Genetic Diseases Resulting from Disordered FGF23/Klotho Biology

Michael J. Econs, MD
Glenn W. Irwin, Jr., Professor of Endocrinology and Metabolism
Director: Division of Endocrinology and Metabolism
Professor of Medicine and Medical and Molecular Genetics
Indiana University School of Medicine
1120 W. Michigan Street
Gatch Clinical Building 459
Indianapolis, Indiana 46202-5111
Telephone: 317-274-1339
Fax: 317-278-0658

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Introduction:

Recent research in Fibroblast Growth Factor 23 (FGF23) and Klotho biology has led to an explosion of our knowledge of FGF23 and Klotho mediated disorders. While some of these disorders are rare, physicians treating metabolic bone disease will see significant numbers of these patients and all of these disorders can be debilitating. New insights into the pathophysiology of these diseases have important therapeutic implications and novel therapies are in clinical development, making it imperative that physicians taking care of these patients understand these newly discovered physiologic insights. This review will highlight several genetic diseases caused by FGF23 and Klotho excess or deficiency. It will not include hypophosphatemic disorders resulting from defects in renal phosphate transporters (most commonly mutations in the gene coding for NPT2c) nor disorders of generalized proximal tubular dysfunction (Fanconi syndrome) as they are not FGF23/Klotho mediated.

The human FGF23 gene codes for a 249 amino acid protein that is responsible for the majority of genetic hypo- and hyperphosphatemic disorders [1, 2]. It is one of 3 endocrine FGFs (the others being FGF19 and FGF21) and is produced in bone by osteocytes and osteoblasts [3]. FGF23 down-regulates expression of the sodium dependent phosphate cotransporters (NPT2a and NPT2c) in the renal proximal tubule, decreasing reabsorption of phosphate and, thereby, decreasing blood phosphate concentrations [4]. FGF23 also decreases expression of CYP27B1 (1 αhydroxylase) and increases expression of CYP24A1 (24 hydroxylase), allowing regulation of I,25 dihydroxyvitamin D (1,25 $(OH)_2$ D) production and degradation respectively, and influencing intestinal phosphate transport as well. When a patient is hyperphosphatemic or has high phosphate intake, increasing FGF23 causes renal phosphate loss, and decreases 1,25 $(OH)_2$ D (and hence intestinal phosphate transport) to restore phosphate homeostasis. In the setting of hypophosphatemia or low phosphate intake, FGF23 gene expression and protein levels decrease allowing increased renal phosphate reabsorption, and increased 1,25 $(OH)_2$ D production, to bring phosphate balance up to appropriate levels. However, in disorders of primary FGF23 excess, the high FGF23 levels drive renal phosphate losses and hypophosphatemia, and the effect on vitamin D metabolism leads to inappropriately normal or frankly low 1,25 $(OH)_2$ D concentrations.

The gene coding for α Klotho, a 1014 amino acid protein, was originally described as an "aging gene" as Klotho hypomorphic mice had a phenotype similar to aging [5]. The name "Klotho" comes from the Greek goddess Klotho, who was thought to spin the thread of life, reflecting the belief that the Klotho hypomorphic mouse displayed an aging phenotype. However, this phenotype resolves when hyperphosphatemia and hypercalcemia is blocked by a vitamin D deficient diet or CYP27B1 deficiency [6, 7]. There are α and β forms of Klotho, coded for by different genes. For purposes of this review "Klotho" will refer to α Klotho. Although there is a soluble isoform, the majority of Klotho found in the circulation is from protein cleavage of the membrane-associated form [8]. Its major effect on phosphate and vitamin D homeostasis comes from its function as a co-receptor for FGF23. Since FGFs play critical roles in development it is thought that co-receptors are necessary for the endocrine FGFs to restrict their binding to tissues they regulate, although in very high concentrations FGF23 may bind FGFR4 without Klotho (see xxxxxx in this issue). Klotho is expressed in kidney (predominantly in the distal tubule), parathyroid, and the choroid plexus [5, 9].

Hypophosphatemic Disorders Due to FGF23 Excess

FGF23 excess leads to decreased renal tubular absorption of phosphate (best measured in patients by TMP/GFR –tubular maximum reabsorption of phosphate divided by the glomerular filtration rate), resulting in hypophosphatemia and inappropriately normal or low 1,25 $(OH)_2$ D concentrations. Although it is recognized that patients with these disorders are usually in steady state (phosphorus absorbed by the GI track equals phosphorus excreted by the

kidney), these disorders are frequently referred to as “renal phosphate wasting disorders”, because excreting phosphate into the urine when a patient or animal is hypophosphatemic is not physiologically appropriate and hence a “waste”.

Autosomal Dominant Hypophosphatemic Rickets (ADHR):

ADHR is a rare disorder characterized by hypophosphatemia due to decreased TMP/GFR and inappropriately normal or low 1,25 $(OH)_2$ D concentrations [10, 11]. We had the privilege of studying a large ADHR kindred, which enabled us to understand the variability in disease expression even before we discovered FGF23, as every ADHR patient in the family has the same mutation. In contrast to other genetic renal phosphate wasting diseases, ADHR has incomplete penetrance, variable age of onset, and the disease may wax and wane in some individuals [10, 12, 13]. In our initial observations we found that our large kindred had two subgroups of patients. One presented during childhood with phosphate wasting, rickets, and lower extremity deformity – similar to XLH patients. The other presented clinically during adolescence or adulthood. These individuals had bone pain, weakness, and insufficiency fractures without lower extremity deformities [10]. The clinical features in people with delayed onset of disease were very similar to tumor induced osteomalacia patients (though none had tumors). In subsequent studies we found waxing and waning of disease manifestations in some ADHR patients. FGF23 concentrations were high when the patients were hypophosphatemic and normal during normophosphatemia [12].

ADHR is caused by mutations that replace the amino acid arginine at positions 176 or 179 [14]. These arginines are within a cleavage site $_{176}RXXR_{179}/S_{180}$ and prevent the protein from being cleaved into inactive fragments [15]. As a result, FGF23 protein accumulates and patients become hypophosphatemic. However, it was puzzling as to why patients couldn’t simply reduce FGF23 expression. The answer came from an unexpected connection between iron and FGF23. Cross sectional and longitudinal studies in ADHR patients demonstrated that when serum iron was low FGF23 concentrations rose, using both the intact FGF23 assay and the C-terminal assay (which measures intact FGF23 plus inactive fragments) [13]. The increased FGF23 concentrations resulted in decreased renal tubular resorption of phosphate and hence hypophosphatemia. In normal individuals without FGF23 mutations, low iron concentrations were associated with increased C-terminal FGF23, but normal intact FGF23 concentrations, with no change in serum phosphorus [13, 16]. Studies in the ADHR mouse provided a detailed mechanism for these observations. On a low iron diet both wild type and R176Q-Fgf23 knock-in mice (the animal model of ADHR) increase Fgf23 mRNA. In concert with our ADHR patients, both intact and C-terminal Fgf23 protein increased in the ADHR mice, which were unable to cleave the mutant protein. In wild type mice iron deficiency also increased Fgf23 protein production, but the WT mice were able to cleave the excess protein into inactive fragments. Thus, C-terminal Fgf23 was elevated, but there was no increase in intact, biologically active Fgf23 [17]. The mechanism is mediated by increased expression of HIF1a [17]. Further studies indicate that hypoxia can also increase Fgf23 mRNA expression and C-terminal Fgf23 with no change in intact Fgf23 [18]. In summary, the human and animal studies demonstrate that iron deficiency and hypoxia increase FGF23 mRNA and protein expression in bone. In normal individuals the excess FGF23 protein is cleaved into inactive fragments, but patients and mice with ADHR causing mutations are unable to cleave FGF23 protein and display elevated serum FGF23 concentrations. These result in the biochemical manifestations of the disease. It is unclear what advantage, if any, FGF23 fragments provide normal individuals in the setting of iron deficiency or hypoxia.

In light of the link between iron deficiency and increased FGF23 expression we initiated a study to determine if low dose oral iron therapy in iron deficient and insufficient ADHR patients can result in disease resolution (clinicaltrials.gov #NCT02233322).

X-linked Hypophosphatemic Rickets (XLH)

XLH is an X-linked dominant disorder with an incidence of approximately 1:20,000, making it the most common renal phosphate wasting disorder. XLH is fully penetrant, but there is wide variation in clinical severity, even within the same genotype. Classically, patients present after they start to walk and are noted to have lower extremity deformity or other skeletal manifestations of rickets. Additional features include short stature, bone pain, pseudofractures, osteoarthritis, stiffness from enthesopathy (calcification of tendons and ligaments), and tooth abscesses [19]. As patients age, enthesopathy and osteoarthritis become disabling features of the disease, limiting mobility [20, 21].

XLH is due to inactivating mutations in the PHEX (phosphate regulating gene with homologies to endopeptidases, on the X chromosome) gene, which codes for a 749 amino acid member of the neutral endopeptidase family [22]. It has a single transmembrane domain and a small cytoplasmic domain, with most of the protein being extracellular. In general, members of this protein family cleave small proteins in the extracellular space. However, PHEX does not degrade FGF23, but instead these inactivating mutations result in increased FGF23 expression [3]. Although beyond the scope of this review, evidence is not convincing that males are more severely affected than females. Serum phosphorus and TMP/GFR are similar between males and females [23]. Likewise, there are no differences in serum phosphorus, alkaline phosphatase, or Fgf23 concentrations in hemizygous (male), heterozygous (female), and homozygous (female) Phex mutant mice [24]. Because one X chromosome is turned off in each female cell, half of the osteocytes in a female XLH patient or heterozygous Phex mutant female mouse have a normal PHEX gene and the other half have the mutant gene. These cells overall make as much FGF23 as a male, in whom every cell has the mutant PHEX. Additionally, the relationship between iron and FGF23 is similar in XLH patients to normal individuals. While XLH patients start with higher intact and C-terminal FGF23 concentrations, iron deficiency only increases the C-terminal concentrations, while intact FGF23 is unrelated to serum iron in XLH [25]. Moreover, treating XLH patients with 1,25 $(OH)_2$ D and phosphate further increases FGF23 concentrations, despite not increasing serum phosphorus into the normal range [26, 27]. These observations give circumstantial evidence that PHEX mutations may give rise to an abnormal phosphate set point.

To test this hypothesis further we crossed mice with Phex mutations with Galnt3 null mice. GALNT3/Galnt3 O-glycosylates FGF23 to protect the protein from being cleaved/inactivated before secretion. GALNT3 mutations lead to tumoral calcinosis [28], a disorder characterized by low intact (biologically active) FGF23, hyperphosphatemia and soft tissue calcifications (see below). Phex mutant mice displayed hypophosphatemia and elevated total (intact plus fragments) and intact Fgf23 concentrations whereas Galnt3-null mice were hyperphosphatemic with low intact Fgf23 concentrations, but had high Fgf23 mRNA expression and high total Fgf23 concentrations (almost all inactive fragments) [29]. The Phex/Galnt3 double mutant was still hypophosphatemic, with serum phosphate concentrations slightly, but significantly higher than Phex mutant (Figure 1). In the absence of altered phosphate responsiveness, Fgf23 mRNA expression in the double mutant would be expected to be similar to the Phex mutant. Instead, in the presence of a slightly improved, but still low serum phosphate the double mutant markedly upregulated Fgf23 gene expression even higher than the Phex mutant mouse (5 and 24 fold higher than 4 and 12 week old Phex mutant mice, respectively) [30]. These levels were 57 and 113 fold higher than 4 and 12 week old control mice, and the total Fgf23 protein expression was similar to mRNA expression. These data, in concert with the above mentioned observations, support the concept that Phex mutant mice and XLH patients may have an altered phosphate set point. This is clinically important because XLH patients may respond to any corrections in serum phosphate concentrations by increasing FGF23 concentrations – essentially fighting attempts to improve their biochemical profile.

Autosomal Recessive Hypophosphatemic Rickets (ARHR)

ARHR is caused by mutations in Dentin Matrix Protein (DMP1) [31, 32], ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) [33], or FAM20C (Family with sequence similarity 20C, [34]). Inactivating mutations in all 3 genes result in increased FGF23 concentrations, causing hypophosphatemia and inappropriately normal 1,25 $(OH)_2$ D concentrations. DMP1 mutations result in a syndrome essentially identical to XLH. Inactivating mutations in ENPP1 also cause infantile arterial calcification, and ossification of the posterior longitudinal ligament of the spine. ARHR type 3 is a variant of Raine's syndrome due to FAM20C inactivating mutations. Knockout and "knockdown of FAM20C leads to marked decrease in DMP1 and increase in FGF23, which results in hypophosphatemia [35]. Further work indicates that FAM20C phosphorylates FGF23 at serine 180 to prevent O-glycosylation of FGF23, leading to increased cleavage of the intact hormone [36]. Thus, inactivating mutations may increase FGF23 levels directly as well as by decreasing DMP1 expression.

Tumor Induced Osteomalacia (TIO)

While not a genetic syndrome, TIO is another form of FGF23 mediated hypophosphatemia. These tumors secrete FGF23, causing hypophosphatemia with inappropriately suppressed 1,25 $(OH)_2$ D concentrations, which results in osteomalacia [37]. While many different types of tumors can cause TIO, phosphaturic mesenchymal tumor of the mixed connective tissue type is the most common cause [38]. Clinically, weakness, fatigue and bone pain can be striking and, if diagnosis is delayed, many patients are wheelchair bound [39]. Of interest and potential significance, disorders resulting in delayed onset of elevated FGF23 concentrations and hypophosphatemia (TIO and some patients with ADHR) frequently present with weakness and fatigue, while in those that present early in life (XLH and other patients with ADHR) weakness is not a predominant complaint.

Hypophosphatemia Due to Klotho Overexpression

As an example of how a single patient can shed light on mammalian biology, Brownstein et al [40] described a patient who had a translocation with a breakpoint adjacent to the Klotho gene. This resulted in elevated circulating levels of the cleaved□Klotho protein. This patient had markedly increased serum FGF23 concentrations with hypophosphatemia and hyperparathyroidism requiring partial parathyroid resection [40]. Further studies showed that cleaved□Klotho administered to mice via an adeno-associated virus resulted in a marked increase in Fgf23 and hypophosphatemia due to increased Fgf23 mRNA in bone [41]. In combination, these human and mouse studies demonstrate a relationship between cleaved Klotho and FGF23 that is not fully understood.

Hyperphosphatemic Disorders Due to FGF23 or Klotho Deficiency: Tumoral Calcinosis

Familial tumoral calcinosis is the converse of the hypophosphatemic disorders discussed above. Affected individuals have hyperphosphatemia and increased or inappropriately normal 1,25 $(OH)_2$ D concentrations [42]. Calcium and PTH are usually within the normal range. Patients frequently present with calcified soft tissue masses, which can vary from small to massive (Figure 2). There is a characteristic dental abnormality [43] and vascular calcifications can be present [42]. Hyperostosis-hyperphosphatemia syndrome was originally thought to be a separate disorder, however, it is a tumoral calcinosis variant [42, 44, 45]. Tumoral calcinosis was previously thought to occur predominantly in people of African ancestry. However, it is now known to occur in other ethnic groups. There are 3 forms of familial tumoral calcinosis. All have recessive inheritance. Mutations in GALNT3 (UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactos-aminyl transferase-3) and FGF23 have been described that result in excessive cleavage of FGF23 protein into biologically inactive fragments [46-48]. A single

case of a Klotho Histidine193Arginine (H193R) mutation, resulting in FGF23 resistance has been reported [49].

Inactivating mutations in GALNT3 cause TC [28]. GALNT3 is an enzyme found in the Golgi that O-glycosylates FGF23 to protect the protein from being cleaved into inactive fragments [48]. Since the C-terminal FGF23 assays measure both inactive fragments and intact FGF23 these patients have very high levels measured by this assay as hyperphosphatemia increases expression of FGF23 mRNA and protein, which is cleaved into inactive fragments. However, levels of full length, biologically active FGF23 (as measured by intact assays) are low, but measureable [45, 48]. FGF23 mutations can also cause familial tumoral calcinosis. So far, all described TC- causing FGF23 mutations are missense mutations in the N terminal domain and are thought to destabilize the protein [46, 47, 50]. Indeed, the biochemical profile is identical to that of patients with GALNT3 mutations: hyperphosphatemia, increased 1,25 $(OH)_2$ D, marked elevations in FGF23 fragments, but low intact FGF23 concentrations. In light of data demonstrating that the FGF23 null mouse only lives 10-12 weeks, it is possible that complete absence of FGF23 may not be compatible with survival in humans. Since mutations in either GALNT3 or FGF23 result in inadequate serum concentrations of intact, biologically active FGF23 it would make physiologic sense to administer FGF23 to these patients, much like giving insulin to patients with type 1 diabetes. Unfortunately, practical considerations of making intact, clinical grade FGF23 protein and the expense of the necessary preclinical and clinical research have prevented these studies from progressing. Current medical therapy continues to be dietary phosphate restriction, phosphate binders, acetazolamide [51-53], and (rarely) probenecid [54], none of which have fully satisfactory outcomes.

Tumoral Calcinosis Due to Missense Mutations in Klotho.

As noted above, Klotho, which is primarily expressed in the kidney, parathyroid, and choroid plexus, functions as a co-receptor for FGF23 [55, 56]. We reported a patient who was homozygous for a H193R Klotho mutation, resulting in FGF23 resistance [49]. Her intact FGF23 concentration was 16,140 pg/ml (normal <70 pg/ml) and she displayed hyperphosphatemia and hypercalcemia with elevated 1,25 $(OH)_2$ D concentrations. In contrast to the Klotho KO mouse [57] she had increased PTH concentrations due to four gland hyperplasia. She had prominent soft tissue calcifications, including dural and posterior fossa calcifications. Essentially, she had a severe case of tumoral calcinosis, without evidence of premature aging. Although this case is only one patient, she demonstrates the importance of Klotho in human phosphate and vitamin D homeostasis.

Summary

Human genetic disorders affecting FGF23/Klotho biology result in substantial morbidity. While some are quite rare, others are fairly common and providers taking care of metabolic bone disease patients will certainly be responsible for the care of these patients. Studies in patients with these diseases, as well as animal models of these diseases, have provided tremendous insight into their pathophysiology. These insights have led to new therapies, which will be summarized in other reviews in this issue.

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Figure Legends:

Figure 1: Serum phosphorus, C-terminal (total) Fgf23, intact Fgf23, and Fgf23 mRNA expression in male (blue) and female (green) mice at 4 and 12 weeks of age. Galnt3 mutant mice have higher serum phosphorus and C-terminal FGF23, but lower intact FGF23 than wild type mice despite increased FGF23 mRNA expression. Phex mutant mice have higher FGF23 gene expression, C-terminal and intact FGF23, and lower serum phosphorus than wild type mice. Of note, double mutant mice had serum phosphorus concentrations above those of Phex mutant mice, but were still hypophosphatemic. Despite hypophosphatemia, there was a marked increase in Fgf23 mRNA and Fgf23 protein fragments in double mutant mice. (Adapted from [30].)

Figure 2: Radiographs from a patient with familial tumoral calcinosis illustrating marked soft tissue calcifications. (Adapted from [58]).

Figure 1

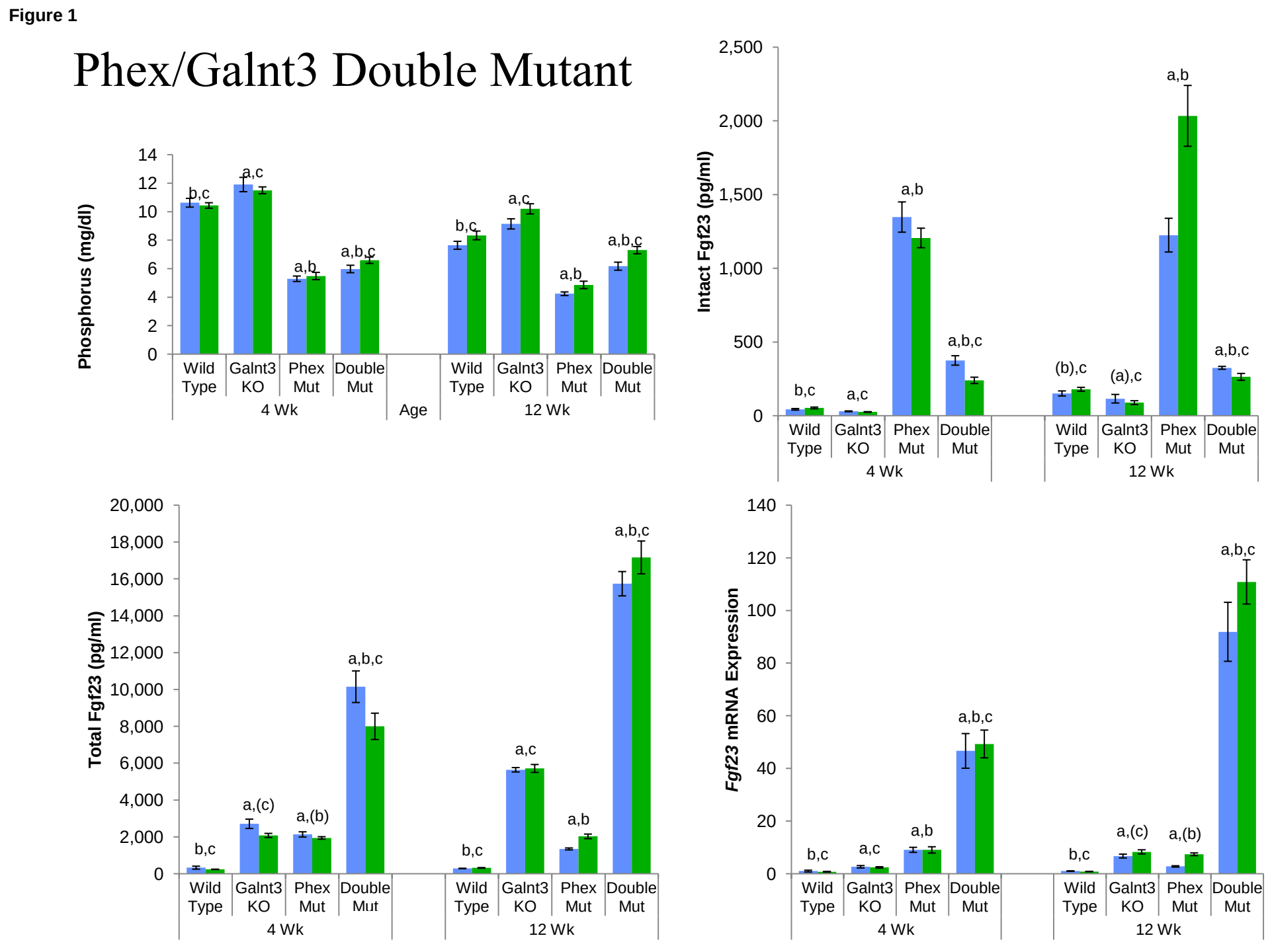
Phex/Galnt3 Double Mutant
Phosphorus (mg/dl)
14
12
10
8
6
4
2
0
b,c
a,c
a,b
a,b,c
b,c
a,c
a,b
a,b,c
Wild Type
Galnt3 KO
Phex Mut
Double Mut
4 Wk
Age
12 Wk
Intact Fgf23 (pg/ml)
2,500
2,000
1,500
1,000
500
0
b,c
a,c
a,b
a,b,c
(b),c
(a),c
a,b
a,b,c
Total Fgf23 (pg/ml)
20,000
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
b,c
a,(c)
a,(b)
a,b,c
b,c
a,c
a,b
a,b,c
Fgf23 mRNA Expression
140
120
100
80
60
40
20
0
b,c
a,c
a,b
a,b,c
b,c
a,(c)
a,(b)
a,b,c

Figure 2