}$ and Na$^{+}$ given as mM in male and female WT, VDR$^{Δ/Δ}$ and Fgf23$^{-/-}$/VDR$^{Δ/Δ}$ compound mutants, all on RD.
Each data column represents the mean ± SEM of five to eight mice each group.
* $p<0.05$ vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

FIG. 9. Comparison of total serum Ca$^{2+}$, K$^{+}$ and Na$^{+}$, given as mmol/L in male and female WT, VDR$^{Δ/Δ}$ mutant mice and Fgf23$^{-/-}$/VDR$^{Δ/Δ}$ compound mutants, all on RD.
Each data column represents the mean ± SEM of six to eight mice in each group.
* $p<0.05$ vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.
# $p<0.05$ vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.

FIG. 10. Comparison of PTH, given as pg/ml in male and female WT, VDR$^{Δ/Δ}$ mutant mice and Fgf23$^{-/-}$/VDR$^{Δ/Δ}$ compound mutants, all on RD.
Each data column represents the mean ± SEM of six to eight mice each group.
* $p<0.05$ vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.
# $p<0.05$ vs. Fgf23$^{-/-}$/VDR$^{Δ/Δ}$ compound mutants, one-way ANOVA followed by Student-Newman-Keuls test.

FIG. 11. Comparison of the relation of urine calcium, potassium, sodium, chloride and phosphate in to creatinine, given as mM/mM in male and female WT, VDR$^{Δ/Δ}$ mutant mice and Fgf23$^{-/-}$/VDR$^{Δ/Δ}$ compound mutants, all on RD.
Each data column represents the mean ± SEM of five to eight mice in each group.
* $p<0.05$ vs. WT,
# $p<0.05$ vs. Fgf23$^{-/-}$/VDR$^{Δ/Δ}$ mutants, one-way ANOVA followed by Student-Newman-Keuls test.
FIG. 12. **Femoral metaphysis** Comparison of total BMD, cortical/subcortical BMD (mg/cm³), trabecular BMD, cortical thickness, endocortical perimeter, periostal perimeter, trabecular area, and total cross-sectional area of the metaphysis in male WT, VDRΔ/Δ and Fgf23−/−/VDRΔ/Δ mice, all on RD. Each data column represents the mean ± SEM of six to eight mice per group.

* p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

# p< 0.05 vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.

FIG. 13. **Femoral shaft** Comparison of total BMD, cort/subcort BMD, trabecular BMD, cortical thickness, endocortical thickness, endocortical perimeter, periostal perimeter, trabecular area, total cross-sectional area of the shaft in male WT, VDRΔ/Δ and Fgf23−/−/VDRΔ/Δ of the male population, given as mg/cm³, mm or mm², all on RD. Each data column represents the mean ± SEM of six to eight mice each group.

* p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

# p< 0.05 vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.


And comparison of mineralizing surface, mineralizing apposition rate and bone formation rate in male WT, VDRΔ/Δ mutant mice and Fgf23−/−/VDRΔ/Δ compound mutants, given as %, μm/d or μm³/μm³/d, all on RD. Each data column represents the mean ± SEM of six to eight mice each group.
FIG. 15 Comparison of bone area/tissue area, bone perimeter/tissue area, number of trabecula/tissue area, number of trabecular/bone area, trabecular area, number of trabecular, trabecular width, trabecular separation in male WT, VDR$^{\text{ΔΔ}}$ mutant mice and $Fgf23^{-/-}$/VDR$^{\text{ΔΔ}}$ compound mutants, all on RD. Each data column represents the mean ± SEM of six to eight mice in each group.

* p< 0.05 vs. WT, one-way ANOVA followed by Student-Newman-Keuls test.

# p< 0.05 vs. VDR mutants, one-way ANOVA followed by Student-Newman-Keuls test.

FIG. 16. A. From left to right: WT, $Fgf23^{-/-}$, VDR$^{\text{ΔΔ}}$ and $Fgf23^{-/-}$/VDR$^{\text{ΔΔ}}$ mutants at the age of 4 weeks (STREICHER et al. 2012). B. Mice out of the present study, from left to right: WT, VDR$^{\text{ΔΔ}}$ mutant and $Fgf23^{-/-}$/VDR$^{\text{ΔΔ}}$ compound mutant at the age of 3 months. C. WT, VDR$^{\text{ΔΔ}}$ mutant and $Fgf23^{-/-}$/VDR$^{\text{ΔΔ}}$ compound mutant at the age of 9 months (ANDRUKHOVA et al. 2011).