HIGH PHOSPHORUS DIET INDUCES VASCULAR CALCIFICATION, A RELATED DECREASE IN BONE MASS AND CHANGES IN THE AORTIC GENE EXPRESSION

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KEYWORDS

High phosphorus diet; vascular calcification; bone mass; vascular wall gene expression; Wnt pathway inhibitors.

ABSTRACT

In chronic kidney disease, hyperphosphatemia has been associated to vascular calcifications. Moreover, the rate and progression of vascular calcification have been related with the reduction of bone mass and osteoporotic fractures, hereby suggesting a strong link between vascular calcification and bone loss. Our aim was to prospectively study the effects of high phosphorus diet on bone mass, vascular calcification and gene expression profile of the arterial wall.

A rat model of 7/8 nephrectomy fed with normal (0.6%) and moderately high (0.9%) phosphorus diet was used. Biochemical parameters, bone mineral density and vascular calcifications were assessed. A microarray analysis of the aortic tissue was also performed to investigate the gene expression profile.

After 20 weeks, the rats fed with a high phosphorus diet showed a significant increase in serum phosphorus, PTH, and creatinine, together with aortic calcification and a decrease in bone mass. The histological analysis of the vascular calcifications showed areas with calcified tissue and the gene expression profile of this calcified tissue showed repression of muscle-related genes and overexpression of bone-related genes, among them, the secreted frizzled related proteins, well-known inhibitors of the Wnt pathway, involved in bone formation.

The study demonstrated prospectively the inverse and direct relationship between vascular calcification and bone mass. In addition, the microarrays findings provide new information on the molecular mechanisms that may link this relationship.

INTRODUCTION

Bone and mineral disorders caused by chronic kidney disease (CKD), particularly by the related poor control of hyperphosphatemia, have been linked to the increased risk of vascular calcification [1, 2]. In addition, the latter has been described as one of the main drivers of mortality in CKD patients [3, 4].

Vascular calcification occurs trough a complex mechanism [5] that consists not only in a simple deposition of calcium and phosphate, but also in a regulated process similar to bone formation which involves loss of vascular calcification inhibitors [6], formation of calcification vesicles [7], and cellular phenotypic changes from vascular smooth muscle cells (VSMCs) to bone-like cells [8]. These new "vascular osteoblasts" are able to express bone-related genes and proteins, such as alkaline phosphatase, Cbfa1/Runx2, osteopontin, osteoprotegerin (OPG) and Msx2, among others [9]. In addition, in the general population and also in CKD patients, the rate and progression of vascular calcification have been associated with an increased risk of bone loss and osteoporotic fractures [10, 11], involving several but not fully understood genetic and molecular mechanisms.

The present experimental study was designed to prospectively assess the effects of high phosphorus diet on bone mass and vascular calcification, while paying attention to the gene expression profile of the arterial wall.

METHODS

Experimental design

Animal model

The study was performed using 4-months old male Wistar rats (n=70). The rats were anaesthetized using methoxi-fluorane, and chronic renal failure (CRF) was induced by surgical 7/8 nephrectomy using the technique modified by Ormrod and Miller [12] by the same technician for all the groups. The nephrectomized rats were subsequently divided in two groups: Group I was fed with normal phosphorus diet (NPD) (0.6% phosphorus, 0.6% calcium and 20% protein content), and group II was fed with high phosphorus diet (HPD) (0.9% phosphorus, 0.6% calcium and 20% protein content) (Panlab, Spain). The rats were housed in wire cages and received diet and water *ad libitum*.

Five rats per group were sacrificed at 8, 16 and 20 weeks after surgery by heart puncture exsanguination. As a result, 6 final subgroups were studied: 3 of them fed with NPD for 8, 16 and 20 weeks (8NPD, 16NPD and 20NPD, respectively), and the other 3 fed with HPD for 8, 16 and 20 weeks (8HPD, 16HPD and 20HPD, respectively). Blood samples, the abdominal aorta and the right tibia were obtained from the 5 rats of the HPD and NPD groups which were sacrificed at 8, 16 and 20 weeks. In addition, one group of rats without nephrectomy was fed with NPD for 20 weeks and used as Reference group (n=9).

The abdominal aortas (the 2 cm segment most proximal to the iliac bifurcation) were extracted and carefully cleaned eliminating the clotted blood. The right tibias were extracted and skin and muscle were peeled off. The *Laboratory Animal Ethics Committee for* of the Universidad de Oviedo approved this protocol.

Biochemical markers

Serum was separated from blood samples by centrifugation at 4,000 RPM and 4ºC. Serum urea, creatinine, calcium and phosphorus were measured using a multi-channel auto analyzer (Hitachi 717) following the manufacturer's protocol. Serum concentration of intact parathormone (iPTH) was measured using an IRMA rat PTH assay with a specific chicken anti-PTH antibody following manufacturer's protocol (Immunotopics).

Aortic calcification analysis

To assess aortic calcifications, a fragment of the undecalcified abdominal aorta was embedded in methyl-methacrylate (Sigma-Aldrich). Five sections of 5 thick were obtained using a Polycut S microtome (Reicher-Jung), and stained following Von Kossa's method [13].

Tartrate resistant acid phosphatase (TRAP) staining was carried out as described previously [14] All sections were evaluated by a blinded pathologist. The sample was considered positive for vascular calcification when calcium deposits were found in all 5μm thick sections.

Microarrays hybridization, analysis and validation

Fragments of the abdominal aorta from the 5 rats sacrificed each time (8, 16 and 20 weeks) were pooled, and homogenized (Ultraturrax, OmniHT) in TRI^{TM} reagent (Sigma-Aldrich). Total RNA was extracted and purified using RNeAsy^{TM} Kit (Qiagen). The RNA integrity was checked using agarose-formaldehyde gels, and the RNA concentration was measured using a VIS-UV spectrophotometer (Nanodrop). cDNA was synthesized using a High Capacity^{TM} kit (Applied Biosystems), and hybridized to Affy RAE_230 cDNA microarrays (Affymetrix) using the adequate quality controls and following the

manufacturer's protocol. To analyze the raw datasets, the data were logged and normalized using the PM/MM (dChip [15]) method .

After the normalization and expression-modelling of the raw data, the first step was to build hierarchical clusters using the Euclidean-Centroid Linkage method. In this step, all genes were included in a preliminary analysis and then, only specific genes grouped by ontology terms, obtained from the Kyoto encyclopaedia of genes and genomes (KEGG), were used. Moreover, samples were grouped into three categories according to the results of Von Kossa staining. In the first category, we included the 20HPD group in which 4 out 5 (80%) animals showed vascular calcifications (VC+++); in the second category, we included the 16HPD group in which 1 of 5 (20%) animals showed vascular calcification (VC+); and in the third category, we included the rest of groups in which none of animals showed vascular calcification (VC-).

Second step: To set gene expression differences among the groups, specific comparisons were performed, using the Reference group as baseline-comparator. We included in the gene list for analysis only those genes which fulfilled the following two criteria: **I)** A fold change greater than 1.5 obtained using both dChip (RMA algorithm) and GGcos (MAS5 algorithm) software packages, and **II)** a time-dependent trend (either increasing or decreasing) referred to the Reference group but mainly centred on the 20HPD group.

Six selected genes of interest from the VC+++ and VC- categories were validated by qRT-PCR, using Taqman^{TM} predeveloped assays (*tropomyosin*: Rn00569447_m1, e*lastin*: Rn01499782_m1, *cathepsin-K*: Rn00580723_m1, *SFRP-1*: Rn01478472_m1, *SFRP-2*: Rn00585549_m1, and *SFRP-4*: Rn01458837_m1) (Applied Biosystems) as described previously [16].

ErmineJ software [17] was also used to perform an OveR score Analysis (ORA test) in order to determine whether the gene ontology (GO) terms were modified in the development of vascular calcification.

Densitometric analysis

The right tibia was fixed with 70% alcohol for one week, then bone mineral density (BMD) was measured by dual energy x-ray absorptiometry (DEXA; Hologic QDR100, adapted to small animals). Three BMD tibiae values were obtained: 1/8 proximal segment (mainly trabecular bone), the remaining 7/8 segment (mainly cortical bone), and the total tibiae, following the procedure detailed in a previous paper [13].

Statistical analysis

A non parametric test (Mann-Whitney) was used to compare biochemical parameters and BMD changes among the groups. A Pearson correlation was employed to study the association between biochemical and BMD parameters. A multivariate logistic regression analysis, adjusted by P, Ca and BMD, was performed to determine which parameters were associated with vascular calcification. Data were expressed as mean ± standard deviation. Differences were considered significant when $p<0.05$. Calculations were performed using the statistical analysis package SPSS 12.0 (SPPS Inc).

RESULTS

Biochemical markers

Serum phosphorus and iPTH levels were significantly higher ($p<0.05$) in all high phosphorus diet (HPD) groups compared to the paired normal phosphorus diet (NPD) groups. In addition, in the HPD groups, both serum phosphorus and iPTH increased over-time. Conversely, serum calcium was lower in the HPD groups compared to the paired NPD groups, achieving statistically significant differences in the 8HPD and 20HPD groups (Table 1).

All groups showed a reduction in renal function compared to the Reference group ($p<0.05$). These changes were more marked in the HPD groups which showed significantly higher levels of serum urea and creatinine than NPD rats ($p<0.05$) (Figure 1). In addition, serum creatinine and urea levels positively correlated with serum phosphorus and PTH ($p<0.005$, $r> 0.9$) and negatively correlated with serum calcium ($p<0.05$, $r> 0.8$).

Aortic analyses

The aortas from the Reference, all NPD, and 8HPD groups did not show macroscopic changes. In the Reference, all NPD, and 8HPD groups, no vascular calcification in the media layer was observed (Figure 2A). Only one rat (20%) from the 16HPD group showed vascular calcifications.

Four out of 5 (80%) rats from the 20HPD group showed macroscopic changes with a rigid appearance (Figure 2B) and calcium deposits in the medial layer. The 5 calcified aortas which were included in the study (1 from 16HPD and 4 from 20HPD) are depicted in Figure 2C. Moreover, the calcified aortas showed osteoid-like tissue and multinucleated cells (Figures 2D and 2E).

The multinucleated cells present surrounding the calcified areas, showed a positive TRAP staining (Figure 2G). In addition no positive TRAP staining was detected in the non calcified aortas (Figure 2F)

In a multivariate adjusted analysis, serum iPTH was the unique parameter positively and independently associated with vascular calcification ($p<0.005$). The mortality reached 50% in the 20HPD group, 40% in the 16HPD and 25% in the 8HPD. In the NPD and Reference groups, mortality reached a mean of 10%.

Relationship between bone mineral density and vascular calcification

The rats from the NPD groups showed normal BMD, with similar values to the Reference group. The rats from the 8HPD and 16HPD groups showed a slight but not significant decrease in BMD. By contrast, rats from the 20HPD group (VC+++) showed significantly lower BMD values compared to the paired 20NPD group (VC-) at all skeletal sites (Table 2); the difference between groups remained after adjusting by body weight ($p<0.05$).

The relationship between trabecular BMD (proximal tibiae) cortical BMD (the remaining 7/8 of tibiae) and vascular calcification is depicted in Figure 3. The rats with vascular calcification had the lowest BMD values, mainly in the area were cortical bone is predominant (Table 2).

Aortic gene expression analysis

To perform a time-course analysis of the aortic gene expression in all groups, "whole-genome" expression microarrays were used. In the first step, the segregation of samples was studied, and then a gene list, containing the statistically deregulated genes compared to the reference group, was build up.

Using unsupervised hierarchical clusters, with all genes and all groups, the 20HPD group was the only group segregated from the other groups, which grouped all together (data not shown). In addition, when classifying the samples according to the severity of vascular calcification, (using VC+++, VC+ and VC- nomenclature), the supervised clusters for "bone-related" and "muscle-related" genes showed a mirror-like expression pattern between the VC+++ and the VC- categories (Figure 4). The VC+ category (only 1 rat with vascular calcification) showed a pattern of expression closer to the VC- rather than to VC+++, particularly in the muscle-related genes.

In the second step, and using the criteria explained in the methods section, 53 genes were found to be differentially expressed (Table 3). In the majority of genes, the most important changes observed in the gene expression (independently of the trend and direction of the change) took place in the VC+++ rats, reinforcing the results obtained with the clusters, and also in agreement with the biochemical, histological, and BMD data.

From the 53 deregulated genes listed in table 3, two muscle-related genes with decreased expression (*tropomyosin 1* and e*lastin*), and 4 bone-related genes with increased expression (*secreted frizzled related protein-1, 2 and 4 [SFRP-1, 2 and 4],* inhibitors of the Wnt pathway [18], and *cathepsin K* [osteoclast marker]), were selected and further validated by qRT-PCR. In agreement with the expression microarrays results, the relative expression of *tropomyosin 1* and Elastin were 0.21±0.02 and 0.52±0.01 respectively whereas the relative expression of *SFRP-1, 2 and 4 and cathepsin K* were 2.01±0.08, 1.77±0.09, 1.49±0.03 and 1.59±0.03 respectively when comparing 20HPD to Reference groups.

The ORA analysis showed that genes involved in the cell cycle, the renewal of the extracellular matrix and ion cotransporters were significantly altered ($p<0.005$).

DISCUSSION

Vascular calcification, bone loss, increased fractures and high risk of mortality are severe and threatening outcomes in the CKD population at all stages [10, 19-21]. The importance of all these factors has been recently stressed and also recognized by K-DIGO with the a new nomenclature for all bone mineral disorders associated to CKD [22]. Elevated serum phosphorus has been described as one of the main pathogenetic players for all these abnormalities [2, 23], particularly in the onset of vascular calcification. Increased PTH synthesis and secretion [24] and impairment of renal function [25] have been also associated with hyperphosphatemia.

In our study, we used our current animal model for experimental uraemia, but applying a moderate increase in the dietary phosphate (50%) in order to mimic the scenario of positive phosphate balance and hyperphosphatemia frequently found in CKD patients [26].

As expected, we found a significant increase in serum phosphorus in rats receiving HPD compared to their matched NPD groups. Since phosphorus and calcium are closely interrelated and regulated [27], serum calcium was found to be significantly lower. Both high phosphorus and low calcium are two well-known stimuli which produce an increase in PTH synthesis, as well as parathyroid gland hyperplasia [28, 29]. In fact, in this experimental model, a marked parathyroid hyperplasia (40-fold increase) (data not shown) and very high serum iPTH levels were found in the HPD groups (particularly after 20 weeks) compared to the NPD groups. In addition, serum iPTH was the only biochemical parameter which was positively and independently associated with vascular calcifications; the higher the serum iPTH, the greater the amount and severity of vascular calcifications. These results are in agreement with the concept that PTH promotes vascular calcification as it has been found in previous experimental studies [30]. Interestingly, the rats which received a diet with a greater content in phosphorus showed a higher spontaneous mortality

[31] throughout the study, reaching 50%, 40% and 25% of mortality in the 20, 16 and 8 HPD groups respectively, compared to a mean of 10% in the matched NPD groups. These findings suggest an important deleterious effect of phosphorus [32].

In agreement with recent studies [33, 34], the HPD group showed a positive association between serum phosphorus and serum creatinine and urea, suggesting a negative effect of phosphorus on the renal function. In the NPD groups, serum creatinine and urea showed a trend to decrease over time (contrary to HPD groups), likely due to the compensatory hypertrophy of the remaining 1/8 of kidney tissue. None of the rats fed with NPD showed vascular calcification

The vascular calcifications observed in the majority of the 20HPD group were widespread and severe (Figures 2C, 2D and 2E). In addition, they showed positive staining for TRAP, a product of the osteoclast activity found only in the calcified aortas, suggesting that the multinucleated cells seen in the wall of the arteries were almost active "osteoclast-like" [9]. Likewise, in the microarray analysis, we observed an increase in Cathepsin-K gene expression, a well known marker of osteoclast activity, also suggesting resorptive activity in the calcified vessels after 20 weeks of HPD feeding.

The inverse relationship between vascular calcification and bone mass/bone activity is well established in the literature mainly in the general population and/or osteoporotic patients but not in CKD patients. In the latter, all the published studies are observational, cross-sectional but never prospective, in addition some of them associate bone with vascular calcification, using data not always obtained at the same time [21, 35-39]. In addition, there is still some controversial results; recent papers has found the extent of vascular calcifications does not appear to be influenced by bone turnover and/or have any association with trabecular bone mass and connectivity when multivariate analysis was performed [40]. In our study, the rats which did not develop vascular calcifications showed a BMD trend

similar to the Reference group while the BMD of the rats from HPD groups was always lower than the matched NPD groups. Furthermore, our study demonstrates for the first time, that only the rats which did develop vascular calcifications showed a significant bone loss and they had the lowest BMD at all sites studied. In agreement with previous findings [41], the most remarkable effect of HPD took place in the cortical bone.

In previous observational papers, no association between calcification scores and abnormalities of mineral metabolism (such as hypercalcemia, hyperphosphatemia, raised Ca × P and hyperparathyroidism) were found [21, 38]. On the contrary, in our study high serum phosphorus and high PTH, both significantly correlated with changes in the aorta and the tibiae. These findings are in agreement with a recent paper [25] which described a negative phosphorus-mediated effect at bone level.

In summary, this is the first time that an experimental study demonstrates that the animals which developed vascular calcification were the only which showed reduction of bone mass, on the contrary bone loss was not observed in the animals which did not develop vascular calcification.

The expression microarrays, already used in previous studies [42], represent a useful tool for the analysis of disorders caused by unknown mechanisms. To clarify the gene expression changes occurring during the progression of vascular calcification, over 31,000 genes have been analyzed by expression microarrays. To our knowledge, this is the first time that this technique is applied to the study of vascular calcification. The microarrays results allowed us to select some candidate genes and pathways for further analyses and they supported the concept of a phenotypic change in the cells, since the hierarchical clusters showed a completely different pattern of expression of bone- and muscle-related genes in the calcified and non calcified aortas. In fact, two important muscle-related genes were

found repressed, supporting the idea of the loss of the muscular phenotype. Moreover, the ORA analysis revealed changes in the ion cotransporters, also found in the gene list.

From the mechanistic point of view, it is well known that SFRPs are inhibitors of the canonical signalling of Wnt pathway; the latter involves ossification, bone formation and also vascular calcification development [43-45]. SFRP-1 knockout mice showed high trabecular bone formation without vascular implications [46]. Shalhoub et al described for the first time a role for SFRPs in cell calcification, showing a decrease of SFRP-3 gene, in an *in vitro* model of VC [47].

Surprisingly, SFRP-3 and SFRP-1, but also SFRP-4 were found overexpressed in our model (not only by expression microarrays but also by qRTPCR performed in aortic tissue), suggesting that SFRP family may play a role, at least, in the late phases of vascular calcification. Thus, the increase of the SFRP-1, 2 and 4 gene expression found in the severely calcified aortas could be interpreted as a defensive response which aim to block the Wnt pathway in order to reduce the mineralization in the calcified aortic wall. As the increase in the SFRP family was not detected during the early stages (weeks 8 or 16), we could hypothesized that this overexpression has been triggered late in this process to avoid a further progression of the vascular calcification. On the other hand, as the SFRPs are secreted proteins, they might be able to reach the bone tissue where they could act like in the vessels trying also to decrease mineralization, resulting in reduction of bone mass. This is a fascinating mechanistic hypothesis that might link the progression of vascular calcification with the reduction of bone mass that merits further and specific studies.

Recently, it has been described that SFRP2 expression is upregulated in the fibrotic phase of myocardial infarction and that SFRP2 null mice have shown reduced fibrosis and improved cardiac function [48]. Interestingly, cardiovascular fibrosis is frequently accompanied by vascular calcification. SFRP-4 has been also described as a potent

phosphaturic agent [49]; thus, we could also speculate that the increase of SFRP-4 gene expression might also be a reactive mechanism to counteract the rise of serum phosphate observed in rats fed with HPD.

In summary, our experimental study prospectively demonstrated the strength of the association between vascular calcification and the reduction in bone mass in a rat model of CKD fed with a moderately high phosphorus diet, showing also that the HPD itself could produce, in the long term, several threatening disorders. The histological and some of the microarrays results complement each other to better understand the changes that occur in the process of vascular calcification. Furthermore, the overexpression of members of the SFRPs family detected by microarrays and qRTPCR, found only when vascular calcification was established and severe, might be indicative of a defensive mechanism triggered in order to reduce or block the activation of the Wnt pathway, with the aim to reduce mineralization of the arterial wall and to avoid progression of vascular calcification.

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Table 1

Bone-related serum parameters in NPD and HPD, and Reference (Ref) groups.

*= $p<0.05$ compared to time-matched NPD groups. N=39

Table 2

BMD values at three different tibia sites in NPD and HPD, and Reference (Ref) groups.

*= $p<0.05$ compared to time-matched NPD groups. N=39

Table 3

Gene expression dataset of the 53 differentially expressed genes sorted by fold change at 20HPD group. Note the time-dependent trend across the different groups. The selected genes of interest are marked in grey. * = 2 probes representing the same gene. **= 3 probes representing the same gene. ***= 4 probes representing the same gene.

Figure 1

Effect of HPD and NPD on serum creatinine and urea in rats with CRF at 8, 16 and 20 weeks. Reference group is represented. *= $p<0.05$ compared to the time-matched HPD groups. N=39

Figure 2

Panel A: Macroscopic image showing the rigid appearance of the entirely calcified aorta of a rat from the 20HPD group. All rats from 20HPD group showed the same macroscopic image of the aorta. Panel B (20X): Von Kossa staining of a non-calcified aorta (similar images were obtained in all rats from Reference, 8NPD, 16NPD, 20NPD and 8HPD groups) Panel C: Images of the calcified aortas included on the study. Panels D (30X) and E (40X): Von Kossa staining of a calcified aorta from the 20HPD group. Similar images were observed in all aortas form the 20HPD group. The arrow points the osteoid tissue (D) and multinucleated cells (E). Panel F (30X) and G (40X). TRAP staining in a section from non-calcified and calcified aorta respectively. The calcified aorta had TRAP activity (red areas), marked by the red zones.

Figure 3

Inverse relationship between vascular calcification and BMD in the 1/8 proximal part of the tibia, and the 7/8 remaining part of tibia. Plain triangles represent rats with vascular calcifications (VC) and the open circles rats with no vascular calcification (No VC).

Figure 4

Hierarchical clusters (heat map) branching the muscle- and bone-related gene families (KEGG criteria) into three categories based on Von Kossa results.

Categories: I) VC+++: Vascular calcification in 80% of the rats (20HPD group); II) VC+: Vascular calcification present in 20% of the rats (16HPD group); and III) VC-: No vascular calcification (20NPD + 16NPD + 8NPD + 8HPD + Reference groups).

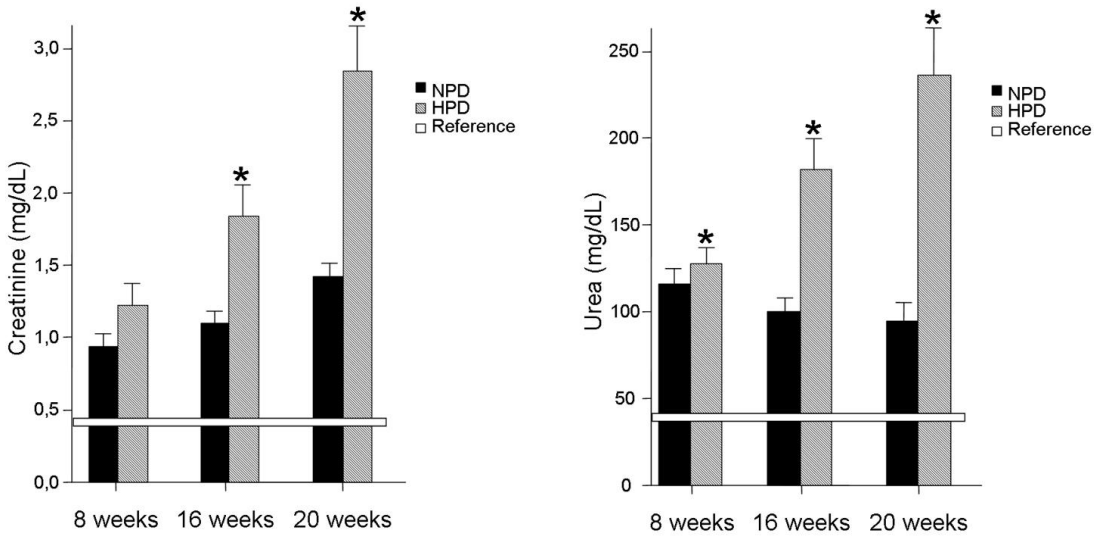
Creatinine (mg/dL)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
NPD
HPD
Reference
8 weeks
16 weeks
20 weeks
Urea (mg/dL)
250
200
150
100
50
0
*
*
NPD
HPD
Reference
8 weeks
16 weeks
20 weeks

Figure 1

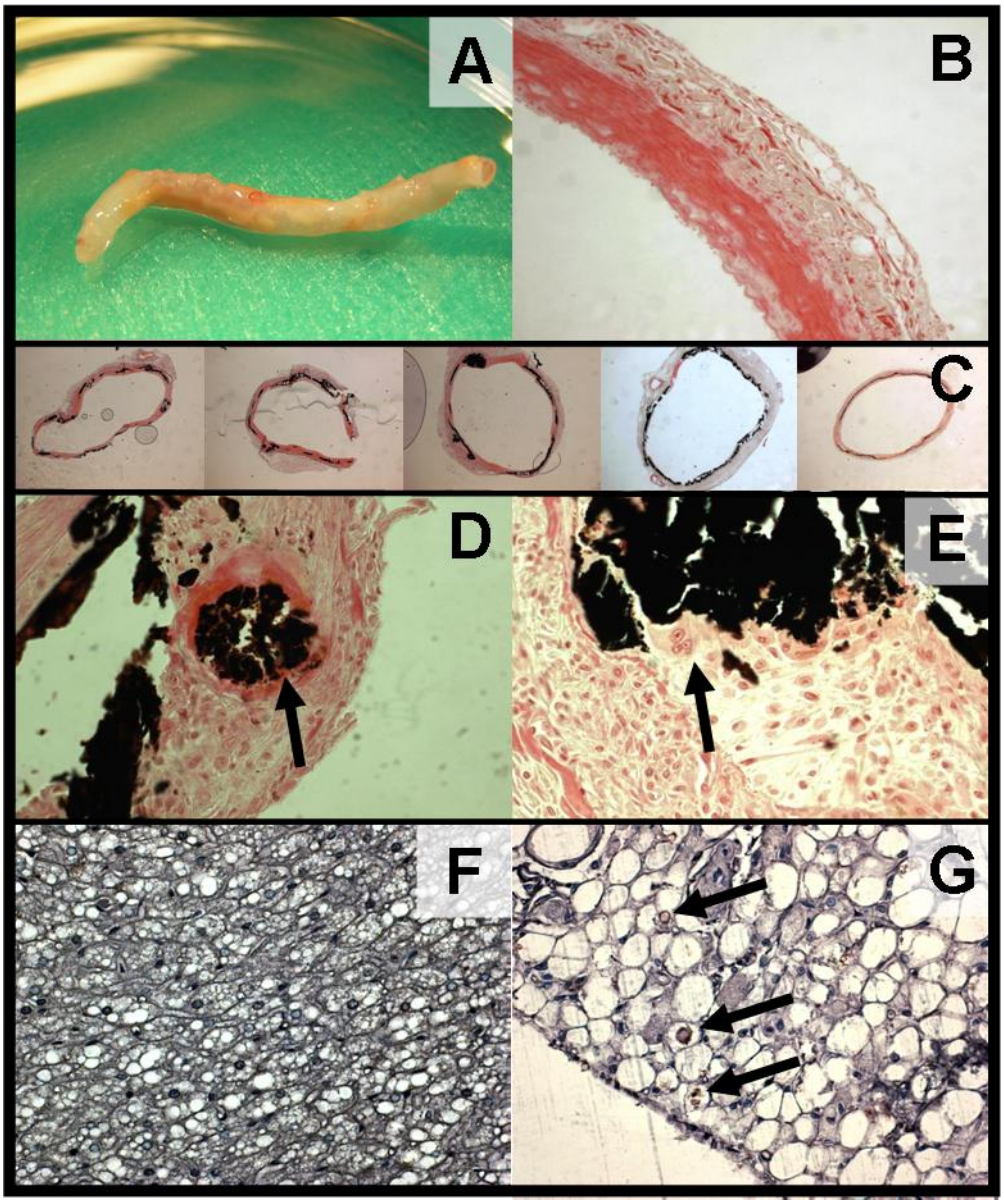

Figure 2

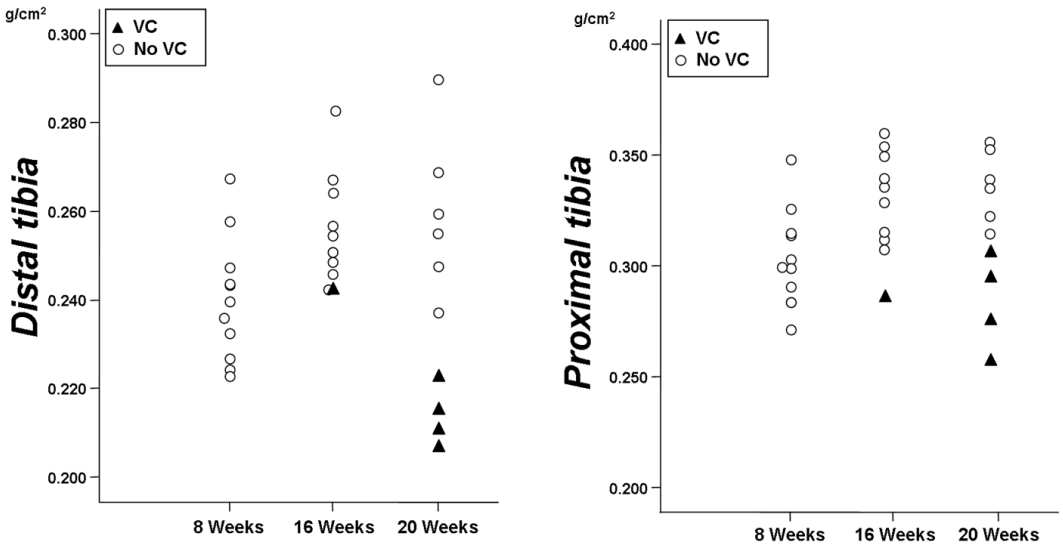
g/cm²
VC
No VC
Distal tibia
0.300
0.280
0.260
0.240
0.220
0.200
8 Weeks
16 Weeks
20 Weeks
g/cm²
VC
No VC
Proximal tibia
0.400
0.350
0.300
0.250
0.200
8 Weeks
16 Weeks
20 Weeks

Figure 3

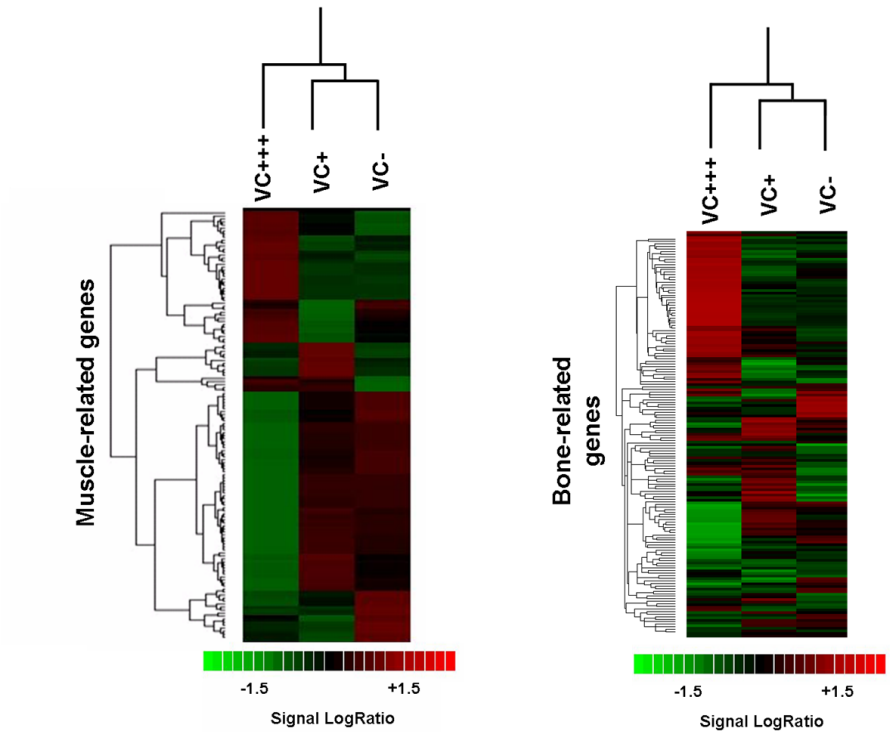
VC+++
VC+
VC-
Muscle-related genes
-1.5
+1.5
Signal LogRatio
VC+++
VC+
VC-
Bone-related genes
-1.5
+1.5
Signal LogRatio

Figure 4