UNIVERSIDAD D CÓRDOBA

Programa de Doctorado en Biociencias y Ciencias Agroalimentarias de la Universidad de Córdoba

INFLUENCE OF DIET CALORIC CONTENT ON PHOSPHATE METABOLISM AND VASCULAR CALCIFICATION

INFLUENCIA DEL CONTENIDO CALÓRICO DE LA DIETA SOBRE EL METABOLISMO DEL FÓSFORO Y LA CALCIFICACIÓN VASCULAR

THESIS presented by RAFAEL RÍOS VARO, Master´s degree in Translational Biomedical Research, for the degree of DOCTOR.

MEMORIA DE TESIS DOCTORAL presentada por RAFAEL RÍOS VARO, Máster en investigación Biomédica Traslacional, para optar al grado de DOCTOR

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TITULO: *Influence of diet caloric content on phosphate metabolism and vascular calcification*

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Department of Animal Medicine and Surgery

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DOCTORAL THESIS

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Por Compendio de Publicaciones

con Mención Internacional

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RAFAEL RÍOS VARO

Córdoba, 2018

D. ESCOLÁSTICO AGUILERA TEJERO, DOCTOR EN VETERINARIA POR LA UNIVERSIDAD DE CÓRDOBA, CATEDRÁTICO DEL DEPARTAMENTO DE MEDICINA Y CIRUGÍA ANIMAL DE LA UNIVERSIDAD DE CÓRDOBA

INFORMA:

Que don RAFAEL RÍOS VARO, Licenciado en Biología y en Bioquímica, ha realizado bajo mi dirección en el Departamento de Medicina y Cirugía Animal de la Universidad de Córdoba, el trabajo titulado: *"Influence of diet caloric content on phosphate metabolism and vascular calcification (Influencia del contenido calórico de la dieta sobre el metabolismo del Fósforo y la Calcificación Vascular)"*, y que a mi criterio dicho trabajo reúne los méritos suficientes para optar al Grado de Doctor.

Y para que así conste y surta los efectos oportunos, firmo el presente informe en Córdoba, a seis de septiembre de dos mil dieciocho.

Dª ANA ISABEL RAYA BERMÚDEZ, DOCTORA EN VETERINARIA POR LA UNIVERSIDAD DE CÓRDOBA, PROFESORA CONTRATADA DOCTOR DEL DEPARTAMENTO DE MEDICINA Y CIRUGÍA ANIMAL DE LA UNIVERSIDAD DE CORDOBA

INFORMA:

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TÍTULO DE LA TESIS: INFLUENCE OF DIET CALORIC CONTENT ON PHOSPHATE METABOLISM AND VASCULAR CALCIFICATION (Influencia del contenido calórico de la dieta sobre el metabolismo del Fósforo y la Calcificación Vascular)

DOCTORANDO/A: RAFAEL RÍOS VARO

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El doctorando Rafael Ríos Varo ha venido colaborando activamente con nuestro grupo de investigación desde el año 2013. Dicha colaboración se inició con su incorporación tras la realización del Máster de Investigación Biomédica Traslacional durante el curso de 2010-2011 y mientras concluía su segunda licenciatura, en Biología, durante los años 2011-2014. Durante este periodo inició una labor investigadora que junto con las Licenciaturas en Biología y en Bioquímica encontraron su continuación académica en los estudios de Doctorado. Su proyecto de Tesis Doctoral está encuadrado en el Programa de Doctorado de la Universidad de Córdoba correspondiente al área de Biociencias y Ciencias Agroalimentarias (P.D. con mención de calidad), estando a su vez adscrito a la línea de investigación Fisiopatología del Metabolismo Mineral.

La presente Tesis Doctoral, llevada a cabo bajo nuestra supervisión y dirección, se ha desarrollado durante el periodo comprendido entre 2013 y 2018. Durante dicho periodo el doctorando ha mostrado una gran dedicación e interés en las tareas de investigación asignadas. De igual modo, ha sido capaz de realizar una labor de investigación con gran validez científica, fruto de la cual se han originado varias publicaciones en revistas de alto interés científico en el área de biociencias.

Además, con objeto de completar su formación y profundizar en el estudio del metabolismo mineral, el doctorando realizó una estancia de tres meses en la Universidad de Londres (University College of London, Reino Unido).

El trabajo realizado en esta Tesis Doctoral ha generado la siguiente producción científica:

a) Publicaciones en revistas científicas indexadas:

Rios R, Pineda C, Lopez I, Muñoz-Castañeda J, Rodriguez M, Aguilera-Tejero E, Raya AI. Phosphorus restriction does not prevent the increase in fibroblast growth factor 23 elicited by high fat diet. *PLoS ONE,* 2018; 13: e0198481.

Rios R, Raya AI, Pineda C, Rodriguez M, Lopez I, Aguilera-Tejero E. Vitamin E protects against extraskeletal calcification in uremic rats fed high fat diets. *BMC Nephrology,* 2017; 18: 374.

Acevedo LM, Raya AI, **Rios R**, Aguilera-Tejero E, Rivero JL. Obesity-induced discrepancy between contractile and metabolic phenotypes in slow- and fast-twitch skeletal muscles of female obese Zucker rats. *Journal of Applied Physiology*, 2017; 123(1): 249-259.

Lopez I, Pineda C, Raya AI, Rodriguez-Ortiz ME, Diaz-Tocados JM, **Rios R**, Rodriguez JM, Aguilera-Tejero E, Almaden Y. Leptin directly stimulates parathyroid hormone secretion. *Endocrine*, 2017; 56(3): 675-678.

Raya AI, **Rios R**, Pineda C, Rodriguez-Ortiz ME, Diez E, Almaden Y, Muñoz-Castañeda JR, Rodriguez M, Aguilera-Tejero E, Lopez I. Energy-dense diets increase FGF23, lead to phosphorus retention and promote vascular calcifications in rats. *Scientific Reports*, 2016; 6: 36881.

b) Artículos enviados a revistas científicas indexadas:

Pineda C, **Rios R**, Raya AI, Rodriguez M, Aguilera-Tejero E, Lopez I. Differential effect of high phosphorus intake on FGF21 in rats fed normo- and hypocaloric diets. *Nutrients* (under review, revised version submitted on August 27, 2018)

c) Comunicaciones científicas en Congresos Internacionales

Lopez I, Esquinas P, **Rios-Varo R**, Pineda C, Raya AI, Rodriguez M, Aguilera-Tejero E. Restriction of Both P and Caloric Intake Decrease Renal Damage Induced by Cafeteria-Style Diet. ASN kidney week. New Orleans 2017, USA.

López I, Pineda C, Raya AI, Rodriguez-Ortiz ME, Diaz-Tocados JM, **Ríos R**, Rodríguez M, Aguilera-Tejero E, Almaden Y. Leptin directly stimulates parathyroid hormone secretion. European Society of Endrocrinology. 18th European Congress of Endocrinology. Munich 2016. Germany.

Raya AI, **Ríos R**, Pineda C, López I, Rodríguez M, Aguilera-Tejero E. Dietary intake of high calories and high phosphate prometes renal lesión in rats. 52nd ERA-EDTA Congress. London 2015, United Kingdom.

Raya AI, Pineda C, **Ríos R**, Montes de Oca A, Rodríguez M, Aguilera E, López I. High fat diet induce calcification in nephrectomized rats. ASN kidney week. Philadelphia 2014, USA.

Raya AI, Pineda CM; Guerrero F; **Ríos R**, Aguilera E, Peralta A, López I. Hypercaloric diet promotes vascular calcification in uremic rats. 51st ERA-EDTA Congress. Amsterdam 2014, The Netherlands.

d) Comunicaciones científicas en Congresos Nacionales

Rios R, Pineda C, López I, Aguilera E, Raya AI. Influencia de la restricción de fósforo en la dieta sobre el perfil lipídico de ratas. VI Congreso científico de jóvenes investigadores en formación de la UCO y V Congreso científico de jóvenes investigadores en formación en Agroalimentación CeiA3, Córdoba 2018.

Esquinas P, **Rios R**, Pineda C, Raya AI, Rodriguez JM, Aguilera-Tejero E, López I. La restricción calórica previene el daño renal en ratas alimentadas con dietas ricas en fósforo. XLVII Congreso Nacional de la S.E.N., Burgos 2017.

Raya AI, **Ríos R**, Pineda C, López I, Rodríguez M, Aguilera-Tejero E. Renal damage induced by diets rich in fat and phosphate. VI Jornadas de Jóvenes Investigadores (IMIBIC), Córdoba 2015.

Ríos R, Pineda C, Aguilera E, López I, Rodríguez M, Raya AI. Niveles elevados de fósforo en la dieta producen daño renal incipiente en ratas. IV Congreso científico de jóvenes investigadores en formación de la UCO y III Congreso científico de jóvenes investigadores en formación en Agroalimentación CeiA3, Córdoba 2014.

Ríos R, Pineda C, Rodríguez M, López I, Aguilera E, Raya AI. Fractional excretion of phosphate is increased in rats fed hypercaloric diet. V jornadas de jóvenes investigadores (IMIBIC), Córdoba 2014

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 6 de SEPTIEMBRE de 2018

Firma del/de los director/es

Fdo.: Dr. ESCOLÁSTICO AGUILERA TEJERO

Fdo.:Dra. ANA ISABEL RAYA BERMÚDEZ

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DOCTORAL THESIS
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RAFAEL RÍOS VARO
Córdoba, 2018

CONTENTS

1. Introduction

Obesity and its associated complications (metabolic syndrome) are a major health concern due to their influence on quality of life, morbidity and mortality. In addition, they represent a significant cost to Health Systems (Popkin et al. 2012; Wang et al. 2011). The etiopathogenesis of obesity/metabolic syndrome (OB/MS) is multifactorial and includes genetic factors, sedentary life style and over-nutrition (Gonzalez-Bulnes et al. 2016; Riccardi et al. 2004). Regarding the latter, intake of energy dense foods (fast food) is a substantial problem in the Western World, that is associated to OB/MS both in children and adults (Salazar et al. 2006). In addition to the increase in body weight, OB/MS is characterized by changes in blood profile that include: dyslipidemia (increased plasma concentrations of triglycerides and cholesterol) (Sastre et al. 2013), type II diabetes (increased blood glucose and insulin), and changes in circulating adipokines (e.g. hyperleptinemia) (Cheng et al. 2012). Rodent models for the investigation of OB/MS include rats or mice with genetic predisposition to develop obesity, usually hyperphagic animals like the Zucker or OLEFT rats (Wang et al. 2014). A different strategy is the use of wild-type animals fed calorie dense diets that usually are rich in fat, i.e. high fat diets (Chooi, 2013).

Mineral metabolism pivots around the homeostasis of two major minerals: calcium (Ca) and phosphorus (P). In vertebrates, body Ca and P is preferentially located in the skeleton. These minerals, particularly P, are also abundant in the intracellular compartment (Jüpnner, 2011). Extracellular concentrations of Ca and P are tightly regulated by a complex hormonal system that includes: parathyroid hormone (PTH), calcitriol (CTR) and fibroblast growth factor 23 (FGF23) (Quinn et al. 2013).

Parathyroid hormone is secreted by the chief cells of the parathyroid glands in response to hypocalcemia and/or hyperphosphatemia. The main action of PTH is to increase extracellular Ca, by extracting Ca from bone and by limiting urinary excretion of Ca

(Rodriguez et al. 2012). Indirectly, PTH promotes intestinal absorption of Ca secondary to PTH stimulation of CTR synthesis. In addition, PTH reduces plasma P by increasing phosphaturia (Knöpfel et al. 2017)

Calcitriol is the main metabolite of vitamin D. Vitamin D (cholecalciferol) is ingested with food and, in some species, may also be synthesized in the skin. Subsequently, cholecalciferol undergoes hydroxylation in the liver giving rise to 25-cholecalciferol (calcidiol), a vitamin D metabolite with minor biological activity but that is very useful as a marker of vitamin D intake (Blomberg-Jensen et al. 2010). Thereafter, calcidiol is hydroxylated again in the kidney to form 1,25-dihydroxyvitamin D (calcitriol), the most active vitamin D metabolite. Calcitriol is further metabolized to other compounds that have slight activity, like 24,25-dihydroxyvitamin D (Honkakoski and Negishi, 2000). Calcitriol actions are aimed to elevate both extracellular Ca and P by increasing bone resorption and intestinal absorption of these minerals. As mentioned above, CTR production is stimulated by PTH and, in turn, CTR also regulates PTH – it does inhibit PTH synthesis and proliferation of parathyroid cells. Therefore, a feed-back loop that regulates CTR and PTH is established between the kidneys and the parathyroid glands (Prosser and Jones, 2004).

Fibroblast growth factor 23 (FGF23), a recently discovered phosphatonin, is synthetized by osteocytes/osteoblasts to promote phosphaturia. To increase urinary excretion of P, FGF23 needs to bind to its receptors (FGFRs) and to a co-receptor, alfa-klotho (commonly named just klotho) (Muñoz-Castañeda et al. 2017; Hu et al. 2010). Fibroblast growth factor 23 is regulated by both CTR and PTH. Calcitriol is a major stimulant of FGF23 synthesis and in turn, FGF23 inhibits CTR production. Thus a second feed-back loop can be identified between kidney and bone (Martin et al. 2010). Parathyroid hormone is also known to stimulate FGF23 synthesis, in part by a direct action and in part through the increased synthesis of CTR (Silver et al. 2012). The influence of FGF23 on PTH is more controversial, although most studies

point out to an inhibitory effect of FGF23 on PTH (Kawakami et al. 2017; Krajisnik et al. 2007). This generates the third feed-back loop in mineral metabolism that connects bone and the parathyroid glands. In addition to its contribution to mineral metabolism, FGF23 is an important metabolite because different studies have demonstrated a direct relationship between circulating levels of FGF23 and cardiovascular mortality both in uremic patients and in the general population (Ali et al. 2014; Stompor, 2014).

Focussing on P metabolism, the main topic of this Thesis, P enters the body through the gastrointestinal tract after being ingested with foods (Marks et al. 2013). Intestinal absorption of P takes place mainly in the small intestine both by passive and active transport mechanisms in which are implicated sodium-phosphate co-transporters (NaPis), mainly NaPiIIb (Lee and Marks, 2015) Part of the ingested P is eliminated with the feces. The absorbed P enters blood where it is kept relatively constant at levels around 1 mM by moving P inside the cells and into the skeleton and by excreting P, mainly by urine. Extracellular P is maintained within physiologic limits by the combined action of PTH (that tends to decrease plasma P by promoting phosphaturia), CTR (that elevates plasma P by facilitating enteric absorption of P and movement of P from bone to the extracellular compartment) and FGF23 (that, like PTH, decreases plasma P by increasing phosphaturia) (Rafi and Razzaque, 2017). Thus, urinary excretion of P plays a central role in P homeostasis and is achieved by inhibiting P reabsorption in the proximal tubules. Both PTH and FGF23 down-regulate the main sodium-phosphate transporter at renal level, NaPiIIa, thus favoring urinary excretion of P (Tani et al. 2007).

Although traditionally have been considered independent regulatory systems, there is a growing evidence pointing out to a close relationship between mineral metabolism and energy metabolism. Examples of this association include studies that link obesity with osteoporosis (Migliaccio et al. 2011), calcium intake (Tremblay and

Gilbert, 2011) and hyperparathyroidism (Cheng et al. 2011). Leptin, an adipokine that is elevated in obese individuals, has been shown to stimulate parathyroid hormone secretion (Lopez et al. 2017). Calcitriol is also related to obesity since vitamin D-receptor knockout rodents display a lean phenotype and are resistant to obesity (Narvaez et al. 2009). Furthermore, mineral metabolism is interrelated with glucose homeostasis -magnesium has protective actions against metabolic syndrome and hypomagnesemia is associated to hyperglycemia and hyperinsulinemia (Guerrero-Romero et al. 2008).

More specifically, P metabolism is also related to energy metabolism. The influence of P on glucose and lipid metabolism has been known for a long time. In humans, hypophosphatemia has been associated to glucose intolerance due to tissue insensitivity to insulin (DeFronzo and Lang, 1980) and glucose disposal rate has been reported to increase after phosphate infusion (Nowicki et al. 1996). Experiments in rats have shown that dietary P deprivation stimulates liver gluconeogenesis and glucogenolysis suggesting that the liver may be implicated in the modulation of glucose homeostasis induced by P deficiency (Xie et al. 1999; Xie et al. 2000). In mice, dietary P restriction has been reported to induce lipid accumulation in the liver through dysregulation of genes involved in the hepatic metabolism of cholesterol (Tanaka et al. 2013). More recently, Abuduli et al. (2016) have described with detail the influence of dietary phosphate on glucose and lipid metabolism, demonstrating an effect not only of P restriction but also of increased P intake. Their data show that a high P diet improves glucose regulation, down-regulates hepatic lipid synthesis and increases the expression of proteins that prevent visceral fat accumulation. The relationship between dietary P and energy metabolism is bidirectional. As explained above, P can regulate energy metabolism but it is also known that energy intake modulates parameters related to P regulation. Leptin, an adipokine that has a profound influence on the regulation of energy intake and that is

consistently increased in obesity (Pan and Myers, 2018), has been shown to regulate the synthesis and secretion of FGF23 (Tsuji et al. 2010).

Western diets that promote OB/MS due to their high caloric content are also typically rich in P (Popking et al. 2012; Wang et al. 2011). In addition to proteins, the use of P as a food additive increases the P content of many processed foods (Sarathy et al. 2008; Uribarri and Calvo, 2003). Moreover, the P used as an additive is inorganic P which is much more readily absorbed in the intestine than the P naturally contained in foods (Frommelt et al. 2014). As a consequence, individuals eating processed fast food are often exposed to both and excess of calories and P. Since P is readily excreted by urine, P overload is not critical when the kidneys are healthy. However, with decreased renal function (either due to disease or to aging) P overload can represent a significant problem (Chen et al. 2004; Muñoz-Castañeda et al. 2017).

Chronic kidney disease (CKD), characterized by a progressive decline in renal function, may be related to OB/MS. In fact, diabetic nephropathy is a major cause of CKD in humans (Gheith et al. 2016). Independent of its etiology, CKD always results in severe changes in mineral metabolism that are usually referred to as secondary hyperparathyroidism (SHPTH) or chronic kidney disease-metabolic bone disease (CKD-MBD) (Cozzolino et al. 2005). Patients with CKD, cannot correctly eliminate P by urine and this leads to a tendency to hyperphosphatemia. Renal synthesis of CTR is also compromised in CKD and the decreased CTR concentrations determine a tendency to hypocalcemia. To maintain extracellular P and Ca within physiologic levels the parathyroid glands increase synthesis and secretion of PTH that, in addition to increase phosphaturia, promotes bone resorption to restore extracellular Ca levels. Progression of CKD results in aggravation of SHPTH and a progressive demineralization of the skeleton (Migliaccio et al. 2011). Phosphaturia is further enhanced

during CKD by increased secretion of FGF23. Thus the hormonal profile of CKD patients is typically characterized by elevated plasma concentrations of PTH and FGF23 and decreased concentrations of CTR (Rodriguez et al. 2012).

One of the major complications of CKD-MBD is the development of extraskeletal calcification. Mineralization of soft tissues, particularly vascular calcification (VC), is very important because it is directly related to cardiovascular morbidity and mortality in CKD patients (Block et al. 2004). Uremic VC affects mainly the tunica media (arteriosclerosis) and it is closely related to elevated concentrations of extracellular P (Villa-Bellosta et al. 2011). During the process of VC, in addition to mineral deposition, vascular smooth muscle cells (VSMCs) undergo a phenotypic transformation by which they lose expression of muscular genes (e.g. alfa-actin) and gain expression of osteogenic genes (osterix, sclerotin, RunX2, etc.) (Evrard et al. 2015).

The mechanisms by which OB/MS may contribute to the severity of VC are more likely multifactorial and include changes in lipid and adipokine profile (Irribarren et al. 2007), diabetes mellitus (Towler et al. 1998), inflammation (Guerrero et al. 2011) and oxidative stress (Mody et al. 2001). Recent studies in obese Zucker rats fed normocaloric diets have demonstrated that oxidative stress plays an important role in the development of VC. Moreover in these studies treatment with vitamin E significantly reduced extraskeletal calcifications (Peralta-Ramirez et al. 2014).

REFERENCES

Abuduli, M., Ohminami, H., Otani, T., Kubo, H., Ueda, H., Kawai, Y., Masuda, M., Yamanaka-Okumura, H., Sakaue, H., Yamamoto, H., Takeda, E. and Taketani, Y. 2016. Effects of dietary phosphate on glucose and lipid metabolism. *Am. J. Physiol. Endocrinol. Metab.*, 310:E526-38.

Ali, F.N., Falkner, B., Gidding, S.S., Price, H.E., Keith, S.W. and Langman, C.B. 2014. Fibroblast growth factor-23 in obese, normotensive adolescents is associated with adverse cardiac structure. *J. Pediatr.*, 165:738-43

Block, G.A., Klassen, P.S., Lazarus, J.M., Ofsthun, N., Lowrie, E.G. and Chertow, G.M. 2004. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J. Am. Soc. Nephrol.*, 15:2208-18.

Blomberg-Jensen, M., Andersen, C.B., Nielsen, J.E., Bagi, P., Jørgensen, A., Juul, A. and Leffers, H. 2010 Expression of the vitamin D receptor, 25-hydroxylases, 1alpha-hydroxylase and 24-hydroxylase in the human kidney and renal clear cell cancer. *J. Steroid Biochem. Mol. Biol.*, 121:376-82.

Chen, J., Muntner, P., Hamm, L.L., Jones, D.W., Batuman, V., Fonseca, V., Whelton, P.K. and He, J. 2004. The metabolic syndrome and chronic kidney disease in U.S. adults. *Ann. Intern. Med.*, 140:167-74.

Cheng, S.P., Doherty, G.M., Chang, Y.C. and Liu, C.L. 2011. Leptin: the link between overweight and primary hyperparathyroidism? *Med. Hypotheses*, 76:94-6.

Cozzolino, M., Brancaccio, D., Gallieni, M. and Slatopolsky, E. 2005. Pathogenesis of vascular calcification in chronic kidney disease. *Kidney Int.*, 68:429-36.

DeFronzo, R.A. and Lang, R. 1980. Hypophosphatemia and glucose intolerance: evidence for tissue insensitivity to insulin. *N. Engl. J. Med.*, 303:1259-63.

Evrard, S., Delanaye, P., Kamel, S., Cristol, J.P., Cavalier, E. 2015. Vascular calcification: from pathophysiology to biomarkers. Clinica Chimica Acta, 438:401-14.

Frommelt, L., Bielohuby, M., Stoehr, B.J.M., Menhofer, D., Bidlingmaier, M. and Kienzle, E. 2014. Effects of low-carbohydrate, high-fat diets on apparent digestibility of minerals and trace elements in rats. *Nutrition*, 30:869-75.

Gheith, O., Farouk, N., Nampoory, N., Halim, M.A. and Al-Otaibi, T. 2016. Diabetic kidney disease: world wide different of prevalence and risk factors. *J. Nephropharmacol.*, 5:49-56

Gonzalez-Bulnes, A., Astiz, S., Ovilo, C., Garcia-Contreras, C. and Vazquez-Gomez, M. 2016. Nature and Nurture in the Early-Life Origins of Metabolic Syndrome. *Curr. Pharm. Biotechnol.*, 17:573-86.

Guerrero-Romero, F., Rascón-Pacheco, R.A., Rodríguez-Morán, M., de la Peña, J.E. and Wacher, N. 2008. Hypomagnesaemia and risk for metabolic glucose disorders: a 10-year follow-up study. *Eur. J. Clin. Invest.*, 38:389-96.

Guerrero, F., Montes de Oca, A., Aguilera-Tejero, E., Zafra, R., Rodriguez, M. and Lopez, I. 2011. The effect of vitamin D derivatives on vascular calcification associated to inflammation. *Nephrol. Dial. Transplant.*, 27:2206-12.

Honkakoski, P. and Negishi, M. 2000. Regulation of cytochrome P450 (CYP) genes by nuclear receptor. *Biochem. J.*, 347:231-7.

Hu, M.C., Shi, M., Zhang, J., Pastor, J., Nakatani, T., Lanske, B., Razzaque, M.S., Rosenblatt, K.P., Baum, M.G., Kuro-o, M. and Moe, O.W. 2010. Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J.*, 24:3438-50

Iribarren, C., Husson, G., Go, A.S., Lo, J.C., Fair, J.M., Rubin, G.D., Hlatky, M.A. and Fortmann, S.P. 2007. Plasma leptin levels and coronary artery calcification in older adults. *J. Clin. Endocrinol. Metab.*, 92:729-32.

Jüppner, H. 2011. Phosphate and FGF-23. *Kidney Int. Suppl.*, 79:S24-7

Kawakami, K., Takeshita, A., Furushima, K., Miyajima, M., Hatamura, I., Kuro-o, M., Furuta, Y. and Sakaguchi, K. 2017. Persistent fibroblast growth factor 23 signalling in the parathyroid glands for secondary hyperparathyroidism in mice with chronic kidney disease. *Sci. Rep.*, 7:40534.

Knöpfel, T., Pastor-Arroyo, E.M., Schnitzbauer, U., Kratschmar, D.V., Odermatt, A., Pellegrini, G., Hernando, N. and Wagner, C.A. 2017. The intestinal phosphate transport NaPi-IIb (Slc34a2) is required to protect bone during dietary phosphate restriction. *Sci. Rep.*, 7:11018.

Krajisnik, T., Björklund, P., Marsell, R., Ljunggren, Ö., Akerström, G., Jonsson, K.B., Westin, G. and Larsson, T.E. 2007. Fiboblast growth factor-23

regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cell. *J. endocrinol.*, 195:125-31.

Lee, C.Y. 2013. The effect of High-Fat Diet-Iduced pathophysiological Canges in the gut on obesity: What should be the ideal treatment? *Clinical and Translational Gastroenterology*, 4e39

Lee, G.L. and Marks, J. 2015. Intestinal phosphate transport: a therapeutic target in chronic kidney disease and beyond? *Pedriatr. Nephrol.*, 30:363-71.

Lopez, I., Pineda, C., Raya, A.I., Rodriguez-Ortiz, M.E., Diaz-Tocados, J.M., Rios, R., Rodriguez, J.M., Aguilera-Tejero, E. and Almaden, Y. 2017. Leptin directly stimulates parathyroid hormone secretion. *Endocrine*, 56:675-8.

Marks, J., Debnam, E.S. and Unwin, R.J. 2013. The role of the gastrointestinal tract in phosphate homeostasis in health and chronic kidney disease. *Curr. Opin. Nephrol. Hypertens.*, 22:481-7.

Martin, A., David, V. and Quarles, L.D. 2012. Regulation and function of the FGF23/klotho endocrine pathways. *Physiol. Rev.*, 92:131-55.

Migliaccio, S., Greco, E.A., Fornari, R., Donini, L.M. and Lenzi, A. 2011. Is obesity in women protective against osteoporosis? *Diabetes Metab. Syndr. Obes.*, 4:273-82.

Mody, N., Parhami, F., Sarafian, T.A. and Demer, L.L. 2001. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic. Biol. Med.*, 31:509-19.

Muñoz-Castañeda, J.R., Herencia, C., Pendón-Ruiz de Mier, M.V., Rodriguez-Ortiz, M.E., Diaz-Tocados, J.M., Vergara, N., Martínez-Moreno, J.M., Salmerón, M.D., Richards, W.G., Felsenfeld, A., Kuro-o, M., Almaden, Y. and Rodríguez, M. 2017. Differential regulation of renal Klotho and FGFR1 in normal and uremic rats. *FASEB J.*, 31:3858-67.

Narvaez, C.J., Matthews, D., Broun, E., Chan, M. and Welsh, J. 2009. Lean phenotype and resistance to diet-induced obesity in vitamin D receptor knockout mice correlates with induction of uncoupling protein-1 in white adipose tissue. *Endocrinology*, 150:651-61.

Nowicki, M., Fliser, D., Fode, P. and Ritz, E. 1996. Changes in plasma phosphate levels influence insulin sensitivity under euglycemic conditions. *J. Clin. Endocrinol. Metab.*, 81:156-9.

Pan, W.W. and Myers, M.G.Jr. 2018. Leptin and the maintenance of elevated body weight. *Nat. Rev. Neurosci.*, 19:95-105.

Peralta-Ramírez, A., Montes de Oca, A., Raya, A.I., Pineda, C., López, I., Guerrero, F., Díez, E., Muñoz-Castañeda, J.R., Martinez, J., Almaden, Y., Rodriguez, M. and Aguilera-Tejero E. 2014. Vitamin E protection of obesity-enhanced vascular calcification in uremic rats. *Am. J. Physiol. Renal Physiol.*, 306:F422-9.

Popkin, B.M., Adair, L.S. and Ng, S.W. 2012. Global nutrition transition and the pandemic of obesity in developing countries. *Nutr. Rev.*, 70:3-21.

Prosser, D.E. and Jones, G. 2004. Enzymes involved in the activation and inactivation of vitamin D. TRENDS in Biochem. Sci., 29:664-73.

Quinn, S.J., Thomsen, A.R.B., Pang, J.L., Kantham, L., Bräuner-Osborne, H., Pollak, M., Goltzman, D. and Brown, E.M. 2012. Interactions between calcium and phosphorus in the regulation of the production of fibroblast growht factor 23 in vivo. *Am. J. Physiol. Encocrinol. Metab.*, 304: E310-20

Rafi, S.K. and Razzaque, M.S. 2017. Hormonal regulation of phosphorus homeostasis: Parathyroid Hormone, Fibroblast Growth Factor 23, and klotho. *Nutr. and Health*, 29-44

Riccardi, G., Giacco, R. and Rivellese, A.A. 2004. Dietary fat, insulin sensitivity and the metabolic syndrome. *Clin. Nutr.*, 23:447-56.

Rodriguez, M., Lopez, I., Muñoz, J., Aguilera-Tejero, E. and Almaden, Y. 2012. FGF23 and mineral metabolism, implication in CKD-MBD. *Nefrología*, 32:275-8

Salazar-Martinez, E., Allen, B., Fernandez-Ortega, C., Torres-Mejia, G., Galal, O. and Lazcano-Ponce, E. 2006. Overweight and obesity status among adolescents from Mexico and Egypt. *Arch. Med. Res.*, 37:535-42.

Sarathy, S., Sullivan, C., Leon, J.B., Sehgal, A.R. 2008. Fast food, phosphorus-containing additives, and the renal diet. *J. Ren. Nutr.*, 18:466-70.

Sastre, C., Rubio-Navarro, A., Buendía, I., Gómez-Guerrero, C., Blanco, J., Mas, S., Egido, J., Blanco-Colio, L.M., Ortiz, A. and Moreno, J.A. 2013. Hyperlipidemia-associated renal damage decreases Klotho expression in kidneys from ApoE knockout mice. *PLoS One*, 8:e83713.

Silver, J., Rodriguez, M. and Slatopolsky, E. 2012. FGF23 and PTH – double agents at the hearth of CKD. *Nephrol. Dial. Transplant.*, 27:1715-20.

Stompor, T. 2014. Coronary artery calcification in chronic kidney disease: An update. *World J. Cardiol.*, 6:115-29.

Tanaka, S., Yamamoto, H., Nakahashi, O., Kagawa, T., Ishiguro, M., Masuda, M., Kozai, M., Ikeda, S., Taketani, Y. and Takeda, E. 2013. Dietary phosphate restriction induces hepatic lipid accumulation through dysregulation of cholesterol metabolism in mice. *Nutr. Res.*, 33:586-93.

Tani, Y., Sato, T., Yamanaka-Okumura, H., Yamamoto, H., Arai, H., Sawada, N., Genjida, K., Taketani, Y. And Takeda, E. 2007. Effects of prolonged high phosphorus diet on phosphorus and calcium balance in rats. *J. Clin. Biochem. Nutr.*, 40:221-8.

Towler, D.A., Bidder, M., Latifi, T., Coleman, T. and Semenkovich, C.F. 1998. Diet-induced diabetes activates an osteogenic gene regulatory program in the aortas of low density lipoprotein receptor-deficient mice. *J. Biol. Chem.*, 273:30427-34.

Tremblay, A. and Gilbert, J.A. 2011. Human obesity: is insufficient calcium/dairy intake part of the problem? *J. Am. Coll. Nutr.*, 30:449S-53S.

Tsuji, K., Maeda, T., Kawane, T., Matsunuma, A. and Horiuchi, N. 2010. Leptin stimulates fibroblast growth factor 23 expression in bone and suppresses renal 1alpha,25-dihydroxyvitamin D3 synthesis in leptin-deficient mice. *J. Bone Miner. Res.*, 25:1711-23.

Uribarri, J. and Calvo, M.S. 2003. Hidden sources of phosphorus in the typical American diet: does it matter in nephrology? *Semin. Dial.*, 16:186-8.

Villa-Bellosta, R., Millan, A. and Sorribas, V. 2011. Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *Am. J. Physiol. Cell Physiol.*, 300:C210-20.

Wang, B., Chandrasekera, P.C. and Pippin, J.J. 2014. Leptin- and Leptin Receptor-Deficient Rodent Models: Relevance for Human Type 2 Diabetes. *Curr. Diabetes Rev.*, 10:131-45.

Wang, Y.C., McPherson, K., Marsh, T., Gortmaker, S.L. and Brown, M. 2011. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet.* , 378:815-25.

Xie, W., Li, Y., Mechin, M.C. and van de Werve, G. 1999. Up-regulation of liver glucose-6-phosphatase in rats fed with a Pi-deficient diet. *Biochem. J.*, 343:393-6.

Xie, W., Tran, T.L., Finegood, D.T., van de Werve, G. 2000. Dietary Pi deprivation in rats affects liver cAMP, glycogen, key steps of gluconeogenesis and glucose production. *Biochem. J.*, 352:227-32.

2. Aims of the Study

This Thesis is based on the general Hypothesis that Mineral Metabolism and Energy Metabolism are linked. More specifically it was hypothesized that abundant energy intake in the form of high fat diets would modify mineral metabolism (blood minerals, calciotropic hormones, soft tissue mineralization) both in healthy individuals and in subjects with decreased renal function.

To test this hypothesis the following objectives were proposed:

1. To investigate the effect of feeding a high fat diet on phosphorus balance in rats with normal and decreased renal function.
2. To evaluate the influence of feeding a high fat diet on the axis fibroblast growth factor 23/alfa-klotho in rats with normal and decreased renal function.
3. To investigate changes in calciotropic hormones (parathyroid hormone, calcitriol, calcidiol, etc.) after feeding high fat diets to rats with normal and decreased renal function.
4. To evaluate the influence of feeding a high fat diet on the development of vascular calcification in uremic rats with and without calcitriol treatment.
5. To investigate the influence of vitamin E supplementation on uremic vascular calcification in rats fed high fat diets.
6. To evaluate whether reducing phosphorus load by feeding a diet with low phosphorus content would prevent the increase in fibroblast growth factor 23 elicited by high fat diet.

Estos objetivos se relacionan con los siguientes trabajos:

- Scientific Report, 6: 36881, 2016: Objetivos 1, 2, 3 y 4
- BMC Nephrology, 18: 374, 2017: Objetivos 2, 3, 4 y 5
- PLos ONE, 13: e0198481, 2018: Objetivos 2 y 6

3. Article 1

Energy-dense diets increase FGF23, lead to phosphorus retention and promote vascular calcifications in rats

ABSTRACT

Rats with normal renal function (Experiment 1, n=12) and uninephrectomized (1/2Nx) rats (Experiment 2, n=12) were fed diets with normal P (NP) and either normal (NF) or high fat (HF). Rats with intact renal function (Experiment 3, n=12) were also fed NF or HF diets with high P (HP). Additionally, uremic (5/6Nx) rats (n=16) were fed HP diets with NF or HF. Feeding the HF diets resulted in significant elevation of plasma FGF23 vs rats fed NF diets: Experiment 1, 593±126 vs 157±28 pg/ml ($p<0.01$); Experiment 2, 538±105 vs 250±18 pg/ml ($p<0.05$); Experiment 3, 971±118 vs 534±40 pg/ml ($p<0.01$). Rats fed HF diets showed P retention and decreased renal klotho (ratio klotho/actin) vs rats fed NF diets: Experiment 1, 0.75±0.06 vs 0.97±0.02 ($p<0.01$); Experiment 2, 0.69±0.07 vs 1.12±0.08 ($p<0.01$); Experiment 3, 0.57±0.19 vs 1.16±0.15 ($p<0.05$). Uremic rats fed HF diet showed more severe vascular calcification (VC) than rats fed NF diet (aortic Ca=6.3±1.4 vs 1.4±0.1 mg/g tissue, $p<0.001$). In conclusion, energy-rich diets increased plasma levels of FGF23, a known risk factor of cardiovascular morbidity and mortality. Even though FGF23 has major phosphaturic actions, feeding HF diets resulted in P retention, likely secondary to decreased renal klotho, and aggravated uremic VC.

INTRODUCTION

Energy-dense foods (fast-foods) are implicated in the etiopathogenesis of dysmetabolic syndrome, a significant and growing health problem both in Western and developing countries (Popking et al. 2012; Wang et al. 2011). In addition to their high caloric content, these foods are usually rich in phosphate (P), which is widely used as a food additive (Sarathy et al. 2008; Uribarri and Calvo, 2003). Moreover, the high fat content of fast-food is likely to increase P digestibility (Frommelt et al. 2014), further aggravating P load. Phosphate overload may cause an increase in fibroblast growth factor 23 (FGF23), which is a known factor of cardiovascular risk (Panwar et al. 2015). Although there is an increasing awareness of the detrimental effects of the P content of fast-food, the influence of the caloric content of the diet on P balance has not been studied in detail.

Phosphate overload may be tolerated when renal function is intact; however, a decline in renal function, which is associated to high-fat intake and type II diabetes (Chen et al. 2004), compromises P handling. In fact, in patients with chronic kidney disease (CKD), elevated serum phosphate plays a major role in the development of vascular calcification (VC) (Block et al. 2004). Phosphate promotes VC through a series of mechanisms, including increased serum CaxP product, which leads to precipitation of calcium salts, and phenotypic transdifferentiation of vascular smooth muscle cells (VSMCs) to osteogenic cells (Giachelli, 2009; Villa-Bellosta et al. 2011).

We hypothesized that feeding a diet with high caloric content (high fat diet) would cause an increase in FGF23 in an attempt to control P balance, and would promote VC when renal function is impaired. Thus, the objectives of the present study were:

- To evaluate changes in FGF23 and P balance induced by high fat diets in rats with normal and reduced renal function; and,

- To investigate the influence of high fat diets on the development of VC in uremic rats.

MATERIAL AND METHODS

Ethics approval. All experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba (Cordoba, Spain). All protocols were carried out in accordance with the approved guidelines. They followed the guidelines laid down by the Higher Council of Scientific Research of Spain following the normal procedures directing animal welfare (Real Decreto 223/88, BOE of 18 of March) and adhered to the recommendations included in the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, NIH) and European laws and regulations on protection of animals, under the advice of specialized personnel.

Animals, surgical procedures and diets. Three months-old Wistar rats, provided by the Animal Housing Facilities of the University of Cordoba (Cordoba, Spain), were housed with a 12h/12h light/dark cycle. Appropriate measures were taken to ensure animal welfare and to address the basic behavioral and physiological needs of rats. Renal function was reduced by either 1/2 nephrectomy (Nx) or 5/6 Nx. The former was performed by unilateral Nx while the 5/6 Nx was accomplished in a two-step procedure that reduces the original renal mass by five-sixths leading to uremia. These procedures are described in detail elsewhere (Lopez et al. 2006). Briefly, animals were anesthetized using xylazine (5 mg/kg, ip) and ketamine (80 mg/kg, ip). For the first step of the 5/6 Nx, a 5- to 8-mm incision was made on the left mediolateral surface of the abdomen. The left kidney was exposed, and the two poles (2/3 of renal mass) were ablated. The kidney was inspected and returned to an anatomically neutral position within the peritoneal cavity. The abdominal wall and skin incisions were closed with sutures, and the rat was placed back into its home cage. After 1 week of recovery, in the second step, the animal was reanesthetized and a 5- to 8-mm incision was made on the right mediolateral surface of

the abdomen. The right kidney was exposed and unencapsulated, the renal pedicle was clamped and ligated, and the kidney was removed. The ligated pedicle was returned to a neutral anatomical position and the abdomen and skin incisions closed with suture materials. The procedure for 1/2 Nx was identical to the second step of the 5/6 Nx. Phentanyl (0.2 mg/kg, ip) was used as analgesic agent.

Diets with two P concentrations were used in the experiments: normal P (0.6%) diet (NP) and high P (1.2%) diet (HP). Independent of their P content, diets had either a normal fat content (NF diet) with a 5% fat concentration that provided Metabolizable Energy = 3518 kcal/kg (Altromin C 1000, Altromin Spezialfutter GmbH, Germany) or a high fat content (HF diet) with a 35% fat concentration that provided Metabolizable Energy = 5241 kcal/kg (Altromin C 1090-60, Altromin Spezialfutter GmbH, Germany). All diets had 0.6% of Ca and 500 IU/g of vitamin D.

Experimental design. *Studies on P balance*. These experiments were conducted with the rats housed in metabolic cages, allowing daily control of food and water intake and collection of urine and feces. During the first week the animals were adapted to the cages and received the control diet with 0.6% P and normal fat (NP-NF). Then, rats were switched to experimental diets in three Experiments:

- Experiment 1- Rats with intact renal function were allotted to 2 groups (n=6 each). Rats in group 1 were fed normal P and normal fat diet (NP-NF) and rats in group 2 were fed normal P and high fat diet (NP-HF) for 30 days. Water and food intake were recorded daily during the whole experiment. During the last week of the trial feces and urine were collected daily to assess P balance. At day 30, rats were sacrificed by exsanguination under general anesthesia (thiopental, 20 mg/kg, ip) to obtain blood and tissue samples.

- Experiment 2- Rats with reduced (1/2 Nx) renal function (n=12) were fed either normal P and normal fat (NP-NF, n=6) or normal P and high fat (NP-HF, n=6) diet for 30 days, following the same protocol as in Experiment 1.
- Experiment 3- Rats with intact renal function were fed HP diet (1.2% P) and were allotted to 2 groups (n=6 each). Rats in group 1 were fed normal fat diet (HP-NF) and rats in group 2 were fed high fat diet (HP-HF) for 30 days, following the same protocol as in Experiments 1 and 2.
- *Studies on vascular calcification (VC)*. These studies (Experiment 4) were aimed at evaluating the effect of feeding HF diet on the development of VC in uremic rats. Sixteen rats were fed a NP diet with either normal fat content (NP-NF, n=6) or high fat content (NP-HF, n=10) for 45 days. At day 45 the first step of the 5/6 Nx was conducted and a week later the 5/6 Nx was completed (day 52). After the second step of 5/6 Nx rats were switched to a HP (1.2%) diet. The fat content of the diet was maintained unchanged; thus rats received HP-NF (n=6) or HP-HF (n=10) diets. Rats were sacrificed at day 112 to obtain blood and tissue samples.

Blood chemistries. Blood for chemistry analyses was obtained at the time of sacrifice (intraperitoneal thiopental anesthesia plus exsanguination), from the abdominal aorta. Blood for measurements of ionized calcium levels was collected in heparinized syringes and immediately analyzed using a Ciba-Corning 634 ISE Ca2+/pH Analyzer (Ciba-Corning, Essex, England). Afterwards, plasma was separated by centrifugation and stored at –20° C until assayed. Plasma creatinine, urea, triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol and phosphorus were measured by spectrophotometry (BioSystems SA, Barcelona, Spain). ELISA tests were used to quantify

plasma FGF23 (Kainos Laboratories, Tokyo, Japan) and PTH (Immutopics, San Clemente, CA). Radioimmunoassay (Immunodiagnostic Systems Ltd, Boldon, UK) was used in plasma samples to determine 25-hydroxyvitamin D (calcidiol) and 1,25-dihydroxyvitamin D (calcitriol).

Urine and feces analysis. Fecal samples were dried, ashed and demineralized with 0.6 mmol/l HNO3 solution. Fecal P was measured by inductively coupled plasma mass spectrophotometry (ICP-MS, Perkin Elmer Elan DRC-e, Walthan, Massachusetts, USA). Urinary phosphorus was measured by spectrophotometry (BioSystems SA, Barcelona, Spain). Net intestinal absorption (equation (1)) and P balance (equation (2)) were calculated as follows:

(1) P net absorption (mg/day) = P intake – P fecal excretion
(2) P balance mg/day = P net absorption – P urinary excretion

Protein extraction and Western blot analysis. Proteins were isolated from renal tissue by using a lysis buffer containing HEPES (10 mmol/l), KCl (10 mmol/l), EDTA (0.1 mmol/l), EGTA (0.1 mmol/l), DTT (1 mmol/l), PMSF (0.5 mmol/l), protease inhibitor cocktail (70 µg/ml), and I-Gepal CA-630 (0.6%), pH 7.9 (Sigma Aldrich, St. Louis, MO). Protein concentration was determined by Bradford method. For Western Blot analysis, 50 µg of protein were electrophoresed on a 10% SDS-polyacrylamide gel (Invitrogen, Carlsbad, CA) and electrophoretically transferred (Transfer Systems, BioRad, Hercules, CA) from the gels onto nitrocellulose membranes (Invitrogen). The following steps were performed with gentle shaking. Membranes were incubated in TTBS-L solution [20 mM Tris-HCl (pH 7.6), 0.2% Tween 20, 150 mM NaCl] (Sigma Aldrich), and 5% nonfat dry milk (Bio-Rad) at room temperature for 1 hour to avoid nonspecific binding. Membranes were then washed with TTBS buffer (the same composition as TTBS-L

without nonfat dry milk) and incubated for 2 hours at room temperature with a mouse anti-FGFR1 antibody (GeneTex, Irvine, CA; 0.5 µg/ml) or rat anti-klotho antibody (Alpha Diagnostic Int., San Antonio, TX; 0.5 µg/ml). The membranes were then washed with TTBS buffer and immunolabeled using a peroxidase-conjugated secondary antibody (1:5000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Finally, they were revealed on autoradiographic film using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ). Beta-Actin was used as housekeeping protein to ensure equal loading of the gels. Protein levels were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Real-Time-Polymerase Chain Reaction (RT-PCR). Study of bone FGF23 and aortic Runx-2, Osterix and Sclerostin mRNA was performed by Quantitative Real-Time PCR. Tibial bone or aorta were disrupted using liquid nitrogen and grinded thoroughly with a mortar. Aortic total RNA was extracted from the lysate using RNA extraction kit (RNeasy Fibrous Tissue Mini Kit, Qiagen, Germany). Bone RNA was extracted using the chloroform and isopropanol precipitation method and a treatment with DNAse I Amplification Grade (Sigma-Aldrich). Fifty ng of total RNA were used to analyze mRNA expression in the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA). RT-PCR was performed in one step, using the QuantiTect SYBR Green RT-PCR kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Primers for Runx-2, Osterix and GAPDH were designed with the free Oligo 7 software. The Sclerostin primer was purchased to Integrated DNA Technologies, Inc (San Diego, CA, USA). Primer sequences are listed below:

FGF23- F: 5'-TTGGATCGTATCACTTCAGC-3',
R: 5'-TGCTTCGGTGACAGGTAG-3';
Runx-2- F: 5'-CGG GAATGATGAGAACTACTC-3',
R: 5'-GCG GTCAGAGAACAAACTAGGT-3';
Osterix- F: 5'-GTACGGCAAGGCTTCGCATCTGA-3',
R: 5´-TCAAGTGGTCGCTTCGGGTAAAG-3';
GAPDH- F: 5'-AGGGCTGCCTTCTCTTGTGAC-3',
R: 5'-TGGGTAGAATCATACTGGAACATGTAG-3'.

The expression of target genes was normalized to GAPDH as housekeeping and calculated according to the 2Δ(ΔCT) method.

Assessment of vascular calcification (VC). Following sacrifice, the thoracic aorta, stomach and lungs were dissected and processed to study mineral content. Calcification was studied by measuring the tissue calcium and phosphorus content. The tissues were demineralized in 10% formic acid (aorta) and 150 mM HCl (stomach and lungs). The calcium and phosphorus content was measured in the supernatant according to a method previously described (Lopez et al. 2006). Fresh tissue was also fixed in 10% buffered formalin, embedded in paraffin, and cut into 3 μm sections. Paraffin-embedded sections of the aorta, lung and stomach were stained by the Von Kossa method to evaluate mineralization.

Statistics. Values are expressed as the mean ± standard error (SE). The difference between means for two different groups was determined by t-test; the difference between means for three or more groups was assessed by Analysis of Variance (ANOVA). Fisher Least Significant Difference (LSD) test was used as a post-hoc procedure. A correlation study was carried out using the Pearson test. $p<0.05$ was considered significant.

RESULTS

Studies on P balance. Phosphorus balance was studied in rats with intact and reduced (1/2Nx) renal function fed diets with normal P (NP) and either normal fat (NF) or high fat (HF); and in rats with normal renal function fed high P (HP) diets with NF or HF. In all experiments, rats fed HF diets reduced their food intake when compared when rats fed NF diets. Therefore rats fed HF diets ingested less P than rats fed NF diets. However, the ingested P was more readily absorbed in rats fed HF diets than in rats fed NF diets. This resulted in almost the same net absorption of P and thus in a nearly identical P load in NF vs HF groups: rats with intact renal function fed NP, 44.5±3.2 mg/day vs 44.1±2.4 mg/day; 1/2Nx rats fed NP, 42.1±1.6 mg/day vs 47.3±2.0 mg/day; rats with intact renal function fed HP, 94.5±6.5 mg/day vs 95.9±4.2 mg/day. Urinary P excretion was not different in rats fed HF or NF and NP. In rats fed HP, P excretion by urine was lower ($p<0.05$) in rats fed HF diet, 85.0±3.6 mg/day, than in rats fed NF diet, 101.3±4.0 mg/day. In rats with intact renal function fed NP, a non-significant tendency to P retention was detected with HF diet, 14.8±1.8 mg/day vs 12.9±2.2 mg/day in rats fed NF diet. Phosphorus retention in rats on HF diets became significant ($p<0.05$) in 1/2Nx rats fed NP, 19.3±1.8 mg/day vs 10.0±2.2 mg/day in rats on NF diet, and in rats fed HP, 10.9±4.4mg/day vs -6.8±5.2 mg/day in rats on NF diet (Table 1).

In the groups receiving NP, even though P load was similar, rats fed HF diets showed consistently higher plasma FGF23 concentrations than rats fed NF diets: rats with intact renal function fed NP, 593±126 pg/ml vs 157±28 pg/ml ($p<0.01$), and 1/2Nx rats fed NP, 538±105 pg/ml vs 250±18 pg/ml ($p<0.05$). Feeding a HP diet resulted in higher FGF23 levels than feeding NP diet and rats fed HP-HF diet also had significantly ($p<0.01$) elevated FGF23, 995±120 pg/ml, when compared with rats fed HP-NF diet, 547±40 pg/ml (Fig. 1). HF diets elicited increased expression of FGF23 in bone. In a subset of rats fed NP, bone mRNA FGF23/GAPDH ratio increased 3 times in rats fed HF

(n=6) vs rats fed NF (n=6) diets (3.6±0.9 vs 1.1±0.5, $p<0.05$). It is interesting to note that even though plasma FGF23 concentrations were significantly higher in rats fed HF diets than in rats fed NF diets, urinary excretion of P was not increased in the animals fed HF diets (Table 1)

	Experiment 1		Experiment 2		Experiment 3	
	NF (n=6)	**HF** (n=6)	**NF** (n=6)	**HF** (n=6)	**NF** (n=6)	**HF** (n=6)
P Intake (mg/day)	93.06 ± 5.15	64.08 ± 3.16[a]	82.60 ± 3.73	66.24 ± 1.48[a]	156.40 ± 7.06	135.00 ± 5.08[a]
P Fecal Excretion (mg/day)	48.59 ± 2.32	20.02 ± 1.41[a]	43.04 ± 1.49	18.09 ± 1.93[a]	61.97 ± 3.42	39.03 ± 1.00[a]
P Net Absorption (mg/day)	44.47 ± 3.23	44.05 ± 2.44	42.14 ± 1.59	47.31 ± 2.04	94.46 ± 6.47	95.93 ± 4.22
P Urinary Excretion (mg/day)	31.60 ± 1.79	29.25 ± 2.02	29.50 ± 1.82	30.64 ± 1.54	101.29 ± 4.03	85.04 ± 3.63[a]
P Balance (mg/day)	12.86 ± 2.18	14.79 ± 1.81	10.04 ± 2.24	19.34 ± 1.76[a]	-6.83 ± 5.17	10.90 ± 4.44[a]

Table 1. Parameters related to phosphorus (P) balance in rats fed diets with normal fat (NF) and high fat (HF) content in Experiments 1-3. Experiment 1 (n=12): rats with intact renal function fed 0.6% P, Experiment 2 (n=12): 1/2Nx rats fed 0.6% P, Experiment 3 (n=12): rats with intact renal function fed 1.2% P. [a]$p < 0.05$ vs NF in the same Experiment.

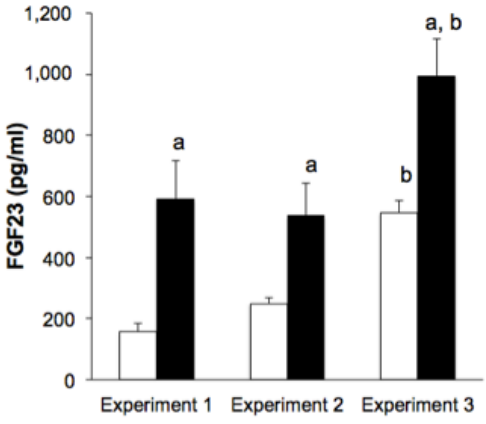

Fig. 1. Influence of the fat content of the diet on fibroblast growth factor 23 (FGF23). Plasma concentrations of FGF23 in rats fed normal fat (NF, white bars □) or high fat (HF, black bars ■) diets in the studies on P balance. Experiment 1 (n=12): rats with intact renal function fed 0.6% P, Experiment 2 (n=12): 1/2Nx rats fed 0.6% P, Experiment 3 (n=12): rats with intact renal function fed 1.2% P. $^{a}p < 0.05$ vs NF in the same Experiment, $^{b}p < 0.05$ vs NF in Experiment 1.

No significant differences between NF and HF groups were found in plasma concentrations of P, iCa, creatinine, total cholesterol, triglycerides and PTH. As expected, 1/2 Nx rats had higher plasma creatinine and urea. Similarly, rats fed HP tended to show higher PTH values. Plasma concentrations of calcidiol were similar in all groups and were not influenced by the increased caloric content in HF diets, except in rats fed HP-HF diet which had higher calcidiol. However, feeding HF diets resulted in significant reductions in plasma concentrations of calcitriol when compared with rats fed NF diets: rats with intact renal function fed NP, 11.3±1.1 pg/ml vs 82.2±11.7 pg/ml ($p<0.001$); 1/2Nx rats fed NP, 20.8±10.8 pg/ml vs 117.1±23.7 pg/ml ($p<0.01$); rats with intact renal function fed HP, 74.8±25.9 pg/ml vs 143.0±11.8 pg/ml ($p<0.05$) (Table 2).

Figure 2 shows the levels of klotho and FGFR1 proteins in the kidney. Changes in renal klotho were well correlated with changes in

plasma FGF23 concentrations. A significant decrease in renal klotho (ratio klotho/β-actin) was identified in all rats fed HF diets: rats with intact renal function fed NP, 0.75±0.06 vs 0.97±0.02 in rats fed NF diet ($p<0.01$); 1/2Nx rats fed NP, 0.69±0.07 vs 1.12±0.08 in rats fed NF diet ($p<0.01$); and rats with intact renal function fed HP, 0.57±0.19 vs 1.16±0.15 in rats fed NF diet ($p<0.05$) (Fig. 2A). Feeding HF diets did not influence FGFR1 expression in the kidney (Fig. 2B).

	Experiment 1		Experiment 2		Experiment 3	
	NF (n=6)	**HF** (n=6)	**NF** (n=6)	**HF** (n=6)	**NF** (n=6)	**HF** (n=6)
Creatinine (mg/dl)	0.58 ± 0.02	0.55 ± 0.02	0.66 ± 0.02[b]	0.72 ± 0.02[b]	0.57 ± 0.01	0.57 ± 0.01
Urea (mg/dl)	22.05 ± 2.75	20.28 ± 1.60	62.85 ± 4.55[b]	51.84 ± 5.97[b]	25.13 ± 2.07	22.94 ± 2.76
Triglycerides (mg/dl)	40.92 ± 5.44	45.11 ± 5.29	39.64 ± 4.61	40.78 ± 3.25	32.17 ± 4.17	40.50 ± 5.66
Total cholesterol (mg/dl)	50.21 ± 3.79	51.57 ± 2.80	48.11 ± 2.69	47.10 ± 2.92	44.71 ± 4.11	52.70 ± 2.92
iCalcium (mmol/l)	1.33 ± 0.01	1.35 ± 0.01	1.28 ± 0.01	1.27 ± 0.01	1.27 ± 0.01	1.26 ± 0.01
Phosphorus (mg/dl)	5.21 ± 0.33	5.93 ± 0.34	5.44 ± 0.21	5.66 ± 0.27	4.98 ± 0.26	6.19 ± 0.52
PTH (pg/ml)	16.99 ± 5.74	11.52 ± 2.88	23.86 ± 6.58	23.44 ± 7.66	25.31 ± 6.10	46.59 ± 14.57[b]
Calcidiol (ng/ml)	30.29 ± 2.33	25.65 ± 3.43	40.85 ± 4.52	42.17 ± 2.21	30.72 ± 2.87	64.34 ± 5.47[a,b]
Calcitriol (pg/ml)	82.19 ± 11.68	11.33 ± 1.11[a]	117.10 ± 23.70	20.78 ± 10.78[a]	143.00 ± 11.78	74.79 ± 25.92[a]

Table 2. Blood biochemistry in rats fed diets with normal fat (NF) and high fat (HF) content in Experiments 1-3. Experiment 1 (n=12): rats with intact renal function fed 0.6% P, Experiment 2 (n=12): 1/2Nx rats fed 0.6% P, Experiment 3 (n=12): rats with intact renal function fed 1.2% P. [a]$p < 0.05$ vs NF in the same Experiment, [b]$p < 0.05$ vs NF in Experiment 1.

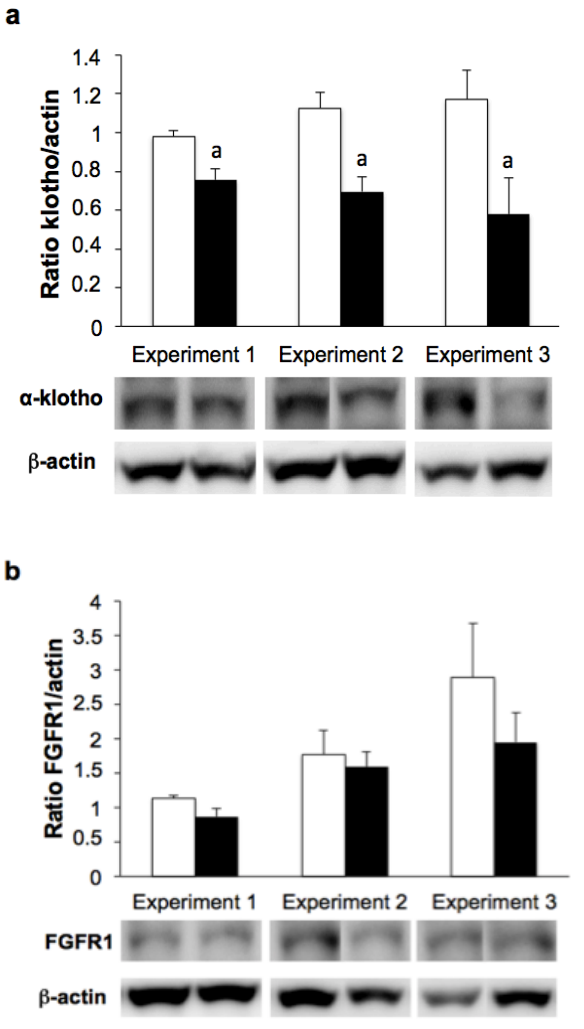

Fig. 2. Influence of the fat content of the diet on renal klotho and fibroblast growth factor receptor 1 (FGFR1). (A) Western Blots of klotho and (B) FGFR1 obtained from renal tissue of rats fed normal fat (NF, white bars □) or high fat (HF, black bars ■) diets in the studies on P balance. Experiment 1 (n=12): rats with intact renal function fed 0.6% P, Experiment 2 (n=12): 1/2Nx rats fed 0.6% P, Experiment 3 (n=12): rats with intact renal function fed 1.2% P. $^{a}p < 0.05$ vs NF in the same Experiment.

Studies on vascular calcification (VC). Uremic rats fed HP-NF diet only experienced very minor mineral deposition in vascular tissue: aortic calcium=1.4±0.1 mg/g tissue, aortic phosphorus=0.1±0.1

mg/g tissue. However, 5/6 Nx rats on HP-HF diet showed consistent increases in both aortic calcium (6.3±1.4 mg/g tissue) and phosphorus (12.4±7.2 mg/g tissue), $p<0.001$ vs NF diet. Similar results were obtained in other soft tissues and extraskeletal calcification was also confirmed by von Kossa staining (Fig. 3). Feeding HF diets not only resulted in mineral deposition but also in a phenotypic transdifferentiation of the vessel wall that was evidenced by an increase in the expression of osteogenic genes. Thus, mRNA Runx-2/GAPDH ratio was increased by 2.8 fold, mRNA/GAPDH Osterix was increased by 2.4 fold and mRNA Sclerostin/GAPDH was increased by 3.8 fold in rats fed HF diet when compared with rats fed NF diet (Fig. 4).

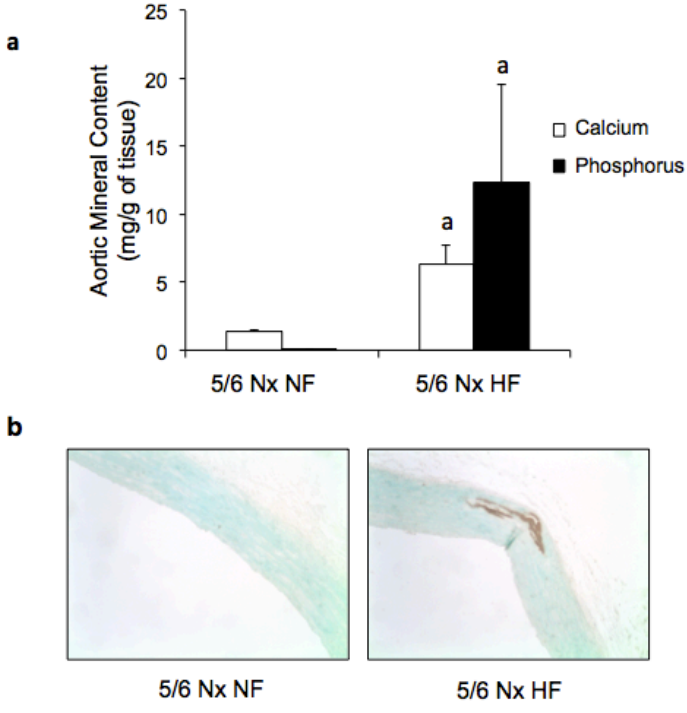

Fig. 3. Influence of the fat content of the diet on vascular calcification. (A) Calcium (white bars □) and phosphorus (black bars ■) content (mg/g of tissue) in the aortas of uremic (5/6 Nx) rats fed high 1.2% P and either normal fat (NF, n=6) or high fat (HF, n=10) diets in Experiment 4. (B) Von Kossa stained sections of the aortas of 5/6Nx rats fed high 1.2% P and either NF (left) or HF (right). Calcification foci are stained in brown. $^{a}p < 0.05$ vs NF.

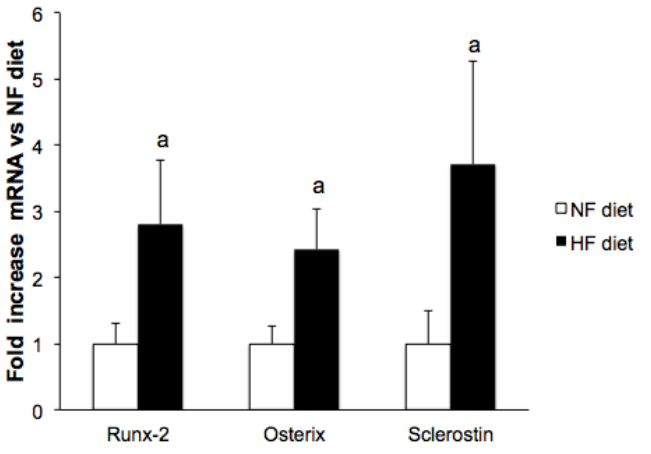

Fig. 4. Influence of the fat content of the diet on the expression of osteogenic genes in aortic tissue. Levels of mRNA Runx-2/GAPDH, mRNA Osterix/GAPDH and mRNA Sclerostin/GAPDH in the aortas of uremic (5/6 Nx) rats fed 1.2% P and either normal fat (NF, white bars □) or high fat (HF, black bars ■) diets in Experiment 4 (n= 6 NF and 10 HF). $^{a}p < 0.05$ vs NF.

No differences between uremic rats fed NF and HF diets were observed in plasma creatinine, urea, iCa, P and PTH. Plasma triglycerides, LDL cholesterol and HDL cholesterol tended to be elevated in rats fed HF diet but significant differences with rats fed NF diet were only found in total cholesterol (111.9±6.4 vs 84.2±3.5 mg/dl). Plasma FGF23 was significantly higher (11,366±1,639 vs 4,799±2,402 pg/ml, $p<0.05$) in rats fed HF diet (Table 3) and was correlated with aortic calcium ($r=0.640$, $p=0.001$). The increase in FGF23 was accompanied by a marked but non-significant decrease in the expression of klotho (ratio klotho/β-actin) in the remnant kidney of 5/6 Nx rats, from 1.00±0.39 in rats fed NF diet to 0.55±0.16 in rats fed HF diet.

	Experiment 4	
	NF (n=6)	**HF** (n=10)
Creatinine (mg/dl)	0.91 ± 0.09	1.01 ± 0.10
Urea (mg/dl)	88.56 ± 30.51	80.02 ± 8.72
Triglycerides (mg/dl)	44.71 ± 10.15	58.11 ± 13.88
Total cholesterol (mg/dl)	84.15 ± 3.51	111.88 ± 6.41[a]
LDL cholesterol (mg/dl)	15.68 ± 3.11	18.35 ± 2.06
HDL cholesterol (mg/dl)	37.51 ± 8.39	45.87 ± 3.89
iCalcium (mmol/l)	1.09 ± 0.03	1.11 ± 0.01
Phosphorus (mg/dl)	6.1 ± 1.4	7.8 ± 1.2
FGF23 (pg/ml)	4,799 ± 2,402	11,366 ± 1,639[a]
PTH (pg/ml)	468 ± 116	476 ± 54

Table 3. Blood biochemistry in uremic (5/6 Nx) rats fed 1.2% P and either normal fat (NF) or high fat (HF) diets in Experiment 4. [a]$p < 0.05$ vs NF.

DISCUSSION

This study was designed to investigate the effect of feeding energy-dense diets on P balance and VC in rats with normal and reduced renal function. Our results show that rats fed diets with high fat content decreased renal klotho expression, developed P retention due to impaired renal excretion of P, and increased plasma FGF23 concentrations. Moreover, uremic rats fed HF diets showed more severe VC than their normocaloric-fed counterparts.

The study experimental design was based on comparing the effect of diets with identical P concentration and different caloric content. For this purpose a high-fat diet, which is commercialized to induce obesity in rodents, was used. As previously reported (Ainslie et al. 2011), during the experiments the rats fed HF diets reduced food consumption to maintain a constant caloric intake (around 50 kcal/day). Thus, after eating HF or NF diets for 30 days, no differences in body weight were observed between groups. This allowed to study the effect of the caloric content of the diet independent of obesity-mediated mechanisms (e.g. adipokines and other inflammatory mediators released by fat tissue). Although the reduction in food intake in rats fed HF diets resulted in a decreased P intake, in agreement with previous reports (Frommelt et al. 2014) the intestinal absorption of P was increased in rats fed HF diets. The decrease in P intake was compensated by the increase in intestinal absorption of P in rats fed HF diets resulting in almost identical P load in HF vs NF groups.

Feeding HF diets was consistently associated to increases in plasma FGF23 concentrations in all experimental groups. Although FGF23 has been reported to be increased in obese people (Marsell et al. 2009) and a recent study identified energy intake as a potential predictor of plasma FGF23 concentrations (di Giussepe et al. 2015), to the best of our knowledge this is the first report demonstrating a direct relationship between ingestion of a high calories/high fat diet and increases in plasma concentrations of FGF23. Circulating levels of

FGF23 are a prognostic factor for cardiovascular disease in patients with CKD (Scialla et al. 2014) and in the general population (Kestenbaum et al. 2014). Thus, the demonstration of elevated FGF23 after feeding energy dense diets provides additional insight into the deleterious effect of these diets on cardiovascular health.

One of the most intriguing findings of the present work is that the increase in FGF23 elicited by HF diets was not accompanied by an increase in renal excretion of P. These data strongly suggest resistance to the phosphaturic action of FGF23. To promote phosphaturia in the kidney tubule, FGF23 needs to bind to the klotho-FGFR complex (Kuro-o, 2010). Therefore, the demonstration of reduced klotho protein levels in the kidneys of rats fed HF diet may explain the resistance to the phosphaturic action of FGF23 found in the present study. Renal klotho has been reported to be decreased in hyperlipidemic ApoE knockout mice and the authors suggested that hyperlipidemia-associated kidney injury might be responsible for the decreased renal expression of klotho (Sastre et al. 2013). While the renal toxicity of high fat diets is well documented (Boini et al. 2014), additional mechanisms might be involved in the decrease of klotho induced by feeding HF diets. Klotho intervenes in energy regulation at different levels and klotho knockout mice display a lean phenotype (Razzaque, 2012). Moreover, rats subjected to caloric restriction have been shown to increase renal klotho mRNA expression and klotho protein (Miyazaqui et al. 2010). Thus, in addition to renal toxic effects, energy-dense diets may directly downregulate renal klotho. Klotho down-regulation by high caloric intake could have significant health consequences since, as shown in this study, it will lead to impaired renal excretion of P and P retention.

Feeding HF diets resulted in low plasma calcitriol concentrations and calcitriol was significantly lower in rats fed HF diets than in rats fed NF diets. The vitamin D content was identical in HF and NF diets. As explained above, the rats fed HF diets ingested less food and this might have resulted in decreased vitamin D intake; but, on the

other hand, diets with high fat content have been reported to increase vitamin D absorption by the intestine (Dawson-Hudges et al. 2015). In any case, plasma calcidiol concentrations were not decreased in rats fed HF diets. Since calcidiol is the vitamin D metabolite that best reflects nutritional intake (Holick, 2007), the origin of the decreased plasma calcitriol in rats fed HF diets cannot be attributed to decreased dietary intake of vitamin D. The most likely explanation for the decreased calcitriol is a reduction in calcitriol synthesis secondary to the increase in FGF23, which is known to inhibit the 1-alpha-hydroxylase activity in the kidney (Shimada et al. 2004). The decrease in serum calcitriol after eating diets with high fat content may also have significant health implications because vitamin D deficiency is associated with cardiovascular disease, diabetes and metabolic syndrome (Dobnig et al. 2008; Parker et al. 2010).

Extraskeletal calcification is a common feature in uremic patients and VC represents an important contributor to the high rate of cardiovascular mortality associated to CKD (Block et al. 2004). The inability to eliminate P plays a major role in the development of uremic VC (Giachelli, 2009). Data from Experiments 1-3 indicated that HF diets resulted in P retention particularly when rats were fed HP and, to a lesser extent, with moderate (1/2 Nx) reduction in renal function. In Experiment 4 we evaluated the effect of feeding HP-HF diets to uremic (5/6 Nx) rats. In contrast to rats fed HP-NF diets, uremic rats fed HP-HF diets showed upregulation of osteogenic genes in vascular tissue and marked VC. In addition to P retention, feeding HF diets also resulted in moderate dislipidemia which may also have contributed to the development of VC. Our data demonstrate that although P retention associated to the ingestion of fast-food can be tolerated when renal function is normal, in individuals with a significant decrease in renal function ingestion of high-fat diets may lead to VC.

CONCLUSION

In conclusion, after being fed with high fat diets rats showed decreased renal klotho expression, increased circulating levels of FGF23 and decreased plasma concentrations of calcitriol. Reduced klotho resulted in resistance to the phosphaturic action of FGF23 and P retention. Moreover, uremic rats fed hypercaloric/high fat diets developed more severe extraosseous calcifications than their normocaloric-fed counterparts.

ACKNOWLEDGMENTS

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REFERENCES

Ainslie, D.A., Proietto, J., Fam, B.C. and Thorburn, A.W. 2000. Short-term, high-fat diets lower circulating leptin concentrations in rats. *Am. J. Clin. Nutr*., 71:438-42.

Block, G.A., Klassen, P.S., Lazarus, J.M., Ofsthun, N., Lowrie, E.G. and Chertow, G.M. 2004. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J. Am. Soc. Nephrol*., 15:2208-18.

Boini, K.M., Xia, M., Abais, J.M., Li, G., Pitzer, A.L., Gehr, T.W., Zhang, Y. And Li, P.L. 2014. Activation of inflammasomes in podocyte injury of mice on the high fat diet: Effects of ASC gene deletion and silencing. *Biochim. Biophys. Acta*, 1843:836-45.

Chen, J., Muntner, P., Hamm, L.L., Jones, D.W., Batuman, V., Fonseca, V., Whelton, P.K. and He, J. 2004. The metabolic syndrome and chronic kidney disease in U.S. adults. *Ann. Intern. Med.*, 140:167-74.

Dawson-Hughes, B., Harris, S.S., Lichtenstein, A.H., Dolnikowski, G., Palermo, N.J. and Rasmussen, H. 2015. Dietary fat increases vitamin D-3 absorption. *J. Acad. Nutr. Diet*., 115:225-30.

di Giuseppe, R., Kühn, T., Hirche, F., Buijsse, B., Dierkes, J., Fritsche, A., Kaaks, R., Boeing, H., Stangl, G.I. and Weikert, C. 2015. Potential Predictors of Plasma Fibroblast Growth Factor 23 Concentrations: Cross-Sectional Analysis in the EPIC-Germany Study. *PloS One*, 10:e0133580.

Dobnig, H., Pilz, S., Scharnagl, H., Renner, W., Seelhorst, U. Wellnitz, B., Kinkeldei, J., Boehm, B.O., Weihrauch, G. and Maerz, W. 2008. Independent association of low serum 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality. *Arch. Intern. Med.*, 168:1340-9.

Frommelt, L., Bielohuby, M., Stoehr, B.J.M., Menhofer, D., Bidlingmaier, M. and Kienzle, E. 2014. Effects of low-carbohydrate, high-fat diets on apparent digestibility of minerals and trace elements in rats. *Nutrition*, 30:869-75.

Giachelli, C.M. 2009. The emerging role of phosphate in vascular calcification. *Kidney Int.*, 75:890-897.

Holick, M.F. 2007. Vitamin D deficiency. *N. Engl. J. Med*., 357:266-81.

Kestenbaum, B., Sachs, M.C., Hoofnagle, A.N., Siscovick, D.S., Ix, J.H., Robinson-Cohen, C., Lima, J.A.C, Polak, J.F., Blondon, M., Ruzinski, J., Rock, D. and de Boer, I.H. 2014. Fibroblast growth factor-23 and cardiovascular disease

in the general population: the Multi-Ethnic Study of Atherosclerosis. *Circ. Heart Fail*, 7:409-17.

Kuro-o, M. 2010. Overview of the FGF23-Klotho axis. *Pediatr. Nephrol.*, 25:583-90.

Lopez, I., Aguilera-Tejero, E., Mendoza, F.J., Almaden, Y., Perez, J., Martin, D. And Rodriguez, M. 2006. Calcimimetic R-568 decreases extraosseous calcifications in uremic rats treated with calcitriol. *J. Am. Soc. Nephrol.*, 17:795-804.

Marsell, R., Mirza, M.A.I., Mallmin, H., Karlsson, M., Mellström, D., Orwoll, E., Ohlsson, O., Jonsson, K.B., Ljunggren, Ö. and Larsson, T.E. 2009. Relation between fibroblast growth factor-23, body weight and bone mineral density in elderly men. *Osteoporos Int.*, 20:1167-73.

Miyazaki, T., Takenaka, T., Inoue, T., Sato, M., Hanyu, M., Eiki, Y., Nodera, M., Yanagisawa, H, Ohno, Y., Shibazaki, S. and Suzuki, H. 2010. Klotho expression is induced by calorie restriction in adult male rats. *Trace Nutr. Res*, 27:92-6.

Panwar, B., Jenny, N.S., Howard, V.J., Wadley, V.G., Muntner, P., Kissela, B.M., Judd, S.E. and Gutiérrez, O.M. 2015. Fibroblast growth factor 23 and risk of incident stroke in community-living adults. *Stroke*, 46:322-8.

Parker, J., Hashmi, O., Dutton, D., Mavrodaris, A., Stranges, S., Kandala, N.B., Clarke, A. and Franco, O.H. 2010. Levels of vitamin D and cardiometabolic disorders: systematic review and meta-analysis. *Maturitas*, 65:225-36.

Popkin, B.M., Adair, L.S. and Ng, S.W. 2012. Global nutrition transition and the pandemic of obesity in developing countries. *Nutr. Rev.*, 70:3-21.

Razzaque, M.S. 2012. The role of Klotho in energy metabolism. *Nat. Rev. Endocrinol.*, 8:579-87.

Sarathy, S., Sullivan, C., Leon, J.B., Sehgal, A.R. 2008. Fast food, phosphorus-containing additives, and the renal diet. *J. Ren. Nutr.*, 18:466-70.

Sastre, C., Rubio-Navarro, A., Buendía, I., Gómez-Guerrero, C., Blanco, J., Mas, S., Egido, J., Blanco-Colio, L.M., Ortiz, A. And Moreno, J.A. 2013. Hyperlipidemia-associated renal damage decreases Klotho expression in kidneys from ApoE knockout mice. *PLoS One*, 8:e83713.

Scialla, J.J., Xie, H., Rahman, M., Anderson, A.H., Isakova, T., Ojo, A., Zhang, X., Nessel, L., Hamano, T., Grunwald, J.E., Raj, D.S., Yang, W, He, J., Lash, J.P.,

Go, A.S., Kusek, J.W., Feldman, H. and Wolf, M. 2014. Fibroblast growth factor-23 and cardiovascular events in CKD. *J. Am. Soc. Nephrol.*, 25:349-60.

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., Fujita, T., Nakahara, K., Fukumoto, S. and Yamashita, T. 2014. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J. Bone Miner. Res.*, 19:429-35.

Uribarri, J. and Calvo, M.S. 2003. Hidden sources of phosphorus in the typical American diet: does it matter in nephrology? *Semin. Dial.*, 16:186-8.

Villa-Bellosta, R., Millan, A. and Sorribas, V. 2011. Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *Am. J. Physiol. Cell Physiol.*, 300:C210-20.

Wang, Y.C., McPherson, K., Marsh, T., Gortmaker, S.L. and Brown, M. 2011. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet.*, 378:815-25.

4. Article 2

Vitamin E protects against extraskeletal calcification in uremic rats fed high fat diets.

ABSTRACT

High fat diets are implicated in the pathogenesis of metabolic syndrome, obesity and renal disease. Previous studies have revealed that high fat diets promote vascular calcification in uremic rats. Moreover, vitamin E has been shown to prevent uremic calcifications in genetically obese Zucker rats fed standard diet. The objective of this study was to investigate the influence of vitamin E supplementation on the development of extraskeletal calcifications in non-obese (wild type) uremic in rats fed high fat diets.

Wistar rats (n=32) were preconditioned by feeding either a normal (NF) or high fat (HF) diet for 45 days and subsequently were subjected to 5/6 nephrectomy (Nx). Just before performing the first Nx step, a blood sample (Pre-Nx) was obtained. After Nx rats were switched to a diet with 0.9% phosphorus and supplemented with calcitriol. Also, after Nx, half of the rats from each group (NF and HF) were treated with vitamin E (VitE) in the diet (30000 mg/kg) and the other half were maintained on basic VitE requirements (27 mg/kg). Thus, rats were allotted to four experimental groups: Nx-NF (n=8), Nx-NF-VitE (n=8), Nx-HF (n=8) and Nx-HF-VitE (n=8). At the time of sacrifice (day 66), blood and tissue samples were obtained.

Feeding a HF diet for 45 days did not increase body weight but elicited hyperglycemia, hypertriglyceridemia, an increase in plasma fibroblast growth factor 23 and a reduction in plasma calcitriol concentrations. After Nx, rats fed HF diet showed substantial extraskeletal calcification with aortic calcium content that was higher than in rats fed NF diet. Supplementation with VitE significantly ($p<0.05$) reduced aortic (from 38.4±8.8 to 16.5±1.4 mg/g), gastric (from 5.6±2.7 to 1.2±0.4 mg/g) and pulmonary (from 1.8±0.3 to 0.3±0.2 mg/g) calcium content in rats on HF diets.

Uremic rats fed HF diets developed more severe extraosseous calcifications than their normocaloric-fed counterparts and dietary VitE supplementation protected against uremic calcifications in rats fed HF

diets. Thus, eating energy-rich foods should be discouraged in patients with renal disease and their deleterious effect may be ameliorated with adequate antioxidant supply.

INTRODUCTION

Energy-dense food with a high fat content is implicated in the pathogenesis of obesity and metabolic syndrome (OB/MS), a significant and growing health problem both in Western and developing countries (Salazar et al. 2006; Wang et al. 2011). The mechanisms involved in the pathogenesis of OB/MS are complex and include genetic predisposition, sedentary lifestyle and excesive intake of sugar- and fat rich foods (Gonzalez-Bulnes et al. 2016; Riccardi et al. 2004). The relationship between OB/MS and renal disease is well known and can be explained by a variety of indirect mechanisms, including hypertension and type II diabetes (Chen et al. 2004; Hall et al. 2003), and direct mechanisms, like increased glomerular capillary wall tension, changes in podocytes, down-regulation of the Sirt 1-adiponectin axis, etc. (Wickman and Kramer, 2013).

However, the influence of OB/MS on vascular calcifications (VC), one of the major contributors to cardiovascular mortality in patients with chronic kidney disease (CKD) (Block et al. 2004), has not been studied with detail. When subjected to nephrectomy, obese Zucker rats have been reported to develop more severe extraskeletal calcifications than lean Zucker rats. In this experimental model, the influence of OB/MS on calcifications seems to be partly mediated by oxidative stress and calcifications can be prevented by treatment with vitamin E (VitE) (Peralta-Ramírez et al. 2014).

Moreover, we have recently shown that uremic rats fed high fat (HF) diets also develop more severe VC than their counterparts fed normal fat (NF) diets. Phosphorus (P) retention, secondary to renal klotho and fibroblast growth factor 23 (FGF23) dysregulation, seems to be implicated in the procalcifying effect of HF diet (Raya et al. 2016). In patients with CKD, elevated serum P plays a major role in the development of VC (Ritter and Slatopolsky, 2016). High extracellular P concentrations promote VC through a series of mechanisms, including phenotypic transdifferentiation of vascular smooth muscle cells

(VSMCs) to osteogenic cells and increased serum calcium (Ca)xP product, which facilitates the deposition of Ca salts (Giachelli, 2009; Villa-Bellosta et al. 2011). Since high P also has been reported to promote oxidative stress in VSMCs (Di Marco et al. 2008), it would be important to determine whether antioxidant therapy with VitE is able to ameliorate calcifications in uremic rats fed a diet rich in fat.

We hypothesized that, as already shown in the model of genetic-obese Zucker rat, supplemental VitE might have a beneficial effect on uremic VC in non-obese wild type rats fed energy-dense diets. Thus, the objective of this study was to investigate the influence of VitE supplementation on uremic VC in rats fed HF diets.

MATERIAL AND METHODS

Ethics Approval. All experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba (Cordoba, Spain). All protocols were carried out in accordance with the approved guidelines. They followed the guidelines laid down by the Higher Council of Scientific Research of Spain following the normal procedures directing animal welfare (Real Decreto 223/88, BOE of 18 of March) and adhered to the recommendations included in the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, NIH) and European laws and regulations on protection of animals, under the advice of specialized personnel.

Animals and Surgical Procedures. Three months-old Wistar rats (n=32), provided by the Animal Housing Facilities of the University of Cordoba (Cordoba, Spain), were housed with a 12h/12h light/dark cycle, and given ad libitum access to a standard diet with normal Ca=0.6% and P=0.6%. Uremia was induced by 5/6 nephrectomy (Nx), a two-step procedure that reduces the original renal mass by five-sixths. Briefly, animals were anesthetized using xylazine (5 mg/kg, ip) and ketamine (80 mg/kg, ip). For the first step of the 5/6 Nx, a 5- to 8-mm incision was made on the left mediolateral surface of the abdomen. The left kidney was exposed, and the two poles (2/3 of renal mass) were ablated. The kidney was inspected and returned to an anatomically neutral position within the peritoneal cavity. The abdominal wall and skin incisions were closed with sutures, and the rat was placed back into its home cage. After 1 week of recovery, in the second step, the animal was reanesthetized and a 5- to 8-mm incision was made on the right mediolateral surface of the abdomen. The right kidney was exposed and unencapsulated, the renal pedicle was clamped and ligated, and the kidney was removed. The ligated pedicle was returned to a neutral anatomical position and the abdomen and skin incisions

closed with suture materials. Fentanyl (0.2 mg/kg, ip) was used as analgesic agent. After the second surgery, the mineral content of the diet was changed to a diet containing Ca=0.6% and P= 0.9% (Altromin Spezialfutter GmbH, Germany). Rats were supplemented with calcitriol, 80 ng/kg ip every other day (Calcijex, Abbot, Madrid, Spain). This dose of calcitriol has been previously shown to be effective in controlling secondary hyperparathyroidism. Independent of their P content, diets had either a normal fat content (NF diet) with a 5% fat concentration that provided Metabolizable Energy = 3518 kcal/kg (Altromin C 1000, Altromin Spezialfutter GmbH, Germany) or a high fat content (HF diet) with a 35% fat concentration that provided Metabolizable Energy = 5241 kcal/kg (Altromin C 1090-60, Altromin Spezialfutter GmbH, Germany). Sacrifice was performed by aortic puncture and exsanguination of the anesthetized (sodium thiopental, ip) rat.

Experimental Design. Sixteen rats were maintained for 45 days on NF diet and 16 rats on HF diet (both diets with 0.6% Ca and 0.6% P). At day 45, rats were subjected to Nx. Just before performing the first Nx step, a blood sample (Pre-Nx) was obtained from these 32 rats to assess the influence of the caloric content of the diet on metabolic status without the confounding effects of uremia. After the second step of Nx, at day 52, rats were switched to a 0.9%P diet and treated with calcitriol, as described above. The caloric content of the diets was maintained as before and the rats continued eating either NF or HF till the end of the experiments. Also, after Nx, half of the rats from each group (NF and HF) were supplemented with VitE in the diet (30000 mg/kg) and the other half were maintained on basic VitE requirements (27 mg/kg). Thus, the four experimental groups were: Nx-NF (n=8), Nx-NF-VitE (n=8), Nx-HF (n=8) and Nx-HF-VitE (n=8). Rats were scheduled for sacrifice at day 80, four weeks after Nx, but due to the rapid deterioration experienced by the rats fed HF, sacrifice was performed at day 66 (Fig. 1).

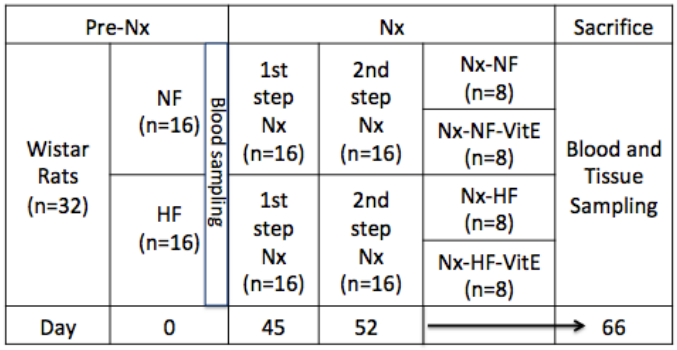

Fig. 1. Diagram of the experimental design. Nx = 5/6 nephrectomy, NF = normal fat diet (5% fat), HF = high fat diet (35% fat), VitE = vitamin E added to the diet (30000 mg/kg).

Assessment of vascular calcification. Following sacrifice, the thoracic aorta, stomach and lungs were dissected and processed to study mineral content. Vascular and soft tissue calcification was studied by histology and by measuring the tissue Ca content. Samples of the abdominal aorta, the right lung, and the stomach were fixed in 10% buffered formalin and subsequently sectioned and stained for mineralization by the Von Kossa method. Another portion of the aorta was demineralized in 10% formic acid, and the arterial tissue Ca content was measured in the supernatant. Quantification of tissue mineral content was performed as described previously (Lopez et al. 2006). Briefly, the stomach and the left lung from each rat were placed into separate 50-ml tubes. Twenty milliliters of 150 mM HCl was added to each tube. The tubes were mixed by inversion for 24 h at room temperature, and Ca was measured in the acid extract. Ca concentration in the acid extracts was measured by spectrophotometry (BioSystems SA, Barcelona, Spain).

Blood Chemistries. Blood for chemistry analyses was obtained either in anesthetized (inhaled sevofluorane) rats, from the jugular vein, or at the time of sacrifice (thiopental anesthesia plus

exanguination), from the abdominal aorta. Blood for measurements of ionized Ca levels was collected in heparinized syringes and immediately analyzed using a Ciba-Corning 634 ISE Ca2+/pH Analyzer (Ciba-Corning, Essex, England). Afterwards, plasma was separated by centrifugation and stored at –20° C until assayed. Plasma creatinine, P, glucose, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides were measured by spectrophotometry (BioSystems SA, Barcelona, Spain). ELISA tests were used to quantify plasma FGF23 (Kainos Laboratories, Tokyo, Japan) and parathyroid hormone (PTH) (Immutopics, San Clemente, CA). Radioimmunoassay was used in plasma samples to determine insulin (Millipore, St. Charles, MO, USA), leptin (Millipore, St. Charles, MO, USA) and calcitriol (Immunodiagnostic Systems Ltd, Boldon, UK).

Statistics. Values are expressed as the mean ± standard error (SE). The difference between means for two different groups was determined by t-test; the difference between means for three or more groups was assessed by Analysis of Variance (ANOVA). Fisher Least Significant Difference (LSD) test was used as a post-hoc procedure. A correlation study was carried out using the Pearson test. $p<0.05$ was considered significant.

RESULTS

Before inducing uremia, body weight was similar in rats fed NF (198.8±5.6 g) and in rats fed HF (202.5±5.1 g). The rats that were fed HF diets did not increase body weight because they ingested less food: 10.7±0.4 g/day than the rats fed NF diets: 13.0±0.5 g/day. The differences in food intake between NF and HF diets were significant ($p<0.05$). After Nx, body weight decreased both in rats fed NF (189.4±3.5 g) and in rats fed HF (178.5±3.1 g).

No differences in plasma creatinine were found between the rats that were fed NF and HF diets prior to Nx. However, rats fed HF diets had significantly higher glucose (134.6±2.6 vs 125.4±1.8 mg/dl), LDL cholesterol (6.6±0.3 vs 5.0±0.4 mg/dl) and triglycerides (72.9±5.9 vs 50.3±3.2 mg/dl). Plasma concentrations of FGF23 were higher in rats fed HF diet, 926±121pg/ml, than in rats fed NF diet, 217±17 pg/ml, $p<0.05$. No differences in plasma leptin and PTH were found between rats fed NF and HF diet. Prior to Nx, plasma concentrations of calcitriol were decreased in rats fed HF, 11.3±1.2 pg/ml, when compared with rats fed NF, 77.7±9.2 pg/ml, $p<0.001$.

Nephrectomy resulted in an increase in creatinine in all experimental groups. At the time of sacrifice plasma creatinine was significantly higher in rats fed HF diet (1.8±0.2 mg/dl) than in rats fed NF diet (1.3±0.1 mg/dl). Treatment with VitE reduced plasma creatinine and the difference was significant in the rats fed NF diet (1.0±0.1 mg/dl). Although plasma Ca was reduced in all uremic rats, higher values were recorded in the rats fed NF diet (1.14±0.02 mmol/l). Plasma P was higher in the rats fed HF diet, 10.8±1.4 mg/dl vs 6.6±1.0 in the rats fed NF diet. VitE supplementation reduced P levels to 8.5±0.5 mg/dl (HF diet) and 4.2±0.4 mg/dl (NF diet). When compared with the non-uremic controls, Nx rats had higher glucose, insulin and cholesterol (total, LDL and HDL) concentrations. Plasma triglycerides were decreased in Nx groups and rats receiving supplemental VitE had the lowest triglycerides levels ($p<0.05$ vs non-supplemented rats). As

expected, the phosphaturic factor FGF23 was increased in all Nx groups and was higher in rats fed HF diets than in rats fed NF diets. Plasma PTH values, which in Nx rats were controlled by treatment with calcitriol, were not influenced by either diet caloric content or VitE supplementation. Uremic rats, that were treated with calcitriol, had mean plasma calcitriol concentrations ranging from 48.5 to 64.6 pg/ml and no significant differences were found between the four experimental groups (Table 1).

Rats fed HF diet showed substantial aortic calcification with calcium content that was significantly higher than in rats fed NF diet (38.4±8.8 mg/g tissue vs 8.7±4.2 mg/g tissue, $p<0.001$). Supplementation with VitE reduced aortic calcium content in rats on HF diets to 16.5±1.4 mg/g tissue ($p=0.018$). Differences in aortic calcification between experimental groups were also evident by histology with Von Kossa staining (Fig. 2).

	PreNx-NF	PreNx-HF	Nx-NF	Nx-NF-VitE	Nx-HF	Nx-HF-VitE
Creatinine (mg/dl)	0.6 ± 0.1[a]	0.5 ± 0.1[a]	1.3 ± 0.1[b]	1.0 ± 0.1[d]	1.8 ± 0.2 [c]	1.5 ± 0.2 [b]
Calcium (mmol/l)	1.30 ± 0.02 [a]	1.29 ± 0.02 [a]	1.14 ± 0.02 [b]	1.04 ± 0.02 [c]	1.01 ± 0.02 [c]	1.00 ± 0.02 [c]
Phosphorus (mg/dl)	3.9 ± 0.4 [a]	4.1 ± 0.2 [a]	6.6 ± 1.0 [a]	4.2 ± 0.4 [a]	10.8 ± 1.4 [b]	8.5 ± 0.5 [b]
Glucose (mg/dl)	125.4 ± 1.8 [a]	134.6 ± 2.6 [b]	167.8 ± 5.6 [c]	165.3 ± 5.9 [c]	158.2 ± 6.4 [c]	145.0 ± 2.3 [b]
Insulin (ng/ml)	0.21 ± 0.04 [a]	0.16 ± 0.02 [a]	0.75 ± 0.18 [b]	0.52 ± 0.09 [b]	0.32 ± 0.14 [a]	0.71 ± 0.21 [b]
Total Cholesterol (mg/dl)	60.2 ± 2.2 [a]	64.5 ± 3.0 [a]	114.4 ± 6.6 [b]	68.8 ± 6.8 [a]	113.2 ± 5.4 [b]	117.5 ± 10.6 [b]
LDL Cholesterol (mg/dl)	5.0 ± 0.4 [a]	6.6 ± 0.3 [b]	7.6 ± 1.3 [b]	4.6 ± 0.6 [a]	9.6 ± 1.6 [c]	10.5 ± 2.1[c]
HDL Cholesterol (mg/dl)	23.1 ± 1.0 [a]	25.9 ± 1.9 [a]	44.6 ± 2.0 [b]	32.8 ± 2.2 [c]	48.8 ± 3.7 [b]	44.5 ± 4.4 [b]
Triglycerides (mg/dl)	50.3 ± 3.2 [a]	72.9 ± 5.9 [b]	32.6 ± 3.2 [c]	14.1 ± 1.2 [d]	35.2 ± 3.5 [c]	48.3 ± 10.7 [a]
FGF23 (pg/ml)	217 ± 17 [a]	926 ± 121[b]	8984 ± 2333 [c]	5055 ± 1588 [c]	20492 ± 3906 [d]	24579 ± 7879 [d]
Leptin (ng/ml)	1.4 ± 0.2 [a]	1.4 ± 0.2 [a]	0.7 ± 0.3 [b]	1.4 ± 0.4 [a]	1.8 ± 0.4[a]	0.8 ± 0.5 [a]
PTH (pg/ml)	70.9 ± 7.6 [a]	55.1 ± 2.5 [a]	59.4 ± 3.9 [a]	59.3 ± 4.2 [a]	64.3 ± 6.9 [a]	71.1 ± 14.2 [a]
Calcitriol (pg/ml)	77.7 ± 9.2 [a]	11.3 ± 1.2 [b]	54.3 ± 8.7 [a]	48.5 ± 6.9 [a]	64.6 ± 5.1 [a]	48.7 ± 12.9 [a]

Table 1. Blood biochemistry from the study rats. For each parameter, values with different superscript are significantly ($p<0.05$) different

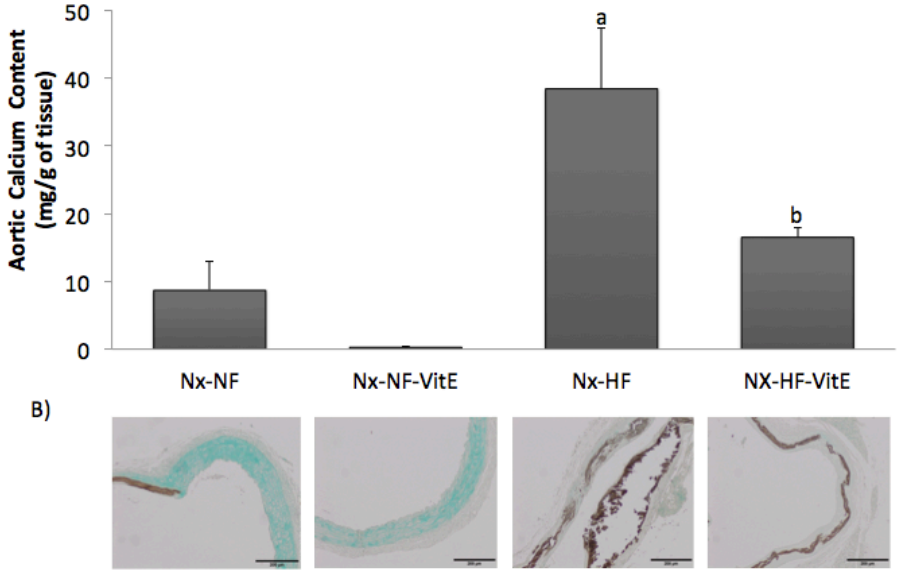

Fig. 2. (A) Calcium content (mg/g of tissue) in the aortas of rats from the four experimental groups. (B) Representative von Kossa stained aortic tissue sections from the same rats where mineral deposits are depicted by the brown pigment. Nx-NF = uremic rats treated with calcitriol (80 ng/kg ip eod) fed normal fat diet with 0.9% phosphorus (n=8), Nx-NF-VitE = uremic rats treated with calcitriol (80 ng/kg ip eod) fed normal fat diet with 0.9% phosphorus and supplemented with vitamin E, 30000 mg/kg (n=8), Nx-HF = uremic rats treated with calcitriol (80 ng/kg ip eod) fed a high fat diet with 0.9% phosphorus (n=8), Nx-HF-VitE = uremic rats treated with calcitriol (80 ng/kg ip eod) fed high fat diet with 0.9% phosphorus and supplemented with vitamin E, 30000 mg/kg (n=8). $^{a}p<0.05$ vs Nx-NF, $^{b}p<0.05$ vs Nx-HF.

Mineral deposition in stomach and lung was less severe than in aorta and only was obvious in rats fed HF. Thus Nx-HF rats had a calcium content of 5.6±2.7 mg/g in the stomach and 1.8±0.3 mg/g in the lungs. Dietary VitE supplementation resulted in a significant decrease in soft tissue calcification both in stomach, 1.2±0.4 mg/g ($p=0.024$), and in the lungs, 0.3±0.2 mg/g ($p<0.001$). Von Kossa staining of stomach and lung tissue sections also demonstrated the presence of calcifications in

rats fed HF and their attenuation with dietary vitamin E (Fig. 3 and Fig. 4).

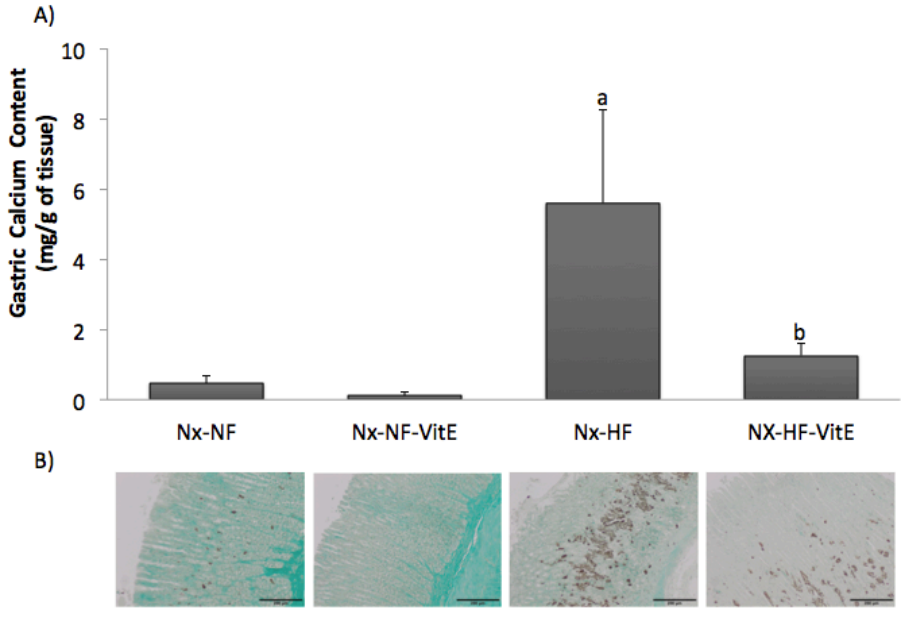

Fig. 3. (A) Calcium content (mg/g of tissue) in the stomachs of rats from the four experimental groups. (B) Representative von Kossa stained gastric tissue sections from the same rats where mineral deposits are depicted by the brown pigment. Nx-NF = uremic rats treated with calcitriol (80 ng/kg ip eod) fed normal fat diet with 0.9% phosphorus (n=8), Nx-NF-VitE = uremic rats treated with calcitriol (80 ng/kg ip eod) fed normal fat diet with 0.9% phosphorus and supplemented with vitamin E, 30,000 mg/kg (n=8), Nx-HF = uremic rats treated with calcitriol (80 ng/kg ip eod) fed a high fat diet with 0.9% phosphorus (n=8), Nx-HF-VitE = uremic rats treated with calcitriol (80 ng/kg ip eod) fed high fat diet with 0.9% phosphorus and supplemented with vitamin E, 30,000 mg/kg (n=8). $^{a}p<0.05$ vs Nx-NF, $^{b}p<0.05$ vs Nx-HF.

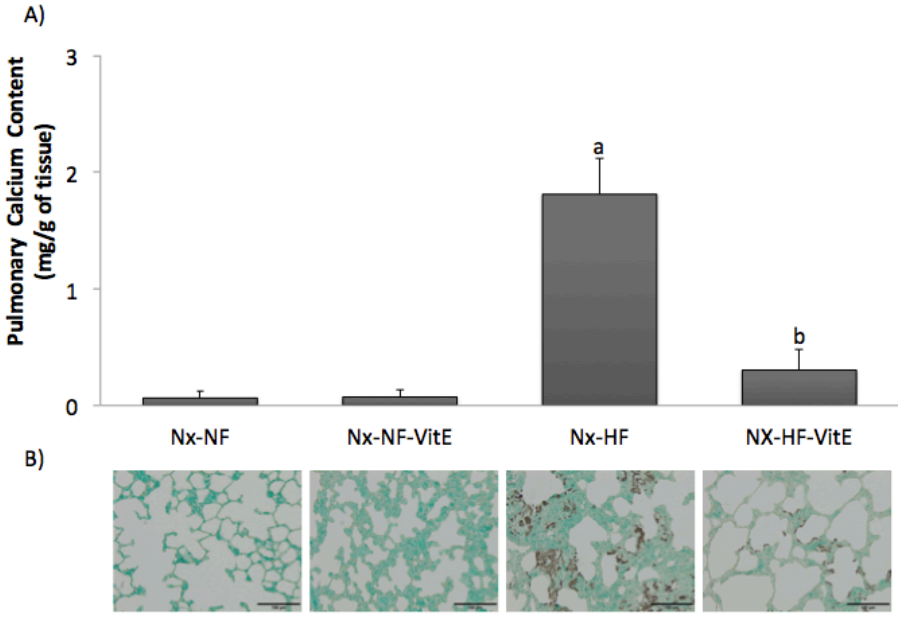

Fig. 4. (A) Calcium content (mg/g of tissue) in the lungs of rats from the four experimental groups. (B) Representative von Kossa stained pulmonary tissue sections from the same rats where mineral deposits are depicted by the brown pigment. Nx-NF = uremic rats treated with calcitriol (80 ng/kg ip eod) fed normal fat diet with 0.9% phosphorus (n=8), Nx-NF-VitE = uremic rats treated with calcitriol (80 ng/kg ip eod) fed normal fat diet with 0.9% phosphorus and supplemented with vitamin E, 30000 mg/kg (n=8), Nx-HF = uremic rats treated with calcitriol (80 ng/kg ip eod) fed a high fat diet with 0.9% phosphorus (n=8), Nx-HF-VitE = uremic rats treated with calcitriol (80 ng/kg ip eod) fed high fat diet with 0.9% phosphorus and supplemented with vitamin E, 30000 mg/kg (n=8). $^{a}p<0.05$ vs Nx-NF, $^{b}p<0.05$ vs Nx-HF.

DISCUSSION

This study was designed to investigate the influence of supplementing VitE on the development of extraskeletal calcifications in uremic rats fed diets with HF content. Our results confirm a previous report of a pro-calcifying effect of HF diets in uremic rats and demonstrate that dietary VitE supplementation protects against uremic calcifications in rats fed HF diets.

During the study the rats fed HF adapted food consumption to maintain a caloric intake similar to rats fed NF (around 50 kcal/day), thus no increase in body weight and plasma leptin concentrations was observed in rats fed HF. This allowed to study the effect of the fat content of the diet independent of obesity-mediated mechanisms (e.g. adipokines and other inflammatory mediators released by fat tissue). At the time of Nx the animals fed HF diet were not obese but they showed biochemical signs of metabolic syndrome: hyperglycemia, hypertriglyceridemia and increased LDL. At this stage, with a background of metabolic changes induced by high caloric intake, we had the opportunity to explore the impact of renal failure on VC and the influence of VitE.

The dose of vitamin E used in the experiments was chosen based on previous studies (Peralta-Ramírez et al. 2014). Rats ingested around 300 mg of vitamin E per day which is the upper limit of what is considered safe for long term administration of vitamin E to humans (Rutkowsky and Grzegorczyk, 2012). Although it is difficult to extrapolate the effect of this dose of vitamin E to humans, none of the side effects described in people after vitamin E overdose (diarrhea, skin inflammation, hyperglycemia, hyperlipidemia) were observed in the present study.

Our results confirm previous data showing that preconditioning with HF diet predisposes rats to develop extraskeletal calcifications associated to renal failure (Raya et al. 2016). This finding highlights the potential deleterious effect on VC of energy-dense diets,

even in the absence of obesity. Moreover, treatment with VitE reduced the severity of extraskeletal calcifications in rats fed HF. VitE has been previously shown to be effective in preventing VC in rats with genetic obesity (Peralta-Ramírez et al. 2014). The results of the present study expand these data to a population of non-obese, although metabolically challenged, rats and demonstrate that obesity per se is not a requisite for the deleterious effect of HF diets on VC in uremic rats. When compared with a earlier report in which genetic obese Zucker rats fed a NF diet were subjected to the same experimental protocol, VC was much more severe in the wild type rats fed HF diet of the current study (Peralta-Ramírez et al. 2014). In fact, the present study was originally designed to last 4 weeks after Nx (same time frame than the previous study with Zucker rats) but the rapid deterioration of the rats fed HF diets required to terminate the study, for humane reasons, 14 days after Nx.

Although it was not a primary objective of this study, the data obtained also provide interesting insights about the role of calcitriol on VC in the context of feeding HF diets. Prior to Nx, rats fed HF diets had very low plasma calcitriol concentrations. A decrease in plasma calcitriol, without changes in plasma calcidiol, has been previously reported in rats with normal renal function after feeding HF diets (Raya et al. 2016). Since FGF23 is known to decrease calcitriol synthesis through inhibition of 1-alpha-hydroxylase activity in the kidney (Shimada et al. 2004), these low calcitriol concentrations have been linked to the increase in FGF23 elicited by HF diets (Raya et al. 2016). In uremic rats calcitriol should be further reduced by the decrease in renal mass and by the elevation in FGF23 secondary to kidney disease (Rodriguez et al. 2014). Vitamin D deficiency is associated with cardiovascular disease (Dobnig et al. 2008; Norman and Powell, 2014; Parker et al. 2010) and calcitriol treatment has been reported to improve survival of CKD patients (Messa et al. 2015; Zheng et al. 2013). Thus, it could be speculated that treatment with calcitriol may have

beneficial effects on uremic rats fed HF. However, when comparing the results of this study with previous data in which rats fed HF were not treated with calcitriol (Raya et al. 2016), calcifications were much more severe after calcitriol treatment. Therefore, the results of this study demonstrate that even though rats fed HF diets have very low plasma calcitriol concentrations, treatment with calcitriol at doses sufficient to control secondary hyperparathyroidism is clearly deleterious. The negative actions of calcitriol are probably related to an increased P load in animals with impaired P excretion derived from feeding HF diets (Raya et al. 2016), which would override any beneficial effect that restoring plasma calcitriol concentrations may have on vascular health. In fact, the data of this study raise the intriguing question of whether the decrease in plasma calcitriol observed after feeding HF diets might act as a protective mechanism in rats that already have difficulty in excreting P.

The decrease in phosphate levels in uremic rats treated with vitamin E is probably related to the reduction in extraskeletal calcification: nephrocalcinosis was less severe in animals treated with vitamin E thus allowing their remnant kidneys to be more efficient in phosphate excretion.

The mechanisms to explain the detrimental effect of HF diets on VC are probably multifactorial. In previous studies with obese Zucker rats we have shown that oxidative stress plays an important role in obesity-associated VC (Peralta-Ramírez et al. 2014). The results of the present study also support a role of oxidative stress in HF diet-induced calcifications, because treatment with VitE substantially decreased VC. In addition to any direct pro-oxidant action of feeding HF, HF diet-induced P retention may also contribute to oxidative stress (Di Marco et al. 2008). Thus, VitE treatment may help to modulate the adverse pro-oxidant effects of P retention secondary to feeding HF. Nonetheless since the study was performed in an animal model of

uremia, caution should be taken when extrapolating the results to the human situation.

CONCLUSIONS

In conclusion, uremic rats fed HF diets develop more severe extraosseous calcifications than their normocaloric-fed counterparts and dietary VitE supplementation protects against uremic calcifications in rats fed HF diets. Thus, eating energy-rich foods should be discouraged in patients with renal disease and their deleterious effect may be ameliorated with adequate antioxidant supply.

ACKNOWLEDGEMENTS

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REFERENCES

Block, G.A., Klassen, P.S., Lazarus, J.M., Ofsthun, N., Lowrie, E.G. and Chertow, G.M. 2004. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J. Am. Soc. Nephrol.*, 15:2208-18.

Chen, J., Muntner, P., Hamm, L.L., Jones, D.W., Batuman, V., Fonseca, V., Whelton, P.K. and He, J. 2004. The metabolic syndrome and chronic kidney disease in U.S. adults. *Ann. Intern. Med.*, 140:167-74.

Di Marco, G.S., Hausberg M, Hillebrand U, Rustemeyer P, Wittkowski W, Lang D, Pavenstädt H. 2008. Increased inorganic phosphate induces human endothelial cell apoptosis in vitro. *Am. J. Physiol. Renal Physiol.*, 294:F1381-7.

Dobnig, H., Pilz, S., Scharnagl, H., Renner, W., Seelhorst, U. Wellnitz, B., Kinkeldei, J., Boehm, B.O., Weihrauch, G. and Maerz, W. 2008. Independent association of low serum 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality. *Arch. Intern. Med.*, 168:1340-9.

Giachelli, C.M. 2009. The emerging role of phosphate in vascular calcification. *Kidney Int.*, 75:890-897.

Gonzalez-Bulnes, A., Astiz, S., Ovilo, C., Garcia-Contreras, C. and Vazquez-Gomez, M. 2016. Nature and Nurture in the Early-Life Origins of Metabolic Syndrome. *Curr. Pharm. Biotechnol.*, 17:573-86.

Hall, J.E., Kuo, J.J., da Silva, A.A., dePaula, R.B., Liu, J. and Tallam, L. 2003. Obesity-associated hypertension and kidney disease. *Curr. Opin. Nephrol. Hypertens.*, 12:195-200.

Lopez, I., Aguilera-Tejero, E., Mendoza, F.J., Almaden, Y., Perez, J., Martin, D. And Rodriguez, M. 2006. Calcimimetic R-568 decreases extraosseous calcifications in uremic rats treated with calcitriol. *J. Am. Soc. Nephrol.*, 17:795-804.

Messa, P., Cozzolino, M., Brancaccio, D., Cannella, G., Malberti, F., Costanzo, A.M., di Luzio Paparatti, U., Festa, V., Gualberti, G., Mazzaferro, S. and FARO Study Group. 2015. Effect of VDRA on survival in incident hemodialysis patients: results of the FARO-2 observational study. *BMC Nephrol.*, 16:11.

Norman, P.E. and Powell, J.T. 2014. Vitamin D and cardiovascular disease. *Circ. Res.*, 114:379-93.

Parker, J., Hashmi, O., Dutton, D., Mavrodaris, A., Stranges, S., Kandala, N.B., Clarke, A. and Franco, O.H. 2010. Levels of vitamin D and cardiometabolic disorders: systematic review and meta-analysis. *Maturitas*, 65:225-36.

Peralta-Ramírez, A., Montes de Oca, A., Raya, A.I., Pineda, C., López, I., Guerrero, F., Díez, E., Muñoz-Castañeda, J.R., Martinez, J., Almaden, Y., Rodriguez, M. and Aguilera-Tejero E. 2014. Vitamin E protection of obesity-enhanced vascular calcification in uremic rats. Am. J. Physiol. Renal Physiol., 306:F422-9.

Raya, A.I., Rios, R., Pineda, C., Rodriguez-Ortiz, M.E., Diez, E., Almaden, Y., Muñoz-Castañeda, J.R., Rodriguez, M., Aguilera-Tejero, E. and Lopez, I. 2016. Energy-dense diets increase FGF23, lead to phosphorus retention and promote vascular calcifications in rats. *Sci. Rep.*, 6:36881.

Riccardi, G., Giacco, R. and Rivellese, A.A. 2004. Dietary fat, insulin sensitivity and the metabolic syndrome. *Clin. Nutr.*, 23:447-56.

Ritter, C.S. and Slatopolsky, E. 2016. Phosphate toxicity in CKD: the killer among us. *Clin. J. Am. Soc. Nephrol.*, 11:1088-100.

Rodriguez, M., Muñoz-Castañeda, J.R. and Almaden, Y. 2014. Therapeutic use of calcitriol. *Curr. Vasc. Pharmacol.*, 12:294-9.

Rutkowski, M. and Grzegorczyk K. 2012. Adverse effects of antioxidative vitamins. Int. J. Occup. Med. Environ. Health, 25:105-21.

Salazar-Martinez, E., Allen, B., Fernandez-Ortega, C., Torres-Mejia, G., Galal, O. and Lazcano-Ponce, E. 2006. Overweight and obesity status among adolescents from Mexico and Egypt. *Arch. Med. Res.*, 37:535-42.

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., Fujita, T., Nakahara, K., Fukumoto, S. and Yamashita, T. 2014. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J. Bone Miner. Res.*, 19:429-35.

Villa-Bellosta, R., Millan, A. and Sorribas, V. 2011. Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *Am. J. Physiol. Cell Physiol.*, 300:C210-20.

Wang, Y.C., McPherson, K., Marsh, T., Gortmaker, S.L. and Brown, M. 2011. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet.* , 378:815-25.

Wickman, C. and Kramer, H. 2013. Obesity and kidney disease: potential mechanisms. *Semin. Nephrol.*, 33:14-22.

Zheng, Z., Shi, H., Jia, J., Li, D. and Lin, S. 2013. Vitamin D supplementation and mortality risk in chronic kidney disease: a meta-analysis of 20 observational studies. *BMC Nephrol.*, 14:199.

5. Article 3

Phosphorus restriction does not prevent the increase in fibroblast growth factor 23 elicited by high fat diet.

ABSTRACT

This study was designed to evaluate the influence of phosphorus (P) restriction on the deleterious effects of high fat diets on mineral metabolism. Twenty-four rats were allotted to 3 groups (n=8 each) that were fed different diets for 7 months. Rats in group 1 were fed normal fat-normal P (0.6%) diet (NF-NP), rats in group 2 were fed high fat- normal P diet (HF-NP) and rats in group 3 were fed high fat-low P (0.2%) diet (HF-LP).

Blood, urine and tissues were collected at the end of the experiments. When compared with the control group (NF-NP), rats fed HF diets showed increases in body weight, and in plasma concentrations of triglycerides and leptin, and decreased plasma calcitriol concentrations. In rats fed HF-NP plasma fibroblast growth factor 23 (FGF23) was higher (279.6±39.4 pg/ml vs 160.6±25.0 pg/ml, $p=0.018$) and renal klotho (ratio klotho/GAPDH) was lower (0.75±0.06 vs 1.06±0.08, $p<0.01$) than in rats fed NF-NP. Phosphorus restriction did not normalize plasma FGF23 or renal klotho; in fact, rats fed HF-LP, that only ingested an average of 22.9 mg/day of P, had higher FGF23 (214.7±32.4 pg/ml) concentrations than rats fed NF-NP (160.6±25.0 pg/ml), that ingested and average of 74.4 mg/day of P over a 7 month period. In conclusion, our results demonstrate that severe P restriction over a prolonged period of time (7 months) does not normalize the increase in circulating FGF23 induced by HF diets. These data indicate that the deleterious effects of high fat diet on the FGF23/klotho axis are not eliminated by reduced P intake.

INTRODUCTION

Obesity and its related metabolic complications represent a major health concern, not only due to their influence on morbidity and mortality but also for the high healthcare cost associated to this disease (Popkin et al. 2012; Wang et al. 2011). A major factor in the development of obesity is excessive caloric intake which is favored by the ingestion of energy-dense processed foods (fast food). In addition to their high caloric concentration, processed foods are often rich in phosphate (P) (Sarathy et al. 2012). Phosphate is a common food additive and the inorganic P added to processed foods is more readily absorbed than organic P (Uribarri and Calvo, 2003). Moreover, the high fat content of many fast foods also enhances intestinal P absorption (Frommelt et al. 2014). Therefore, individuals eating fast food often ingest an overload of P that must be excreted by the kidney to maintain P homeostasis.

Fibroblast growth factor 23 (FGF23) is a major phosphaturic hormone. In the kidney, FGF23 promotes P excretion after binding to its receptor (FGFR1) and co-receptor (klotho) (Kuro-o, 2010). Increased levels of FGF23, which are usually found in hyperphosphatemic uremic patients, have been reported as a risk factor of cardiovascular mortality (Scialla et al. 2014). The relationship between high FGF23 and mortality is not restricted to patients with kidney disease but has also been identified in the general population (Kestenbaum et al. 2014; Panwar et al. 2015).

An increase in circulating levels of FGF23 has been reported in rats fed high fat diets (Raya et al. 2016). The mechanism for increased FGF23 production seems to be related to decreased expression of renal klotho after feeding high fat diet. Klotho down-regulation generates tubular resistance to FGF23 and thus more FGF23 is needed for P excretion (Kanbay et al. 2017). Additionally, a recent study has shown that an excessive tubular load of P down-regulates the expression of klotho in the kidney. Renal klotho was reduced in rats with increased

tubular load of P and was higher in uremic rats on a low P diet than in uremic rats in a high P diet (Muñoz-Castañeda et al. 2017).

We hypothesize that reducing P load by feeding a diet with low P content would prevent the decrease in renal klotho and the increase in FGF23 elicited by high fat diet. Thus the main objective of this study was to evaluate the influence of P restriction on the deleterious effects of high fat diets on mineral metabolism. Additionally, since previous studies (Raya et al. 2016) were of short duration (1 month), a secondary objective was to evaluate the influence of high fat diets on FGF23 over a more extended period of time (7 months).

MATERIAL AND METHODS

Ethics approval. All experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba (Cordoba, Spain). All protocols were carried out in accordance with the approved guidelines. They followed the guiding principle laid down by the Higher Council of Scientific Research of Spain following the normal procedures directing animal welfare (Real Decreto 223/88, BOE of 18 of March) and adhered to the recommendations included in the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, NIH) and European laws and regulations on protection of animals, under the advice of specialized personnel.

Animals and diets. Twenty-four 2 months-old Wistar rats, provided by the Animal Housing Facilities of the University of Cordoba (Cordoba, Spain), were housed with a 12h/12h light/dark cycle. Appropriate measures were taken to ensure animal welfare and to address the basic behavioral and physiological needs of rats.

Two diets with normal P (NP=0.6%) were used in the experiments: normal fat content diet (NF-NP) with a 5% fat concentration that provided Metabolizable Energy = 3518 kcal/kg (Altromin C 1090-10, AltrominSpezialfutter GmbH, Germany) and a high fat content diet (HF-NP) with a 35% fat concentration that provided Metabolizable Energy = 5241 kcal/kg (Altromin C 1090-60, AltrominSpezialfutter GmbH, Germany). In addition, a HF diet with low P (LP=0.2%) was also used (HF-LP). All diets contained 0.6% of Ca and 500 IU/g of vitamin D.

Experimental design. Rats were allotted to 3 groups (n=8 each) which were fed ad libitum the study diets for 7 months. Rats in group 1 were fed NF-NP, rats in group 2 were fed HF-NP and rats in group 3 were fed HF-LP. During the last week of the trial the rats were

housed in metabolic cages, allowing daily control of food and water intake and collection of urine. At the end of the experiment, rats were sacrificed by exsanguination under general anesthesia (inhaled sevoflurane) to obtain blood and tissue samples.

Blood chemistries. Blood was collected from the abdominal aorta at the time of sacrifice. Blood glucose was measured immediately after collection with a blood glucose meter (Bayer Consumer Care AG, Basel, Switzerland). Afterwards, plasma was separated by centrifugation and stored at –20° C until assayed. Plasma concentrations of total cholesterol, triglycerides, urea, creatinine, calcium and phosphorus were measured by spectrophotometry (BioSystems SA, Barcelona, Spain). ELISA tests were used to quantify plasma intact FGF23 (Immutopics, San Clemente, CA), leptin (Millipore Corporation, Billerica, MA, USA), tumor necrosis factor alpha (TNFα) (eBioscience, Bender MedSystems GmbH, Vienna, Austria) and parathyroid hormone (PTH) (Immutopics, San Clemente, CA). Radioimmunoassay (Immunodiagnostic Systems Ltd, Boldon, UK) was used in plasma samples to determine 1,25-dihydroxyvitamin D (calcitriol).

Urine chemistries. Urine phosphorus concentrations were measured by spectrophotometry (BioSystems SA, Barcelona, Spain).

Real-Time-Polymerase Chain Reaction (RT-PCR). Analyses of renal klotho mRNA were performed by quantitative Real-Time PCR. Kidney tissue was disrupted using liquid nitrogen and grinded thoroughly with a mortar. Renal total RNA was extracted with using chloroform and isopropanol precipitation method and a treatment with DNAse I amplification Grade (Sigma-Aldrich) and quantified by spectrophotometry (ND-1000, Nanodrop Technologies, Wilmington, DE). Fifty ng of total RNA were used to analyze mRNA expression in the

Light Cycler thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA). RT-PCR was performed in one step, with the QuantiTect SYBR Green RT-PCR kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Rat primers for membrane Klotho were designed with the free Oligo 7 software (http://www.oligo.net/), and the sequence is:

α-klotho F: 5'- CTCTGAAAGCCTACGTGTTGG -3',
R: 5'- TAGAAACGAGATGAAGGCCAG -3';
GAPDH- F: 5'-AGGGCTGCCTTCTCTTGTGAC-3',
R: 5'-TGGGTAGAATCATACTGGAACATGTAG-3'.

Results were normalized to that of GAPDH. Quantification of relative expression was determined by the 2Δ(ΔCT) method.

Protein extraction and Western blot analysis. Proteins were isolated from renal tissue by using a lysis buffer containing HEPES (10 mmol/l), KCl (10 mmol/l), EDTA (0.1 mmol/l), EGTA (0.1 mmol/l), DTT (1 mmol/l), PMSF (0.5 mmol/l), protease inhibitor cocktail (70 µg/ml), and I-Gepal CA-630 (0.6%), pH 7.9 (Sigma Aldrich, St. Louis, MO). Protein concentration was determined by Bradford method. For Western Blot analysis, 50 µg of protein (klotho) or 100 µg (NaPiIIa) of protein were electrophoresed on a 10% SDS-polyacrilamide gel (Invitrogen, Carlsbad, CA) and electrophoretically transferred (Transfer Systems, BioRad, Hercules, CA) from the gels onto nitrocellulose membranes (Invitrogen). The following steps were performed with gentle shaking. Membranes were incubated in TTBS-L solution [20 mM Tris-HCl (pH 7.6), 0.2% Tween 20, 150 mM NaCl] (Sigma Aldrich), and 5% nonfat dry milk (Bio-Rad) at room temperature for 1 hour to avoid nonspecific binding. Membranes were then washed with TTBS buffer (the same composition as TTBS-L without nonfat dry milk) and incubated overnight at 4ºC temperature with a rat anti-klotho antibody (Alpha Diagnostic Int, San Antonio, TX; 0.5 µg/ml) or a rabbit polyclonal

anti-NaPiIIa antibody (Abcam plc, Cambridge, UK; 1 µg/ml). The membranes were then washed with TTBS buffer and immunolabeled using a peroxidase-conjugated secondary antibody (1:5000 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA). Finally, they were revealed on autoradiographic film using ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ). GAPDH was used as housekeeping protein to ensure equal loading of the gels. Protein levels were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Renal Histopathology. Kidney tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned and processed for staining with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Masson's Trichrome and Von Kossa stains. Lesions were scored using a semi quantitative scale graded from 0-3: 0 (absent), 1 (slight), 2 (moderate) or 3 (severe). This scale was constructed by a previous scanning of all the samples under study in which the more severe lesions were identified and were assigned a value=3. For statistical analysis lesions were grouped into three categories: a) glomerular lesions (glomerular retraction and sclerosis), b) tubular lesions (tubular atrophy, tubular hyperplasia and thickening of basement membrane) and c) interstitial lesions (interstitial edema and inflammatory infiltrate). Analyses were performed in a blind manner.

Statistics. Values are expressed as the mean ± standard error (SE). The difference between means for the three experimental groups was assessed by ANOVA. Fisher LSD test was used as a post-hoc procedure. A correlation study was carried out using the Pearson test. $p<0.05$ was considered significant.

RESULTS

At the beginning of the study all rats had similar body weight that ranged between 239.4±1.7 and 251.2±3.3 g. During the 7 months that lasted the experiment rats experienced an increase in body weight that was more accentuated in the groups fed a HF diet. Phosphorus restriction attenuated the increase in body weight in rats fed HF, but the differences were small and non-significant (Fig 1). Mean daily intake of P (mg/day) was not influenced by the amount of ingested fat but, obviously, was much lower ($p<0.001$) in the P restricted group: NF-NP = 74.4±6.3, HF-NP = 77.9±3.7, HF-LP = 22.9±1.4.

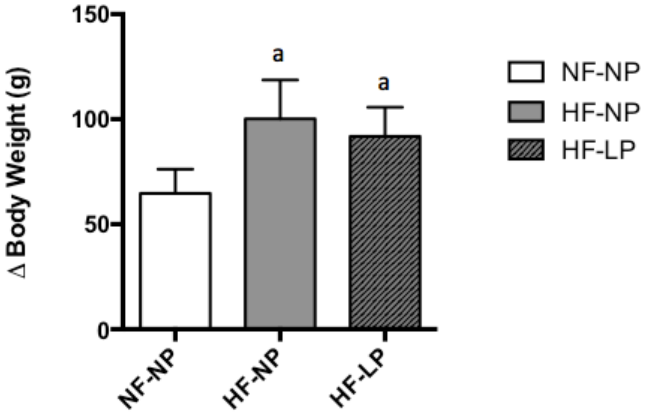

Fig. 1. Body Weight. Increase in body weight after the 7 months that lasted the experiments in rats (n=8 per group) fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). [a]$p<0.05$ vs NF-NP.

Blood glucose and cholesterol concentrations were not different between groups. However, plasma triglycerides were higher in rats fed HF than in rats fed NF: HF-NP = 0.94±0.1 mmol/l vs NF-NP = 0.58±0.1 mmol/l, $p=0.002$. Phosphorus restriction led to a non-significant decrease in triglyceride concentrations in HF rats. Plasma leptin concentrations were also higher ($p<0.05$) in rats fed HF (5.2±0.8 and 5.8±0.5 ng/ml) than in rats fed NF (3.9±0.5 ng/ml) and were not influenced by P restriction; these data are depicted in Table 1

	NF-NP (n=8)	HF-NP (n=8)	HF-LP (n=8)
Glucose (mmol/l)	6.43 ± 0.3	5.54 ± 0.3	5.76 ± 0.2
Total Cholesterol (mmol/l)	1.31 ± 0.2	1.28 ± 0.2	1.37 ± 0.1
Triglycerides (mmol/l)	0.58 ± 0.1	0.94 ± 0.1[a]	0.63 ± 0.0
Leptin (ng/ml)	3.9 ± 0.5	5.2 ± 0.8[a]	5.8 ± 0.5[a]
Calcium (mmol/l)	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.1
Phosphorus (mmol/l)	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
PTH (pmol/l)	29.8 ± 6.5	27.5 ± 6.0	21.0 ± 1.6
Calcitriol (pmol/l)	50.7 ± 21.5	5.3 ± 0.9[a]	12.3 ± 1.9[a]
Urea (mmol/l)	3.98 ± 0.51	4.98 ± 0.54	4.99 ± 0.44
Creatinine (µmol/l)	65 ± 4	73 ± 3	81 ± 2
TNFα (pg/ml)	62.7 ± 4.6	75.5 ± 7.6	77.7 ± 4.5

Table 1. Blood biochemistry. Plasma concentrations of parameters related to energy metabolism, mineral metabolism, renal function, and inflammation in rats fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). [a]$p<0.05$ vs NF-NP, [b]$p<0.05$ vs HF-NP.

Plasma Ca concentrations were not different in the study groups. Plasma P was not influenced by the caloric content of the diet: NF-NP = 1.2±0.1 mmol/l vs HF-NP = 1.0±0.1 mmol/l. Phosphorus restriction did not modify plasma P in rats fed HF (1.0±0.1 mmol/l). Plasma PTH was not different in HF vs NF groups but tended to be decreased in the HF-LP group. Plasma calcitriol concentrations were higher in rats fed normal fat: NF-NP = 50.7±21.5 pmol/l vs HF-NP = 5.3±0.9 pmol/l, $p<0.05$. Phosphorus restriction led to a modest increase in plasma calcitriol in rats fed high fat, HF-LP = 12.3±1.9 pmol/l, $p<0.05$ vs HF-NP. No significant differences in either urea or creatinine were observed between the experimental groups, although rats fed HF had slightly higher values of both parameters. Plasma concentration of TNFα tended to be higher in rats fed HF (75.5±7.6 and 77.7±4.5 pg/ml)

when compared with rats fed NF (62.7±4.6 pg/ml) but differences were not significant (Table 1).

Plasma concentrations of FGF23 were higher in the HF groups but significant differences were only found between HF-NP, 279.6±39.4 pg/ml and NF-NP, 160.6±25.0 pg/ml ($p=0.018$). A non-significant tendency to decreased FGF23 was detected in P restricted rats (Fig 2A).

Renal klotho, both at the mRNA and protein levels, changed in accordance, but in opposite direction, to FGF23. Thus, mRNA (ratio klotho/GAPDH) was lower ($p<0.01$) in rats fed HF-NP (0.65±0.09) than in rats fed NF-NP (1.06±0.13). A significant ($p<0.01$) decrease in renal klotho protein (ratio klotho/GAPDH) was also found in the HF-NP group (0.75±0.06) when compared with the NF-NP group (1.06±0.08) (Fig 3).

Urinary excretion of P was higher ($p<0.01$) in rats fed HF-NP (33.5±1.7 mg/day) than in rats fed NF-NP (23.8±0.8 mg/day). Rats fed HF-LP excreted very little P (3.5±0.6 mg/day), $p<0.01$ vs NP groups (Fig 2B). Renal NaPiIIa (ratio NaPiIIa/GAPDH) was decreased ($p<0.01$) in rats fed HF-NP (0.59±0.05) when compared with rats fed NF-NP (1.04±0.04). Phosphorus restriction restored NaPiIIa expression in rats fed HF to values (0.94±0.09) than were not different from rats fed NF (Fig 2C).

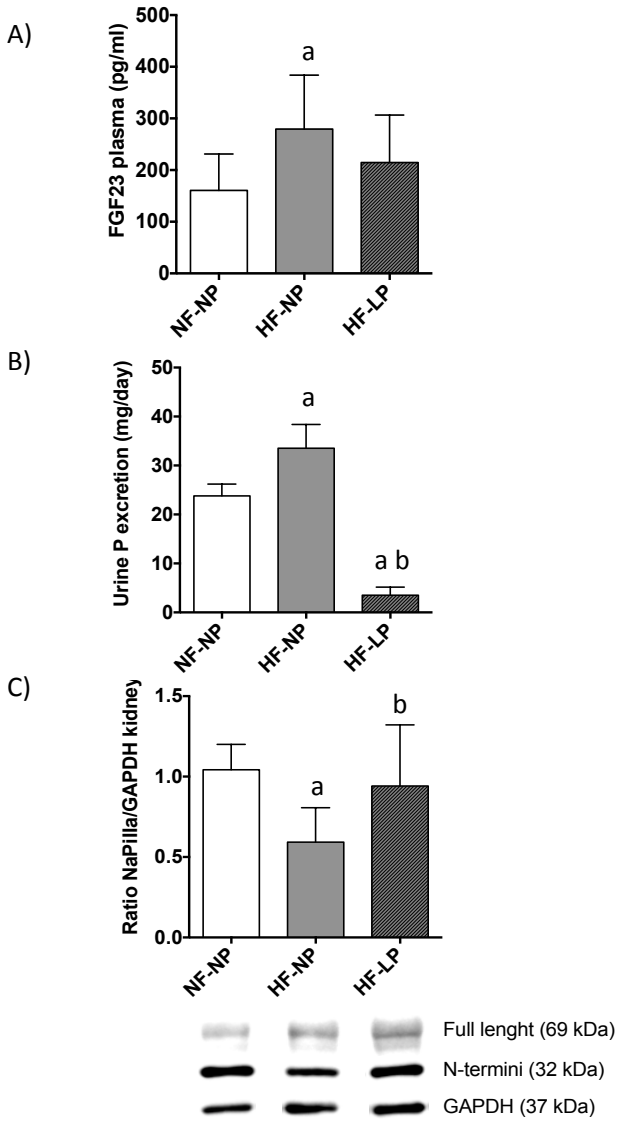

Fig. 2. Plasma FGF23, Urinary excretion of P and Renal NaPiIIa. (A) Circulating concentrations of FGF23 in rats (n=8 per group) fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). (B) Urinary excretion of P (mg/day) in rats (n=8 per group) fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). (C) Expression of NaPiIIa in the kidney of rats (n=8 per group) fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). $^{a}p<0.05$ vs NF-NP, $^{b}p<0.05$ vs HF-NP

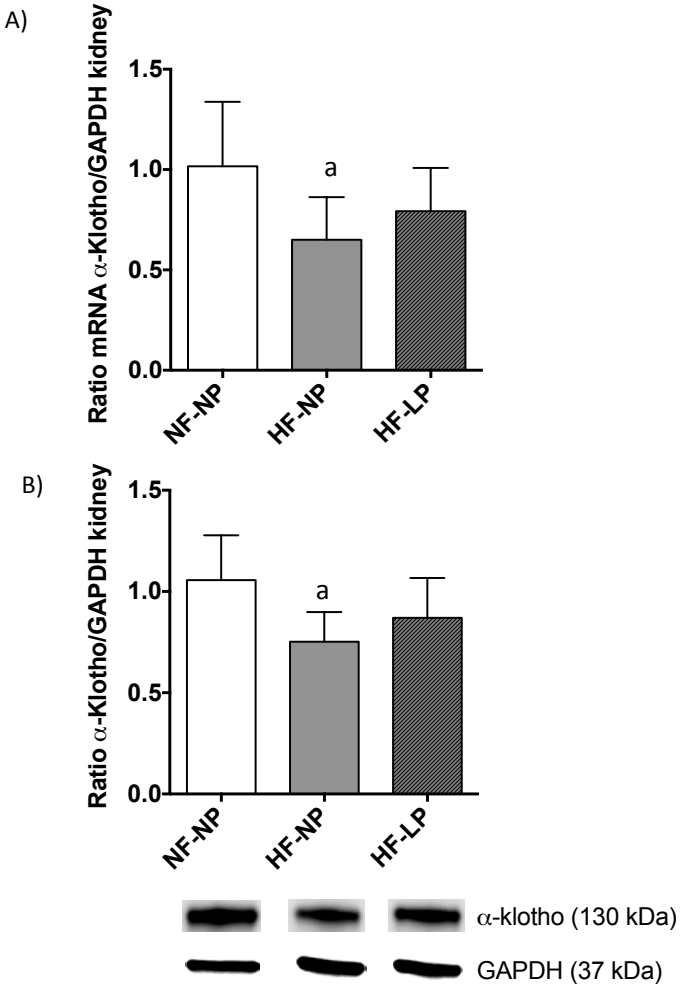

Fig. 3. Renal klotho. Klotho mRNA (A) and protein (B) in the kidneys of rats (n=8 per group) fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). [a]p<0.05 vs NF-NP.

In rats fed HF, the amount of P ingested was well correlated (r2 = 0.663) with urinary excretion of P (Fig 4A). However, no significant correlation was found between ingested P and either plasma P or FGF23 (Fig 4B and 4C).

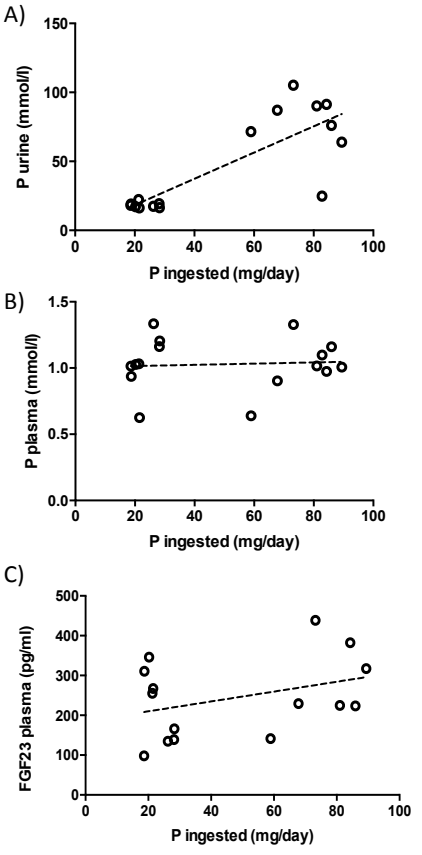

Fig. 4. Diet vs Urine & Plasma P and FGF23. Correlation between P ingested and (A) P urine (r^2=0.663, p<0.001), (B) P plasma (r^2=0.004, p=0.806), and (C) FGF23 plasma (r^2=0.130, p=0.187) in rats fed high fat diets with normal and low phosphorus content.

Histopathological studies demonstrated the presence of subtle renal lesions in rats fed HF diets. The number of retracted and sclerotic glomeruli was significantly higher in rats fed HF and P restriction did not influence glomerular damage. Similarly, rats fed HF showed more tubular atrophy and hyperplasia, as well as thickening of the basement membrane than rats fed NF. Phosphorus restriction tended to attenuate tubular lesions but the differences were not significant. Interstitial

edema and infiltrate were also more prominent in rats fed HF and, in this case, P restriction significantly attenuated interstitial pathology. (Table 2). Neither fibrosis nor nephrocalcinosis were detected in any experimental group.

	NF-NP (n=8)	HF-NP (n=8)	HF-LP (n=8)
Glomerular lesions	0.5 ± 0.1	1.1 ± 0.3[a]	1.3 ± 0.4[a]
Tubular lesions	0.1 ± 0.1	1.2 ± 0.1[a]	0.9 ± 0.2[a]
Interstitial lesions	0.4 ± 0.1	0.8 ± 0.1[a]	0.5 ± 0.1[b]

Table 2. Renal histopathology. Glomerular retraction and sclerosis (Glomerular lesions); tubular atrophy, hyperplasia and thickening of basement membrane (Tubular lesions); and interstitial edema and infiltrate (Interstitial lesions) in rats fed diets with normal fat and normal phosphorus (NF-NP), high fat and normal phosphorus (HF-NP) and high fat and low phosphorus (HF-LP). Semiquantitative scale (0-3). [a]$p<0.05$ vs NF-NP, [b]$p<0.05$ vs HF-NP.

DISCUSSION

This study was designed to investigate the effect of restricting P intake on the changes in mineral metabolism elicited by HF diets. Our results demonstrate that marked P restriction over a prolonged period of time (7 months) does not normalize the elevated circulating FGF23 levels induced by HF diets. These data indicate that the deleterious effects of high fat diet on the FGF23/klotho axis are not eliminated by reduced P intake.

The increase in plasma concentrations of FGF23 detected in the present study after feeding HF diets confirms previous results which were obtained over a much shorter period of time (1 month) (Raya et al. 2016). It is interesting to note that the magnitude of changes in both plasma FGF23 and renal klotho was similar after 1 and 7 months of exposure to HF diets. Thus the effect of HF diets on these parameters does not seem to be progressive.

As it has been previously reported, the mechanism for increased FGF23 in rats fed HF diets is likely related to the decrease in renal klotho. Renal klotho has been shown to decrease in response to HF diets both in Wistar rats (Raya et al. 2016) and in APoE knockout mice (Sastre et al. 2013). When renal klotho is decreased, tubular resistance to FGF23 action ensues and more FGF23 is needed to maintain phosphaturia, consequently resulting in an increase in circulating levels of FGF23 (Kanbay et al. 2017). In addition, feeding high fat and obesity may elicit systemic inflammation (Poret et al. 2018) and renal injury (Wickman and Kramer, 2013) which could also influence FGF23 (Kanbay et al. 2017). In the present study, a tendency to increased TNFα and minor renal lesions were observed in rats fed HF. Therefore, in addition to the reduction in renal klotho, both inflammation and renal damage may have played a marginal role in the increase in FGF23.

Based on recent published data that demonstrated that klotho expression in the kidney is regulated by the P load in the renal tubule

(Muñoz-Castañeda et al. 2017), it was hypothesized that reducing P intake, by feeding a 0.2% P diet, would restore renal klotho and decrease circulating FGF23 levels in rats fed HF. Our results show that P restriction resulted in a discrete increase in renal klotho expression in rats fed HF diets. A small and non-significant decrease in circulating levels of FGF23 was also observed in rats fed HF diets after P restriction. These results support previous data on the role of tubular P load on klotho but, on the other hand, demonstrate that P restriction is not able to fully compensate the changes in FGF23 elicited by feeding a HF diet. In fact, in this model the stimulatory effect of HF diet on FGF23 overcame the inhibitory effect of P restriction and the present data point towards a preferential regulation of FGF23 by fat intake rather than by P intake, as demonstrated by direct comparison of HF-LP and NF-NP groups. This contention is illustrated by the fact that rats fed HF-LP, that only ingested an average of 22.9 mg/day of P, had higher FGF23 concentrations than rats fed NF-NP, that ingested and average of 74.4 mg/day of P over a 7 month period.

The main physiologic role of FGF23 is to enhance phosphaturia (Kuro-o, 2010) and this is accomplished by down-regulating P transporters, mainly NaPiIIa, in the renal tubule. The reduction of NaPiIIa restricts P reabsorption and promotes urinary excretion of P (Biber et al. 2009). In our study, NaPiIIa was regulated in accordance to the changes in FGF23. Therefore, feeding HF resulted in a significant decrease in NaPiIIa and increased urinary excretion of P. Interestingly, in rats fed HF, P restriction increased NaPiIIa to almost to the same level than in rats fed NF, contrary to what was observed with FGF23. These data are in agreement with previous reports suggesting that, in addition to FGF23, NaPiIIa may be directly regulated by P (Bourgeois et al. 2013).

It is surprising that high fat intake has such a profound influence on FGF23. It remains to be determined to what point FGF23 regulation by fat intake is an epiphenomenon or a reflection of further,

and as yet not clarified, physiologic actions of FGF23. FGF23 plays a pivotal role in mineral metabolism and is consistently increased in patients with chronic kidney disease (Kovesdy and Quarles, 2016). In addition, FGF23 has been reported to be increased in obese people (Marsell et al. 2009) and a recent study identified energy intake as a potential predictor of plasma FGF23 concentrations (di Giusseppe et al. 2015). In this context it is relevant to point out that klotho has been shown to be implicated in energy metabolism (Miyazaqui et al. 2010; Razzaque, 2012).

Parameters related to energy intake may influence the increase in FGF23 after feeding HF diets. One factor that might be relevant is the increase in plasma leptin concentrations observed in rats fed HF. Leptin has been reported to stimulate FGF23 secretion by osteocytes (Tsuji et al. 2010). The effect of leptin is mediated by up-regulation of the stimulatory action of calcitriol on skeletal synthesis of FGF23 (Saini et al. 2013). However, in the present study, in agreement with previous data (Raya et al. 2016; Rios et al. 2017), calcitriol levels were very low in rats fed HF diets deeming unlikely a leptin-mediated stimulatory effect of calcitriol as responsible for the increase in FGF23. Actually, since FGF23 is known to decrease calcitriol synthesis through inhibition of 1-alpha-hydroxylase activity in the kidney (Shimada et al. 2004), it seems more likely that the high FGF23 may be responsible for the low calcitriol concentrations in rats fed HF diets.

A remarkable finding of this study is that plasma concentrations of P did not change in rats fed different amounts of P. Plasma P was maintained constant by adjusting urinary excretion of P which showed an excellent correlation with P ingestion. However, it is interesting to note that FGF23 had a poor correlation with ingested P and that many rats fed HF-LP had high FGF23 concentrations, even though their urinary excretion of P was consistently low. This finding also points toward a regulation of FGF23 by high fat diet, independent of P intake.

CONCLUSION

In conclusion, the results of this study indicate that dietary P restriction did not prevent the increase in FGF23 elicited by feeding HF diets.

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REFERENCES

Biber, J., Hernando, N., Forster, I. and Murer, H. 2009. Regulation of phosphate transport in the proximal tubules. *Pflugers Arch. – Eur. J. Physiol.*, 458:39-52.

Bourgeois, S., Capuano, P., Stange, G., Mühlemann, R., Murer, H., Biber, J. and Wagner, C. 2013. The phosphate transporter NaPi-IIa determines the rapid adaptation to dietary phosphate intake in the mouse irrespective of persistently high FGF23 levels. *Pflugers Arch. – Eur. J. Physiol.*, 465:1557-72

di Giuseppe, R., Kühn, T., Hirche, F., Buijsse, B., Dierkes, J., Fritsche, A., Kaaks, R., Boeing, H., Stangl, G.I. and Weikert, C. 2015. Potential Predictors of Plasma Fibroblast Growth Factor 23 Concentrations: Cross-Sectional Analysis in the EPIC-Germany Study. *PloS One*, 10:e0133580.

Frommelt, L., Bielohuby, M., Stoehr, B.J.M., Menhofer, D., Bidlingmaier, M. and Kienzle, E. 2014. Effects of low-carbohydrate, high-fat diets on apparent digestibility of minerals and trace elements in rats. *Nutrition*, 30:869-75.

Kanbay, M., Vervoelt, M., Cozzolino, M., Siriopol, D., Covic, A., Goldsmith, D. and Solak, Y. 2017. Novel faces of fibroblast growth factor 23 (FGF23): iron deficiency, inflammation, insulin resistance, left ventricular hypertrophy, proteinuria and acute kidney injury. *Calcif. Tissue Int.*, 100:217-28

Kestenbaum, B., Sachs, M.C., Hoofnagle, A.N., Siscovick, D.S., Ix, J.H., Robinson-Cohen, C., Lima, J.A.C, Polak, J.F., Blondon, M., Ruzinski, J., Rock, D. and de Boer, I.H. 2014. Fibroblast growth factor-23 and cardiovascular disease in the general population: the Multi-Ethnic Study of Atherosclerosis. *Circ. Heart Fail*, 7:409-17.

Kovesdy, C.P. and Quarles, L.D. 2016. FGF23 from bench to bedside. *Am. J. Physiol, Renal Physiol.*, 310:F1168-74.

Kuro-o, M. 2010. Overview of the FGF23-Klotho axis. *Pediatr. Nephrol.*, 25:583-90.

Marsell, R., Mirza, M.A.I., Mallmin, H., Karlsson, M., Mellström, D., Orwoll, E., Ohlsson, O., Jonsson, K.B., Ljunggren, Ö. and Larsson, T.E. 2009. Relation between fibroblast growth factor-23, body weight and bone mineral density in elderly men. *Osteoporos Int.*, 20:1167-73.

Miyazaki, T., Takenaka, T., Inoue, T., Sato, M., Hanyu, M., Eiki, Y., Nodera, M., Yanagisawa, H, Ohno, Y., Shibazaki, S. and Suzuki, H. 2010. Klotho expression is induced by calorie restriction in adult male rats. *Trace Nutr. Res*, 27:92-6.

Muñoz-Castañeda, J.R., Herencia, C., Pendón-Ruiz de Mier, M.V., Rodriguez-Ortiz, M.E., Diaz-Tocados, J.M., Vergara, N., Martínez-Moreno, J.M., Salmerón, M.D., Richards, W.G., Felsenfeld, A., Kuro-o, M., Almaden, Y. and Rodríguez, M. 2017. Differential regulation of renal Klotho and FGFR1 in normal and uremic rats. *FASEB J.*, 31:3858-67.

Panwar, B., Jenny, N.S., Howard, V.J., Wadley, V.G., Muntner, P., Kissela, B.M., Judd, S.E. and Gutiérrez, O.M. 2015. Fibroblast growth factor 23 and risk of incident stroke in community-living adults. *Stroke*, 46:322-8.

Popkin, B.M., Adair, L.S. and Ng, S.W. 2012. Global nutrition transition and the pandemic of obesity in developing countries. *Nutr. Rev.*, 70:3-21.

Poret, J.M., Souza-Smith, F., Marcell, S.J., Gaudet, D.A., Tzeng, T.H., Braymer, H.D., Harrison-Bernad, L.M. and Primeaux, D. 2018. High fat diet comsumption differentially affects adipose tissue inflammation and adipocyte size in obesity-prone and obesity-resistant rats. *Int. J. Obes. (Lond.)*, 42:535-41.

Raya, A.I., Rios, R., Pineda, C., Rodriguez-Ortiz, M.E., Diez, E., Almaden, Y., Muñoz-Castañeda, J.R., Rodriguez, M., Aguilera-Tejero, E. and Lopez, I. 2016. Energy-dense diets increase FGF23, lead to phosphorus retention and promote vascular calcifications in rats. *Sci. Rep.*, 6:36881.

Razzaque, M.S. 2012. The role of Klotho in energy metabolism. *Nat. Rev. Endocrinol.*, 8:579-87.

Rios, R., Raya, A.I., Pineda, C., Rodriguez, M., Lopez, I. and Aguilera-Tejero, E. 2017. Vitamin E protects against extraskeletal calcification in uremic rats fed high fat diets. *BMC Nephrol.*, 18:374.

Saini, R.K., Kaneko, I., Jurutka, P.W., Forster, R., Hsieh, A., Hsieh, J.C., Haussler, M.R. and Withfield, G.K. 2013. 1,25-dihydroxyvitamin D_3 regulation of fibroblast growth factor-23 expression in bone cells: evidence for primary and secondary mechanisms modulated by leptin and interleukin-6. *Calcif. Tissue Int.*, 92:339-53.

Sarathy, S., Sullivan, C., Leon, J.B., Sehgal, A.R. 2008. Fast food, phosphorus-containing additives, and the renal diet. *J. Ren. Nutr.*, 18:466-70.

Sastre, C., Rubio-Navarro, A., Buendia, I., Gomez-Guerrero, C., Blanco, C., Mas, S., Egido, J., Blanco-Colio, L.M., Ortiz, A. and Moreno, J.A. 2013. Hyperlipidemia-associated renal damage decreases klotho expression in kidneys from ApoE knockout mice. *PloS One*, 8:e83713.

Scialla, J.J., Xie, H., Rahman, M., Anderson, A.H., Isakova, T., Ojo, A., Zhang, X., Nessel, L., Hamano, T., Grunwald, J.E., Raj, D.S., Yang, W, He, J., Lash, J.P., Go, A.S., Kusek, J.W., Feldman, H. and Wolf, M. 2014. Fibroblast growth factor-23 and cardiovascular events in CKD. *J. Am. Soc. Nephrol.*, 25:349-60.

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., Fujita, T., Nakahara, K., Fukumoto, S. and Yamashita, T. 2014. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J. Bone Miner. Res.*, 19:429-35.

Tsuji, K., Maeda, T., Kawane, T., Matsunuma, A. and Horiuchi, N. 2010. Leptin stimulates fibroblast growth factor 23 expression in bone and suppresses renal 1alpha,25-dihydroxyvitamin D_3 synthesis in leptin-deficient mice. *J. Bone Miner. Res.*, 25:1711-23.

Uribarri, J. and Calvo, M.S. 2003. Hidden sources of phosphorus in the typical American diet: does it matter in nephrology? *Semin. Dial.*, 16:186-8.

Wang, Y.C., McPherson, K., Marsh, T., Gortmaker, S.L. and Brown, M. 2011. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet.*, 378:815-25.

Wickman, C. and Kramer, H. 2013. Obesity and kidney disease: potential mechanisms. *Semin. Nephrol.*, 33:14-22.

6. Conclusions

1. Feeding a high fat diet resulted in phosphorus retention in rats with reduced renal function (1/2 nephrectomy) fed normal phosphorus (0.6%) and in rats with normal renal function fed high phosphorus (1.2%) diets.

2. In rats with intact or reduced (1/2 nephrectomy) renal function, high fat intake resulted in a decreased expression of renal alfa-klotho and an increase in both the plasma concentrations of fibroblast growth factor 23 and the bone expression of fibroblast growth factor 23.

3. Ingestion of high fat diets resulted in consistent decreases in circulating concentrations of 1,25(OH)2-vitamin D in rats with normal and reduced (1/2 nephrectomy) renal function.

4. Uremic rats fed high fat diets developed more severe extraskeletal calcifications than uremic rats fed normal fat diets.

5. Vitamin E supplementation attenuated uremic vascular calcification in rats fed high fat diets.

6. Reducing phosphorus load, by feeding a diet with low phosphorus (0.2%) content, did not prevent the increase in fibroblast growth factor 23 elicited by high fat diets.

Estas conclusiones se relacionan con los siguientes trabajos:

- Scientific Report, 6: 36881, 2016: Conclusión 1, 2, 3 y 4
- BMC Nephrology, 18: 374, 2017: Conclusión 2, 3, 4 y 5
- PLos ONE, 13: e0198481, 2018: Conclusión 2 y 6

7. Summary

SUMMARY

To investigate the effect of feeding a high fat (HF) diet on phosphorus (P) balance, studies were conducted in rats with normal renal function fed normal (0.6%) and elevated (1.2%) P and in uninephrectomized rats fed 0.6% P. Rats were fed either a normal fat (NF) or HF diet for 30 days. At the end of the experiments rats fed HF did not differ from rats fed NF in body weight, renal function and lipid profile. When compared with rats fed NF, rats fed HF that received 1.2% P and uninephrectomized rats showed P retention. In addition, an increase in fibroblast growth factor 23 (FGF23), both circulating levels and bone expression, was detected in all groups fed HF. The increase in FGF23 was likely secondary to a decrease in renal expression of alfa-klotho observed in the rats fed HF. All rats fed HF also showed reduced plasma calcitriol and normal plasma calcidiol concentrations. In uremic rats (5/6 nephrectomy) fed 0.9% P, HF diet intake resulted in more severe extraskeletal calcifications than in their NF fed counterparts.

In a second set of experiments, the influence of HF diet on vascular and extraskeletal calcification was evaluated with more detail. In this study rats were fed either NF or HF diets, with 0.6% P, for 45 days, before performing 5/6 nephrectomy. After nephrectomy, rats were switched to diets (NF or HF) with 1.2% P and were treated with calcitriol (80 ng/kg on alternate days). Half of the rats in each feeding schedule (NF or HF) were supplemented with vitamin E (30000 mg/kg). Prior to nephrectomy, after 45 days feeding HF, rats did not increase body weight but showed moderate hyperglycemia and dyslipidemia. As in previous experiments, plasma FGF23 was increased and calcitriol was decreased in rats fed HF. After Nx, rats fed HF diet showed substantial extraskeletal calcification. Uremic rats fed HF diets developed more severe extraosseous calcifications than uremic rats fed NF. Dietary vitamin E supplementation protected against uremic calcifications in rats fed HF diets.

To evaluate whether reducing P load by feeding a diet with low P content would prevent the increase in FGF23 elicited by high fat diet, rats were fed HF diets with normal (0.6%) or low (0.2%) P for 7 months. At the end of the experiments, when compared with rats fed NF, rats fed HF had increased their body weight and their plasma levels of triglycerides and leptin. In addition, these rats showed a tendency to reduced renal function and increased inflammatory status. As in previous experiments, feeding HF resulted in increased plasma concentrations of FGF23 and decreased plasma concentrations of calcitriol. Phosphorus restriction did not normalize the plasma concentrations of FGF23 and did not restore the decreased levels of renal alfa-klotho in rats fed HF. When comparing the groups fed 0.6% P, urinary excretion of P was higher and renal expression of the sodium-phosphate co-transporter NaPiIIa was lower in rats fed HF. Urinary excretion of P was very low in rats fed 0.2% P even though these rats had elevated FGF23 concentrations secondary to HF feeding.

RESUMEN

Para investigar el efecto de la alimentación con dieta alta en grasa (AG) sobre el balance de fósforo (P), se llevaron a cabo estudios en ratas con función renal normal alimentadas con P normal (0.6%) y elevado (1.2%) y en ratas uninefrectomizadas alimentadas con 0.6% P. Las ratas fueron alimentadas con una dieta con un contenido normal de grasa (NG) o AG durante 30 días. Al final de los experimentos, las ratas alimentadas con AG no difirieron de las ratas alimentadas con NG en peso corporal, función renal ni perfil lipídico. Cuando se compararon con ratas alimentadas con NG, las ratas alimentadas con AG que recibieron 1,2% de P y las ratas uninefrectomizadas mostraron retención de P. Además, se detectó un aumento en el factor de crecimiento de fibroblastos 23 (FGF23), tanto en los niveles circulantes como en la expresión ósea, en todos los grupos alimentados con AG. El aumento de FGF23 fue probablemente secundario a la disminución en la expresión renal de alfa-klotho observado en las ratas alimentadas con AG. Todas las ratas alimentadas con AG también mostraron reducción de calcitriol plasmático y concentraciones normales de calcidiol en plasma. En ratas urémicas (nefrectomía 5/6) alimentadas con 0,9% de P, la ingesta de dieta AG dio lugar a calcificaciones extraesqueléticas más graves que en las alimentadas con NG.

En un segundo conjunto de experimentos, la influencia de la dieta AG sobre la calcificación vascular y extraesquelética se evaluó con más detalle. En este estudio, las ratas fueron alimentadas con dietas NG o AG, con 0,6% de P, durante 45 días, antes de realizar una nefrectomía (Nx) 5/6. Después de la Nx, las ratas se alimentaron con dietas (NG o AG) con 1,2% de P y se trataron con calcitriol (80 ng/kg en días alternos). La mitad de las ratas de cada grupo de alimentación (NG o AG) se trataron con vitamina E (30000 mg / kg). Durante los 45 días previos a la Nx, las ratas no aumentaron el peso corporal, pero mostraron hiperglucemia moderada y dislipidemia. Como en experimentos previos, el FGF23 aumentó y el calcitriol disminuyó en

ratas alimentadas con AG. Después de Nx, las ratas alimentadas con dieta AG mostraron calcificación extraesquelética sustancial. Las ratas urémicas alimentadas con dietas AG desarrollaron calcificaciones extraóseas más severas que las ratas urémicas alimentadas con NG. La suplementación dietética con vitamina E protege contra las calcificaciones urémicas en ratas alimentadas con dieta AG.

Para evaluar si la reducción de la carga de P mediante alimentación con dieta con bajo contenido de P evitaría el aumento de FGF23 provocado por una dieta AG, se alimentaron las ratas con dietas AG con P normal (0.6%) o P bajo (0.2%) durante 7 meses. Al final de los experimentos, cuando se compararon con ratas alimentadas con NG, las ratas alimentadas con AG aumentaron su peso corporal y sus niveles plasmáticos de triglicéridos y leptina. Además, estas ratas mostraron una tendencia a disminuir la función renal y aumentar el estado inflamatorio. Como en experimentos previos, la alimentación de AG dio como resultado un aumento de las concentraciones plasmáticas de FGF23 y una disminución de las concentraciones plasmáticas de calcitriol. La restricción de fósforo no normalizó las concentraciones plasmáticas de FGF23 y no restableció los niveles disminuidos de alfa-klotho renal en ratas alimentadas con AG. Al comparar los grupos alimentados con 0.6% P, la excreción urinaria de P fue mayor y la expresión renal del cotransportador de fósforo-sodio, NaPiIIa, fue menor en las ratas alimentadas con AG. La excreción urinaria de P fue muy baja en ratas alimentadas con 0,2% de P aunque estas ratas tenían concentraciones elevadas de FGF23 secundarias a la alimentación con AG.

8. Appendix

LIST OF ABBREVIATIONS

Ca - Calcium

CKD - Chronic Kidney Disease

CTR - Calcitriol (1,25-dihydroxyvitamin D)

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic Acid

EGTA - Ethylene Glycol Tetraacetic Acid

eod - every other day

FGF23 - Fibroblast Growth Factor 23

FGFR1 - Fibroblast Growth Factor Receptor 1

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

HEPES - Hydroxyethyl piperazineethanesulfonic acid

HDL - High Density Lipoprotein

HF - High Fat

HP - High Phosphorus

ip - intraperitoneal

KCl - Potasium Chloride

LSD – Least significant difference

LDL - Low Density Lipoprotein

LP - Low Phosphorus

NaCl – Sodium Chloride

NaPiIIa - Sodium-Phosphate co-transporter type 2a

NF - Normal Fat

NP - Normal Phosphorus

Nx - Nephrectomy

OB/MS - Obesity/Metabolic Syndrome

P - Phosphorus

PMSF - Phenylmethane Sulfonyl Fluoride

PTH - Parathyroid Hormone

RT-PCR - Reverse Transcription Polymerase Chain Reaction

SHPTH - Secondary Hyperparathyroidism

TG - Triglycerides

TTBS- Tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20)

VC - Vascular Calcification

VitE - Vitamin E

VSMCs - Vascular Smooth Muscle Cells

LIST OF PUBLICATIONS

Energy-dense diets increase FGF23, lead to phosphorus retention and promote vascular calcifications in rats
Ana I. Raya, Rafael Rios, Carmen Pineda, Maria E. Rodriguez-Ortiz, Elisa Diez, Yolanda Almaden, Juan R. Muñoz-Castañeda, Mariano Rodriguez, Escolastico Aguilera-Tejero & Ignacio Lopez

Scientific Reports, 6: 36881, 2016.

Vitamin E protects against extraskeletal calcification in uremic rats fed high fat diets.
Rafael Rios, Ana I. Raya, Carmen Pineda, Mariano Rodriguez, Ignacio Lopez & Escolastico Aguilera-Tejero

BMC Nephrology, 18: 374, 2017.

Phosphorus restriction does not prevent the increase in fibroblast growth factor 23 elicited by high fat diet.
Rafael Rios, Carmen Pineda, Ignacio Lopez, Juan R. Muñoz-Castañeda, Mariano Rodriguez, Escolastico Aguilera-Tejero & Ana I. Raya

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