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Siempre he creído que si trabajas, los resultados vendrán solos. No hago las cosas a medias, porque sé que si lo hago entonces solo puedo esperar tener resultados a medias.

Michael Jordan

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Abreviaturas

ABREVIATURAS

A: Absorbancia
ADN: Ácido desoxirribonucleico
ADNc: ADN copia
AKT: Serín Treonín proteína kinasa inespecifica
ARN: Ácido ribonucleico
ARNm: ARN mensajero
ARNr: ARN ribosómico
BSA: Albúmina sérica bovina
BMP: Proteína morfogenética ósea
CaSR: Receptor sensor de calcio
CaxP: Producto calcio fósforo
CBFA-1/Runx2: Factor activador del núcleo 1/Factor transcripción relacionado a Run2
CMLV: Células de músculo liso vascular
CKD-MBD: Alteraciones óseas y minerales en la ERC
C_T: Ciclo umbral
DKK-1: Dickkopf -1
DMO: Densidad mineral ósea
DXA: Densitómetro radiológico digital de doble energía
EGFR: Receptor del factor de crecimiento epidérmico
ERC: Enfermedad renal crónica
ERK: Kinasa regulada extracelularmente
FGF23: Factor de crecimiento fibroblástico 23
FGFR: Receptor de FGF
HEK293: Celulas de riñón embrionario humano 293
HPD: Dieta alta en fósforo
GAPDH: Gliceraldehído 3-fosfato deshidrogenasa
IL: Interleuquina
IRMA: Ensayo immunorradiométrico
kDa: KiloDaltons
MAPK: Quinasa de la proteína activada por mitógeno
Mod sHPT: Hiperparatiroidismo secundario moderado
Mod/sev sHPT: Hiperparatiroidismo secundario moderado-severo
Na-Pi II: Co-transportador de sodio y fosfato tipo II
NPD: Dieta normal en fósforo
NFκ-B: Factor nuclear κ B
ODR: Osteodistrofia renal
OMPF: 3-*O*-metil fluorescein fosfato
OPG: Osteoprotegerina
PBS: Tampón fosfato salino

PCNA:	Antígeno nuclear de proliferación celular
PCR:	Reacción en cadena de la polimerasa
PTH:	Parathormona
PTHR1:	Receptor de PTH tipo 1
PTHrP:	Péptido relacionado con la PTH
qRT-PCR:	PCR cuantitativa a tiempo real
RANK:	Receptor activador de NFκ-B
RANKL:	Ligando del receptor activador de NFκ-B
ROS:	Especies reactivas de oxigeno
RXR:	Receptor X retinoide
SDS:	Dodecil sulfato sódico
Sev sHPT:	Hiperparatiroidismo secundario severo
sHPT:	Hiperparatiroidismo secundario
TGF-β:	Factor de crecimiento transformante beta
TNF-α:	Factor de necrosis tumoral alfa
TRAP:	Fosfatasa ácida tartrato resistente
U.R.:	Unidades relativas
VDR:	Receptor de vitamina D

Tabla de contenido

TABLA DE ILUSTRACIONES

Introducción

El riñón y el hueso son los principales reguladores de la homeostasis del calcio y del fósforo. El riñón, a través de la captación activa o el filtrado de calcio y fósforo a nivel del túbulo proximal, es capaz de regular la homeostasis de dichos elementos. Por su parte, el hueso actúa como principal reservorio de calcio y fósforo pudiendo ser movilizados si así fuera necesario. La parathormona (PTH), la 1,25-dihidroxivitamina D (calcitriol), y el factor de crecimiento fibroblástico 23 (FGF23) son las principales hormonas involucradas en la regulación de calcio y fósforo.

Aspectos del metabolismo mineral asociados con el concepto de enfermedad renal crónica

La enfermedad renal crónica (ERC) se define como una disminución progresiva del filtrado glomerular que se asocia, a medida que la insuficiencia renal avanza, con la pérdida de las funciones ejercidas por el riñón. Entre estas funciones destacan la función hormonal, la regulación de la homeostasis del medio interno, la excreción de sustancias derivadas del metabolismo nitrogenado y otras funciones metabólicas. Recientemente, las distintas fases de progresión de la ERC han sido divididas en 5 estadios, desde ERC estadio 1, la forma más leve, hasta estadio 5, la forma más avanzada de la ERC. El deterioro progresivo de la función renal determina la aparición de un conjunto de cambios hormonales y metabólicos entre los que destacan las alteraciones del metabolismo del calcio, de la vitamina D y PTH y del equilibrio ácido base, así como la acumulación en sangre de un conjunto de sustancias no bien definidas que se engloban con el término de *toxinas urémicas*.

El mantenimiento de la homeostasis del calcio y el fósforo es un proceso complejo que se produce fundamentalmente a tres niveles: hueso, intestino y riñón (1) y depende de factores endocrinos y paracrinos, dentro de los primeros se encuentran las denominadas hormonas calciotropas: PTH, calcitriol y FGF23 (2).

En el riñón en condiciones normales, la PTH y el FGF23 favorecen la excreción tubular de fósforo, a la par la PTH impide la excreción de calcio. La PTH tambien estimula la 1-α-hidroxilasa renal y con ello la síntesis de calcitriol (3), que por un lado favorece la absorción intestinal de calcio y de fósforo y por otro, inhibe la síntesis de PTH. En individuos con función renal normal o con ERC grado 1, 2 ó 3, todos los mecanismos interrelacionados de regulación se encuentran activos, y el efecto combinado del aumento de PTH y FGF23 se traduce en un incremento de los niveles séricos de calcio y un descenso de los de fósforo (Ilustración 1).

Debido al descenso de la función renal, disminuye la excreción de fósforo, hecho que se traduce en ligeros aumentos del fósforo sérico que tiene dos efectos, uno a nivel de la glándula paratiroides aumentando la síntesis de PTH (4) y el otro a nivel óseo estimulando la síntesis de FGF23 (5, 6). Los incrementos en PTH y FGF23 favorecen la excreción de fósforo a nivel del túbulo renal; estos cambios logran compensar parcialmente la retención de fósforo secundaria al descenso de función renal. Además, el FGF23 ejerce otras dos acciones importantes, a nivel renal inhibe la síntesis de 1-α-hidroxilasa y en consecuencia la producción de calcitriol y a nivel de la glándula paratiroides inhibe la síntesis y secreción de PTH (7, 8).

A medida que la ERC progresa y alcanza estadios más avanzados, estos mecanismos de regulación van progresivamente alcanzando su nivel máximo de regulación y a partir de estadios avanzados de ERC grado 4 y 5 ya no son suficientes, la homeostasis mineral se encuentra seriamente comprometida y pequeñas reducciones en la función renal son capaces de provocar importantes desequilibrios metabólicos (9). En estos casos se observa una bajada de los niveles de calcio séricos al desaparecer la señal del Calcitriol y una subida de los niveles de fósforo séricos, al bajar la masa renal funcionante y verse comprometida la excreción fósforo a pesar de la presencia de altos niveles de FGF23 y PTH. Los altos niveles de PTH y FGF23 característicos de estas etapas, además se han asociado con perdidas óseas y mortalidad (10, 11).

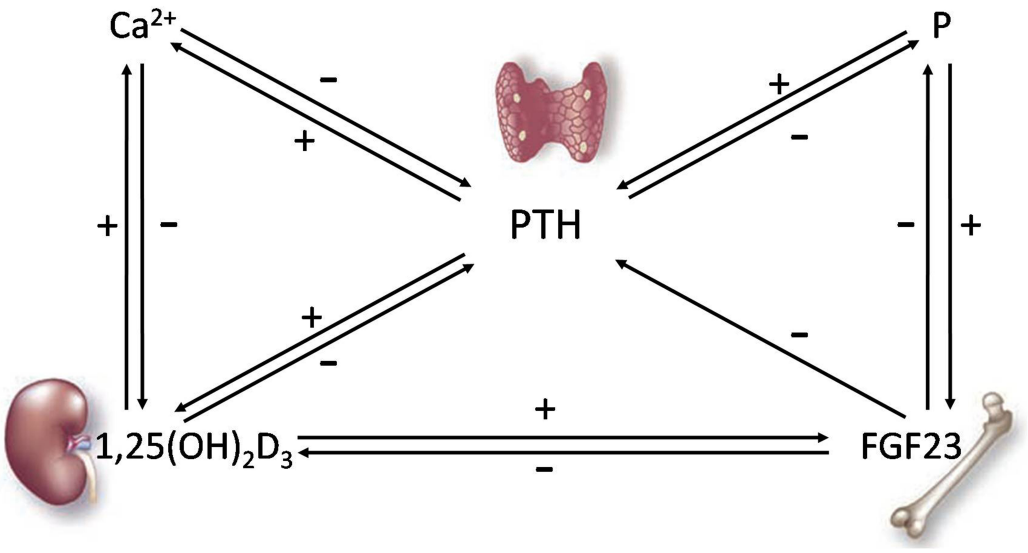

Ilustración 1. Interrelaciones entre calcio (Ca2+) y fósforo (P) y sus hormonas, PTH, FGF23 y calcitriol (1,25(OH)2D3). (Adaptado de Silver y col. (12)).

Por tanto, en la ERC avanzada, los mecanismos de la homeostasis mineral se ver seriamente comprometidos, mostrando un cuadro con serias consecuencias para el hueso y algunos tejidos blandos (fragilidad ósea, calcificaciones vasculares y valvulares y calcifilaxis) (1, 13-15).

Durante sesenta años, el conjunto de estas alteraciones del metabolismo óseo y mineral asociadas con la ERC se han definido como osteodistrofia renal (ODR) (16); término que englobaba, no sólo la respuesta espontánea del hueso frente a la ERC a consecuencia del hiperparatiroidismo secundario, sino también a las distintas condiciones clínicas y terapéuticas del paciente (17). Actualmente, el término ODR ha sido reemplazado por el término "alteraciones óseas y minerales en la ERC" (en inglés CKD-MBD) (18) que integra todas las alteraciones bioquímicas, esqueléticas y calcificaciones cardíacas y vasculares que ocurren como consecuencia de las alteraciones del metabolismo mineral en la ERC. El término ODR ha quedado restringido a las alteraciones histológicas de morfología y arquitectura ósea propias de la ERC.

En esta tesis, nos centraremos en el estudio de dos de las alteraciones del metabolismo mineral asociadas a la enfermedad renal crónica: el hiperparatiroidismo secundario y la calcificación vascular, prestando atención a los cambios de expresión de genes y proteínas en las fases mas avanzadas de ambas alteraciones. Además, se estudio la relación entre las calcificaciones vasculares y la pérdida de masa ósea.

Hiperparatiroidismo secundario en la ERC

Generalidades

Durante la progresión de la ERC, la hiperfunción de la glándula paratiroides es una frecuente manifestación y tiene como consecuencia el incremento de los niveles sistémicos de PTH. A lo largo de los años, se han ido identificando un cúmulo de factores que colaboran en el desarrollo y la progresión del hiperparatiroidismo secundario, condicionando un aumento importante de la morbi-mortalidad en pacientes con ERC grados 3-5 (14).

El primer factor patogénico descrito como desencadenante del hiperparatiroidismo secundario fue la hipocalcemia (19). Los niveles de calcio pueden verse alterados por distintas causas, pero el principal responsable de esta hipocalcemia es el descenso en la síntesis de calcitriol como consecuencia de una menor producción de 1-α-hidroxilasa a nivel tubular renal debido a la pérdida de parénquima renal funcionante (19, 20). Este descenso de los niveles de calcitriol reduce la absorción intestinal de calcio favoreciendo la tendencia al descenso de los niveles plasmáticos del mismo. La disminución de calcio sérico estimula la síntesis y secreción de PTH en las glándulas paratiroides y esta hormona ejerce su efecto directo en el riñón y en el hueso e indirecto en el tracto digestivo a través del calcitriol estimulando mecanismos que intentan normalizar el calcio sérico. A su vez, el calcitriol de forma independiente del calcio, tiene efecto regulador sobre la síntesis de PTH (21, 22).

En los años sesenta, se confirmó que la hiperfosforemia también tenía un papel relevante en la etiopatogenia del hiperparatiroidismo secundario (19, 23). En un principio, el incremento en la secreción de PTH se atribuyó a la capacidad del fósforo para disminuir los niveles de calcio sérico (24). Sin embargo, a lo largo de la década siguiente se comprobó que la estimulación de la PTH se produce también a otros niveles. Por un lado, el fósforo inhibe la síntesis de 1-α-hidroxilasa renal y con ello la síntesis de calcitriol (25, 26). Además, el fósforo tiene un efecto directo e independiente del calcio sobre la síntesis de PTH (19, 26, 27) y sobre la proliferación de las células de la glándula paratiroides (28, 29).

Factores reguladores de la PTH

La regulación de la PTH es un mecanismo complejo en la que intervienen diversos factores como calcio, calcitriol, fósforo y factor de crecimiento fibroblástico 23 (FGF23).

El calcio iónico extracelular es el principal regulador de la función paratiroidea. Niveles bajos de calcio extracelular estimulan la secreción de PTH en cuestión de segundos, mientras que niveles elevados inhiben rápidamente la liberación de la hormona y favorecen su degradación dentro de las propias células paratiroideas. Estos hallazgos hicieron sospechar sobre la existencia de un receptor sensor de calcio (o receptor sensible a calcio) en la membrana de las células paratiroideas, capaz de regular la secreción de PTH en función de los niveles de calcio extracelular. En 1993 dicho receptor fue clonado y caracterizado en glándulas paratiroides bovinas, demostrando tener no sólo propiedades de receptor sino también de sensar el calcio (CaSR) (30). El CaSR es un receptor de la familia de receptores acoplados a proteínas G formado por un largo dominio extracelular de 612 aminoácidos. A continuación posee un dominio de anclaje a la membrana formado por siete hélices transmembrana, característico de la superfamilia de receptores acoplados a proteínas G y finalmente, un dominio citoplasmático que corresponde con el extremo carboxilo de la molécula.

Aún no se conocen en su totalidad los mecanismos moleculares por los que el CaSR ejerce sus acciones, aunque sí se ha demostrado que es capaz de activar múltiples cascadas de señalización. Los agonistas del CaSR activan las fosfolipasas C, A_2 y D tanto en células renales de embrión humano (HEK293) transfectadas con el CaSR, como en células paratiroides bovinas en cultivo (31). El aumento del calcio extracelular provoca la activación de la fosfolipasa C a través de la subunidad Gq del receptor. La fosfolipasa C metaboliza al fosfatidil inositol 4,5 difosfato liberando inositol 1,4,5 trifosfato. Esto produce un aumento del calcio citosólico a través de la movilización de los depósitos celulares así como de la activación de canales de calcio voltaje-dependientes y de otros canales no específicos. Por otro lado, la subunidad Gi del CaSR inhibe la

adenilato ciclasa, y con ello disminuyen los niveles de AMP cíclico. El aumento del calcio iónico extracelular también produce incrementos en la liberación de ácido araquidónico en células HEK293 transfectadas con el CaSR pero no en las mismas células no transfectadas, indicando que el CaSR está implicado en la activación de la fosfolipasa A_2 (31). Del mismo modo, el calcio iónico extracelular también es capaz de incrementar la formación de fosfatidilbutanol, un marcador de activación de la fosfolipasa D, demostrando que el CaSR está implicado en la activación de dicha señal intracelular. Como resultado final de la activación del CaSR se produce la inhibición de la liberación y síntesis de PTH.

Además de su papel en la regulación del metabolismo de la PTH, el CaSR y el calcio tienen un efecto inhibitorio sobre la proliferación celular de la glándula paratiroides. Existe una relación inversa entre la hiperplasia de la glándula paratiroides de ratas urémicas y el descenso en los niveles de ARN mensajero (ARNm) y proteína de CaSR, demostrando la importancia que tiene el CaSR en el desarrollo, progresión y función de las glándulas paratiroides en la ERC (32).

El calcitriol tiene un efecto inhibitorio directo sobre la secreción de PTH, disminuyendo a nivel transcripcional la síntesis del ARNm de la PTH. El calcitriol actúa sobre la glándula paratiroides a través de su receptor específico, el VDR, un receptor de alta afinidad y especificidad que pertenece a la familia de los receptores esteroideos/tiroideos. En el VDR se reconocen 2 dominios fundamentales, uno de unión al ADN y otro de unión al ligando (calcitriol). Una vez formado el complejo hormona-receptor, se produce la translocación del complejo calcitriol-VDR al núcleo de la célula formando un heterodímero con el receptor X-retinoico (RXR). El complejo calcitriol-VDR-RXR se une a elementos de respuesta a vitamina D localizados en la región promotora del gen de la PTH, bloqueando su transcripción (21).

En el hiperparatiroidismo secundario de la insuficiencia renal crónica, se observa una disminución significativa de la expresión de VDR (33). Esta reducción es parcialmente responsable de una menor respuesta al calcitriol y por tanto de un inadecuado control inhibitorio en la transcripción del gen de la PTH;

el resultado es un aumento en los niveles de PTH. En el hiperparatiroidismo secundario severo, el crecimiento glandular es predominantemente nodular, en las áreas nodulares se observa una disminución en la densidad de receptores VDR que es en gran parte responsable de la falta de respuesta al tratamiento con calcitriol (34).

El calcitriol es también un importante regulador del crecimiento celular. Niveles bajos de calcitriol favorecen el crecimiento de la glándula paratiroides, dicho efecto estaría debido, al menos en parte, a cambios en el calcio sérico. El déficit de calcitriol ejerce los mismos efectos que una dieta baja en calcio (29). Sin embargo se ha observado que el calcitriol puede ejercer efecto antiproliferativo independiente del calcio, este se acompaña de un freno en el incremento del factor de crecimiento transformante alfa (TGF-α), principal responsable del desarrollo de hiperplasia en la glándula paratiroides. (35).

El fósforo es otro de los factores que actúa directamente sobre las glándulas paratiroides regulando la síntesis y secreción de PTH. El aumento en los niveles de fósforo sérico produce un importante incremento en la secreción de PTH (4, 27) y además, es capaz de regular a nivel post-transcripcional la expresión del gen de la PTH (36). En este proceso estarían implicadas diversas proteínas citosólicas, capaces de unirse a secuencias específicas en la región 3´ no traducida de la molécula de ARNm, que estabilizarían el transcrito de PTH y evitarían su degradación. Por el contrario, en presencia de fósforo bajo, se produciría un descenso de estos factores protectores y se inestabilizaría el ARNm, favoreciendo su degradación (37). Además, estudios recientes (38), han demostrado *in vivo* que el fósforo también puede influenciar de forma aguda la secreción de PTH por mecanismos independientes del calcio y el calcitriol.

El fósforo también ha demostrado ser un importante estímulo proliferativo para las células de la glándula paratiroides; numerosos estudios han demostrado que en animales con insuficiencia renal crónica, una dieta alta en fósforo ocasiona una hiperplasia de las glándulas paratiroides; esto ocurre sin que se observen cambios en los niveles de calcio y calcitriol (27, 29, 39). El mecanismo por el cual el fósforo estimula la hiperplasia de paratiroides guarda

relación con un aumento en TGF-a. El aumento de este último requiere la activación del receptor del factor de crecimiento epidérmico (EGFR), glicoproteína de membrana de 170 kDa con actividad intrínseca de tiroquinasa que se encuentra presente en condiciones normales en las glándulas paratiroides. En varios carcinomas humanos como asimismo en alteraciones prolifertivas no neoplásicas (40), se observa una expresión aumentada de TGF-α y EGFR que condiciona un círculo vicioso autocrino que resulta en un incremento progresivo de TGF-α y consecuentemente en un aumento de la hiperplasia glandular (40).

Además del efecto de los reguladores clásicos de la función paratiroidea, recientemente ha sido descrito que el Factor de Crecimiento Fibroblástico 23 (FGF23) es otro importante regulador de los niveles de PTH. El FGF23 es sintetizado fundamentalmente en osteocitos pero también en osteoblastos como respuesta a niveles elevados de fósforo y a los incrementos de calcitriol (41, 42). El FGF23 actúa sobre sus tejidos diana, entre ellos paratiroides y riñón, a través de receptores FGFR pero siempre en presencia de su correceptor, klotho (43), una proteína transmembrana que actúa como cofactor del FGF23 facilitando la unión de FGF23 a su receptor. Por tanto, la acción del FGF23 en todos los tejidos estará determinada no sólo por la presencia de su receptor, con el que forma un heterodímero, sino también por la presencia obligada de klotho. Una vez que FGF23 reconoce el complejo FGFR-klotho, se une a él, desencadenando la activación de la vía de las MAPKs. El FGF23, miembro de una gran familia de factores de crecimiento fibroblástico, ha sido descrito como el más potente para aumentar la excreción de fósforo urinario por su capacidad de inhibir en el túbulo proximal la reabsorción de fosfato dependiente del transpotador Na-Pi. Además, el FGF23 también inhibe en el riñón la síntesis de 1-alfa-hidroxilasa con el consiguiente descenso en la activación del calcidiol a calcitriol. Recientemente se ha descrito que la glándula paratiroides es un importante órgano diana del FGF23 (8) en el que FGF23 actúa directamente activando la vía de las kinasas activadas por mitógeno (MAPKs), disminuyendo la expresión génica y la secreción de la PTH.

El posible efecto del FGF23 sobre la PTH ha sido un tema controvertido. Se ha mencionado que el FGF23 podría estimular e inhibir la PTH, mientras que un trabajo con ratones transgénicos que sobre-expresaban FGF23 mostraba hipofosforemia y niveles bajos de calcitriol y PTH (44), otros trabajos también con ratones transgénicos con sobreexpresión de FGF23 mostraron hiperplasia de la glándula paratiroides que se correlacionaba con altos niveles de PTH (45, 46).

No obstante, es conocido que en pacientes con ERC los niveles de FGF23 y de PTH se encuentran elevados (47) y que los primeros se correlacionan con progresión del fallo renal (48, 49), desarrollo de hiperparatiroidismo secundario (50), y mortalidad en pacientes diálisis (51).

Todo ello parecería indicar que el FGF23 podría estimular la PTH, sin embargo, trabajos recientes han demostrado que el FGF23, tanto *in vivo* como *in vitro,* es capaz de inhibir la síntesis y secreción de PTH a través de la activación de la vía de las MAPK (8, 52). Además del riñón y de las glándulas paratiroides, otros posibles órganos diana del FGF23 son la glándula pituitaria y el plexo coroideo cerebral, ambos expresan klotho y FGFR1, pero la función de FGF23 en estos tejidos es desconocida por el momento.

Hiperparatiroidismo secundario severo

En el hiperparatiroidismo secundario severo, la glándula paratiroides ve como su sensibilidad las factores reguladores clásicos, como el Calcio o el Calcitriol, se ve seriamente disminuida y hay una secreción descontrolada de PTH. Esto ha sido relacionado en los ultimos años con el descenso en los niveles del CaSR y del VDR (32, 53). Ademas, en etapas avanzadas de ERC, la coexistencia de niveles elevados de FGF23 y PTH en pacientes urémicos con hiperparatiroidismo secundario sugieren que el FGF23 no es capaz de ejercer su supuesto efecto inhbitorio sobre la PTH cuando la regulación de la glándula paratiroides esta comprometida debido a la uremia. Se ha descrito recientemente que esta carencia es consecuencia de un descenso en los niveles

de klotho y FGFR1 en la glándula paratiroides urémica en estudios in vitro en in vivo, que colaborarían a esa "resistencia" al efecto del FGF23 (54-56).

En los últimos años se han hecho grandes aportes al conocimiento de los mecanismos involucrados en la progresión del hiperparatiroidismo secundario, tanto en su fisiopatología como en sus bases moleculares. Sin embargo, el manejo clínico de este cuadro sigue siendo complejo y difícil, especialmente en el hiperparatiroidismo secundario severo en el que hay una desregulación en los mecanismos a cargo del control de la proliferación glandular y de la secreción de PTH.

Calcificaciones vasculares

Virchow en el siglo XIX describió por primera vez la aparición de calcificaciones metastásicas o ectópicas en pacientes con ERC. Contiguglia y col. en 1973 mostraron que el Ca procedente de los vasos de pacientes urémicos eran cristales de hidroxiapatita, los mismos que los del esqueleto (57).

La calcificación vascular es un proceso íntimamente ligado al envejecimiento. Sin embargo, en las ultimas décadas, varios estudios comenzaron a mostrar que un porcentaje muy elevado de pacientes con ERC presentaban calcificaciones vasculares (58), con mayor frecuencia y severidad que la población general, incluso en pacientes jóvenes menores de 30 años (13), con el consecuente impacto en la morbi-mortalidad de estos pacientes. Es conocida la relación entre las calcificaciones vasculares y la mortalidad cardiovascular, principal causa de muerte en pacientes en diálisis con más del 50 % de los fallecimientos (59-66).

El mecanismo por el cual se produce la calcificación vascular es complejo y no totalmente comprendido. Inicialmente se pensaba que consistía en una simple precipitación de Ca y P en un apropiado microambiente (67). Varias líneas de evidencia indicaron, sin embargo que los mecanismos pasivos discurrían a la par que ciertos mecanismos activos. Durante este proceso activo las células de músculo vascular liso (CMVL), debido a la acción de ciertos promotores de la calcificación, sufren un mayor grado de apoptosis, formación de vesículas y cambian el fenotipo de células musculares lisas de la pared arterial a células similares a osteoblastos, induciendo formación de matriz y también atrayendo factores locales envueltos en el proceso de mineralización (68-70). Sin embargo, en mamíferos y humanos, las concentraciones séricas de Ca y P exceden en varias veces la solubilidad del producto calcio-fósforo (CaxP), sin embargo, no existe precipitación dentro de los vasos. Este hecho remarca claramente la importancia del papel que juegan los inhibidores fisiológicos de la calcificación

que contrabalancean el efecto bien conocido de los promotores de la calcificación.

La lista de promotores e inhibidores del proceso de calcificación es larga y se incrementa cada año (Ilustración 2) (71-73).

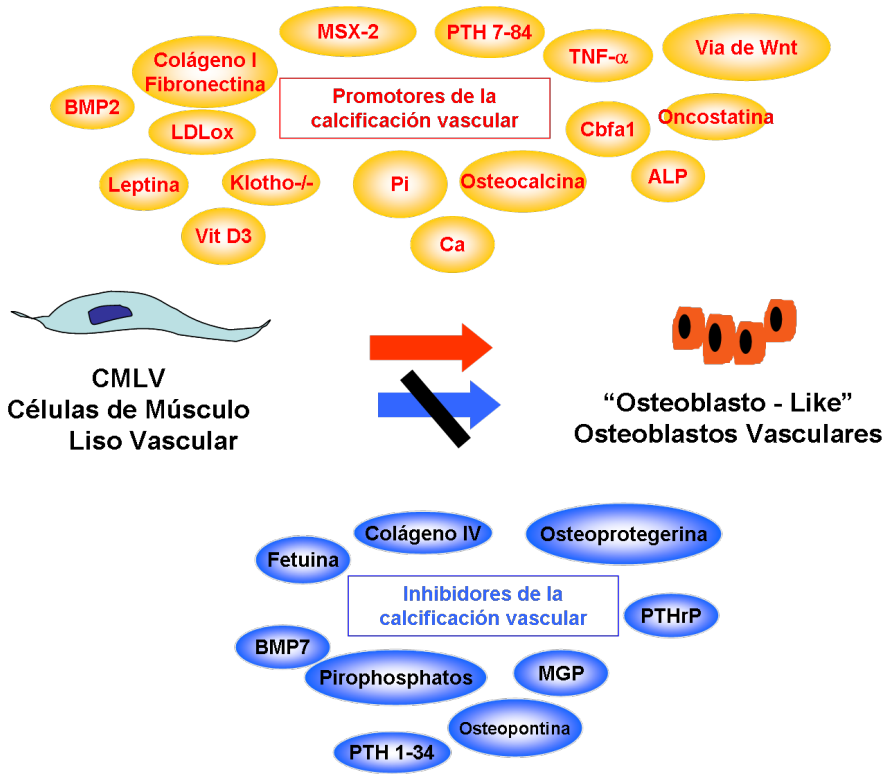

Ilustración 2. Promotores e inhibidores de la calcificación vascular. *Adaptado de "Alteraciones del metabolismo óseo y mineral en la enfermedad renal crónica: avances en patogenia, diagnóstico y tratamiento"*

La hiperfosfatemia es el factor que de forma más constante se ha asociado con incremento de las calcificaciones vasculares y mortalidad en pacientes en HD (74). Con respecto al producto CaxP parece claro que su aumento se relaciona con mayor mortalidad global, por cardiopatía isquémica y por muerte súbita (75, 76).

El papel de la vitamina D como factor de riesgo merece una particular atención ya que dosis altas de metabolitos de vitamina D se han asociado experimental y clínicamente con un incremento en las calcificaciones vasculares y

la mortalidad (77, 78), mientras que la administración de metabolitos activos de vitamina D a dosis menores.

Promotores de Calcificación Vascular

Factores del metabolismo mineral

Fósforo y calcio

El fósforo sérico alto es uno de los factores de riesgo más importantes relacionados con la uremia, asociados a la calcificación vascular en pacientes con insuficiencia renal crónica y en la población general (59). Es bien sabido que los altos niveles de fósforo sérico agravan el hiperparatiroidismo secundario y también reducen la actividad de la enzima 1-alfa-hidroxilasa, que a su vez, disminuye los niveles séricos de calcitriol. Una cuestión importante que debe ser respondida es "cómo los incrementos en el fósforo sérico activan varios mecanismos en los vasos" desembocando en el desarrollo de calcificaciones vasculares.

El fósforo, además de ser un elemento mineral extracelular, también se ha descrito como un jugador importante en los mecanismos de señalización de las CMLV (79). No sólo colabora con el calcio en la mineralización de la matriz, sino que también es capaz de actuar como un "mensajero intracelular secundario", activando varias vías moleculares relacionadas con la formación de hueso. Penetra en las células a través de un canal específico dependiente de Na llamado Pit-1; de hecho, el bloqueo de Pit-1 previene la calcificación vascular en un modelo in vitro (80). En experimentos in vitro han demostrado que niveles intracelulares elevados de fósforo pueden actuar directamente en la trascripción de genes relacionados con el hueso, como CBFA-1/RUNX2 y osteocalcina, lo que resulta en la activación de varias vías osteogénicas en las CMLV, dando lugar a los consabidos cambios fenotípicos, pasando de CMLV a células óseas (81). Además, en ratas urémicas con alto fósforo sérico, se ha sugerido que la

calcificación vascular en la capa media es causada, al menos en parte, por incrementos en las formas vasculares de CBFA-1/RUNX2 y Pit-1 (82). CBFA-1/RUNX2 promueve la expresión de una de las familias más importantes de las proteínas implicadas en las calcificaciones vasculares: la proteínas morfogenéticas óseas (BMPs), de las que se hablará mas adelante en este capitulo.

Vitamina D

El descubrimiento de que las CMLV expresan el receptor de la Vitamina D (VDR), hizo pensar que la unión del metabolito activo de la vitamina D, $1,25(OH)_2D_3$, podría tener efectos sobre las propias CMLV. De hecho, la $1,25(OH)_2D_3$ regula al alza el VDR e incrementa la carga de calcio en las CMLV de rata en cultivo (83) y de aorta de conejo (84). Otros estudios mostraron que concentraciones de 10^{-9} M de $1,25(OH)_2D_3$ inducían la proliferación de las CMLV (85), migración y un aumento de la expresión de osteopontina (77). También se demostró que una concentración de 1,2µmol/L inducía cambios morfológicos en las CMLV de aorta de rata, incluida la progresión de un fenotipo contráctil a uno "óseo" con un incremento la producción de factores que promueven la calcificación *in vitro*.

Jono *et al* demostraron que la $1,25(OH)_2D_3$ a concentraciones comprendidas entre 10^{-7} y 10^{-9} M induce un incremento de la calcificación dosis dependiente en CMLV bovinas *in vitro*. Esto va acompañado de un incremento similar en la actividad fosfatasa alcalina y una regulación a la baja del péptido relacionado con la PTH (PTHrp) (78). Este cambio fenotípico permite a las CMLV sintetizar proteínas específicas de osteoblastos que incrementan el grado de calcificación. Entre ellas podemos destacar la expresión de factores de transcripción como CBFa1, osteocalcina (86) y de moléculas solubles como el RANKL o BMP4 (87).

Factores moleculares

Eje BMP-Wnt

Dentro de los factores moleculares que promueven el desarrollo de la calcificación vascular se deben destacar en primer lugar la familia de las Bone Morphogenetic Proteins (BMPs), formada por al menos 30 proteinas con propiedades de inducción ósea que tienen un papel importante en el desarrollo de la organogénesis en varios tejidos. Forman parte de la superfamilia del TGF-b y señalizan tras la unión a sus receptores transmembrana. Los receptores de las BMP (tipo I y tipo II) funcionan de manera cooperativa y tras la unión de la proteína al receptor tipo II, se produce la activación del tipo I. El resultado es un aumento de la transcripción génica a través de fosforilación de proteínas y de la traslocación al núcleo de factores de transcripción de la familia de los Smad.

La expresión de BMPs se ha venido detectando detectado en placas ateroscleróticas calcificadas (88) desde hace mas de 18 años. Además, BMP2, BMP4 y BMP6 se han encontrado en áreas de calcificación vascular de origen uremico (89). Estudios posteriores han demostrado que BMP2 inhibe la proliferación de las CMLV estimuladas con suero uremico (90) tras lo que desciende la expresión de marcadores de célula muscular lisa, favoreciendo la transición fenotipica. In vitro, estimula la captación de fósforo, el fenotipo osteoblástico y la calcificación. El papel de BMP4 no está tan estudiado, aunque se ha demostrado que induce calcificación de CMLV y está implicado en la calcificación vascular inducida por RANKL (87).

Entre los mecanismos moleculares que se desencadenan después de sobre expresión de miembros de la familia de las BMPs durante la calcificación vascular, se encuentra la inducción directa de la expresión de los factores de transcripción osteoblásticos tales como MSX2 y CBFA-1/RUNX2, estableciendo una inter-regulacion entre BMPs y dichos factores que aun permanece poco clara (91, 92).

Se ha demostrado que el eje "BMP-MSX-2" también recluta a componentes de la familia de proteínas Winngless / ints (Wnt's) (93),

tradicionalmente asociada con la formación de hueso. Recientemente, la vía de señalización Wnt, incluyendo sus inhibidores, también se ha añadido a la larga lista de factores paracrinos "promotores de la calcificación vascular" (94), debido a varios estudios que han detectado que junto al desarrollo de la calcificación se observa aparición de diversos componentes de esta vía de señalización en áreas calcificadas (95-98). De los estudios en los que se ha visto una expresión de los inhibidores de Wnt tales como secreted frizzled proteins-2 y 4 (SFRPs) y dickkopf-1 (DDK1) en áreas severamente calcificadas se desprende una interesante hipótesis que postula que dichos inhibidores de la via de Wnt pueden ser factores que juegan un importante papel en la comunicación vaso-hueso, que será tratada mas adelante en esta tesis.

Estrés oxidativo

El estrés oxidativo consta de una serie de sistemas enzimaticos acoplados encargados de la depuración de especies reactivas de oxigeno (ROS) como el ión superoxido o el peróxido de hidrógeno, finalizando todas estas reacciones en la producción de agua como producto final. La subida de ROS y ciertas modificaciones en los niveles y actividad de diversas enzimas relacionadas con estrés oxidativo se ha relacionado también con el eje BMP-MSX-Wnt y por ello con la inflamación y la calcificación vascular. Si bien recientemente se ha ahondado en los mecanismos del estrés oxidativo que se ven afectados y/o regulados durante la calcificación vascular y el estrés oxidativo y los mecanismos de señalización intracelular que comporta han adquirido un papel relevante, diversos aspectos permanecen aun poco estudiados.

CMVL cultivadas con H_2O_2, presentaron calcificaciones, a través de la estimulación directa de CBFA-1/RUNX2 (99) con un aumento en los niveles de NADPH oxidasa, lo que demuestra que el estrés oxidativo puede actuar como un promotor de la calcificación vascular. Ademas, se ha descrito una posible implicación de las enzimas relacionadas con el metabolismo del óxido nitrico. Además, en estudios in vivo han demostrado que algunos de los antioxidantes pueden prevenir las calcificaciones vasculares (100). Recientes estudios han

arrojado luz sobre el papel del receptor de los productos avanzados de glicosilación (RAGE) (101) como un mediador fundamental en la respuesta inflamatoria que desencadena la liberación de especies reactivas de oxígeno durante la calcificación vascular.

Otros factores

Klotho es un correceptor del factor de crecimiento de fibroblastos 23 (FGF-23), una hormona fosfatúrica, y que controla la excreción de fósforo (102), entre otras funciones. Ratones KO para el gen de klotho mostraron aceleración del envejecimiento, con calcificación ectópica generalizada, incluyendo calcificaciones vasculares. Los mecanismos por los que FGF-23/klotho afectan a las calcificaciones vasculares no se entienden completamente, si bien estudios recientes sugieren que este eje controla directamente la excreción de fosfatos y por ello parte de la homeostasis mineral, y otros pasos importantes en el metabolismo de la vitamina D y la PTH indirectamente, lo que, a su vez, puede ser también responsable de los efectos vasculares.

Defectos en los genes codificantes para FGF-23 y klotho provocan perturbaciones varias, incluyendo la calcificación vascular y la pérdida de hueso.

Inhibidores de la calcificación vascular

Fetuína A

En el suero, la más abundante de los inhibidores de la calcificación vascular son fetuin-A (alfa 2-glicoproteína-Heremans Schmid), OPG y la proteína gla de la matriz. La Fetuína-A es un conocido inhibidor de la osteogénesis (103), capaz de inhibir la calcificación vascular. Los ratones KO para Fetuína-A desarrollan espontáneamente una generalizada calcificación de tejidos blandos, incluyendo una significativa calcificación de miocardio. En estos ratones, la calcificación

vascular se asoció a la regulación al alza de los pro-fibrótico factor TGF-beta (104).

Proteina Gla de la Matriz (MGP)

La MGP es una pequeña proteína se secreta al medio extracelular y que sufre fundamentalmente dos modificaciones post-traslacionales dependientes algunas de ellas de la vitamina K (105). Aunque su mecanismo molecular preciso no se conoce, la evidencia científica acumulada demuestra que tiene un papel fundamental inhibiendo la calcificación de tejidos blandos. Las primeras evidencias se acumularos al constatar que el tratamiento de ratas con un antagonista de la vitamina K (warfarina) provocaba extensas calcificaciones del cartílago provocando un crecimiento anormal. Además los animales KO para la MGP mueren por ruptura aórtica debido a extensas calcificaciones vasculares.

El mecanismo preciso por el cual MGP inhibe la calcificación vascular no se conoce, pero se han sugerido varias posibilidades. Price et al sugiere que la MGP se une a los cristales de hidroxiapatita impidiendo su crecimiento (106). Además, se ha demostrado que la MGP es capaz de inhibir la diferenciación fenotípica de las CMLV en células de hueso. Así, los animales KO de MGP muestran una pérdida de marcadores de CMLV con un aumento de la expresión de Cbfa1 y osteopontina (107). Los estudios de Shanahan en los que encuentra menor expresión de MGP en arterias de pacientes diabéticos con esclerosis de Mönckeberg apoyan esta teoría (108).

Osteoprotegerina (OPG)

Es un miembro de la familia de receptores de los factores de necrosis tumoral (TNF-R), que ha sido identificado como regulador de la resorción ósea (37). OPG funciona como un receptor soluble, señuelo de ligando (RANKL) del receptor activador del factor nuclear – κB (RANK) (7). OPG es además receptor del ligando inductor de la apoptosis relacionado con el factor de la necrosis tumoral (TRAIL), que es un potente inductor de apoptosis.

La primera evidencia de que este sistema estaba implicado en la calcificación vascular derivó del estudio del ratón KO para OPG, el cual presenta osteoporosis y calcificaciones de la aorta y arterias renales (109). Se ha demostrado que OPG inhibe la calcificación vascular en ratas in vivo provocada tanto por vitamina D como por warfarina (110). Recientemente se han producido progresos en el entendimiento del modo de acción de OPG evitando la calcificación vascular, implicando NFkB y BMP-4 (87).

Vías de señalización implicadas en la calcificación vascular

Es bien sabido que las CMLV son capaces de conservar un poder de diferenciación osteogénica debido en parte a ambas líneas celulares tienen como origen embrionario el mesodermo (107). Como ha sido mencionado anteriormente, una pléyade estudios han mostrado que el proceso de calcificación vascular es un proceso mas allá de la precipitación físico-química, sino que es un proceso altamente regulado. Se han identificado diversos factores osteogénicos que aumentan su expresión y actividad y se han convertido por tanto en marcadores de calcificación vascular clásicos, como el factor de transcripción osteoblástico Runx-2 o las BMP-2/4. Sin embargo, durante la ultima década, diversos estudios han tenido como objetivo el estudio de los mecanismos moleculares desencadenados en la célula en respuesta a diversos estímulos como desregulaciones en el metabolismo mineral, aumento del estrés oxidativo, envejecimiento, inflamación crónica o alta glucosa, que tienen como consecuencia ultima, la diferenciación osteogénica (111).

En esta sección nos centraremos en las vías de señalización secundarias a las señales que provienen de las afectaciones del metabolismo mineral, el estrés oxidativo y el envejecimiento asociados a la enfermedad renal crónica.

Sobrecarga de fósforo y calcio.

Es bien sabido que ambos desordenes, frecuentemente caracterizados como hiperfosfatemia e hipercalcemia, son dos de las presentaciones clínicas mas

frecuentes en la enfermedad crónica, como consecuencia de la perdida de capacidad regulatoria del riñon. Esto tiene como consecuencia la presencia de un entorno mineralizante en la circulación sanguinea. Como ha sido descrito anteriormente, el fósoforo es clásicamente el principal promotor de la calcificacion vascular. Sin embargo, la mayoria de mecanismos y vias de señalización acopladas a la entrada de fósoforo al interior celular permanecen desconocidos en gran parte (Ilustración 3).

Recientemente se ha asociado la entrada de fósforo a la iniciación de la apoptosis y de la liberación de vesículas de matriz que contienen hidroxiapatita y se ha sugerido el eje Gas-Ax1/Pi3K/AKT (112, 113) como la vía de señalización activada por fósforo que desemboca en apoptosis y liberación de vesículas. La vía de señalización de MAPK ERK/p38 también ha sido recientemente relacionada a los efectos intracelulares del fósforo (114, 115), pero los efectores primarios, directamente relacionados a la sobrecarga directa de fósforo y que hace que se activen estas vías aun permanecen sin dilucidar.

El efecto de la sobrecarga de calcio es a dos niveles. El calcio puede entrar a la célula a través de los "canales de calcio dependientes de voltaje" o "canales de calcio operados por depósitos" que puede resultar en la movilización de Calcio de los depósitos intracelulares que desembocaría en una regulación génica diferencial a través del factor de transcripción dependiente de Calcio/AMP cíclico (CREB) (116). El papel del calcio como segundo mensajero intracelular como consecuencia de la sobrecarga de calcio esta aun poco claro, aunque recientemente se ha descrito que el calcio altera el metabolismo de las Anexinas que se unen a calcio A2, A5 y A6 para promover la liberación de vesículas de matriz (117). El otro tipo de acción del calcio es a través de su receptor, expresado en las CMLV (118). Recientemente, el CaSR ha sido descrito como un factor principal en el desarrollo de calcificaciones vasculares, reclutando miembros del sistema MAPK (119).

Especies reactivas de oxigeno y envejecimiento

La mayoría de estudios que han tenido como objetivo el papel de ROS en la patobiología de la calcificación vascular han adjudicado un papel central al peroxido de hidrogeno en la activación de las vías de señalización que desencadenan la respuesta osteogénica. Se ha demostrado que el peroxido de hidrogeno por si mismo es capaz de activar la vía AKT y aumentar la tasa de transcripción de CBFA-1/RUNX2 (99). Sin embargo, la razón del acumulo intracelular de peroxido de hidrógeno permanece poco clara.

La acumulación de los productos avanzados de oxidación de proteínas y el LDL oxidado es frecuente en la uremia y la diabetes y se han asociado a la activación de la vía p38/MAPK y a la inflamación a través de TNF-alfa (120, 121).

Recientemente, una articulo publicado por Raughnaut y cols. (122), han sugerido un novedoso mecanismo implicado en la relación calcificación vascular-ROS. Este mecanismo comprende diversos aspectos relacionados con la senescencia y el envejecimiento prematuro, describiendo por primera vez que el estrés oxidativo puede influir en los niveles de la metaloproteasa Zmpste-24 (123). Esta proteasa pertenece al sistema de procesamiento y maduración de la LaminaA/C, componente de la envuelta nuclear, cuyos defectos se asocian al envejecimiento prematuro y síndromes de progeria. La presencia de ROS inhibe la expresión de Zpmste-24, provocando una acumulación de Prelamina A y por ello, una senescencia y daño en el DNA acelerados en las CMLV, favoreciendo así ciertos aspectos implicados en la diferenciación osteogénica.

Además el envejecimiento y senescencia de las CMLV ha sido asociado también una desregulación de citoquinas inflamatorias como IL-6, afectando a la via de NF-KappaB (124).

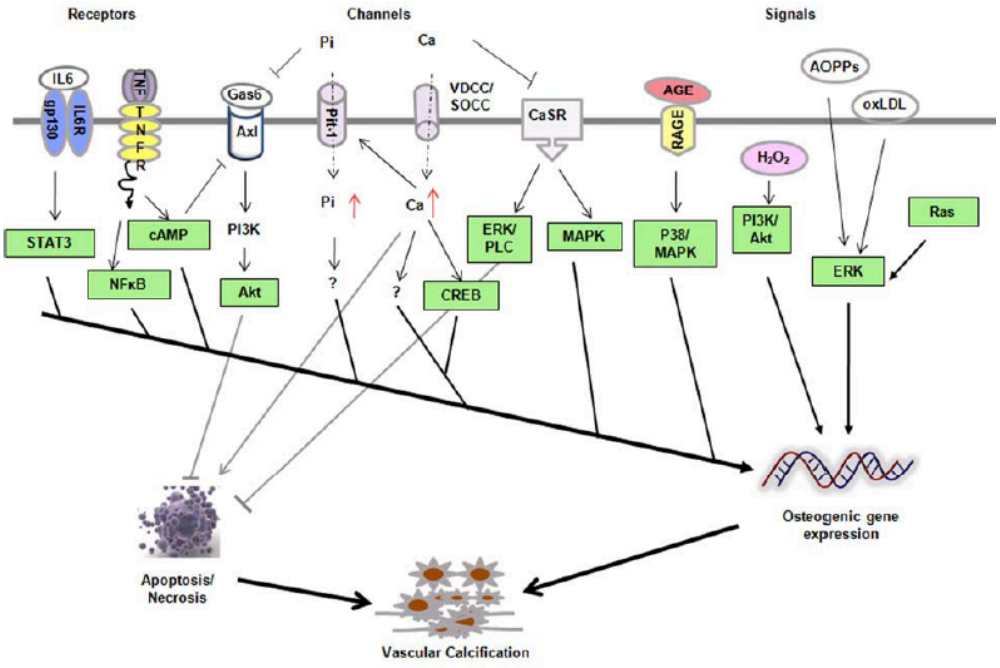

Ilustración 3. Esquema que representa las diferentes vías de señalizaron implicadas en la regulación de la diferenciación osteogénica en las células de músculo liso vascular. Adaptado de Liu y cols. (111).

Relación entre calcificaciones vasculares y pérdida de masa ósea

La relación entre las calcificaciones vasculares y la osteoporosis se observó desde hace mucho tiempo ya que los pacientes con osteoporosis presentaban frecuentemente enfermedades vasculares, incluyendo aterosclerosis y calcificaciones arteriales. De hecho, la práctica clínica diaria revela que el paciente hospitalizado por fracturas osteoporóticas sufre de eventos cardiovasculares como infarto agudo de miocardio o ACV (125).

Existen estudios epidemiológicos donde se demuestra la coexistencia de las calcificaciones vasculares con la pérdida de masa ósea, sugiriendo una posible relación entre osteoporosis y aterosclerosis. En el estudio Framingham, las mujeres con la masa ósea más baja, determinada por una radiografía sobre el área cortical de los metacarpianos de las manos, presentaban una mayor

incidencia de enfermedad arterial coronaria en un periodo de 30 años (126). En este mismo estudio se mostró que la pérdida ósea cortical se asociaba con la progresión de calcificaciones ateroescleróticas en mujeres (127). La masa ósea baja también se asoció con calcificaciones aórticas en población general, detectadas con radiología convencional (127). En un estudio en 2348 mujeres posmenopáusicas de California reveló que las calcificaciones aórticas representan un potente predictor para DMO baja y fracturas de fragilidad (128). Un análisis de un subgrupo de este estudio reveló una asociación entre calcificación aterosclerótica y pérdida ósea vertebral. Otro estudio prospectivo de 2662 mujeres posmenopáusicas de Dinamarca mostró que las calcificaciones aórticas se asociaron de forma significativa con DMO baja y pérdida rápida de masa ósea del fémur proximal (129).

En la línea de este paralelismo epidemiológico, se han sugerido mecanismos moleculares comunes en la patogénesis de la osteoporosis y de las enfermedades vasculares, especialmente calcificaciones arteriales incluyendo mecanismos relacionados con el envejecimiento, inflamación, equilibrio entre formación y resorción ósea, estrés oxidativo y ERC. La osteoporosis de bajo remodelado característica del envejecimiento no es el único desorden del metabolismo óseo que ha sido asociado a la calcificación vascular. En pacientes en diferentes etapas de ERC, el fósforo sérico esta fuertemente asociado con las calcificaciones vasculares junto con pérdida de masa ósea. Además, concentraciones altas de fósforo sérico han sido descritas como uno de los factores patogénicos mas importantes que inducen calcificación vascular (66, 69).

Por el contrario, los niveles altos de PTH presentan un papel que es todavía controvertido al respecto de su papel como promotores de calcificación vascular. (130). Un metanálisis reciente destaco e fósforo sérico por encima del calcio y PTH como un potente promotor de la calcificación vascular y por lo tanto causa de eventos cardiovasculares y mortalidad (131).

Datos experimentales han mostrado claramente que células del músculo liso vasculares en presencia de diversos estímulos, modifican su fenotipo a células similares a osteoblastos capaces de inducir calcificaciones vasculares.

La generación y caracterización de los ratones knockout de genes relacionados con el metabolismo óseo han aportado información importante ya que, algunos de estos modelos animales combinan fenotipos esqueléticos y vasculares (67). Entre las proteínas implicadas se encuentran la MGP, la osteopontina, la fetuina A, Smad 6, la OPG y klotho (132)

Recientemente, ratas urémicas alimentadas con dieta alta en fósforo y ratones KO para el receptor de LDL alimentados con dieta alta en grasas mostraron un aumento significativo de la calificación vascular y una bajada de la masa ósea. Un análisis genómico mostró que las áreas con calcificación vascular severa mostraban aumentos significativos de inhibidores de la vía de Wnt, concretamente, algunos miembros de la familia de secreted frizzled-related proteins (SFRPs) (98, 133-135). Además, la inducción de nefritis se asoció con la sobre-regulación de SFRP4, SFRP2 y DDK1 en la capa adventicia (136). Este incremento en inhibidores de la vía de Wnt en áreas severamente calcificadas podría ser indicativo del desencadenamiento de mecanismos defensivos en la pared e la aorta para contrarrestar la formación ósea o intentar atenuar la mineralización en el vaso. Debido a que muchos de estos inhibidores son secretados, podrían actuar no solo en el vaso, sino también viajar al hueso, promoviendo en parte la perdida de masa ósea, constituyendo un grupo de nuevos factores implicados en esta relación.

Hipótesis y Objetivos

Hipótesis de trabajo:

El estudio de los mecanismos implicados en el desarrollo de las manifestaciones típicas de las alteraciones del metabolismo óseo y mineral en el escenario de la enfermedad renal crónica es altamente complejo. Los mecanismos por los cuales la sobrecarga de fósforo interviene en el desarrollo de el hiperparatiroidismo secundario y la relación calcificación vascular-pérdida de masa ósea todavía permanecen poco claros, dando valor al uso de técnicas basadas en la genómica para la identificación de nuevos factores que puedan jugar un papel tanto en su desarrollo como en los mecanismos defensivos desatados en respuesta a condiciones severas.

Por ello, en esta tesis se empleo un modelo animal de ratas urémicas alimentadas con una dieta alta en fósforo y una aproximación genómica, cuyo fin fue identificar nuevos factores implicados en el el hiperparatiroidismo secundario y la calcificación vascular. Una vez identificados estos factores, se llevaron a cabo experimentos funcionales con el fin de identificar los mecanismos de acción y las relaciones existentes entre estas alteraciones.

Objetivos:

1. Analizar el efecto de la sobrecarga de fósforo en el desarrollo del hiperparatiroidismo secundario.

Objetivos específicos:

1.1 Analizar y validar el modelo animal empleado

1.2 Analizar los resultados del perfil de expresión génica en los diferentes estadios de hiperparatiroidismo secundario.

1.3 Estudio de la relación entre la expresión génica incrementada de las Dusps y los niveles de ERK y pERK 1/2

1.4 Analizar el papel de las Dusps en el efecto del FGF23 sobre la secreción de PTH

2. Analizar el efecto de la sobrecarga de fósforo en el desarrollo de la calcificación vascular.

Objetivos específicos:

2.1 Analizar y validar el modelo animal empleado

2.2 Estudio de la calcificación vascular desde un punto de vista histológico

2.3 Analizar los resultados del perfil de expresión génica en los diferentes grupos de estudio.

2.4 Estudio de la relación entre calcificaciones vasculares y pérdida de masa ósea.

3. Analizar el papel de diversos antioxidantes en la calcificación vascular *in vitro*.

Objetivos específicos:

3.1 Validar el modelo empleado

3.2 Analizar el papel de diversos antioxidantes en la mineralización in vitro

Material y Métodos

Modelo animal y diseño experimental general

Para realizar todos los experimentos se utilizaron 85 ratas Wistar macho de 4 meses de edad respectivamente procedentes del animalario de la Universidad de Oviedo donde se mantuvieron estabulados en una misma dependencia, con una temperatura de entre 20 y 24°C, suministrándoles comida y bebida *ad libitum*. Como alimento se les proporcionó una dieta estándar (dieta A04, Panlab SL) que contenía 0,6% de fósforo y 0,6% de calcio (dieta NPD, por sus siglas en ingles) o una dieta alta en fósforo que contenía 0,9% de fósforo y 0,6% de calcio (dieta HPD por sus siglas en inglés) (dieta A06, Panlab SL).

Los animales fueron manipulados en todo momento de acuerdo a lo dispuesto en la normativa legal vigente y, en particular, al Real Decreto 1201/2005 sobre protección de animales de experimentación y otros fines científicos. Los ensayos con animales fueron aprobados por el Comité Ético de Experimentación Animal de la Universidad de Oviedo.

Los sacrificios de los animales se hicieron a las 4, 8, 12, 16 y 20 semanas desde la nefrectomía, mediante anestesia con CO2 y posterior punción cardiaca, formando por lo tanto 20 grupos formados por un mínimo de 5 ratas cada uno, como esta detallado en la Ilustración 4.

A su vez, se incluyó un grupo de animales normales sin nefrectomía y con dieta normal.

Se recogieron todos los tejidos especificados según la seccion Tejidos recogidos en el sacrificio. Almacenamiento.

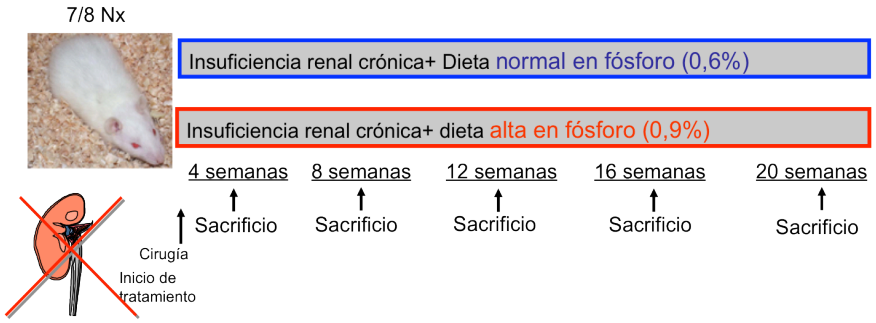

Ilustración 4. Diseño del estudio.

Diseño experimental I; hiperparatiroidismo secundario

Para simplificar el diseño del estudio, se construyeron tres grupos en base a los criterios: tiempo de exposición a la dieta alta en fósforo y valores séricos de PTH, resultando en la formación de 3 grupos grupos denominados "hiperparatirodismo secundario moderado" (Mod sHPTH), que incluye los animales pertenecientes a los grupos con ERC alimentados con dieta normal en fósforo; "hiperparatiroidismo secundario moderado/severo" (Mod/sev sHPT), que incluye a los animales con ERC alimentados con dieta alta en fósforo durante 4, 8 y 12 semanas e "hiperatairoidismo secundario muy severo" (sev sHPT), ", que incluye a los animales con ERC alimentados con dieta alta en fósforo durante 16 y 20 semanas. Se incluyó un grupo referencia de animales sin nefrectomía (Referencia) y alimentados con dieta normal.

Diseño experimental II; calcificación vascular

En el caso del estudio de la calcificación vascular, unicamente se incluyeron en el estudio los grupos de animales alimentados con dieta alta en fósforo durante 8, 16 y 20 semanas (8HPD, 16HPD y 20HPD) y sus correspondientes grupos controles, es decir, los grupos de animales alimentados con dieta normal en fósforo durante 8, 16 y 20 semanas (8NPD, 16NPD y 20NPD) . Se incluyó un grupo referencia de animales sin nefrectomía y alimentados con dieta normal.

Tejidos recogidos en el sacrificio. Almacenamiento

Al sacrificio se recogieron los siguientes tejidos y muestras necesarias para llevar a cabo las distintas determinaciones, almacenándose tal y como se detalla a continuación:

- Se extrajo sangre del corazón y se separó el suero que se almacenó a -70°C hasta ser utilizado para llevar a cabo las distintas determinaciones analíticas.
- Las glándulas paratiroides se sumergieron en un inhibidor de ARNasas (*RNA later,* Ambion) a -20°C hasta su posterior utilización para estudios moleculares.
- La arteria aorta se dividio en varios fragmentos. Uno de ellos se sumergió en un inhibidor de ARNasas (*RNA later*) a -20°C hasta su posterior utilización para estudios de genómica, otro se sumergió en alcohol al 70% un periodo mínimo de 1 semana para posterior análisis histológico y otro se congeló directamente a -70°C para su análisis proteómico.
- La tibia derecha se sumergió en alcohol al 70% un periodo mínimo de 1 semana para posterior análisis densitométrico. Tras realizar la densitometría, el tercio proximal de la misma fue incluido en metil-metacrilato.

Determinaciones analíticas

Análisis bioquímicos generales

La cuantificación de parámetros bioquímicos generales en suero, calcio (Ca), fósforo (P), creatinina (Cr), urea y proteínas totales se realizó mediante las técnicas estándar, utilizando un autoanalizador multicanal (Hitachi 717, Boehringer Mannheim). La determinación de FGF23 sérico en el modelo *in vivo* y

de la secreción de PTH en los experimentos de cultivo de glándulas paratiroides *ex vivo* se realizó con kits de ELISA según las instrucciones del fabricante (Immutopics 60-6300y 60-2300 respectivamente)

Determinaciones mediante radioinmunonanálisis

La determinación de la PTH intacta sérica en el modelo animal se llevó a cabo mediante radioinmunoensayo utilizando un kit comercial específico para rata (IRMA Rat PTH, Immutopics), con un coeficiente de variación intraensayo inferior al 4,4% y de interensayo inferior al 4,8%.

Inducción de enfermedad renal crónica

En la mayoría de estudios (137-139), la ERC se induce mediante nefrectomía 5/6 en dos tiempos quirúrgicos, con una semana de intervalo en la actuación sobre uno u otro riñón. No obstante, en nuestro laboratorio esta técnica fue modificada, ampliándose la reserción del riñón hasta una nefrectomía 7/8, según el modelo descrito por Ormrod y Miller (140), (Ilustración 5). El objetivo fue lograr un mayor grado de insuficiencia renal. La intervención se realizó, además, en un único tiempo, tras observarse un grado de insuficiencia renal y una mortalidad similar –e incluso menor– a la que se obtenía con la realización en dos tiempos, pero con la ventaja del consiguiente ahorro de tiempo (141).

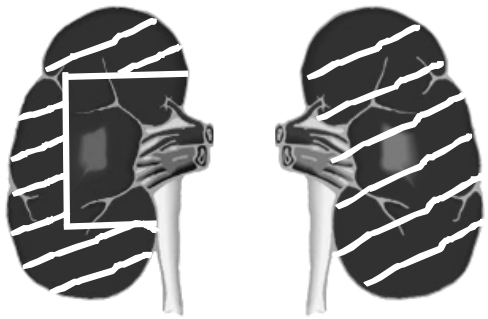

Ilustración 5. Modelo de ERC propuesto por Ormrod y Miller. *La parte rayada representa el tejido que se secciona.*

En primer lugar se realizó la nefrectomía total del lado derecho. Para ello, se rasuró al animal en la región lumbar y se practicó una incisión de los planos cutáneo y muscular. Una vez localizado el riñón, se procedió a la extirpación total del mismo, previa ligadura del pedículo renal. Finalmente se suturó el músculo y la piel. A continuación se realizó la nefrectomía contra-lateral. Tras localizar el riñón, se practicó la sección de los polos superior e inferior, así como del tercio externo del parénquima renal. Por último, y del mismo modo que se hizo con el riñón derecho, se suturó el músculo y la piel.

Como anestésico se utilizó una combinación de medetomidina (Dontor, Orion Corporation) y ketamina (Ketolar, Warner-Lambert Company) por vía intraperitoneal, administrada a una dosis de 0,16 mg/kg y 42 mg/kg respectivamente. Como antagonista del anestésico se utilizó atimepazol (Antisedan, Orion Corporation) a una dosis de 0,08 mg/kg. Durante la intervención, y con objeto de minimizar la hemorragia se utilizó un vasoconstrictor (Espongostan Film, Intersurgical España), mediante compresión en el lugar de corte.

Extracción de las glándulas paratiroides

Debido a la dificultad que supone identificar y aislar las glándulas paratiroides, algunos autores utilizan la tiroparatiroidectomía (142). En nuestro caso, pese a las dificultades, se optó por la paratiroidectomía. Para proceder con la extracción de las glándulas paratiroides, el animal se anestesió introduciéndolo en una urna con CO_2 y una vez anestesiado, se desangró por punción cardiaca. Se colocó la rata en decúbito prono sobre un tablero cubierto por una sábana de quirófano y se abrieron las patas delanteras a modo de cruz enganchándolas al tablero para que no se cerrasen. Se hizo una incisión en la línea media a lo largo de todo el cuello, desde el esternón a la mandíbula y lateralmente siguiendo el borde superior de las clavículas hacia afuera. La grasa y el tejido celular subcutáneo se retiraron hacia los lados quedando las dos glándulas salivales al descubierto.

A partir de este momento la manipulación se realizó bajo un estereomicroscopio (Olympus SZ-ST) con una fuente de luz fría Leica L2 (Leica), con el fin de ampliar la zona y mejorar el campo de visión sin desecarlo por el calor de una luz convencional. Una vez identificados los dos haces del músculo esternocleidomastoideo se cortaron y separaron lateralmente. Los dos haces del músculo esternohiodeo que protegen la tráquea se seccionaron y se separaron en su parte inferior dejando a la vista la tráquea, el tiroides y, a lo largo del trayecto dorso lateral de cada lóbulo tiroideo, un músculo muy delgado debajo del cual se encuentran unas formaciones nacaradas de consistencia dura que son las glándulas paratiroides que fueron aisladas del tejido tiroideo y extraídas convenientemente (Ilustración 6).

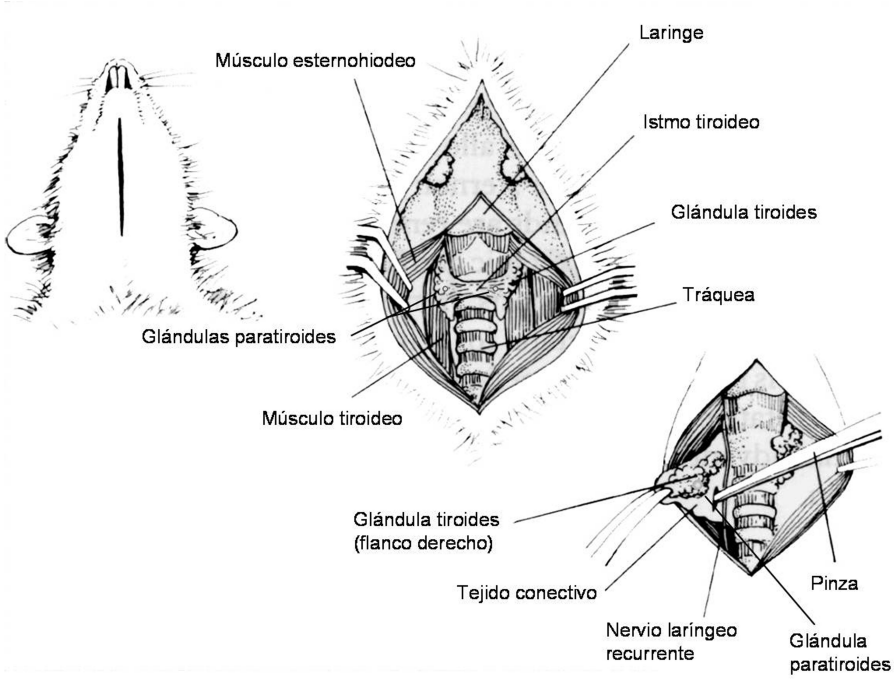

Ilustración 6. Aspecto del campo quirúrgico una vez abierto el cuello de la rata. *Si las secciones se hacen más amplias no es necesario el uso de separadores (Adaptado de Waynforth H. B.(143)).*

Extracción de la arteria Aorta

Para proceder con la extracción de la arteria Aorta, el animal se anestesió introduciéndolo en una urna con CO_2 y una vez anestesiado, se desangró por punción cardiaca. Se colocó la rata en decúbito prono sobre un tablero cubierto

por una sábana de quirófano y se abrieron las patas delanteras a modo de cruz enganchándolas al tablero para que no se cerrasen. Se hizo una incisión en la línea media a lo largo de todo el tronco, desde el cuello hasta el abdomen.

Una vez abierto el campo, se localizó la arteria Aorta; una vez localizada se procedió a su extracción y posterior limpieza de restos de grasa y sangre en una placa *petri* hasta conseguir la aorta lo mas limpia posible.

Valoración de la calcificación vascular.

Para el estudio histológico se utilizó un fragmento de la Aorta abdominal. En la preparación de las muestras se siguieron los siguientes pasos:

Fijación. Las muestras se sumergieron en alcohol al 70% y se mantuvieron a temperatura ambiente hasta el momento de su procesamiento (24 horas como mínimo).

Deshidratación e infiltración en metil-metacrilato. Incluye los siguientes pasos:

- 24 horas en acetona al 75%.
- 24 horas en acetona absoluta.
- 2° paso de 24 horas en acetona absoluta.
- 24 horas en solución de acetona absoluta y de metil-metacrilato (monómero) (1:1).
- 24 horas en metil-metacrilato (monómero).
- 2° paso de 24 horas en metil-metacrilato (monómero).

Inclusión: Las muestras se introdujeron en tubos de cristal con polimetilmetacrilato parcialmente polimerizado y se taparon con Parafilm (American National Can.). Tras permanecer durante 4 días a 4°C, se dejaron a temperatura ambiente y una vez atemperadas, se destaparon y se dejaron

endurecer durante un periodo de 2 semanas. Una vez comprobada la dureza casi total del metacrilato, se introdujeron 12 horas en una estufa a 37-40°C. Completada la polimerización, se rompió el tubo de cristal, obteniéndose un bloque cilíndrico de metacrilato con la muestra incluida en su interior.

Corte: Antes de colocar el cilindro de metacrilato en el micrótomo, se pulió la cara de corte con una pulidora de probetas metalográficas hasta alcanzar la profundidad deseada, para evitar el desgaste innecesario de las cuchillas. Los cortes se realizaron con un microtomo Polycut S (Reichert-Jung), usando una cuchilla de acero con filo de carburo de tungsteno. Para la tinción de Von-Kossa el grosor de los cortes fue de 3 micras. Los cortes fueron colocados sobre portaobjetos tratados previamente con gelatina y a continuación fueron reblandecidos con etanol al 95% para poder ser estirados y aplanados con un pincel. Después se recubrieron con una lámina de polietileno y se colocaron en una prensa durante 12 horas a 37°C.

Desplastificación: Los cortes se desplastificaron en metil-acetato durante 30 minutos y después se hidrataron en soluciones decrecientes de alcohol, siendo el último paso su lavado en agua destilada.

Tinción: Para los estudios histológicos se realizó la tinción Von Kossa-Ponceau con Xilidina-Orange G. Esta tinción permite distinguir el osteoide de la matriz mineralizada. El osteoide se tiñe de rojo y el hueso mineralizado de negro. El protocolo que se siguió fue el siguiente:

- Nitrato de plata al 3%, 5 minutos
- Agua destilada, 5 minutos, dos lavados
- Formol-carbonato de sodio, 5 minutos
- Agua corriente, 5 minutos
- Sulfato de sodio al 5%, 5 minutos
- Agua corriente, 10 minutos.

- Ponceau de Xilidina, 45 minutos
- Lavado breve en ácido acético
- Lavado breve en agua destilada
- Ácido fosfotúngstico-Orange G, 7 minutos
- Lavado breve en ácido acético al 1%
- Lavado breve en agua destilada
- Deshidratar sumergiendo en soluciones crecientes de alcohol y montar

Diagnóstico cualitativo histológico

El diagnóstico cualitativo histológico se llevó a cabo de forma ciega por un patólogo experimentado en el metabolismo óseo, usando el equipo representado en la Ilustración 7 .

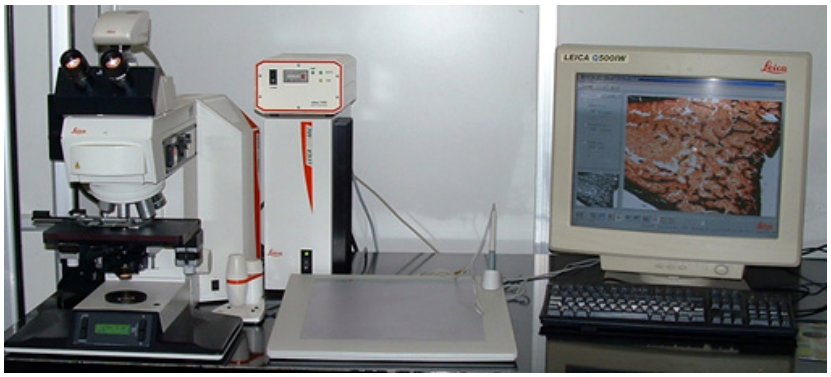

Ilustración 7. Equipo utilizado para la evaluación de la presencia de calcificaciones vasculares

Densitometría ósea

Se utilizó un densitómetro radiológico digital de doble energía (DXA) (Hologic QDR-1000). El aparato consta de: a) Unidad de exploración, integrada por un tubo de rayos X, un sistema de detección que se desplaza sincrónicamente a lo largo de la superficie de exploración donde se sitúa el paciente, y un ordenador encargado de gobernar el sistema, producir la digitalización y análisis de imagen y finalmente aportar los datos densitométricos y b) Consola de control, provista de monitor de visualización de imágenes, teclado de control del ordenador, impresora y un sistema de conservación de datos en disco óptico.

El sistema de rayos X está formado por un tubo emisor y un filtro de samario que permite el paso de una emisión pulsátil de dos haces de 70 y 140 KeV de energía. El sistema de generación de imagen se basa en una escala digital proporcional al contaje de radiación efectuado por el cristal de ioduro sódico en cada punto, que posteriormente se refleja en pantalla en una escala de grises. Antes de cada exploración, y de forma automática se lleva a cabo un proceso de autocalibrado mediante un disco interno con cuatro estándares de densidad diferente.

El cálculo de densidad se realiza a través de un proceso matemático que se inicia con la diferenciación del tejido óseo respecto a los tejidos blandos (diferencial de la captación del haz de baja y alta energía), la determinación del área explorada y del contenido mineral (calculado a través de la ley general de atenuación), y con el cociente de ambos, la densidad por unidad de superficie.

Procedimiento operativo

En las exploraciones realizadas en ratas en segmento lumbar y en tibia se utilizó un colimador sobre la fuente de salida de rayos X y un software específico para animales de pequeño tamaño que permitió aumentar el poder de resolución.

Finalizada la exploración y con la imagen digitalizada en pantalla se procedió al análisis de la misma. Se seleccionó el campo útil de exploración, delimitándose de forma automática o manual los diferentes subsectores: segmento proximal y distal de la tibia. A continuación el sistema informático procedió a realizar las diferentes operaciones matemáticas para calcular la densidad.

Ilustración 8. Imagen densitométrica realizadas en rata a nivel de tibia.

Las determinaciones densitométricas se llevaron a cabo en los segmentos aislados, colocando estos en recipientes que contenían 3 cm de harina de trigo, cantidad necesaria para igualar los cocientes de atenuación de los dos haces de energía que producen los tejidos blandos en las densitometrías in vivo. La utilización de la harina de trigo ofreció además la ventaja adicional de permitir un perfecto posicionamiento del hueso.

En la Ilustración 8 se muestran un ejemplo de imágen densitométrica de rata realizadas a nivel de tibia.

Una vez finalizado el proceso en cada zona explorada y en los subsectores establecidos se obtuvieron los valores del área (cm2), contenido mineral óseo (g), y densidad mineral ósea referida al área de proyección (g/cm2).

Genómica

La extracción del ARN se realizo siguiendo el protocolo detallado en la sección Extracción de ARN. La calidad y cantidad del ARN purificado se estimaron mediante chequeo de una alícuota en un gel de agarosa y medida

espectrofotométrica. A partir de 2μg de cada una de las muestras, se sintetizó cDNA con el *One-Cycle cDNA Synthesis kit (*Affymetrix), siguiendo el protocolo del *Expression Analysis Technical Manual* de Affymetrix. A partir de este cDNA se sintetizó cRNA siguiendo el protocolo del *IVT Labeling kit* (Affymetrix). El cRNA así sintetizado, se purificó con el *GeneChip Sample Cleanup Module* (Affymetrix). Una vez sintetizado y purificado, se fragmentó el cRNA (15 μg de cada preparación) para preparar las mezclas de hibridación.

Una vez observadas las imágenes de cada chip, se controlaron parámetros fundamentales, para valorar la calidad de las mezclas de hibridación: la presencia de los *spike controls* y la relación 3'/5' de los genes *housekeeping*.

Como paso previo a la comparación de los arrays se llevó a cabo el escalado de los datos de cada array, con el fin de minimizar las discrepancias debidas a variables tales como la preparación de la muestra, la hibridación, etc. Usando el software GCOS 1.2 (Affymetrix), todos los arrays se escalaron definiendo como la intensidad media el valor arbitrario de 200. Los factores de escalado aplicados a cada muestra de este experimento estaban dentro de los límites establecidos por Affymetrix indicando la posibilidad de realizar comparaciones entre arrays y aportando fiabilidad a los resultados.

Posteriormente se utilizo el software dChip (144, 145) para leer los archivos de imágenes de los arrays (Archivos CDF y CEL) y normalizar la expresión de los arrays usando el sistema "perfect march/mismatch" (PM/MM), especificando la posición de los genes Housekeeping en el array. Después se procedió al filtrado de los datos para eliminar "outlayers" y centrar el análisis en los genes sometidos a grandes variaciones. Posteriormente se procedió a una clusterización jerárquica usando el algoritmo Centroid-Linkage y las listas de genes (clusterización jerárquica supervisada) definidas por la Kyoto Enciclopaedia of Genes and Genomes (KEGG) (146).

A continuación, se compraron las muestras correspondientes a cada experimento usando siempre el algoritmo "False Discovery Rate" con el fin de evitar falsos positivos. Se originaron entonces diversos archivos, en los que además de el valor de cambio del gen y el p valor correspondiente a su

comparación, se incluyeron los términos de anotación de funciones biológicas para asignar una significación real biológica a cada gen. Tras un cribado mediante el software IPA (Ingenuity Pathway Analysis) (Ingenuity Systems Inc.), se construyeron las listas de genes correspondientes, aplicando un cribado final en el que se seleccionaron los genes con una tasa de cambio mayor o menor que 2 o -2 respectivamente.

Cultivos in vitro

Cultivos de glándulas paratiroides

Para el cultivo *ex vivo* se utilizaron un total de 96 ratas Wistar macho de 4 meses de edad. Una vez extraídas, las glándulas paratiroides se depositaron en cestas de propileno con una membrana de Nylon semipermeable de 12 µm de poro acopladas a placas de cultivo de 12 mm de diámetro (Costar®). Este sistema facilita el manejo de las glándulas y evita pérdidas de tejido durante el periodo de cultivo

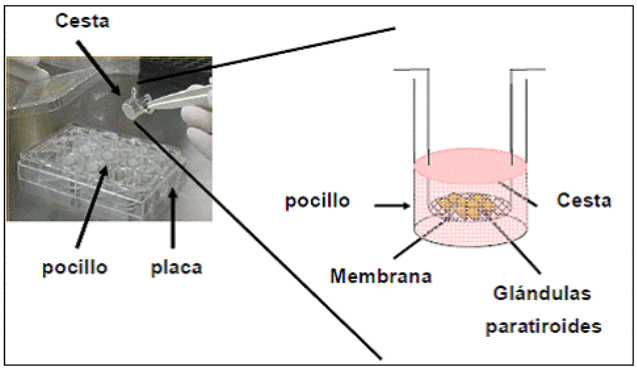

Ilustración 9. Modelo de cultivo de tejido paratiroideo. *Las glándulas se depositan sobre cestas y se introducen en placas de 12 mm de diámetro.*

Las glándulas se cultivaron en un incubador (mod. Stuart SI60D, Bibby Scientific Limited) a 37°C y en agitación constante. A cada pocillo se le añadieron 2 mL de un medio de cultivo no comercial, descrito por Almadén y col (130). Dicho medio se preparaba fresco antes de cada experimento y contenía: NaCl 125 mM, KCl 5,9 mM, $MgCl_2$ 1,2 mM, glucosa 12 mM, HEPES 25 mM, albúmina sérica bovina (BSA) 0,1%, (todo ello de Sigma-Aldrich), L-glutamina 4 mM,

penicilina 100 U/mL, estreptomicina 100 μg/mL, piruvato sódico 1 mM (todo ello de Biochrom AG, Berlín, Alemania) e insulina 0,1 U/mL (Novo Nordisk A/S) ajustado a pH 7,4. El fósforo se añadió como una mezcla de NaH_2PO_4 y Na_2HPO_4 (Sigma-Aldrich) en una proporción 1:2. De este modo se consigue una concentración final de fósforo en el medio de 1 mM. Finalmente, se añadió $CaCl_2$ (Sigma-Aldrich) a concentraciones variables (0,6 mM o 1,2mM) en función de cada experimento.

Para cada uno de los 5 experimentos (realizados por sextuplicado) se utilizaron 4 glándulas paratiroides divididas de forma aleatoria. Antes de comenzar el ensayo (0 horas), las glándulas se lavaron durante 8 horas a 37°C en agitación constante con 2 mL de medio de cultivo con una concentración de calcio 1,2 mM, con el fin de permitir la estabilización de la secreción de PTH tal y como se había observado en previos trabajos (27, 130, 147). Tras el período de lavado, se tomo una muestra de 250μL de medio para la determinación de la PTH basal. Para estimular la secrecion de PTH, se sustituyó el medio de cultivo por medio fresco con una concentración de calcio de 0,6 mM en todos los grupos menos en uno que se mantuvo con 1,2mM de Ca y se usó como grupo referencia. A los grupos estimulados se les añadio FGF23, FGF23 y un inhibidor de la fosforilación de ERK químico (UO126) e inhibidores de la fosforilación de ERK biológicos (Dusps recombinantes).

Por lo tanto se formaron los siguientes grupos: a)1,2mM Ca; b) 0,6 mM Ca; c) 0,6 mM Ca + FGF23 100 ng/mL (2629-FG, R&D Systems); d) 0,6 mM Ca + FGF23 100 ng/mL + UO126 1 μM (UO126, #U-120, Sigma Aldrich), y e) 0,6 mM Ca + FGF23 100 ng/mL + mezcla Dusps recombinantes a una concentración de 1μL/mL cada una (AK-020, Biomol).

La actividad de las Dusps estuvo en el intervalo 5-90 U/μg . La actividad fue ensayada mediante el método de la hidrólisis del 3-*O*-metil fluorescein fosfato (OMFP) a 30°C. Las condiciones fueron: 1 μg enzima por 100 μl en 100 mM Tris-HCl, pH 8.2, 40 mM NaCl, 1 mM DTT, 20% glicerol, 0.5 mM OMFP. Una unidad fue igual a 1 pmol de fosfato hidrolizado de OMFP por minuto. Una vez

finalizado el experimento, se recogieron 250 µl de medio de cultivo para el análisis de la PTH secretada y las glándulas fueron recogidas y conservadas

Cultivos de células de músculo liso vascular. Calcificación vascular in vitro.

Para llevar a cabo el estudio *in vitro* de la calcificación vascular se usó un cultivo primario de celulas de músculo liso vascular de pases no superiores a 5. Las células se cultivaron, siempre por triplicado para condición experimental y analisis, en placas de cultivo 10 cm de diámetro hasta 60% de confluencia en medio de cultivo DMEM:F12 1:1 con suero descomplementado al 10% (Sigma-Aldrich), penicilina 100 U/mL y estreptomicina 100 µg/mL (Biochrom AG) a 37°C en una atmósfera húmeda con 5% de CO_2. Alcanzada la subconfluencia, se les cambió el medio de cultivo por medio suplementado con BSA al 0,25% durante 24 horas. Tras este período de adaptación las células se cultivaron con el medio calcificante, que consiste en medio suplementado con BSA y con concentraciones finales de Ca y P de 2 y 3 mM respectivamente durante 8 días. Además del medio calcificante, se añadieron diversos antioxidantes (todos de Sigma Aldrich) curcumina (C7727), silbina (02000585), resveratrol (R5010), alpha-tocoferol (258024), L-ascorbato sódico (A4034) y Trolox™ (238813). Tras ese período las células fueron teñidas con rojo de Alizarina, analizadas mediante citometría y se recogieron los extractos proteicos para el Western Blot.

Tecnicas Comunes in vitro.

Extracción de ARN

La extracción de ARN total se llevó a cabo en diferentes tejidos y células: glándulas paratiroides, útero, riñón, tibia y células UMR106. En las paratiroides, tanto en el estudio *in vitro* como en el *in vivo* y dado el pequeño tamaño de las mismas, el ARN total se extrajo partiendo no de glándulas individuales sino de un conjunto de 8 glándulas. Sin embargo, en el útero, la tibia y el riñón el ARN se

extrajo de forma independiente en cada animal. En el caso del cultivo *in vitro* de osteoblastos el ARN total se extrajo de una placa de 10 cm de diámetro en subconfluencia.

Para la extracción de ARN total se empleó una modificación de la técnica en un solo paso descrita por Chomczynski y col.(148) Este método se basa en el uso de una mezcla de isotiocianato de guanidina y fenol en una solución monofásica (TRI REAGENT™, Sigma-Aldrich).

El hueso, por su dureza, previo a la homogenización con el reactivo, se sumergió en nitrógeno líquido y se trituró con la ayuda de un mortero. Los tejidos y cultivos celulares se disgregaron en 1 mL de reactivo TRI™ utilizando un homogeneizador de tejidos acoplado a una cuchilla de 5 mm (OMNI International). El ARN total se extrajo siguiendo las instrucciones del fabricante. La concentración y la pureza del ARN se determinaron con un espectrofotómetro UV-VIS (Nanodrop Tech.) midiendo la absorbancia (A) a 260 y 280 ηm. En una solución de ARN puro, este cociente tiene un valor teórico de 2,0. Un valor inferior sería indicativo de contaminación con proteínas o fenol durante el proceso de extracción. En nuestro caso, se descartaron aquellas muestras con una relación A260/A280 menor de1,5.

Síntesis de ADN copia

La obtención de los ADNc se llevó a cabo a partir de 1 µg de ARN total extraído previamente utilizando el kit comercial High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) siguiendo las instrucciones del fabricante. El ADNc obtenido se mantuvo a -20°C hasta su utilización.

PCR cuantitativa a tiempo real

Los experimentos de qRT-PCR se realizaron en un sistema de PCR a tiempo real modelo ABI Prism 7000 (Applied Biosystems). Para analizar la

expresión de los genes problema se utilizaron ensayos prediseñados Taqman® Gene Expression Assays detallados en la Ilustración 10

CaSR	Rn 00566496_m1x2
Catepsina K	Rn 00580723_m1
Dusp 6	Rn 00672017_g1
Dusp 7	Rn 01103513_m1
Dusp 5	Rn 00683448_m1
Elastina	Rn 01499782_m1
GAPDH	Rn99999916_m1
Klotho	Rn 00580123_m1
PPIA	Rn00690933_m1
PTH	Rn00566882_m1
SFRP1	Rn01478472_m1
SFRP-2	Rn 01458837_m1
SFRP-4	Rn 00585549_m1
Sm-22	Rn 00580659_m1
Tropomosina 1	Rn 00569447_m1
VDR	Rn 00566976_m1x2

Ilustración 10. Lista de los ensayos taqman usados ordenados alfabeticamente.

Para llevar a cabo el análisis de la expresión diferencial es necesario normalizar los resultados frente a un gen constitutivo. En este caso, los resultados se normalizaron frente al gen constitutivo ARN ribosómico GAPDH y PPIA Todas las secuencias se detectaron con reactivos Taqman® (Taqman® Universal PCR Master Mix, Applied Biosystems).

Para cuantificar la expresión relativa de los genes en todos los casos se utilizó una aproximación conocida como comparación del ciclo umbral (149). El término ciclo umbral (CT) se refiere al ciclo de PCR en el cual la fluorescencia emitida por la muestra supera un valor arbitrario predefinido (umbral), por encima del cual se considera que hay amplificación. Este método permite, de

forma rápida y sencilla, la cuantificación relativa de la expresión de un gen en una muestra problema respecto a una muestra calibradora. Para comparar ambas muestras es necesario además estandarizar la cantidad de ADNc molde en las reacciones de PCR. Por ello, los resultados deben normalizarse frente a un gen constitutivo, que actuaría como control endógeno.

Western Blot

En la extracción de proteínas de los cultivos celulares, el tampón de extracción utilizado fue uno de características estándar (RIPA) cuya composición era: Tris-HCl 50 mM, NaCl 150 mM, NP-40 1%, deoxicolato sódico 0,5%, EDTA 1,0 mM y SDS 0,1%. La muestra de células se sonicó durante 1 minuto a 4°C para evitar la degradación de las proteínas y se sometió a ultracentrifugación a 14000 G durante 20 minutos a 4°C. Tras ese período se recogieron las proteínas presentes en el sobrenadante de la muestra. La cuantificación de las proteínas se realizó mediante el método estándar de Bradford (Bio-Rad) (150). Las proteínas ya cuantificadas se almacenaron a -70°C hasta su posterior utilización.

Los diferentes extractos proteicos se sometieron a electroforesis en geles de acrilamida de 0,75 mm de grosor en condiciones desnaturalizantes (SDS-PAGE) con un tampón de electroforesis, según el método descrito por Laemmli y col.(151) cargandose en los geles 30 µg de proteína. En cada uno de los geles se cargó una calle con marcadores de peso molecular para identificar las proteínas problema (Rainbow™ Molecular Weight Markers, GE Healthcare). Las proteínas separadas por su peso molecular se transfirieron a una membrana de nitrocelulosa (Hybond™ P membrane, GE Healthcare) utilizando un tampón de transferencia. La transferencia se llevó a cabo en frío y en agitación durante 1 hora a un voltaje constante de 100 v.

Una vez finalizada la transferencia, las membranas fueron bloqueadas durante 1 hora a temperatura ambiente con leche en polvo desnatada al 5% en un tampón fosfato salino (PBS) para evitar uniones inespecíficas.

Transcurrido ese tiempo, las membranas se mantuvieron toda la noche con el anticuerpo primario correspondiente en la dilución 1:100 para Cbfa1/Runx2 (ab54868, Abcam), 1:1000 para SOD-2 (sc30080, Santa Cruz Biotechnology), y 1:30.000 para GAPDH (sc25778, Santa Cruz Biotechnology).

Al día siguiente, la membrana se sometió a 3 lavados de 10 minutos cada uno con una solución de lavado compuesta por PBS y Tween-20 (Sigma-Aldrich) y seguidamente la membrana se incubó durante 1 hora a temperatura ambiente con el anticuerpo secundario frente a ratón (401215; Calbiochem) y frente a conejo (sc 2001; Santa Cruz Biotech) ligado a peroxidasa específico para cada anticuerpo primario disuelto adecuadamente en solución de lavado . La membrana se sometió a otros 3 lavados de 10 minutos cada uno con solución de lavado y posteriormente se llevó a cabo la detección de la proteína mediante detección cromógena mediante el kit comercial Pierce® ECL Western Blotting Substrate (Thermo Scientific).

El revelado se llevo a cabo usando la equipacion Chemidoc XRS+ (Bio Rad). La cuantificación relativa de la intensidad de las bandas obtenidas en el Western Blot se realizó con el programa informático Image Lab (Bio Rad).

Tinción inmunohistoquímica

Las tinciones inmunohistoquímicas se llevaron a cabo sobre cortes de tejido parafinado de 5 µm de espesor de glándulas paratiroides cultivadas *in vitro* y de glándulas paratiroides y útero de ratas normales utilizando los sistemas EnVision+® (Dako Cytomation) y rat ABC Staining System (Santa Cruz Biotechnology), siguiendo las instrucciones del fabricante. La contratinción se llevó a cabo con hematoxilina (Sigma-Aldrich).

En el estudio *ex vivo* de glándulas paratiroides cultivadas con FGF23 o la mezcla de FGF23 y los inhibidores de las MAPK, para la detección de ERK y pERK se utilizaron dos anticuerpos policlonales de conejo (9102 y 43765 respectivamente; Cell Signalling)

Para cada glándula se analizaron tres cortes no consecutivos. Todas las muestras se procesaron a la vez con objeto de homogenizar las condiciones de trabajo (grado de desenmascaramiento del antígeno y tiempos de incubación con los anticuerpos primario y secundario y el sustrato).

Tinción Rojo de Alizarina

Para los estudios de mineralización in vitro, se realizó la tinción rojo de Alizarina. Esta tinción permite la matriz mineralizada ya que se tiñe de rojo. El protocolo que se siguió fue el siguiente:

- Lavado de células con PBS, 3 veces.
- Fijación con formaldehído al 10% en PBS (P6148; Sigma Aldrich), 1 hora a 4°C
- Agua corriente, 5 minutos
- Rojo de alizarina, 5 minutos (A 5533; Sigma Aldrich)
- Agua corriente, 5 minutos

Cuantificación de la tinción sobre las placas teñidas (de 6 pocillos; Costar).

- Acido acético al 10% (400μL) e incubar 30 minutos en agitador orbital.
- Calentar a 85° C durante 10 minutos
- Transferir a hielo
- Centrifugar 20.000 Gs durante 15 minutos
- Transferir 375 μL del sobrenadante a tubos nuevos
- Neutralizar el ácido acético con 150μL de amoniaco al 10%
- Leer la absorbancia a 405nm (

Evaluación de la tinción

La evaluación de la tinción y la toma de microfotografías se llevaron a cabo con el equipo Olympus CKX-41 (Olympus)

Citometría de flujo

DCFH-DA

Las células fueron recogidas mediante incubación con tripsina cuando se encontraban a un 60% de confluencia. Luego fueron resuspendidas en tampón de Hank's para ser contadas. Tras el recuento fueron centrifugadas a 500 x g 5' y resuspendidas a una concentración final de 250.000 células /ml en una solución 500 nM de DCFH-DA en tampón de Hank e incubadas durante 30 min a 37 °C y 5% de CO_2.

Posteriormente fueron centrifugadas y resuspendidas en tampón de Hank y se midió su fluorescencia a 485(ex)/530(em) nm en el servicio de Citometría de los Servicios Cientifico-Técnicos de la Universidad de Oviedo usando el equipo Cytomics FC500.

Análisis estadístico

El análisis estadístico de los resultados se realizó con el paquete informático SPSS 12.0 (SPSS Inc) para Windows. Para el análisis estadístico de los marcadores bioquímicos, así como el peso corporal, el peso del útero, la DMO a nivel de tibia proximal, los resultados obtenidos de qRT-PCR y Western Blot se empleó la prueba paramétrica de la t de Student (t-Student). Las correlaciones entre diferentes parámetros séricos fueron realizadas utilizando el coeficiente de

correlación de Pearson (r). En todos los casos se consideró como significación estadística una $p<0,05$.

Resultados

A continuación, se incluyen las 7 publicaciones derivadas de los estudios que tienen mayor relación con el conjunto de experimentos que se presentan en esta tesis doctoral que han sido divididas en 3 apartados. En el anexo final se incluyen otro 3 trabajos en los que el autor ha participado durante el periodo de realización de la Tesis doctoral. De ellos, dos tienen relación directa con el tema de la Tesis doctoral, pero no pertenecen al cuerpo central de a misma.

Primer apartado: papel de la sobrecarga de fósforo en la CKD-MBD:

Publicacion 1: Pathogenesis of bone and mineral related disorders in chronic kidney disease: key role of hyperphosphatemia. J Ren Care. 2009 Mar;35 Suppl 1:34-8.

Esta revisión describe el importante papel de la sobrecarga de fósforo en el escenario de las alteraciones del metabolismo óseo en la enfermedad renal crónica y supone el punto de partida para el desarrollo de los trabajos de esta tesis.

Segundo apartado: sobrecarga de fósforo e hiperparatiroidismo secundario:

Publicación 2. Severe Hyperplasia and Lack of Response to FGF23 of Uremic Parathyroid Glands is Associated with Increased Dual Specificity Phosphatases Gene Expression. Manuscrito en revisión en el Journal of Bone and Mineral Research.

Esta publicación original, apoyada por la publicación 1 del Anexo (pagina 230) es el núcleo principal de este apartado, en el que, tras revisar los modelos existentes para estudiar el metabolismo de la glándula paratiroides y colaborar activamente en la descripción de nuevos mecanismos implicados en la regulación de la paratiroides (Publicación 2 del Anexo, Pagina 233), se llevaron a cabo experimentos enfocados a determinar el perfil de expresión génica del desarrollo del hiperparatiroidismo secundario. Estos experimentos sirvieron como base para proponer, mediante experimentos funcionales usando modelos ex vivo, la existencia de nuevos factores que pueden regular este proceso.

Tercer apartado: sobrecarga de fósforo y calcificación vascular-pérdida de masa ósea.

Publicaciones 3 y 4:

3.- Vascular calcification in patients with chronic kidney disease: types, clinical impact and pathogenesis. Med Princ Pract. 2011;20(3):203-12. Epub 2011 Mar 29.

4.- Mecanismos de calcificación vascular en la enfermedad renal crónica. Pablo Román García y Jose Manuel Valdivielso Revilla. Capitulo del libro "Alteraciones del metabolismo óseo y mineral en la enfermedad renal crónica:

avances en patogenia, diagnóstico y tratamiento", editado por Jorge Cannata Andia. Wolters Kluwer. ISBN 978-84-96921-81-8

Estas revisiones describen el complejo escenario de las calcificaciones vasculares, con especial énfasis sobre el fósforo como promotor de la calcificación vascular, suponiendo el punto de partida para el desarrollo de los trabajos originales de este apartado. Además, ambas revisiones describen la relación entre calcificaciones vasculares y pérdida de masa ósea, que será el punto clave de la siguiente publicación.

Publicación 5. High Phosphorus Diet Induces Vascular Calcification, a Related Decrease In Bone Mass and Changes in the Aortic Gene Expression. Bone. 2010 Jan;46(1):121-8.

Esta publicación original describe la relación entre calcificaciones vasculares y pérdida de masa ósea usando un modelo de ratas urémicas. Además, se describe, mediante técnicas genómicas, la posible implicación de nuevos factores en las etapas mas severas de la calcificación vascular, que podrían estar también implicados en la desmineralización ósea, constituyendo uno de los nexos entre calcificación vascular y pérdida de masa ósea, aspectos revisados en la publicación número 6.

Publicación 6. The connections between vascular calcification and bone health. Keith Hruska, Pablo Roman-Garcia and Jorge B Cannata-Andia. Aceptada con cambios menores para su publicación en "Nephrology, dialysis and transplantation."

Esta revisión, en colaboración con un grupo estadounidense, pone especial énfasis en la relación vaso-hueso, describiendo los factores patogenéticos compartidos entre ambas alteraciones, tomando como base los resultados e hipótesis de la publicación 5.

Publicacion 7: Vascular calcification and natural antioxidants: A possible Benefit. Aceptado para su publicación en Journal of Nephrology.

Esta publicación original, en colaboración con un grupo nacional, confirma el importante papel que el estrés oxidativo juega en la mineralización in vitro. También pone de relevo la complejidad en las acciones de los antioxidantes y sugiere un papel beneficioso para la curcumina.

En total, el factor de impacto generado suma: 10,797 por la publicaciones incluidas en los resultados mas 14,46 por las incluidas en el anexo. En total, el factor de impacto de esta tesis alcanza 25,517, 38 citaciones y un factor H de 3.

Publicación 1: Pathogenesis of bone and mineral related disorders in chronic kidney disease: key role of hyperphosphatemia.

PATHOGENESIS OF BONE AND MINERAL RELATED DISORDERS IN CHRONIC KIDNEY DISEASE: KEY ROLE OF HYPERPHOSPHATEMIA

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Román-García P., Carrillo-López N., Cannata-Andía J.B. (2009). Pathogenesis of bone and mineral related disorders in chronic kidney disease: key role of hyperphosphatemia. *Journal of Renal Care* **35**(s1), 34–38.

SUMMARY
This paper reviews the pathogenesis of hyperphosphataemia and its role in the regulation of parathyroid hormone synthesis and parathyroid cell proliferation in chronic kidney disease. The association between hyperphosphaemia and vascular calcification, and the interventions that can be used to control plasma phosphate are also discussed.

KEY WORDS Hyperphosphatemia • Bone disease • CKD-MBD • Bone fractures • Pathogenesis hyperparathyroidism • Calcifications

PATHOGENESIS OF SECONDARY HYPERPARATHYROIDISM: A KEY COMPONENT OF MINERAL AND BONE DISORDERS OF CHRONIC KIDNEY DISEASE

The parathyroid glands play a critical role in the development of chronic kidney disease (CKD)-mineral and bone disorders (MBD), including vascular calcification fractures and poor outcomes (Cannata-Andia *et al.* 2006). Calcium, calcitriol and phosphorus are the main regulators of the parathyroid hormone (PTH) synthesis and secretion (Locatelli *et al.* 2002a).

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Calcium, the main factor involved in the regulation of PTH, acts through its specific receptor, the Calcium-sensing receptor (CaR), located on the surface of the chief cells of the parathyroid glands. The parathyroid glands are very sensitive to changes in serum calcium concentration. Small decreases in extracellular calcium are rapidly sensed by CaR leading within seconds or minutes, to increments in PTH secretion (Slatopolsky 1998). Chronic hypocalcemia, acting post-transcriptionally, stimulates PTH gene expression and subsequent PTH synthesis within hours to days, and the proliferation of parathyroid cells occurs over days to weeks (Naveh-Many *et al.* 1995). Likewise, small increases in extracellular calcium concentration are also sensed by CaR causing a decrease in PTH (Silver & Levi 2005).

Calcitriol, the natural active vitamin D metabolite, acts on the parathyroid gland through the nuclear vitamin D receptor (VDR). When calcitriol binds the VDR, the complex calcitriol-VDR is translocated to the nucleus where PTH gene transcription and PTH synthesis are repressed (Pike 1994; Naveh-Many & Silver 1998). The mechanisms governing the synthesis of PTH in the parathyroid gland are complex and still not fully understood. Although the nuclear vitamin D receptor (VDR) can suppress PTH gene transcription, PTH is also regulated post-transcriptionally and the PTH transcript has greater stability under conditions of low calcium and high phosphorus (Moallem *et al.* 1998).

In CKD, the decreased number of vitamin D receptors (VDR) and the resistance to the action of vitamin D are of great importance in the pathogenesis of secondary hyperparathyroidism (SHPT) (Slatopolsky *et al.* 1999a). This phenomenon is

more evident in the nodular than in the diffuse forms of parathyroid hyperplasia. As a result, low serum 1.25 $(OH)_2D_3$ and the decreased VDR concentration in the parathyroid lead to the overexpression of the PTH gene and to increases in PTH-mRNA.

Calcium and calcitriol not only regulate PTH synthesis and secretion, but also its expression on its own receptors. The relationships between calcium, calcitriol, CaR and VDR have been studied in great depth by several authors. The extracellular calcium seems not to influence the CaR but it can upregulate VDR, by contrast, calcitriol is able to upregulate both, CaR and VDR expression (Garfia *et al.* 2002; Carrillo-Lopez *et al.* 2008).

As CKD progresses towards stage 5, SHPT increases in severity, resulting in the proliferation of parathyroid cells and the development of diffuse hyperplasia. This is accompanied by a decrease in CaR and VDR expression (Kifor *et al.* 1996). As vitamin D is a potent inhibitor of PTH synthesis, a reduction in VDR expression might also inhibit the vitamin D-mediated signals that suppress PTH synthesis and release, although this has not yet been demonstrated experimentally. It is the hyperplasic nodules of the parathyroid gland that show the greatest decrease in both CaR and VDR expression (Gogusev *et al.* 1997), rendering them less responsive to circulating calcium. As parathyroid cells are transformed into a severe nodular hyperplasic state, a decline in VDR expression reduces the efficiency of VDR activators in up-regulating the transcription of the CaR gene and in inhibiting parathyroid cell proliferation.

Besides disturbances in calcium and calcitriol, phosphorus metabolism also plays a critical role in the development of CKD-MBD abnormalities. High phosphorus triggers not only secondary hyperparathyroidism, but also all the other mineral disorders associated with CKD which are related to the high prevalence of cardiovascular mortality (Block *et al.* 2004). All the available data clearly stress the paramount importance of adequately controlling hyperphosphatemia, "a silent killer" in CKD patients (Amann *et al.* 1999).

PATHOGENESIS AND CONSEQUENCES OF HYPERPHOSPHATEMIA

The kidneys are the main regulators of human phosphate homeostasis and they have the complete machinery to maintain the normal phosphate balance in day-to-day life. While the kidney removes phosphorus from circulating plasma, bone acts as a reservoir of phosphate. In CKD patients, the ability of kidneys to remove phosphorus is reduced, resulting in hyperphosphatemia (Craver *et al.* 2007). Moreover, in CKD there is a reduction of calcitriol production which contributes to the decrease in the serum calcium levels (Panda *et al.* 2004). Low serum calcitriol and calcium levels together with high serum phosphorus stimulate PTH secretion, which in turn, reduces renal phosphate reabsorption to maintain phosphate homeostasis.

A new hormone, a phosphaturic hormone, FGF-23 (Schiavi & Kumar 2004) has been found recently to be involved in the complex regulation of phosphate homeostasis. In advanced CKD when serum phosphorus is high, FGF-23 synthesis is stimulated, as a result, there is an increase in phosphorus excretion as well as inhibition of calcitriol production (Shimada *et al.* 2004).

The most well-known consequences of hyperphosphatemia are its effects on the parathyroid glands, which in turn affects bone metabolism. High serum phosphate levels impair calcitriol synthesis, increase the skeletal resistance to PTH and also increase PTH synthesis. In addition, recent studies have shown that hyperphosphatemia increases parathyroid cell proliferation and it may reduce the calcium sensing receptor expression. *In vitro* studies demonstrate that high phosphorus levels increase PTH synthesis and secretion at the post-transcriptional level (Almaden *et al.* 1996). The parathyroid gene expression response to changes in serum phosphate concentration is regulated by mechanism similar to that already described for the calcium. High serum phosphate—as does low calcium—increases the stability of PTH-mRNA. In addition, phosphorus also increases parathyroid cell proliferation (Slatopolsky *et al.* 1999b).

Furthermore, high serum PTH levels have also been shown to increase vascular calcification and the risk of mortality. Due to the close relationship between high serum phosphate and PTH, it may be also speculated that PTH effect on mortality may also be indirectly reflecting the effect of hyperphosphatemia. All these negative effects of high serum phosphate levels explain why, in the dialysis scenario, serum PTH levels are the strongest risk factor associated with the severity of secondary hyperparathyroidism (Cannata-Andia *et al.* 2006).

HYPERPHOSPHATEMIA AND VASCULAR CALCIFICATION

The predisposition of patients with CKD to vascular calcification was mentioned for the first time in the 19th century, since then many studies have addressed this matter. If the series published in the last three decades are reviewed, it can be observed that 40–92% of the patients undergoing dialysis treatment, including young patients, have vascular calcification.

Hyperphosphatemia may play an important role in the development of calcification and cardiovascular alterations in patients with CKD. This has a great relevance from both the epidemiological and the clinical perspective. Between 40 and 60% of patients undergoing dialysis have high levels of serum phosphate and vascular mortality is nearly 50% (Cannata-Andía & Rodriguez-García 2002). Hyperphosphatemia may influence vascular calcification, arterial rigidity (London *et al.* 2007) and risk of death; in addition, it worsens secondary hyperparathyroidism.

It has been also demonstrated that vascular calcifications in some localities were associated with increased risk of vertebral fractures (Rodriguez Garcia *et al.* 2005) and the progression of vascular calcification has been associated with a more rapid bone loss and greater number of prevalent and incident vertebral fractures (Rodriguez Garcia *et al.* 2005; Naves *et al.* 2006; Rodriguez Garcia *et al.* 2008).

It is well known that patients with CKD have multiple risk factors that facilitate the development of all types of calcification; some of them are modifiable with the possibility to reduce calcification. Within this group of modifiable risk factors, one of the most relevant is hyperphosphatemia (Cannata-Andía & Rodriguez-García 2002). It is known that certain stimuli, including phosphate, play a central role in the differentiation of the smooth muscle cell to osteoblast-like cells (Giachelli 2004). The mechanisms by which the process of vascular calcification is produced is complex and it does consist, not in a simple precipitation of calcium and phosphate, but in a mixed, active and modifiable process, in which the final product is a calcification which has similar characteristics to bone tissue, and occurs in several conditions such as atherosclerosis, cardiac valvular disease and uremic arteriolopathy.

In brief the active process in which phosphorus plays a main role consists in "activating" bone-related genes and "deactivating" muscle-related genes in the vascular cells (VSMCs), leading to the already mentioned change of phenotype which undergoes changes of gene and protein expression (i.e. Bone Morphogenetic Proteins) (Hruska *et al.* 2005) in the cells of the vessel wall (Roman-Garcia *et al.* 2008). This is a very active area in which new findings are enriching knowledge almost every week. As a result, new relationships between bone biology and vascular calcification have been set by clinical and basic studies (Schoppet *et al.* 2008) which are not covered by this review.

HOW TO MANAGE HYPERPHOSPHATEMIA AND REDUCE RISKS

According to what has been described, it is evident that the control of hyperphosphatemia in advanced CKD is of utmost importance. Diet, adequate use of phosphate-binding agents and different dialysis strategies can be used. These strategies, which are currently used in combination, can have favourable consequences in the control of plasma phosphate, but they can be flawed by side effects.

THE ROLE OF THE DIET

It is important to stress the need of maintaining a diet low in phosphorus, not exceeding 1g/kg/day of protein, this will allow maintenance of adequate nutritional status. The side effects that may result from severe, uncontrolled, protein and phosphorus restriction in the diet should not be underestimated, especially in the stage of advanced CKD where a progressive worsening of the appetite, a reduced protein and calorie intake, can contribute to malnutrition. On the other hand, compliance with dietary restriction is not easily achieved in most patients in the long run, since it involves major changes in lifestyle (Takeda *et al.* 2007).

Nephrologists should have a good knowledge of the dietary habits of their patients. A diet rich in proteins is also usually rich in phosphorus. However, proteins with very different phosphorus content can provide equivalent nutritional value as can be seen from the difference in phosphorus content among meat, cheese and eggs (Cannata-Andía & Rodriguez-García 2002). Egg white is an excellent example of food with a high level of protein value but low phosphorus content. Thus, the dietary advice, which is often considered to be of minor importance, plays an important role in the management of hyperphosphatemia. Unfortunately, the effectiveness of this strategy is limited due to the lack and heterogeneous

distribution of dietitians in the CKD units in Europe (Locatelli *et al.* 2002b).

THE ROLE OF PHOSPHATE-BINDING AGENTS

It is important to stress that prescription of these compounds should be individualised, adapting their use to the dietary habits of each patient. It must be taken into account that the phosphate-binders should only be taken with those foods with a phosphorus content that justifies the need for their administration. They should be taken during meals, (before the meal is better than after). It is also very important that the patient understands why the phosphate binder has been prescribed.

Aluminium hydroxide, which has been widely used for many years, is the most potent but also the most toxic phosphate binder (Cannata-Andia and Fernandez-Martin 2002). For this reason in the 1980s, it was almost replaced by calcium salts. When the use of latter became widespread, disadvantages started also to become apparent. It has been shown that the use of calcium salts, mainly exceeding 1.5 g of calcium daily, increases the risk of vascular calcification, and also can lead to a greater rigidity (London *et al.* 2007) of the arteries. This consequent greater risk of cardiovascular events is crucial for patients undergoing dialysis.

These adverse events have limited the use of the calcium-containing phosphate-binders, and stimulated the development and production of new-generation phosphate binders, such as sevelamer and lanthanum carbonate which are specifically analysed in other contributions of this supplement.

THE EFFECT OF THE DIALYSIS REGIME

The dialysis regime and modality can influence phosphate levels. It has been reported that there is better control of serum-phosphate in peritoneal dialysis and in long and more frequent dialysis sessions in haemodialysis. However, some of these approaches that are also addressed in other papers of this supplement may have many practical limitations in the daily management of hyperphosphatemia.

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CONFLICT OF INTEREST

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REFERENCES

Almaden Y., Canalejo A., Hernandez A. *et al*. (1996). Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands in vitro. *Journal of Bone Mineral Research* **11**, 970-976.

Amann K., Gross M.L., London G.M. *et al*. (1999). Hyperphosphataemia–a silent killer of patients with renal failure? *Nephrology Dialysis Transplantation* **14**, 2085-2087.

Block G.A., Klassen P.S., Lazarus J.M. *et al*. (2004). Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *Journal of American Society of Nephrology* **15**, 2208-2218.

Cannata-Andia J.B. & Fernandez-Martin J.L. (2002). The clinical impact of aluminium overload in renal failure. *Nephrology Dialysis Transplantation* **17**(Suppl 2), 9-12.

Cannata-Andia J.B., Rodriguez-Garcia M., Carrillo-Lopez N. *et al*. (2006). Vascular calcifications: pathogenesis, management, and impact on clinical outcomes. *Journal of American Society of Nephrology* **17**, S267-S273.

Carrillo-Lopez N., Alvarez-Hernandez D., Gonzalez-Suarez I. *et al*. (2008). Simultaneous changes in the calcium-sensing receptor and the vitamin D receptor under the influence of calcium and calcitriol. *Nephrology Dialysis Transplantation* **23**, 3479-3484.

Craver L., Marco M.P., Martinez I. *et al*. (2007). Mineral metabolism parameters throughout chronic kidney disease stages 1-5–achievement of K/Doqi target ranges. *Nephrology Dialysis Transplantation* **22**, 1171-1176.

Garfia B., Canadillas S., Canalejo A. *et al*. (2002). Regulation of parathyroid vitamin D receptor expression by extracellular calcium. *Journal of American Society of Nephrology* **13**, 2945-2952.

Giachelli C.M. (2004). Vascular calcification mechanisms. *Journal of American Society of Nephrology* **15**, 2959-2964.

Gogusev J., Duchambon P., Hory B. *et al*. (1997). Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. *Kidney International* **51**, 328-336.

Hruska K.A., Mathew S., Davies M.R. *et al*. (2005). Connections between vascular calcification and progression of chronic kidney disease: therapeutic alternatives. *Kidney International* (Suppl)S142-S151.

Kifor O., Moore F.D., Jr, Wang P., *et al*. (1996). Reduced immunostaining for the extracellular Ca2+-sensing receptor in primary and uremic secondary hyperparathyroidism. *Journal of Clinical Endocrinology Metabolism* **81**, 1598-1606.

Locatelli F., Cannata-Andia J.B., Drüeke T.B. *et al*. (2002a). Management of disturbances of calcium and phosphate metabolism in chronic renal insufficiency, with emphasis on the control of hyperphosphataemia. *Nephrology Dialysis Transplantation* **17**, 723-731.

Locatelli F., Fouque D., Heimburger O. *et al*. (2002b). Nutritional status in dialysis patients: a European consensus. *Nephrology Dialysis Transplantation* **17**, 563-572.

London G., Marchais S., Guerin A. *et al*. (2007). Arterial hypertension, chronic renal insufficiency and dialysis. *Nephrology Therapy* **3**(Suppl 3), S156-S161.

Moallem E., Kilav R., Silver J. *et al*. (1998). RNA-protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. *Journal of Biological Chemistry* **273**, 5253-5259.

Naveh-Many T., Rahamimov R., Livni N. *et al*. (1995). Parathyroid cell proliferation in normal and chronic renal failure rats. The effects of calcium, phosphate, and vitamin D. *Journal of Clinical Investigation* **96**, 1786-1793.

Naveh-Many T. & Silver J. (1998). Regulation of parathyroid hormone gene expression and secretion by vitamin D. In *Vitamin D: Physiology, Molecular Biology and Clinical Applications,* (ed Holick M. F.). Totowa: Humana Press Inc.

Naves M., Rodriguez-Garcia M., Diaz-Lopez J.B. *et al*. (2008). Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. *Osteoporos International* **19**, 1161-1166.

Panda D.K., Miao D., Bolivar I. *et al*. (2004). Inactivation of the 25-hydroxyvitamin D 1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. *Journal of Biological Chemistry* **279**, 16754-16766.

Pike J. W. (1994). Vitamin D: receptors and the mechanism of action of 1,25-dihydroxyvitamin D3. In *Vitamin Receptors: Vitamins As Ligands in Cell Communication,* (ed Dakshinamurti K.). New York: Cambridge University Press.

Rodriguez Garcia M., Gomez Alonso C., Diaz-Lopez B. *et al*. (2008). Vascular calcifications, vertebral fractures and mortality in haemodialysis patients. *Nephrology Dialysis Transplantation*, In Press.

Rodriguez Garcia M., Naves Diaz M. & Cannata Andia J. B. (2005). Bone metabolism, vascular calcifications and mortality: associations beyond mere coincidence. *Journal of Nephrology* **18**, 458-463.

Roman-Garcia P., Carrillo-Lopez N., Naves Diaz M. *et al*. (2008). Vascular calcification implies a differential gene expression with deregulation in the SFRP family. *Nephrology Dialysis Transplantation Plus,* 1 ii22 (Free Comunication At XLIII Era-Edta Congress 2008).

Schiavi S.C. & Kumar R. (2004). The phosphatonin pathway: new insights in phosphate homeostasis. *Kidney International* **65**, 1-14.

Schoppet M., Shroff R.C., Hofbauer L.C. *et al*. (2008). Exploring the biology of vascular calcification in chronic kidney disease: what's circulating? *Kidney International* **73**, 384-390.

Shimada T., Kakitani M., Yamazaki Y. *et al*. (2004). Targeted ablation of FGF23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *Journal of Clinical Investigation* **113**, 561-568.

Silver J. & Levi R. (2005). Regulation of PTH synthesis and secretion relevant to the management of secondary hyperparathyroidism in chronic kidney disease. *Kidney International* (Suppl)S8-S12.

Slatopolsky E. (1998). The role of calcium, phosphorus and vitamin D metabolism in the development of secondary hyperparathyroidism. *Nephrology Dialysis Transplantation* **13**(Suppl 3), 3-8.

Slatopolsky E., Brown A. & Dusso A. (1999a). Pathogenesis of secondary hyperparathyroidism. *Kidney International* **73**(Suppl), S14-S19.

Slatopolsky E., Dusso A. & Brown A.J. (1999b). The role of phosphorus in the development of secondary hyperparathyroidism and parathyroid cell proliferation in chronic renal failure. *American Journal of Medical Science* **317**, 370-376.

Takeda E., Yamamoto H., Nishida Y. *et al*. (2007). Phosphate restriction in diet therapy. *Contributions to Nephrology* **155**, 113-124.

Publicación 2: Severe Hyperplasia and Lack of Response to FGF23 of Uremic Parathyroid Glands is Associated with Increased Dual Specificity Phosphatases Gene Expression

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Severe Hyperplasia and Lack of Response to FGF23 of Uremic Parathyroid Glands is Associated with Increased Dual-Specificity Phosphatases Gene Expression

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Keywords:	Parathyroid < DISORDERS OF CALCIUM/PHOSPHATE METABOLISM, METABOLIC BONE DISEASE, Arrays < GENE/GENETIC RESEARCH, PTH/PTHrP < HORMONES and RECEPTORS, PARATHYROID HORMONE AND PARATHYROID GLAND
Abstract:	Phosphate load accelerates the progression of secondary hyperparathyroidism (sHPT). In advanced stages of sHPT there is a marked hyperplasia and resistance to classical regulatory factors such as calcium or calcitriol but also to novel factors such as FGF23, which suppresses PTH secretion by an ERK-dependent mechanism. Nephrectomized rats (7/8) were fed with a high or normal phosphorus diet for different periods of time in order to induce sHPT. Biochemical parameters, parathyroid gland microarrays, RT-qPCR, and immunohistochemistry for ERK and pERK were performed. To test the role of dual-specificity phosphatases (Dusps) on parathyroid gland regulation, normal parathyroid glands were cultured with FGF23 and Dusps

	Uremic rats fed with a high phosphorus diet (HPD) showed more severe sHPT, higher serum FGF23 levels, decreased parathyroid Klotho gene expression, and higher mortality. In sHPT, parathyroid microarrays displayed a widespread gene expression downregulation; only a few genes overexpressed, among them, Dusp5 and -6. In very severe sHPT, a significant reduction in pERK (the target of Dusp), and a significant increase of Dusp5 and -6 gene expression were observed. In ex vivo experiments with parathyroid glands, Dusps partially blocked the effect of FGF23 on PTH secretion, suggesting that Dusps might play a role in parathyroid regulation. The overexpression of Dusps and the inactivation of ERK found in the in vivo studies, together with the results obtained in stimulated parathyroid glands in culture, which showed that Dusps decreased the FGF23 effect on the parathyroid glands, might be indicative of the defense mechanism triggered to counteract hyperplasia, a mechanism that can also contribute to the resistance to the effect of FGF23 on parathyroid gland observed in advanced forms of CKD.

Severe Hyperplasia and Lack of Response to FGF23 of Uremic Parathyroid Glands is Associated with Increased Dual-Specificity Phosphatases Gene Expression

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ABSTRACT

Phosphate load accelerates the progression of secondary hyperparathyroidism (sHPT). In advanced stages of sHPT there is a marked hyperplasia and resistance to classical regulatory factors such as calcium or calcitriol but also to novel factors such as FGF23, which suppresses PTH secretion by an ERK-dependent mechanism.

Nephrectomized rats (7/8) were fed with a high or normal phosphorus diet for different periods of time in order to induce sHPT. Biochemical parameters, parathyroid gland microarrays, RT-qPCR, and immunohistochemistry for ERK and pERK were performed. To test the role of dual-specificity phosphatases (Dusps) on parathyroid gland regulation, normal parathyroid glands were cultured with FGF23 and Dusps.

Uremic rats fed with a high phosphorus diet (HPD) showed more severe sHPT, higher serum FGF23 levels, decreased parathyroid Klotho gene expression, and higher mortality. In sHPT, parathyroid microarrays displayed a widespread gene expression downregulation; only a few genes overexpressed, among them, Dusp5 and -6. In very severe sHPT, a significant reduction in pERK (the target of Dusp), and a significant increase of Dusp5 and -6 gene expression were observed. In *ex vivo* experiments with parathyroid glands, Dusps partially blocked the effect of FGF23 on PTH secretion, suggesting that Dusps might play a role in parathyroid regulation.

The overexpression of Dusps and the inactivation of ERK found in the *in vivo* studies, together with the results obtained in stimulated parathyroid glands in culture, which showed that Dusps decreased the FGF23 effect on the parathyroid glands, might be indicative of the defense mechanism triggered to counteract hyperplasia, a mechanism that can also contribute to the resistance to the effect of FGF23 on parathyroid gland observed in advanced forms of CKD.

INTRODUCTION

Hyperplasia of the parathyroid glands is a common finding in patients with chronic kidney disease (CKD). Excess of dietary phosphate accelerates the progression of secondary hyperparathyroidism (sHPT) (1) and increases vascular calcification as well.(2,3) In mild and early stages of CKD, parathyroid hyperplasia is, at least, partly reversible;(4-6) in advanced and late stages, however, the enlarged parathyroid glands do not respond adequately to increments in serum calcium, high doses of active vitamin D or high levels of fibroblast growth factor 23 (FGF23) due to several abnormalities, including the decreased expression of the calcium sensing receptor (CaSR), the vitamin D receptor (VDR), Klotho, and the FGF receptor 1 (FGFR1), which lead to a parathyroid resistance to their current regulatory factors.(7-9)

In addition to the well-known role of calcium and calcitriol in parathyroid regulation, it has been demonstrated that FGF23 also suppresses PTH secretion by activating the extracellular signal-regulated kinase (ERK) / mitogen-activated protein kinase (MAPK) pathway in the parathyroid glands.(8) However, in sHPT, the inhibitory effect of FGF23 on PTH synthesis is not observed.(8,10) The absence of FGF23 inhibition of PTH synthesis has been recently associated to the low levels of Klotho found in parathyroid glands from uremic patients or animals.(9,11)

The present experimental study was designed to assess parathyroid hyperplasia, secondary to CKD, by analyzing the gene expression profile changes across different stages of hyperparathyroidism. In addition, we also aimed to elucidate whether regulators of MAPK signaling (Dusps) might play a role in the FGF23/MAPK signaling in the parathyroid glands.

MATERIAL AND METHODS

Animal model

The study was performed in 4-month old male Wistar rats. Rats were anaesthetized using methoxi-fluorane, and chronic renal failure was induced by surgical 7/8 nephrectomy.(12) The nephrectomized rats were subsequently divided into two groups: group I was fed with an NPD (0.6% phosphorus, 0.6% calcium, and 20% protein content), and group II was fed with a HPD (0.9% phosphorus, 0.6% calcium, and 20% protein content) (Panlab). Rats were housed in wire cages and received diet and water *ad libitum*.

Five rats from the HPD and NDP groups were sacrificed by heart puncture exsanguination after 4, 8, 12, 16, and 20 weeks of follow-up. At sacrifice, blood samples and parathyroid glands were obtained.(13) An additional group of rats without nephrectomy was fed with an NPD for 20 weeks and used as Reference group (n=9). The total number of groups studied was 11 (5 rats per group) and the total number of animals analyzed was 59.

As the aim of the present study was to investigate parathyroid gland regulation across different stages of hyperparathyroidism, we decided to use PTH values as the main parameter to form the 3 groups of study: “Moderate sHPT”, which included serum PTH values below 150 pg/mL; “Moderate-Severe sHPT”, which included values between 151 and 1,000 pg/mL; and “Very Severe sHPT", which included values above 1,001 pg/mL. “Moderate sHPT” included all rats from the NPD subgroups, “Moderate-Severe sHPT” included rats from the HPD weeks 4, 8 and 12 subgroups, and “Very Severe sHPT” included rats from the HPD weeks 16 and 20. subgroups This strategy was also used to analyze and compare the rest of parameters.

The *Laboratory Animal Ethics Committee* of the Universidad de Oviedo approved this protocol.

Biochemical markers

Serum was separated from blood samples by centrifugation at 4,000 rpm at 4 ºC. Serum urea, creatinine, calcium, and phosphorus were measured using a multi-channel Auto Analyzer (Hitachi 717) following the manufacturer's protocol. Serum PTH was measured using an immunoradiometric rat PTH assay with a specific chicken anti-PTH antibody, following the manufacturer's protocol (Immutopics) (Normal ranges 10-30 pg/dL)). Serum FGF23 was measured using an enzyme-linked immuno-sorbent assay following the manufacturer's protocol (60-6300, Immutopics) (Normal ranges 800-1000).

Gene expression microarrays

The parathyroid glands (one per rat) from each subgroup were pooled and homogenized (Ultraturrax, OmniHT) in TRI™ reagent (Sigma-Aldrich). Total RNA was extracted and purified using an RNeasy™ Kit (Qiagen). RNA integrity was checked using agarose-formaldehyde gels, and RNA concentration was measured using a VIS-UV spectrophotometer (Nanodrop). cDNA was synthesized with a High Capacity™ kit (Applied Biosystems), and hybridized to one Affy RAE_230 cDNA microarray (Affymetrix) following the required quality controls and the manufacturer's protocol. The array included all rat genome genes (31,099 probes per array). As a result of the pooling, we obtained one array per each of the 11 subgroups and we formed the groups following the criteria explained above.

To analyze the raw datasets, data were logarithmically transformed and normalized using the PerfectMatch/MisMatch method (dChip(14)), with the Reference group as baseline-comparator. After the normalization and expression-modeling of the raw data, we followed a two-stepwise process. In the first step, in order to investigate specific differences among the samples in selected genes, hierarchical clusters were built using the Euclidean-Centroid

Linkage method. Two types of clusters were built: a) unsupervised clusters, in which all genes were included in order to investigate general differences in the pattern of expression among the samples, and b) supervised clusters, in which specific genes grouped by ontology terms (obtained from the Kyoto Encyclopaedia of Genes and Genomes [KEGG]) were included. In the second step, different comparisons were performed (Moderate *vs*. Very Severe sHPT, Moderate *vs*. Moderate-Severe sHPT, and Moderate-Severe *vs*. Very Severe sHPT) using ANOVA and Student's T tests. Afterwards, a fold change was adjudicated using a false discovery rate (FDR) algorithm to avoid false positives. Only the probes with fold changes >+2 or <-2 were selected.

RT-qPCR

Genes of interest selected from the microarrays were validated by RT-qPCR (ABI Prism 7000), using Taqman™ predeveloped assays: Dusp 6 (Rn_00518185_m1) and Dusp 5 (Rn_0683448_m1). In addition, other genes related to sHPT such as PTH (Rn00566882_m1), CaSR (Rn 00566496_m1), Klotho (Rn00580132_m1), and VDR (Rn00566976_m1) were analyzed. As endogenous control, 18S (Eukaryotic 18s rRNA Endogenous Control Reagent Applied Biosystems) and GAPDH (Rn99999916_m1) were used.

Immunohistochemistry

The presence of ERK and pERK in parathyroid tissue was also determined by IHQ in 5-µm-thick serial sections from paraffin-embedded parathyroid glands using specific antibodies (Cell Signaling Tech ref #9106 and #9102, respectively) and hematoxylin counterstaining (HistoStainPlus IHC detection Kit™, Invitrogen) following the manufacturer's instructions. A negative control without primary antibody was used. For the quantification of the staining, a Leica CTR-Mic microscope coupled to image analysis software (Leica

Q500IW) was used. Briefly, the image of each gland was converted to grayscale; then, utilizing the optical density function of the software, pixels that fell within a designed threshold were used and counted; as a result, we obtained a mean of gray color. Brown *et al.* described the complete method.(15)

Parathyroid tissue culture

Parathyroid glands were obtained from 3-month-old male Wistar rats (n=96), with normal renal function, as described(13). The *Laboratory Animal Ethics Committee* of the Universidad de Oviedo approved this protocol.

The glands were washed for 8 hours in standard medium[17] containing 1.2 mM Ca. After the washing period, the glands were treated for an additional 24 hours with the standard medium or with a medium containing 0.6 mM calcium, alone or in combination with different factors. This very same approach has been published in previous studies related to PTH synthesis and secretion (16). As a result, 5 groups were obtained: a) 1.2 mM Ca; b) 0.6 mM Ca; c) 0.6 mM Ca + FGF23 100 ng/mL (2629-FG, R&D Systems); d) 0.6 mM Ca + FGF23 100 ng/mL + UO1261 μM (UO126, #U-120, Sigma Aldrich), and e) 0.6 mM Ca + FGF23 100 ng/mL + mixture of recombinant Dusps at 1μL/mL each (AK-020, Biomol). The activity of the Dusps ranged between an interval of 5-90 U/μg depending on the specific Dusp. It was assayed by 3-*O*-methyl fluorescein phosphate (OMFP) hydrolysis at 30°C. The assay conditions were:1 μg enzyme per 100 μl in 100 mM Tris-HCl, pH 8.2, 40 mM NaCl, 1 mM DTT, 20% glycerol, 0.5 mM OMFP. One unit was equal to 1 pmol of phosphate hydrolyzed from OMFP per minute. Six independent experiments were performed in each group.

PTH secretion was measured in the culture media at the beginning and at the end of all experiments using an enzyme-linked immuno-sorbent assay following the manufacturer's protocol (60-2500, Immunotopics).

Statistical analysis

Student's t-test was used to compare biochemical parameters among the groups. Pearson's test was used to study the correlations among the biochemical parameters. Data were expressed as mean ± standard deviation. Differences were considered significant when $p<0.05$. Calculations were performed using the statistical analysis package SPSS 12.0 (SPSS Inc).

RESULTS

Effects of high phosphate diet in uremic rats

Different degrees of sHPT were induced in uremic rats by feeding with a high (HPD) or normal phosphorus diet (NPD) for different time periods.

The Moderate sHPT group was composed of all uremic rats fed with NPD, and the Moderate-Severe and Very Severe sHPT groups were composed of rats fed with HPD. In general terms, a longer exposure to HPD led to a more severe sHPT. The mortality of rats reached 50%, 40%, and 25% in the Very Severe, Moderate-Severe, and Moderate sHPT groups, respectively. Serum PTH, phosphorus, and FGF23 levels were significantly higher in the Moderate-Severe and Very Severe sHPT groups compared to the other two groups (Figures 1A, 1B, 1D). Similarly to what has been shown in previous studies the subtotal nephrectomy was not enough to induce moderate or severe forms of secondary hyperparathyroidism. (17-20)

In addition, significantly lower serum calcium levels were observed in the Moderate-Severe and Very Severe sHPT groups (Figure 1C).

Serum FGF23 levels correlated positively with serum PTH ($r=0.83$, $p<0.01$, Figure 2A) and with serum phosphorus ($r=0.76$, $p<0.05$, Figure 2B).

The decrease in renal function was more marked in the Very Severe sHPT group, which showed significantly higher levels of serum urea and creatinine compared to the Moderate and Moderate-Severe sHPT groups (data not shown). In fact, serum creatinine ($r=0.88$, $p<0.001$, Figure 2C) and urea ($r=0.77$, $p<0.001$, Figure 2D) levels positively correlated with serum PTH levels.

Gene expression analyses

In the first step, unsupervised hierarchical clusters (HC) (including all probes [31,099] and the different groups) showed that the Very Severe sHPT group was the only one segregated from the others (data not shown). By contrast, in the case of mineral metabolism-associated genes, the Very Severe sHPT group showed a remarkably different pattern of expression and clustered separately from the Moderate and Moderate-Severe sHPT groups (Figure 3).

In the second step, using the criteria explained in the Concise Methods section, 136 probes were found to be differentially expressed in the "Very Severe *vs*. Moderate sHPT" comparison, 32 in the "Moderate-Severe *vs*. Moderate sHPT" comparison, and 10 in the "Very Severe *vs*. Moderate-Severe sHPT" comparison. The lists of differentially expressed probes are shown in Tables 1, 2, and 3, respectively.

Overall, a widespread downregulation of gene expression was observed, involving more than 80% of the genes. Only a minor percentage of genes were upregulated. Among the latter, the dual-specificity phosphatase 6 (Dusp6) gene showed the highest overexpression in all the comparisons. Dual-specificity phosphatase 5 (Dusp5) was also upregulated in the "Very Severe *vs.* Moderate sHPT" comparison.

The quantitative real time PCR (RT-qPCR) experiments confirmed a very high upregulation of Dusp6 gene expression in Very Severe sHPT (mean: 43-fold increase relative to the Reference Group) and also in Moderate-Severe sHPT (mean: 3.27-fold increase). Dusp5 gene expression also showed a significant 7.09-fold upregulation in Very Severe sHPT but not in the Moderate and Moderate-Severe sHPT subgroups (Figure 4).

Microarray analysis did not disclose significant differences in the gene expression of "classical" parathyroid regulators such as calcium sensing receptor (CaSR), VDR, or PTH. This is not a surprising result given that microarray analysis is a screening technology and the

strict criteria applied to prevent false positives may have increased the number of false negative findings or the number of genes below the threshold, including the aforementioned "classical" genes. In RT-qPCR analyses of the same parathyroid samples, CaSR, VDR and Klotho gene expression were also significantly reduced in the Very Severe sHPT group, whilst PTH was progressively upregulated (Figure 5). The difference in Klotho expression between Moderate and Moderate-Severe Groups was not statistically significant and it was under the limit of detection of the qRT-PCR (1.89 fold increase).

Activation of ERK in parathyroid glands

Dusps are protein phosphatases that dephosphorylate a specific MAPK, inhibiting its function. In particular, Dusp5 and Dusp6 specifically dephosphorylate and inactivate phospho-ERK-1 and -2 (pERK 1/2). Therefore, we analyzed the activation of ERK by immunohistochemistry (IHQ) analysis of ERK and pERK 1/2 in paraffin-embedded parathyroid tissue. ERK staining was similar in all sHPT groups and the Reference group. However, the intensity of pERK staining increased significantly in Moderate sHPT when compared to the Reference group. Interestingly, pERK decreased as the severity of sHPT increased, and it was significantly reduced in the Very Severe sHPT group compared to the Moderate sHPT group (Figures 6A, 6B).

Evaluation of the role of Dusps on PTH secretion

In order to test whether Dusps might have a regulatory effect on parathyroid function, additional experiments were performed using parathyroid glands from normal rats cultured *ex vivo*.

Parathyroid glands cultured for 24 hours in a medium containing 0.6 mM calcium showed the expected significant increase in PTH secretion compared to the glands cultured in a standard medium (1.2 mM calcium). By contrast, when glands were cultured in a medium containing 0.6 mM calcium + FGF23 100 ng/mL, PTH secretion decreased significantly, down to the levels observed when using the standard medium. The effect of FGF23 on PTH secretion was ablated by the addition of a chemical ERK 1/2 inhibitor (UO126, 1μM) and, consequently, PTH secretion was restored completely. The effect of FGF23 in PTH secretion was also partially blocked by adding Dusps, which are the biological inhibitors of the phosphorylation of ERK, resulting in a partial but significant restoration of PTH secretion (Figure 7).

DISCUSSION

Secondary hyperparathyroidism, vascular calcification, bone loss, and an increased fracture rate are severe and threatening outcomes in the CKD population at all stages.(21) Elevated serum phosphorus has been described as a major pathogenic player associated with sHPT progression, impairment of renal function, vascular calcification, and high risk of mortality.(2,22-24) We observed that, in agreement with previous findings, that the degree of severity of sHPT was proportional to the time of exposure to a high phosphorus diet,(25,26) in addition we also observed that the high phosphorus diet impaired renal function and increased mortality. As in other studies, we have also found a significant increase of FGF23 levels in all groups (compared to reference), which were higher in more severe sHPT; in fact, a direct correlation between serum FGF23 and PTH was found.(27,28) Both facts are frequently interpreted as a loss of the capability of FGF23 to inhibit PTH secretion, -the resistance to FGF23 concept-. Furthermore, serum FGF23 and phosphorus levels were also associated. The positive correlation of serum phosphorus with FGF23 and PTH, together with the higher values of these biochemical parameters observed in rats with the lowest renal function, may be indicative of a compensatory phosphaturic response via FGF23 and PTH, which was unable to fully compensate the outcome of decreased glomerular phosphate filtration.

To our knowledge, the gene expression microarray analysis shown in this study is the first carried out in parathyroid glands from rats. It clearly revealed that sHPT progression is characterized by a widespread gene expression downregulation, as opposed to the gene expression upregulation observed in primary hyperparathyroidism.(29,30) Since the Very Severe sHPT clustered separately from the Moderate-Severe and Moderate sHPT groups (when the samples were clustered using mineral metabolism-associated genes), we can hypothesize that the more important changes in gene expression took place in advanced stages

of sHPT. In addition, our molecular findings are consistent with previous data obtained in patients with severe nodular secondary and tertiary HPT(30) and also with current clinical responses observed in advanced CKD patients, in whom mainly mild to moderate sHPT, but not the more severe forms of sHPT, are responsive to current therapies targeting the classical regulators.

In advanced CKD, the abnormal control of PTH secretion has been partly attributed to CaSR and VDR downregulation.(7,26) We did not find a significant deregulation of these genes using microarrays. It is known that microarray analysis has several limitations such as the underestimation or overestimation of gene expressions.(31) As described in the results, we used a very restrictive approach to avoid false positives and as a consequence some of the "classical" genes known to be deregulated in CKD may have been automatically placed below the threshold. In addition, glands with severe degrees of sHPT show multiple genetic and molecular aberrations(30,32), which might mask the expected changes in the "classical" genes. Possibly, the main changes in these genes needs to be evaluated in the early stages of secondary hyperparathyroidism.

Validation by RT-qPCR, which is used as a supporting stepwise technique, is a widely accepted approach because it is far more sensitive and capable of detecting minor changes in specific genes. , In fact, we did find significant upregulation in PTH and downregulation in CaSR, VDR, and Klotho gene expression in the Very Severe sHPT group when using RT-qPCR. These findings coincide with previous publications, which also have found a degree of downregulation in the CaSR close to 50% or even less. (15,33,34)

In our study, we found that the lack of the inhibitory effect of FGF23 on PTH secretion was associated to a downregulation of Klotho gene expression. Similar findings were reported by 3 different previous studies(9,35,36) even though a recent paper described an upregulation of Klotho in the parathyroid glands of uremic patients with severe sHPT(37). In addition,

results from genetically modified mouse models suggest taht FGF23 may be an indirect regulator of the parathyroid function(38). These controversial results are difficult to conciliate but could be partly explained by the difficulties associated to the quantification and interpretation of Klotho expression and the differences among the animal models used to investigate the role of FGF23 in mineral metabolism.(10,39)

Interestingly, in all comparisons (and irrespective of the groups compared), the repression of gene expression was the most common finding; only a very few genes were found to be overexpressed. Among them, Dusp6 was the one gene that always showed the highest degree of overexpression as confirmed by RT-qPCR.

Dusps constitute a subclass of the protein phosphatase super-family that dephosphorylates MAPKs. Despite MAPK signalling can be deactivated by changes in location or scaffolding, to our knowledge, the Dusp Family is the unique phosphatase system in charge of the dephosphorilation of MAPK. (40) The members of the Dusp family exhibit high specificity against a single MAPK. A good example of their high specificity is the fact that Dusp6 and Dusp5 dephosphorylate and inactivate pERK-1 and -2 (1/2) but not other subclasses of MAPK such as JNK or p38.(41-43) The MAPK/ERK pathway is a very complex signaling mechanism which regulates mainly the cell cycle and proliferation.(44) In the parathyroid gland, the MAPK/ERK pathway is activated with the development of sHPT(8,45,46) but is also part of the FGF23 intracellular signaling pathway that suppresses PTH secretion. In particular, ERK1/2 is required for FGF23-induced PTH suppression; in addition, ERK1/2 inhibitors prevent this suppression, and ERK inactivation has been recently described in FGF23-refractory parathyroid glands from uremic rats. (8,36)

According to our results, a plausible explanation for the somewhat surprising dynamics of ERK is that in the initial stages of hyperparathyroidism, an increase of ERK phosphorylation (a trigger to “start hyperproliferation”) can be observed. As

hyperparathyroidism progresses to more severe forms, parathyroid cells would increase Dusps gene expression in order to reduce the high activity of the parahyroid gland through increased ERK phosphorylation; as a result of this counteracting mechanism, a decrease of pERK levels is observed. This mechanism has been previously described in lung cancer and myocardial cells.(47,48)

Interestingly, inactivation of ERK by the increased Dusps gene expression may contribute to the fact that FGF23 appears to have little suppressive effect on PTH secretion in severe forms CKD. Indeed, several studies support that Dusp6 plays a key role as a specific negative-feedback regulator of "FGF-activated" ERK1/2 signaling.(49)

To mechanistically confirm whether Dusps might play a role in FGF23 signaling, we performed additional experiments culturing normal parathyroid glands with FGF23 alone or in combination with UO126 (an ERK inhibitor) and recombinant Dusps. Following previous published results, parathyroid glands were cultured with 0.6 mM calcium to secure PTH stimulation.(16,50) As expected, low calcium levels increased PTH secretion compared to standard calcium levels, which clearly suppress PTH. FGF23 reduced low calcium-induced PTH secretion in normal glands, to values similar to observed when using 1.2 mM calcium concentration, as previously described.(8) The chemical ERK1/2 inhibitor (UO126) prevented the suppressive effect of FGF23 on PTH secretion and, interestingly, the addition of recombinant Dusps also prevented the FGF23 effect on PTH secretion. These findings strongly suggest that Dusps might regulate FGF23 signaling through MAPK inhibition. The fact that Dusp5 is a target of p53 (51) represents a feasible mechanism by which p53, activated in response to the observed DNA damage (29,32) might negatively regulate cell-cycle progression by downregulating mitogen- or stress-activated protein kinases. The latter supports the hypothesis that Dusps could be implicated in the counter-response to severe

hyperplasia, likely via p53 activation, having an additional effect which would be the "shut down" of FGF23 signaling in the parathyroid glands.

In summary, rats fed with a HPD showed a marked reduction in renal function, severe sHPT, high levels of FGF23, and a higher mortality. Despite the parathyroid gene expression downregulation was the predominant finding associated to sHPT, we observed a striking overexpression of Dusp6 and inactivation of the MAPK/ERK pathway. The latter might reflect a defensive mechanism triggered to counteract sHPT progression. In addition, the *ex vivo* results strongly suggest that increases in Dusp, could contribute to the parathyroid FGF23 resistance. Altogether, our results allowed us to postulate that the overexpression of Dusps, with the associated inactivation of ERK, might play a regulatory role in parathyroid regulation in sHPT, partially blocking the intracellular FGF23 signaling pathway.

Bibliography

1. Slatopolsky E 1998 The role of calcium, phosphorus and vitamin D metabolism in the development of secondary hyperparathyroidism. Nephrol Dial Transplant **13 Suppl 3:**3-8.
2. Roman-Garcia P, Carrillo-Lopez N, Fernandez-Martin JL, Naves-Diaz M, Ruiz-Torres MP, Cannata-Andia JB 2010 High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. Bone **46**(1)**:**121-128.
3. Giachelli CM 2004 Vascular calcification mechanisms. J Am Soc Nephrol **15**(12)**:**2959-2964.
4. Slatopolsky E 2003 New developments in hyperphosphatemia management. J Am Soc Nephrol **14**(9 Suppl 4)**:**S297-299.
5. Naveh-Many T, Rahamimov R, Livni N, Silver J 1995 Parathyroid cell proliferation in normal and chronic renal failure rats. The effects of calcium, phosphate, and vitamin D. J Clin Invest **96**(4)**:**1786-1793.
6. Gutierrez O, Isakova T, Rhee E, Shah A, Holmes J, Collerone G, Juppner H, Wolf M 2005 Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. J Am Soc Nephrol **16**(7)**:**2205-2215.
7. Rodriguez M, Canalejo A, Garfia B, Aguilera E, Almaden Y 2002 Pathogenesis of refractory secondary hyperparathyroidism. Kidney Int Suppl (80)**:**155-160.
8. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro OM, Mohammadi M, Sirkis R, Naveh-Many T, Silver J 2007 The parathyroid is a target organ for FGF23 in rats. J Clin Invest **117**(12)**:**4003-4008.
9. Galitzer H, Ben-Dov IZ, Silver J, Naveh-Many T 2010 Parathyroid cell resistance to fibroblast growth factor 23 in secondary hyperparathyroidism of chronic kidney disease. Kidney Int **77**(3)**:**211-218.
10. Lafage-Proust MH 2010 Does the downregulation of the FGF23 signaling pathway in hyperplastic parathyroid glands contribute to refractory secondary hyperparathyroidism in CKD patients? Kidney Int **77**(5)**:**390-392.
11. Komaba H, Goto S, Fujii H, Hamada Y, Kobayashi A, Shibuya K, Tominaga Y, Otsuki N, Nibu K, Nakagawa K, Tsugawa N, Okano T, Kitazawa R, Fukagawa M, Kita T 2010 Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients. Kidney Int **77**(3)**:**232-238.
12. Naves-Diaz M, Carrillo-Lopez N, Rodriguez-Rodriguez A, Braga S, Fernandez-Coto T, Lopez-Novoa JM, Lopez-Hernandez F, Cannata-Andia JB 2010 Differential effects of 17beta-estradiol and raloxifene on bone and lipid metabolism in rats with chronic kidney disease and estrogen insufficiency. Menopause.
13. Alvarez-Hernandez D, Gonzalez-Suarez I, Naves M, Carrillo-Lopez N, Fdez-Coto T, Fernandez-Martin JL, Cannata-Andia JB 2005 Long-term response of cultured rat parathyroid glands to calcium and calcitriol: the effect of cryopreservation. J Nephrol **18**(2)**:**141-147.
14. Li C, Wong WH 2001 Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A **98**(1)**:**31-36.
15. Brown AJ, Ritter CS, Finch JL, Slatopolsky EA 1999 Decreased calcium-sensing receptor expression in hyperplastic parathyroid glands of uremic rats: role of dietary phosphate. Kidney Int **55**(4)**:**1284-1292.
16. Carrillo-Lopez N, Alvarez-Hernandez D, Gonzalez-Suarez I, Roman-Garcia P, Valdivielso JM, Fernandez-Martin JL, Cannata-Andia JB 2008 Simultaneous changes

in the calcium-sensing receptor and the vitamin D receptor under the influence of calcium and calcitriol. Nephrol Dial Transplant **23**(11)**:**3479-3484.
17. Sanchez CP, He YZ 2007 Bone growth during daily or intermittent calcitriol treatment during renal failure with advanced secondary hyperparathyroidism. Kidney Int **72**(5)**:**582-591.
18. Mizobuchi M, Finch JL, Martin DR, Slatopolsky E 2007 Differential effects of vitamin D receptor activators on vascular calcification in uremic rats. Kidney Int **72**(6)**:**709-715.
19. Lopez I, Mendoza FJ, Aguilera-Tejero E, Perez J, Guerrero F, Martin D, Rodriguez M 2008 The effect of calcitriol, paricalcitol, and a calcimimetic on extraosseous calcifications in uremic rats. Kidney Int **73**(3)**:**300-307.
20. Saji F, Shiizaki K, Shimada S, Okada T, Kunimoto K, Sakaguchi T, Hatamura I, Shigematsu T 2009 Regulation of fibroblast growth factor 23 production in bone in uremic rats. Nephron Physiol **111**(4)**:**p59-66.
21. London GM, Marchais SJ, Guerin AP, Boutouyrie P, Metivier F, de Vernejoul MC 2008 Association of bone activity, calcium load, aortic stiffness, and calcifications in ESRD. J Am Soc Nephrol **19**(9)**:**1827-1835.
22. Mathew S, Tustison KS, Sugatani T, Chaudhary LR, Rifas L, Hruska KA 2008 The Mechanism of Phosphorus as a Cardiovascular Risk Factor in CKD. J Am Soc Nephrol **19**(6)**:**1092-1105.
23. Cannata-Andia JB, Rodriguez-Garcia M, Carrillo-Lopez N, Naves-Diaz M, Diaz-Lopez B 2006 Vascular calcifications: pathogenesis, management, and impact on clinical outcomes. J Am Soc Nephrol **17**(12 Suppl 3)**:**S267-273.
24. Huttunen MM, Tillman I, Viljakainen HT, Tuukkanen J, Peng Z, Pekkinen M, Lamberg-Allardt CJ 2007 High dietary phosphate intake reduces bone strength in the growing rat skeleton. J Bone Miner Res **22**(1)**:**83-92.
25. Almaden Y, Hernandez A, Torregrosa V, Canalejo A, Sabate L, Fernandez Cruz L, Campistol JM, Torres A, Rodriguez M 1998 High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue in vitro. J Am Soc Nephrol **9**(10)**:**1845-1852.
26. Silver J 2000 Molecular mechanisms of secondary hyperparathyroidism. Nephrol Dial Transplant **15 Suppl 5:**2-7.
27. Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, Smith K, Lee H, Thadhani R, Juppner H, Wolf M 2008 Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med **359**(6)**:**584-592.
28. Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM 2004 Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol **15**(8)**:**2208-2218.
29. Santamaria I, Alvarez-Hernandez D, Cannata-Andia JB 2005 Genetics and molecular disorders in severe secondary hyperparathyroidism: lessons from rna and microarray studies. J Nephrol **18**(4)**:**469-473.
30. Santamaria I, Alvarez-Hernandez D, Jofre R, Polo JR, Menarguez J, Cannata-Andia JB 2005 Progression of secondary hyperparathyroidism involves deregulation of genes related to DNA and RNA stability. Kidney Int **67**(6)**:**2267-2279.
31. Zhang M, Zhang L, Zou J, Yao C, Xiao H, Liu Q, Wang J, Wang D, Wang C, Guo Z 2009 Evaluating reproducibility of differential expression discoveries in microarray studies by considering correlated molecular changes. Bioinformatics **25**(13)**:**1662-1668.
32. Afonso S, Santamaria I, Guinsburg ME, Gomez AO, Miranda JL, Jofre R, Menarguez J, Cannata-Andia J, Cigudosa JC 2003 Chromosomal aberrations, the consequence of

refractory hyperparathyroidism: its relationship with biochemical parameters. Kidney Int Suppl (85):S32-38.
33. Canadillas S, Canalejo A, Santamaria R, Rodriguez ME, Estepa JC, Martin-Malo A, Bravo J, Ramos B, Aguilera-Tejero E, Rodriguez M, Almaden Y 2005 Calcium-sensing receptor expression and parathyroid hormone secretion in hyperplastic parathyroid glands from humans. J Am Soc Nephrol **16**(7):2190-2197.
34. Ritter CS, Finch JL, Slatopolsky EA, Brown AJ 2001 Parathyroid hyperplasia in uremic rats precedes down-regulation of the calcium receptor. Kidney Int **60**(5):1737-1744.
35. Komaba H, Goto S, Fujii H, Hamada Y, Kobayashi A, Shibuya K, Tominaga Y, Otsuki N, Nibu K, Nakagawa K, Tsugawa N, Okano T, Kitazawa R, Fukagawa M 2010 Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients. Kidney Int **77**(3):232-238.
36. Canalejo R, Canalejo A, Martinez-Moreno JM, Rodriguez-Ortiz ME, Estepa JC, Mendoza FJ, Munoz-Castaneda JR, Shalhoub V, Almaden Y, Rodriguez M 2010 FGF23 Fails to Inhibit Uremic Parathyroid Glands. J Am Soc Nephrol.
37. Hofman-Bang J, Martuseviciene G, Santini MA, Olgaard K, Lewin E 2010 Increased parathyroid expression of klotho in uremic rats. Kidney Int **78**(11):1119-1127.
38. Stubbs JR, Liu S, Tang W, Zhou J, Wang Y, Yao X, Quarles LD 2007 Role of hyperphosphatemia and 1,25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. J Am Soc Nephrol **18**(7):2116-2124.
39. Kuro OM 2011 Phosphate and Klotho. Kidney Int Suppl (121):S20-23.
40. Patterson KI, Brummer T, O'Brien PM, Daly RJ 2009 Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem J **418**(3):475-489.
41. Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM 1996 Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. Embo J **15**(14):3621-3632.
42. Shin DY, Ishibashi T, Choi TS, Chung E, Chung IY, Aaronson SA, Bottaro DP 1997 A novel human ERK phosphatase regulates H-ras and v-raf signal transduction. Oncogene **14**(22):2633-2639.
43. Owens DM, Keyse SM 2007 Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene **26**(22):3203-3213.
44. Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA, McCubrey JA 2003 Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia **17**(7):1263-1293.
45. Canadillas S, Canalejo R, Rodriguez-Ortiz ME, Martinez-Moreno JM, Estepa JC, Zafra R, Perez J, Munoz-Castaneda JR, Canalejo A, Rodriguez M, Almaden Y 2010 The up-regulation of the parathyroid VDR expression by extracellular calcium is mediated by the ERK1/2-MAPK signaling pathway. Am J Physiol Renal Physiol.
46. Parisi E, Almaden Y, Ibarz M, Panizo S, Cardus A, Rodriguez M, Fernandez E, Valdivielso JM 2009 N-methyl-D-aspartate receptors are expressed in rat parathyroid gland and regulate PTH secretion. Am J Physiol Renal Physiol **296**(6):F1291-1296.
47. Zhang Z, Kobayashi S, Borczuk AC, Leidner RS, Laframboise T, Levine AD, Halmos B 2010 Dual specificity phosphatase 6 (DUSP6) is an ETS-regulated negative feedback mediator of oncogenic ERK signaling in lung cancer cells. Carcinogenesis **31**(4):577-586.
48. Maillet M, Purcell NH, Sargent MA, York AJ, Bueno OF, Molkentin JD 2008 DUSP6 (MKP3) null mice show enhanced ERK1/2 phosphorylation at baseline and increased

myocyte proliferation in the heart affecting disease susceptibility. J Biol Chem **283**(45)**:**31246-31255.
49. Ekerot M, Stavridis MP, Delavaine L, Mitchell MP, Staples C, Owens DM, Keenan ID, Dickinson RJ, Storey KG, Keyse SM 2008 Negative-feedback regulation of FGF signalling by DUSP6/MKP-3 is driven by ERK1/2 and mediated by Ets factor binding to a conserved site within the DUSP6/MKP-3 gene promoter. Biochem J **412**(2)**:**287-298.
50. Gonzalez-Suarez I, Alvarez-Hernandez D, Carrillo-Lopez N, Naves-Diaz M, Luis Fernandez-Martin J, Cannata-Andia JB 2005 Aluminum posttranscriptional regulation of parathyroid hormone synthesis: a role for the calcium-sensing receptor. Kidney Int **68**(6)**:**2484-2496.
51. Ueda K, Arakawa H, Nakamura Y 2003 Dual-specificity phosphatase 5 (DUSP5) as a direct transcriptional target of tumor suppressor p53. Oncogene **22**(36)**:**5586-5591.

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LEGENDS

Figure 1

Mean ± standard deviation of A) serum PTH, B) serum phosphorus, C) serum calcium, D) serum FGF23 from the Reference, Moderate (MOD sHPT), Moderate-Severe (MOD/SEV sHPT) and Very Severe sHPT (Very SEV sHPT) groups.

a= $p<0.01$ compared to Reference group.

b= $p<0.05$ compared to Moderate sHPT group.

c= $p<0.05$ compared to Moderate sHPT group.

Figure 2

Graphical representation of the Pearson correlations between A) serum PTH and FGF23, B) serum FGF23 and phosphorus, C) serum PTH and creatinine, and D) serum PTH and urea. Units are mg/dL for creatinine and urea, and pg/mL for PTH and FGF23.

Figure 3

Hierarchical clusters of all groups using only those genes related to mineral metabolism. MOD = Moderate sHPT, MOD/SEV = Moderate-Severe sHPT and Very SEV = Very Severe sHPT.

Figure 4

Parathyroid gland Dusp 6 and Dusp 5 mRNA levels (RT-qPCR). Data represent the mean of relative expression from the Reference, Moderate (MOD sHPT), Moderate-Severe (MOD/SEV sHPT) and Very Severe sHPT (Very SEV sHPT) groups.

Mean ± standard deviation values are shown. Reference group was considered as baseline.

# $p<0.05$ when comparing *vs.* Moderate sHPT group. * $p<0.05$ when comparing *vs.* Moderate-Severe group.

Figure 5

Parathyroid gland PTH, CaSR, VDR, and Klotho mRNA levels measured by RT-qPCR in the Moderate (MOD sHPT), Moderate-Severe (MOD/SEV sHPT), Very Severe sHPT (Very SEV sHPT), and Reference group, which was given a reference value of 1. Data represent the mean of relative expression from the subgroups within the three groups. Mean ± standard deviation values are shown.

* $p<0.05$ when comparing *vs.* Moderate sHPT group.

Figure 6

Parathyroid gland total ERK 1/2 and p ERK 1/2 protein staining.

A) Representative immunohistochemistry images from the Reference, Moderate (MOD sHPT), Moderate-Severe (MOD/SEV sHPT), and Very Severe sHPT (Very SEV sHPT) groups.

B) Quantification of the levels of total ERK and pERK in parathyroid glands. Relative units (R.U.) are cells/mm^2. Reference group values for ERK and pERK are 197 ±10.7 and 213 ± 4.69 respectively. Mean ± standard deviation values are shown.

* p<0.05 when comparing Very Severe *vs.* Moderate sHPT groups. ** p<0.05 when comparing Moderate sHPT *vs.* Reference groups.

probe set	gene	Accession	fold change	P value
1372208_at	protein phosphatase 1, regulatory (inhibitor) subunit 1B	AA942959	-15,51	0,002337
1370124_at	metallothionein 3	NM_053968	-14,73	0,000024
1378692_at	paired box gene 8	BF564165	-12,4	0,001508
1373631_at	RAP1, GTPase activating protein 1	BF284067	-12,29	0,007627
1374070_at	glutathione peroxidase 2 /// glutathione peroxidase 2	AA800587	-11,61	0,026228
1379390_at	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-a	AA891414	-10,29	0,011152
1367904_at	regulated endocrine-specific protein 18	NM_019278	-9,95	0,023551
1371048_at	forkhead box E1 (thyroid transcription factor 2) /// forkhead b	Y11321	-9,86	0,011032
1391194_at	sal-like 1 (Drosophila) (predicted)	BG377337	-9,83	0,012785
1370764_a_a	thyroglobulin	M35965	-9,78	0,001381
1373915_at	dystrophia myotonica-protein kinase (predicted)	AI044427	-9,4	0,00063
1370775_a_a	calcitonin/calcitonin-related polypeptide, alpha	M11597	-9,16	0,020795
1393335_at	EGF-like-domain, multiple 6	BF418373	-8,98	0,000275
1370973_at	sodium channel, voltage-gated, type VII, alpha	BF285019	-8,87	0,000088
1369784_at	thyroid peroxidase /// thyroid peroxidase	NM_019353	-8,78	0,001976
1389470_at	complement factor B /// complement factor B	AI639117	-8,72	0,002546
1371010_at	thyroid transcription factor 1 /// thyroid transcription factor 1	BF389361	-8,31	0,000371
1370384_a_a	prolactin receptor	M57668	-8,14	0,000106
1377353_a_a	tumor necrosis factor (ligand) superfamily, member 13	AA800814	-8,01	0,018282

1374775_at	antigen identified by monoclonal antibody Ki-67 (predicted)	AI714002	2,15	0,03131
1369156_at	fyn-related kinase	NM_024368	2,28	0,023371
1373658_at	Rac GTPase-activating protein 1 (predicted) /// Rac GTPase	AI409259	2,3	0,008934
1383447_at	ets variant gene 5 (ets-related molecule) (predicted)	AI101323	2,35	0,031435
1371875_at	mannosidase, beta A, lysosomal	BM388852	2,46	0,007069
1380775_at	M-phase phosphoprotein 1 (predicted)	BE110723	2,46	0,011388
1382122_at	Ets variant gene 5 (ets-related molecule) (predicted)	BE113124	2,6	0,008857
1370391_at	cellular retinoic acid binding protein 2	U23407	2,71	0,012111
1388253_at	Stearoyl-Coenzyme A desaturase 2	M15114	2,79	0,022646
1389403_at	bone morphogenetic protein 7 /// bone morphogenetic protei	AI013715	2,83	0,002196
1367776_at	cell division cycle 2 homolog A (S, pombe) /// cell division cy	NM_019296	2,86	0,039293
1389566_at	cyclin B2 /// cyclin B2	AW253821	2,86	0,007195
1388484_at	ubiquitin-conjugating enzyme	BI296084	2,99	0,009501
1369436_at	cholinergic receptor, nicotinic, alpha polypeptide 10	NM_022639	3,01	0,044713
1370449_at	purinergic receptor P2Y, G-protein coupled, 14	U76206	3,12	0,009019
1390918_at	GH regulated TBC protein 1	BE099056	3,19	0,015337
1369972_at	serine (or cysteine) peptidase inhibitor, clade B, member 5 //	NM_057108	3,27	0,033223
1378658_at	chloride channel calcium activated 6	BI292185	3,39	0,00023
1377006_at	Chaperonin subunit 6a (zeta)	AA875047	3,47	0,001908
1375788_at	Ribosomal protein L7	BM388719	3,52	0,000331
1368266_at	arginase 1 /// arginase 1	NM_017134	3,55	0,000472
1368124_at	dual specificity phosphatase 5	NM_133578	3,57	0,034395
1381533_at	Rho family GTPase 1	AI144754	3,68	0,03904
1382803_at	mitogen activated protein kinase kinase kinase kinase 1 (pre	AI237423	3,78	0,003315
1390797_at	lymphocyte cytosolic protein 2	BF282471	3,82	0,026496
1370090_at	lymphocyte cytosolic protein 2	NM_130421	3,89	0,003265
1369525_at	GATA binding protein 3	NM_133293	3,98	0,000253
1389408_at	ribonucleotide reductase M2 (mapped) /// ribonucleotide redi	BG379338	3,98	0,041589
1390358_at	calcium channel, voltage-dependent, alpha 2/delta 3 subunit	BF405996	6,3	0,028359
1370064_at	presenilin 2	AB004454	8,25	0,03509
1387024_at	dual specificity phosphatase 6	NM_053883	9,74	0,032946
1382778_at	Dual specificity phosphatase 6	AI231350	9,92	0,014481
1377064_at	dual specificity phosphatase 6	AI602811	11,72	0,031855

Table 2

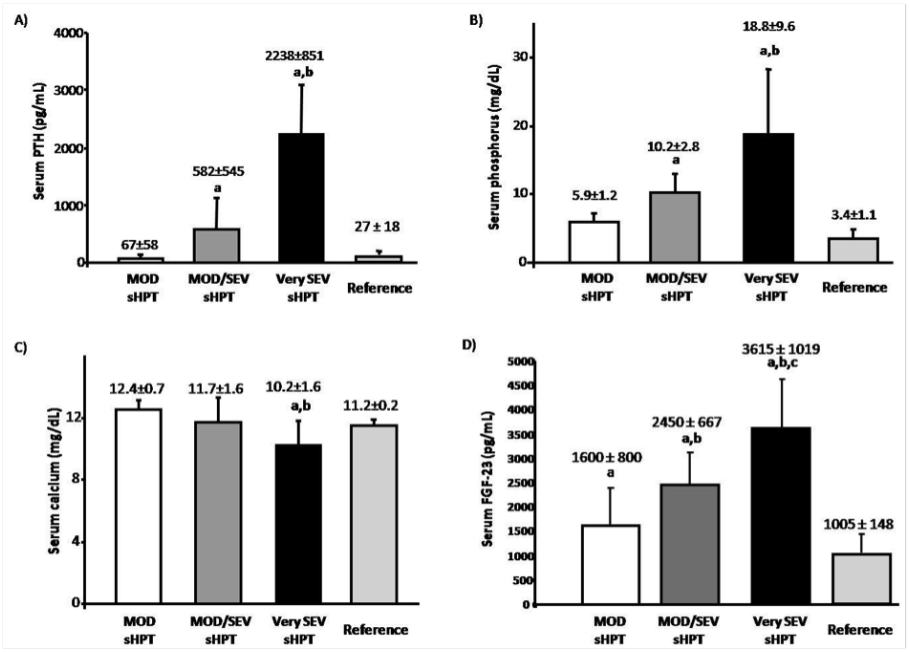
Gene expression dataset of the differentially expressed probes sorted by fold change, corresponding to the comparison of the Moderate-Severe *vs.* Moderate sHPT groups.

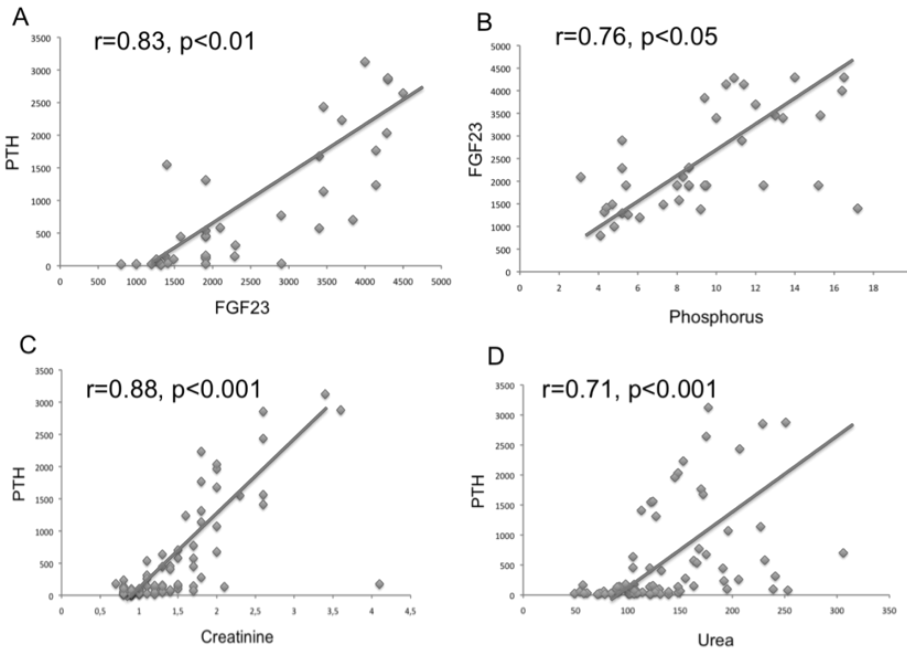
probe set	gene	Accession	fold change	P value
1382882_x_a	Carnitine palmitoyltransferase 1a, liver	AA963228	-5,42	0,020994
1369765_at	achaete-scute complex homolog-like 1 (Drosophila)	NM_022384	-3,72	0,017102
1368887_at	cadherin 22	NM_019161	-3,6	0,001011
1383165_at	Syncollin	BM390462	-3,54	0,006321
1370229_at	N-myc downstream regulated gene 4	BG666709	-2,9	0,036277
1387315_at	surfactant associated protein D	NM_012878	-2,83	0,002683
1370973_at	sodium channel, voltage-gated, type VII, alpha	BF285019	-2,8	0,006471
1390672_at	candidate mediator of the p53-dependent G2 arrest /// ca	BG381258	-2,73	0,039653
1387022_at	aldehyde dehydrogenase family 1, member A1 /// aldehyc	NM_022407	-2,65	0,026217
1368711_at	forkhead box A2	NM_012743	-2,64	0,028757
1367904_at	regulated endocrine-specific protein 18	NM_019278	-2,61	0,036564
1373780_at	tetraspanin 1	BE349699	-2,51	0,031956
1383413_at	Gup1, glycerol uptake/transporter homolog (yeast)	AW531481	-2,41	0,005787
1372208_at	protein phosphatase 1, regulatory (inhibitor) subunit 1B	AA942959	-2,38	0,032622
1370372_at	RASD family, member 2	AF134409	-2,35	0,00424
1389688_at	Leprecan-like 1	BM384373	-2,24	0,011393
1370963_at	growth arrest specific 7	AJ131902	-2,23	0,002619
1372016_at	growth arrest and DNA-damage-inducible 45 beta /// grov	BI287978	-2,21	0,028853
1370815_at	neurofilament, heavy polypeptide	AF031879	-2,21	0,013385
1371112_at	ret proto-oncogene /// ret proto-oncogene	AJ299017	-2,18	0,004354
1398269_at	netrin 1	NM_053731	-2,11	0,017218
1391559_at	TLC domain containing 1	AW525904	-2,11	0,048893
1387145_at	gap junction membrane channel protein beta 1 /// gap jun	NM_017251	-2,09	0,049952
1384515_at	hairy/enhancer-of-split related with YRPW motif 2	BF388057	-2,09	0,049809
1377783_at	angiopoietin 4 (predicted)	BI294141	-2,08	0,011101
1373631_at	RAP1, GTPase activating protein 1	BF284067	-2,07	0,043726
1374630_at	chloride intracellular channel 3	AI234249	-2,05	0,034222
1375951_at	thrombomodulin	AA818521	-2,03	0,024088
1382659_at	mannose receptor, C type 2 (predicted)	BF289229	-2,02	0,045442
1373991_at	Potassium inwardly-rectifying channel	AI411366	-2,01	0,045591
1378658_at	chloride channel calcium activated 6	BI292185	3,15	0,012157
1382778_at	Dual specificity phosphatase 6	AI231350	3,8	0,033501

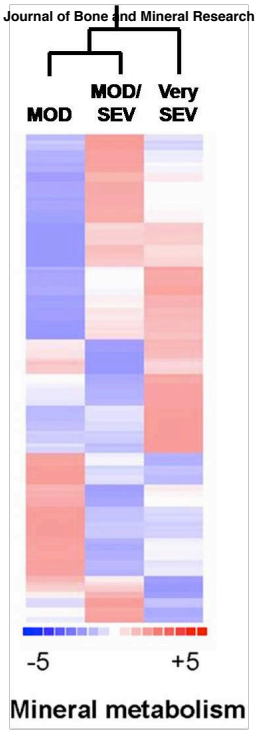
Table 3

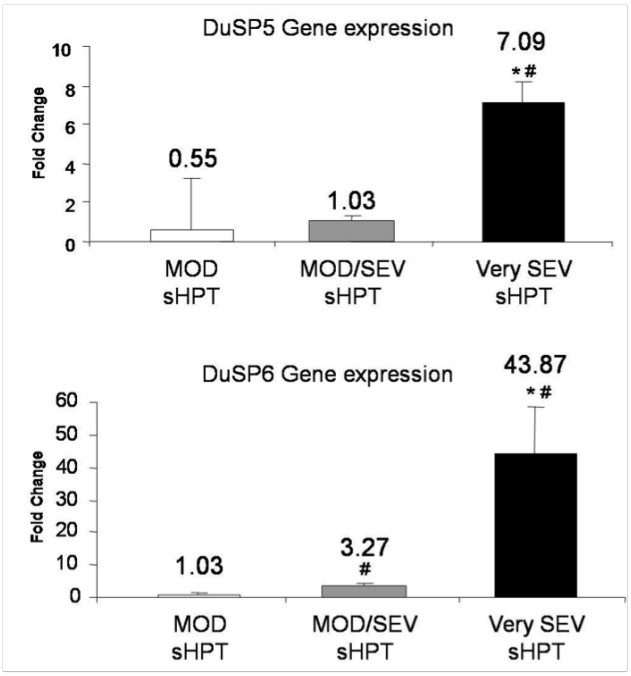
Gene expression dataset of the differentially expressed probes sorted by fold change, corresponding to the comparison of the Very Severe *vs.* Moderate-Severe sHPT groups.

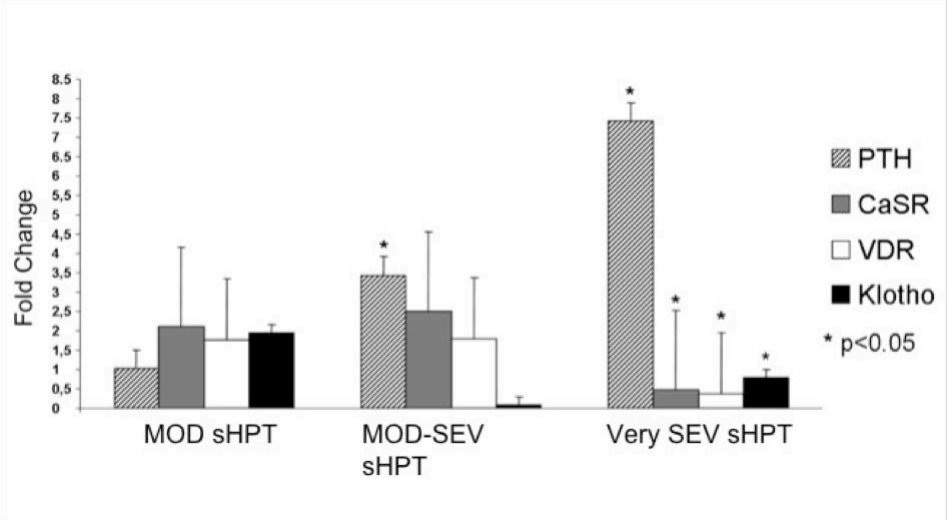
probe set	gene	Accession	fold change	P value
1370775_a_a	calcitonin/calcitonin-related polypeptide, alpha	M11597	-4,6	0,008461
1368080_at	response gene to complement 32	NM_054008	-3,88	0,045345
1383695_at	vasoactive intestinal peptide receptor 1	BI301509	-2,7	0,028991
1386921_at	carboxypeptidase E	NM_013128	-2,63	0,018966
1370963_at	growth arrest specific 7	AJ131902	-2,46	0,023424
1370449_at	purinergic receptor P2Y, G-protein coupled, 14	U76206	2,07	0,006677
1368266_at	arginase 1 /// arginase 1	NM_017134	2,1	0,018493
1377064_at	dual specificity phosphatase 6	AI602811	2,88	0,044266
1382778_at	Dual specificity phosphatase 6	AI231350	2,95	0,016609
1387024_at	dual specificity phosphatase 6	NM_053883	3,14	0,042671

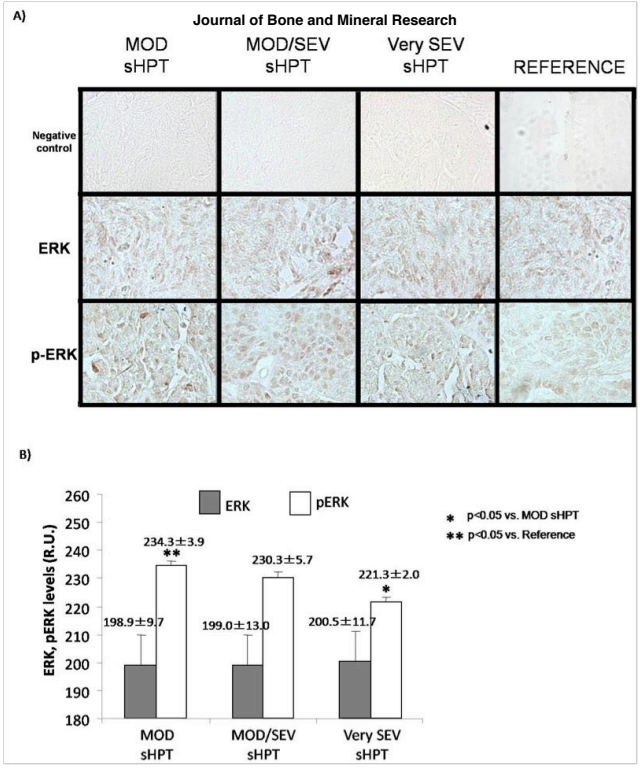

A)
Serum PTH (pg/mL)
67±58
582±545
a
2238±851
a,b
27 ± 18
MOD sHPT
MOD/SEV sHPT
Very SEV sHPT
Reference
B)
Serum phosphorus (mg/dL)
5.9±1.2
10.2±2.8
a
18.8±9.6
a,b
3.4±1.1
C)
Serum calcium (mg/dL)
12.4±0.7
11.7±1.6
10.2±1.6
a,b
11.2±0.2
D)
Serum FGF-23 (pg/mL)
1600 ± 800
a
2450 ± 667
a,b
3615 ± 1019
a,b,c
1005 ± 148

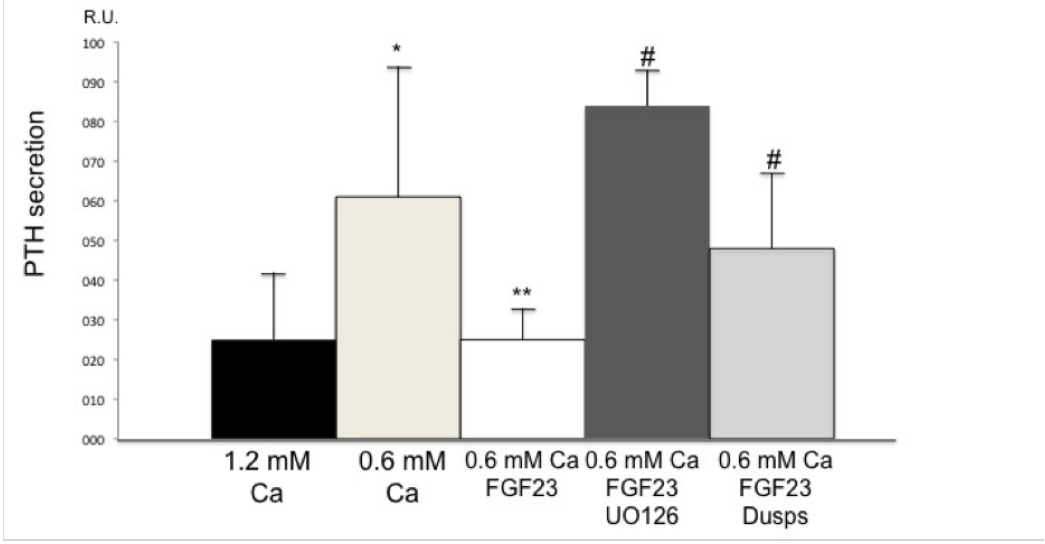

A
r=0.83, p<0.01
PTH
FGF23
B
r=0.76, p<0.05
FGF23
Phosphorus
C
r=0.88, p<0.001
PTH
Creatinine
D
r=0.71, p<0.001
PTH
Urea


MOD
MOD/ SEV
Very SEV
-5
+5
Mineral metabolism


DuSP5 Gene expression
Fold Change
10
8
6
4
2
0
0.55
1.03
7.09
*#
MOD sHPT
MOD/SEV sHPT
Very SEV sHPT
DuSP6 Gene expression
Fold Change
60
50
40
30
20
10
0
1.03
3.27
#
43.87
*#
MOD sHPT
MOD/SEV sHPT
Very SEV sHPT


Fold Change
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
MOD sHPT
MOD-SEV sHPT
Very SEV sHPT
PTH
CaSR
VDR
Klotho
* p<0.05


A)
MOD sHPT
MOD/SEV sHPT
Very SEV sHPT
REFERENCE
Negative control
ERK
p-ERK
B)
ERK
pERK
* p<0.05 vs. MOD sHPT
** p<0.05 vs. Reference
ERK, pERK levels (R.U.)
260
250
240
230
220
210
200
190
180
198.9±9.7
234.3±3.9
199.0±13.0
230.3±5.7
200.5±11.7
221.3±2.0
MOD sHPT
MOD/SEV sHPT
Very SEV sHPT


R.U.
PTH secretion
100
090
080
070
060
050
040
030
020
010
000
*
**
#
#
1.2 mM Ca
0.6 mM Ca
0.6 mM Ca FGF23
0.6 mM Ca FGF23 UO126
0.6 mM Ca FGF23 Dusps

Publicación 3: Vascular calcification in patients with chronic kidney disease: types, clinical impact and pathogenesis.

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Vascular Calcification in Patients with Chronic Kidney Disease: Types, Clinical Impact and Pathogenesis

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Key Words
Vascular calcification · Chronic kidney disease · Secondary hyperparathyroidism · Bone loss vascular-bone links

Abstract
Vascular calcification plays a major role in cardiovascular disease, which is one of the main causes of mortality in chronic kidney disease patients. Vascular calcification is determined by prevalent traditional and uraemia-related (non-traditional) risk factors. It occurs mainly in the arteries, which are classified into three types according to their size and structural characteristics. In addition, vascular calcification has been associated with bone loss and fractures in chronic kidney disease patients and the general population, stressing the fact that both disorders can share pathogenetic pathways. The strategies to control vascular calcification involve several measures, chief among them the control of hyperphosphataemia. Furthermore, it has been recently described that strategies that reduce bone resorption and increase bone mineralization may decrease the risk of vascular calcifications; however, this approach still remains controversial. The mechanisms involved in vascular calcification are complex and not yet fully understood. Phosphorus plays a major role, while other factors related to bone formation have been recently identified.

Vascular Calcification in Chronic Kidney Disease: Types and Risk Factors

In recent years, the main cause of morbidity and mortality in chronic kidney disease (CKD) patients has been judged to be cardiovascular disease. Recent studies suggest that vascular calcification plays a major role in cardiovascular disease in dialysis patients. Following the recommendations of the Kidney Disease: Improving Global Outcomes Foundation [1, 2], kidney damage is defined as structural or functional kidney abnormalities, which are accompanied by other abnormalities such as vascular calcification, bone loss and fractures, parathyroid dysfunction and several biochemical abnormalities, especially in serum PTH, calcium, phosphorus and alkaline phosphatase. All together are today known as CKD bone and mineral disorders [2].

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Clinical Impact of Vascular Calcification

As previously mentioned, CKD patients exhibit a very high percentage of vascular calcifications [13–16], leading to cardiovascular disease, decreased life expectancy and mortality [17] even in the earliest phases of CKD. Russo et al. [18] showed that 40% of patients with CKD (mean glomerular filtration rate 33 ml/min/1.73 m^2) suffered from calcification of the coronary arteries compared with 13% of controls within a similar age range but with normal renal function. Kramer et al. [19] found a significant positive association between the presence of coronary calcifications and renal failure, an association which increased dramatically in CKD diabetic patients.

CKD patients develop vascular calcification even at early ages [16] and almost in all localizations in a greater proportion than the general population. They are frequently localized in high-calibre arteries, such as the aorta (79%), medium arteries (70.5%), including coronary arteries [11], and also in small calibre arteries (20.2%) [20]. These differences may reflect the heterogeneity of the three categories of arteries studied, which also imply relevant functional changes in the vasculature [21]. The calcification of the cardiac valves involves also a high risk of cardiovascular dysfunction [22] in both general and haemodialysis (HD) populations.

In a recent study, prevalent aortic calcifications were significantly higher in HD patients (79%) than in a random-based general population of the same age, sex and region (37.5%) [20]. Other reports have shown similar results in HD patients [23] in which age was positively associated with vascular calcification in large and medium-calibre arteries. Time on HD and total time on renal replacement therapy have been positively associated with vascular calcification, particularly in medium-calibre arteries: each new year on renal replacement therapy increased the risk of having vascular calcifications by approximately 15% [24]. Therefore, the time spent on dialysis is an important risk factor for medial and intimal arterial calcifications in CKD patients [25].

In addition to information available for the HD patients, the Framingham study has shown that vascular calcification is also an independent predictor of vascular morbidity and mortality in the general population [26].

Links between Vascular Calcification and Bone Disorders

Vascular calcification, bone loss and fragility fractures are very common disorders associated with aging, both in patients with CKD [16, 27, 28] and in the general population [29–32]. In recent years, several epidemiological studies have drawn attention to the relationship between vascular calcification and bone health [31–34]. Even though the pathogenetic factors linking vascular calcifications and bone fragility are not fully understood, recent studies have shown that vascular calcification in some localizations were associated with an increased risk of fragility fractures in both general and HD populations [20, 35, 36].

Table 2. Strategies to reduce vascular calcification

Balanced diet, with low salt and saturated fats intake
Regular physical exercise
Smoking abstinence
Low alcohol intake
Control of serum phosphorus and calcium levels
Use of physiologic doses of vitamin D
Treatment of secondary hyperparathyroidism
Lipid-lowering therapy with statins
Treatment of hypertension
Rigorous control of diabetes mellitus
Caution with acenocumarol or warfarin treatments

Strategies to Reduce Vascular Calcifications

Any strategy designed to reduce the impact of vascular calcifications has to begin with primary prevention measures to control cardiovascular risk factors. In the particular case of CKD, it is imperative to avoid further kidney damage. In this respect, it is crucial to promote a healthy lifestyle, with a balanced diet, regular physical exercise, smoking abstinence and a low alcohol intake. Once vascular calcifications appear, secondary prevention must aim to reduce their complications, intensifying previous measures and initiating the appropriate drug therapy. Particular aspects of the pharmacological approach are discussed below.

Theoretically, any kind of intervention aiming to reduce vascular calcification should curtail the influence of factors that promote calcifications and/or augment the effects of factors that may inhibit calcifications [37]. Most strategies to reduce vascular calcifications have focused on the most common modifiable risk factors such as hyperphosphataemia, hypercalcaemia, the CaxP product, hyperparathyroidism, smoking, dyslipidaemia or hypertension (table 2).

Table 1. Risk factors associated to vascular calcification in CKD patients

Traditional risk factors
Hypertension
Dyslipidaemia
Diabetes mellitus
Smoking
Older age
Family history of premature coronary heart disease
Uraemia-related and non-traditional risk factors
Time on dialysis
Hyperphosphatemia
High calcium-phosphorus product
Hyperparathyroidism and hypoparathyroidism
High dosage of vitamin D metabolites
Low fetuin-A
Anaemia
Poor nutrition (low albumin)
Chronic inflammation (CRP, IL-1, IL-6, TNF-α)
Hyperhomocysteinaemia
Advanced glycated end-products

The predisposition of patients with CKD towards developing vascular calcification was mentioned for the first time back in the 19th century; since then, many studies have addressed this important aspect. Vascular calcification occurs mainly in the arteries, which can be classified into three types according to their size and structure: elastic or large-calibre arteries, muscular or medium-calibre arteries and small-calibre arteries.

Elastic or large-calibre arteries show a relatively thin wall in proportion to their diameter. The tunica media is rather thick and contains more elastic fibres than smooth muscle; the adventitia tends to be fairly thin. Through the elastic arteries, blood is conducted from the heart to the distribution arteries. Large vessels like the aorta, subclavia and common carotid arteries are included in this group.

Muscular or medium-calibre arteries have a tunica media which contains a great proportion of smooth muscle fibres; they are capable of withstanding further vasodilatation and vasoconstriction to adjust the volume of blood to accommodate perfusion requirements. Medium-calibre arteries include the axillary, brachial, radial, coronary, femoral and tibial arteries.

Finally, small-calibre arteries are less than 2 mm thick and their tunica media contains only smooth muscle fibres. In these vessels, luminal size variations, caused by vasoconstriction and vasodilatation of smooth muscle cells, are responsible for regulating the local blood flow and perfusion pressure. This group includes the palmar arch and the digital arteries, among others.

The classical description of arterial calcification specifies it may occur in two locations: the intima and the media layers [3]. Nevertheless, this classical concept is not fully accepted by all authors [4, 5].

Intimal calcification begins and progresses under the influence of both genetic and lifestyle circumstances throughout a person's lifetime. Intimal calcification is associated with a sequence of atherosclerotic events that include endothelial dysfunction, intimal edema, lipid cell formation and the migration of leukocytes and macrophages that can in turn cause a plaque rupture, thus leading to the formation of the thrombus [6]. Atherosclerotic lesions have a patchy distribution along the length of the artery and may cause local stenoses and occlusions. Furthermore, it is characterized by chronic arterial inflammation exacerbated by alterations in lipid metabolism [7] and other well-characterized risk factors, including hypertension, diabetes, dyslipidemia [8, 9], obesity, smoking and a family history of premature coronary heart disease.

Calcification of the media occurs in the elastic lamina of large-calibre and medium- to small-size arteries; it seems to be independent of atherosclerosis, but both can coexist. This type of calcification was known initially as Monckeberg sclerosis and it can be seen radiographically as railroads [10]. It typically affects arteries such as visceral abdominal, thyroid and lung [10], but it is also extremely common in the aorta, limb and femoral arteries. Calcification of the media increases linearly with age. It is frequently observed in patients with metabolic abnormalities such as hypervitaminosis D, CKD and diabetes [11].

Table 1 summarizes the most prevalent traditional, uraemia-related and non-traditional risk factors for vascular calcification in CKD patients. Like in the general population, traditional cardiovascular risk factors, present in a large proportion of patients with CKD, are responsible to a great extent for the progression of vascular calcifications. Among non-traditional cardiovascular risk factors, including uraemia-related risk factors, hyperphosphatemia and the dialysis vintage are the risk factors more strongly associated with increased vascular calcification and mortality [12]. Elevated CRP and IL-6, as expression of chronic inflammation, have also been frequently associated with vascular calcification.

Control of Hyperphosphataemia, Hypercalcaemia and CaxP Product

Disturbances in serum phosphorus, calcium and the calcium-phosphorus product are frequently seen in CKD patients and are implicated in the promotion of vascular calcification as well as in an increased death risk [37]. Because dietary restriction of phosphorus and intermittent dialysis are not usually effective in controlling serum phosphorus, most patients with CKD stage 5 show a high prevalence of hyperphosphataemia with its known implications in the pathogenesis of secondary hyperparathyroidism, cardiovascular alterations and mortality. As mentioned before, in vivo and in vitro studies shed light on the role of phosphorus as a promoter of vascular calcification, demonstrating that the control of phosphorus should be a priority in clinical practice.

Calcium-based phosphate binders such as calcium acetate and calcium carbonate have replaced aluminium hydroxide as the most widely prescribed phosphate binders. The possible negative role of calcium overload from these binders on the progression of vascular calcifications has led to the progressive but not total reduction of calcium- and aluminium-based phosphate binders in favour of new calcium- and aluminium-free phosphate binders (sevelamer hydrochloride, sevelamer carbonate and lanthanum carbonate). These new compounds have been shown to have reduced hypercalcaemic adverse events in comparison to calcium-based phosphate binders [38].

An experimental study demonstrated that treatment with sevelamer in rats decreased renal calcification compared to rats that received calcium carbonate and untreated rats [39]. In addition, a clinical trial showed that sevelamer reduced the progression of both coronary and aortic calcifications compared to calcium carbonate [40]. However, the mechanism of the beneficial effect of sevelamer on the progression of calcification is still not fully understood. One possible mechanism is based on the reduction of the calcium load; however, reduced vascular calcification may also result from reductions in total and LDL cholesterol, which occur during treatment with sevelamer [38]. In fact, two recent studies have shed doubts on the role of phosphate binders in the progression of calcification [41, 42].

Control of Secondary Hyperparathyroidism: Vitamin D and Calcimimetics

The use of vitamin D metabolites is a challenging subject that still remains controversial. The current treatment of secondary hyperparathyroidism in dialysis patients includes suppression of PTH with supraphysiologic doses of vitamin D or its analogues. Although it is widely known that a high dosage of vitamin D metabolites favours the onset and progression of vascular calcifications, several studies have paradoxically demonstrated a long-term beneficial effect of vitamin D on vascular calcifications. Low vitamin D status is associated with a higher prevalence of vascular calcifications, bone and mineral disturbances, susceptibility to some infections, higher risk of autoimmune diseases, some malignancies and many other complications [43].

Observational studies in patients on HD and in the general population have also demonstrated a lower morbidity and a cardiovascular survival advantage in patients who are treated with vitamin D receptor activators [44, 45].

A major breakthrough in the management of calcium and phosphate metabolism in dialysis patients was achieved recently with the introduction of calcimimetics. These compounds were the first agents introduced to lower PTH with advantageous effects on serum calcium and phosphate. It has been demonstrated experimentally that the calcimimetic R568 reduces aortic calcifications and mortality in rats in which aortic calcifications were induced using a high dose of calcitriol [46]. Moreover, another experimental study showed that calcimimetics may even favour the regression of vascular calcification [47].

Control of Dyslipidemia

Dyslipidemia, particularly increased LDL cholesterol, has been implicated in the progression of vascular calcifications. In addition, in the general population, the beneficial effect of lowering LDL cholesterol levels on the progression of calcification has been reported by several groups [48, 49]. As mentioned previously, patients who were treated with sevelamer showed a significant decrease in LDL cholesterol levels [40], which may explain the beneficial effects in the progression of cardiovascular calcification. It is known that the rapid progression of coronary arterial calcification in HD patients is associated with higher triglycerides and lower HDL cholesterol levels [50].

Control of Blood Pressure

Hypertension is a modifiable risk factor for vascular calcifications in both the general population and CKD patients. Several studies in patients with end-stage renal disease and essential hypertension have shown that arterial stiffening is an independent predictor of mortality.

As arteries become stiffer, the pulse wave velocity increases and it is responsible for a rapid return of wave reflections from the periphery to the ascending aorta during systole, which causes an abnormal rise of aortic systolic blood pressure with decreased diastolic blood pressure and high pulse pressure. Increased wave reflections and high pulse pressure are independent risk factors for mortality in end-stage renal disease patients [51].

Diabetes

Diabetes is a disease that is known to be complicated by heterogeneous metabolic risk factors, such as hyperglycemia, dyslipidemia, insulin resistance, glycation, oxidative and carbonic stress, and tissue hypoxia. In the non-uremic population, vascular calcification occurs more frequently in diabetics. In CKD patients, vascular calcification in diabetics has been reported to be more prevalent and more advanced than in non-diabetics [52]. Several studies emphasize the importance of glycaemic control in the prevention of the development and progression of vascular calcification in diabetic CKD patients [53].

Factors That Decrease Vascular Calcification

Although vascular calcification is very common in patients with CKD, it is absent in a non-negligible percentage of patients (close to 20%) despite a similar exposure to the known factors that promote calcification [54]. As mentioned before, inhibitors of the precipitation of calcium and phosphate must be playing a major role in preventing extra-osseous calcification. Unfortunately, the therapeutic potential of these inhibitors of calcification has not been explored in clinical trials. Because MGP requires vitamin K for γ-carboxylation, an acquired vitamin K deficiency by the use of warfarin or acenocumarol may predispose towards vascular calcification [55].

Clinical and experimental studies have consistently established a positive association between arterial calcification and bone resorption [56, 57]. Consequently, it can be hypothesized that treatment strategies that simultaneously reduce bone resorption and increase bone mineralization may decrease the risk of vascular calcifications.

Bisphosphonates, used as standard therapy for osteoporosis, inhibit the experimentally induced vascular calcification, offering perspectives for the treatment of vascular calcification. The exact mechanism by which bisphosphonates inhibit arterial calcification is not entirely understood. One possibility is an indirect effect through inhibition of bone resorption, which would reduce the efflux of calcium and phosphate out of the bone, resulting in a decreased performance of the substrates required to form hydroxyapatite in the arterial wall [58].

Bisphosphonates have been demonstrated to reduce vascular calcification in experimental models [59], but also in CKD in a reduced group of HD patients [60]. Nevertheless, the use of bisphosphonates, particularly in CKD patients with underlying renal osteodystrophy, should be carefully considered as they are still in the research phase [58]. It has been recently described that in uremic rats treated with bisphosphonates, there was a strong correlation between inhibition of aortic calcification and bone mineralization, suggesting that bisphosphonates may not be able to prevent vascular calcification without inhibiting bone formation [61].

Even though new strategies may improve the management of vascular diseases and, more specifically, may have a positive impact on the high prevalence of vascular calcifications, the more effective approach is still that involving the best possible control of mineral and bone metabolism and inflammatory parameters [11].

We need more experimental, epidemiological and randomised clinical studies designed to ascertain the effects of the newly available bone-vascular active drugs on the bone and cardiovascular systems.

Molecular Aspects Related to Vascular Calcification

Until recent years, vascular calcification was considered the result of a simple precipitation of the circulating calcium and phosphate. However, the mechanisms by which the process of vascular calcification is produced are complex; it does not consist of a simple precipitation of calcium and phosphate, it involves active and modifiable processes that will be discussed later in this review. The final result is the formation of bone structures inside the artery wall [62, 63]. This regulated process involves several changes, such as the decrease in vascular calcification inhibitors [64], increase in vascular calcification promoters, formation of calcification vesicles [65], and, as a result, the induction of a cellular phenotypic change: from vascular smooth muscle cells (VSMCs) to bone-like cells [66, 67]. Interestingly enough and in line with several epidemiological studies, the increase in bone-like cells in the vessels has been reported to be associated to a decrease in bone mass and mineralization [11, 35, 68].

Thanks to the advances in molecular biology, a great number of mechanisms have been extensively investigated, and several inhibitors and promoters of vascular cal-

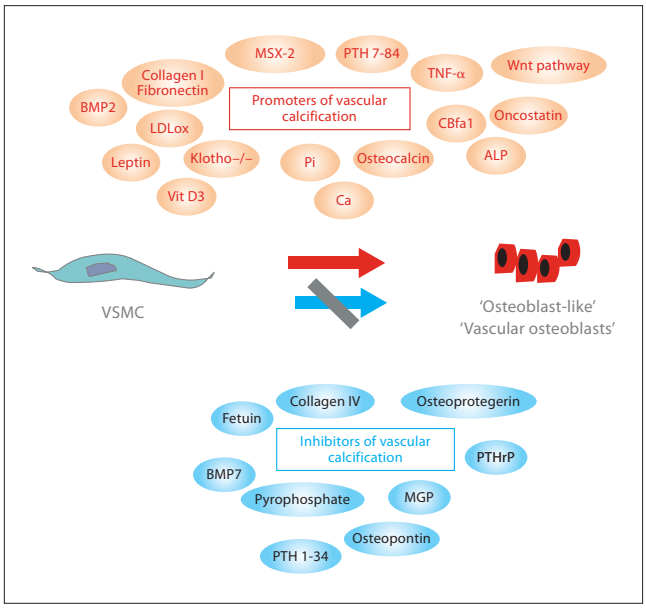

Fig. 1. Promoters and inhibitors of vascular calcification.

cification have been described (fig. 1). Among the latter, phosphorus and calcium play a relevant role. In humans and other mammals, serum concentrations of calcium and phosphate exceed the calcium-phosphate solubility product; thus, the likelihood of precipitation is high. Nevertheless, in both young and adult populations, intra-vessel precipitation is uncommon, clearly stressing the important role played by the vascular calcification inhibitors in preventing calcium/phosphate precipitation and deposition.

Promoters of Vascular Calcification: Phosphorus, Bone Morphogenetic Protein-Msx-2-Wnt Axis, Inflammation and Oxidative Stress

High serum phosphorus is the most important uremia-related, non-traditional risk factor associated with vascular calcification in CKD patients and the general population [17]. It is well known that high serum phosphorus levels stimulate parathyroid activity, decreasing the levels of calcium-sensing receptor and vitamin D receptor; it also lowers the activity of 1-α-hydroxylase, consequently decreasing serum calcitriol levels.

In addition, phosphorus is also capable of acting as a secondary intracellular messenger, activating several molecular pathways related to bone formation. It reaches the intracellular space via a specific Na-dependent channel called Pit-1 and exerts some interesting actions; in fact, the blockade of Pit-1 prevents vascular calcification [69].

In vitro experiments have demonstrated that elevated intracellular phosphate levels may directly increase an important bone-specific transcription factor core-binding factor α (Cbfa-1), resulting in the activation of several osteogenic pathways in the VSMCs, which leads to phenotypic changes of VSMCs into bone-like cells [70, 71].

Focusing on the downstream actions of Cbfa-1, it promotes the expression of osteocalcin and alkaline phosphatase in the vasculature. In addition, one of the most important families of proteins involved in mineralization and vascular calcification, the bone morphogenetic proteins (BMPs) are activated by Cbfa-1. The BMP family, especially the 2 and 4 members, have been described as potent promoters of vascular calcification, as they can re-

cruit other bone-related players such as Msx-2 and Wnt-related proteins [72–74]. Inflammation is also an important factor involved in the pathogenesis of vascular calcification development [75].

Oxidative stress has also been related to vascular calcification. VSMCs cultured with H_2O_2 developed calcification via stimulation of Cbfa-1 [76]. In addition, in vivo studies have shown that some antioxidants can prevent vascular calcification [77]. In agreement with these experimental analyses, clinical studies have shown that serum levels of oxidized LDL, advanced oxidation protein products and urine levels of F-2 isosprostanes (biomarkers of oxidative stress) may all be considered as risk factors for vascular and valvular calcification [25, 78].

Inhibitors of Vascular Calcification: Pyrophosphates, Fetuin A and Osteoprotegerin

Pyrophosphates (PPi) are located in the vascular matrix and they are supposed to preserve the aortic VSMC phenotype, thanks to the inhibition of calcium carbonate formation, hence inhibiting calcium phosphate crystal formation; PPi inhibits the change of VSMCs into bone-like cells [79, 80].

In serum, the most abundant inhibitors of vascular calcification are fetuin-A (α2-Heremans-Schmid glycoprotein), osteoprotegerin (OPG) and matrix-gla protein. Fetuin-A is a known inhibitor of osteogenesis [81], capable of inhibiting vascular calcification [82]. Fetuin-A knockout mice spontaneously develop widespread soft tissue calcification, including significant myocardial calcification, findings associated to the upregulation of the profibrotic factor TGF-β [83].

OPG inhibits osteoclast differentiation, modulating bone resorption through its action as a decoy receptor of RANKL. OPG-null mice develop early-onset osteoporosis and severe medial layer calcification [84], suggesting that OPG acts as an inhibitor of in vivo vascular calcification. OPG was shown to inhibit ALP activity in aortic tissue and prevent the progression of medial layer vascular calcification [85]. The importance of the OPG/RANKL axis in vascular calcification has been recently shown; the increase in vascular calcium content was parallel to an increase in the RANKL and BMP4 expression [86].

Other Players

Klotho, a co-receptor of fibroblast growth factor 23 (FGF-23), among other functions, has a phosphaturic function [87, 88]. The knockout mice for the Klotho gene showed accelerating aging with widespread ectopic calcification, including vascular calcifications. The mechanisms by which fibroblast growth factor 23/Klotho affect vascular calcification may involve phosphate excretion as well as vitamin D and PTH leading to vascular calcification and bone loss [89–92].

Advanced glycation end-products (AGEs) are chemical modifications of proteins and lipids that become non-enzymatically glycated after contact with carbohydrates [93]. The generation of AGEs is a continous in vivo process and their accumulation increases with aging and diseases, specially diabetes [94]. AGEs accumulate in the vessel wall and contribute to the development of atherosclerosis through the formation of cross-links between molecules in the basement membrane of the extracellular matrix, involving different cell-surface receptors, especially RAGE [95]. Furthermore, recent studies have shown a correlation between the accumulation of AGEs in bone and increased fracture risk, even with normal bone mineral density [96–98]. AGEs may induce abnormal cross-links in the collagen proteins causing bone fragility [99, 100]. Furthermore, they may decrease osteoblast function and increase osteoclast activity, alter the function of the osteoblasts and increase the activity of the osteoclasts [101].

In summary, vascular calcification, an established highly prevalent finding of CKD, known since the beginning of renal replacement therapy, has recently re-emerged as a key complication of CKD, and great efforts are currently underway to clarify the morphological, functional and molecular aspects of this disorder.

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References

1 Levey AS, Eckardt KU, Tsukamoto Y, Levin A, Coresh J, Rossert J, De Zeeuw D, Hostetter TH, Lameire N, Eknoyan G: Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2005;67:2089–2100.

2 Eknoyan G, Lameire N, Barsoum R, Eckardt KU, Levin A, Levin N, Locatelli F, MacLeod A, Vanholder R, Walker R, Wang H: The burden of kidney disease: improving global outcomes. Kidney Int 2004;66:1310–1314.

3 Amann K: Media calcification and intima calcification are distinct entities in chronic kidney disease. Clin J Am Soc Nephrol 2008; 3:1599–1605.
4 Micheletti RG, Fishbein GA, Currier JS, Singer EJ, Fishbein MC: Calcification of the internal elastic lamina of coronary arteries. Mod Pathol 2008;21:1019–1028.
5 McCullough PA, Agrawal V, Danielewicz E, Abela GS: Accelerated atherosclerotic calcification and monckeberg's sclerosis: a continuum of advanced vascular pathology in chronic kidney disease. Clin J Am Soc Nephrol 2008;3:1585–1598.
6 Ross R: Atherosclerosis – an inflammatory disease. N Engl J Med 1999;340:115–126.
7 Libby P: Inflammation in atherosclerosis. Nature 2002;420:868–874.
8 Sessa R, Cipriani P, Di Pietro M, Schiavoni G, Santino I, Del Piano M: Chlamydia pneumoniae and chronic diseases with a great impact on public health. Int J Immunopathol Pharmacol 2008;21:1041–1043.
9 Garcia-Lopez E, Carrero JJ, Suliman ME, Lindholm B, Stenvinkel P: Risk factors for cardiovascular disease in patients undergoing peritoneal dialysis. Perit Dial Int 2007; 27(suppl 2):S205–S209.
10 Doherty TM, Fitzpatrick LA, Shaheen A, Rajavashisth TB, Detrano RC: Genetic determinants of arterial calcification associated with atherosclerosis. Mayo Clin Proc 2004; 79:197–210.
11 Cannata-Andia JB, Rodriguez-Garcia M, Carrillo-Lopez N, Naves-Diaz M, Diaz-Lopez B: Vascular calcifications: Pathogenesis, management, and impact on clinical outcomes. J Am Soc Nephrol 2006;17:S267–273.
12 Roman-Garcia P, Carrillo-Lopez N, Cannata-Andia JB: Pathogenesis of bone and mineral related disorders in chronic kidney disease: key role of hyperphosphatemia. J Ren Care 2009;35(suppl 1):34–38.
13 Goldsmith DJ, Covic A, Sambrook PA, Ackrill P: Vascular calcification in long-term haemodialysis patients in a single unit: a retrospective analysis. Nephron 1997;77:37–43.
14 Mazzaferro S, Coen G, Bandini S, Borgatti PP, Ciaccheri M, Diacinti D, Ferranti E, Lusenti T, Mancini G, Monducci I, et al: Role of ageing, chronic renal failure and dialysis in the calcification of mitral annulus. Nephrol Dial Transplant 1993;8:335–340.
15 Maher ER, Young G, Smyth-Walsh B, Pugh S, Curtis JR: Aortic and mitral valve calcification in patients with end-stage renal disease. Lancet 1987;2:875–877.
16 Goodman WG, Goldin J, Kuizon BD, Yoon C, Gales B, Sider D, Wang Y, Chung J, Emerick A, Greaser L, Elashoff RM, Salusky IB: Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med 2000;342: 1478–1483.
17 Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM: Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol 2004; 15:2208–2218.
18 Russo D, Palmiero G, De Blasio AP, Balletta MM, Andreucci VE: Coronary artery calcification in patients with crf not undergoing dialysis. Am J Kidney Dis 2004;44:1024–1030.
19 Kramer H, Toto R, Peshock R, Cooper R, Victor R: Association between chronic kidney disease and coronary artery calcification: the Dallas Heart Study. J Am Soc Nephrol 2005;16:507–513.
20 Rodriguez-Garcia M, Gomez-Alonso C, Naves-Diaz M, Diaz-Lopez JB, Diaz-Corte C, Cannata-Andia JB: Vascular calcifications, vertebral fractures and mortality in haemodialysis patients. Nephrol Dial Transplant 2009;24:239–246.
21 Dalager S, Paaske WP, Kristensen IB, Laurberg JM, Falk E: Artery-related differences in atherosclerosis expression: Implications for atherogenesis and dynamics in intima-media thickness. Stroke 2007;38:2698–2705.
22 Ribeiro S, Ramos A, Brandao A, Rebelo JR, Guerra A, Resina C, Vila-Lobos A, Carvalho F, Remedio F, Ribeiro F: Cardiac valve calcification in haemodialysis patients: role of calcium-phosphate metabolism. Nephrol Dial Transplant 1998;13:2037–2040.
23 Okuda K, Kobayashi S, Hayashi H, Ohtake H, Nakajima Y, Yoshida K, Kashima H, Irie Y: Case-control study of calcification of the hepatic artery in chronic hemodialysis patients: comparison with the abdominal aorta and splenic artery. J Gastroenterol Hepatol 2002;17:91–95.
24 Yuen D, Pierratos A, Richardson RM, Chan CT: The natural history of coronary calcification progression in a cohort of nocturnal haemodialysis patients. Nephrol Dial Transplant 2006;21:1407–1412.
25 Krasniak A, Drozdz M, Pasowicz M, Chmiel G, Michalek M, Szumilak D, Podolec P, Klimeczek P, Konieczynska M, Wicher-Muniak E, Tracz W, Khoa TN, Souberbielle JC, Drueke TB, Sulowicz W: Factors involved in vascular calcification and atherosclerosis in maintenance haemodialysis patients. Nephrol Dial Transplant 2007;22:515–521.
26 Wilson PW, Kauppila LI, O'Donnell CJ, Kiel DP, Hannan M, Polak JM, Cupples LA: Abdominal aortic calcific deposits are an important predictor of vascular morbidity and mortality. Circulation 2001;103:1529–1534.
27 Goldsmith D, Ritz E, Covic A: Vascular calcification: a stiff challenge for the nephrologist: does preventing bone disease cause arterial disease? Kidney Int 2004;66:1315–1333.
28 London GM, Marty C, Marchais SJ, Guerin AP, Metivier F, de Vernejoul MC: Arterial calcifications and bone histomorphometry in end-stage renal disease. J Am Soc Nephrol 2004;15:1943–1951.
29 O'Neill TW, Felsenberg D, Varlow J, Cooper C, Kanis JA, Silman AJ: The prevalence of vertebral deformity in European men and women: the European Vertebral Osteoporosis study. J Bone Miner Res 1996;11:1010–1018.
30 Naves Diaz M, Diaz Lopez JB, Gomez Alonso C, Altadill Arregui A, Rodriguez Rebollar A, Cannata Andia JB: Study of incidence of osteoporotic fractures in a cohort of individuals older than 50 years from Asturias, Spain, after a 6 year follow-up period. Med Clin (Barc) 2000;115:650–653.
31 Kiel DP, Kauppila LI, Cupples LA, Hannan MT, O'Donnell CJ, Wilson PW: Bone loss and the progression of abdominal aortic calcification over a 25 year period: The Framingham heart study. Calcif Tissue Int 2001; 68:271–276.
32 Naves Diaz M, Diaz Lopez JB, Gomez C, Rodriguez Rebollar A, Rodriguez Garcia M, Cannata Andia JB: Association between current bone metabolism parameters and the progression of vascular calcification in men and women. J Bone Miner Res 2004;19: S-365.
33 Frye MA, Melton LJ, 3rd, Bryant SC, Fitzpatrick LA, Wahner HW, Schwartz RS, Riggs BL: Osteoporosis and calcification of the aorta. Bone Miner 1992;19:185–194.
34 Voigt MT, San Valentin R, Forrest KY, Nevitt MC, Cauley JA: Bone mineral density and aortic calcification: the study of osteoporotic fractures. J Am Geriatr Soc 1997;45:140–145.
35 Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, Gomez-Alonso C, Cannata-Andia JB: Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. Osteoporos Int 2008;19: 1161–1166.
36 Schulz E, Arfai K, Liu X, Sayre J, Gilsanz V: Aortic calcification and the risk of osteoporosis and fractures. J Clin Endocrinol Metab 2004;89:4246–4253.
37 Qunibi WY: Reducing the burden of cardiovascular calcification in patients with chronic kidney disease. J Am Soc Nephrol 2005;16 (suppl 2):S95–102.
38 Bleyer AJ, Burke SK, Dillon M, Garrett B, Kant KS, Lynch D, Rahman SN, Schoenfeld P, Teitelbaum I, Zeig S, Slatopolsky E: A comparison of the calcium-free phosphate binder sevelamer hydrochloride with calcium acetate in the treatment of hyperphosphatemia in hemodialysis patients. Am J Kidney Dis 1999;33:694–701.
39 Cozzolino M, Staniforth ME, Liapis H, Finch J, Burke SK, Dusso AS, Slatopolsky E: Sevelamer hydrochloride attenuates kidney and cardiovascular calcifications in long-term experimental uremia. Kidney Int 2003; 64:1653–1661.
40 Chertow GM, Burke SK, Raggi P: Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients. Kidney Int 2002;62:245–252.

41 Qunibi W, Moustafa M, Muenz LR, He DY, Kessler PD, Diaz-Buxo JA, Budoff M: A 1-year randomized trial of calcium acetate versus sevelamer on progression of coronary artery calcification in hemodialysis patients with comparable lipid control: The Calcium Acetate Renagel Evaluation-2 (CARE-2) study. Am J Kidney Dis 2008;51:952–965.
42 Barreto DV, Barreto Fde C, de Carvalho AB, Cuppari L, Draibe SA, Dalboni MA, Moyses RM, Neves KR, Jorgetti V, Miname M, Santos RD, Canziani ME: Phosphate binder impact on bone remodeling and coronary calcification – results from the BRIC study. Nephron Clin Pract 2008;110:c273–283.
43 Holick MF: Vitamin D deficiency. N Engl J Med 2007;357:266–281.
44 Naves-Diaz M, Alvarez-Hernandez D, Passlick-Deetjen J, Guinsburg A, Marelli C, Rodriguez-Puyol D, Cannata-Andia JB: Oral active vitamin d is associated with improved survival in hemodialysis patients. Kidney Int 2008;74:1070–1078.
45 Teng M, Wolf M, Ofsthun MN, Lazarus JM, Hernan MA, Camargo CA Jr, Thadhani R: Activated injectable vitamin D and hemodialysis survival: a historical cohort study. J Am Soc Nephrol 2005;16:1115–1125.
46 Lopez I, Aguilera-Tejero E, Mendoza FJ, Almaden Y, Perez J, Martin D, Rodriguez M: Calcimimetic r-568 decreases extraosseous calcifications in uremic rats treated with calcitriol. J Am Soc Nephrol 2006;17:795–804.
47 Lopez I, Mendoza FJ, Guerrero F, Almaden Y, Henley C, Aguilera-Tejero E, Rodriguez M: The calcimimetic AMG 641 accelerates regression of extraosseous calcifications in uremic rats. Am J Physiol Renal Physiol 2009;296:F1376–F1385.
48 Budoff MJ, Lane KL, Bakhsheshi H, Mao S, Grassmann BO, Friedman BC, Brundage BH: Rates of progression of coronary calcium by electron beam tomography. Am J Cardiol 2000;86:8–11.
49 Callister TQ, Raggi P, Cooil B, Lippolis NJ, Russo DJ: Effect of HMG-CoA reductase inhibitors on coronary artery disease as assessed by electron-beam computed tomography. N Engl J Med 1998;339:1972–1978.
50 Tamashiro M, Iseki K, Sunagawa O, Inoue T, Higa S, Afuso H, Fukiyama K: Significant association between the progression of coronary artery calcification and dyslipidemia in patients on chronic hemodialysis. Am J Kidney Dis 2001;38:64–69.
51 London GM, Guerin AP, Marchais SJ, Metivier F, Pannier B, Adda H: Arterial media calcification in end-stage renal disease: Impact on all-cause and cardiovascular mortality. Nephrol Dial Transplant 2003;18:1731–1740.
52 Ishimura E, Okuno S, Taniwaki H, Kizu A, Tsuchida T, Shioi A, Shoji T, Tabata T, Inaba M, Nishizawa Y: Different risk factors for vascular calcification in end-stage renal disease between diabetics and nondiabetics: the respective importance of glycemic and phosphate control. Kidney Blood Press Res 2008; 31:10–15.
53 Ishimura E, Okuno S, Kitatani K, Kim M, Shoji T, Nakatani T, Inaba M, Nishizawa Y: Different risk factors for peripheral vascular calcification between diabetic and non-diabetic haemodialysis patients – importance of glycaemic control. Diabetologia 2002;45: 1446–1448.
54 Block GA, Raggi P, Bellasi A, Kooienga L, Spiegel DM: Mortality effect of coronary calcification and phosphate binder choice in incident hemodialysis patients. Kidney Int 2007;71:438–441.
55 Holden RM, Booth SL: Vascular calcification in chronic kidney disease: the role of vitamin K. Nat Clin Pract Nephrol 2007;3:522–523.
56 Persy V, De Broe M, Ketteler M: Bisphosphonates prevent experimental vascular calcification: treat the bone to cure the vessels? Kidney Int 2006;70:1537–1538.
57 Hofbauer LC, Brueck CC, Shanahan CM, Schoppet M, Dobnig H: Vascular calcification and osteoporosis – from clinical observation towards molecular understanding. Osteoporos Int 2007;18:251–259.
58 Neven EG, De Broe ME, D'Haese PC: Prevention of vascular calcification with bisphosphonates without affecting bone mineralization: a new challenge? Kidney Int 2009; 75:580–582.
59 Price PA, Faus SA, Williamson MK: Bisphosphonates alendronate and ibandronate inhibit artery calcification at doses comparable to those that inhibit bone resorption. Arterioscler Thromb Vasc Biol 2001;21:817–824.
60 Nitta K, Akiba T, Suzuki K, Uchida K, Watanabe R, Majima K, Aoki T, Nihei H: Effects of cyclic intermittent etidronate therapy on coronary artery calcification in patients receiving long-term hemodialysis. Am J Kidney Dis 2004;44:680–688.
61 Lomashvili KA, Monier-Faugere MC, Wang X, Malluche HH, O'Neill WC: Effect of bisphosphonates on vascular calcification and bone metabolism in experimental renal failure. Kidney Int 2009;75:617–625.
62 Schoppet M, Shroff RC, Hofbauer LC, Shanahan CM: Exploring the biology of vascular calcification in chronic kidney disease: what's circulating? Kidney Int 2008;73:384–390.
63 Moe SM, Chen NX: Mechanisms of vascular calcification in chronic kidney disease. J Am Soc Nephrol 2008;19:213–216.
64 Giachelli CM: Vascular calcification mechanisms. J Am Soc Nephrol 2004;15:2959–2964.
65 Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, Jahnen-Dechent W, Weissberg PL, Shanahan CM: Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. J Am Soc Nephrol 2004;15: 2857–2867.
66 Wu-Wong JR, Nakane M, Ma J, Ruan X, Kroeger PE: Effects of vitamin D analogs on gene expression profiling in human coronary artery smooth muscle cells. Atherosclerosis 2006;186:20–28.
67 Hruska KA, Mathew S, Lund R, Qiu P, Pratt R: Hyperphosphatemia of chronic kidney disease. Kidney Int 2008;74:148–157.
68 Rodriguez Garcia M, Naves Diaz M, Cannata Andia JB: Bone metabolism, vascular calcifications and mortality: associations beyond mere coincidence. J Nephrol 2005;18: 458–463.
69 Li X, Yang HY, Giachelli CM: Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. Circ Res 2006;98:905–912.
70 Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM: Phosphate regulation of vascular smooth muscle cell calcification. Circ Res 2000; 87:E10–E17.
71 Mizobuchi M, Ogata H, Hatamura I, Koiwa F, Saji F, Shiizaki K, Negi S, Kinugasa E, Ooshima A, Koshikawa S, Akizawa T: Up-regulation of Cbfa1 and Pit-1 in calcified artery of uraemic rats with severe hyperphosphataemia and secondary hyperparathyroidism. Nephrol Dial Transplant 2006;21: 911–916.
72 Al-Aly Z, Shao JS, Lai CF, Huang E, Cai J, Behrmann A, Cheng SL, Towler DA: Aortic Msx2-Wnt calcification cascade is regulated by TNF-α-dependent signals in diabetic Ldlr–/– mice. Arterioscler Thromb Vasc Biol 2007;27:2589–2596.
73 Towler DA, Shao JS, Cheng SL, Pingsterhaus JM, Loewy AP: Osteogenic regulation of vascular calcification. Ann NY Acad Sci 2006; 1068:327–333.
74 Roman-Garcia P, Carrillo-Lopez N, Fernandez-Martin JL, Naves-Diaz M, Ruiz-Torres MP, Cannata-Andia JB: High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. Bone 2010;46:121–128.
75 Shao JS, Cheng SL, Pingsterhaus JM, Charlton-Kachigian N, Loewy AP, Towler DA: Msx2 promotes cardiovascular calcification by activating paracrine wnt signals. J Clin Invest 2005;115:1210–1220.

76 Byon CH, Javed A, Dai Q, Kappes JC, Clemens TL, Darley-Usmar VM, McDonald JM, Chen Y: Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by Akt signaling. J Biol Chem 2008;283:15319–15327.
77 Liberman M, Bassi E, Martinatti MK, Lario FC, Wosniak J Jr., Pomerantzeff PM, Laurindo FR: Oxidant generation predominates around calcifying foci and enhances progression of aortic valve calcification. Arterioscler Thromb Vasc Biol 2008;28:463–470.
78 Oberg BP, McMenamin E, Lucas FL, McMonagle E, Morrow J, Ikizler TA, Himmelfarb J: Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int 2004;65:1009–1016.
79 Johnson K, Polewski M, van Etten D, Terkeltaub R: Chondrogenesis mediated by PPi depletion promotes spontaneous aortic calcification in NPP1–/– mice. Arterioscler Thromb Vasc Biol 2005;25:686–691.
80 Rutsch F, Ruf N, Vaingankar S, Toliat MR, Suk A, Hohne W, Schauer G, Lehmann M, Roscioli T, Schnabel D, Epplen JT, Knisely A, Superti-Furga A, McGill J, Filippone M, Sinaiko AR, Vallance H, Hinrichs B, Smith W, Ferre M, Terkeltaub R, Nurnberg P: Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. Nat Genet 2003;34:379–381.
81 Binkert C, Demetriou M, Sukhu B, Szweras M, Tenenbaum HC, Dennis JW: Regulation of osteogenesis by fetuin. J Biol Chem 1999;274:28514–28520.
82 Westenfeld R, Jahnen-Dechent W, Ketteler M: Vascular calcification and fetuin-A deficiency in chronic kidney disease. Trends Cardiovasc Med 2007;17:124–128.
83 Merx MW, Schafer C, Westenfeld R, Brandenburg V, Hidajat S, Weber C, Ketteler M, Jahnen-Dechent W: Myocardial stiffness, cardiac remodeling, and diastolic dysfunction in calcification-prone fetuin-A-deficient mice. J Am Soc Nephrol 2005;16:3357–3364.
84 Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS: Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998;12:1260–1268.
85 Orita Y, Yamamoto H, Kohno N, Sugihara M, Honda H, Kawamata S, Mito S, Soe NN, Yoshizumi M: Role of osteoprotegerin in arterial calcification: development of new animal model. Arterioscler Thromb Vasc Biol 2007;27:2058–2064.
86 Panizo S, Cardus A, Encinas M, Parisi E, Valcheva P, Lopez-Ongil S, Coll B, Fernandez E, Valdivielso JM: RANKL increases vascular smooth muscle cell calcification through a RANK-BMP4-dependent pathway. Circ Res 2009;104:1041–1048.
87 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima YI: Mutation of the mouse Klotho gene leads to a syndrome resembling ageing. Nature 1997;390:45–51.
88 Memon F, El-Abbadi M, Nakatani T, Taguchi T, Lanske B, Razzaque MS: Does Fgf23-klotho activity influence vascular and soft tissue calcification through regulating mineral ion metabolism? Kidney Int 2008;74:566–570.
89 Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M: Suppression of aging in mice by the hormone Klotho. Science 2005;309:1829–1833.
90 Kurosu H, Ogawa Y, Miyoshi M, Yamamoto M, Nandi A, Rosenblatt KP, Baum MG, Schiavi S, Hu MC, Moe OW, Kuro-o M: Regulation of fibroblast growth factor-23 signaling by Klotho. J Biol Chem 2006;281:6120–6123.
91 Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M, Sirkis R, Naveh-Many T, Silver J: The parathyroid is a target organ for FGF23 in rats. J Clin Invest 2007;117:4003–4008.
92 Torres PU, Prie D, Molina-Bletry V, Beck L, Silve C, Friedlander G: Klotho: an antiaging protein involved in mineral and vitamin D metabolism. Kidney Int 2007;71:730–737.
93 Singh R, Barden A, Mori T, Beilin L: Advanced glycation end-products: a review. Diabetologia 2001;44:129–146.
94 Brownlee M: Advanced protein glycosylation in diabetes and aging. Annu Rev Med 1995;46:223–234.
95 Goldin A, Beckman JA, Schmidt AM, Creager MA: Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation 2006;114:597–605.
96 Yamamoto M, Yamaguchi T, Yamauchi M, Yano S, Sugimoto T: Serum pentosidine levels are positively associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes. J Clin Endocrinol Metab 2008;93:1013–1019.
97 Schwartz AV, Garnero P, Hillier TA, Sellmeyer DE, Strotmeyer ES, Feingold KR, Resnick HE, Tylavsky FA, Black DM, Cummings SR, Harris TB, Bauer DC: Pentosidine and increased fracture risk in older adults with type 2 diabetes. J Clin Endocrinol Metab 2009;94:2380–2386.
98 Shiraki M, Kuroda T, Tanaka S, Saito M, Fukunaga M, Nakamura T: Nonenzymatic collagen cross-links induced by glycoxidation (pentosidine) predicts vertebral fractures. J Bone Miner Metab 2008;26:93–100.
99 Vashishth D, Gibson GJ, Khoury JI, Schaffler MB, Kimura J, Fyhrie DP: Influence of nonenzymatic glycation on biomechanical properties of cortical bone. Bone 2001;28:195–201.
100 Tang SY, Zeenath U, Vashishth D: Effects of non-enzymatic glycation on cancellous bone fragility. Bone 2007;40:1144–1151.
101 Franke S, Siggelkow H, Wolf G, Hein G: Advanced glycation endproducts influence the mrna expression of rage, rankl and various osteoblastic genes in human osteoblasts. Arch Physiol Biochem 2007;113:154–161.

Publicación 4: Mecanismo de calcificación vascular en la enfermedad renal crónica.

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Mecanismos de calcificación vascular en la enfermedad renal crónica

Pablo Román García y José Manuel Valdivielso Revilla

Introducción

La enfermedad renal crónica (ERC) se define en la actualidad según las recomendaciones de las guías de la fundación *Kidney Disease: Improving Global Outcomes* (K/DIGO)[1]. Es conocida la relación entre las calcificaciones vasculares y la mortalidad cardiovascular, principal causa de muerte en pacientes en diálisis con más del 50% de los fallecimientos[2]. Los pacientes en hemodiálisis (HD) crónica presentan de dos a cinco veces más calcificaciones de las arterias coronarias que los individuos de su misma edad de la población general[3].

Virchow, en el siglo XIX, describió por primera vez la aparición de calcificaciones metastásicas o ectópicas en pacientes con ERC. Contiguglia y cols. mostraron en 1973 que el calcio procedente de los vasos de pacientes urémicos eran cristales de hidroxiapatita, los mismos que los del esqueleto[4]. En las últimas décadas, varios estudios comenzaron a mostrar que un porcentaje muy elevado de pacientes con ERC presentaban calcificaciones vasculares[5], con mayor frecuencia y gravedad que la población general, incluso en pacientes jóvenes menores de 30 años[6], con el consecuente impacto en la morbimortalidad de estos pacientes.

El mecanismo por el cual se produce la calcificación vascular es complejo y no bien comprendido. Inicialmente, se pensaba que consistía en una simple precipitación de Ca y P en un microambiente apropiado[7]. Varias líneas de evidencia indicaron, sin embargo, que los mecanismos pasivos discurrían a la par que ciertos mecanismos activos. Durante este proceso activo, células de músculo liso vascular (CMLV), debido a la acción de ciertos promotores de la calcificación, sufren un mayor grado de apoptosis, formación de vesículas y cambian el fenotipo de células musculares lisas de la pared arterial a células similares a osteoblastos, lo que induce la formación de matriz y también atrae factores locales implicados en el proceso de mineralización. Sin embargo, en mamíferos y humanos, las concentraciones séricas de Ca y P exceden en varias veces la solubilidad del producto calcio-fósforo (Ca x P). No obstante, no existe precipitación dentro de los vasos. Este hecho remarca claramente la importancia del papel que juegan los inhibidores fisiológicos de la calcificación que contrabalancean el efecto bien conocido de los promotores de la calcificación.

La lista de promotores e inhibidores del proceso de calcificación es larga y se incrementa cada año[8-10] (Figura 1).

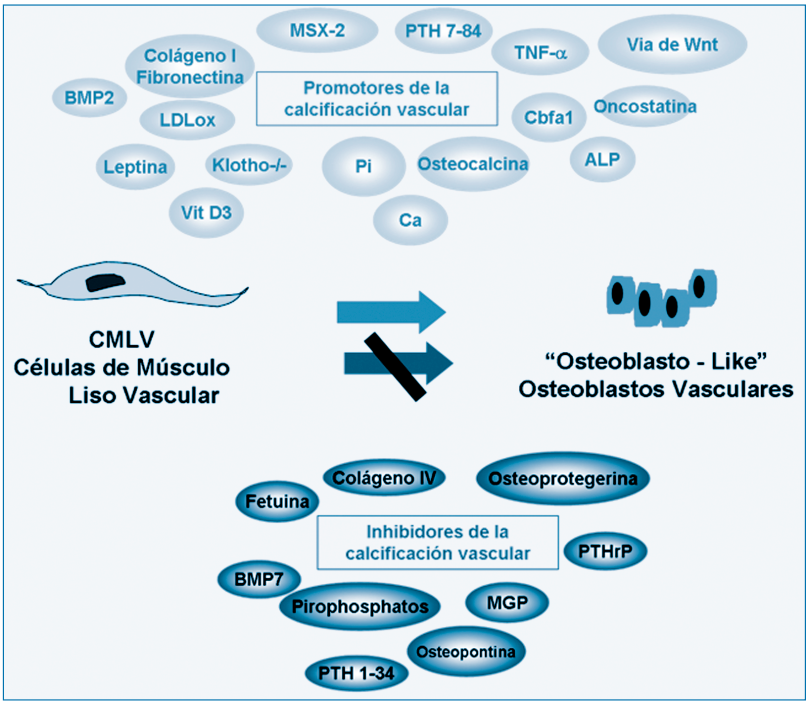

Figura 1. *Promotores e inhibidores de la calcificación vascular.*

La hiperfosfatemia es el factor que, de forma más constante, se ha asociado con incremento de las calcificaciones vasculares y mortalidad en pacientes en HD[11]. Con respecto al producto Ca x P, parece claro que su aumento se relaciona con mayor mortalidad global, por cardiopatía isquémica y por muerte súbita[12,13].

El papel de la vitamina D como factor de riesgo merece una particular atención, ya que dosis altas de metabolitos de vitamina D se han asociado experimental y clínicamente a un incremento en las calcificaciones vasculares y la mortalidad[14,15], mientras que la administración de metabolitos activos de vitamina D se asocia a una mayor protección frente a estas manifestaciones.

Promotores de calcificación vascular

Fósforo y calcio

El fósforo sérico alto es uno de los factores de riesgo más importantes relacionados con la uremia, asociados a la calcificación vascular en pacientes con insuficiencia renal crónica y en la población general[2]. Es bien sabido que los altos niveles de fósforo sérico agravan el hiperparatiroidismo secundario y también reducen la actividad de la enzima 1α-hidroxilasa, que, a su vez, disminuye los niveles séricos de calcitriol.

Una cuestión importante que debe ser respondida es cómo los incrementos en el fós-

foro sérico activan varios mecanismos en los vasos, desembocando en el desarrollo de calcificaciones vasculares.

El fósforo, además de ser un elemento mineral extracelular, también se ha descrito como un jugador importante en los mecanismos de señalización de las CMLV[16]. No sólo colabora con el calcio en la mineralización de la matriz, sino que también es capaz de actuar como un «mensajero intracelular secundario», activando varias vías moleculares relacionadas con la formación de hueso. Penetra en las células a través de un canal específico dependiente de Na llamado Pit-1; de hecho, el bloqueo de Pit-1 previene la calcificación vascular en un modelo *in vitro*[17]. Experimentos *in vitro* han demostrado que niveles intracelulares elevados de fósforo pueden actuar directamente en la transcripción de genes relacionados con el hueso, como core binding factor α-1 (CBFA-1) y osteocalcina, lo que resulta en la activación de varias vías osteogénicas en las CMLV, dando lugar a los consabidos cambios fenotípicos, pasando de CMLV a células óseas[18]. Además, en ratas urémicas con alto fósforo sérico, se ha sugerido que la calcificación vascular en la capa media es causada, al menos en parte, por incrementos en las formas vasculares de CBFA-1 y Pit-1[19]. CBFA-1 promueve la expresión de una de las familias más importantes de las proteínas implicadas en las calcificaciones vasculares: las proteínas morfogenéticas óseas (BMP, por sus siglas en inglés), de las que se hablará más adelante en este capítulo.

Vitamina D

El descubrimiento de que las CMLV expresan el receptor de la vitamina D (VDR) hizo pensar que la unión del metabolito activo de la vitamina D, $1,25(OH)_2D_3$, podría tener efectos sobre las propias CMLV. De hecho, la $1,25(OH)_2D_3$ regula por incremento el VDR y aumenta la carga de calcio en las CMLV de rata en cultivo[20] y de aorta de conejo[21]. Otros estudios mostraron que concentraciones de 10^{-9} M de $1,25(OH)_2D_3$ inducían la proliferación de las CMLV[22], la migración y un aumento de la expresión de osteopontina[14]. También se demostró que una concentración de 1,2 µmol/l inducía cambios morfológicos en las CMLV de aorta de rata, incluída la progresión de un fenotipo «contráctil» a uno «óseo» con un incremento la producción de factores que promueven la calcificación *in vitro*[23].

Jono y cols. demostraron que la $1,25(OH)_2D_3$ en concentraciones comprendidas entre 10^{-7} y 10^{-9} M induce un incremento de la calcificación dosis dependiente en CMLV bovinas *in vitro*. Esto va acompañado de un incremento similar en la actividad de la fosfatasa alcalina y una regulación a la baja del péptido relacionado con la PTH (PTHrp)[15]. Este cambio fenotípico permite a las CMLV sintetizar proteínas específicas de osteoblastos que incrementan el grado de calcificación[24]. Entre ellas, podemos destacar la expresión de factores de transcripción como CBFA-1, osteocalcina[25] y de moléculas solubles como el RANKL o BMP4[26].

Estudios *in vivo* han demostrado que, aparte de la capacidad de la vitamina D de inducir el cambio fenotípico en las CMLV, otro factor importante es el efecto hiperfosfatémico e hipercalcémico de dosis altas de vitamina D. Así, altas dosis de vitamina D y nicotina han sido usadas como un modelo de calcificación vascular[27]. Este modelo acusa un aumento de la calcificación de las capas medias de las

arterias, lo que incrementa la rigidez arterial. Un dato más a favor del papel fundamental del fósforo en la calcificación arterial inducida por vitamina D proviene de estudios *in vivo* con animales KO para FGF23[28]. En estos animales, que tienen niveles altos de vitamina D e hiperfosfatemia junto con calcificación vascular, el uso de una dieta pobre en P evitó la calcificación vascular. Sin embargo, dosis bajas no hipercalcemiantes ni hiperfosforemiantes de calcitriol han sido capaces de producir calcificación arterial en animales con insuficiencia renal crónica[25]. Por el contrario, en animales con un modelo de aterosclerosis, dosis bajas de calcitriol y paricalcitol protegían de la calcificación vascular[29].

Proteínas morfogenéticas óseas (BMP)

Las BMP son un grupo de al menos 30 proteínas con propiedades de inducción ósea que tienen un papel importante en el desarrollo de la organogénesis en varios tejidos. Forman parte de la superfamilia del TGF-beta y activan, tras la unión, a sus receptores transmembrana. Los receptores de las BMP (tipo I y tipo II) funcionan de manera cooperativa y, tras la unión de la proteína al receptor tipo II, se produce la activación del tipo I. El resultado es un aumento de la transcripción génica a través de la fosforilación de proteínas y de la traslocación al núcleo de factores de transcripción de la familia de los Smad.

La expresión de las BMP se ha detectado en placas ateroscleróticas[30]. Además, se han encontrado BMP2, BMP4 y BMP6 en áreas de calcificación vascular[31]. Estudios posteriores han demostrado que la BMP2 inhibe la proliferación de las CMLV estimuladas con suero[32], tras lo que desciende la expresión de marcadores de célula muscular lisa. Además, como se ha dicho anteriormente, es capaz de inducir la expresión de MSX2 y CBFA-1, y de inducir calcificación vascular *in vivo*. *In vitro*, estimula la captación de fósforo, el fenotipo osteoblástico y la calcificación. El papel de la BMP4 no está tan estudiado, aunque se ha demostrado que induce calcificación de CMLV y está implicada en la calcificación vascular inducida por RANKL[26].

Contrariamente a otras BMP, la BMP7 es una proteína que protege de la calcificación vascular promoviendo el fenotipo de CMLV. Además, en un modelo de calcificación vascular en animales con aterosclerosis, el tratamiento con BMP7 fue capaz de reducir los niveles de calcificación vascular[33].

Eje Wnt-Msx2

Estudios con CMLV cultivadas en medio con alto contenido de fósforo, han demostrado que la BMP2 estimula la expresión vascular del factor de transcripción Msx-2[34], otro gen relacionado con el hueso. Se ha demostrado que el eje «BMP-MSX-2» también recluta a componentes de la familia de proteínas Wingless/ints (Wnts)[35], tradicionalmente asociadas a la formación de hueso. Recientemente, la vía de señalización Wnt, incluyendo sus inhibidores, también se ha añadido a la larga lista de factores paracrinos «promotores de la calcificación vascular»[36].

Estrés oxidativo

El estrés oxidativo se ha relacionado también con el eje BMP-MSX-Wnt, la inflamación y la calcificación vascular. CMVL cultivadas con H_2O_2 presentaron calcificaciones, a través de la estimulación directa de

CBFA-1[37], lo que demuestra que el estrés oxidativo puede actuar como un promotor de la calcificación vascular. Además, estudios *in vivo* han demostrado que algunos de los antioxidantes pueden prevenir las calcificaciones vasculares[38].

Inhibidores de calcificación vascular

Fetuína–A

En el suero, los inhibidores de la calcificación vascular más abundantes son la fetuína-A (alfa 2-glicoproteína-Heremans Schmid), la OPG y la proteína gla de la matriz[31]. La fetuína-A es un conocido inhibidor de la osteogénesis[40], capaz de inhibir la calcificación vascular. Los ratones KO para fetuína-A desarrollan espontáneamente una calcificación generalizada de tejidos blandos, incluyendo una significativa calcificación de miocardio. En estos ratones, la calcificación vascular se asoció a la regulación al alza de los factores profibróticos TGF-beta[41].

Proteína gla de la matriz (MGP)

La MGP es una pequeña proteína que se secreta al medio extracelular y que sufre fundamentalmente dos modificaciones postraslacionales, dependientes algunas de ellas de la vitamina K[42], factor de creciente importancia en el desarrollo de calcificación vascular. Aunque su mecanismo molecular preciso no se conoce, la evidencia científica acumulada demuestra que tiene un papel fundamental en la inhibición de la calcificación de tejidos blandos. Las primeras evidencias se acumularon al constatar que el tratamiento de ratas con un antagonista de la vitamina K (warfarina) provocaba extensas calcificaciones del cartílago, provocando un crecimiento anormal. Además, los animales KO para la MGP mueren por ruptura aórtica debido a extensas calcificaciones vasculares.

El mecanismo preciso por el cual la MGP inhibe la calcificación vascular no se conoce, pero se han sugerido varias posibilidades. Price y cols. sugieren que la MGP se une a los cristales de hidroxiapatita impidiendo su crecimiento[43]. Además, se ha demostrado que la MGP es capaz de inhibir la diferenciación fenotípica de las CMLV en células de hueso. Así, los animales KO de MGP muestran una pérdida de marcadores de CMLV con un aumento de la expresión de Cbfa1 y osteopontina[44]. Los estudios de Shanahan, en los que encuentra menor expresión de la MGP en arterias de pacientes diabéticos con esclerosis de Mönckeberg apoyan esta teoría[45].

Osteoprotegerina (OPG)

Es un miembro de la familia de receptores de los factores de necrosis tumoral (TNF-R), que ha sido identificado como regulador de la resorción ósea[37]. La OPG funciona como un receptor soluble, señuelo de ligando (RANKL) del receptor activador del factor nuclear – B (RANK)[7]. La OPG es, además, receptor del ligando inductor de la apoptosis relacionado con el factor de la necrosis tumoral (TRAIL), que es un potente inductor de apoptosis.

La primera evidencia de que este sistema estaba implicado en la calcificación vascular derivó del estudio del ratón KO para la OPG, el cual presentaba osteoporosis y calcificaciones de la aorta y arterias renales[46]. Se ha demostrado que la OPG inhibe la calcificación vascular en ratas *in vivo* provocada tanto por la vitamina D como por warfarina[47]. Recientemente, se han producido progresos en el entendimiento del modo de acción de la OPG evitando la calcificación vascular, que implica a NFkB y BMP4[26].

Otros factores

Klotho es un correceptor del factor de crecimiento fibroblástico 23 (FGF23), una hormona fosfatúrica, y controla la excreción de fósforo[48], entre otras funciones.

Los defectos en los genes codificantes para FGF23 y Klotho provocan perturbaciones varias, incluyendo la calcificación vascular y la pérdida de hueso. Ratones KO para el gen de Klotho mostraron aceleración del envejecimiento, con calcificación ectópica generalizada, incluyendo calcificaciones vasculares. Los mecanismos por los que FGF23/Klotho afectan a las calcificaciones vasculares no se entienden completamente, si bien estudios recientes sugieren que este eje controla directamente la excreción de fosfatos, y por ello también, parte de la homeostasis mineral y otros pasos importantes en el metabolismo de la vitamina D y la PTH indirectamente, lo que, a su vez, puede ser también responsable de los efectos vasculares.

Recientemente el papel de los pirofosfatos y los bifosfonatos, no sólo en la prevención sino en la reversión de las calcificaciones vasculares, es objeto de nuevas y prometedoras investigaciones.

Bibliografía

1. Eknoyan G, Lameire N, Barsoum R, et al.The burden of kidney disease: improving global outcomes. Kidney Int 2004; 66(4):1310-1314.
2. Block GA, Klassen PS, Lazarus JM, et al. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol 2004; 15(8):2208-2218.
3. Moe SM, Chen NX. Pathophysiology of vascular calcification in chronic kidney disease. Circ Res 2004: 95(6):560-567.
4. Contiguglia SR, Alfrey AC, Miller NL, et al. Nature of soft tissue calcification in uremia. Kidney Int 1973; 4(3):229-235.
5. Goldsmith DJ, Covic A, Sambrook PA, Ackrill P. Vascular calcification in long-term haemodialysis patients in a single unit: a retrospective analysis. Nephron 1997; 77(1):37-43.
6. Goodman WG, Goldin J, Kuizon BD, et al. Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med 2000; 342(20):1478-1483.
7. Hofbauer LC, Brueck CC, Shanahan CM, et al. Vascular calcification and osteoporosis-from clinical observation towards molecular understanding. Osteoporos Int 2007; 18(3):251-259.
8. Giachelli CM, Jono S, Shioi A, et al. Vascular calcification and inorganic phosphate. Am J Kidney Dis 2001; 38(4 Suppl 1):S34-S37.
9. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. Jama 2004; 292(4):490-495.
10. Moe SM, O'Neill KD, Duan D, et al. Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins. Kidney Int 2002; 61(2):638-647.
11. Naves M GA, Marelli C, Tejada J, et al. Relative Risk (RR) of Death According to Serum Ca, P and PTH. Results from a Large Sample of Dialysis Patients from Latin America Followed for up to 54 Months. The CORES Study (abstract). J Am Soc Nephrol 2005; 16:728A.
12. Ganesh SK, Stack AG, Levin NW, et al. Association of elevated serum PO(4), Ca x PO(4) product, and parathyroid hormone with cardiac mortality risk in chronic hemodialysis patients. J Am Soc Nephrol 2001; 12(10):2131-2138.
13. Block GA, Hulbert-Shearon TE, Levin NW, Port FK. Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. Am J Kidney Dis 1998; 31(4):607-617.
14. Rebsamen MC, Sun J, Norman AW, Liao JK. 1alpha, 25-dihydroxyvitamin D_3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. Circ Res 2002; 91(1):17-24.
15. Jono S, Nishizawa Y, Shioi A, Morii H. 1,25-Dihydroxyvitamin D_3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. Circulation 1998; 98(13):1302-1306.
16. Villa-Bellosta R, Bogaert YE, Levi M, Sorribas V. Characterization of phosphate transport in rat vascular smooth muscle cells: implications for vascular calcifica-

tion. Arterioscler Thromb Vasc Biol 2007; 27(5):1030-1036.
17. Li X, Yang HY, Giachelli CM. Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. Circ Res 2006; 98(7):905-912.
18. Jono S, McKee MD, Murry CE, et al. Phosphate regulation of vascular smooth muscle cell calcification. Circ Res 2000; 87(7):E10-E17.
19. Mizobuchi M, Ogata H, Hatamura I, et al. Up-regulation of Cbfa1 and Pit-1 in calcified artery of uraemic rats with severe hyperphosphataemia and secondary hyperparathyroidism. Nephrol Dial Transplant 2006; 21(4):911-916.
20. Inoue T, Kawashima H. 1,25-Dihydroxyvitamin D_3 stimulates 45Ca2+-uptake by cultured vascular smooth muscle cells derived from rat aorta. Biochem Biophys Res Commun 1988; 152(3):1388-1394.
21. Rajasree S, Umashankar PR, Lal AV, et al.1,25-dihydroxyvitamin D3 receptor is upregulated in aortic smooth muscle cells during hypervitaminosis D. Life Sci 2002; 70(15):1777-1788.
22. Cardus A, Panizo S, Valdivielso JM, et al. 1,25-dihydroxyvitamin D3 regulates VEGF production through a vitamin D response element in the VEGF promoter. Atherosclerosis 2009; 204(1):85-89.
23. Tukaj C, Kubasik-Juraniec J, Kraszpulski M. Morphological changes of aortal smooth muscle cells exposed to calcitriol in culture. Med Sci Monit 2000; 6(4):668-674.
24. Cardus A, Panizo S, Valdivielso JM. Differential effects of vitamin D analogs on vascular calcification. J Bone Miner Res 2007; 22(6):860-866.
25. Mizobuchi M, Finch JL, Martin DR, Slatopolsky E. Differential effects of vitamin D receptor activators on vascular calcification in uremic rats. Kidney Int 2007; 72(6):709-715.
26. Panizo S, Cardus A, Valdivielso JM. RANKL increases vascular smooth muscle cell calcification through a RANK-BMP4-dependent pathway. Circ Res 2009; 104(9):1041-1048.
27. Niederhoffer N, Lartaud-Idjouadiene I, Giummelly P. Calcification of medial elastic fibers and aortic elasticity. Hypertension 1997; 29(4):999-1006.
28. Stubbs JR, Liu S, Tang W, et al. Role of hyperphosphatemia and 1,25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. J Am Soc Nephrol 2007; 18(7):2116-2124.
29. Mathew S, Lund RJ, Chaudhary LR, et al. Vitamin D Receptor Activators Can Protect against Vascular Calcification. J Am Soc Nephrol 2008; 19:1509-1519.
30. Bostrom K, Watson KE, Horn S, et al. Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Invest 1993; 91(4):1800-1809.
31. Dhore CR, Cleutjens JP, Lutgens E, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2001; 21(12):1998-2003.
32. Nakaoka T, Gonda K, Ogita T, et al. Inhibition of rat vascular smooth muscle proliferation in vitro and in vivo by bone morphogenetic protein-2. J Clin Invest 1997; 100(11):2824-2832.
33. Dorai H, Vukicevic S, Sampath TK. Bone morphogenetic protein-7 (osteogenic protein-1) inhibits smooth muscle cell proliferation and stimulates the expression of markers that are characteristic of SMC phenotype in vitro. J Cell Physiol 2000; 184(1):37-45.
34. Mathew S, Tustison KS, Sugatani T, et al. The Mechanism of Phosphorus as a Cardiovascular Risk Factor in CKD. J Am Soc Nephrol 2008; 19(6):1092-1105.
35. Shao JS, Cheng SL, Pingsterhaus JM, et al. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. J Clin Invest 2005; 115(5):1210-1220.
36. Shao JS, Cai J, Towler DA. Molecular mechanisms of vascular calcification: lessons learned from the aorta. Arterioscler Thromb Vasc Biol 2006; 26(7):1423-1430.
37. Byon CH, Javed A, Dai Q, et al. Oxidative Stress Induces Vascular Calcification through Modulation of the Osteogenic Transcription Factor Runx2 by AKT Signaling. J Biol Chem 2008; 283(22):15319-15327.
38. Liberman M, Bassi E, Martinatti MK, et al. Oxidant generation predominates around calcifying foci and enhances progression of aortic valve calcification. Arterioscler Thromb Vasc Biol 2008; 28(3):463-470.
39. Krasniak A, Drozdz M, Pasowicz M, et al. Factors involved in vascular calcification and atherosclerosis in maintenance haemodialysis patients. Nephrol Dial Transplant 2007; 22(2):515-521.
40. Binkert C, Demetriou M, Sukhu B, et al. Regulation of osteogenesis by fetuin. J Biol Chem 1999; 274(40):28514-28520.
41. Merx MW, Schafer C, Westenfeld R, et al. Myocardial stiffness, cardiac remodeling, and diastolic dysfunction in calcification-prone fetuin-A-deficient mice. J Am Soc Nephrol 2005; 16(11):3357-3364.
42. Berkner KL, Runge KW. The physiology of vitamin K nutriture and vitamin K-dependent protein function in atherosclerosis. J Thromb Haemost 2004; 2(12):2118-2132.
43. Price PA, Faus SA, Williamson MK. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. Arterioscler Thromb Vasc Biol 1998; 18(9):1400-1407.
44. Steitz SA, Speer MY, Curinga G, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. Circ Res 2001; 89(12):1147-5114.
45. Shanahan CM, Cary NR, Salisbury JR, et al. Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for

smooth muscle cell-mediated vascular calcification. Circulation 1999; 100(21):2168-2176.
46. Bucay N, Sarosi I, Dunstan CR, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998; 12(9):1260-1268.
47. Price PA, June HH, Buckley JR, Williamson MK. Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. Arterioscler Thromb Vasc Biol 2001; 21(10):1610-1616.
48. Kuro-o M, Matsumura Y, Aizawa H, et al. Mutation of the mouse Klotho gene leads to a syndrome resembling ageing. Nature 1997; 390(6655):45-51.

Publicación 5: High Phosphorus Diet Induces Vascular Calcification, a Related Decrease In Bone Mass and Changes in the Aortic Gene Expression

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High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression

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ABSTRACT

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

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

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

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

Introduction

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

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

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

Methods

Experimental design

Animal model

The study was performed using 4-month-old male Wistar rats ($n=70$). The rats were anaesthetized using methoxi-fluorane, and chronic renal failure (CRF) was induced by surgical 7/8 nephrectomy using the technique modified by Ormrod and Miller [12] by the same technician for all the groups. The nephrectomized rats were subsequently divided in two groups: Group I was fed with normal phosphorus diet (NPD) (0.6% phosphorus, 0.6% calcium and 20%

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protein content), and group II was fed with high phosphorus diet (HPD) (0.9% phosphorus, 0.6% calcium and 20% protein content) (Panlab, Spain). The rats were housed in wire cages and received diet and water *ad libitum*.

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

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

Biochemical markers

Serum was separated from blood samples by centrifugation at 4000 RPM and 4C. Serum urea, creatinine, calcium and phosphorus were measured using a multi-channel auto analyzer (Hitachi 717) following the manufacturer's protocol. Serum concentration of intact parathormone (iPTH) was measured using an IRMA rat PTH assay with a specific chicken anti-PTH antibody following manufacturer's protocol (Immunotopics).

Aortic calcification analysis

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

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

Microarrays hybridization, analysis and validation

Fragments of the abdominal aorta from the 5 rats sacrificed each time (8, 16 and 20 weeks) were pooled, and homogenized (Ultraturrax, OmniHT) in TRI™ reagent (Sigma-Aldrich). Total RNA was extracted and purified using RNeAsy™ Kit (Qiagen). The RNA integrity was checked using agarose-formaldehyde gels, and the RNA concentration was measured using a VIS-UV spectrophotometer (Nanodrop). cDNA was synthesized using a High Capacity™ kit (Applied Biosystems), and hybridized to Affy RAE_230 cDNA microarrays (Affymetrix) using the adequate quality controls and following the manufacturer's protocol. To analyze the raw datasets, the data were logged and normalized using the PM/MM (dChip [15]) method.

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

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

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

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

Densitometric analysis

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

Statistical analysis

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

Results

Biochemical markers

Serum phosphorus and iPTH levels were significantly higher ($p<0.05$) in all high phosphorus diet (HPD) groups compared to the

Table 1
Bone-related serum parameters in NPD and HPD, and reference (Ref) groups.

	Serum P, mg/dL		PTH, pg/dL		Serum Ca, mg/dL	
	Mean	SD	Mean	SD	Mean	SD
8NPD	5.7	1.2	37.8	22.0	12.1	0.2
16NPD	5.0	0.5	65.6	54.1	11.7	0.5
20NPD	5.6	0.7	82.2	57.6	12.5	0.6
8HPD	8.7*	0.8	283.6*	233.7	11.6*	0.4
16HPD	11.9*	2.7	1314.0*	781.7	11.3	2.1
20HPD	14.1*	2.4	2461.2*	717.0	10.9*	0.1
Ref	4.9	0.9	44.2	39.1	11.5	0.6

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

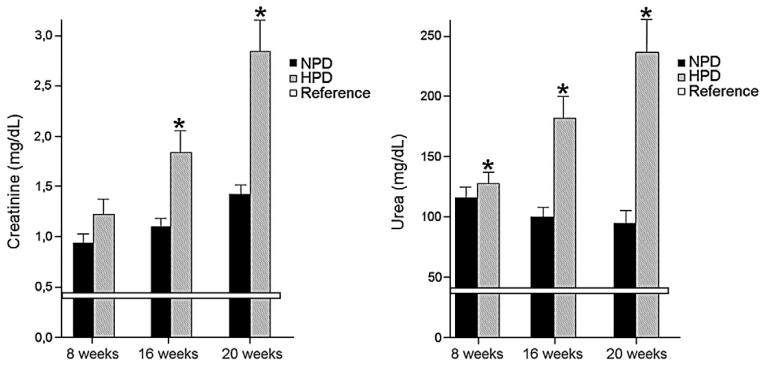

Fig. 1. Effect of HPD and NPD on serum creatinine and urea in rats with CRF at 8, 16 and 20 weeks. Reference group is represented. *$p<0.05$ compared to the time-matched HF groups. $N=39$.

paired normal phosphorus diet (NPD) groups. In addition, in the HPD groups, both serum phosphorus and iPTH increased over-time. Conversely, serum calcium was lower in the HPD groups compared to the paired NPD groups, achieving statistically significant differences in the 8HPD and 20HPD groups (Table 1).

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

Aortic analyses

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

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

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

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

Relationship between bone mineral density and vascular calcification

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

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

Aortic gene expression analysis

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

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

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

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

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

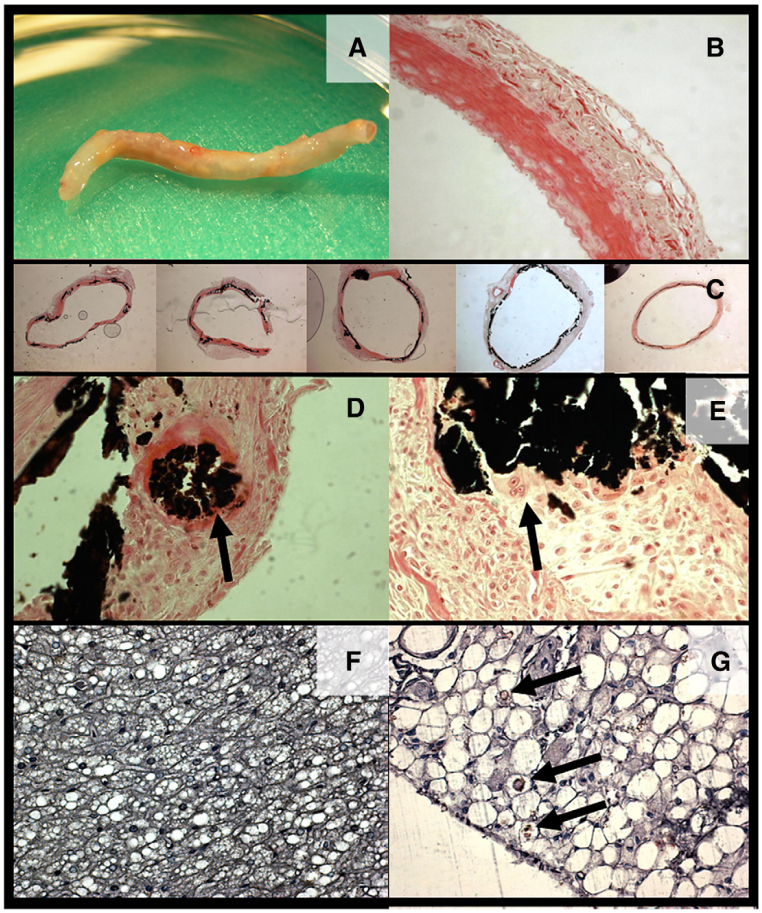

Fig. 2. Panel A: macroscopic image showing the rigid appearance of the entirely calcified aorta of a rat from the 20HPD group. All rats from 20HPD group showed the same macroscopic image of the aorta. Panel B (20×): von Kossa staining of a non-calcified aorta (similar images were obtained in all rats from reference, 8NPD, 16NPD, 20NPD and 8HPD groups). Panel C: images of the calcified aortas included on the study. Panels D (30×) and E (40×): von Kossa staining of a calcified aorta from the 20HPD group. Similar images were observed in all aortas form the 20HPD group. The arrow points the osteoid tissue (D) and multinucleated cells (E). Panel F (30×) and G (40×). TRAP staining in a section from non-calcified and calcified aorta, respectively. The calcified aorta had TRAP activity (red areas), marked by the red zones.

Discussion

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

In our study, we used our current animal model for experimental uraemia, but applying a moderate increase in the dietary phosphate

Table 2
BMD values at three different tibia sites in NPD and HPD, and reference (Ref) groups.

	Total tibia		Proximal tibia		Distal tibia	
	Mean	SD	Mean	SD	Mean	SD
8NPD	0.258	0.015	0.308	0.014	0.246	0.015
16NPD	0.277	0.015	0.329	0.020	0.265	0.014
20NPD	0.283	0.006	0.338	0.014	0.268	0.005
8HPD	0.247	0.009	0.297	0.009	0.235	0.013
16HPD	0.271	0.017	0.340	0.042	0.252	0.012
20HPD	0.237*	0.020	0.296*	0.031	0.222*	0.019
Ref	0.289	0.012	0.337	0.022	0.277	0.010

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

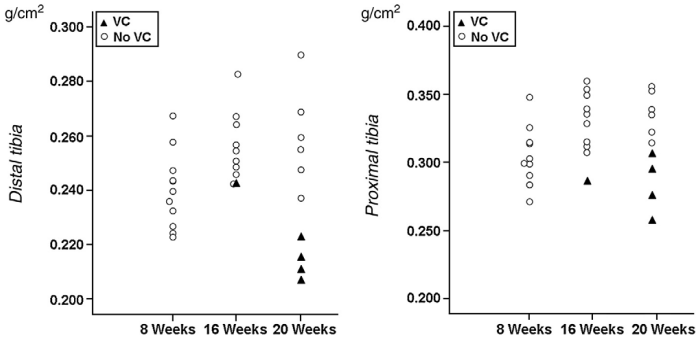

Fig. 3. Inverse relationship between vascular calcification and BMD in the 1/8 proximal part of the tibia, and the 7/8 remaining part of tibia. Plain triangles represent rats wi vascular calcifications (VC) and the open circles rats with no vascular calcification (No VC).

(50%) in order to mimic the scenario of positive phosphate balance and hyperphosphatemia frequently found in CKD patients [26].

As expected, we found a significant increase in serum phosphorus in rats receiving HPD compared to their matched NPD groups. Since phosphorus and calcium are closely interrelated and regulated [27], serum calcium was found to be significantly lower. Both high phosphorus and low calcium are two well-known stimuli which produce an increase in PTH synthesis, as well as parathyroid gland hyperplasia [28,29]. In fact, in this experimental model, a marked parathyroid hyperplasia (40-fold increase) (data not shown) and very high serum iPTH levels were found in the HPD groups (particularl after 20 weeks) compared to the NPD groups. In addition, serum iPT was the only biochemical parameter which was positively an independently associated with vascular calcifications; the higher th serum iPTH, the greater the amount and severity of vascul calcifications. These results are in agreement with the concept th PTH promotes vascular calcification as it has been found in previou experimental studies [30]. Interestingly, the rats which received a di with a greater content in phosphorus showed a higher spontaneou mortality [31] throughout the study, reaching 50%, 40% and 25% c

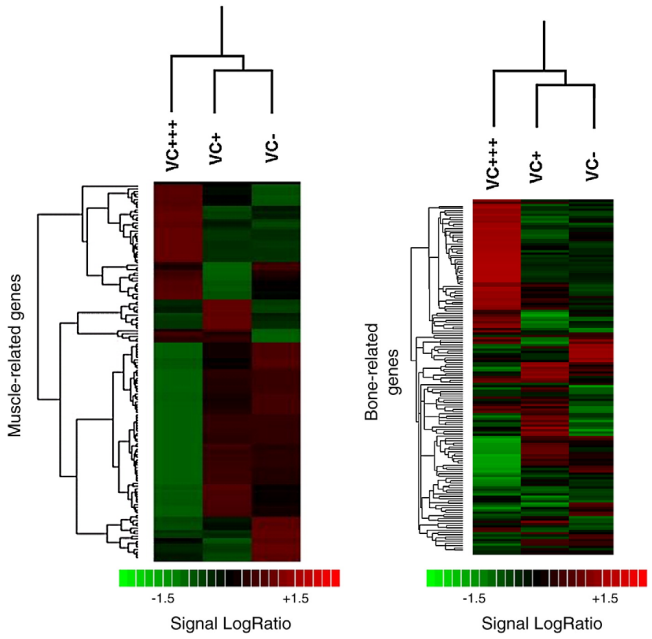

Fig. 4. Hierarchical clusters (heat map) branching the muscle- and bone-related gene families (KEGG criteria) into three categories based on Von Kossa results. Categories: (VC++: vascular calcification in 80% of the rats (20HPD group); (II) VC+: vascular calcification present in 20% of the rats (16HPD group); and (III) VC−: no vascular calcificatio (20NPD+ 16NPD+ 8NPD+ 8HPD+ reference groups).

Table 3
Gene expression dataset of the 53 differentially expressed genes sorted by fold change at 20HPD group.

Name	Acc. #	Symbol	Fold change					
			8NPD	16NPD	20NPD	8HPD	16HPD	20HPD
Fatty acid synthase /// fatty acid synthase	NM_017332	Fasn	-0.3	-1.8	-1.1	-1.6	-2.7	-3.9
Solute carrier family 22 (organic cation transporter). member 2	NM_031584	Slc22a2	0.1	0.1	0.1	-0.1	0	-2.8
Acetyl-coenzyme A carboxylase alpha	NM_022193	Acac	-0.3	-1.4	-0.9	-0.9	-1.8	-2.1
FXYD domain-containing ion transport regulator 7	NM_022008	Fxyd7	-0.3	-0.1	-0.2	-0.6	0	-2.1
Heat shock 70kD protein 1B (mapped)	BI278231	Hs701b	-0.7	-0.6	-0.7	-0.5	-1.1	-2.1
Elastin *	J04035	Eln	-0.3	-0.2	-0.3	-0.4	0.5	-2
Malic enzyme 1 *	NM_012600	Me1	0	-1	-0.5	-1.1	-1.2	-2
RAS homolog gene family, member C (predicted)	AW915147	Rhot1	-0.1	-0.5	-0.3	-0.3	-0.1	-2
LRP16 protein	BI295768	LRP16	-0.7	-0.7	-0.7	-0.7	-0.4	-1.9
Myosin binding protein C, fast-type (predicted)	AW533848	Mybpc2	-0.9	-1.3	-1.1	-1.5	-0.9	-1.8
RAB3A interacting protein	NM_017313	RABIN3	-0.3	-0.1	-0.2	-0.3	-0.2	-1.8
Solute carrier family 2 (facilitated glucose transporter), member 4 *	NM_012751	Glut4	0	-0.5	-0.3	-0.6	-0.5	-1.8
Acetyl-Coenzyme A dehydrogenase, long-chain	NM_012819	Acadl	-0.2	-0.8	-0.5	-0.6	-0.7	-1.7
G protein-coupled receptor associatedsorting protein 1	NM_134386	pips	-0.8	-0.3	-0.6	-0.7	-0.5	-1.7
Glycerol-3-phosphate dehydrogenase 2, mitochondrial	U08027	mGPD	-0.2	-0.6	-0.4	-1.1	-0.8	-1.7
Muscle glycogen phosphorylase	BI275633	Muscpho	-0.4	-0.3	-0.5	-0.8	-0.3	-1.7
Tropomyosin 1, alpha ***	NM_019131	Tpm1	-0.7	-0.5	-0.6	-0.5	0.2	-1.7
ATP-binding cassette. sub-family G (WHITE), member 4	NM_053502	Abcg1	-0.5	0.1	-0.2	-0.2	0.2	-1.6
Glycerol-3-phosphate dehydrogenase 1 (soluble)	NM_022215	Gpd3	0	-1.3	-0.8	-1.1	-1.6	-1.6
ATPase. Na+/K+ transporting, beta 2 polypeptide	U45946	ATP1B2	-0.6	-0.3	-0.5	-0.7	-0.4	-1.5
ATP-binding cassette, sub-family A	AI502224	ABC1	-0.2	-0.1	-0.2	-0.3	-0.1	-1.5
Desmin	NM_022531	Des	-0.1	0.2	0.0	0	0.3	-1.5
Myomesin 1 (skelemin) 185kDa	BF284487	Myom1	0.1	0.1	-0.1	-0.4	-0.1	-1.5
Potassium voltage gated channel. Shab-related subfamily, member 1	NM_013186	Kcnb1	-0.6	-0.7	-0.7	-0.7	-0.6	-1.5
Procollagen. type V, alpha 3	NM_053638	Idh3a	-0.2	-1	-0.7	-0.9	-0.8	-1.5
Cadherin 13	NM_138889	Cdh13	0	0.1	0.1	0.2	0.4	1.5
Cadherin 23 (oto cadherin)	NM_053644	Cdh23	0.7	0.3	0.3	-0.1	0.5	1.5
Serine (or cysteine) peptidase inhibitor, clade G, member 1	AW915763	Spin2c	0.1	0.1	0.1	0.2	0.2	1.5
Cytochrome P450, family 1. subfamily b. polypeptide 1	NM_012940	Cyp1b1	-0.2	-0.1	-0.2	0.2	0	1.6
Programmed cell death 4	NM_017154	Xdh	0	-0.1	-0.1	0	0	1.6
Secreted frizzled-related protein 2 *	BF396545	Sfrp2	0.4	0.1	0.3	0.3	0.3	1.6
Cathepsin C *	NM_017097	Ctsc	0.4	-0.2	0.2	0.3	0	1.7
Cathepsin K	NM_031560	Ctsk	0.5	0.1	0.4	0.7	0.7	1.7
Cyclin D1 **	BI295861	Ccnd1	-0.1	0.4	0.2	-0.1	0.9	1.7
Integrin. alpha 6	AA955091	Itga6	-0.4	-0.6	-0.5	-0.4	-0.2	1.7
Cytochrome P450, family 4, subfamily b, polypeptide 1	M29853	Cyp4b1	-0.4	-0.7	-0.7	-0.3	0	1.8
Integrin alpha L	BI289767	Itall	-0.2	0.4	0.5	0.1	0.3	1.8
Procollagen C-endopeptidase enhancer protein	NM_019237	Pcolce	0.5	0.3	0.3	0.7	0.3	1.8
Cathepsin E	NM_012938	Ctse	-1	0.1	-0.4	-0.4	0.1	1.9
Cathepsin S	NM_017320	Ctss	0.3	0.2	0.4	0.6	0.4	2
Serine (or cysteine) proteinase inhibitor. clade B. member 1a	NM_057108	Serpinb1a	0.1	0.3	0.0	0.4	0.4	2
Procollagen, type XI, alpha 1***	AI136248	Col11a2	-0.5	0.3	0.3	0.2	0.3	2.1
Integrin beta 2	AI177292	Intb2	-0.2	-0.1	0.2	0.3	-0.1	2.2
Secreted frizzled-related protein 1	AW144660	Sfrp1	-0.2	0.5	0.2	-0.1	0.1	2.2
Cathepsin Z	AA849399	Ctsz	0.1	0.1	-0.5	0.1	0.2	2.3
Integrin binding sialoprotein	NM_012587	Ibsp	0	0	-0.1	-0.3	1.4	2.5
Cyclin B2 /// cyclin B2	AW253821	Ccnb2	0.7	0.6	0.1	1	0.3	2.6
Cadherin 22	NM_019161	Cdh22	0.7	0.1	0.5	0.7	0.3	2.8
Cytochrome P450, family 2. subfamily e. polypeptide 1	NM_031543	Cyp2e1	-1.2	-0.2	-0.5	0.4	2.3	3
Cyclin A2 /// cyclin A2	AA998516	Ccna2	0.6	0.6	0.3	1.7	0.9	3.2
Runt related transcription factor 1	NM_017325	Runx1	0.3	-0.1	0.1	-0.1	0.5	3.3
Secreted frizzled-related protein 4	AF220608	Sfrp4	-0.6	-0.9	-0.1	0.2	-0.2	3.4
Serine (or cysteine) peptidase inhibitor. clade A, member 3N	NM_031531	Spin2c	-0.4	0.2	-0.2	-0.1	0.1	4

The selected genes of interest are marked in grey. *2 probes representing the same gene. **3 probes representing the same gene. ***4 probes representing the same gene. Note the time-dependent trend across the different groups.

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

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

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

The inverse relationship between vascular calcification and bone mass/bone activity is well established in the literature mainly in the general population and/or osteoporotic patients but not in CKD patients. In the latter, all the published studies are observational, cross-sectional but never prospective, in addition some of them

associate bone with vascular calcification, using data not always obtained at the same time [21,35-39]. In addition, there is still some controversial results; recent papers has found the extent of vascular calcifications does not appear to be influenced by bone turnover and/or have any association with trabecular bone mass and connectivity when multivariate analysis was performed [40]. In our study, the rats which did not develop vascular calcifications showed a BMD trend similar to the reference group while the BMD of the rats from HPD groups was always lower than the matched NPD groups. Furthermore, our study demonstrates for the first time, that only the rats which did develop vascular calcifications showed a significant bone loss and they had the lowest BMD at all sites studied. In agreement with previous findings [41], the most remarkable effect of HPD took place in the cortical bone.

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

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

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

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

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

Recently, it has been described that SFRP2 expression is upregu lated in the fibrotic phase of myocardial infarction and that SFRP2 nu mice have shown reduced fibrosis and improved cardiac function [48 Interestingly, cardiovascular fibrosis is frequently accompanied b vascular calcification. SFRP-4 has been also described as a poter phosphaturic agent [49]; thus, we could also speculate that th increase of SFRP-4 gene expression might also be a reactiv mechanism to counteract the rise of serum phosphate observed i rats fed with HPD.

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

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References

[1] Cannata-Andia JB, Rodriguez-Garcia M, Carrillo-Lopez N, Naves-Diaz M, Dia Lopez B. Vascular calcifications: pathogenesis, management, and impact c clinical outcomes. J Am Soc Nephrol 2006;17:S267–73.
[2] Moe SM, Chen NX. Mechanisms of vascular calcification in chronic kidney diseas J Am Soc Nephrol 2008;19:213–6.
[3] Cannata-Andia JB, Rodriguez-Garcia M. Hyperphosphataemia as a cardiovascul risk factor—how to manage the problem. Nephrol Dial Transplant 2002;1 (Suppl 11):16–9.
[4] Block GA, Port FK. Re-evaluation of risks associated with hyperphosphatemia ar hyperparathyroidism in dialysis patients: recommendations for a change management. Am J Kidney Dis 2000;35:1226–37.
[5] Schoppet M, Shroff RC, Hofbauer LC, Shanahan CM. Exploring the biology vascular calcification in chronic kidney disease: what's circulating? Kidney I 2008;73:384–90.
[6] Giachelli CM. Vascular calcification mechanisms. J Am Soc Nephrol 2004;1 2959–64.
[7] Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, et Human vascular smooth muscle cells undergo vesicle-mediated calcification response to changes in extracellular calcium and phosphate concentrations: potential mechanism for accelerated vascular calcification in ESRD. J Am S Nephrol 2004;15:2857–67.
[8] Neven E, Dauwe S, De Broe ME, D'Haese PC, Persy V. Endochondral bone formatic is involved in media calcification in rats and in men. Kidney Int 2007;72:574–8
[9] Shao JS, Cai J, Towler DA. Molecular mechanisms of vascular calcification: lesso learned from the aorta. Arterioscler Thromb Vasc Biol 2006;26:1423–30.
[10] Rodriguez-Garcia M, Gomez-Alonso C, Naves-Diaz M, Diaz-Lopez JB, Diaz-Corte Cannata-Andia JB. Vascular calcifications, vertebral fractures and mortality haemodialysis patients. Nephrol Dial Transplant 2009;24:239–46.
[11] Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, Gomez-Alonso C, Cannata-Andia J Progression of vascular calcifications is associated with greater bone loss ar increased bone fractures. Osteoporos Int 2008;19:1161–6.

[12] Ormrod D, Miller T. Experimental uremia. Description of a model producing varying degrees of stable uremia. Nephron 1980;26:249–54.
[13] Gomez-Alonso C, Menendez-Rodriguez P, Virgos-Soriano MJ, Fernandez-Martin JL, Fernandez-Coto MT, Cannata-Andia JB. Aluminum-induced osteogenesis in osteopenic rats with normal renal function. Calcif Tissue Int 1999;64:534–41.
[14] Alvarez-Garcia O, Carbajo-Perez E, Garcia E, Gil H, Molinos I, Rodriguez J, et al. Rapamycin retards growth and causes marked alterations in the growth plate of young rats. Pediatr Nephrol 2007;22:954–61.
[15] Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A 2001;98:31–6.
[16] Carrillo-Lopez N, Alvarez-Hernandez D, Gonzalez-Suarez I, Roman-Garcia P, Valdivielso JM, Fernandez-Martin JL, et al. Simultaneous changes in the calcium-sensing receptor and the vitamin D receptor under the influence of calcium and calcitriol. Nephrol Dial Transplant 2008;23:3479–84.
[17] Lee HK, Braynen W, Keshav K, Pavlidis P. ErmineJ: tool for functional analysis of gene expression data sets. BMC Bioinformatics 2005;6:269.
[18] Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. J Cell Sci 2003;116:2627–34.
[19] Matias PJ, Ferreira C, Jorge C, Borges M, Aires I, Amaral T, et al. 25-Hydroxyvitamin D3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. Nephrol Dial Transplant 2009;24:611–8.
[20] Naves-Diaz M, Alvarez-Hernandez D, Passlick-Deetjen J, Guinsburg A, Marelli C, Rodriguez-Puyol D, et al. Oral active vitamin D is associated with improved survival in hemodialysis patients. Kidney Int 2008;74:1070–8.
[21] London GM, Marchais SJ, Guerin AP, Boutouyrie P, Metivier F, de Vernejoul MC. Association of bone activity, calcium load, aortic stiffness, and calcifications in ESRD. J Am Soc Nephrol 2008;19:1827–35.
[22] Moe S, Drueke T, Cunningham J, Goodman W, Martin K, Olgaard K, et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from kidney disease: improving global outcomes (KDIGO). Kidney Int 2006;69: 1945–53.
[23] Moe SM, Chen NX. Pathophysiology of vascular calcification in chronic kidney disease. Circ Res 2004;95:560–7.
[24] Almaden Y, Canalejo A, Hernandez A, Ballesteros E, Garcia-Navarro S, Torres A, et al. Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands in vitro. J Bone Miner Res 1996;11:970–6.
[25] Huttunen MM, Tillman I, Viljakainen HT, Tuukkanen J, Peng Z, Pekkinen M, et al. High dietary phosphate intake reduces bone strength in the growing rat skeleton. J Bone Miner Res 2007;22:83–92.
[26] Roman-Garcia P, Carrillo-Lopez N, Cannata-Andia JB. Pathogenesis of bone and mineral related disorders in chronic kidney disease: key role of hyperphosphatemia. J Ren Care 2009;35(Suppl 1):34–8.
[27] Silver J, Kilav R, Sela-Brown A, Naveh-Many T. Molecular mechanisms of secondary hyperparathyroidism. Pediatr Nephrol 2000;14:626–8.
[28] Silver J. Molecular mechanisms of secondary hyperparathyroidism. Nephrol Dial Transplant 2000;15(Suppl 5):2–7.
[29] Almaden Y, Hernandez A, Torregrosa V, Canalejo A, Sabate L, Fernandez Cruz L, et al. High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue in vitro. J Am Soc Nephrol 1998;9: 1845–52.
[30] Neves KR, Graciolli FG, dos Reis LM, Graciolli RG, Neves CL, Magalhaes AO, et al. Vascular calcification: contribution of parathyroid hormone in renal failure. Kidney Int 2007;71:1262–70.
[31] Cozzolino M, Staniforth ME, Liapis H, Finch J, Burke SK, Dusso AS, et al. Sevelamer hydrochloride attenuates kidney and cardiovascular calcifications in long-term experimental uremia. Kidney Int 2003;64:1653–61.
[32] Burke SK. Phosphate is a uremic toxin. J Ren Nutr 2008;18:27–32.
[33] Neves KR, Graciolli FG, dos Reis LM, Pasqualucci CA, Moyses RM, Jorgetti V. Adverse effects of hyperphosphatemia on myocardial hypertrophy, renal function, and bone in rats with renal failure. Kidney Int 2004;66:2237–44.
[34] Voormolen N, Noordzij M, Grootendorst DC, Beetz I, Sijpkens YW, van Manen JG, et al. High plasma phosphate as a risk factor for decline in renal function and mortality in pre-dialysis patients. Nephrol Dial Transplant 2007;22:2909–16.
[35] Adragao T, Herberth J, Monier-Faugere MC, Branscum AJ, Ferreira A, Frazao JM, et al. Low bone volume-a risk factor for coronary calcifications in hemodialysis patients. Clin J Am Soc Nephrol 2009;4:450–5.
[36] Braun J, Oldendorf M, Moshage W, Heidler R, Zeitler E, Luft FC. Electron beam computed tomography in the evaluation of cardiac calcification in chronic dialysis patients. Am J Kidney Dis 1996;27:394–401.
[37] Raggi P, James G, Burke SK, Bommer J, Chasan-Taber S, Holzer H, et al. Decrease in thoracic vertebral bone attenuation with calcium-based phosphate binders in hemodialysis. J Bone Miner Res 2005;20:764–72.
[38] Toussaint ND, Lau KK, Strauss BJ, Polkinghorne KR, Kerr PG. Associations between vascular calcification, arterial stiffness and bone mineral density in chronic kidney disease. Nephrol Dial Transplant 2008;23:586–93.
[39] London GM, Marty C, Marchais SJ, Guerin AP, Metivier F, de Vernejoul MC. Arterial calcifications and bone histomorphometry in end-stage renal disease. J Am Soc Nephrol 2004;15:1943–51.
[40] Coen G, Ballanti P, Mantella D, Manni M, Lippi B, Pierantozzi A, et al. Bone turnover, osteopenia and vascular calcifications in hemodialysis patients. A histomorphometric and multislice CT study. Am J Nephrol 2009;29:145–52.
[41] Bilezikian JP. Bone strength in primary hyperparathyroidism. Osteoporos Int 2003;14(Suppl 5):S113–5 discussion S115–7.
[42] Santamaria I, Alvarez-Hernandez D, Jofre R, Polo JR, Menarguez J, Cannata-Andia JB. Progression of secondary hyperparathyroidism involves deregulation of genes related to DNA and RNA stability. Kidney Int 2005;67:2267–79.
[43] Holmen SL, Giambernardi TA, Zylstra CR, Buckner-Berghuis BD, Resau JH, Hess JF, et al. Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. J Bone Miner Res 2004;19:2033–40.
[44] Al-Aly Z, Shao JS, Lai CF, Huang E, Cai J, Behrmann A, et al. Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr-/- mice. Arterioscler Thromb Vasc Biol 2007;27:2589–96.
[45] Towler DA, Shao JS, Cheng SL, Pingsterhaus JM, Loewy AP. Osteogenic regulation of vascular calcification. Ann N Y Acad Sci 2006;1068:327–33.
[46] Bodine PV, Zhao W, Kharode YP, Bex FJ, Lambert AJ, Goad MB, et al. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol 2004;18:1222–37.
[47] Shalhoub V, Shatzen E, Henley C, Boedigheimer M, McNinch J, Manoukian R, et al. Calcification inhibitors and Wnt signaling proteins are implicated in bovine artery smooth muscle cell calcification in the presence of phosphate and vitamin D sterols. Calcif Tissue Int 2006;79:431–42.
[48] Kobayashi K, Luo M, Zhang Y, Wilkes DC, Ge G, Grieskamp T, et al. Secreted Frizzled-related protein 2 is a procollagen C proteinase enhancer with a role in fibrosis associated with myocardial infarction. Nat Cell Biol 2009;11:46–55.
[49] Berndt T, Craig TA, Bowe AE, Vassiliadis J, Reczek D, Finnegan R, et al. Secreted frizzled-related protein 4 is a potent tumor-derived phosphaturic agent. J Clin Invest 2003;112:785–94.

Publicación 6: The connections between vascular calcification and bone health.

Aceptada "con cambios" para su publicación en Nephrology, Dialysis and Transplantation.

The connections between vascular calcification and bone health

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Abstract

Vascular calcification, bone loss and increased fracture risk are age-associated disorders. Several epidemiological studies have suggested a relationship between vascular calcification, impaired bone metabolism and increased mortality. So far, this relationship had been underestimated as osteoporosis and vascular calcification have been considered non-modifiable disorders of aging. Recent data suggest that this association is not simply an artefact of age, stressing that the coincidence of vascular calcification with low bone activity and osteoporosis could be biologically linked.

During the vascular calcification development, the transition of vascular smooth muscle cells towards an osteoblast-like phenotype promotes the release of the vesicular structures, and mineralization within these structures is promoted by several players, including those related to mineral metabolism, like phosphorus or PTH, which influence the expression of osteogenic factors.

However, an intriguing question is whether the presence of well established and severe vascular calcification can have an impact on bone metabolism, thus demonstrating true cross-talk between these tissues. Some evidence is now emerging, suggesting that some inhibitors of the Wnt pathway, such as secreted frizzled proteins 2 and 4 and DKK-1, may play a role linking vascular calcification and bone loss.

An additional important question to answer, from the patient's perspective, is whether or not progression of vascular calcification can be prevented or restricted. Several evidences suggest serum phosphorus control as a main target to maintain normal bone turnover and protect against vascular calcification.

Introduction

Vascular calcification, bone loss and increased fracture risk are disorders associated with ageing, both in patients with chronic kidney disease (CKD) [21,22,32,33,38,46,53] and in the general population [28,44,45,47]. Furthermore, several epidemiological studies suggest a relationship between vascular calcification, impaired bone metabolism and increased mortality [9,19,28,52,63]. The relationship between bone metabolism and vascular calcification was a component of the decision made by the Kidney Disease: Improving Global Outcomes (KDIGO) foundation to adopt a new nomenclature, the CKD–Mineral and Bone Disorder (CKD–MBD), for a syndrome of CKD incorporating disturbances in mineral metabolism, vascular calcification, renal osteodystophy and excessive mortality [40]. This review is focused on the system biology between the skeleton, mineral metabolism and vascular calcification that is disordered in the CKD-MBD.

Bone turnover and vascular calcification

The association between bone fragility and vascular calcification has been made repeatedly since a significant inverse correlation between bone mineral density and aortic calcification was reported 20 years ago [19]. However, this association was probably underestimated because osteoporosis and vascular calcification were considered non-modifiable disorders of aging. Recent data suggest that this association is not simply an artefact of age. The role of ageing cannot be completely dismissed, but the clinical coincidence of vascular calcification with low bone activity and osteoporosis suggests that there are direct biological links between arteriosclerosis and osteoporosis, and the coincidence is supported by pathological science.

In support of this concept, a study published in 2004 demonstrated that patients with the highest degree of aortic calcification had the lowest bone density [55]. In the same cohort followed for 2 years, bone loss was greater in patients with progressive vascular calcification [55]. In agreement with these results, a recent study from one of our groups has shown that

after 4 years of follow up, subjects with the most severe aortic calcification had not only a lower bone mass, but also a higher incidence of new osteoporotic fractures [45].

In addition, another recent study from the same group, involving patients on haemodialysis, demonstrated that vascular calcification of the large and medium calibre arteries was associated with an increased risk of vertebral fractures [53]. Both vascular calcification and vertebral fractures were associated with increased mortality in women participating in this study.

A relationship between vascular calcification and low bone turnover assessed by histomorphometric markers has also been demonstrated in haemodialysis patients [33] (Table 1). Preliminary data have demonstrated a negative relationship between low bone turnover and the degree of coronary artery calcification [5]. Data demonstrating an inverse relationship between mineralized bone volume and both coronary calcification and vascular stiffness have also been recently published [1].

Despite all of this evidence, the relationship between low bone turnover and vascular calcification remains under debate. A recent publication found that vascular calcification was not influenced by bone turnover when multivariate analysis was performed [12]. This is likely due to the fact that both high and low turnover was assessed. In fact, it is not bone turnover itself that is related to vascular calcification, but rather that bone resorption is in excess of bone formation, which can occur at any rate of turnover. This concept has been proven in the several phase three osteoporosis trials, especially with denosumab, wherein, inhibition of bone resorption and equalization with formation results in a major reduction of the serum calcium and phosphorus. These results demonstrate that the serum calcium and phosphorus, although normal in concentration, are being controlled through excess bone resorption. In agreement with this concept, it has been reported that the correction of the balance in bone turnover, either high or low, protects against the progression of vascular calcification [6]. This is

in agreement with translational studies demonstrating that stimulation of bone formation in CKD stage 3-4 corrected hyperphosphatemia [15]. Overall, most of the epidemiological, clinical and translational evidence strongly suggest that the incidence and progression of vascular calcification is inversely related to bone mass and positively related with the degree of mineralized bone loss, and thus with the incidence and prevalence of osteoporotic fragility fractures.

The low turnover osteoporosis of aging is not the only disorder of mineral metabolism that has been linked to vascular calcification. In patients with different stages of CKD, the serum phosphorus is strongly associated with increased vascular calcification and decreased bone strength. Indeed, abnormally high serum phosphorus concentrations have been described as one of the main pathogenetic factors inducing vascular calcification [41,42]. In contrast PTH levels have and have not been associated with vascular calcification [4]. A recent metanalysis demonstrates that of the serum phosphorus, calcium and PTH, only phosphorus is associated with cardiovascular events and mortality associated with vascular calcification [48].

Pathophysiology of vascular calcification

Vascular calcification in patients with CKD occurs through precipitation of calcium phosphate as a consequence of unstable supersaturation of the exchangable calcium and phosphate pools. However, the process is not solely a passive one related to precipitation from the extracellular fluid surrounding vascular smooth muscle cells (VSMCs) of the vascular walls. Rather VSMCs undergo a transition away from their contractile functional state, expressing markers of their osteoblast cousins and develop an exchangable calcium/phosphorus pool analogous to the site of bone formation wherein calcification of the skeleton occurs. The analogy to bone formation is especially strong in atherosclerotic calcification of the neointima stimulated by CKD [37]. In the other form of vascular calcification stimulated by CKD, medial arterial calcification, the process represents a complex set of steps in which the normal inhibitors of calcification are diminished and concentrations of calcium and phosphorus

produce unstable supersaturation leading to crystal formation and vascular calcification. Calcification appears to be initiated by the release of vesicular structures from VSMCs that contain hydroxyapatite [57]. The transition of VSMCs towards the osteoblastic phenotype promotes the release of the vesicular structures, and mineralization within these structures is promoted by expression of osteoblastic proteins. Osteoblastic morphogens, the bone morphogenetic proteins (BMP)-2 and BMP-4, transcription factors, core-binding factor 1 (Cbfa-1, also know as Runx2), a key transcription factor in osteoblast differentiation, and bone proteins such as alkaline phosphatase and osteocalcin are all components of the osteoblastic transition of VSMCs [37].

The factors that are involved in this change in VSMC phenotype have been the focus of much research in recent years, with evidence suggesting that it is driven both by an increase in factors that promote this change and a decrease in factors that inhibit it. In recent times, a host of these calcification promoters and inhibitors have been identified, some of which may be systemic and others localized (Figure 1). The relative importance of these factors is unclear, and it is likely that some play more of a role in the progression of soft tissue calcification rather than in its initiation.

Several factors related to mineral metabolism have been shown to promote calcification. For example, it has been demonstrated *in vitro* that calcium and (in particular) phosphorus levels induced VSMC calcification [51]. As stated above, serum phosphorus seems to be particularly important in the development of vascular calcification. Indeed, serum phosphorus may well be the link between bone turnover and vascular calcification. When bone turnover is low, as in the adynamic bone disorder, the size of the exchangable phosphorus pool is reduced leading to larger excursions in the concentrations associated with intake. In addition, bone resorption is in excess of formation serving to block the resovoir function of the skeleton for excess phosphorus. In the case of high bone turnover, as in secondary hyperparathyroidism, phosphorus is released from bone and again the reservoir function of the skeleton is

compromised. Stimulation of skeletal anabolism and increasing bone formation rates above rates of resorption reduce hyperphosphatemia, demonstrating restoration of the resovoir function of the skeleton. In these studieswith BMP-7 as the anabolic principle vascular calcification was reduced in part by movement of phosphorus into the skeleton [15,16]. As renal excretion of phosphorus was not increased in these studies, the decrease in phosphorus levels must have been due to the increase in bone formation. Treatment with phosphate binders, which may serve to decrease the supersaturation of the exchangeable Ca-Pi pool, has also been shown to prevent vascular calcification in mouse models [36].

Phosphorus has also been shown to directly stimulate the osteoblastic transition of VSMC in CKD (26,27,31,32). The mechanism of phosphorus in inducing calcification has been explored. Knockdown of the putative phosphate sensor, the sodium-dependent phosphate co-transporter, Pit-1, by siRNA inhibited phosphorus-stimulated mineralization of VSMCs [31]. This indicated that vascular calcification may be regulated by cellular uptake of phosphorus. Extracellular Pi signaling or increased intracellular phosphorus stimulate VSMCs to undergo transition to an osteoblastic phenotype, expressing Runx2, Msx2 and osterix the critical osteoblast transcription factors promoting the expression of the osteoblast transcriptome [20] and stimulating matrix vesicles (Figure 2). An additional role for serum phosphorus (and calcium) may be to promote VSMC apoptosis, contributing to the initiation of calcification, since apoptotic bodies may function similarly to matrix vesicles in heterotopic mineralization.

The concept of the vasculature under the influence of osteoblastic transition acting as a new reservoir for phosphorus since deposition is in excess or resorption may explain why vascular calcification is present before hyperphosphataemia is detected [17,34]. Vascular calcification has been detected in 47% of non-diabetic patients with Stage 4 CKD [58] and up to 94% of predialysis patients with diabetes [39], but hyperphosphataemia usually only manifests in Stage 4 and 5 CKD [13]. Data suggest that fibroblast growth factor (FGF)-23 levels increase early in CKD and may be a marker of increased phosphorus load ahead of the development of

hyperphosphataemia [17]. Current evidence on the association of FGF-23 with vascular calcification is mixed, but recent studies demonstrate a positive and independent association with aortic calcification especially in early CKD in translational models and in patients [43]. Elevations in FGF23, a hormone secreted by the osteocyte, in early CKD indicate that the skeleton has been affected by renal damage. Then becomes what is the signal for the osteocyte to secrete FGF23? While this remains to be proven, changes in the Ca-Pi exchangeable pool are leading contenders. Thus in CKD prior to hyperphosphatemia, the changes in the systemic environment produced by a high phosphorus load and a blocked skeletal reservoir, leads to vascular calcification which acts together with increased renal excretion to maintain normal serum phosphorus concentrations.

Other mineral metabolism parameters that may contribute to the development and progression of vascular calcification include vitamin D and PTH. VSMCs express vitamin D receptors [26] and pharmacological calcitriol doses induce matrix mineralization of VSMCs *in vitro* [27]. However, the physiological function of vitamin D receptor activation in VSMCs is inhibitory to matrix mineralization through stimulation of smooth muscle differentiation and repression of osteoblastic transition [35]. Patients with CKD generally have low levels of vitamin D and treatment with vitamin D has been shown to reduce mortality [29]. Both vitamin D/calcitriol deficiency and pharmacological doses of active vitamin D analogues stimulate vascular calcification, suggesting a biphasic dose response and underscoring the protective inhibitory physiological actions of calcitriol.

The role of PTH is also unclear. PTH fragment 1–34 has been shown to inhibit calcification in a murine model of atherosclerotic vascular calcification [56], but PTH 7–84 may act to increase vascular calcification [62] and high PTH levels are often associated with high calcification scores [12]. Experiments performed by Jorgetti and collaborators demonstrated that PTH itself is not able to induce vascular calcification, but has a synergistic effect with the phosphorus, probably due to an indirect and deleterious effect associated with bone remodelling and

osteoclastic activity [23]. A recent meta-analysis of factors related to vascular calcification and mortality has reinforced the role of Pi as a cardiovascular risk factor but failed to indentify the role of PTH [48].

Soft tissue calcification occurs in some patients with CKD well before mineral metabolism is impaired, and recent studies demonstrate onset of vascular calcification in Stage 2 CKD before stimulation of osteoblastic transition is demonstrable. Uraemic serum has been shown to induce osteoblast-like changes in VSMCs, even when the effect of phosphorus is restricted by blocking Pit-1 [42]. Inflammation and reactive oxygen species are two factors that have been associated with vascular calcification. It has been demonstrated that tumour necrosis factor-α promotes osteogenesis of aortic smooth muscle cells *in vitro* [60] and calcification scores correlate with concentrations of C-reactive protein and interleukin 6 [30]. In addition, hydrogen peroxide has been reported to stimulate Cbfa-1 directly [8]. Another candidate is BMP-2, which is high in uraemic serum [10]; it increases osteoblastic differentiation of calcifying cells and may also reduce expression of matrix Gla protein, a calcification inhibitor [65]. Leptin is another molecule that has been shown to induce calcification *in vitro* [60]. In summary, there are many molecules that may be present in uraemic serum that promote vascular calcification and it is unlikely that there is only one definitive initiating factor. Additionally, CKD may inhibit VSMC differentiation, a permissive step for osteoblastic transition.

Vascular calcification inducing bone loss

It is clear that impaired bone metabolism and its consequences have an important role in the development of vascular calcification. However, an intriguing question is whether the presence of well established and severe vascular calcification can have an impact on bone metabolism, thus demonstrating true cross-talk between these tissues. Some evidence is now emerging.

In a recent study, rats fed a high phosphorus diet and the high fat fed LDLR-/- mice showed a significant increase in aortic calcification and a decrease in bone mass [54]. A microarray analysis of the areas with severe vascular calcification showed over-expression of the family of secreted frizzled-related proteins (SFRPs) [48]. The SFRPs are circulating wingless/int (Wnt) protein inhibitors. Induction of interstitial nephritis is associated with up-regulation of SFRP4, SFRP2, and DDK1 in the vascular adventia [59]. SFRPs and DKK1 are inhibitors of the canonical signalling Wnt pathway, which is actively involved in bone formation and vascular calcification [2,25,61]. This increase in SFRPs in areas of severe vascular calcification may be indicative of a wall artery defensive mechanism triggered in order to block the activation of the Wnt pathway, with the aim of attenuating mineralization in the calcified aortic wall. As the SFRPs are secreted circulating proteins, they can act not only locally in the artery wall to reduce mineralization, but also in bone to impair mineralization, resulting in reduced bone mass.

This new and challenging feedback hypothesis may help to explain the results observed in epidemiological studies of the general and CKD population. The evidence suggests that more severe cases of progressive vascular calcification were associated to greater bone loss, and more frequent bone fractures.

The need for further research

The association between impaired bone health and vascular calcification has sparked tremendous research effort in recent years. However, it is clear that for some questions, definitive answers are still being sought.

Can we definitely say that vascular calcification is a consequence of low bone turnover? In our view, it is clear that low bone turnover represents an environment that encourages vascular calcification, particularly given the established role of phosphorus in promoting vascular calcification. It can be speculated that patients with aging or CKD and adynamic bone disorder are particularly at risk of the damaging effects of phosphorus. The strong emerging consensus from observational studies suggests that Pi is a cardiovascular risk, and this risk is heightened in the aged osteoporotic or CKD patient. Low bone turnover is a powerful trigger for the development of abnormalities in the exchangable Pi pool that stimulates vascular calcification. However, increased bone turnover is also present in the CKD population, and this is also likely to increase the risk of vascular calcification, again via the resulting impaired calcium and phosphorus metabolism. No doubt other factors also trigger calcification, and inflammation may be particularly important in patients with diabetes.

One of the most important questions to answer from the patient's perspective is whether or not progression of vascular calcification can be prevented or restricted. In considering this, it is helpful that more is now known about the pathogenesis of this potentially fatal complication of CKD and clear modifiable targets are being identified. Efforts to maintain normal mineral metabolism, and thus bone health, are at the heart of strategies to prevent soft tissue calcification. A clear target is the control of serum phosphorus. Several phosphate binders are available, some of which contain calcium. Whether calcium-based binders contribute to the progression of vascular calcification has been a matter of much debate. Some studies have shown that non-calcium-based binders may attenuate vascular calcification in comparison with calcium-based agents [7,11], whereas others have not [49]. It has been suggested that the use of calcium-based agents may be of particular concern in patients with

adynamic bone disease [32]. Given the clearer evidence for the role of phosphorus, physicians should perhaps give greater consideration to the ability of phosphate binders to reduce serum phosphorus levels and maintain good bone health. Treatment with lanthanum carbonate has been shown to lead to beneficial changes in bone histomorphometry in patients with either high or low turnover bone disease [14]. As phosphorus load appears to increase ahead of the development of hyperphosphataemia this could conceivably contribute to calcification; phosphorus restriction before hyperphosphataemia occurs is therefore an intriguing prospect. Studies assessing the effect of phosphate binders in patients with normal serum phosphorus levels are ongoing and the results will be of interest.

The effect of vitamin D treatments on vascular calcification in patients with CKD is still unclear, but several studies have shown a survival benefit associated with vitamin D. This benefit seems more evident with low–dose treatment, in a range of physiological replacement [46]. Some evidence suggests that the calcimimetic cinacalcet may protect against vascular calcification in patients on dialysis [3], but the clinical evidence is as yet limited. A recent study investigated the use of cinacalcet plus low-dose vitamin D therapy, compared with vitamin D therapy alone, on coronary artery calcification [18]. Results showed a trend towards attenuation of the progression of coronary artery calcification, although the difference in calcification scores between groups did not reach statistical significance [50]. Any beneficial effects of cinacalcet may be confined to patients on dialysis; in the early stages of CKD, the actions of cinacalcet on PTH lead to an unwanted increase in serum phosphorus levels [18].

Agents that act directly on bone may also be effective in attenuating calcification. Preclinical studies have suggested the potential for inhibition of the receptor activator of NF-kappaβ ligand [24], and a potential role for the skeletal anabolic BMP-7 [16].

Summary

There is good evidence to suggest that impaired bone turnover, particularly low bone turnover, promotes the progression of vascular calcification. Several factors have been identified as possible links between bone and calcifying soft tissues, but a greater understanding of the key determinants of vascular calcification is still required. Maintenance of good bone health appears to be critical to maintaining good cardiovascular health in patients with CKD. Intriguingly, the original rationale for controlling serum phosphorus levels was to maintain bone health and it would appear that we have to focus again on this aspect of treatment to reduce cardiovascular mortality. Phosphate binders offer an effective approach to maintaining normal bone turnover and are likely to help to protect against vascular calcification.

Disclosures

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Author's contributions
JCA designed and wrote the revision, PRG wrote the revision and the figure 1 and KH designed and wrote the revision and made figure 2 and Table1

References

1. Adragao T, Herberth J, Monier-Faugere MC, et al. Low bone volume--a risk factor for coronary calcifications in hemodialysis patients. Clin J Am Soc Nephrol 2009; 4:450-5.

2. Al-Aly Z, Shao JS, Lai CF, et al. Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr-/- mice. Arterioscler Thromb Vasc Biol 2007; 27:2589-96.

3. Aladren Regidor MJ. Cinacalcet reduces vascular and soft tissue calcification in secondary hyperparathyroidism (SHPT) in hemodialysis patients. Clin Nephrol 2009; 71:207-13.

4. Almaden Y, Canalejo A, Hernandez A, et al. Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands in vitro. J Bone Miner Res 1996; 11:970-6.

5. Asci G, Ozkahya M, Duman S, et al. The link between cardiovascular and bone disease in hemodialysis patients. Nephrol Dial Transplant Plus 2007; 22:iv217 (Free comunication at XLIII ERA-EDTA Congress 2007).

6. Barreto DV, Barreto Fde C, Carvalho AB, et al. Association of changes in bone remodeling and coronary calcification in hemodialysis patients: a prospective study. Am J Kidney Dis 2008; 52:1139-50.

7. Block GA, Spiegel DM, Ehrlich J, et al. Effects of sevelamer and calcium on coronary artery calcification in patients new to hemodialysis. Kidney Int 2005; 68:1815-24.

8. Byon CH, Javed A, Dai Q, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. J Biol Chem 2008; 283:15319-27.

9. Cannata-Andia JB, Rodriguez-Garcia M, Carrillo-Lopez N, et al. Vascular calcifications: pathogenesis, management, and impact on clinical outcomes. J Am Soc Nephrol 2006; 17:S267-73.

10. Chen NX, Duan D, O'Neill KD, et al. The mechanisms of uremic serum-induced expression of bone matrix proteins in bovine vascular smooth muscle cells. Kidney Int 2006; 70:1046-53.

11. Chertow GM, Burke SK, Raggi P. Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients. Kidney Int 2002; 62:245-52.

12. Coen G, Ballanti P, Mantella D, et al. Bone turnover, osteopenia and vascular calcifications in hemodialysis patients. A histomorphometric and multislice CT study. Am J Nephrol 2009; 29:145-52.

13. Craver L, Marco MP, Martinez I, et al. Mineral metabolism parameters throughout chronic kidney disease stages 1-5 - achievement of K/DOQI target ranges. Nephrol Dial Transplant 2007; 22:1171-6.

14. D'Haese PC, Spasovski GB, Sikole A, et al. A multicenter study on the effects of lanthanum carbonate (Fosrenol) and calcium carbonate on renal bone disease in dialysis patients. Kidney Int Suppl 2003:S73-8.

15. Davies MR, Lund RJ, Hruska KA. BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. J Am Soc Nephrol 2003; 14:1559-67.

16. Davies MR, Lund RJ, Mathew S, et al. Low turnover osteodystrophy and vascular calcification are amenable to skeletal anabolism in an animal model of chronic kidney disease and the metabolic syndrome. J Am Soc Nephrol 2005; 16:917-28.

17. Fang Y, Zhang Y, Mathew S, et al. Early Chronic Kidney Disease (CKD) Stimulates Vascular Calcification (VC) and Decreased Bone Formation Rates Prior to Positive Phosphate Balance. J Am Soc Nephrol 2009; 20:Free communication.

18. Floege J, Raggi P, Block GA, et al. Study design and subject baseline characteristics in the ADVANCE Study: effects of cinacalcet on vascular calcification in haemodialysis patients. Nephrol Dial Transplant 2010; 25:1916-23.

19. Frye MA, Melton LJ, 3rd, Bryant SC, et al. Osteoporosis and calcification of the aorta. Bone Miner 1992; 19:185-94.

20. Giachelli CM. Vascular calcification mechanisms. J Am Soc Nephrol 2004; 15:2959-64.

21. Goldsmith D, Ritz E, Covic A. Vascular calcification: a stiff challenge for the nephrologist: does preventing bone disease cause arterial disease? Kidney Int 2004; 66:1315-33.

22. Goodman WG, Goldin J, Kuizon BD, et al. Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med 2000; 342:1478-83.

23. Graciolli FG, Neves KR, dos Reis LM, et al. Phosphorus overload and PTH induce aortic expression of Runx2 in experimental uraemia. Nephrol Dial Transplant 2009; 24:1416-21.

24. Helas S, Goettsch C, Schoppet M, et al. Inhibition of receptor activator of NF-kappaB ligand by denosumab attenuates vascular calcium deposition in mice. Am J Pathol 2009; 175:473-8.

25. Holmen SL, Giambernardi TA, Zylstra CR, et al. Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. J Bone Miner Res 2004; 19:2033-40.

26. Inoue T, Kawashima H. 1,25-Dihydroxyvitamin D3 stimulates 45Ca2+-uptake by cultured vascular smooth muscle cells derived from rat aorta. Biochem Biophys Res Commun 1988; 152:1388-94.

27. Jono S, Nishizawa Y, Shioi A, et al. 1,25-Dihydroxyvitamin D3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. Circulation 1998; 98:1302-6.

28. Kiel DP, Kauppila LI, Cupples LA, et al. Bone loss and the progression of abdominal aortic calcification over a 25 year period: the Framingham Heart Study. Calcif Tissue Int 2001; 68:271-6.

29. Kovesdy CP, Ahmadzadeh S, Anderson JE, et al. Association of activated vitamin D treatment and mortality in chronic kidney disease. Arch Intern Med 2008; 168:397-403.

30. Krasniak A, Drozdz M, Pasowicz M, et al. Factors involved in vascular calcification and atherosclerosis in maintenance haemodialysis patients. Nephrol Dial Transplant 2007; 22:515-21.

31. Li X, Yang HY, Giachelli CM. Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. Circ Res 2006; 98:905-12.

32. London GM, Marchais SJ, Guerin AP, et al. Association of bone activity, calcium load, aortic stiffness, and calcifications in ESRD. J Am Soc Nephrol 2008; 19:1827-35.

33. London GM, Marty C, Marchais SJ, et al. Arterial calcifications and bone histomorphometry in end-stage renal disease. J Am Soc Nephrol 2004; 15:1943-51.

34. Mathew S, Lund R, Hruska K. Mechanisms of the Inverse Relationship between Vascular Calcification (VC) and Bone Mineral Density (BMD) in Chronic Kidney Disease (CKD). J Am Soc Nephrol 2008; 19:Free comunication.

35. Mathew S, Lund RJ, Chaudhary LR, et al. Vitamin D receptor activators can protect against vascular calcification. J Am Soc Nephrol 2008; 19:1509-19.

36. Mathew S, Lund RJ, Strebeck F, et al. Reversal of the adynamic bone disorder and decreased vascular calcification in chronic kidney disease by sevelamer carbonate therapy. J Am Soc Nephrol 2007; 18:122-30.

37. Mathew S, Tustison KS, Sugatani T, et al. The mechanism of phosphorus as a cardiovascular risk factor in CKD. J Am Soc Nephrol 2008; 19:1092-105.

38. Matias PJ, Ferreira C, Jorge C, et al. 25-Hydroxyvitamin D3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. Nephrol Dial Transplant 2009; 24:611-8.

39. Merjanian R, Budoff M, Adler S, et al. Coronary artery, aortic wall, and valvular calcification in nondialyzed individuals with type 2 diabetes and renal disease. Kidney Int 2003; 64:263-71.

40. Moe S, Drueke T, Cunningham J, et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2006; 69:1945-53.

41. Moe SM, Chen NX. Mechanisms of vascular calcification in chronic kidney disease. J Am Soc Nephrol 2008; 19:213-6.

42. Moe SM, Chen NX. Pathophysiology of vascular calcification in chronic kidney disease. Circ Res 2004; 95:560-7.

43. Nasrallah MM, El-Shehaby AR, Salem MM, et al. Fibroblast growth factor-23 (FGF-23) is independently correlated to aortic calcification in haemodialysis patients. Nephrol Dial Transplant 2010; 25:2679-85.

44. Naves Diaz M, Diaz Lopez JB, Gomez Alonso C, et al. [Study of incidence of osteoporotic fractures in a cohort of individuals older than 50 years from Asturias, Spain, after a 6 year follow-up period]. Med Clin (Barc) 2000; 115:650-3.

45. Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, et al. Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. Osteoporos Int 2008; 19:1161-6.

46. Naves-Diaz M, Alvarez-Hernandez D, Passlick-Deetjen J, et al. Oral active vitamin D is associated with improved survival in hemodialysis patients. Kidney Int 2008; 74:1070-8.

47. O'Neill TW, Felsenberg D, Varlow J, et al. The prevalence of vertebral deformity in european men and women: the European Vertebral Osteoporosis Study. J Bone Miner Res 1996; 11:1010-8.

48. Palmer SC, Hayen A, Macaskill P, et al. Serum levels of phosphorus, parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. Jama 2011; 305:1119-27.

58. Sigrist M, Bungay P, Taal MW, et al. Vascular calcification and cardiovascular function in chronic kidney disease. Nephrol Dial Transplant 2006; 21:707-14.

59. Surendran K, Schiavi S, Hruska KA. Wnt-dependent beta-catenin signaling is activated after unilateral ureteral obstruction, and recombinant secreted frizzled-related protein 4 alters the progression of renal fibrosis. J Am Soc Nephrol 2005; 16:2373-84.

60. Tintut Y, Patel J, Parhami F, et al. Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. Circulation 2000; 102:2636-42.

61. Towler DA, Shao JS, Cheng SL, et al. Osteogenic regulation of vascular calcification. Ann N Y Acad Sci 2006; 1068:327-33.

62. Vattikuti R, Towler DA. Osteogenic regulation of vascular calcification: an early perspective. Am J Physiol Endocrinol Metab 2004; 286:E686-96.

63. Vogt MT, San Valentin R, Forrest KY, et al. Bone mineral density and aortic calcification: the Study of Osteoporotic Fractures. J Am Geriatr Soc 1997; 45:140-5.

64. Yang H, Curinga G, Giachelli CM. Elevated extracellular calcium levels induce smooth muscle cell matrix mineralization in vitro. Kidney Int 2004; 66:2293-9.

65. Zebboudj AF, Shin V, Bostrom K. Matrix GLA protein and BMP-2 regulate osteoinduction in calcifying vascular cells. J Cell Biochem 2003; 90:756-65.

TABLE LEGEND

Table 1. Correlation of bone histomorphometric parameters and parathyroid hormone levels with calcification score. Adapted from London *et al.* 2004 [33] with permission.

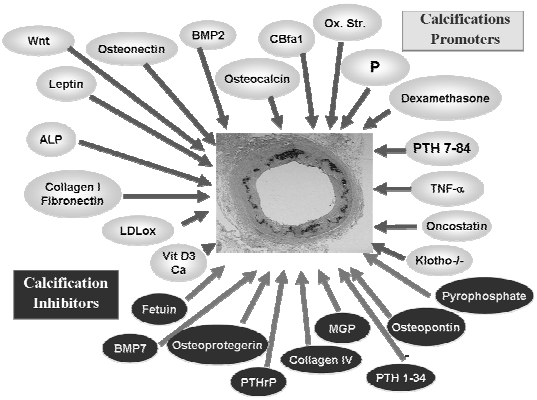
Range 0–4, where 0 = no calcification detected and 4 = generalized calcification in all arterial segments examined. PTH, parathyroid hormone.

FIGURE LEGENDS

Fig. 1. Promoters and inhibitors of vascular calcification Modified from a picture produced by Professor G. London with permission.

ALP, alkaline phosphatase; BMP, bone morphogenetic protein; Ca, calcium; CBfa1, core-binding factor 1 (also known as Runx2); LDLox, oxidized low-density lipoprotein; MGP, matrix GLA protein; P, phosphorus; PTH, parathyroid hormone; PTHrP, parathyroid-hormone-related protein; TNF-α, tumour necrosis factor alpha; Vit D_3, calcitriol; Wnt, wingless/int proteins.

Fig. 2. The active role of phosphorus in vascular calcification. Adapted from Yang *et al.* 2004 [64] with permission; Ca, calcium; Na, sodium; P, phosphorus; Pit-1, sodium-dependent phosphate co-transporter; SMC, smooth muscle cell; VSMC, vascular smooth muscle cell.


Calcifications Promoters
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Fibronectin
TNF-α
LDLox
Oncostatin
Vit D3
Ca
Klotho-/-
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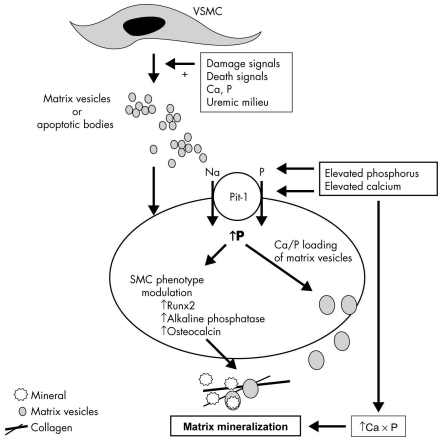
VSMC
Damage signals
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or
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Na
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Pit-1
Elevated phosphorus
Elevated calcium
↑P
Ca/P loading
of matrix vesicles
SMC phenotype
modulation
↑Runx2
↑Alkaline phosphatase
↑Osteocalcin
Mineral
Matrix vesicles
Collagen
Matrix mineralization
↑Ca × P

Parameter	Arterial calcification score*					Trend (*P* value)
	0	1	2	3	4	
Osteoclast resorption (%)	3.4	2.31	1.81	1.73	0	0.0001
Osteoclasts (/mm^2)	1.4	1.88	1.62	1.12	0.12	0.002
Osteoblast surface (%)	13.4	11.9	9.56	4.11	1.00	0.0001
PTH (pg/mL)	388	567	316	202	71	0.0001

Publicacion 7: Natural antioxidants and vascular calcification: A possible benefit?

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Abstract: Several studies have demonstrated the impact of vascular calcification on morbidity and mortality in general population and chronic kidney disease. The process of vascular calcification involves complex mechanisms including the overexpression of genes and proteins associated with mineralization and increments of reactive oxygen species (ROS). Taking into account the previous findings, we decided to analyze in vitro the likely inhibitory effect of natural antioxidants in the process of vascular calcification.
Primary vascular smooth muscle cells (VSMCs) were cultured with either normal medium or normal medium supplemented with calcium and phosphorus in combination with several antioxidants. Mineralization, intracellular reactive oxygen species (ROS) levels and the protein expression of Runx2 and Mn-Superoxide Dismutase-2 (SOD-2) were investigated.
Curcumin and sylibyn were the more effective inhibiting both ROS increase and VSMC mineralization. Curcumin was able to prevent the increase in Runx2 expression, but did not modify SOD-2 expression in the VSMCs cultured with the P + Ca medium.
These findings support the convenience of performing further studies in this field as some antioxidants might have potential benefits in the management of vascular calcification.

Suggested Reviewers:

Opposed Reviewers:

Natural antioxidants and vascular calcification: A posible benefit?

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Short Title: Antioxidants and vascular calcification

Abstract

Several studies have demonstrated the impact of vascular calcification on morbidity and mortality in general population and chronic kidney disease. The process of vascular calcification involves complex mechanisms including the overexpression of genes and proteins associated with mineralization and increments of reactive oxygen species (ROS). Taking into account the previous findings, we decided to analyze *in*

vitro the likely inhibitory effect of natural antioxidants in the process of vascular calcification.

Primary vascular smooth muscle cells (VSMCs) were cultured with either normal medium or normal medium supplemented with calcium and phosphorus in combination with several antioxidants. Mineralization, intracellular reactive oxygen species (ROS) levels and the protein expression of Runx2 and Mn-Superoxide Dismutase-2 (SOD-2) were investigated.

Curcumin and sylibyn were the more effective inhibiting both ROS increase and VSMC mineralization. Curcumin was able to prevent the increase in Runx2 expression, but did not modify SOD-2 expression in the VSMCs cultured with the P + Ca medium.

These findings support the convenience of performing further studies in this field as some antioxidants might have potential benefits in the management of vascular calcification.

Introduction

Several studies have demonstrated the impact of vascular calcification on morbidity and mortality in general population and chronic kidney disease (CKD) (1, 2). The process of vascular calcification involves not only the physical deposition of calcium and phosphate but also a series of complex mechanisms including the overexpression of genes and proteins associated with mineralization (3). In addition, it has been shown that vascular calcification is also related with increments of reactive oxygen species (ROS) (4). In fact, hydrogen peroxide, one of the most common types of ROS, has shown to be able to induce the expression of the osteoblastic transcription factor Runx2 (5).

Taking into account the previous findings, we decided to analyze *in vitro* the likely inhibitory effect of natural antioxidants in the process of vascular calcification.

Methods

Primary vascular smooth muscle cells (VSMCs) from Wistar rats were cultured during 8 days with either normal medium (DMEM:F12 1:1 with 0.1% of bovine serum albumin) or normal medium supplemented with Ca and P (P+Ca medium) at concentrations of 2 and 3 mM respectively. The latter were also cultured adding to the P+Ca medium the following natural antioxidants (all from Sigma); curcumin (C7727), silybin (02000585), resveratrol (R5010), alpha-tocopherol (258024), sodium L-ascorbate (A4034), and Trolox™ (238813). The antioxidant concentrations were selected from previous publications (6). Cell cultures were studied always under subconfluency conditions.

Mineralization was investigated using Alizarin red staining and quantified following described procedures (7).

Intracellular reactive oxygen species (ROS) levels were measured using flow cytometry and dihydrochlorofluorescein diacetate (DHCF-DA) (Molecular Probes). Briefly, cells were cultured in normal or P + Ca medium plus antioxidants during 8 days. Afterwards, the cells were starved and incubated during 60 min. with the probe and immediately analyzed in a flow cytometer (Cytomics FC500). The results were expressed as percentage of fluorescent cells after the incubation with the different culture mediums.

The protein expression of Runx2 and Mn-Superoxide Dismutase-2 (SOD-2) was measured by Western blotting using a standard protocol. Antibody dilutions were 1:100 for Runx2 (ab54868, Abcam), 1:1000 for SOD-2 (sc30080, Santa Cruz Biotechnology), and 1:30.000 for GAPDH (sc25778, Santa Cruz Biotechnology).

Results

A significant increase in mineralization was observed when VSMCs were cultured with P+Ca medium during 8 days. The effects of antioxidants were heterogeneus (Table1). Only curcumin (5μM) and silybin (50μM) were able to reduce mineralization by 30 and 35%, respectively (Figure 1) when compared to the VSMC cultured with P + Ca alone.

Parallel to these changes, the levels of ROS increased 88% in the VSMCs were cultured with the P + Ca medium. In fact, the addition of curcumin and silybin significantly decreased ROS up to 82 % and 64 %, respectively. Interestingly, after the incubation with curcumin, the levels of ROS were close to the levels observed in the control group (Table 2).

As curcumin was the more effective inhibitor of both ROS and VSMC mineralization, we investigated the effect of this antioxidant in Runx2 and SOD-2 expression. Curcumin was able to prevent the increase in Runx2 expression, but did not modify SOD-2 expression in the VSMCs cultured with the P + Ca medium (Figure 2).

Discussion

Vascular calcification is a threatening frequent complication particularly in the scenario of CKD. In recent years several studies have tried to better understand the mechanisms implicated in the genesis and regulation of this disorder.

Recently, it has been shown that ROS may play a role in the process of vascular calcification showing that ROS signalling affects the expression of the osteoblastic proteins involved in the initiation of vascular calcification (8). In agreement with this effect, Miller *et al* (9) demonstrated the critical role of ROS in the pathogenesis of several forms of valvular calcification. Furthermore, in humans, increments in ROS in the calcific aortic valvular stenosis have been associated to reductions in the defensive mechanisms responsible of removal several ROS, including hydrogen peroxide (H_2O_2).

Several other works have also stressed the importance of H_2O_2 as a second messenger involved in oxidative stress, which in turns may increase VSMCs mineralization at least partly by a direct upregulation of Runx2 expression (4).

Our study shows that a medium supplemented with Ca and P (3 and 2mM respectively) was able to both induce increase of ROS and mineralization in primary cultures of VSMCs. Even tough these results and others show that oxidative stress is currently involved in the process of vascular calcification, the heteregoneus response to several antioxidants shows the complexity of this process as only curcumin and sylibin were able to reduce VSMC mineralization.

Curcumin is the active ingredient of the traditional herbal remedy and dietary spice turmeric (*Curcuma longa*) (10), and silybin is the major active constituent of the compound silymarin, a potent detoxifying agent**.** As it has been shown both, we found that silybin but especially curcumin, decreased the ROS levels to those found in control

group. ROS was measured using fluorescent probes that are highly specific for H_2O_2, so we can hypothesize that curcumin and sylibin reduce specifically H_2O_2 levels. SOD-2 levels (responsible of the scavenge of superoxide ion into oxygen and H_2O_2) were increased in the P + Ca group, but there were no changes in the curcumin-treated group. This finding might indicate that curcumin could act specifically on H_2O_2 levels but not in other type of ROS located upstream in this cascade.

In conclusion, our results confirm that mineralization of VSMCs is associated with increments in oxidative stress. Curcumin and sylibin two well-known antioxidants of different origin with a variety of described actions (11), were able to decrease ROS. Curcumin decreased runx-2 without modifying SOD-2, suggesting that this antioxidant may directly reduce the H_2O_2 levels. These findings support the convenience of performing further studies in this field as some antioxidants might have potential benefits in the management of vascular calcification.

Bibliography

1. Goodman, WG: Vascular calcification in end-stage renal disease. *J Nephrol,* 15 Suppl 6**:** S82-5, 2002.
2. Moe, SM & Chen, NX: Mechanisms of vascular calcification in chronic kidney disease. *J Am Soc Nephrol,* 19**:** 213-6, 2008.
3. Steitz, SA, Speer, MY, Curinga, G, Yang, HY, Haynes, P, Aebersold, R, Schinke, T, Karsenty, G & Giachelli, CM: Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circ Res,* 89**:** 1147-54, 2001.
4. Sutra, T, Morena, M, Bargnoux, AS, Caporiccio, B, Canaud, B & Cristol, JP: Superoxide production: a procalcifying cell signalling event in osteoblastic differentiation of vascular smooth muscle cells exposed to calcification media. *Free Radic Res,* 42**:** 789-97, 2008.
5. Byon, CH, Javed, A, Dai, Q, Kappes, JC, Clemens, TL, Darley-Usmar, VM, McDonald, JM & Chen, Y: Oxidative Stress Induces Vascular Calcification through Modulation of the Osteogenic Transcription Factor Runx2 by AKT Signaling. *J Biol Chem,* 283**:** 15319-15327, 2008.
6. Bisht, K, Wagner, KH & Bulmer, AC: Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto- and DNA-protective dietary compounds. *Toxicology,* 278**:** 88-100, 2010.
7. Gregory, CA, Gunn, WG, Peister, A & Prockop, DJ: An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem,* 329**:** 77-84, 2004.
8. Towler, DA: Oxidation, inflammation, and aortic valve calcification peroxide paves an osteogenic path. *J Am Coll Cardiol,* 52**:** 851-4, 2008.
9. Miller, JD, Chu, Y, Brooks, RM, Richenbacher, WE, Pena-Silva, R & Heistad, DD: Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. *J Am Coll Cardiol,* 52**:** 843-50, 2008.
10. Kim, YS, Ahn, Y, Hong, MH, Joo, SY, Kim, KH, Sohn, IS, Park, HW, Hong, YJ, Kim, JH, Kim, W, Jeong, MH, Cho, JG, Park, JC & Kang, JC: Curcumin attenuates inflammatory responses of TNF-alpha-stimulated human endothelial cells. *J Cardiovasc Pharmacol,* 50**:** 41-9, 2007.
11. Pesakhov, S, Khanin, M, Studzinski, GP & Danilenko, M: Distinct combinatorial effects of the plant polyphenols curcumin, carnosic acid, and silibinin on proliferation and apoptosis in acute myeloid leukemia cells. *Nutr Cancer,* 62**:** 811-24, 2010.

Legends

Table 1:

Alizarin red quantification. Calcifying group (P+Ca) was used as reference.

Table 2:

Intracellular ROS quantification by flow cytometry. Mean of three measurements with DHCF, expressed as percentaje of fluorescent cells, and relative to phosphorus group (P), is depicted for each group.

Figure 1:

Microphotography of alizarin red staining (100X). A) Control group; B) P+Ca group; C) P+Ca+Silibyn 50μM; D) P+Ca+Curcumin 5μM

Figure 2:

Western Blot of Runx-2 and SOD-2 of VSMCs cultures with a calcifying media or a calcifying media supplemented with Curcumin 5μM.

Table 1

Table 1:

Alizarin red quantification.Calcifying group (P+Ca) was used as reference.

	CONTROL	P+Ca	P+Ca+ Asc 100µM	P+Ca+ Troll 100µM	P+Ca+ Sili 50µM
Relative Units	0.013	1	1.13	1.18	0.64
T Test vsP+Ca	***<0.0001***	--	*0.97*	*0.18*	***0.008***

	P+Ca+ Resv 50µM	P+Ca+ Toco500µM	P+Ca+ Toco100µM	P+Ca+ Curc 5µM
Relative Units	1.22	1.02	1.04	0.70
T Test vsP+Ca	*0.09*	*0.67*	*0.57*	***0.012***

Table 2

Table 2:

Intracellular ROS quantification by flow cytometry. Mean of three measurements with DHCF, expressed as percentaje of fluorescent cells, and relative to phosphorus group (P), is depicted for each group.

	CONTROL	**P+Ca**	**P+Ca+ Curc 5μM**	**P+Ca+ Sili 50μM**
Relative Units	0.12	1.00	0.18	0.36
T-Test vs P+Ca	*0.0034*	--	*0.001*	*0.001*
T-test vs Control	--	*0.003*	*0.73*	*0.06*

Figure 1
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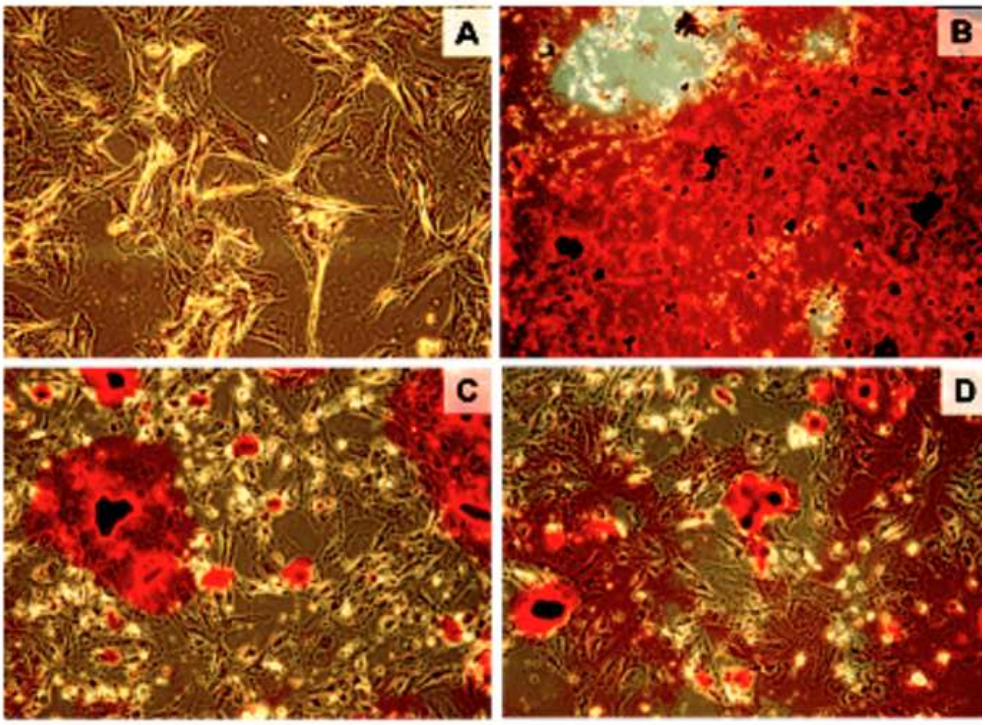

Figure 2
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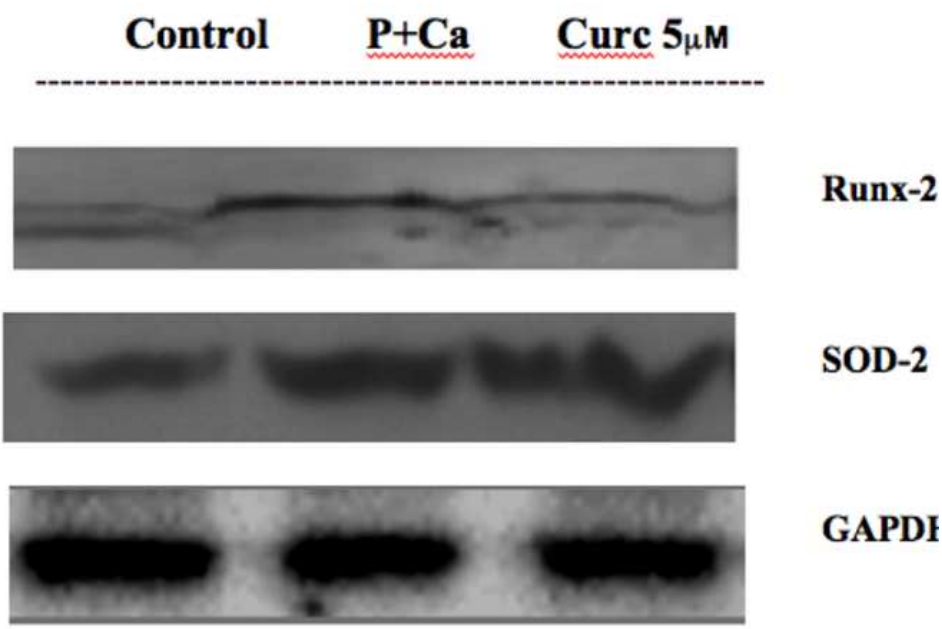

Discusión

Inicialmente el concepto de enfermedad ósea metabólica asociada a la ERC venía conociéndose como osteodistrofia renal . Hoy en día osteodistrofia renal se refiere a las alteraciones de la morfología y arquitectura ósea propias de la ERC. El termino CKD-MBD integra todas las alteraciones bioquímicas, esqueléticas y calcificaciones extraesqueléticas que ocurren como consecuencia de las alteraciones del metabolismo mineral en la ERC (18). El escenario de la CKD-MBD es altamente complejo y se caracteriza por mostrar una, o la combinación de varias de las siguientes manifestaciones:

1) Anormalidades del calcio (Ca), fósforo (P), hormona paratiroidea (PTH) y vitamina D.

2) Alteraciones en el remodelado, mineralización, volumen, crecimiento o fragilidad del esqueleto.

3) Calcificaciones cardio-vasculares o de otros tejidos blandos

Tanto los estudios clínicos en humanos como los estudios experimentales *in vivo* e *in vitro* han sido y son cruciales para comprender mejor el desarrollo de las diversas manifestaciones y las posibles aproximaciones terapéuticas en este cambiante escenario, tanto en condiciones fisiológicas como en situaciones patológicas, como la ERC. En el estudio de estos complejos, pero a la vez interconectados, desordenes del metabolismo mineral en la ERC, las técnicas de cribado o "screening" molecular, como los microarrays de expresión o la proteómica han sido fundamentales para describir dianas y/o nuevos mecanismos.

En la discusión de los resultados de la tesis doctoral, se abordará un primer apartado centrado en los posibles nuevos factores moleculares implicados en el el hiperparatiroidismo secundario encuadrado dentro del apartado 1 de la CKD-MBD (anormalidades del fósforo y de la PTH), remarcando la aportación de los diferentes modelos *in vivo* e *in vitro* empleados, incluyendo microarrays de expresión en el estudio de la glándula paratiroides. Posteriormente, la discusión se centrará en remarcar la importancia de los resultados derivados del estudio de la relación entre la calcificación vascular y la

perdida de masa ósea como consecuencia de la sobrecarga de fósforo, englobando los apartados 1, 2 y 3 de la CKD-MBD. Esta parte de la tesis también ha tenido como base una aproximación genómica, descritos en los apartados 3 y 4 de la sección *Resultados*, que contienen los trabajos originales presentados en la tesis doctoral. Finalmente se discutirá el papel del estrés oxidativo en la calcificación vascular, descrito en el apartado 5 de la sección Publicaciones.

Hiperparatiroidismo secundario severo; factores implicados.

El fósforo elevado, una de las principales características de la ERC es el principal factor patogenético en el desarrollo del hiperparatiroidismo secundario (sHPT, por sus siglas en inglés) (4, 152). En nuestro laboratorio, para estudiar diferentes etapas en el desarrollo del sHPT, se desarrolló un modelo murino de enfermedad renal crónica, en el cual, los animales eran nefrectomizados (5/6) y alimentados con una dieta con un contenido moderadamente alto en fósforo (50% mas que en la dieta normal) y sacrificados a diferentes tiempos. Para simplificar el diseño del estudio, se construyeron tres grupos en base al criterio: "tiempo de exposición a la dieta alta en fósforo" y "valores séricos de PTH", resultando en la formación de los grupos denominados "hiperparatirodismo secundario moderado" (Mod sHPT), "hiperparatiroidismo secundario moderado/severo" (Mod/Sev sHPT) e "hiperparatiroidismo secundario muy severo" (Sev sHPT), incluyendo un grupo referencia (Ref) de animales sin nefrectomía y alimentados con dieta normal.

Como primer resultado, observamos que la dieta alta en fósforo (HPD por sus sigas en inglés) afectaba gravemente a la función renal a la par que incrementaba la mortalidad, como se ha descrito profusamente en la bibliografía en pacientes en hemodiálisis (153-155). De acuerdo con otros estudios, se encontró el FGF23 significativamente incrementado en todos los grupos que habían recibido HPD comparados con los que habían recibido una dieta normal en fósforo (NPD por sus siglas en inglés) (51, 59) (Ilustración 11).

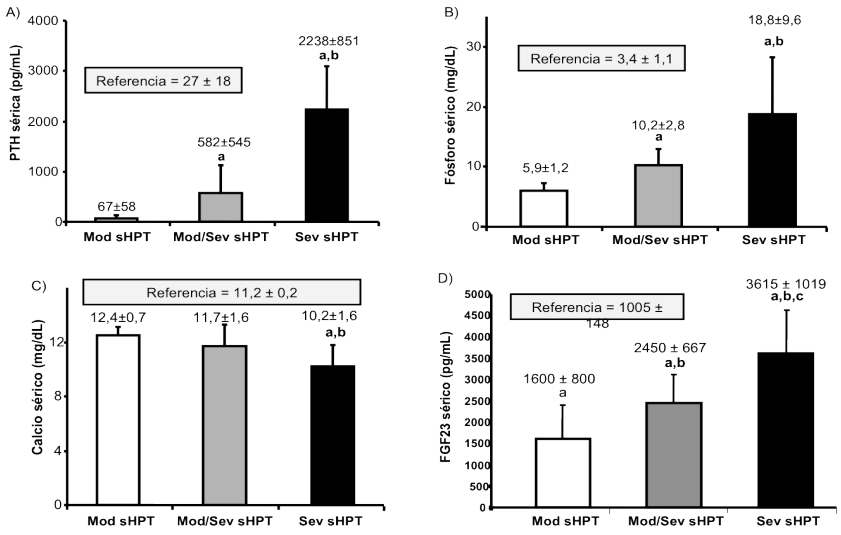

Ilustración 11. Representación gráfica de la media y desviación estándar de los valores bioquímicos correspondientes a PTH (A), fósforo (B), calcio (C) y FGF23 (D) de los diferentes grupos de estudio. a= p<0,05 comparado frente al grupo Referencia, b= p<0,05 comparado frente al grupo Mod sHPT, c= p<0,05 comparado frente al grupo Mod/Sev sHPT

Interesantemente, los niveles séricos de PTH y de FGF23 se correlacionaron significativamente. La correlación positiva entre fósforo, PTH y FGF23 puede ser interpretada como una respuesta fosfatúrica compensatoria en respuesta a la sobrecarga de fósforo en la dieta, pero que no es capaz de llevarse a cabo debido al daño renal provocado (

Ilustración 12).

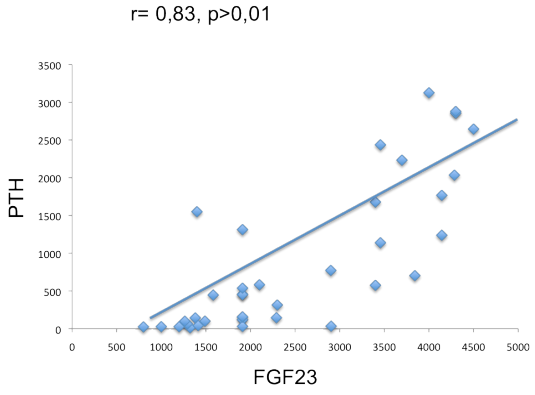

Ilustración 12. Representación grafica de la correlación de Pearson entre los valores bioquímicos de FGF23 y PTH

Para estudiar estas diferentes etapas en el desarrollo del sHPT, se emplearon microarrays de expresión por primera vez en gandulas paratiroides de rata.

En un primer paso, se comprobó que, como se ha descrito en la clínica, la gandula mantiene cierto grado de control hasta etapas muy tardías del desarrollo del sHPT. Esto se puede ver en los cladogramas jerárquicos, en los que, usando solo genes que pertenecen al metabolismo óseo, el grupo "hiperparatiroidismo secundario muy severo" se separó de los otros dos, indicando un perfil de expresión de estos genes similar en los grupos "hiperparatirodismo secundario moderado" e "hiperparatiroidismo secundario moderado/severo" (Ilustración 13).

Ilustración 13. Cladograma jerárquico usando los genes relacionados con metabolismo mineral, representando la clasificación de los diferentes grupos de estudios agrupados según la severidad del hiperparatiroidismo secundario. Sev representa el grupo Sev sHPT, Mo/Sev el grupo Mod/Sev sHPT y Mo el grupo Mod sHPT.

A continuación, se generaron las listas de genes. En estas listas, la mayoría de genes significativamente modificados aparecían fuertemente reprimidos, con sólo unos pocos genes mostrando expresión incrementada. La represión generalizada ya se había descrito anteriormente en nuestro grupo como una característica típica del desarrollo del sHPT, en la que la glándula paratiroides acaba por sufrir una total perdida de control de los procesos celulares básicos, acumulando diversos daños en los mecanismos de reparación del material genético (156, 157). De manera interesante, entre los genes desregulados encontrados mediante el análisis genómico, no se encontraban los típicos genes encargados de la regulación básica de la función paratiroidea, tales como el receptor/sensor de calcio (CaSR), el receptor de la Vitamina D (VDR), el gen de la PTH o klotho. La perdida del control sobre la glándula paratiroides se ha atribuido a descensos en el CaSR y el VDR, por lo que la circunstancia de que estos genes no apareciesen en las listas fue chocante.

Esta carencia de resultados en los genes clásicos involucrados en la regulación de la glándula paratiroides puede ser explicada desde varios puntos de vista. Los microarrays de expresión pueden dar lugar la sobreestimación de los cambios en las expresión de determinados genes, por lo que el análisis de las listas desde un punto de vista funcional cobra vital importancia (158). Además, los algoritmos empleados fueron altamente restrictivos para evitar los falsos positivos, por lo que estos genes clásicos pueden haber quedado fuera de las listas debido a que otros genes han sido detectados como mas robustamente desregulados. Finalmente, las glandulas muestras múltiples aberraciones moleculares (157, 159) y en los conjuntos ("pools") de glándulas usados para las muestras se detecto una cierta dispersión en cuanto a valores de PTH, por lo que posiblemente los cambios en estos genes, si pretenden ser detectados mediante microarrays, deberían investigarse en etapas muy determinadas y en grupos altamente homogéneos.

Sin embargo, por PCR cuantitativa (qRT-PCR), técnica mas sensible que los microarrays, si se detectaron cambios significativos en estos genes, especialmente en las etapas mas avanzadas del sHPT, sugiriendo de nuevo que la

glándula mantiene cierto grado de control hasta las ultimas etapas del desarrollo del sHPT, como esta detallado en la Ilustración 14.

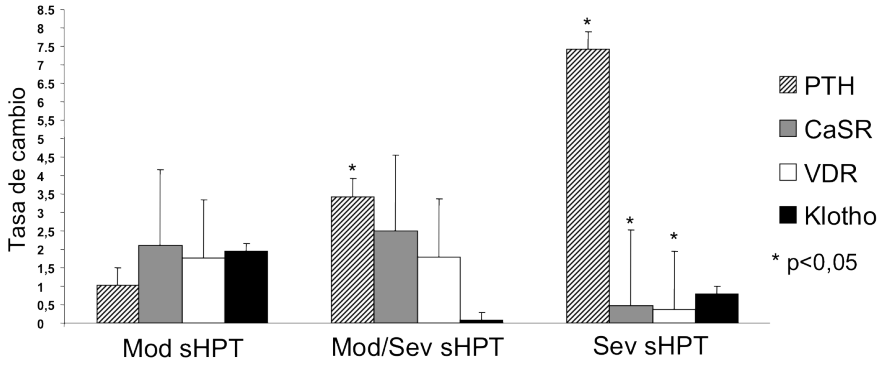

Ilustración 14. Representación gráfica de la tasa de cambio (determinada por qRT-PCR) de los genes PTH, CaSR, VDR y klotho en las glándulas paratiroides de los diferentes grupos de estudio.

*= p<0,05 comparado frente grupo Mod sHPT

Entre los genes de expresión incrementada, la presencia de varios genes pertenecientes a la familia de las Fosfatasas de especificidad dual (Dusp por sus siglas en inglés) fue estudiada en mas detalle.

Las proteínas codificadas por estos genes son las principales encargadas de desactivar específicamente la vía de las kinasas de regulación extracelular (ERK), pero no otras subclases de las MAPK como p38, a través de la defosforilación de pERK (160-163) , interviniendo por tanto en la regulación de esta vía, altamente implicada, en lo que a metabolismo paratiroideo se refiere en hiperproliferación y señalización de FGF23, entre otras.

Se comprobó no solo mediante microarrays, sino mediante qRT-PCR que estos genes estaban altamente sobre-expresados conforme avanzaba el desarrollo del sHPT, por lo que los niveles de pERK, su objetivo biológico, deberían estar inversamente relacionados. Se realizo una tinción inmunohistoquímica para ERK y pERK sobre cortes de glándulas paratiroides para comprobar si existía una relación directa entre niveles elevados de Dusps y niveles disminuidos de pERK en los diferentes grupos (Ilustración 15).

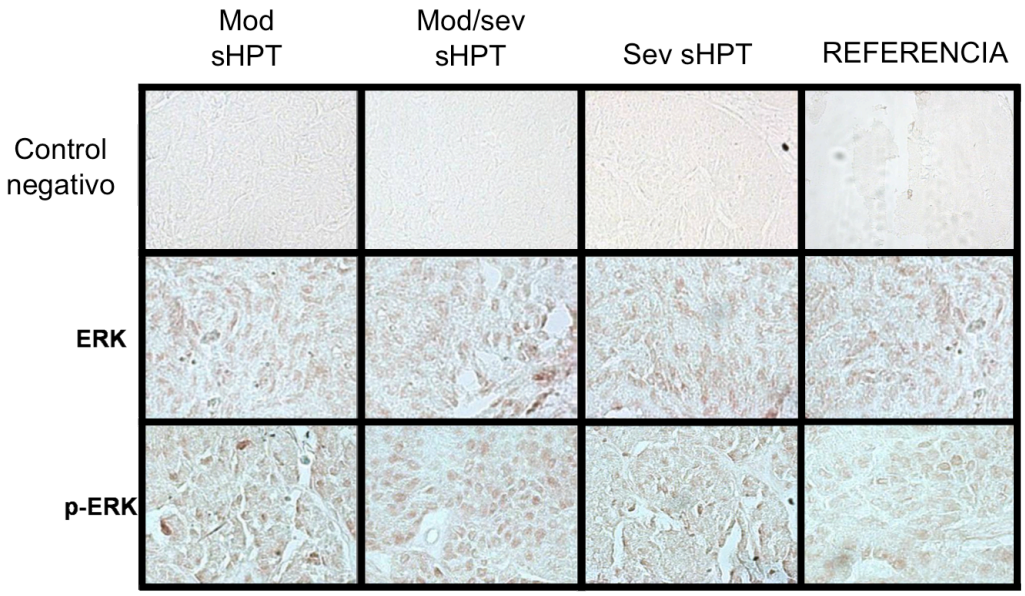

Ilustración 15. Tinción inmunohistoquímica de ERK y pERK en cortes de glándulas paratiroides de los diferentes grupos de estudio.

Esta dinámica de ERK (Ilustración 16) es de alguna manera, sorprendente, pero es lógico pensar que la señal hiperproliferativa se diese al inicio del desarrollo del sHPT, desencadenándose procesos destinados a frenar ese crecimiento en las etapas mas tardías, para intentar mantener la integridad glandular e intentar frenar el excesivo crecimiento como ultimo mecanismo de defensa.

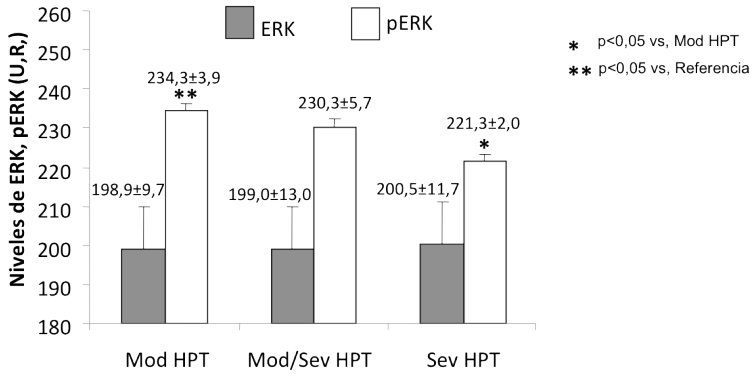

Ilustración 16. Cuantificación de los niveles de ERK y pERK en las glándulas paratiroides de los diferentes grupos de estudio.

El desencadenamiento de estos mecanismos que combaten la hiperproliferación, podría afectar también a la señalización de FGF23 de la glándula paratiroides. Como esta descrito en la introducción, el FGF23 es capaz de inhibir la síntesis y secreción de PTH, actuando a través de la vía de las MAPK (8, 164). Tanto la correlación entre los valores de PTH y FGF23 como la presencia de valores muy elevados de FGF23 en el grupo "hiperparatiroidismo secundario muy severo", son frecuentemente interpretados como la perdida de la capacidad de la glándula paratiroides de reaccionar al FGF23, acuñando el termino "resistencia a FGF23". Esta teoria esta apoyada por la correlacion observada en la Ilustración 12.

De acuerdo con lo anterior, si en las etapas mas avanzadas del sHPT se desencadenan procesos destinados a desactivar la hiperproliferación a través de la desactivación de la vía de las MAPK, secundariamente, esto podría redundar en que la señal de FGF23 no se transmitiese correctamente aguas abajo, colaborando a la resistencia a FGF23.

Un hecho a favor de esta hipótesis es que hasta la fecha, la falta de efecto de FGF23 sobre la PTH ha sido achacado a la regulación a la baja de los receptores de FGF23, klotho y FGFR1 (54, 56), si bien esto ha sido puesto en duda en un reciente articulo (165).

Interesantemente, klotho y FGFR1, al igual que otros receptores que controlan el metabolismo glandular, no sufren un descenso en sus niveles total, lo que implicaria la presencia de otros factores en esta resistencia al FGF23. Además, existen altos niveles de FGF23 circulante, lo que debiese resultar en un cierto efecto del FGF23 sobre la PTH. Pero tanto en la práctica clínica como en los experimentos in vivo, este efecto inhibitorio del FGF23 sobre la PTH no existe, siendo el FGF23 totalmente incapaz de actuar en la glándula paratiroides, lo que sugiere, que ademas de la bajada de los receptores, aguas abajo debería haber otros factores que colaboren a esta resistencia al FGF23. Estos factores pueden ser las Dusps, que incrementadas dramáticamente en las etapas tardías del sHPT, primariamente como ultimo mecanismo de defensa ante la hiperproliferación de la glándula, podría secundariamente desactivar la señal

proveniente de klotho/FGFR1. Interesantemente, Dusp6 ha sido relacionada con la desactivación de la señal trasmitida por los FGFR (166) .

Para confirmar que las Dusps pueden colaborar a desactivar la señalización ejercida por FGF23, se diseño un experimento usando cultivo de glándulas paratiroides *ex vivo*. Glándulas normales fueron cultivadas con 0,6 mM de Calcio para asegurar la secreción de PTH, siguiendo diseños de estudios publicados previamente (167, 168).

Glándulas normales "estimuladas" fueron cultivadas con FGF23, observándose una reducción significativa de la secreción de PTH, medida como porcentaje de cambio respecto a la condiciones iniciales. Cuando al FGF23 se le añadío un inhibidor químico de las MAPK, UO126, la secreción elevada de PTH fue completamente restaurada, desactivando el efecto supresor del FGF23 y dejando la secreción de PTH al nivel de las glándulas cultivadas con el medio control, 1,2mM de Calcio. Cuando se sustituyó el inhibidor químico por los inhibidores biológicos, las Dusps, la secreción de PTH fue parcialmente restaurada, demostrando que las Dusps recombinantes, si bien no alcanzan u n efecto ran potente como el inhibidor químico, son capaces de desactivar parcialmente la señal ejercida por el FGF23 en las glándulas paratiroides en lo que a secreción de PTH se refiere, como se puede ver en la Ilustración 17.

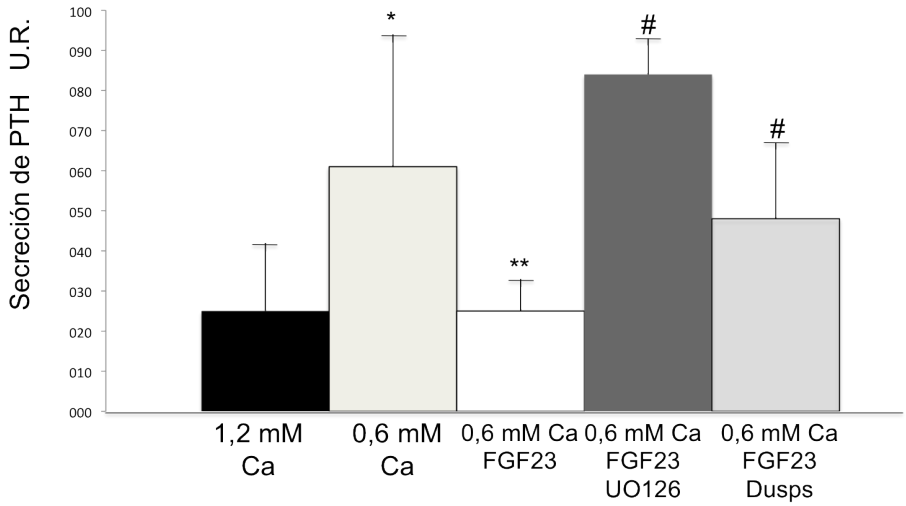

Ilustración 17. Secreción de PTH de glándulas paratiroides normales cultivadas con diferentes tratamientos. Las unidades son porcentaje de cambio de secreción respecto a la condición inicial. *= p<0,05 comparado frente al grupo 1,2mM Ca **= p<0,05 comparado frente al grupo 0,6mM Ca . # = p<0,05 comparado frente al grupo 0,6mM Ca + FGF23

El hecho de que Dusp 5 es un objetivo de p53 (169) representa un mecanismo plausible por el que se explicaría el porqué de esta sobre-regulación de las Dusps. Una característica del sHPT, previamente publicada por nuestro grupo, es que las glándulas acumulan aberraciones cromosómicas y fallos en los mecanismos de reparación del ADN y ARN (156, 159), lo que podría desembocar en una fuerte activación de p53. Esta activación de p53 seria el estimulo primario para el mecanismo de defensa en etapas muy avanzadas, ejercido a través de la sobre-expresión de las Dusps y posterior desactivación de MAPK, que secundariamente afectaría a la señalización (previamente comprometida a causa del descenso en los receptores) de FGF23.

Como resumen de esta sección, en el desarrollo del hiperparatiroidismo secundario severo, la represión génica constituye el hecho mas generalizado en la glándula paratiroides. Sin embargo, como mecanismo de defensa, la glándula paratiroides sobre expresa ciertos genes de la familia de las Dusps, intentando frenar la señal hiperproliferativa ejercida por ERK/pERK. Como hecho secundario a este desactivacion de las señal de las MAPK, la transmisión de la

señal de FGF23 se ve seriamente comprometida y las glándulas paratiroides de las ratas con sHPT severo no son capaces de responder a FGF23 y frenar sus secreción de PTH.

Relación entre calcificación vascular y pérdida de masa ósea

La calcificación de los tejidos blandos ocurre en una gran proporción de pacientes con ERC y su descripción data del siglo XIX. En la ERC, las lesiones se observan fundamentalmente en la capa media pero también en la intima, pudiendo afectar el flujo así como la rigidez vascular con el consecuente aumento en la presión arterial y la velocidad de la onda de pulso.

Las calcificaciones vasculares en la intima arterial suelen estar asociadas a la existencia de placas ateroescleróticas previas. Afectan la capa media de las arterias de mediano calibre, la aorta y las coronarias, con una disposición concéntrica del Ca en las células del músculo liso vascular, produciendo rigidez y arterioesclerosis. En estas calcificaciones, se da una diferenciación fenotípica similar a la de las células óseas, produciendo una marcada disminución en la capacidad contráctil de las células musculares. Las complicaciones vasculares suelen preceder a las alteraciones propias del hueso que ocurren mas tardíamente y de forma insidiosa (170). Aunque todas las formas histológicas de osteodistrofia renal se han asociado con una mayor prevalencia de calcificaciones vasculares, la de mayor impacto es la que se observa en la osteodistrofia renal de bajo recambio.

El fósforo elevado, una de las principales características de la ERC es también el principal factor patogenético en el desarrollo de la calcificación vascular en la población con ERC en todas sus etapas (66, 69). Además, en nuestro modelo, la sobrecarga de fósforo es el principal responsable del desarrollo del hiperparatiroidismo secundario (130) como ha sido descrito en el apartado anterior de esta tesis, pero también ha sido relacionado con la perdida de función renal (171).

La asociación entre calcificación vascular y descalcificación ósea data de hace 20 años (172). De hecho, la sobrecarga de fósforo es uno de los posibles factores patogenéticos comunes entre ambas alteraciones. Cuando el recambio óseo es bajo, como en la enfermedad adinámica ósea, el tamaño del "almacén"

de fósforo se reduce, resultando en elevados niveles de fósforo en sangre asociados con la ingesta. Además, la resorción ósea, es, en exceso de formación ósea, un factor que altera la función de reservorio del esqueleto, quedando altamente comprometido. Cuando el recambio es alto, como en el hiperparatiroidismo secundario el fósforo es liberado desde el hueso y de nuevo, se altera la función de reservorio del esqueleto, quedando altamente comprometido. Se demostró que la estimulación de formación ósea mediante la administración de BMP-7, reduce la hiperfosfatemia, restaura la función de reservorio del esqueleto, y se reduce la calcificación vascular (173, 174). Como la función renal en estos estudios permaneció inalterada, la reducción de los niveles de fósforo sérico se debe únicamente al incremento de la formación ósea.

En nuestro modelo experimental, anteriormente descrito, las ratas que recibieron la dieta alta en fósforo (HPD por sus siglas en inglés) mostraron un incremente significativo del fósforo sérico respecto de las ratas que recibieron una dieta normal en fósforo (NPD por sus siglas en ingles) en todos los tiempos de estudio (8, 16 y 20 semanas) (Ilustración 18). Debido a que el fósforo y el calcio están altamente inter-relacionados, el calcio sérico estaba significativamente reducido (175). Tanto el fósforo elevado como el calcio disminuido son dos estímulos para la secreción de PTH y la hiperplasia de la glándula paratiroides. (4, 152).

	P sérico mg/dL		PTH sérica pg/dL		Ca sérico mg/dL	
	Media	DE	Media	DE	Media	DE
8NPD	**5,7**	1,2	**37,8**	22,0	**12,1**	0,2
16NPD	**5,0**	0,5	**65,6**	54,1	**11,7**	0,5
20NPD	**5,6**	0,7	**82,2**	57,6	**12,5**	0,6
8HPD	**8,7***	0,8	**283,6***	233,7	**11,6***	0,4
16HPD	**11,9***	2,7	**1314,0***	781,7	**11,3**	2,1
20HPD	**14,1***	2,4	**2461,2***	717,0	**10,9***	0,1
Ref	**4,9**	0,9	**44,2**	39,1	**11,5**	0,6

Ilustración 18. Media y desviación estándar de los parámetros bioquímicos clásicos del metabolismo óseo en los grupos de estudio. *= p<0,05 comparado frente al grupo Referencia (Ref)

Interesantemente y unido con la sección anterior de esta discusión, la PTH sérica fue el único parámetro bioquímico significativamente asociado con la presencia de calcificación vascular. Un estudio ha adjudicado un papel importante en el desarrollo de las calcificaciones vasculares a la PTH independientemente del fósforo (176). Esta hipótesis resalta la mas importante limitación de este parte del estudio, que es que no ha sido posible separar el efecto de la sobrecarga de fósforo de la PTH elevada sobre la calcificación vascular.

Al respecto de la calcificación vascular, el 20% de los animales que recibieron HPD durate 16 semanas (16HPD(desarrollaron calcificaciones vasculares, desde el punto de vista de la tincion de Von Kossa. Como puede verse en la Ilustración 19 las calcificaciones vasculares observadas en la mayoría (80%) de los animales alimentados con HPD durante 20 semanas (20HPD) fueron generalizadas y severas. Además, las ratas de este grupo que mostraron calcificación vascular, también mostraron una tinción positiva para fosfatasa alcalina tartrato-resistente (TRAP), un producto de la actividad de los osteoclastos que se encontró sólo en las zonas severamente calcificadas, lo que

sugiere que las células multinucleadas se vieron en la pared de estas arterias, eran células "osteoclastos-like" (94) .

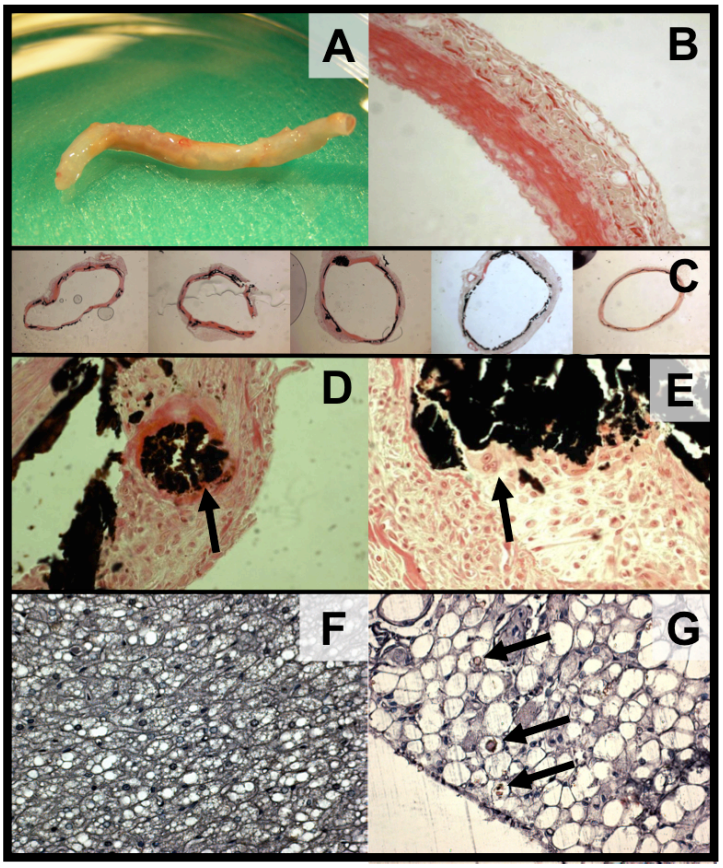

Ilustración 19. Representación grafica de diversos aspectos de la calcificación vascular. A) imagen representativa de la rigidez de la arteria aorta proveniente de un animal del grupo 20HPD. B) Imagen histológica de la tinción de von Kossa de una aorta de un animal que no sufrió calcificación vascular. C) Imágenes histológicas de la tinción de von Kossa de las 5 aortas pertenecientes a todos los animales incluidos en el grupo 20HPD. D y E) presencia de zonas severamente calcificadas, semejantes a osteiode y células multinucleadas en una aorta severamente calcificada respectivamente. F y G) Tinción TRAP de tejido aórtico no calcificado y severamente calcificado respectivamente.

Asimismo, en el análisis genómico, se observó un aumento en la expresión génica de catepsina-K, un conocido marcador de la actividad de osteoclastos (177), sugiriendo también que existía cierta actividad de reabsorción

en los vasos severamente calcificados después de 20 semanas de alimentación HPD. Este aspecto será mas extensamente tratado mas adelante en esta discusión.

La relación entre la calcificación vascular y la bajada de la masa ósea está bien establecida en la literatura, sobre todo en la población general y / o pacientes con osteoporosis, pero no en los pacientes con ERC. En este último caso, todos los estudios publicados son observacionales o transversales, pero nunca prospectivos; además, algunos de ellos asociaron la calcificación vascular con la masa ósea a partir de datos no siempre obtenidos al mismo tiempo (178-183). Además, existen algunos resultados controvertidos; estudios recientes han encontrado, usando análisis multivariante, que la extensión de las calcificaciones vasculares no parece estar influenciada por el recambio óseo ni tiene alguna relación con la masa ósea trabecular (184).

En nuestro estudio, respecto a la densidad mineral ósea (DMO), las ratas que no desarrollaron calcificaciones vasculares mostraron una tendencia similar a la observada en el grupo de referencia, mientras que la densidad mineral ósea de las ratas de los grupos que recibieron HPD fue siempre inferior al sus pares de los grupos NPD. Además, nuestro estudio demuestra por primera vez, que sólo las ratas que se desarrollaron calcificaciones vasculares mostraron una pérdida significativa de hueso y tenían la menor densidad mineral ósea ambos sitios estudiados (Ilustración 20) (Ilustración 21).

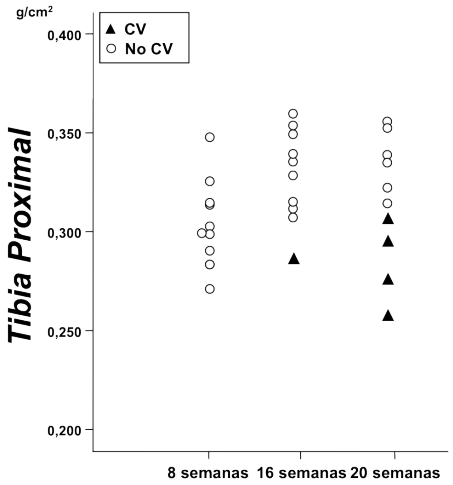

Ilustración 20. Representación gráfica de la masa ósea a nivel proximal en los grupos de estudio. Los animales positivos calcificación vascular por von Kossa están representados con triangulo negro.

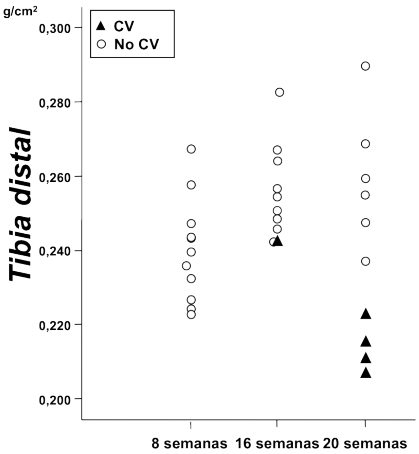

Ilustración 21. Representación gráfica de la masa ósea a nivel distal (altamente relacionada con la parte cortical del hueso) en los grupos de estudio. Los animales positivos calcificación vascular por von Kossa están representados con triangulo negro

De acuerdo con los resultados anteriores (185), el efecto mas remarcable de la HPD tuvo lugar en la parte cortical del hueso. En anteriores trabajos observacionales, no se encontró asociación entre las puntuaciones (scores) de la calcificación y alteraciones del metabolismo mineral (como la hipercalcemia, la hiperfosfatemia, producto Ca x P e hiperparatiroidismo) (181, 182). Por el

contrario, en nuestro estudio con altos niveles de fósforo y PTH séricos, se observó, una correlación significativa entre ambos parámetros bioquímicos y cambios en la aorta y la tibia. Estos resultados están de acuerdo con un estudio reciente (171), que describía un efecto del fósforo negativo a nivel óseo.

En resumen, esta es la primera vez que un estudio experimental demuestra que los animales que desarrollaron calcificación vascular fueron las únicos que mostraron una reducción significativa de la masa ósea; por el contrario, la pérdida de hueso no se observó en los animales que no desarrollaron la calcificación vascular.

En esta sección, de nuevo se emplearon técnicas de genómica para analizar las calcificaciones vasculares durante diferentes etapas de su desarrollo, usando los conocimientos adquiridos en estudios previos de nuestro laboratorio (157), representando una herramienta útil para el análisis de las alteraciones del metabolismo mineral, fundamentalmente las que tiene que ver con la sobrecarga de fósforo. Para aclarar los cambios de expresión génica que ocurren durante la progresión de la calcificación vascular, más de 31.000 genes fueron analizados a la vez en los diferentes grupos de estudio. A nuestro entender, esta es la primera vez que se aplica esta técnica al estudio de la calcificación vascular *in vivo*.

Los resultados de microarrays nos permitieron seleccionar algunos genes candidatos y vías de señalización, para profundizar el análisis. Los resultados mas preliminares apoyaron la idea de un cambio fenotípico de las células, ya que los cladogramas jerárquicos (Ilustración 22) (Ilustración 23) mostraron un patrón completamente diferente en la expresión de genes relacionados con el hueso y músculo entre los grupos que había sufrido calcificación aórtica o no.

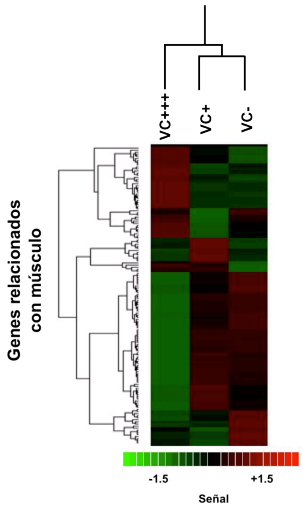

Ilustración 22. Cladograma jerárquico usando los genes relacionados con músculo, representando la clasificación de los diferentes grupos de estudios agrupados según la severidad de la calcificación vascular. VC+++ representa el grupo 20HPD, VC+ el grupo 16HPD y VC- el resto de grupos de estudio.

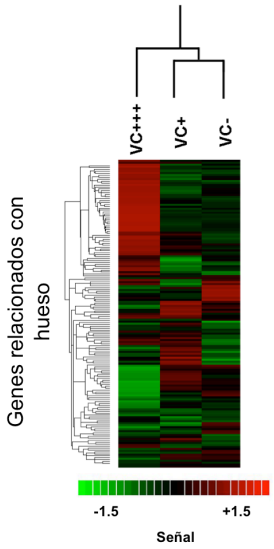

Ilustración 23 Cladograma jerárquico usando los genes relacionados con hueso, representando la clasificación de los diferentes grupos de estudios agrupados según la severidad de la calcificación vascular. VC+++ representa el grupo 20HPD, VC+ el grupo 16HPD y VC- el resto de grupos de estudio

De hecho, en las listas generadas a continuación, dos importantes genes relacionados con el músculo se encontraron altamente reprimidos y fueron validados por qRT-PCR; esto apoyaba a la idea de la pérdida del fenotipo muscular.

Desde el punto de vista molecular, es bien sabido que las proteínas relacionadas con frizzled secretadas (SFRP por sus siglas en ingles) son inhibidores de la vía de señalización Wnt, altamente implicada en la osificación, formación ósea y también en el desarrollo de la calcificación vascular (133-135). El ratón KO para SFRP-1 mostró una alta tasa de formación ósea, como se esperaría al eliminar un inhibidor de la formación ósea, pero no tenia implicaciones vasculares conocidas (186). Un estudio de 2006 describió por primera vez un papel para las SFRPs en la calcificación vascular *in vitro*, mostrando una inhibición del gen SFRP-3(97).

Sorprendentemente, los genes SFRP-1, 2 y 4 se encontraron sobreexpresados en nuestro modelo (no sólo por los microarrays de expresión, sino también por qRT-PCR realizada en el tejido aórtico), lo que sugiere que la familia de SFRPs podría jugar un papel, al menos, en las fases finales de la calcificación vascular.

Esta situación es sorprendente, puesto que lo esperable consistiría en una reducción de su expresión, que favoreciese la formación ósea. Por ello, el aumento de la expresión génica SFRP-1, 2 y 4 encontrado en las aortas severamente calcificadas podría interpretarse como una respuesta defensiva que tiene como objetivo bloquear la vía de Wnt con el fin de reducir/combatir la mineralización en la pared aórtica calcificada. Dado que el incremento en la familia SFRPs no se detectó durante las primeras etapas (semanas 8 y 16), se podría hipotetizar que esta sobreexpresión se ha activado al final de este proceso para evitar una mayor progresión de la calcificación vascular y mantener cierta integridad vascular (Ilustración 24).

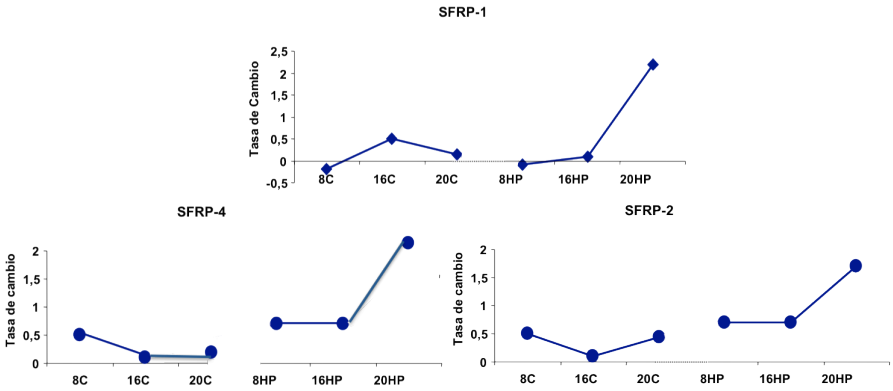

Ilustración 24. Evolución de la expresión génica de SFRP-1, 2 y 4 en los diferentes grupos de estudio.

Por otro lado, como las SFRPs son proteínas secretadas, podrían ser capaces de alcanzar el tejido óseo, donde podrían actuar como en los vasos tratando también de reducir la mineralización, lo que resulta en la reducción de la masa ósea. Esta es una hipótesis fascinante que podría vincular la progresión de la calcificación vascular con la reducción de la masa ósea que necesita mas estudios específicos. Curiosamente, la inducción de nefritis intersticial se asoció con una sobre-expresión de SFRP4, SFRP2, y DDK1 en la capa adventicia vascular y una elevada carga de DKK1 circulante (136).

Interesantemente, la fibrosis cardiovascular se ha asociado frecuentemente a la aparición de calcificaciones vasculares. Se ha descrito que la expresión de SFRP2 se encuentra aumentada en la fase de fibrosis de infarto de miocardio y que ratones KO para SFRP2 han mostrado reducción de la fibrosis y mejoras en la función cardíaca (187). SFRP-4 tiene un alto poder fosfatúrico (188); por lo que se podría especular que el aumento en la expresión de SFRP4 también podría formar parte de una respuesta destinada a frenar el incremento de fósforo sérico.

En resumen, este estudio experimental prospectivo demostró la fuerza de la asociación entre la calcificación vascular y la reducción de la masa ósea en un

modelo de ratas con ERC alimentadas con una dieta moderadamente alta en fósforo, que muestra también que la HPD produce, a largo plazo, varias alteraciones graves del metabolismo óseo altamente relacionadas con la mortalidad.

Los resultados histológicos y de los microarrays se complementan entre sí y ayudan a comprender mejor los cambios que se producen en el proceso de calcificación vascular y la magnitud de la severidad observada en las ultimas fases de este experimento. La sobreexpresión de los miembros de la familia SFRPs detectada por microarrays y qRT-PCR, que sólo fue encontrada cuando se estableció la calcificación vascular severa, podría ser indicativa de un mecanismo de defensa activa con el fin de reducir o bloquear la activación de la vía Wnt, con el objetivo para reducir la mineralización de la pared arterial, conservar cierta funcionalidad muscular y evitar la progresión de la calcificación vascular.

Estrés oxidativo en la calcificación vascular

La mortalidad de los pacientes con enfermedad renal crónica (ERC) es significativamente mayor que la de la población general, siendo la principal causa de muerte las enfermedades cardiovasculares. Dado que se ha postulado que existe una relación entre estrés oxidativo y problemas cardiovasculares, se ha propuesto la existencia de un desequilibrio redox en los pacientes con ERC. El estrés oxidativo se define como una alteración en el balance entre la producción de substancias pro-oxidantes y la capacidad de organismo de neutralizar estas substancias. En esta situación, se produce la oxidación de diversas macromoléculas, lo que da como resultado alteraciones en la estructura y en la función de células y tejidos. La uremia, y los tratamientos que se utilizan en la misma, alteran significativamente este proceso, de forma que suele existir una menor calcificación de los huesos y, en muchas ocasiones, calcificaciones anormales en las paredes vasculares. El estrés oxidativo puede jugar un papel clave en la génesis de esta desregulación, fundamentalmente a tres niveles, alterando la homeostasis calcio-fósforo, modificando el comportamiento del tejido óseo o induciendo alteraciones fenotípicas en los vasos sanguíneos, siendo esta última el objeto de esta sección.

En los últimos años varios estudios han tratado de comprender mejor los mecanismos implicados en la génesis y la regulación de la calcificación vascular y se ha demostrado que las especies reactivas de oxigeno (ROS por sus siglas en inglés) juegan un papel principal en el proceso de calcificación vascular, demostrando que la señalización intracelular a través de ROS afecta a la expresión de las proteínas típicamente óseas, implicadas en la iniciación de las enfermedades vasculares (189).

De acuerdo con este efecto, se ha demostrado el papel fundamental de ROS en la patógena de varias formas de calcificación valvular (190). Además, en humanos, los incrementos de ROS en la estenosis y calcificación valvular, se han asociado a la reducción de los mecanismos defensivos responsables de la eliminación de varios tipos de ROS, incluyendo peróxido de hidrógeno (H_2O_2). Los estudios no

son concluyentes, quizá por la complejidad de este tipo de estudios o por la variabilidad en la propia administración de antioxidantes (191). La importancia de H_2O_2 como un segundo mensajero ha sido descrita en los últimos años, demostrando que puede aumentar directamente la expresión de Cbfa-1/Runx2 expresión, a través de la vía de AKT, promoviendo así al mineralización (99). Además se ha visto que presencia de elevados niveles de H_2O_2 se relaciona con la senescencia vascular acelerada a través de procesos que implican el procesamiento de ciertos componentes de la envuelta nuclear (122), lo que sugiere que estos mecanismos también podrían estar implicados en el desarrollo de la calcificación vascular, ya que el envejecimiento, la senescencia y la calcificación vascular son procesos altamente afines. Sin embargo, muchos aspectos relacionados con el estrés oxidativo y la calcificación vascular, como los mecanismos por los que se acumulan los diferentes tipos de ROS cuando las CMLV se transdiferencian a células óseas o las capacidades ciertamente paradójicas de diversos antioxidantes aun permanecen poco claros (100).

Estudios que no son objeto de esta tesis mostraron, usando un modelo animal similar al descrito y analizando la calcificación vascular mediante técnicas de proteómica, que el desarrollo de la calcificación vascular cursa con una alta desregulación en proteínas que juegan papeles importantes en el metabolismo del estrés oxidativo. En la Ilustración 25 se muestra la lista de proteínas resultado de comparar el grupo Referencia, el 16HPD y el 20HPD entre si. El perfil de expresión diferencial muestra una desregulación a la alta o a la baja de la SOD-2, GPX, o anhidrasa carbónica 3.

Por lo tanto, este estudio sirvió de partida para analizar el papel de los antioxidantes en un modelo de calcificación *in vitro*.

Símbolo	Nombre	20HP/Ref T test	Conciente 20HP/Ref	16HP/Ref T Test	Cociente 16HP/Ref	20HP/16HP T Test	Cociente 20HP/16HP	1-ANOVA	MA Si
PPIA_RAT	*Peptidyl-prolyl cis-trans isomerase A (Ciclophilin A)*	*0,0086*	*-1,17*			*0,048*	*-1,14*	*0,0091*	
PSME1_MOUSE	Proteasome activator complex subunit 1	0,016	1,5			0,023	1,36	0,0053	
MLRN_RAT	Myosin regulatory light chain 2, smooth muscle isoform	0,0088	-6,78			0,0076	-5,65	0,0085	
TPM2_RAT	Tropomyosin beta chain					0,028	-2,77	0,0036	
ACTC_RAT	Actin aortic smooth muscle					0,023	-2,14	0,00023	
CAPG_RAT	Macrophage-capping protein	0,001	3,45	0,028	1,59	0,0043	2,16	0,0035	
DEST_RAT	Destrin	*0,038*	*-1,93*			0,0052	-2,58	0,0087	
MYL6_RAT	Myosin light polypeptide 6	0,023	-2,71			0,016	-2,37	0,0095	
TAGL_RAT	Transgelin	0,03	-2,06					0,0083	
TPM3_RAT	Tropomyosin alpha-3 chain	0,0031	3,16			0,043	2,03	0,0044	
ACADM_RAT	Medium chain specific acyl CoA dehydrogenase mithocondrial precursor	0,037	-2,11					0,0026	
ALDR_RAT	Aldose reductase					0,025	-2,14	0,004	
CAH3_RAT	Carbonic anhidrase 3	0,0042	4,86			0,037	2,73	0,0069	
GPX3_RAT	Glutathione peroxidase 3 precursor	0,0052	-2,76	0,0021	-2,17			0,0079	
GSTM2_RAT	Glutathione S-transferase Mu2	0,018	-2,38			0,0055	-2,45	0,007	
IDPH_BOVIN	Isocitrate dehydrogenase NADP mitochondrial precursor			0,029	1,4			0,0026	
SBP1_RAT	Selenium binding protein 1	0,017	-1,54			0,0087	-1,57	0,0017	
SODM_RAT	Superoxide dismutase [Mn], mitochondrial precursor	0,0087	2,14	0,011	1,28	0,033	1,67	0,0081	
TKT_RAT	Transketolase	0,0041	-1,62	0,011	-1,6			0,001	
ALBU_RAT	Albumin			0,037	-2,61	0,022	1,65	0,0013	
APOE_RAT	Apolipoprotein E precursor	*0,0015*	*-1,92*			0,00097	-2,26	0,0044	
K1C10_RAT	Keratin type I cytoeskeletal 10	0,034	1,62					0,0022	
PRAX_MOUSE	Periaxin	*0,027*	*1,39*	0,04	1,41			0,0019	
S10A4_RAT	Protein S100-A4	*0,015*	*2,66*			0,035	2,13	0,01	
TRFE_RAT	Serotransferrin precursor	0,044	-2,19	0,024	-2,94			0,00042	

Ilustración 25. Lista de proteínas desreguladas en diversas etapas de la calcificación vascular

Nuestro estudio *in vitro* muestra que un medio calcificante, es decir, suplementado con Ca y P (3 y 2 mm, respectivamente) fue capaz de inducir el aumento de los ROS y la mineralización en cultivos primarios de CMLV. Estos resultados junto con otros (193), muestran que el estrés oxidativo está altamente asociado al propio proceso de calcificación vascular. En cambio, la heterogeneidad en la respuesta al tratamiento con varios antioxidantes muestra la complejidad de este proceso y como sólo la curcumina y silibina fueron capaces de reducir la mineralización de las CMLV (Ilustración 26).

	CONTROL	P+Ca	P+Ca+ Asc 100μM	P+Ca+ Troll 100μM	P+Ca+ Sili 50μM
Unidades relativas	0,013	1	1,13	1,18	0,64
Test T vs P+Ca	***<0,0001***	–	*0,97*	*0,18*	***0,008***

	P+Ca+ Resv 50μM	P+Ca+ Toco 500μM	P+Ca+ Toco100μM	P+Ca+ Curc 5μM
Unidades relativas	1,22	1,02	1,04	0,70
Test T vs P+Ca	*0,09*	*0,67*	*0,57*	***0,012***

Ilustración 26. Cuantificación de la tinción de rojo de Alizarina en los grupos de estudio.

La curcumina es el ingrediente activo de los remedios tradicionales a base de hierbas y especia cúrcuma dietética (*Cúrcuma longa*) y la silibina es el principal constituyente activo de la silimarina, compuesto cuya principal característica es un potente poder desintoxicante (194).

La curcumina pero no la silibina fueron capaces prevenir el aumento de los niveles de ROS comparados frente al grupo control.

	CONTROL	P+Ca	P+Ca+ Curc 5μM	P+Ca+ Sili 50μM
Unidades relativas	0.12	1.00	0.18	0.36
Test T vs P+Ca	*0.0034*	*--*	*0.001*	***0.001***
Test T vs Control	*--*	*0.003*	*0.73*	*0.06*

Ilustración 27. Cuantificación relativa del numero de células positivas para tinción con DHCF-DA mediante citometría de flujo.

La presencia de ROS se midió utilizando sondas fluorescentes relativamente específicas para H_2O_2, por lo que podemos hipotetizar que la curcumina disminuyó los niveles de H_2O_2. Los niveles de SOD-2 (responsable de la transformación de iones superóxido en oxígeno en H_2O_2 y agua) se incrementaron en el grupo P + Ca, pero no hubo cambios en el grupo tratado con la curcumina. Este hallazgo podría indicar que la curcumina actuaría modificando los niveles de H_2O_2, pero no en otro tipo de ROS situado aguas arriba de la cascada.

En conclusión, nuestros resultados confirman que la mineralización de CMLV se asocia con incrementos en el estrés oxidativo. La curcumina y silibina, dos conocidos antioxidantes de origen diferentes con una variedad de acciones descritas, fueron capaces de disminuir ROS y prevenir por ello la calcificación vascular. La curcumina disminuyó la expresión de Cbfa-1/Runx-2 sin modificar SOD-2, lo que sugiere que este antioxidante podría reducir directamente los niveles de H_2O_2.

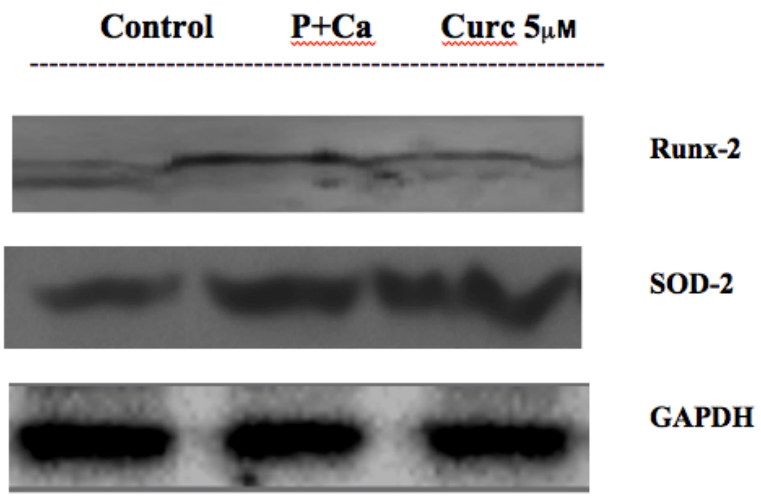

Ilustración 28. Western Blot para Cbfa-1/Runx2 y SOD-2 de extractos proteicos de CMLV cultivadas con medio control, calcificante (P+Ca) o calcificante con curcumina (Curc 5μM)

Estos resultados apoyan la conveniencia de realizar más estudios en este campo ya que algunos antioxidantes podrían tener potenciales beneficios en la gestión de la calcificación vascular.

Conclusiones

1. **El modelo de ratas urémicas con sobrecarga de fósforo se caracterizó por un aumento progresivo de los valores séricos de fósforo, PTH, FGF23, urea y creatinina y un moderado descenso de calcio.**
2. **La sobrecarga de fósforo en un modelo de ratas urémicas indujo diversos estadios de hiperparatiroidismo secundario, desde leve-moderado a severo.**
3. **Los diversos estadios de hiperparatiroidismo secundario se caracterizaron por una progresiva y generalizada regulación a la baja de la expresión génica.**
4. **Los genes del receptor sensor de calcio, receptor de Vitamina D y klotho sufrieron una inhibición significativa de su expresión en estadios avanzados de hiperparatiroidismo secundario.**
5. **En los estadios mas avanzados del hiperparatiroidismo secundario, el análisis del perfil de expresión génica mostró una regulación al alza de la familia de las "Dual Specificity Phosphatases".**
6. **La regulación al alza de las "Dual Specificity Phosphatases" se asoció con una bajada de los niveles de pERK.**
7. **Las "Dual Specificity Phosphatases" fueron capaces de suprimir parcialmente el efecto del FGF23 sobre la secrecion de PTH en glándulas paratiroides en cultivo.**
8. **La sobrecarga prolongada (de 16 a 20 semanas) de fósforo en un modelo de ratas urémicas se asoció a la presencia de calcificación vascular.**
9. **A las 20 semanas, se detectó actividad fosfatasa ácida tartrato resistente y expresión génica de catepsina K en las aortas calcificadas de ratas urémicas.**
10. **El análisis del perfil de expresión génica mostró una inhibición de tropomiosina y elastina y una regulación al alza de familia de las "Secreted Related Frizzled Proteins" tras 20 semanas.**
11. **Los animales que mostraron calcificación vascular manifiesta y severa fueron los que mostraron los valores de masa ósea mas bajos**
12. **La curcumina y la silibina fueron capaces de prevenir la mineralización en un modelo de calcificación vascular *in* vitro estimulado con un exceso de**

fósforo y calcio.

13. **La curcumina fue capaz de prevenir la subida de las especies reactivas de oxigeno, pero no el aumento de superoxido Dismutasa 2 en la mineralización *in vitro*.**

Bibliografía

1. Goodman, WG, Coburn, JW, Slatopolsky, E, Salusky, IB & Quarles, LD: Renal osteodystrophy in adults and children. In: *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism.* Quinta ed. edited by FAVUS, M. J., Washington, American Society for Bone and Mineral Research, 2003, pp 430-447.
2. Silver, J & HM, K: *Principles of Bone Biology,* San Diego, CA, Academic Press 1996.
3. Mannstadt, M & Drueke, TB: Parathyroid hormone receptors: from cloning to physiological, physiopathological and clinical implications. *Nephrologie,* 18**:** 5-10, 1997.
4. Almaden, Y, Hernandez, A, Torregrosa, V, Canalejo, A, Sabate, L, Fernandez Cruz, L, Campistol, JM, Torres, A & Rodriguez, M: High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue in vitro. *J Am Soc Nephrol,* 9**:** 1845-52, 1998.
5. Weber, TJ, Liu, S, Indridason, OS & Quarles, LD: Serum FGF23 levels in normal and disordered phosphorus homeostasis. *J Bone Miner Res,* 18**:** 1227-34, 2003.
6. Shimada, T, Kakitani, M, Yamazaki, Y, Hasegawa, H, Takeuchi, Y, Fujita, T, Fukumoto, S, Tomizuka, K & Yamashita, T: Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest,* 113**:** 561-8, 2004.
7. Shimada, T, Hasegawa, H, Yamazaki, Y, Muto, T, Hino, R, Takeuchi, Y, Fujita, T, Nakahara, K, Fukumoto, S & Yamashita, T: FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res,* 19**:** 429-35, 2004.
8. Ben-Dov, IZ, Galitzer, H, Lavi-Moshayoff, V, Goetz, R, Kuro-o, M, Mohammadi, M, Sirkis, R, Naveh-Many, T & Silver, J: The parathyroid is a target organ for FGF23 in rats. *J Clin Invest,* 117**:** 4003-8, 2007.
9. Rodriguez Puyol, D & Praga, M: Causas de la Insuficiencia Renal Crónica y sus mecanismos de progresión. In: *Nefrología clínica.* edited by HERNANDO AVENDANO, L., Madrid, Ed Panamericana, 1998, pp 535-546.
10. Wolf, M, Molnar, MZ, Amaral, AP, Czira, ME, Rudas, A, Ujszaszi, A, Kiss, I, Rosivall, L, Kosa, J, Lakatos, P, Kovesdy, CP & Mucsi, I: Elevated Fibroblast Growth Factor 23 is a Risk Factor for Kidney Transplant Loss and Mortality. *J Am Soc Nephrol,* 22**:** 956-66, 2011.
11. Isakova, T, Wahl, P, Vargas, GS, Gutierrez, OM, Scialla, J, Xie, H, Appleby, D, Nessel, L, Bellovich, K, Chen, J, Hamm, L, Gadegbeku, C, Horwitz, E, Townsend, RR, Anderson, CA, Lash, JP, Hsu, CY, Leonard, MB & Wolf, M: Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int*, 2011.
12. Silver, J & Naveh-Many, T: Phosphate and the parathyroid. *Kidney Int,* 75**:** 898-905, 2009.
13. Goodman, WG, Goldin, J, Kuizon, BD, Yoon, C, Gales, B, Sider, D, Wang, Y, Chung, J, Emerick, A, Greaser, L, Elashoff, RM & Salusky, IB: Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med,* 342**:** 1478-83, 2000.
14. Avram, MM, Sreedhara, R, Avram, DK, Muchnick, RA & Fein, P: Enrollment parathyroid hormone level is a new marker of survival in hemodialysis and peritoneal dialysis therapy for uremia. *Am J Kidney Dis,* 28**:** 924-30, 1996.
15. Rostand, SG & Drueke, TB: Parathyroid hormone, vitamin D, and cardiovascular disease in chronic renal failure. *Kidney Int,* 56**:** 383-92, 1999.
16. Cannata J: Osteodistrofia renal: evolución histórica. *Nefrologia,* 18 (supl 2)**:** 3-7, 1998.
17. Mucsi, I & Hercz, G: Relative hypoparathyroidism and adynamic bone disease. *Am J Med Sci,* 317**:** 405-9, 1999.
18. Moe, S, Drueke, T, Cunningham, J, Goodman, W, Martin, K, Olgaard, K, Ott, S, Sprague, S, Lameire, N & Eknoyan, G: Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int,* 69**:** 1945-53, 2006.

19. Martin, KJ & Slatopolsky, E: The parathyroids in renal disease. In: *The parathyroids, basic and clinical concepts.* edited by BILEZIKIAN JP, M. R. Y. L. M., New York, Raven Press, 1994, pp 711-720.
20. Naveh-Many, T & Silver, J: Regulation of parathyroid hormone gene expression and secretion by vitamin D. In: *Vitamin D: Physiology, Molecular Biology and Clinical Applications.* edited by HOLICK, M. F., Totowa, Humana Press Inc., 1998, pp 217-237.
21. Silver, J, Naveh-Many, T, Mayer, H, Schmelzer, HJ & Popovtzer, MM: Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat. *J Clin Invest,* 78: 1296-301, 1986.
22. Russell, J, Lettieri, D & Sherwood, LM: The effects of 1,25-dihydroxyvitamin D3 on the synthesis of pre-proparathyroid hormone mRNA. *Trans Assoc Am Physicians,* 99: 189-96, 1986.
23. Slatopolsky, E, Caglar, S, Gradowska, L, Canterbury, J, Reiss, E & Bricker, NS: On the prevention of secondary hyperparathyroidism in experimental chronic renal disease using "proportional reduction" of dietary phosphorus intake. *Kidney Int,* 2: 147-51, 1972.
24. Reiss, E, Canterbury, JM, Bercovitz, MA & Kaplan, EL: The role of phosphate in the secretion of parathyroid hormone in man. *J Clin Invest,* 49: 2146-2149, 1970.
25. Tanaka, Y & Deluca, HF: The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. *Arch Biochem Biophys,* 154: 566-74, 1973.
26. Lopez-Hilker, S, Dusso, AS, Rapp, NS, Martin, KJ & Slatopolsky, E: Phosphorus restriction reverses hyperparathyroidism in uremia independent of changes in calcium and calcitriol. *Am J Physiol,* 259: F432-7, 1990.
27. Slatopolsky, E, Finch, J, Denda, M, Ritter, C, Zhong, M, Dusso, A, MacDonald, PN & Brown, AJ: Phosphorus restriction prevents parathyroid gland growth. High phosphorus directly stimulates PTH secretion in vitro. *J Clin Invest,* 97: 2534-40, 1996.
28. Wang, Q, Palnitkar, S & Parfitt, AM: Parathyroid cell proliferation in the rat: effect of age and of phosphate administration and recovery. *Endocrinology,* 137: 4558-62, 1996.
29. Naveh-Many, T, Rahamimov, R, Livni, N & Silver, J: Parathyroid cell proliferation in normal and chronic renal failure rats. The effects of calcium, phosphate, and vitamin D. *J Clin Invest,* 96: 1786-93, 1995.
30. Brown, EM, Gamba, G, Riccardi, D, Lombardi, M, Butters, R, Kifor, O, Sun, A, Hediger, MA, Lytton, J & Hebert, SC: Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature,* 366: 575-80, 1993.
31. Kifor, O, Diaz, R, Butters, R & Brown, EM: The Ca2+-sensing receptor (CaR) activates phospholipases C, A2, and D in bovine parathyroid and CaR-transfected, human embryonic kidney (HEK293) cells. *J Bone Miner Res,* 12: 715-25, 1997.
32. Brown, AJ, Ritter, CS, Finch, JL & Slatopolsky, EA: Decreased calcium-sensing receptor expression in hyperplastic parathyroid glands of uremic rats: role of dietary phosphate. *Kidney Int,* 55: 1284-92, 1999.
33. Brown, AJ, Dusso, A, Lopez-Hilker, S, Lewis-Finch, J, Grooms, P & Slatopolsky, E: 1,25-(OH)2D receptors are decreased in parathyroid glands from chronically uremic dogs. *Kidney Int,* 35: 19-23, 1989.
34. Fukuda, N, Tanaka, H, Tominaga, Y, Fukagawa, M, Kurokawa, K & Seino, Y: Decreased 1,25-dihydroxyvitamin D3 receptor density is associated with a more severe form of parathyroid hyperplasia in chronic uremic patients. *J Clin Invest,* 92: 1436-43, 1993.
35. Dusso, A, Cozzolino, M, Lu, Y, Sato, T & Slatopolsky, E: 1,25-Dihydroxyvitamin D downregulation of TGFalpha/EGFR expression and growth signaling: a mechanism for the antiproliferative actions of the sterol in parathyroid hyperplasia of renal failure. *J Steroid Biochem Mol Biol,* 89-90: 507-11, 2004.
36. Moallem, E, Kilav, R, Silver, J & Naveh-Many, T: RNA-Protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. *J Biol Chem,* 27: 5253-9, 1998.

37. Silver, J, Kilav, R & Naveh-Many, T: Mechanisms of secondary hyperparathyroidism. *Am J Physiol Renal Physiol,* 283: F367-76, 2002.
38. Martin, DR, Ritter, CS, Slatopolsky, E & Brown, AJ: Acute regulation of parathyroid hormone by dietary phosphate. *Am J Physiol Endocrinol Metab,* 289: E729-34, 2005.
39. Almaden, Y, Felsenfeld, AJ, Rodriguez, M, Canadillas, S, Luque, F, Bas, A, Bravo, J, Torregrosa, V, Palma, A, Ramos, B, Sanchez, C, Martin-Malo, A & Canalejo, A: Proliferation in hyperplastic human and normal rat parathyroid glands: role of phosphate, calcitriol, and gender. *Kidney Int,* 64: 2311-7, 2003.
40. Studer, H & Derwahl, M: Mechanisms of nonneoplastic endocrine hyperplasia--a changing concept: a review focused on the thyroid gland. *Endocr Rev,* 16: 411-26, 1995.
41. Kolek, OI, Hines, ER, Jones, MD, LeSueur, LK, Lipko, MA, Kiela, PR, Collins, JF, Haussler, MR & Ghishan, FK: 1alpha,25-Dihydroxyvitamin D3 upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport. *Am J Physiol Gastrointest Liver Physiol,* 289: G1036-42, 2005.
42. Liu, S, Tang, W, Zhou, J, Stubbs, JR, Luo, Q, Pi, M & Quarles, LD: Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *J Am Soc Nephrol,* 17: 1305-15, 2006.
43. Urakawa, I, Yamazaki, Y, Shimada, T, Iijima, K, Hasegawa, H, Okawa, K, Fujita, T, Fukumoto, S & Yamashita, T: Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature,* 444: 770-4, 2006.
44. Shimada, T, Urakawa, I, Yamazaki, Y, Hasegawa, H, Hino, R, Yoneya, T, Takeuchi, Y, Fujita, T, Fukumoto, S & Yamashita, T: FGF-23 transgenic mice demonstrate hypophosphatemic rickets with reduced expression of sodium phosphate cotransporter type IIa. *Biochem Biophys Res Commun,* 314: 409-14, 2004.
45. Bai, X, Miao, D, Li, J, Goltzman, D & Karaplis, AC: Transgenic mice overexpressing human fibroblast growth factor 23 (R176Q) delineate a putative role for parathyroid hormone in renal phosphate wasting disorders. *Endocrinology,* 145: 5269-79, 2004.
46. Larsson, T, Marsell, R, Schipani, E, Ohlsson, C, Ljunggren, O, Tenenhouse, HS, Juppner, H & Jonsson, KB: Transgenic mice expressing fibroblast growth factor 23 under the control of the alpha1(I) collagen promoter exhibit growth retardation, osteomalacia, and disturbed phosphate homeostasis. *Endocrinology,* 145: 3087-94, 2004.
47. Imanishi, Y, Inaba, M, Nakatsuka, K, Nagasue, K, Okuno, S, Yoshihara, A, Miura, M, Miyauchi, A, Kobayashi, K, Miki, T, Shoji, T, Ishimura, E & Nishizawa, Y: FGF-23 in patients with end-stage renal disease on hemodialysis. *Kidney Int,* 65: 1943-6, 2004.
48. Gutierrez, O, Isakova, T, Rhee, E, Shah, A, Holmes, J, Collerone, G, Juppner, H & Wolf, M: Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. *J Am Soc Nephrol,* 16: 2205-15, 2005.
49. Fliser, D, Kollerits, B, Neyer, U, Ankerst, DP, Lhotta, K, Lingenhel, A, Ritz, E, Kronenberg, F, Kuen, E, Konig, P, Kraatz, G, Mann, JF, Muller, GA, Kohler, H & Riegler, P: Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the Mild to Moderate Kidney Disease (MMKD) Study. *J Am Soc Nephrol,* 18: 2600-8, 2007.
50. Nakanishi, S, Kazama, JJ, Nii-Kono, T, Omori, K, Yamashita, T, Fukumoto, S, Gejyo, F, Shigematsu, T & Fukagawa, M: Serum fibroblast growth factor-23 levels predict the future refractory hyperparathyroidism in dialysis patients. *Kidney Int,* 67: 1171-8, 2005.
51. Gutierrez, OM, Mannstadt, M, Isakova, T, Rauh-Hain, JA, Tamez, H, Shah, A, Smith, K, Lee, H, Thadhani, R, Juppner, H & Wolf, M: Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. *N Engl J Med,* 359: 584-92, 2008.
52. Krajisnik, T, Bjorklund, P, Marsell, R, Ljunggren, O, Akerstrom, G, Jonsson, KB, Westin, G & Larsson, TE: Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cells. *J Endocrinol,* 195: 125-31, 2007.

53. Garfia, B, Canadillas, S, Canalejo, A, Luque, F, Siendones, E, Quesada, M, Almaden, Y, Aguilera-Tejero, E & Rodriguez, M: Regulation of parathyroid vitamin D receptor expression by extracellular calcium. *J Am Soc Nephrol,* 13: 2945-52, 2002.
54. Canalejo, R, Canalejo, A, Martinez-Moreno, JM, Rodriguez-Ortiz, ME, Estepa, JC, Mendoza, FJ, Munoz-Castaneda, JR, Shalhoub, V, Almaden, Y & Rodriguez, M: FGF23 fails to inhibit uremic parathyroid glands. *J Am Soc Nephrol,* 21: 1125-35, 2010.
55. Komaba, H, Goto, S, Fujii, H, Hamada, Y, Kobayashi, A, Shibuya, K, Tominaga, Y, Otsuki, N, Nibu, KI, Nakagawa, K, Tsugawa, N, Okano, T, Kitazawa, R & Fukagawa, M: Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients. *Kidney Int*, 2009.
56. Galitzer, H, Ben-Dov, IZ, Silver, J & Naveh-Many, T: Parathyroid cell resistance to fibroblast growth factor 23 in secondary hyperparathyroidism of chronic kidney disease. *Kidney Int,* 77: 211-8, 2010.
57. Contiguglia, SR, Alfrey, AC, Miller, NL, Runnells, DE & Le Geros, RZ: Nature of soft tissue calcification in uremia. *Kidney Int,* 4: 229-35, 1973.
58. Goldsmith, DJ, Covic, A, Sambrook, PA & Ackrill, P: Vascular calcification in long-term haemodialysis patients in a single unit: a retrospective analysis. *Nephron,* 77: 37-43, 1997.
59. Block, GA, Klassen, PS, Lazarus, JM, Ofsthun, N, Lowrie, EG & Chertow, GM: Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *J Am Soc Nephrol,* 15: 2208-18, 2004.
60. Schiffrin, EL, Lipman, ML & Mann, JF: Chronic kidney disease: effects on the cardiovascular system. *Circulation,* 116: 85-97, 2007.
61. Cannata-Andia, JB, Rodriguez-Garcia, M, Carrillo-Lopez, N, Naves-Diaz, M & Diaz-Lopez, B: Vascular calcifications: pathogenesis, management, and impact on clinical outcomes. *J Am Soc Nephrol,* 17: S267-73, 2006.
62. Zoccali, C, Mallamaci, F & Tripepi, G: Novel cardiovascular risk factors in end-stage renal disease. *J Am Soc Nephrol,* 15 Suppl 1: S77-80, 2004.
63. Haydar, AA, Covic, A, Colhoun, H, Rubens, M & Goldsmith, DJ: Coronary artery calcification and aortic pulse wave velocity in chronic kidney disease patients. *Kidney Int,* 65: 1790-4, 2004.
64. Floege, J & Ketteler, M: Vascular calcification in patients with end-stage renal disease. *Nephrol Dial Transplant,* 19 Suppl 5: V59-66, 2004.
65. Rodriguez Garcia, M, Gomez Alonso, C, Diaz-Lopez, B, Naves Diaz, M & Cannata Andia, JB: Vascular calcifications, vertebral fractures and mortality in haemodialysis patients. *Nephrol Dial Transplant*: In Press, 2008.
66. Moe, SM & Chen, NX: Pathophysiology of vascular calcification in chronic kidney disease. *Circ Res,* 95: 560-7, 2004.
67. Hofbauer, LC, Brueck, CC, Shanahan, CM, Schoppet, M & Dobnig, H: Vascular calcification and osteoporosis-from clinical observation towards molecular understanding. *Osteoporos Int,* 18: 251-9, 2007.
68. Moe, SM: Vascular calcification: Hardening of the evidence. *Kidney Int,* 70: 1535-7, 2006.
69. Moe, SM & Chen, NX: Mechanisms of vascular calcification in chronic kidney disease. *J Am Soc Nephrol,* 19: 213-6, 2008.
70. Giachelli, CM: Vascular calcification mechanisms. *J Am Soc Nephrol,* 15: 2959-64, 2004.
71. Giachelli, CM, Jono, S, Shioi, A, Nishizawa, Y, Mori, K & Morii, H: Vascular calcification and inorganic phosphate. *Am J Kidney Dis,* 38: S34-7, 2001.
72. Hofbauer, LC & Schoppet, M: Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. *Jama,* 292: 490-5, 2004.
73. Moe, SM, O'Neill, KD, Duan, D, Ahmed, S, Chen, NX, Leapman, SB, Fineberg, N & Kopecky, K: Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins. *Kidney Int,* 61: 638-47, 2002.

74. Naves M, GA, Marelli C, Tejada J, Silvestri F, Passlick-Deetjen J, Cannata-Andia J: Relative Risk (RR) of Death According to Serum Ca, P and PTH. Results from a Large Sample of Dialysis Patients from Latin America Followed for up to 54 Months. The CORES Study (abstract). *J Am Soc Nephrol,* 16**:** 728A, 2005.
75. Ganesh, SK, Stack, AG, Levin, NW, Hulbert-Shearon, T & Port, FK: Association of elevated serum PO(4), Ca x PO(4) product, and parathyroid hormone with cardiac mortality risk in chronic hemodialysis patients. *J Am Soc Nephrol,* 12**:** 2131-8, 2001.
76. Block, GA, Hulbert-Shearon, TE, Levin, NW & Port, FK: Association of serum phosphorus and calcium x phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis,* 31**:** 607-17, 1998.
77. Rebsamen, MC, Sun, J, Norman, AW & Liao, JK: 1alpha,25-dihydroxyvitamin D3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. *Circ Res,* 91**:** 17-24, 2002.
78. Jono, S, Nishizawa, Y, Shioi, A & Morii, H: 1,25-Dihydroxyvitamin D3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. *Circulation,* 98**:** 1302-6, 1998.
79. Villa-Bellosta, R, Bogaert, YE, Levi, M & Sorribas, V: Characterization of phosphate transport in rat vascular smooth muscle cells: implications for vascular calcification. *Arterioscler Thromb Vasc Biol,* 27**:** 1030-6, 2007.
80. Li, X, Yang, HY & Giachelli, CM: Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. *Circ Res,* 98**:** 905-12, 2006.
81. Jono, S, McKee, MD, Murry, CE, Shioi, A, Nishizawa, Y, Mori, K, Morii, H & Giachelli, CM: Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res,* 87**:** E10-7, 2000.
82. Mizobuchi, M, Ogata, H, Hatamura, I, Koiwa, F, Saji, F, Shiizaki, K, Negi, S, Kinugasa, E, Ooshima, A, Koshikawa, S & Akizawa, T: Up-regulation of Cbfa1 and Pit-1 in calcified artery of uraemic rats with severe hyperphosphataemia and secondary hyperparathyroidism. *Nephrol Dial Transplant,* 21**:** 911-6, 2006.
83. Inoue, T & Kawashima, H: 1,25-Dihydroxyvitamin D3 stimulates 45Ca2+-uptake by cultured vascular smooth muscle cells derived from rat aorta. *Biochem Biophys Res Commun,* 152**:** 1388-94, 1988.
84. Rajasree, S, Umashankar, PR, Lal, AV, Sarma, PS & Kartha, CC: 1,25-dihydroxyvitamin D3 receptor is upregulated in aortic smooth muscle cells during hypervitaminosis D. *Life Sci,* 70**:** 1777-88, 2002.
85. Cardus, A, Panizo, S, Encinas, M, Dolcet, X, Gallego, C, Aldea, M, Fernandez, E & Valdivielso, JM: 1,25-dihydroxyvitamin D3 regulates VEGF production through a vitamin D response element in the VEGF promoter. *Atherosclerosis,* 204**:** 85-9, 2009.
86. Mizobuchi, M, Finch, JL, Martin, DR & Slatopolsky, E: Differential effects of vitamin D receptor activators on vascular calcification in uremic rats. *Kidney Int,* 72**:** 709-15, 2007.
87. Panizo, S, Cardus, A, Encinas, M, Parisi, E, Valcheva, P, Lopez-Ongil, S, Coll, B, Fernandez, E & Valdivielso, JM: RANKL increases vascular smooth muscle cell calcification through a RANK-BMP4-dependent pathway. *Circ Res,* 104**:** 1041-8, 2009.
88. Bostrom, K, Watson, KE, Horn, S, Wortham, C, Herman, IM & Demer, LL: Bone morphogenetic protein expression in human atherosclerotic lesions. *J Clin Invest,* 91**:** 1800-9, 1993.
89. Dhore, CR, Cleutjens, JP, Lutgens, E, Cleutjens, KB, Geusens, PP, Kitslaar, PJ, Tordoir, JH, Spronk, HM, Vermeer, C & Daemen, MJ: Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol,* 21**:** 1998-2003, 2001.
90. Nakaoka, T, Gonda, K, Ogita, T, Otawara-Hamamoto, Y, Okabe, F, Kira, Y, Harii, K, Miyazono, K, Takuwa, Y & Fujita, T: Inhibition of rat vascular smooth muscle proliferation in vitro and in vivo by bone morphogenetic protein-2. *J Clin Invest,* 100**:** 2824-32, 1997.

91. Lee, MH, Kim, YJ, Kim, HJ, Park, HD, Kang, AR, Kyung, HM, Sung, JH, Wozney, JM & Ryoo, HM: BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. *J Biol Chem,* 278: 34387-94, 2003.
92. Mathew, S, Tustison, KS, Sugatani, T, Chaudhary, LR, Rifas, L & Hruska, KA: The Mechanism of Phosphorus as a Cardiovascular Risk Factor in CKD. *J Am Soc Nephrol,* 19: 1092-105, 2008.
93. Shao, JS, Cheng, SL, Pingsterhaus, JM, Charlton-Kachigian, N, Loewy, AP & Towler, DA: Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. *J Clin Invest,* 115: 1210-20, 2005.
94. Shao, JS, Cai, J & Towler, DA: Molecular mechanisms of vascular calcification: lessons learned from the aorta. *Arterioscler Thromb Vasc Biol,* 26: 1423-30, 2006.
95. Lund, RJ, Davies, MR, Brown, AJ & Hruska, KA: Successful treatment of an adynamic bone disorder with bone morphogenetic protein-7 in a renal ablation model. *J Am Soc Nephrol,* 15: 359-69, 2004.
96. Shao, JS, Aly, ZA, Lai, CF, Cheng, SL, Cai, J, Huang, E, Behrmann, A & Towler, DA: Vascular Bmp Msx2 Wnt signaling and oxidative stress in arterial calcification. *Ann N Y Acad Sci,* 1117: 40-50, 2007.
97. Shalhoub, V, Shatzen, E, Henley, C, Boedigheimer, M, McNinch, J, Manoukian, R, Damore, M, Fitzpatrick, D, Haas, K, Twomey, B, Kiaei, P, Ward, S, Lacey, DL & Martin, D: Calcification inhibitors and Wnt signaling proteins are implicated in bovine artery smooth muscle cell calcification in the presence of phosphate and vitamin D sterols. *Calcif Tissue Int,* 79: 431-42, 2006.
98. Roman-Garcia, P, Carrillo-Lopez, N, Fernandez-Martin, JL, Naves-Diaz, M, Ruiz-Torres, MP & Cannata-Andia, JB: High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. *Bone,* 46: 121-8, 2010.
99. Byon, CH, Javed, A, Dai, Q, Kappes, JC, Clemens, TL, Darley-Usmar, VM, McDonald, JM & Chen, Y: Oxidative Stress Induces Vascular Calcification through Modulation of the Osteogenic Transcription Factor Runx2 by AKT Signaling. *J Biol Chem,* 283: 15319-15327, 2008.
100. Liberman, M, Bassi, E, Martinatti, MK, Lario, FC, Wosniak, J, Jr., Pomerantzeff, PM & Laurindo, FR: Oxidant generation predominates around calcifying foci and enhances progression of aortic valve calcification. *Arterioscler Thromb Vasc Biol,* 28: 463-70, 2008.
101. Hofmann Bowman, MA, Gawdzik, J, Bukhari, U, Husain, AN, Toth, PT, Kim, G, Earley, J & McNally, EM: S100A12 in vascular smooth muscle accelerates vascular calcification in apolipoprotein E-null mice by activating an osteogenic gene regulatory program. *Arterioscler Thromb Vasc Biol,* 31: 337-44, 2011.
102. Kuro-o, M, Matsumura, Y, Aizawa, H, Kawaguchi, H, Suga, T, Utsugi, T, Ohyama, Y, Kurabayashi, M, Kaname, T, Kume, E, Iwasaki, H, Iida, A, Shiraki-Iida, T, Nishikawa, S, Nagai, R & Nabeshima, YI: Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature,* 390: 45-51, 1997.
103. Binkert, C, Demetriou, M, Sukhu, B, Szweras, M, Tenenbaum, HC & Dennis, JW: Regulation of osteogenesis by fetuin. *J Biol Chem,* 274: 28514-20, 1999.
104. Merx, MW, Schafer, C, Westenfeld, R, Brandenburg, V, Hidajat, S, Weber, C, Ketteler, M & Jahnen-Dechent, W: Myocardial stiffness, cardiac remodeling, and diastolic dysfunction in calcification-prone fetuin-A-deficient mice. *J Am Soc Nephrol,* 16: 3357-64, 2005.
105. Berkner, KL & Runge, KW: The physiology of vitamin K nutriture and vitamin K-dependent protein function in atherosclerosis. *J Thromb Haemost,* 2: 2118-32, 2004.
106. Price, PA, Faus, SA & Williamson, MK: Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol,* 18: 1400-7, 1998.

107. Steitz, SA, Speer, MY, Curinga, G, Yang, HY, Haynes, P, Aebersold, R, Schinke, T, Karsenty, G & Giachelli, CM: Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circ Res,* 89: 1147-54, 2001.
108. Shanahan, CM, Cary, NR, Salisbury, JR, Proudfoot, D, Weissberg, PL & Edmonds, ME: Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. *Circulation,* 100: 2168-76, 1999.
109. Bucay, N, Sarosi, I, Dunstan, CR, Morony, S, Tarpley, J, Capparelli, C, Scully, S, Tan, HL, Xu, W, Lacey, DL, Boyle, WJ & Simonet, WS: osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev,* 12: 1260-8, 1998.
110. Price, PA, June, HH, Buckley, JR & Williamson, MK: Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. *Arterioscler Thromb Vasc Biol,* 21: 1610-6, 2001.
111. Liu, Y & Shanahan, CM: Signalling pathways and vascular calcification. *Front Biosci,* 16: 1302-14, 2011.
112. Son, BK, Kozaki, K, Iijima, K, Eto, M, Kojima, T, Ota, H, Senda, Y, Maemura, K, Nakano, T, Akishita, M & Ouchi, Y: Statins protect human aortic smooth muscle cells from inorganic phosphate-induced calcification by restoring Gas6-Axl survival pathway. *Circ Res,* 98: 1024-31, 2006.
113. Son, BK, Kozaki, K, Iijima, K, Eto, M, Nakano, T, Akishita, M & Ouchi, Y: Gas6/Axl-PI3K/Akt pathway plays a central role in the effect of statins on inorganic phosphate-induced calcification of vascular smooth muscle cells. *Eur J Pharmacol,* 556: 1-8, 2007.
114. Speer, MY, Yang, HY, Brabb, T, Leaf, E, Look, A, Lin, WL, Frutkin, A, Dichek, D & Giachelli, CM: Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res,* 104: 733-41, 2009.
115. Abedin, M, Lim, J, Tang, TB, Park, D, Demer, LL & Tintut, Y: N-3 fatty acids inhibit vascular calcification via the p38-mitogen-activated protein kinase and peroxisome proliferator-activated receptor-gamma pathways. *Circ Res,* 98: 727-9, 2006.
116. Pulver-Kaste, RA, Barlow, CA, Bond, J, Watson, A, Penar, PL, Tranmer, B & Lounsbury, KM: Ca2+ source-dependent transcription of CRE-containing genes in vascular smooth muscle. *Am J Physiol Heart Circ Physiol,* 291: H97-105, 2006.
117. Kapustin, AN, Davies, JD, Reynolds, JL, McNair, R, Jones, GT, Sidibe, A, Schurgers, LJ, Skepper, JN, Proudfoot, D, Mayr, M & Shanahan, CM: Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circ Res,* 109: e1-e12, 2011.
118. Molostvov, G, James, S, Fletcher, S, Bennett, J, Lehnert, H, Bland, R & Zehnder, D: Extracellular calcium-sensing receptor is functionally expressed in human artery. *Am J Physiol Renal Physiol,* 293: F946-55, 2007.
119. Caudrillier, A, Mentaverri, R, Brazier, M, Kamel, S & Massy, ZA: Calcium-sensing receptor as a potential modulator of vascular calcification in chronic kidney disease. *J Nephrol,* 23: 17-22, 2010.
120. Bear, M, Butcher, M & Shaughnessy, SG: Oxidized low-density lipoprotein acts synergistically with beta-glycerophosphate to induce osteoblast differentiation in primary cultures of vascular smooth muscle cells. *J Cell Biochem,* 105: 185-93, 2008.
121. Tanikawa, T, Okada, Y, Tanikawa, R & Tanaka, Y: Advanced glycation end products induce calcification of vascular smooth muscle cells through RAGE/p38 MAPK. *J Vasc Res,* 46: 572-80, 2009.
122. Ragnauth, CD, Warren, DT, Liu, Y, McNair, R, Tajsic, T, Figg, N, Shroff, R, Skepper, J & Shanahan, CM: Prelamin A acts to accelerate smooth muscle cell senescence and is a novel biomarker of human vascular aging. *Circulation,* 121: 2200-10, 2010.
123. Varela, I, Cadinanos, J, Pendas, AM, Gutierrez-Fernandez, A, Folgueras, AR, Sanchez, LM, Zhou, Z, Rodriguez, FJ, Stewart, CL, Vega, JA, Tryggvason, K, Freije, JM & Lopez-

Otin, C: Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation. *Nature,* 437: 564-8, 2005.
124. Shao, JS, Cheng, SL, Sadhu, J & Towler, DA: Inflammation and the osteogenic regulation of vascular calcification: a review and perspective. *Hypertension,* 55: 579-92, 2010.
125. Hofbauer, LC, Brueck, CC, Shanahan, CM, Schoppet, M & Dobnig, H: Vascular calcification and osteoporosis--from clinical observation towards molecular understanding. *Osteoporos Int,* 18: 251-9, 2007.
126. Samelson, EJ, Kiel, DP, Broe, KE, Zhang, Y, Cupples, LA, Hannan, MT, Wilson, PW, Levy, D, Williams, SA & Vaccarino, V: Metacarpal cortical area and risk of coronary heart disease: the Framingham Study. *Am J Epidemiol,* 159: 589-95, 2004.
127. Kiel, DP, Kauppila, LI, Cupples, LA, Hannan, MT, O'Donnell, CJ & Wilson, PW: Bone loss and the progression of abdominal aortic calcification over a 25 year period: the Framingham Heart Study. *Calcif Tissue Int,* 68: 271-6, 2001.
128. Schulz, E, Arfai, K, Liu, X, Sayre, J & Gilsanz, V: Aortic calcification and the risk of osteoporosis and fractures. *J Clin Endocrinol Metab,* 89: 4246-53, 2004.
129. Bagger, YZ, Tanko, LB, Alexandersen, P, Qin, G & Christiansen, C: Radiographic measure of aorta calcification is a site-specific predictor of bone loss and fracture risk at the hip. *J Intern Med,* 259: 598-605, 2006.
130. Almaden, Y, Canalejo, A, Hernandez, A, Ballesteros, E, Garcia-Navarro, S, Torres, A & Rodriguez, M: Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands in vitro. *J Bone Miner Res,* 11: 970-6, 1996.
131. Palmer, SC, Hayen, A, Macaskill, P, Pellegrini, F, Craig, JC, Elder, GJ & Strippoli, GF: Serum levels of phosphorus, parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. *Jama,* 305: 1119-27, 2011.
132. Shanahan, CM: Mechanisms of vascular calcification in renal disease. *Clin Nephrol,* 63: 146-57, 2005.
133. Holmen, SL, Giambernardi, TA, Zylstra, CR, Buckner-Berghuis, BD, Resau, JH, Hess, JF, Glatt, V, Bouxsein, ML, Ai, M, Warman, ML & Williams, BO: Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. *J Bone Miner Res,* 19: 2033-40, 2004.
134. Al-Aly, Z, Shao, JS, Lai, CF, Huang, E, Cai, J, Behrmann, A, Cheng, SL & Towler, DA: Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr-/- mice. *Arterioscler Thromb Vasc Biol,* 27: 2589-96, 2007.
135. Towler, DA, Shao, JS, Cheng, SL, Pingsterhaus, JM & Loewy, AP: Osteogenic regulation of vascular calcification. *Ann N Y Acad Sci,* 1068: 327-33, 2006.
136. Surendran, K, Schiavi, S & Hruska, KA: Wnt-dependent beta-catenin signaling is activated after unilateral ureteral obstruction, and recombinant secreted frizzled-related protein 4 alters the progression of renal fibrosis. *J Am Soc Nephrol,* 16: 2373-84, 2005.
137. Damment, SJ & Shen, V: Assessment of effects of lanthanum carbonate with and without phosphate supplementation on bone mineralization in uremic rats. *Clin Nephrol,* 63: 127-37, 2005.
138. Gadola, L, Noboa, O, Marquez, MN, Rodriguez, MJ, Nin, N, Boggia, J, Ferreiro, A, Garcia, S, Ortega, V, Musto, ML, Ponte, P, Sesser, P, Pizarrosa, C, Ravaglio, S & Vallega, A: Calcium citrate ameliorates the progression of chronic renal injury. *Kidney Int,* 65: 1224-30, 2004.
139. Kang, DH, Nakagawa, T, Feng, L, Watanabe, S, Han, L, Mazzali, M, Truong, L, Harris, R & Johnson, RJ: A role for uric acid in the progression of renal disease. *J Am Soc Nephrol,* 13: 2888-97, 2002.
140. Ormrod, D & Miller, T: Experimental uremia. Description of a model producing varying degrees of stable uremia. *Nephron,* 26: 249-54, 1980.

141. Virgós MJ, Fernández Soto I, Naves M & Cannata JB: Acerca de la insuficiencia renal crónica experimental. *Nefrología,* X: 103-104, 1990.
142. Waynforth, H: *Experimental and surgical technique in the rat,* London, Harcourt Brace Jovanovich 1980.
143. Waynforth, HB: *Specific surgical Operations en Experimental and Surgical Technique in the Rat,* London, Academic Press 1980.
144. Corradi, L, Fato, M, Porro, I, Scaglione, S & Torterolo, L: A Web-based and Grid-enabled dChip version for the analysis of large sets of gene expression data. *BMC Bioinformatics,* 9: 480, 2008.
145. Li, C & Hung Wong, W: Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol,* 2: RESEARCH0032, 2001.
146. Kanehisa, M & Goto, S: KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res,* 28: 27-30, 2000.
147. Alvarez-Hernandez, D, Gonzalez-Suarez, I, Naves, M, Carrillo-Lopez, N, Fdez-Coto, T, Fernandez-Martin, JL & Cannata-Andia, JB: Long-term response of cultured rat parathyroid glands to calcium and calcitriol: the effect of cryopreservation. *J Nephrol,* 18: 141-7, 2005.
148. Chomczynski, P: A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques,* 15: 532-4, 536-7, 1993.
149. Livak, KJ & Schmittgen, TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25: 402-8, 2001.
150. Bradford, MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem,* 72: 248-54, 1976.
151. Laemmli, UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227: 680-5, 1970.
152. Silver, J: Molecular mechanisms of secondary hyperparathyroidism. *Nephrol Dial Transplant,* 15 Suppl 5: 2-7, 2000.
153. Cannata-Andia, JB & Rodriguez-Garcia, M: Hyperphosphataemia as a cardiovascular risk factor -- how to manage the problem. *Nephrol Dial Transplant,* 17 Suppl 11: 16-9, 2002.
154. Coladonato, JA: Control of hyperphosphatemia among patients with ESRD. *J Am Soc Nephrol,* 16 Suppl 2: S107-14, 2005.
155. Ritz, E: The clinical management of hyperphosphatemia. *J Nephrol,* 18: 221-8, 2005.
156. Santamaria, I, Alvarez-Hernandez, D & Cannata-Andia, JB: Genetics and molecular disorders in severe secondary hyperparathyroidism: lessons from rna and microarray studies. *J Nephrol,* 18: 469-73, 2005.
157. Santamaria, I, Alvarez-Hernandez, D, Jofre, R, Polo, JR, Menarguez, J & Cannata-Andia, JB: Progression of secondary hyperparathyroidism involves deregulation of genes related to DNA and RNA stability. *Kidney Int,* 67: 2267-79, 2005.
158. Zhang, M, Zhang, L, Zou, J, Yao, C, Xiao, H, Liu, Q, Wang, J, Wang, D, Wang, C & Guo, Z: Evaluating reproducibility of differential expression discoveries in microarray studies by considering correlated molecular changes. *Bioinformatics,* 25: 1662-8, 2009.
159. Afonso, S, Santamaria, I, Guinsburg, ME, Gomez, AO, Miranda, JL, Jofre, R, Menarguez, J, Cannata-Andia, J & Cigudosa, JC: Chromosomal aberrations, the consequence of refractory hyperparathyroidism: its relationship with biochemical parameters. *Kidney Int Suppl*: S32-8, 2003.
160. Patterson, KI, Brummer, T, O'Brien, PM & Daly, RJ: Dual-specificity phosphatases: critical regulators with diverse cellular targets. *Biochem J,* 418: 475-89, 2009.
161. Groom, LA, Sneddon, AA, Alessi, DR, Dowd, S & Keyse, SM: Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *Embo J,* 15: 3621-32, 1996.

162. Shin, DY, Ishibashi, T, Choi, TS, Chung, E, Chung, IY, Aaronson, SA & Bottaro, DP: A novel human ERK phosphatase regulates H-ras and v-raf signal transduction. *Oncogene,* 14**:** 2633-9, 1997.
163. Owens, DM & Keyse, SM: Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene,* 26**:** 3203-13, 2007.
164. Silver, J & Naveh-Many, T: FGF23 and the parathyroid glands. *Pediatr Nephrol,* 25**:** 2241-5, 2010.
165. Hofman-Bang, J, Martuseviciene, G, Santini, MA, Olgaard, K & Lewin, E: Increased parathyroid expression of klotho in uremic rats. *Kidney Int,* 78**:** 1119-27, 2010.
166. Ekerot, M, Stavridis, MP, Delavaine, L, Mitchell, MP, Staples, C, Owens, DM, Keenan, ID, Dickinson, RJ, Storey, KG & Keyse, SM: Negative-feedback regulation of FGF signalling by DUSP6/MKP-3 is driven by ERK1/2 and mediated by Ets factor binding to a conserved site within the DUSP6/MKP-3 gene promoter. *Biochem J,* 412**:** 287-98, 2008.
167. Carrillo-Lopez, N, Alvarez-Hernandez, D, Gonzalez-Suarez, I, Roman-Garcia, P, Valdivielso, JM, Fernandez-Martin, JL & Cannata-Andia, JB: Simultaneous changes in the calcium-sensing receptor and the vitamin D receptor under the influence of calcium and calcitriol. *Nephrol Dial Transplant,* 23**:** 3479-84, 2008.
168. Gonzalez-Suarez, I, Alvarez-Hernandez, D, Carrillo-Lopez, N, Naves-Diaz, M, Luis Fernandez-Martin, J & Cannata-Andia, JB: Aluminum posttranscriptional regulation of parathyroid hormone synthesis: a role for the calcium-sensing receptor. *Kidney Int,* 68**:** 2484-96, 2005.
169. Ueda, K, Arakawa, H & Nakamura, Y: Dual-specificity phosphatase 5 (DUSP5) as a direct transcriptional target of tumor suppressor p53. *Oncogene,* 22**:** 5586-91, 2003.
170. Hruska, KA, Choi, ET, Memon, I, Davis, TK & Mathew, S: Cardiovascular risk in chronic kidney disease (CKD): the CKD-mineral bone disorder (CKD-MBD). *Pediatr Nephrol,* 25**:** 769-78, 2010.
171. Huttunen, MM, Tillman, I, Viljakainen, HT, Tuukkanen, J, Peng, Z, Pekkinen, M & Lamberg-Allardt, CJ: High dietary phosphate intake reduces bone strength in the growing rat skeleton. *J Bone Miner Res,* 22**:** 83-92, 2007.
172. Frye, MA, Melton, LJ, 3rd, Bryant, SC, Fitzpatrick, LA, Wahner, HW, Schwartz, RS & Riggs, BL: Osteoporosis and calcification of the aorta. *Bone Miner,* 19**:** 185-94, 1992.
173. Davies, MR, Lund, RJ, Mathew, S & Hruska, KA: Low turnover osteodystrophy and vascular calcification are amenable to skeletal anabolism in an animal model of chronic kidney disease and the metabolic syndrome. *J Am Soc Nephrol,* 16**:** 917-28, 2005.
174. Davies, MR, Lund, RJ & Hruska, KA: BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. *J Am Soc Nephrol,* 14**:** 1559-67, 2003.
175. Silver, J, Kilav, R, Sela-Brown, A & Naveh-Many, T: Molecular mechanisms of secondary hyperparathyroidism. *Pediatr Nephrol,* 14**:** 626-8, 2000.
176. Neves, KR, Graciolli, FG, dos Reis, LM, Graciolli, RG, Neves, CL, Magalhaes, AO, Custodio, MR, Batista, DG, Jorgetti, V & Moyses, RM: Vascular calcification: contribution of parathyroid hormone in renal failure. *Kidney Int,* 71**:** 1262-70, 2007.
177. Bossard, MJ, Tomaszek, TA, Thompson, SK, Amegadzie, BY, Hanning, CR, Jones, C, Kurdyla, JT, McNulty, DE, Drake, FH, Gowen, M & Levy, MA: Proteolytic activity of human osteoclast cathepsin K. Expression, purification, activation, and substrate identification. *J Biol Chem,* 271**:** 12517-24, 1996.
178. Adragao, T, Herberth, J, Monier-Faugere, MC, Branscum, AJ, Ferreira, A, Frazao, JM, Dias Curto, J & Malluche, HH: Low bone volume--a risk factor for coronary calcifications in hemodialysis patients. *Clin J Am Soc Nephrol,* 4**:** 450-5, 2009.
179. Braun, J, Oldendorf, M, Moshage, W, Heidler, R, Zeitler, E & Luft, FC: Electron beam computed tomography in the evaluation of cardiac calcification in chronic dialysis patients. *Am J Kidney Dis,* 27**:** 394-401, 1996.

180. Raggi, P, James, G, Burke, SK, Bommer, J, Chasan-Taber, S, Holzer, H, Braun, J & Chertow, GM: Decrease in thoracic vertebral bone attenuation with calcium-based phosphate binders in hemodialysis. *J Bone Miner Res,* 20: 764-72, 2005.
181. Toussaint, ND, Lau, KK, Strauss, BJ, Polkinghorne, KR & Kerr, PG: Associations between vascular calcification, arterial stiffness and bone mineral density in chronic kidney disease. *Nephrol Dial Transplant,* 23: 586-93, 2008.
182. London, GM, Marchais, SJ, Guerin, AP, Boutouyrie, P, Metivier, F & de Vernejoul, MC: Association of bone activity, calcium load, aortic stiffness, and calcifications in ESRD. *J Am Soc Nephrol,* 19: 1827-35, 2008.
183. London, GM, Marty, C, Marchais, SJ, Guerin, AP, Metivier, F & de Vernejoul, MC: Arterial calcifications and bone histomorphometry in end-stage renal disease. *J Am Soc Nephrol,* 15: 1943-51, 2004.
184. Coen, G, Ballanti, P, Mantella, D, Manni, M, Lippi, B, Pierantozzi, A, Di Giulio, S, Pellegrino, L, Romagnoli, A, Simonetti, G & Splendiani, G: Bone turnover, osteopenia and vascular calcifications in hemodialysis patients. A histomorphometric and multislice CT study. *Am J Nephrol,* 29: 145-52, 2009.
185. Bilezikian, JP: Bone strength in primary hyperparathyroidism. *Osteoporos Int,* 14 Suppl 5: S113-5; discussion S115-7, 2003.
186. Bodine, PV, Zhao, W, Kharode, YP, Bex, FJ, Lambert, AJ, Goad, MB, Gaur, T, Stein, GS, Lian, JB & Komm, BS: The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol Endocrinol,* 18: 1222-37, 2004.
187. Kobayashi, K, Luo, M, Zhang, Y, Wilkes, DC, Ge, G, Grieskamp, T, Yamada, C, Liu, TC, Huang, G, Basson, CT, Kispert, A, Greenspan, DS & Sato, TN: Secreted Frizzled-related protein 2 is a procollagen C proteinase enhancer with a role in fibrosis associated with myocardial infarction. *Nat Cell Biol,* 11: 46-55, 2009.
188. Berndt, T, Craig, TA, Bowe, AE, Vassiliadis, J, Reczek, D, Finnegan, R, Jan De Beur, SM, Schiavi, SC & Kumar, R: Secreted frizzled-related protein 4 is a potent tumor-derived phosphaturic agent. *J Clin Invest,* 112: 785-94, 2003.
189. Towler, DA: Oxidation, inflammation, and aortic valve calcification peroxide paves an osteogenic path. *J Am Coll Cardiol,* 52: 851-4, 2008.
190. Miller, JD, Chu, Y, Brooks, RM, Richenbacher, WE, Pena-Silva, R & Heistad, DD: Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. *J Am Coll Cardiol,* 52: 843-50, 2008.
191. Mann, JF, Lonn, EM, Yi, Q, Gerstein, HC, Hoogwerf, BJ, Pogue, J, Bosch, J, Dagenais, GR & Yusuf, S: Effects of vitamin E on cardiovascular outcomes in people with mild-to-moderate renal insufficiency: results of the HOPE study. *Kidney Int,* 65: 1375-80, 2004.
192. Yang, CC, Hsu, SP, Wu, MS, Hsu, SM & Chien, CT: Effects of vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress. *Kidney Int,* 69: 706-14, 2006.
193. Ciceri, P, Volpi, E, Brenna, I, Arnaboldi, L, Neri, L, Brancaccio, D & Cozzolino, M: Combined effects of ascorbic acid and phosphate on rat VSMC osteoblastic differentiation. *Nephrol Dial Transplant*, 2011.
194. Kim, YS, Ahn, Y, Hong, MH, Joo, SY, Kim, KH, Sohn, IS, Park, HW, Hong, YJ, Kim, JH, Kim, W, Jeong, MH, Cho, JG, Park, JC & Kang, JC: Curcumin attenuates inflammatory responses of TNF-alpha-stimulated human endothelial cells. *J Cardiovasc Pharmacol,* 50: 41-9, 2007.

Anexo: otros trabajos publicados durante la Tesis Doctoral

Publicacion I

Review

Expert Opinion

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Parathyroid gland regulation: contribution of the *in vivo* and *in vitro* models

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Importance of the field: The current regulation of parathyroid hormone (PTH) and the development of parathyroid disorders in chronic kidney disease involve complex mechanisms. Factors such as calcium, phosphorous, calcitriol, vitamin D receptor, calcium-sensing receptor and fibroblast growth factor 23 play a key role in the regulatory process in the pathogenesis of secondary hyperparathyroidism.
Areas covered in this review: This review provides an analysis of published results related to the different models and approaches used to study the mechanisms involved in the pathogenesis of secondary hyperparathyroidism. The review includes clinical studies, animal and *ex vivo*/*in vitro* models which have been extensively used in this area.
What the reader will gain: Readers will have an overview of the main findings and progress achieved in the knowledge of the parathyroid function combining the results obtained from the different models used to understand the parathyroid gland regulation.
Take home message: Each of the available models used to study the complex system of parathyroid regulation has advantages and limitations; therefore, it is necessary to combine the information obtained from more than one model in order to have a more complete knowledge of the mechanisms involved in PTH regulation.

Keywords: calcium, FGF23, *in vitro* models, *in vivo* models, parathyroid function, parathyroid hormone, phosphorous, secondary hyperparathyroidism

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1. Introduction

The progression of chronic kidney disease (CKD) leads to a reduction of 1-α hydroxylase in the kidney, which in turn results in low levels of the active form of vitamin D (1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] or calcitriol) impairing calcium absorption in the intestine favoring the reduction in serum calcium [1]. As a result, the decreases in serum calcium stimulate parathyroid hormone (PTH) synthesis and release, which in turn increase bone turnover, bone resorption and stimulate 1-α hydroxylase. All these mechanisms lead to compensatory increases in serum calcium.

In addition, the progressive reduction of the renal function impairs phosphorous excretion, leading to increases in serum phosphorous, which increases fibroblast growth factor 23 (FGF23) and PTH; both mechanisms increase urinary phosphorous excretion. However, FGF23 and PTH exert opposite effects on calcitriol synthesis: FGF23 inhibits 1-α hydroxylase, whereas PTH stimulates it.

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Article highlights.

- Chronic kidney disease-mineral and bone disorders (CKD-MBDs) are very common in dialysis patients. They are characterized by combinations of abnormalities in: i) calcium, phosphorous, parathyroid hormone (PTH) or vitamin D metabolism; ii) bone turnover, mineralization, volume, linear growth or strength and iii) vascular or other soft tissue calcification.
- PTH is secreted by the parathyroid glands and is mainly responsible for the control of calcium homeostasis.
- PTH regulation involves calcium, calcitriol, phosphorous and the recently discovered phosphaturic hormone fibroblast growth factor 23.
- Other drugs used for the treatment of CKD-MBD, such as calcimimetics and aluminum, are also able to suppress PTH levels.
- Clinical studies have provided important contributions to the knowledge of the pathogenesis of secondary hyperparathyroidism and also have allowed to identify the cell pathways and mechanisms involved in the progression of the disease.
- Animal models of CKD have also been useful to study the different factors involved in the regulation of the PTH; in addition, they have been crucial to confirm that phosphorous is one of the main players in PTH regulation.
- *In vitro/ex vivo* models have contributed to a better understanding of the molecular mechanisms involved in the pathogenesis and progression of secondary hyperparathyroidism.
- The combination of the results obtained from clinical and experimental studies has been essential to better understand the parathyroid regulation and the effect of the different therapies on CKD-MBD.

This box summarizes key points contained in the article.

As the renal function decreases, all these complex and tightly interrelated mechanisms of parathyroid gland regulation fail to adequately control the parathyroid gland function. As a result, low serum levels of calcitriol and calcium and phosphorous retention are present at late stages of CKD. Furthermore, at advanced stages of this disorder, CKD stage five patients show severe forms of secondary hyperparathyroidism with diffuse and nodular hyperplasia and a significant reduction in the vitamin D and calcium-sensing receptors (VDR and CaSR) expression with a poor response of the parathyroid glands to calcium changes and vitamin D analogue therapy, and a clear trend towards autonomous (tertiary) parathyroid gland behavior.

Several of the mineral abnormalities end up inducing not only bone disease, but also several cardiovascular disorders, including vascular calcifications and a greater risk of mortality [2]. The recently coined term 'chronic kidney disease-mineral and bone disorder' (CKD-MBD) encompasses all these abnormalities [3]. CKD-MBD includes either one or a combination of: i) calcium, phosphorous, PTH or vitamin D metabolism; ii) bone turnover, mineralization, volume, linear growth or strength and iii) vascular or other soft tissue calcification. There are excellent reviews focused on different aspects of CKD-MBD [4-7]; however, this review deals mainly with aspects related to the parathyroid gland regulation.

1.1 Parathyroid hormone

PTH is synthesized by the parathyroid cells; through the blood stream, PTH reaches the main target sites, the kidney and bone [8]. The main role of PTH is the regulation of calcium homeostasis. Under physiological conditions, osteoblasts are stimulated by PTH via its specific PTH receptor, which then send signals to bone marrow-derived osteoclast precursors to stimulate their fusion, differentiation and activation. The mature and active osteoclasts resorb bone and release calcium to the blood. In addition, PTH provides an additional calcium homeostatic response in order to preserve normal serum calcium levels acting in the kidneys by increasing tubular calcium reabsorption.

PTH can also exert other anabolic actions in bone. Intermittent or pulsatile injections of recombinant PTH, as well as injections with amino-terminal fragments, are able to increase bone formation and bone mass; the latter became the basis for the use of PTH injections to treat osteoporosis [9]. Besides vitamin D, PTH is the only other anabolic bone agent known.

1.2 Parathyroid gland regulation

PTH regulation involves a complex mechanism in which calcium [10], calcitriol [11], phosphorous [12] and FGF23 [13] play a central role.

Both calcium and calcitriol act on the parathyroid cells trough their specific receptors, CaSR and VDR. While the CaSR is a cell-membrane receptor member of the GPCR family, the VDR is a nuclear receptor that, when bound to vitamin D, acts as a transcription factor. The differences in the nature of the two ligands and their receptors lead to two different mechanisms of action with a complementary function on the parathyroid cells.

On one hand, small decreases in extracellular calcium concentrations are rapidly sensed by the CaSR, triggering, within seconds or minutes, an increase in PTH release. Small increases in calcium are also sensed by the CaSR, yielding opposite results [14-16]. If the stimulus persists for longer periods (hours, days), calcium is able to regulate PTH synthesis post-transcriptionally by modifying the mRNA stability through differences in binding of the parathyroid proteins to an element in its 3′-untranslated region (3′-UTR) [17,18]. As a result, the decreases in serum calcium reduce mRNA degradation by increasing its stability and the half-life of mRNA PTH. By contrast, the active form of vitamin D (calcitriol) inhibits the PTH gene transcription resulting in a reduction of the PTH synthesis [19-22].

In CKD, the reduction of renal function and active renal mass, together with the increase in serum FGF23 [23], decrease 1-α hydroxylase synthesis with the consequent reduction in calcitriol synthesis which, in turn, decreases intestinal calcium absorption but also leads to a lower PTH gene transcription suppression. Both mechanisms favor the increase of PTH.

High phosphorous is another factor able to act on the parathyroid cells by increasing PTH synthesis [12,24,25] through a post-transcriptional mechanism stabilizing the PTH mRNA [17]. Finally, calcium, calcitriol and phosphorous are well-known factors involved in parathyroid cell proliferation; thus, abnormalities of these factors may contribute to the development of parathyroid gland hyperplasia [26,27].

Although the effects of calcium, calcitriol and even phosphorous on the parathyroid function take place through specific mechanisms, these three factors also produce additional complementary effects, mainly through their interaction with the CaSR and VDR parathyroid receptors.

The main function of the CaSR is to sense calcium; however, the CaSR expression in parathyroid glands does not seem to be regulated by the extracellular calcium levels [28-30]. In contrast, it has been described that CaSR expression can be regulated by calcitriol, which can increase CaSR even under hypocalcemic conditions [28,31,32]. Also, phosphorous may influence the CaSR expression; in fact, a reduction in the expression of CaSR has been described in the presence of hyperphosphatemia [33-35].

Regarding VDR regulation, contrary to what occurs with calcium and CaSR regulation, calcitriol does regulate its own receptor, VDR, stimulating its synthesis and half-life [28,36,37]. In addition, calcium is also able to modify the VDR expression [28,32,38-40].

Finally, FGF23, initially described as a potent phosphatonin [41], is involved not only in the control of phosphorous but also in the regulation of vitamin D metabolism [42] and PTH synthesis [13]. Elegant studies performed by Silver and coworkers [13] have demonstrated a direct effect of FGF23 on the parathyroid gland through the MAPK pathway, leading to a decrease in PTH synthesis and secretion. In addition, the putative, indirect regulation of PTH by estrogens through FGF23 has been also recently described [43]. The importance of FGF23 in the pathogenesis of secondary hyperparathyroidism is still under investigation; nevertheless, alternative paradigms for the pathogenesis of secondary hyperparathyroidism in CKD involving FGF23 have been recently proposed [44].

1.3 Other important factors involved in PTH secretion

Other factors such as calcimimetics and aluminum can also act on the PTH regulation.

Calcimimetics, the CaSR allosteric modulators recently introduced for the treatment of secondary hyperparathyroidism, act by increasing CaSR sensitivity to extracellular calcium and decreasing PTH synthesis and secretion [45-47]. Moreover, calcimimetics are also able to cooperate with VDR activators, increasing the VDR expression in the parathyroid glands [48]. As a result, the use of calcimimetics not only achieves the known effect on the CaSR, but can also increase the VDR expression.

Finally, several studies have demonstrated the inhibitory effect of aluminum on PTH mRNA levels through complex and combined mechanisms, including a direct action on the CaSR, by reducing its gene expression through a post-transcriptional mechanism [49,50]. It seems clear that the parathyroid gland is a target tissue for aluminum, and part of the direct inhibitory effect of aluminum is also due to its capacity to reduce cell proliferation in the parathyroid gland [50].

Most of the previously described findings have been obtained thanks to the contribution of different *in vivo* and *ex vivo/in vitro* models used to study the regulation of the parathyroid gland function. The remaining part of this review describes and analyzes different models which have been used to investigate the complex mechanisms involved in PTH regulation.

2. Models used to study the parathyroid gland function

2.1 Contribution of human clinical studies

The human clinical studies were mostly aimed to obtain information at the functional, morphological, molecular and genomic levels.

Clinical studies carried out in humans have revealed important aspects of the pathogenesis of secondary hyperparathyroidism. Functional studies carried out in humans have clearly shown that hypocalcemia stimulates PTH secretion, while hypercalcemia suppresses it. The mathematical model which best relates PTH and serum calcium is a sigmoidal curve [51]. The set-point for calcium has been defined as the calcium concentration at which the maximal PTH is reduced by 50% [52]. In the CKD patients with secondary hyperparathyroidism, the set-point of calcium is shifted to the right, meaning that a higher concentration of calcium is necessary to suppress the PTH. This is the result of a sum of factors which end up increasing the size of the parathyroid gland and decreasing VDR and CaSR expression, the main receptors known to be involved in the parathyroid gland regulation [53-56].

This functional relationship between calcium and PTH has been extensively used in humans to investigate the effect of VDR activators and calcimimetics on the parathyroid gland, assuming that if they are effective they should be able to partly or totally correct the abnormal calcium and parathyroid sigmoidal relationship observed in the advanced forms of secondary hyperparathyroidism. So far, both have been able to shift the set-point of calcium to the left, partly recovering parathyroid sensitivity to calcium [57]. Finally, a successful parathyroidectomy has also proved to be effective in reducing PTH levels and shifting the PTH–calcium curve to the left [58].

The importance and contribution of high phosphorous in the pathogenesis of secondary hyperparathyroidism was experimentally demonstrated many years ago [59]. However, since then, human studies have not been able to fully differentiate and dissect the effect of phosphorous independently from the changes in serum calcium [60] and/or calcitriol levels.

As secondary hyperparathyroidism progresses, the parathyroid glands become refractory to medical treatment and parathyroidectomy is frequently needed. Human parathyroid tissue obtained from parathyroidectomies has been used for genetic, genomic and molecular studies aiming to investigate in depth the mechanisms involved in parathyroid gland deregulation in advanced stages of parathyroid hyperplasia.

The most advanced form of this disorder, nodular hyperplasia, has been associated with chromosomal aberrations [61] and also with severe changes in gene expression [62,63]. To study these specific aspects in diffuse and nodular parathyroid glands from renal patients who underwent parathyroidectomy, several techniques have been used, such as comparative genomic hybridization [61], microarrays and bidirectional subtraction library [62]. The combination of these techniques has allowed to demonstrate that the progression of secondary hyperparathyroidism damages several cell pathways at different levels, a fact which partly explains the multiple, complex and integrated cellular mechanisms involved in the progression of the disease [62].

Cell growth is highly promoted in the nodules; DNA stability is severely compromised because the protective mechanism and repair systems fail; RNA synthesis and stability are also in jeopardy. Finally, protein synthesis, processing and destination become clearly hindered due to failures in the folding, assembly and sorting of the polypeptides. All these striking alterations, dominated by the profile of gene repression found in the severe cases of nodular secondary hyperparathyroidism, are almost impossible to control at this late stage of the disease and alert the need of an early approach in the management of secondary hyperparathyroidism in CKD patients [62].

In the nodular severe forms of secondary hyperparathyroidism, the monoclonal growth of the parathyroid gland dominates the scene. A study revealed that 64% of hemodialysis patients with refractory secondary hyperparathyroidism showed at least one parathyroid lesion with monoclonal growth [64], whereas another study found that the monoclonal pattern was present in 58% of hyperplastic nodules in females [65]. However, the genes implicated in the genesis and evolution of secondary hyperparathyroidism monoclonality are not those observed in primary hyperparathyroidism [63].

Despite human studies being very useful to understand some aspects of the pathogenesis and progression of secondary hyperparathyroidism, they show several limitations, mainly due to the lack of homogeneity of the studies. As an example, most of the published works have been carried out in patients with different ages and different degrees of severity of secondary hyperparathyroidism receiving different treatments. Thus, the experimental models have been of great value to complement and further expand the investigation on parathyroid regulation.

2.2 Contribution of the *in vivo* animal models

The main advances in the understanding of the pathogenesis of secondary hyperparathyroidism have been possible mainly thanks to the results obtained using experimental animal models of CKD. Partial nephrectomy has been the most common technique used to produce CKD [26,66-69], although more recently the addition of adenine to the diet has become a current model to study CKD-MBD [47,70]. Five/six or seven/eight nephrectomy induces a moderate renal insufficiency, which in most cases is not enough to develop severe secondary hyperparathyroidism. To increase the magnitude of the latter, the concomitant use of high phosphorous diet was introduced to provide a substantial extra stimulus, resulting in a more severe degree of secondary hyperparathyroidism.

The studies performed following the previously described procedures obtained significant increases in PTH gene expression [71] and severe secondary hyperparathyroidism. The stimulatory effect of high serum phosphorous on PTH is powerful enough to exert its action independently of the serum calcium levels [72], achieving PTH levels 20 – 40 times higher than those obtained using only partial nephrectomy with no phosphorous supplementation [33,73].

The rat model with CKD has been used to describe that the regulation of the parathyroid function by calcium and phosphorous occurs at post-transcriptional level by regulating the binding of proteins to the 3′-UTR of the PTH mRNA [74]. Furthermore, the addition of high phosphorous levels to the diet not only induced a severe parathyroid hyperplasia, but also triggered a reduction in the expression of CaSR in the same areas of the parathyroid gland where an increased cell proliferation was observed [33]. Similar studies have also shown that the increase in cell proliferation rate produced by a high phosphorous diet precedes the downregulation of CaSR [75], suggesting that the mechanisms which stimulate the parathyroid cell proliferation precede the reduction in CaSR expression.

The *in vivo* animal studies also have been fundamental to demonstrate the important effects of calcitriol, calcium and calcimimetics. Calcitriol inhibits PTH gene expression and stimulates VDR expression in parathyroid tissue [76,77]. Despite VDR being the specific receptor for calcitriol and other active vitamin D metabolites, serum calcium levels also influence VDR expression; in fact, a recent study has shown that the higher the serum calcium, the higher the expression of VDR levels [32].

The increased PTH gene expression in experimental uremia has been also reversed using calcimimetics which act via a post-transcriptional mechanism involving the *trans*-acting factor AUF1 present in the parathyroid glands [47].

Regarding regression of the parathyroid hyperplasia after different treatments, the animal models have helped to enhance the knowledge in this area. Calcitriol has shown to decrease cell proliferation and increase apoptosis restoring the levels of CaSR and VDR, leading to a regression of the parathyroid hyperplasia in uremic rats [78]. The direct injection of calcitriol or other vitamin D analogues into hyperplasic glands has also been able to induce cell apoptosis, suggesting this could be a valid, alternative method to reduce the size of the parathyroid

gland [79]. Similarly, calcimimetics have proven to be able to reduce parathyroid cell proliferation and gland size in uremic rats with secondary hyperparathyroidism [80].

The animal models used to study secondary hyperparathyroidism have also been used to analyze other aspects of CKD-MBD, such as vascular and other soft tissue calcifications [81-83]. A recent study demonstrated that, after 20 weeks, rats with CKD receiving a high phosphorous diet developed not only severe secondary hyperparathyroidism but also severe vascular calcifications leading to changes in the gene and protein expression profiles of the calcified aortas [73]. In addition, other recent studies have been useful to show the effects and interactions of drugs such as calcitriol, paricalcitol, calcimimetics and biphosphonates in the production and regression of vascular calcifications [84-86].

One important general limitation of the animal models is that even though several parameters can be controlled, others frequently associated or related to uncontrolled or unmeasured parameters cannot be controlled, leading to undesirable modifications in other factors involved in PTH regulation. In some studies, this issue makes it difficult to reach definitive conclusions about the chronology and/or the importance of the factors and mechanisms studied. To limit the influence of all these uncontrolled factors, other models such as knockout models or *ex vivo/in vitro* models have been extensively used to better understand the regulation of the PTH secretion.

2.3 Contribution of the knockout models

Knockout models have been of great value to make precise the role of several known factors in the pathogenesis of secondary hyperparathyroidism.

CaSR knockout has demonstrated the key role of this membrane protein in calcium homeostasis and PTH regulation [87]. Mice with a partial CaSR knockout are characterized by modest elevations of serum calcium and PTH levels as well as hypocalciuria, whereas mice with complete CaSR knockout show elevated serum calcium and PTH levels, parathyroid hyperplasia, bone abnormalities, retarded growth and premature death, thus, demonstrating the great importance of the CaSR in several aspects of parathyroid gland regulation and bone health.

Recently, the role of the CaSR in maintaining calcium homeostasis in the absence of PTH and consequently the CaSR-regulated PTH secretion has been described, by using single and double knockout mouse models for CaSR and/or PTH [88]. Thus, the double knockout for CaSR and PTH showed high serum calcium levels, similar to those presented in the single CaSR-null mice, supporting the fact that CaSR defends against hypercalcemia independently of its regulation of PTH secretion by increasing the urinary calcium excretion.

On the other hand, several papers have tried to demonstrate the effect of VDR on parathyroid gland regulation by using VDR null mice [89-91]. In all cases, VDR null mice presented severe hypocalcemia and secondary hyperparathyroidism that could be corrected with a high calcium rescue diet, indirectly demonstrating the role of VDR in the normal parathyroid physiology. Furthermore, a recent study with a specific deletion of VDR has provided additional information on the role of this receptor in the control of the parathyroid gland. The deletion induced a moderate increase in PTH levels but also a reduction in the parathyroid CaSR expression, suggesting that the VDR has a limited role in the parathyroid gland regulation [92].

2.4 Contribution of the *ex vivo/in vitro* models

The functional and molecular studies of the response of the parathyroid glands using *ex vivo/in vitro* models have been limited: first, because of the lack of functional parathyroid cell lines and second, because of the limited functional long-term response to calcium observed when isolated parathyroid cells were cultured.

A great number of the *in vitro* studies have been carried out to demonstrate the viability and functionality of primary monolayer parathyroid cell cultures. The methodology followed for this type of culture is simple and there are no important differences in all the published papers [93-98]. Briefly, the parathyroid tissue is minced into small fragments and digested with collagenase in culture medium. Then, the parathyroid cells are released from the tissue to the culture medium, the cells are collected, centrifugated and resuspended in a growth media to work with them.

Unfortunately, despite the simplicity of the procedure to obtain the cells, dispersed or primary monolayer parathyroid cells such as those from bovine often become progressively less responsive to changes in extracellular calcium [93,94] probably due to a rapid decrease in CaSR mRNA and protein levels, a phenomenon observed after a few hours of culture, which seems not to be influenced by the culture conditions (medium serum, calcium or calcitriol).

In contrast, another study has found that proliferating bovine parathyroid cells in early passages preserve their functionality, and they were able to respond to calcium and calcitriol even after 72 h after subconfluency [95]. Under these conditions, it has been found that calcitriol is able to suppress cell proliferation. Similarly, positive results have also been obtained with a human parathyroid cell culture model from uremic patients with secondary hyperparathyroidism [96], in which the parathyroid cells remain viable and functional until the fifth passage, which corresponds approximately to 5 months of follow-up as assessed by persistent responsiveness to changes in extracellular calcium.

In addition, by using this model, it has been found that calcium, calcimimetics and calcitriol were able to decrease parathyroid cell proliferation, whereas phosphorous increased it [97]. Monolayer cultures of bovine parathyroid cells have also been successfully used to study the ability of FGF23 to regulate PTH and 1-α hydroxylase expression [98].

In summary, despite the mentioned controversy and limitations, mainly concerning the study of PTH regulation

by calcium, primary monolayer cell cultures have been of great usefulness to study the other important factors involved in parathyroid gland regulation, such as calcitriol, calcimimetics, phosphorous and FGF23.

To improve the performance of the parathyroid cell culture model, a new approach has been recently used. It consists in the coincubation of the bovine parathyroid cells with a t-tail type I collagen; after 1 – 2 weeks, the parathyroid cells coalesce into a 3D organoid, termed 'pseudoglands'. It has been shown that the CaSR mRNA expression in these pseudoglands decreased after 1 day of incubation; however, later on, this negative effect apparently disappears and the CaSR expression increased becoming almost normal after longer periods of culture. Using this model, it was proven that PTH mRNA can be suppressed by extracellular calcium, demonstrating its usefulness to the study of the calcium-mediated control of the parathyroid gland [99].

In addition, it has been recently described that parathyroid cells in culture were able to produce cell aggregates called 'spheroids' which secreted PTH for > 150 days [100]. These have been recently used in parathyroid cells obtained from patients with secondary hyperparathyroidism in order to suppress the production of PTH by siRNA, a method which provides a useful approach for further studies. The results from the innovative parathyroid spheroid cell culture model stress once more the importance of maintaining a 3D structure in order to have adequate parathyroid cell functionality.

Due to some of the above-mentioned limitations of parathyroid cell culture, non-parathyroid cells have also been used to study some specific mechanisms involved in parathyroid hyperplasia. For example, the human epidermoid carcinoma cell line, A431 which mimics hyperplastic parathyroid cells, has been used to demonstrate that the TGF-α/EGFR system is one of the key elements in the regulation of parathyroid hyperplasia [101,102]. In addition, human embryonic kidney cells (HEK293) co-transfected with bovine CaSR have been used to demonstrate that aluminum is a strong agonist of the CaSR [50]. In addition and more recently, the same kidney cells co-transfected with both human CaSR and human PTH plasmids were used to study the regulation of PTH gene expression by the calcimimetic R568 [103]. The results indicated that in the cells co-transfected with CaSR, the PTH gene expression was regulated by calcium and the calcimimetic R568; conversely, there was no response in cells without CaSR transfection. The PTH CaSR-dependent decreased gene expression observed in these engineered cells occurs via the balanced interactions of the *trans*-acting factors KSRP and AUF1 with PTH mRNA, as already described *in vivo* [47,74].

Despite all progresses, efforts and sophisticated adaptations such as the one described above, parathyroid cell culture is still a model with some limitations when used to study the PTH regulation by calcium. Therefore, some authors have had to use *ex vivo/in vitro* cultures of isolated parathyroid glands to further explore parathyroid regulation. In fact, the latter has become the reference model to analyze the parathyroid molecular mechanisms in response to different stimuli [12,25,104,105].

In this type of *ex vivo/in vitro* culture, the whole rat parathyroid glands are extracted and excised from the surrounding thyroid tissue and then immediately placed in well plates containing the experimental culture medium. In the case of human parathyroid glands, slices of tissue are cultured following the same procedure.

By using this model and culturing parathyroid glands from rats, it has been found that calcium is able to acutely suppress PTH secretion as previously described *in vivo* [12,106]. Similar results were obtained in human hyperplastic parathyroid glands [25]. This model has also allowed for the study of the effect of serum calcium and phosphorous on the parathyroid function [12,106]. In fact, it has been shown that calcium does not influence the expression of its own receptor (CaSR), but, in contrast, it is able to upregulate the parathyroid VDR [28]. On the other hand, the effect of phosphorous on PTH secretion is slower than that observed for calcium [12], increasing PTH mRNA, as demonstrated in parathyroid tissue obtained from parathyroidectomies of hemodialysis and kidney transplant patients [25]. The effect of phosphorous on PTH secretion has also been demonstrated in bovine parathyroid tissue slices but not in dispersed cells, pointing out again the importance of having a 3D architecture in order to obtain adequate functionality in these cells [107].

The use of parathyroid glands from rats has also showed that calcitriol upregulates not only VDR but also CaSR, even in the presence of low calcium levels [28], a finding not described in previous studies [29]. Furthermore, using human parathyroid tissue it has been demonstrated that calcitriol suppresses parathyroid cell proliferation, as long as the phosphorous concentration in the culture medium is normal [108]. In addition, the ability of calcimimetics, alone or in combination with calcitriol, to suppress PTH secretion and to increase VDR expression has also been proven in human and rat hyperplastic parathyroid glands [48], showing that the effect of calcimimetics was exerted, independently of calcium levels, in a concentration-dependent manner.

The *ex vivo/in vitro* parathyroid gland culture model has also been used to demonstrate that aluminum suppresses not only PTH secretion but also PTH mRNA by a post-transcriptional mechanism, acting as a true CaSR agonist [50].

Finally, this model has proven to be useful to test the functionality of fresh and cryopreserved fragments of parathyroid glands which are currently used for the re-implantation of parathyroid tissue [109]. This is a practical matter of great interest because one of the still unsolved problems for surgeons is how to select the best fragments of parathyroid tissue to be re-implanted, either as fresh or cryopreserved. Several techniques have been used to help in this selection but the results have been quite heterogeneous [110-112]. Still, in most cases, the decision has to be made in the

operating theatre based only on the macroscopic appearance of the parathyroid gland and, unfortunately, no definitive solid results on this matter have been achieved.

The functional studies with fresh or cryopreserved parathyroid fragments seems to indicate that fresh tissue preserves almost all biological properties whereas after cryopreservation, the parathyroid glands maintain some functionality but their capacity to fully respond to some effectors, such as calcium, for long periods of time seems to be impaired [113,114].

3. Expert opinion

The study of the parathyroid function regulation is very complex. In the last decades, great advances on the pathogenesis of the disease have been achieved. Human and experimental studies of all types have been crucial to better understand the parathyroid gland behavior in CKD, and also to know that the development and progression of secondary hyperparathyroidism occur due to a combination of factors.

Many aspects of the main factors and feedbacks involved in the parathyroid regulation have been progressively researched and described in the past 4 decades. The late stages of CKD involve intrinsic molecular and genomic changes which are responsible for the morphological disturbances found in the parathyroid glands. Thanks to the combination of the results obtained from all the experimental models, some of them summarized in this review, the knowledge of the parathyroid function has greatly increased.

However, in more recent years, the molecular biology techniques have been crucial to further the knowledge about the parathyroid function. Fortunately, new findings in this field are published each year which allow us to better specify the role and weight of each known factor in the physiological and pathological regulation of the parathyroid gland at the different stages of CKD.

Phosphorous retention, low levels of calcium and calcitriol all stimulate PTH production and parathyroid cell proliferation. In addition, FGF23, a recently discovered player, acts not only as a phosphaturic hormone but also as an important vitamin D and PTH regulator. Future studies might help to add new information about the role of FGF23 in the different stages of CKD. However, with the information available it has been learnt that one of the main advantageous and practical results of the FGF23 action is that it prevents the coexistence of high serum phosphorous and calcitriol serum levels, a coincidence that may end up increasing undesirable outcomes, among others, the possibility of having vascular calcifications.

If we had to pick up one topic in which striking improvements have been recently achieved, in no small measure thanks to the described models, we would choose phosphorous. As a result of the combination of results gathered from epidemiological, clinical and experimental studies, the role of phosphorus and the consequences of its overload have greatly increased in recent years, winning the spotlight when it comes to the general impact on health and its role in the parathyroid regulation and other CKD-MBD disorders, such as vascular function and calcification, cardiovascular disease and mortality.

The introduction of the FGF23 and its multiple interactions, mainly with phosphorous, but also with vitamin D and PTH, has enriched the CKD-MBD constellation. In this area of research, important advances in the coming years should be expected which might complete and limit more precisely the role of FGF23 in the parathyroid regulation across the different stages of CKD.

What clearly emerges from this review is that the intelligent use of the results obtained from different but complementary models, exploring the parathyroid function with a 'bed to the bench' approach, has allowed for a more comprehensive knowledge about the parathyroid regulation in CKD which may be soon translated into practical measures to improve the daily management of CKD-MBD disorders.

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Declaration of interest

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Bibliography

1. Introduction and definition of CKD-MBD and the development of the guideline statements. Kidney Int Suppl 2009;76(113):S3-8
2. Block GA, Klassen PS, Lazarus JM, et al. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. J Am Soc Nephrol 2004;15(8):2208-18
3. Moe S, Drueke T, Cunningham J, et al. Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2006;69(11):1945-53
4. Fadem SZ, Moe SM. Management of chronic kidney disease mineral-bone disorder. Adv Chronic Kidney Dis 2007;14(1):44-53
5. Komaba H, Tanaka M, Fukagawa M. Treatment of chronic kidney disease-mineral and bone disorder (CKD-MBD). Intern Med 2008;47(11):989-94
6. Moe SM, Drueke T, Lameire N, Eknoyan G. Chronic kidney disease-mineral-bone disorder: a new paradigm. Adv Chronic Kidney Dis 2007;14(1):3-12
7. Ogata H, Koiwa F, Kinugasa E, Akizawa T. CKD-MBD: impact on management of kidney disease. Clin Exp Nephrol 2007;11(4):261-8
8. Potts JT, Gardella TJ. Progress, paradox, and potential: parathyroid hormone research over five decades. Ann NY Acad Sci 2007;1117:196-208
9. Zanchetta JR, Bogado CE, Ferretti JL, et al. Effects of teriparatide [recombinant human parathyroid hormone (1-34)] on cortical bone in postmenopausal women with osteoporosis. J Bone Miner Res 2003;18(3):539-43
10. Jüppner H, Kronenberg HM. Parathyroid Hormone. In: Favus MJ, editor, Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism. 5th edition. American Society for Bone and Mineral Research; Washington; 2003. p. 117-24
11. Silver J, Kilav R, Naveh-Many T. Mechanisms of secondary hyperparathyroidism. Am J Physiol Renal Physiol 2002;283(3):F367-76
12. Slatopolsky E, Finch J, Denda M, et al. Phosphorus restriction prevents parathyroid gland growth. High phosphorus directly stimulates PTH secretion in vitro. J Clin Invest 1996;97(11):2534-40
13. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, et al. The parathyroid is a target organ for FGF23 in rats. J Clin Invest 2007;117(12):4003-8
14. Brown EM, Gamba G, Riccardi D, et al. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. Nature 1993;366(6455):575-80
15. Silver J, Levi R. Regulation of PTH synthesis and secretion relevant to the management of secondary hyperparathyroidism in chronic kidney disease. Kidney Int Suppl 2005(95):S8-12
16. Slatopolsky E. The role of calcium, phosphorus and vitamin D metabolism in the development of secondary hyperparathyroidism. Nephrol Dial Transplant 1998;13(Suppl 3):3-8
17. Naveh-Many T, Bell O, Silver J, Kilav R. Cis and trans acting factors in the regulation of parathyroid hormone (PTH) mRNA stability by calcium and phosphate. FEBS Lett 2002;529(1):60-4
18. Nechama M, Uchida T, Yosef-Levi IM, et al. The peptidyl-prolyl isomerase Pin1 determines parathyroid hormone mRNA levels and stability in rat models of secondary hyperparathyroidism. J Clin Invest 2009;119(10):3102-14
19. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. Am J Physiol Renal Physiol 2005;289(1):F8-28
20. Pike JW. Vitamin D: Receptors and the mechanism of action of 1,25-Dihydroxyvitamin D3. In: Dakshinamurti K, editor, Vitamin receptors: vitamins as ligands in cell communication. Cambridge University Press; New York; 1994. p. 59-77
21. Goodman WG. The flavors of vitamin D: tasting the molecular mechanisms. Kidney Int 2004;66(3):1286-7
22. Naveh-Many T, Silver J. Regulation of parathyroid hormone gene expression and secretion by vitamin D. In: Holick MF, editor, Vitamin D: physiology, molecular biology and clinical applications. Humana Press, Inc.; Totowa; 1998. p. 217-37
23. Gutierrez O, Isakova T, Rhee E, et al. Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. J Am Soc Nephrol 2005;16(7):2205-15
24. Lopez-Hilker S, Dusso AS, Rapp NS, et al. Phosphorus restriction reverses hyperparathyroidism in uremia independent of changes in calcium and calcitriol. Am J Physiol 1990;259(3 Pt 2):F432-7
25. Almaden Y, Hernandez A, Torregrosa V, et al. High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue in vitro. J Am Soc Nephrol 1998;9(10):1845-52
26. Naveh-Many T, Rahamimov R, Livni N, Silver J. Parathyroid cell proliferation in normal and chronic renal failure rats. The effects of calcium, phosphate, and vitamin D. J Clin Invest 1995;96(4):1786-93
27. Szabo A, Merke J, Beier E, et al. 1,25(OH) 2 vitamin D3 inhibits parathyroid cell proliferation in experimental uremia. Kidney Int 1989;35(4):1049-56
28. Carrillo-Lopez N, Alvarez-Hernandez D, Gonzalez-Suarez I, et al. Simultaneous changes in the calcium-sensing receptor and the vitamin D receptor under the influence of calcium and calcitriol. Nephrol Dial Transplant 2008;23(11):3479-84
29. Brown AJ, Zhong M, Finch J, et al. Rat calcium-sensing receptor is regulated by vitamin D but not by calcium. Am J Physiol 1996;270(3 Pt 2):F454-60
30. Rogers KV, Dunn CK, Conklin RL, et al. Calcium receptor messenger ribonucleic acid levels in the parathyroid glands and kidney of vitamin D-deficient rats are not regulated by plasma calcium or 1,25-dihydroxyvitamin D3. Endocrinology 1995;136(2):499-504
31. Abukawa H, Mano H, Arakawa T, et al. Tissue specific expression and differential regulation by 1alpha,25-dihydroxyvitamin D3 of the calcium-sensing receptor (CaSR) gene in rat kidney, intestine, and calvaria. Cytotechnology 2001;35(1):81-6
32. Garfia B, Canadillas S, Canalejo A, et al. Regulation of parathyroid vitamin D receptor expression by extracellular calcium. J Am Soc Nephrol 2002;13(12):2945-52
33. Brown AJ, Ritter CS, Finch JL, Slatopolsky EA. Decreased calcium-sensing receptor expression in

hyperplastic parathyroid glands of uremic rats: role of dietary phosphate. Kidney Int 1999;55(4):1284-92

34. Caride AJ, Chini EN, Homma S, et al. mRNAs coding for the calcium-sensing receptor along the rat nephron: effect of a low-phosphate diet. Kidney Blood Press Res 1998;21(5):305-9
35. Hernandez A, Torres A, Concepcion MT, Salido E. Parathyroid gland calcium receptor gene expression is not regulated by increased dietary phosphorus in normal and renal failure rats. Nephrol Dial Transplant 1996;11(Suppl 3):11-4
36. Denda M, Finch J, Brown AJ, et al. 1,25-dihydroxyvitamin D3 and 22-oxacalcitriol prevent the decrease in vitamin D receptor content in the parathyroid glands of uremic rats. Kidney Int 1996;50(1):34-9
37. Wiese RJ, Uhland-Smith A, Ross TK, et al. Up-regulation of the vitamin D receptor in response to 1,25-dihydroxyvitamin D3 results from ligand-induced stabilization. J Biol Chem 1992;267(28):20082-6
38. Maiti A, Beckman MJ. Extracellular calcium is a direct effecter of VDR levels in proximal tubule epithelial cells that counter-balances effects of PTH on renal vitamin D metabolism. J Steroid Biochem Mol Biol 2007;103(3-5):504-8
39. Russell J, Bar A, Sherwood LM, Hurwitz S. Interaction between calcium and 1,25-dihydroxyvitamin D3 in the regulation of preproparathyroid hormone and vitamin D receptor messenger ribonucleic acid in avian parathyroids. Endocrinology 1993;132(6):2639-44
40. Maiti A, Hait NC, Beckman MJ. Extracellular calcium-sensing receptor activation induces vitamin D receptor levels in proximal kidney HK-2G cells by a mechanism that requires phosphorylation of p38alpha MAPK. J Biol Chem 2008;283(1):175-83
41. Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. Nat Genet 2000;26(3):345-8
42. Shimada T, Kakitani M, Yamazaki Y, et al. Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. J Clin Invest 2004;113(4):561-8
43. Carrillo-Lopez N, Roman-Garcia P, Rodriguez-Rebollar A, et al. Indirect regulation of PTH by estrogens may require FGF23. J Am Soc Nephrol 2009;20(9):2009-17
44. Wetmore JB, Quarles LD. Calcimimetics or vitamin D analogs for suppressing parathyroid hormone in end-stage renal disease: time for a paradigm shift? Nature clinical practice 2009;5(1):24-33
45. Rodriguez M, Nemeth E, Martin D. The calcium-sensing receptor: a key factor in the pathogenesis of secondary hyperparathyroidism. Am J Physiol Renal Physiol 2005;288(2):F253-64
46. Nagano N. Pharmacological and clinical properties of calcimimetics: calcium receptor activators that afford an innovative approach to controlling hyperparathyroidism. Pharmacol Ther 2006;109(3):339-65
47. Levi R, Ben-Dov IZ, Lavi-Moshayoff V, et al. Increased parathyroid hormone gene expression in secondary hyperparathyroidism of experimental uremia is reversed by calcimimetics: correlation with posttranslational modification of the trans acting factor AUF1. J Am Soc Nephrol 2006;17(1):107-12
48. Rodriguez ME, Almaden Y, Canadillas S, et al. The calcimimetic R-568 increases vitamin D receptor expression in rat parathyroid glands. Am J Physiol Renal Physiol 2007;292(5):F1390-5
49. Díaz-Corte C, Fernández-Martín JL, Barreto S, et al. Effect of aluminium load on parathyroid hormone synthesis. Nephrol Dial Transplant 2001;16(4):742-5
50. Gonzalez-Suarez I, Alvarez-Hernandez D, Carrillo-Lopez N, et al. Aluminum posttranscriptional regulation of parathyroid hormone synthesis: a role for the calcium-sensing receptor. Kidney Int 2005;68(6):2484-96
51. Mayer GP, Hurst JG. Sigmoidal relationship between parathyroid hormone secretion rate and plasma calcium concentration in calves. Endocrinology 1978;102(4):1036-42
52. Felsenfeld AJ, Llach F. Parathyroid gland function in chronic renal failure. Kidney Int 1993;43(4):771-89
53. Fukuda N, Tanaka H, Tominaga Y, et al. Decreased 1,25-dihydroxyvitamin D3 receptor density is associated with a more severe form of parathyroid hyperplasia in chronic uremic patients. J Clin Invest 1993;92(3):1436-43
54. Korkor AB. Reduced binding of [3H] 1,25-dihydroxyvitamin D3 in the parathyroid glands of patients with renal failure. N Engl J Med 1987;316(25):1573-7
55. Gogusev J, Duchambon P, Hory B, et al. Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. Kidney Int 1997;51(1):328-36
56. Yano S, Sugimoto T, Tsukamoto T, et al. Association of decreased calcium-sensing receptor expression with proliferation of parathyroid cells in secondary hyperparathyroidism. Kidney Int 2000;58(5):1980-6
57. Valle C, Rodriguez M, Santamaria R, et al. Cinacalcet Reduces the Set Point of the PTH-Calcium Curve. J Am Soc Nephrol 2008:2430-36
58. Malberti F, Corradi B, Cosci P, et al. Different effects of calcitriol and parathyroidectomy on the PTH-calcium curve in dialysis patients with severe hyperparathyroidism. Nephrol Dial Transplant 1996;11(1):81-7
59. Slatopolsky E, Bricker NS. The role of phosphorus restriction in the prevention of secondary hyperparathyroidism in chronic renal disease. Kidney Int 1973;4(2):141-5
60. Reiss E, Canterbury JM, Bercovitz MA, Kaplan EL. The role of phosphate in the secretion of parathyroid hormone in man. J Clin Invest 1970;49(11):2146-49
61. Afonso S, Santamaria I, Guinsburg ME, et al. Chromosomal aberrations, the consequence of refractory hyperparathyroidism: its relationship with biochemical parameters. Kidney Int Suppl 2003(85):S32-8
62. Santamaria I, Alvarez-Hernandez D, Jofre R, et al. Progression of secondary hyperparathyroidism involves deregulation of genes related to DNA and RNA stability. Kidney Int 2005;67(6):2267-79
63. Santamaria I, Alvarez-Hernandez D, Cannata-Andia JB. Genetics and molecular disorders in severe secondary hyperparathyroidism: lessons from rna and microarray studies. J Nephrol 2005;18(4):469-73
64. Arnold A, Brown MF, Urena P, et al. Monoclonality of parathyroid tumors in chronic renal failure and in primary parathyroid hyperplasia. J Clin Invest 1995;95(5):2047-53

65. Chudek J, Ritz E, Kovacs G. Genetic abnormalities in parathyroid nodules of uremic patients. Clin Cancer Res 1998;4(1):211-4

66. Ormrod D, Miller T. Experimental uremia. Description of a model producing varying degrees of stable uremia. Nephron 1980;26(5):249-54

67. Merke J, Hugel U, Zlotkowski A, et al. Diminished parathyroid 1,25(OH)2D3 receptors in experimental uremia. Kidney Int 1987;32(3):350-3

68. Lopez I, Aguilera-Tejero E, Mendoza FJ, et al. Calcimimetic R-568 decreases extraosseous calcifications in uremic rats treated with calcitriol. J Am Soc Nephrol 2006;17(3):795-804

69. Virgos MJ, Menendez-Rodriguez P, Serrano M, et al. Insuficiencia renal crónica e hiperparatiroidismo secundario en ratas: Valoración bioquímica e histología. Rev Esp Fisiol 1993;49(4):241-47

70. Tamagaki K, Yuan Q, Ohkawa H, et al. Severe hyperparathyroidism with bone abnormalities and metastatic calcification in rats with adenine-induced uraemia. Nephrol Dial Transplant 2006;21(3):651-9

71. Kilav R, Silver J, Naveh-Many T. Parathyroid hormone gene expression in hypophosphatemic rats. J Clin Invest 1995;96(1):327-33

72. Estepa JC, Aguilera-Tejero E, Lopez I, et al. Effect of phosphate on parathyroid hormone secretion in vivo. J Bone Miner Res 1999;14(11):1848-54

73. Roman-Garcia P, Carrillo-Lopez N, Fernandez-Martin JL, et al. High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. Bone 2009. [Epub ahead of print]

74. Moallem E, Kilav R, Silver J, Naveh-Many T. RNA-Protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. J Biol Chem 1998;273(9):5253-9

75. Ritter CS, Finch JL, Slatopolsky EA, Brown AJ. Parathyroid hyperplasia in uremic rats precedes down-regulation of the calcium receptor. Kidney Int 2001;60(5):1737-44

76. Silver J, Naveh-Many T, Mayer H, et al. Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat. J Clin Invest 1986;78(5):1296-301

77. Naveh-Many T, Marx R, Keshet E, et al. Regulation of 1,25-dihydroxyvitamin D3 receptor gene expression by 1,25-dihydroxyvitamin D3 in the parathyroid in vivo. J Clin Invest 1990;86(6):1968-75

78. Taniguchi M, Tokumoto M, Matsuo D, et al. Parathyroid growth and regression in experimental uremia. Kidney Int 2006;69(3):464-70

79. Shiizaki K, Hatamura I, Negi S, et al. Highly concentrated calcitriol and its analogues induce apoptosis of parathyroid cells and regression of the hyperplastic gland–study in rats. Nephrol Dial Transplant 2008;23(5):1529-36

80. Colloton M, Shatzen E, Miller G, et al. Cinacalcet HCl attenuates parathyroid hyperplasia in a rat model of secondary hyperparathyroidism. Kidney Int 2005;67(2):467-76

81. Cardus A, Panizo S, Parisi E, et al. Differential effects of vitamin D analogs on vascular calcification. J Bone Miner Res 2007;22(6):860-6

82. Moe SM, Chen NX, Seifert MF, et al. A rat model of chronic kidney disease-mineral bone disorder. Kidney Int 2009;75(2):176-84

83. Moe SM, Seifert MF, Chen NX, et al. R-568 reduces ectopic calcification in a rat model of chronic kidney disease-mineral bone disorder (CKD-MBD). Nephrol Dial Transplant 2009;24(8):2371-7

84. Lopez I, Mendoza FJ, Aguilera-Tejero E, et al. The effect of calcitriol, paricalcitol, and a calcimimetic on extraosseous calcifications in uremic rats. Kidney Int 2008;73(3):300-7

85. Lopez I, Mendoza FJ, Guerrero F, et al. The calcimimetic AMG 641 accelerates regression of extraosseous calcifications in uremic rats. Am J Physiol Renal Physiol 2009;296(6):F1376-85

86. Price PA, Roublick AM, Williamson MK. Artery calcification in uremic rats is increased by a low protein diet and prevented by treatment with ibandronate. Kidney Int 2006;70(9):1577-83

87. Ho C, Conner DA, Pollak MR, et al. A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Nat Genet 1995;11(4):389-94

88. Kantham L, Quinn SJ, Egbuna OI, et al. The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion. Am J Physiol 2009;297(4):E915-23

89. Li YC, Amling M, Pirro AE, et al. Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. Endocrinology 1998;139(10):4391-6

90. Song Y, Kato S, Fleet JC. Vitamin D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and ECaC2 and calbindin D9k mRNA. J Nutr 2003;133(2):374-80

91. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, et al. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci USA 2001;98(23):13324-9

92. Meir T, Levi R, Lieben L, et al. Deletion of the vitamin D receptor specifically in the parathyroid demonstrates a limited role for the receptor in parathyroid physiology. Am J Physiol Renal Physiol 2009;297(5):F1192-8

93. Mithal A, Kifor O, Kifor I, et al. The reduced responsiveness of cultured bovine parathyroid cells to extracellular Ca2+ is associated with marked reduction in the expression of extracellular Ca(2+)-sensing receptor messenger ribonucleic acid and protein. Endocrinology 1995;136(7):3087-92

94. Brown AJ, Zhong M, Ritter C, et al. Loss of calcium responsiveness in cultured bovine parathyroid cells is associated with decreased calcium receptor expression. Biochem Biophys Res Commun 1995;212(3):861-7

95. Ishimi Y, Russell J, Sherwood LM. Regulation by calcium and 1,25-(OH)2D3 of cell proliferation and function of bovine parathyroid cells in culture. J Bone Miner Res 1990;5(7):755-60

96. Roussanne MC, Gogusev J, Hory B, et al. Persistence of Ca2+-sensing receptor expression in functionally active, long-term

human parathyroid cell cultures. J Bone Miner Res 1998;13(3):354-62

97. Roussanne MC, Lieberherr M, Souberbielle JC, et al. Human parathyroid cell proliferation in response to calcium, NPS R-467, calcitriol and phosphate. Eur J Clin Invest 2001;31(7):610-6

98. Krajisnik T, Bjorklund P, Marsell R, et al. Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cells. J Endocrinol 2007;195(1):125-31

99. Ritter CS, Slatopolsky E, Santoro S, Brown AJ. Parathyroid cells cultured in collagen matrix retain calcium responsiveness: importance of three-dimensional tissue architecture. J Bone Miner Res 2004;19(3):491-8

100. Kanai G, Kakuta T, Sawada K, et al. Suppression of parathyroid hormone production in vitro and in vivo by RNA interference. Kidney Int 2009;75(5):490-8

101. Arcidiacono MV, Cozzolino M, Spiegel N, et al. Activator protein 2alpha mediates parathyroid TGF-alpha self-induction in secondary hyperparathyroidism. J Am Soc Nephrol 2008;19(10):1919-28

102. Arcidiacono MV, Sato T, Alvarez-Hernandez D, et al. EGFR activation increases parathyroid hyperplasia and calcitriol resistance in kidney disease. J Am Soc Nephrol 2008;19(2):310-20

103. Galitzer H, Lavi-Moshayoff V, Nechama M, et al. The calcium-sensing receptor regulates parathyroid hormone gene expression in transfected HEK293 cells. BMC Biol 2009;7:17

104. Almaden Y, Canalejo A, Ballesteros E, et al. Regulation of arachidonic acid production by intracellular calcium in parathyroid cells: effect of extracellular phosphate. J Am Soc Nephrol 2002;13(3):693-8

105. Alvarez-Hernandez D, Naves M, Santamaria I, et al. Response of parathyroid glands to calcitriol in culture: Is this response mediated by the genetic polymorphisms in vitamin D receptor? Kidney Int Suppl 2003(85):S19-22

106. Almaden Y, Canalejo A, Hernandez A, et al. Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands in vitro. J Bone Miner Res 1996;11(7):970-6

107. Nielsen PK, Feldt-Rasmussen U, Olgaard K. A direct effect in vitro of phosphate on PTH release from bovine parathyroid tissue slices but not from dispersed parathyroid cells. Nephrol Dial Transplant 1996;11(9):1762-8

108. Almaden Y, Felsenfeld AJ, Rodriguez M, et al. Proliferation in hyperplastic human and normal rat parathyroid glands: role of phosphate, calcitriol, and gender. Kidney Int 2003;64(6):2311-7

109. Cohen MS, Dilley WG, Wells SA, et al. Long-term functionality of cryopreserved parathyroid autografts: a 13-year prospective analysis. Surgery 2005;138(6):1033-40

110. Norton JA, Brennan MF, Wells SAJ. Surgical Management of Hyperparathyroidism. In: Bilezikian JP, editor, The parathyroids. Raven Press, Ltd; New York; 1994. p. 531-51

111. Neyer U, Hoerandner H, Haid A, et al. Total parathyroidectomy with autotransplantation in renal hyperparathyroidism: low recurrence after intra-operative tissue selection. Nephrol Dial Transplant 2002;17(4):625-9

112. de Francisco AL, Fresnedo GF, Rodrigo E, et al. Parathyroidectomy in dialysis patients. Kidney Int Suppl 2002(80):161-6

113. Alvarez-Hernandez D, Gonzalez-Suarez I, Carrillo-Lopez N, et al. Viability and functionality of fresh and cryopreserved human hyperplastic parathyroid tissue tested in vitro. Am J Nephrol 2008;28:76-82

114. Alvarez-Hernandez D, Gonzalez-Suarez I, Naves M, et al. Long-term response of cultured rat parathyroid glands to calcium and calcitriol: the effect of cryopreservation. J Nephrol 2005;18(2):141-7

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Publicacion 2

BASIC RESEARCH www.jasn.org

Indirect Regulation of PTH by Estrogens May Require FGF23

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ABSTRACT

The mechanisms by which estrogens modulate PTH are controversial, including whether or not estrogen receptors (ERs) are present in the parathyroid glands. To explore these mechanisms, we combined a rat model of CKD with ovariectomy and exogenous administration of estrogens. We found that estrogen treatment significantly decreased PTH mRNA and serum levels. We did not observe ERα or ERβ mRNA or protein in the parathyroids, suggesting an indirect action of estrogens on PTH regulation. Estrogen treatment significantly decreased serum 1,25$(OH)_2$ vitamin D_3 and phosphorus levels. In addition, estrogens significantly increased fibroblast growth factor 23 (FGF23) mRNA and serum levels. *In vitro*, estrogens led to transcriptional and translational upregulation of FGF23 in osteoblast-like cells in a time- and concentration-dependent manner. These results suggest that estrogens regulate PTH indirectly, possibly through FGF23.

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Estrogen deficiency is the main factor implicated in bone loss in postmenopausal osteoporosis.[1] As a consequence of the lack of estrogens, bone turnover increases, leading to an imbalance between bone formation and bone resorption, favoring the latter.[2,3] This imbalance affects calcium–phosphate metabolism and may increase serum parathyroid hormone (PTH) levels.[4]

Estrogen replacement therapy prevents bone loss and fractures,[5,6] acting directly on bone cells through their specific estrogen receptors (ERs): α and β.[7,8] In addition, in postmenopausal women, estrogens can also reduce PTH serum levels[4,9] through an as of yet poorly understood mechanism.

A possible direct effect of estrogens reducing PTH acting through ERα and ERβ located in the parathyroid cells has been suggested, but the existence of ERα and ERβ in parathyroid tissue is still a controversial issue.[10–13] Estrogens may also decrease PTH secretion by acting on other factors such as calcium,[14,15] 1,25$(OH)_2$ D_3 (calcitriol),[15] and phosphorus,[16,17] among others. Recently, fibroblast growth factor 23 (FGF23), involved in phosphorus and vitamin D metabolism,[18] has been suggested to influence PTH synthesis and secretion.[19]

In women with chronic kidney disease (CKD), little is known about the role that estrogen deficiency plays in the pathogenesis and progression of bone disease.[20,21] Understanding the mechanism through which estrogens act on PTH is also a subject of interest in these patients, because of the high prevalence of secondary parathyroid disorders.[22] Because several aspects of the effects of estrogens on PTH remain unclear, the objective of this study was to investigate the factors and mechanisms involved in the likely effect of estrogens on the parathyroid gland.

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RESULTS

In Vivo Study

Renal Function, Estrogen Replacement, and Bone Mass.
Five different groups of rats were studied: CKD without ovariectomy (OVX), CKD+OVX treated with placebo, CKD+OVX treated with 17β-estradiol (E_2) at doses of 15 and 45 ng/kg/d, and a group of rats with normal renal function without OVX of the same age. No differences in renal function (serum urea and creatinine) were observed among all groups with CKD (Table 1). As expected, the placebo group showed significantly lower estrogen serum levels, uterus weight (UW), and UW/body weight (BW) ratio than the CKD-control group with no OVX. However, with the administration of E_2 (E_2-15 and E_2-45), significantly higher values of UW and UW/BW were observed compared with the placebo group (Table 1).

The placebo group showed significantly lower bone mineral density (BMD) than the CKD-control group. BMD loss was partially prevented with the dose of 15 μg/kg body weight/d of E_2 and totally prevented with the dose of 45 μg/kg body weight/d (Table 1).

Effect of Estrogens on PTH mRNA and Serum Levels.
The placebo group showed a significant increase in the serum intact PTH (iPTH) levels compared with the CKD-control group. The PTH increase was partially and totally blunted with 15 and 45 μg/kg body weight/d doses of E_2, respectively (Figure 1A). Similar results were obtained at the transcriptional level by quantitative real-time RT-PCR (qRT-PCR). A decreasing trend in the PTH mRNA levels with E_2 treatment was observed, achieving similar values as the CKD-control group with the high E_2 dose (45 μg/kg body weight/d; Figure 1B).

Evaluation of the Likely Direct Effect of E_2 on PTH Regulation: Study of ERα and ERβ on Parathyroid Tissue.
To evaluate the putative direct effect of E_2 on PTH regulation, the presence of ERα and ERβ was analyzed by qRT-PCR in each pool of parathyroid glands from the CKD-control, placebo, E_2-15, and E_2-45 groups. No signal for ERα and ERβ was observed in any of the four studied groups.

To double check the absence of ERs on parathyroid tissue, both ERα and ERβ were also assessed using parathyroid glands extracted from normal rats (no CKD, no OVX) at mRNA and protein levels. At the transcriptional level, three independent qRT-PCR experiments and two independent RT-PCR experiments showed no expression of either ERα or ERβ genes in normal parathyroid tissue. As expected, ERα and ERβ transcripts were observed in the uterus, used as a positive control tissue in the qRT-PCR experiments (data not shown) and also in the uterus and tibia used in RT-PCR experiments (Figure 2A).

At the protein level, parathyroid glands did not show ERα and ERβ expression as depicted in the Western blot and im-

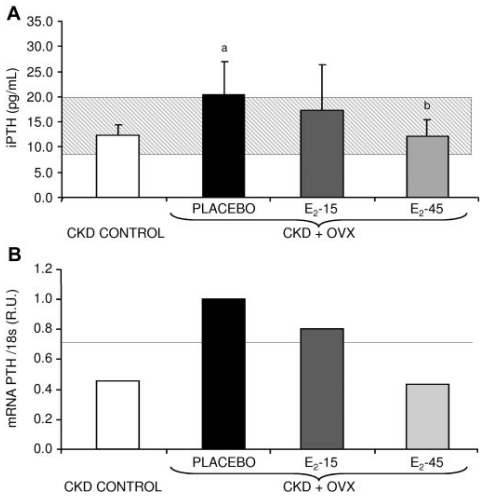

Figure 1. (A) Serum iPTH levels of rats treated with placebo, E_2-15 μg/kg body weight/d, E_2 −45 μg/kg body weight/d, and the CKD-control group. $^aP < 0.05$ compared with CKD-control group and $^bP < 0.05$ compared with placebo group. The gray horizontal bar represents the range (mean ± SD) for the normal group. (B) PTH mRNA levels measured by quantitative real-time RT-PCR. R.U., relative units referred to the placebo group. The horizontal line represents PTH gene expression in the normal group.

Table 1. Serum biochemical markers and BMD in all groups at the end of the study

	Normal Group	CKD Control	CKD + OVX		
			Placebo	E_2-15	E_2-45
Creatinine (mg/dl)	0.6 ± 0.00	1.03 ± 0.08	1.01 ± 0.08	1.06 ± 0.10	0.90 ± 0.04
Urea (mg/dl)	30.60 ± 5.41	69.50 ± 11.03	67.33 ± 3.50	72.00 ± 14.15	65.00 ± 6.04
Uterus weight (mg)	496.00 ± 161.43	494.33 ± 65.58	181.83 ± 122.61[a]	352.38 ± 98.20[ab]	300.00 ± 44.22[ab]
Body weight (g)	310.00 ± 26.05	320.50 ± 24.42	353.00 ± 36.99	312.50 ± 18.63[b]	333.40 ± 9.24
Uterus weight/body weight (mg/g)	1.60 ± 0.48	1.58 ± 0.24	0.53 ± 0.38[a]	1.12 ± 0.27[ab]	0.90 ± 0.14[ab]
17 ß-E_2 (pg/ml)	45.2 ± 22.6	32.03 ± 13.07	12.63 ± 3.16[a]	23.64 ± 13.52[b]	35.86 ± 3.28[b]
Proximal tibia BMD (g/cm^2)	0.28 ± 0.01	0.28 ± 0.02	0.25 ± 0.02[a]	0.26 ± 0.02	0.28 ± 0.02[b]

[a] $P < 0.05$ compared with the CKD-control group.
[b] $P < 0.05$ compared with the placebo group.

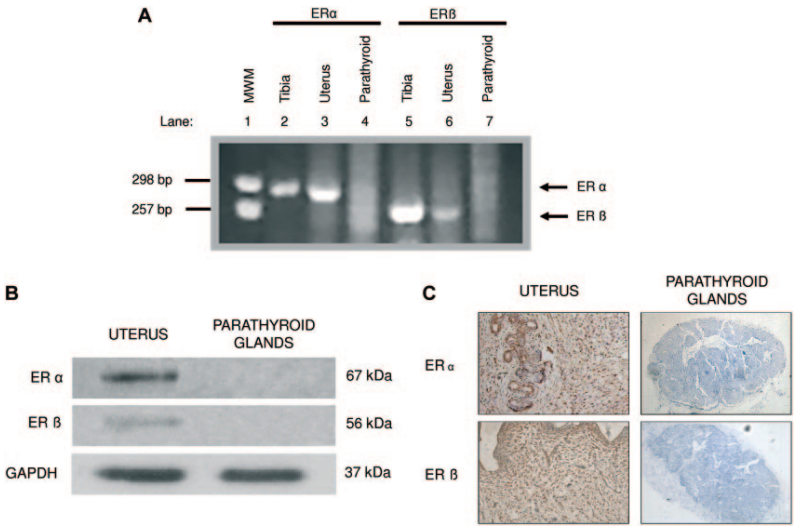

Figure 2. ERα and ERβ detection in parathyroid tissue. (A) Rat ERα (lanes 2, 3, and 4) and ERβ (lanes 5, 6, and 7) RT-PCR in different tissues resolved on agarose gel electrophoresis. Molecular weight markers (MWM; pUC18/HaeIII) (lane 1). (B) Western blot analysis of ERα and ERβ proteins in parathyroid glands and uterus. Anti-GAPDH was used as a loading control. (C) Immunohistochemical staining of the ERα and ERβ in parathyroid glands and uterus. Antibodies dilution: 1:50 for parathyroid tissue and 1:1000 for uterus). Hematoxylin counterstaining (magnification: ×20).

munohistochemistry analyses, despite that, in the latter, we applied a primary antibody concentration for ERα and ERβ 20 times higher than in the uterus tissue to the parathyroid glands. On the contrary, both receptors were highly expressed in the uterus (Figure 2B and C).

Evaluation of the Putative Indirect Effect of E_2 on PTH Regulation.

To study the likely indirect mechanisms involved in the effect of E_2 on PTH mRNA and serum levels, other serum biochemical parameters related to PTH regulation were studied.

E_2 was able to significantly decrease $1.25(OH)_2D_3$ levels in a dose-dependent manner, reaching lower values than the CKD-control group with both doses of E_2 (Figure 3A). E_2 also decreased serum phosphorus compared with the placebo group (Figure 3B), with a similar trend to that observed in the serum PTH results (Figure 1A).

Because of the known effect of FGF23 on phosphate metabolism and PTH function,[18,19] serum FGF23 was also measured. FGF23 mRNA and serum levels decreased in the placebo group compared with the CKD-control group. Interestingly, rats treated with both E_2 doses showed higher FGF23 mRNA and serum levels, achieving higher values than the CKD-control group (Figure 3C and D).

Because FGF23 requires Klotho as a coreceptor, the expression of this gene was measured in the pools of parathyroid glands and individual kidneys from all groups. An estrogen dose-dependent increase in Klotho mRNA levels in the parathyroid glands was observed (Figure 3E). However, no significant differences were found in kidney (data not shown).

In addition, FGF23 serum levels positively correlated with serum E_2 levels ($r = 0.734$, $P = 0.001$) and negatively correlated with serum $1.25(OH)_2D_3$ ($r = -0.791$, $P < 0.001$) in the three groups with CKD+OVX (Figure 4).

In Vitro Study: Direct Effect of E_2 on FGF23

To confirm the finding that E_2 might directly increase FGF23, the effect of E_2 on FGF23 mRNA and protein levels was evaluated using UMR-106 osteoblasts.

E_2 significantly increased FGF23 mRNA levels in a concentration- and time-dependent manner achieving the highest mRNA levels when cells were cultured for 48 h (Figure 5A).

To analyze FGF23 at the protein level, total extracts of proteins from cells cultured with E_2 for 24 and 48 h were subjected to Western blot. A single band of 32 kD corresponding to intact FGF23 was detected in all samples. In addition, FGF23 protein levels increased when both E_2 concentration and time of culture increased, following the same pattern seen in qRT-PCR (Figure 5B).

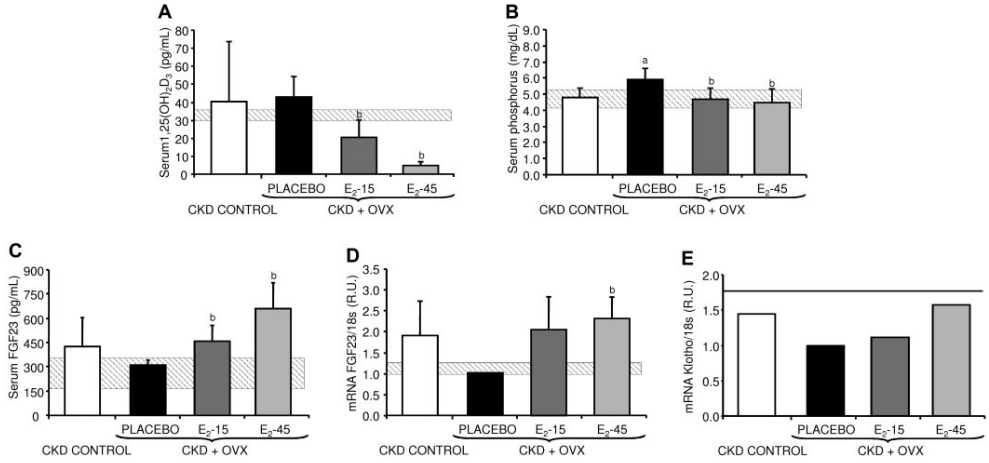

Figure 3. (A) Serum $1,25(OH)_2D_3$, (B) serum phosphorus, and (C) serum FGF23 levels in the CKD-control, placebo, E_2-15, and E_2-45 groups. $^aP < 0.05$ compared with the CKD-control group and $^bP < 0.05$ compared with the placebo group. (D) FGF23 mRNA levels measured by quantitative real-time RT-PCR from tibias of rats from the placebo, E_2-15, E_2-45, and CKD-control groups. R.U., relative units referred to the placebo group. $^aP < 0.05$ compared with the CKD-control group and $^bP < 0.05$ compared with the placebo group. The gray horizontal bars represent the range (mean ± SD) for the normal group. (E) Klotho mRNA levels measured by quantitative real-time RT-PCR. R.U., relative units referred to the placebo group. The horizontal line represents Klotho mRNA levels in the normal group.

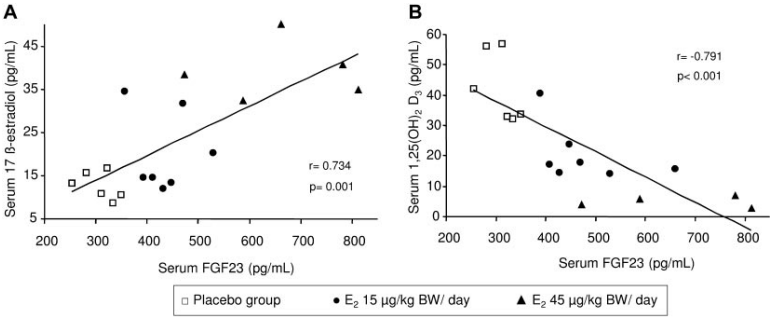

Figure 4. Correlation between (A) serum E_2 *versus* serum FGF23 and (B) serum FGF23 *versus* serum $1.25(OH)_2D_3$. *r*, Pearson coefficient.

DISCUSSION

Estrogen treatment prevents bone loss in postmenopausal women[23] by a direct action on bone cells,[8] but it might act also indirectly influencing the synthesis and secretion of calciotropic hormones, such as PTH,[4] inhibiting PTH-dependent bone resorption.[24] The effect of estrogens on PTH could be direct, acting on ERα and ERβ, in the parathyroids; or indirect, influencing calcium, phosphorus, or other mediators, and then secondarily, reducing PTH levels. In this study, we showed that the parathyroid tissue does not express both ERα and ERβ and that FGF23 is upregulated by estrogens. As a result, we suggest that estrogens would not act on PTH directly but likely indirectly by a mechanism which may involve FGF23.

In our study, estrogen deprivation in CKD rats showed, as expected, a significant decrease in UW/BW ratio and serum estrogen levels, together with a significant decrease in BMD in the most trabecular area of the tibia, caused by the increased bone resorption from estrogen deprivation.[3,5,7,25]

To achieve a hormonal replacement equivalent to that used

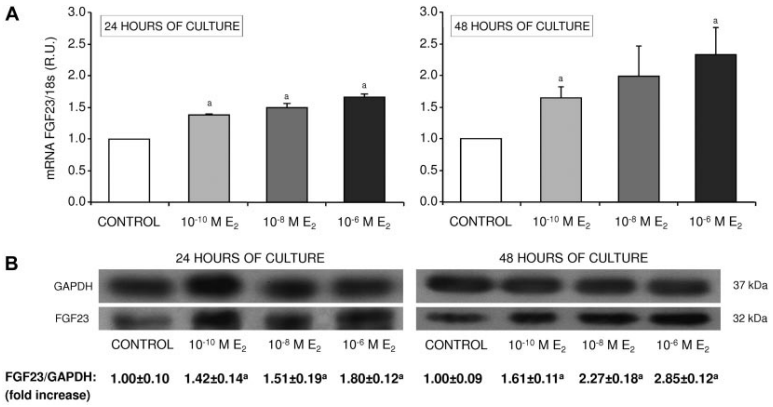

Figure 5. *In vitro* effect of E_2 on FGF23. (A) FGF23 mRNA levels measured by qRT-PCR from UMR-106 cells cultured with vehicle (control) and different concentrations of E_2 (10^{-10}, 10^{-8}, and 10^{-6} M) for 24 and 48 h. R.U., relative units referred to the control group. (B) Representative image of Western blot analysis of FGF23 protein from UMR-106 cells treated with 10^{-10}, 10^{-8}, or 10^{-6} M of E_2 for 24 and 48 h. Anti-GAPDH was used as loading control. Mean ± SD of three independent experiments are shown. $^{a}P < 0.05$ compared with the CKD-control group.

in postmenopausal women, two different estrogen concentrations were tested.[25,26] Both doses were able to significantly increase the UW/BW ratio and the estrogen serum levels compared with the untreated group (Table 1). However, only the high estrogen dose completely reversed the estrogen insufficiency and totally avoided the loss of BMD (Table 1). Estrogen treatment was able not only to prevent BMD loss but also to avoid the increase in PTH mRNA and serum levels compared with the placebo group, achieving similar values to the CKD control group with no OVX (Figure 1). The latter is still a controversial issue, because previous experimental studies[10,14] have shown contradictory results related to the effect of estrogens on PTH, likely explained by methodological differences such as renal function and the age of the rats.

The mechanisms by which estrogens may induce a reduction in PTH synthesis and secretion are still not well understood.[4,27,28] If the parathyroid cells would possess ERs, estrogens might directly influence PTH; however, this aspect is still controversial. Some authors have described the lack of ERs in parathyroid glands,[11–13] and others have reported that parathyroid glands are target organs for estrogens,[10] showing that estrogens increased PTH gene expression. It is not easy to explain these controversial results. However, the different experimental approaches, doses of estrogens tested, periods of treatment, and procedures used for gland removal (parathyroidectomy[11–13] or thyroparathyroidectomy[10]) may explain these conflicting results.

In this study, the presence of ERα and ERβ in parathyroid tissue from the different groups studied, including normal rats, was tested by four different experimental approaches. Despite this careful and meticulous research, the ERs were not found in parathyroid tissue, even when using a primary antibody concentration 20 times higher in parathyroids than in uterus in the immunohistochemistry analysis (Figure 2).

This negative finding supports the hypothesis that the parathyroid glands do not express ERs, and thus estrogens cannot reduce PTH by a direct mechanism. Other receptors, such as the retinoid X receptor, might play a role in the effect of estrogens on different genes,[29–31] but the definitive estrogen signaling pathways seem necessarily to involve the ERs.[32]

Another possible factor involved in PTH regulation by estrogens could be calcitriol, but there is no clear evidence to support this hypothesis. Some experimental data suggest that estrogens do not influence calcitriol levels[14,33,34]; meanwhile, others have shown that estrogens might modulate vitamin D receptor expression[34,35] and decrease serum calcitriol levels.[35,36] In our study, a significant dose-dependent decrease of serum calcitriol and PTH levels was observed with the use of estrogens (Figures 1A and B and 3A); this decrease was strikingly higher in the case of calcitriol. The reduction in serum calcitriol levels should have been accompanied by higher PTH levels[37] unless a third player, in this case estrogens, was interfering in calcitriol–PTH regulation, decreasing both calcitriol and PTH.

Another mechanism by which estrogens can influence PTH levels is decreasing serum phosphorus levels. Several works have described that estrogens can downregulate the kidney sodium–phosphate cotransporter (Na-Pi), increasing phosphorus in urine and causing hypophosphatemia.[17,38,39] In agreement with this view, we found that estrogen administration significantly decreased serum phosphorus levels to values similar to the CKD-control group (Figure 3B). Because phospho-

rus is well known to increase the synthesis and secretion of PTH,[40] the reduction of phosphorus, secondary to the use of estrogens, could have been at least partly responsible for the reduction in PTH levels.

Finally, another possible factor linking the changes observed in phosphorus and PTH is FGF23, which has been identified as one of the most potent phosphatonins able to increase urinary phosphorus[41,42] by inhibiting Na-Pi–dependent phosphate reabsorption in the proximal tubule. In addition, FGF23 also inhibits 1α-hydroxylase, leading to a decrease in calcitriol levels,[43] and it can act directly on the mitogen activating protein kinase (MAPK) pathway of the parathyroid gland, leading to a decrease in PTH synthesis and secretion.[19]

In our study, all of the previously described findings were present. The estrogen-treated rats showed significantly higher serum and bone FGF23 values and significantly lower serum calcitriol, phosphorus, and PTH values, the latter also confirmed by qRT-PCR.

Because the whole set of results strongly suggested that the estrogen effect on PTH may be at least partly driven by FGF23, and FGF23 requires Klotho as a coreceptor to suppress PTH expression and secretion,[19,44] we also measured Klotho gene expression in parathyroid glands. A dose-dependent increase in Klotho mRNA levels was observed in the parathyroid glands from rats treated with estrogens, likely caused by the stimulatory effect of FGF23 on Klotho expression.[19] However, previous findings suggest estrogens potentially suppress Klotho expression in estrogen target organs.[45] The fact that estrogens did not suppress Klotho together with the finding of the lack of ERs in the parathyroid glands are in keeping with our findings that the parathyroid glands are not a direct target tissue for estrogens. No changes in kidney Klotho mRNA levels were found despite that the kidney is a target organ for estrogens. It may be speculated that the high levels of FGF23 may counterbalance or mask the estrogen effect in the kidney.

To further study the direct effect of estrogens on FGF23 metabolism, we performed *in vitro* experiments using osteoblast-like cells. The results showed that estrogens, in the presence of a constant concentration of phosphorus, increase FGF23 levels in a concentration- and time-dependent manner, measured at transcriptional and translational levels (Figure 5A and B). The mechanism by which estrogens signaling stimulate FGF23 is unknown. According to the classical estrogen signaling pathway,[46] the nuclear ERs may bind putative estrogen response elements (EREs) in the gene promoter, acting as transcription factors; however, further studies are needed to fully understand the mechanisms by which estrogens may upregulate FGF23.

Therefore, taking all our experiments together, we postulate that PTH regulation by estrogens is mainly indirect, and FGF23 (a new factor never described as part of this axis until now) may be a candidate for potential factors linking estrogens and PTH. However, further studies are needed to confirm whether FGF23 stimulated by estrogens directly suppresses parathyroid function.

CONCISE METHODS

In Vivo Study: Animals, Drugs, and Experimental Design

Six-month-old female sexually mature Sprague-Dawley rats with a mean BW at the beginning of study of 325 $\pm$ 32 g ($n = 24$) were used. The animals were fed with a standard rodent chow containing 0.6% calcium and 0.6% phosphorus (Panlab, Barcelona, Spain) and housed in wire cages. Water and food administration was *ad libitum*.

E_2 (Innovative Research of America, Sarasota, FL) was dissolved in ethanol and diluted with corn oil to a final volume of 0.8 ml corn oil/kg body weight/injection. The final doses of E_2 administered to rats were 15 and 45 μg/kg body weight/d. This treatment was administered intraperitoneally 5 d/wk for 8 wk. Placebo (0.8 ml corn oil/kg body weight/d) was administered following the same procedure.

CKD was surgically induced using the modified technique by Ormrod and Miller (equivalent to 7/8 nephrectomy).[47] Estrogen deprivation was surgically induced performing bilateral OVX. Both procedures were done in the same intervention using 42 mg/kg of intraperitoneally ketamine (Ketolar; Warner Lambert) and 0.16 mg/kg of medetomidine (Dontor; Orion, Espoo, Finland) as anesthetics. One week after surgery, a total of 20 animals with CKD+OVX were divided into three experimental groups. Group 1 (E_2-15, $n = 8$) received intraperitoneal E_2 (15 μg/kg body weight/d). Group 2 (E_2-45, $n = 5$) received intraperitoneal E_2 (45 μg/kg body weight/d). Group 3 (placebo, $n = 7$) received vehicle (corn oil at 0.8 ml/kg body weight/d) administered through intraperitoneal injections as described. A fourth group with CKD (same procedure) and no OVX was used as the CKD-control group ($n = 4$). A group of rats ($n = 5$) with normal renal function without OVX (normal group) was also included in the study.

After 8 wk of treatment, all rats were killed by exsanguination. Blood samples, tibias, uteri, parathyroid glands, and kidneys were removed and stored frozen at -80 °C until analysis. Blood samples were drawn for serum analyses, the right tibia was removed to perform BMD analyses, and uteri were collected to be weighed and used as a tissue marker of estrogen replacement. Because of the small size of each individual gland and to obtain enough total RNA for quantitation, the parathyroid glands from each group studied were pooled (8, 14, 16, 10, and 10 glands were pooled from the CKD-control, placebo, E_2-15, E_2-45, and normal groups, respectively), and each individual's left tibia and kidney were used to extract total RNA. Parathyroid glands, left tibias, and uteri from normal rats were used both to extract total RNA and proteins. In addition, parathyroid glands and uteri were also embedded in paraffin. The protocol was approved by the Laboratory Animal Ethics Committee of Oviedo University.

In Vitro Study

To study the *in vitro* regulation of FGF23 by E_2, a rat osteosarcoma cell line UMR-106 (Health Protection Agency Culture Collections, Salisbury, UK) was used. UMR-106 cells were grown in phenol red–free αMEM (Sigma-Aldrich, St. Louis, MO) containing 1 mM phosphorus with 10% charcoal-stripped FBS (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin-sulfate (Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere with 5% CO_2. Cells were

grown to subconfluence and were cultured in phenol red–free αMEM containing 0.25% (wt/vol) BSA (culture medium) for 24 h. At the end of this adaptation period, cells were exposed to vehicle (ethanol) or E_2 at 10^{-10}, 10^{-8}, or 10^{-6} M concentrations for 24 or 48 h in culture medium. After the period of exposure, cells were collected to extract total RNA and proteins to measure FGF23.

Analytical and Technical Procedures

Serum Markers and BMD Analysis.
Serum urea, creatinine, calcium, and phosphorus levels were measured using a multichannel autoanalyzer (Hitachi 717; Boehringer Mannheim, Berlin, Germany), serum E_2 levels were measured by RIA (Diagnostic Systems Laboratories, Webster, TX), serum iPTH by IRMA (Rat PTH kit Immunotopics, San Juan Capistrano, CA), serum $1.25(OH)_2D_3$ by RIA (IDS, Boldon, Tyne & Wear, UK), and serum FGF23 with a sandwich ELISA kit (Kainos Laboratories, Tokyo, Japan), following the manufacturer's protocol in all cases.

BMD was measured at the proximal one eighth of the right tibia using dual-energy x-ray absorptiometry (QDR-100; Hologic, Bedford, MA) with software specifically prepared and adapted to small animals.[48]

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time RT-PCR.
Total RNA extraction was performed by the method of Chomczynski.[49] Total RNA concentration and purity were quantified by spectrophotometry UV-Vis (NanoDrop Technologies, Wilmington, DE), measuring the absorbance at 260 and 280 nm. RNA integrity was corroborated using formaldehyde/agarose gels. All RNA samples were stored in RNase-free tubes at −80 °C until analysis.

RT-PCR to synthesize cDNA was performed from 1 μg of total RNA previously extracted from parathyroid, tibia, uterus, kidney, and cell culture samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The cDNAs obtained were stored at −20 °C until required for analysis.

qRT-PCR was performed in the *in vivo* and *in vitro* studies on an ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems). PTH, ERα, ERβ, FGF23, and Klotho genes and rRNA 18s as endogenous control were analyzed using TaqMan pre-Developed assay reagents (TaqMan Gene Expression Assays-On-Demand; Applied Biosystems). All reactions were performed in triplicate, amplifying endogenous and target genes in the same plate. Relative quantitative evaluation of target genes was performed by comparing threshold cycles using $\Delta\Delta C_T$ method, as described previously.[50,51]

Detection by RT-PCR of ERα and ERβ.
Because of the controversy related to the existence of ERα and ERβ in parathyroid glands, the presence of mRNA corresponding to the receptors in parathyroid tissue was also analyzed amplifying cDNA from normal rats with specific oligonucleotides for ERα (forward: 5′-GCA CAA GCG TCA GAG AGA TG-3′; reverse: 5′-GCA CTC TCT TTG CCC AGT TG-3′) and ER β (forward: 5′-GGT GTG GGT ACC GTA TAG TG-3′; reverse: 5′-ATC ATG TGC ACC AGT TCC TTG-3′). The cDNA from normal tibia was used as positive control.

Protein Extraction and Western Blot Analysis.
To enrich the nuclear protein fraction to test the presence of ERα and ERβ, total proteins from a pool of 10 parathyroid glands and uteri (used as a positive control) from normal rats were extracted using a high-salts buffer containing 500 mM NaCl, 50 mM HEPES (pH 7.0), and 1× Protease Inhibitor Cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany).

To analyze the *in vitro* effect of E_2 on FGF23 protein, total proteins from UMR-106 cells exposed for 24 or 48 h to different E_2 concentrations were extracted using a standard RIPA buffer with protease inhibitors.

All samples of proteins were quantified by Bradford's method (Bio-Rad, Hercules, CA).

For the study of ERs proteins, aliquots of 20 μg of protein from the parathyroids and uterus were electrophoresed on SDS-PAGE minigels and transferred to a Hybond P membrane (GE Healthcare UK, Buckinghamshire, UK) following standard protocols.[52] ERα and ERβ proteins were detected with a mouse anti-ER α monoclonal IgG1 antibody (dilution 1:1000; Acris Antibodies, Hiddenhausen, Germany) and a rabbit anti-ER β polyclonal IgG antibody (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Rabbit anti-GAPDH polyclonal antibody (dilution 1:25,000; Santa Cruz Biotechnology) was used as a loading control.

For the *in vitro* FGF23 protein assay, three independent experiments were performed using aliquots containing 30 μg of total proteins from E_2-treated UMR-106 cells. Total proteins were loaded on SDS-PAGE according to the same protocol described for the study of ERs with Western blot. FGF23 protein was detected using a goat anti-FGF23 polyclonal antibody (dilution 1:100; Santa Cruz Biotechnology) and rabbit anti-GAPDH polyclonal antibody (1:20,000; Santa Cruz Biotechnology) was used as load control. In both cases, chromogenic detection was performed with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL). For relative Western blot quantification, Quantity One 1-D Analysis Software *v*4 (Bio-Rad) and a GS-800 Calibrated Densitometer (Bio-Rad) were used.

Immunohistochemistry.
The presence of ERα and ERβ in parathyroid tissue was also determined by immunohistochemistry in 5-μm-thick serial sections from paraffin-embedded parathyroid glands and uteri from normal rats using the same specific antibodies used for Western blot and hematoxylin counterstaining (Dako REAL EnVision; Dako, Carpinteria, CA) following the manufacturer's instructions. For ER detection in uterus tissue, a dilution of 1:1000 of both antibodies was used; however, to increase the sensitivity of the detection of ERα and ERβ in parathyroid tissue, the dilution of both antibodies was 1:50.

Statistical Analysis

Biochemical markers, UW, BW, proximal tibia BMD, qRT-PCR, and Western blot quantitation were statistically analyzed using *t* test. Correlations between serum FGF23, serum E_2, and $1.25(OH)_2D_3$ were performed using the Pearson correlation coefficient (*r*).

The results are expressed as mean ± SD. Differences were considered significant when $P < 0.05$. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL).

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DISCLOSURES

None.

REFERENCES

1. Weitzmann MN, Pacifici R: Estrogen deficiency and bone loss: An inflammatory tale. *J Clin Invest* 116: 1186–1194, 2006
2. Raisz LG: Pathogenesis of osteoporosis: Concepts, conflicts, and prospects. *J Clin Invest* 115: 3318–3325, 2005
3. Manolagas SC: Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 21: 115–137, 2000
4. Khosla S, Atkinson EJ, Melton LJ III, Riggs BL: Effects of age and estrogen status on serum parathyroid hormone levels and biochemical markers of bone turnover in women: A population-based study. *J Clin Endocrinol Metab* 82: 1522–1527, 1997
5. Rodriguez-Rodriguez A, Naves M, Rodriguez-Rebollar A, Gomez C, Braga S, Cannata-Andia JB: Hormonal replacement therapy in an animal model with chronic renal failure and ovariectomy: Biochemical and densitometric study. *Kidney Int* 63[Suppl 85]: 57–61, 2003
6. Pinkerton JV, Dalkin AC: Combination therapy for treatment of osteoporosis: A review. *Am J Obstet Gynecol* 197: 559–565, 2007
7. Riggs BL, Khosla S, Melton III: Sex steroids and the construction and conservation of the adult skeleton. *Endocr Rev* 23: 279–302, 2002
8. Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, Nishina H, Takeda S, Takayanagi H, Metzger D, Kanno J, Takaoka K, Martin TJ, Chambon P, Kato S: Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* 130: 811–823, 2007
9. Stock JL, Coderre JA, Mallette LE: Effects of a short course of estrogen on mineral metabolism in postmenopausal women. *J Clin Endocrinol Metab* 61: 595–600, 1985
10. Naveh-Many T, Almogi G, Livni N, Silver J: Estrogen receptors and biologic response in rat parathyroid tissue and C cells. *J Clin Invest* 90: 2434–2438, 1992
11. Prince RL, MacLaughlin DT, Gaz RD, Neer RM: Lack of evidence for estrogen receptors in human and bovine parathyroid tissue. *J Clin Endocrinol Metab* 72: 1226–1228, 1991
12. Saxe AW, Gibson GW, Russo IH, Gimotty P: Measurement of estrogen and progesterone receptors in abnormal human parathyroid tissue. *Calcif Tissue Int* 51: 344–347, 1992
13. Lim SK, Won YJ, Lee HC, Huh KB, Park YS: A PCR analysis of ERalpha and ERbeta mRNA abundance in rats and the effect of ovariectomy. *J Bone Miner Res* 14: 1189–1196, 1999
14. Liel Y, Shany S, Smirnoff P, Schwartz B: Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. *Endocrinology* 140: 280–285, 1999
15. Ten Bolscher M, Netelenbos JC, Barto R, Van Buuren LM, Van der vijgh, WJ: Estrogen regulation of intestinal calcium absorption in the intact and ovariectomized adult rat. *J Bone Miner Res* 14:1197–1202, 1999
16. Uemura H, Irahara M, Yoneda N, Yasui T, Genjida K, Miyamoto KI, Aono T, Takeda E: Close correlation between estrogen treatment and renal phosphate reabsorption capacity. *J Clin Endocrinol Metab* 85: 1215–1219, 2000
17. Faroqui S, Levi M, Soleimani M, Amlal H: Estrogen downregulates the proximal tubule type IIa sodium phosphate cotransporter causing phosphate wasting and hypophosphatemia. *Kidney Int* 73: 1141–1150, 2008
18. Shimada T, Kakitani M, Yamazaki Y, Hasegawa H, Takeuchi Y, Fujita T, Fukumoto S, Tomizuka K, Yamashita T: Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 113: 561–568, 2004
19. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M, Sirkis R, Naveh-Many T, Silver J: The parathyroid is a target organ for FGF23 in rats. *J Clin Invest* 117: 4003–4008, 2007
20. Holley JL, Schmidt RJ: Hormone replacement therapy in postmenopausal women with end-stage renal disease: A review of the issues. *Semin Dial* 14: 146–149, 2001
21. Weisinger JR, Gonzalez L, Alvarez H, Hernandez E, Carlini RG, Capriles F, Cervino M, Martinis R, Paz-Martinez V, Bellorin-Font E: Role of persistent amenorrhea in bone mineral metabolism of young hemodialyzed women. *Kidney Int* 58: 331–335, 2000
22. Felsenfeld AJ, Rodriguez M, Aguilera-Tejero E: Dynamics of parathyroid hormone secretion in health and secondary hyperparathyroidism. *Clin J Am Soc Nephrol* 2: 1283–1305, 2007
23. Delmas PD: Treatment of postmenopausal osteoporosis. *Lancet* 359: 2018–2026, 2002
24. Cosman F, Shen V, Xie F, Seibel M, Ratcliffe A, Lindsay R: Estrogen protection against bone resorbing effects of parathyroid hormone infusion. Assessment by use of biochemical markers. *Ann Intern Med* 118: 337–343, 1993
25. Naves Diaz M, Rodriguez Rodriguez A, Fernandez Martin JL, Serrano Arias M, Menendez Rodriguez P, Cannata Andia JB: Effects of estradiol, calcitriol and both treatments combined on bone histomorphometry in rats with chronic kidney disease and ovariectomy. *Bone* 41: 614–619, 2007
26. Verhaeghe J, Oloumi G, van Herck E, van Bree R, Dequeker J, Einhorn TA, Bouillon R: Effects of long-term diabetes and/or high-dose 17 beta-estradiol on bone formation, bone mineral density, and strength in ovariectomized rats. *Bone* 20: 421–428, 1997
27. Selby PL, Peacock M: Ethinyl estradiol and norethindrone in the treatment of primary hyperparathyroidism in postmenopausal women. *N Engl J Med* 314: 1481–1485, 1986
28. Marcus R, Madvig P, Crim M, Pont A, Kosek J: Conjugated estrogens in the treatment of postmenopausal women with hyperparathyroidism. *Ann Intern Med* 100: 633–640, 1984
29. Segars JH, Marks MS, Hirschfeld S, Driggers PH, Martinez E, Grippo JF, Brown M, Wahli W, Ozato K: Inhibition of estrogen-responsive gene activation by the retinoid X receptor beta: Evidence for multiple inhibitory pathways. *Mol Cell Biol* 13: 2258–2268, 1993
30. Nunez SB, Medin JA, Braissant O, Kemp L, Wahli W, Ozato K, Segars JH: Retinoid X receptor and peroxisome proliferator-activated receptor activate an estrogen responsive gene independent of the estrogen receptor. *Mol Cell Endocrinol* 127: 27–40, 1997
31. Celli G, Darwiche N, De Luca LM: Estrogen induces retinoid receptor expression in mouse cervical epithelia. *Exp Cell Res* 226: 273–282, 1996
32. Marino M, Galluzzo P, Ascenzi P: Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics* 7: 497–508, 2006
33. Schwartz B, Smirnoff P, Shany S, Liel Y: Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. *Mol Cell Biochem* 203: 87–93, 2000

34. Zhang Y, Lai WP, Wu CF, Favus MJ, Leung PC, Wong MS: Ovariectomy worsens secondary hyperparathyroidism in mature rats during low-Ca diet. *Am J Physiol Endocrinol Metab* 292: E723–E731, 2007
35. Chen C, Noland KA, Kalu DN: Modulation of intestinal vitamin D receptor by ovariectomy, estrogen and growth hormone. *Mech Ageing Dev* 99: 109–122, 1997
36. Colin EM, Van Den Bemd GJ, Van Aken M, Christakos S, De Jonge HR, Deluca HF, Prahl JM, Birkenhager JC, Buurman CJ, Pols HA, Van Leeuwen JP: Evidence for involvement of 17beta-estradiol in intestinal calcium absorption independent of 1,25-dihydroxyvitamin D3 level in the rat. *J Bone Miner Res* 14: 57–64, 1999
37. Goodman WG: The flavors of vitamin D: Tasting the molecular mechanisms. *Kidney Int* 66: 1286–1287, 2004
38. Dick IM, Prince RL: The effect of estrogen on renal phosphorus handling in the rat. *Am J Nephrol* 21: 323–330, 2001
39. Dick IM, Devine A, Beilby J, Prince RL: Effects of endogenous estrogen on renal calcium and phosphate handling in elderly women. *Am J Physiol Endocrinol Metab* 288: E430–E435, 2005
40. Almaden Y, Hernandez A, Torregrosa V, Canalejo A, Sabate L, Fernandez Cruz L, Campistol JM, Torres A, Rodriguez M: High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue in vitro. *J Am Soc Nephrol* 9: 1845–1852, 1998
41. Bai X, Miao D, Li J, Goltzman D, Karaplis AC: Transgenic mice overexpressing human fibroblast growth factor 23 (R176Q) delineate a putative role for parathyroid hormone in renal phosphate wasting disorders. *Endocrinology* 145: 5269–5279, 2004
42. Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, Takeuchi Y, Fujita T, Fukumoto S, Yamashita T: Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. *Proc Natl Acad Sci U S A* 98: 6500–6505, 2001
43. Perwad F, Zhang MY, Tenenhouse HS, Portale AA: Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism in vivo and suppresses 25-hydroxyvitamin D-1alpha-hydroxylase expression in vitro. *Am J Physiol Renal Physiol* 293: F1577–F1583, 2007
44. Nakatani T, Sarraj B, Ohnishi M, Densmore MJ, Taguchi T, Goetz R, Mohammadi M, Lanske B, Razzaque MS: In vivo genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23)-mediated regulation of systemic phosphate homeostasis. *FASEB J* 23: 433–441, 2009
45. Oz OK, Hajibeigi A, Howard K, Cummins CL, van Abel M, Bindels RJ, Word RA, Kuro-o M, Pak CY, Zerwekh JE: Aromatase deficiency causes altered expression of molecules critical for calcium reabsorption in the kidneys of female mice. *J Bone Miner Res* 22: 1893–1902, 2007
46. Parker MG: Transcriptional activation by oestrogen receptors. *Biochem Soc Symp* 63: 45–50, 1998
47. Ormrod D, Miller T: Experimental uremia. Description of a model producing varying degrees of stable uremia. *Nephron* 26: 249–254, 1980
48. Gomez-Alonso C, Menendez-Rodriguez P, Virgos-Soriano MJ, Fernandez-Martin JL, Fernandez-Coto MT, Cannata-Andia JB: Aluminum-induced osteogenesis in osteopenic rats with normal renal function. *Calcif Tissue Int* 64: 534–541, 1999
49. Chomczynski P: A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15: 532–534, 536–537, 1993
50. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402–408, 2001
51. Carrillo-Lopez N, Alvarez-Hernandez D, Gonzalez-Suarez I, Roman-Garcia P, Valdivielso JM, Fernandez-Martin JL, Cannata-Andia JB: Simultaneous changes in the calcium-sensing receptor and the vitamin D receptor under the influence of calcium and calcitriol. *Nephrol Dial Transplant* 23: 3479–3484, 2008
52. Maniatis T, Fistch EF, Sambrook J: *Molecular Cloning*, New York, Cold Spring Harbor Laboratory Press, 1989

Publicación 3

Matrix metalloproteinase 1 promoter polymorphisms and risk of myocardial infarction: a case–control study in a Spanish population

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Objectives **Inherited and acquired risk factors contribute to the development of the atherosclerotic lesion and its most common clinical manifestation, myocardial infarction (MI). Multiple studies have suggested a role for matrix metalloproteinases (MMPs) in atherosclerosis, and several functional polymorphisms in the *MMP-1* gene have been linked to the risk of MI. The aim of this study was to evaluate the association between *MMP-1* promoter polymorphisms and early MI in a Spanish cohort.**

Methods **We carried out a case–control study with 261 unrelated patients who had suffered an MI before 55 years of age and 194 healthy controls, all male and smokers. The genotypes for the three *MMP-1* promoter polymorphisms −1607 1G/2G, −519 A/G, and −340 T/C were determined through PCR–restriction fragment length polymorphism. Allelic, genotypic, and haplotypic frequencies were statistically compared between groups.**

Results **Frequencies of the three polymorphisms did not differ between patients and controls. The −1607 1G/2G and −519 A/G variants were in linkage disequilibrium. Analysis of the haplotype frequencies showed significant associations of the $2G_{-1607}$-G_{-519}-T_{-340} (odds ratio = 2.40; 95% confidence interval = 1.27–4.55; $P < 0.006$) and $1G_{-1607}$-G_{-519}-T_{-340} (odds ratio = 0.68; 95% confidence interval = 0.50–0.94; $P < 0.05$) haplotypes with the risk of early MI.**

Conclusion ***MMP-1*** **promoter polymorphisms are associated with the risk of early MI in a Spanish population of smoking males.** ***Coron Artery Dis*** **20:383–386 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Myocardial infarction (MI) is caused by several inherited and acquired risk factors that predispose to the development of atherosclerotic lesions and plaque rupture [1]. Degradation of the supporting elements of a coronary atherosclerotic plaque weakens its fibrous cap and thereby increases susceptibility to rupture. Matrix metalloproteinases (MMPs) disrupt the extracellular matrix, are present in atherosclerotic plaques, and seem to be more active in unstable lesions. Therefore, MMPs may play an important role in plaque rupture and the posterior remodeling of the vessel wall [2]. In particular, MMP-1 (collagenase-1) has been found in the shoulder of the atherosclerotic plaque, suggesting that an excess of collagenolitic activity could lead to an early plaque rupture [3]. Moreover, ApoE knockout mice expressing human MMP-1 had less advanced atherosclerotic lesions compared with their littermates that do not express MMP-1 [4]. In this way, MMP-1 could play a dual role in coronary artery disease (CAD): on one hand it favors the remodeling of the atherosclerotic lesions, but on the other hand, enhanced MMP-1 expression promotes plaque rupture [5].

The MMP-1 is a candidate to modify the risk of atherosclerosis and MI in humans, and several polymorphisms have been analyzed through case–control studies. Most of them were focused on the 1G/2G insertion–deletion polymorphism at nucleotide position −1607. It has been reported that the insertion allele (2G) has increased gene transcription, compared with the deletion allele [6]. Homozygosis for the 2G allele has been associated with a lower risk for coronary heart disease in a Caucasian population [7] and haplotypes of 1G/2G MMP-1 (1G allele) and 5A/6A MMP-3 have been associated with increased risk of CAD, but not with MI [8]. Other studies could not find any association of this polymorphism with MI [9].

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Other genetic variants in the promoter have also been studied. Associations between haplotypes of the −519 A/G and −340 T/C polymorphisms and MI have been reported among British, Swedish, and Chinese populations [10,11], and the −519 A/G polymorphism has been associated with hypertension [12], a conventional risk factor for CAD. Moreover, concrete haplotypes of these polymorphisms have been linked to increased promoter activities and higher levels of MMP-1 [10], suggesting a relationship between these variants and the increased presence of this protein in atherosclerotic plaques.

Here, we analyzed the effect of these three functional promoter polymorphisms on MI risk in a group of Spanish male patients, all smokers, and who have suffered an early event of MI.

Methods

Patients and controls

We studied 261 unrelated male patients who have survived to a first episode of acute MI before 55 years of age. All these patients were diagnosed between 1998 and 2006 at our Cardiology Department, and MI was defined according to the World Health Organization criteria [13]. These patients underwent coronary angiography for diagnostic purposes, and had at least one atherosclerotic-diseased coronary vessel (a narrowing of >30% of the vessel diameter). All the patients were recruited for a research project on the genetic factors involved in MI [14,15].

The control group consisted of 194 healthy male individuals, aged between 30 and 60 years, recruited through the Blood Bank at our Institution. These controls had not suffered episodes of cardiovascular disease, but they were not angiographically evaluated to exclude the presence of diseased coronary vessels, because coronary angiography is an invasive and potentially dangerous technique, only used for diagnostic/therapeutic purposes among patients with symptoms of CAD.

As early MI is strongly associated with smoking in our population, all the patients and controls were current or former smokers. Those with history of hypertension or a blood pressure greater than 140/90 mmHg in at least four determinations were considered as hypertensives. Those with a total cholesterol value greater than 200 mg/dl were considered as hypercholesterolemics. Patients with a history of diabetes or a basal glycemia greater than 120 mg/dl were excluded. Table 1 shows the main clinical, biochemical, and anthropometric characteristics of patients and controls.

All the participants were Caucasian males from the same region (Asturias, Northern Spain, total population 1 million), and gave their written informed consent to participate in the study, which was approved by the Ethics Committee of Clinical Investigation of Asturias.

Table 1 Characteristics of patients and controls

	MI ($N=261$)	Controls ($N=194$)	P value
Age (years)	46.05 ± 5.97	43.49 ± 7.48	<0.0001
Hypertensives (%)	39	18	<0.0001
Hypercholesterolemics (%)	24.7	6.1	<0.0001
Cholesterol (mg/dl)	216.11 ± 51.15	213.31 ± 37.26	0.12
LDL-C (mg/dl)[a]	145.29 ± 45.86	134.43 ± 36.78	0.35
HDL-C (mg/dl)	34.60 ± 9.96	51.03 ± 14.79	<0.0001
Triglycerides (mg/dl)	181.78 ± 105.66	139.42 ± 95.58	0.78

Data are mean ± standard deviation.
HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MI, myocardial infarction.
[a]Calculated with the Friedewald formula [16].

MMP-1 polymorphisms genotyping

DNA was obtained from 10 ml of peripheral blood following a salting-out method [17]. The three polymorphisms [−1607 1G/2G (rs11292517), −519 A/G (rs1144393), and −340 T/C (rs514921)] were genotyped through PCR–restriction fragment length polymorphism with the following conditions: 1 denaturing cycle at 95°C for 3 min and 30 cycles at 95°C for 30 s, annealing temperature (58, 62, and 60°C, respectively) for 30 s, and 72°C for 30 s. For −519 A/G, the fragment of 399 bp obtained with primers, forward 5′-TTA CAG AGA TGG GGT CTC AC-3′ and reverse 5′-CCA TGG AGT ACT CTT TGA CC-3′, was digested with *Kpn* I. For −1607 1G/2G and −340 T/C, primers with a mismatch were designed: forward 5′-AAG TGT TCT TTG GTC TCT GC-3′, reverse 5′-GGA TTG ATT TGA GAT AAG TCA GAT C-3′, and forward 5′-TGT GTG GAG AAA CCT GTA GGA C-3′, reverse 5′-TCA CTT GGT GTT GCA ATG TC-3′, pairs for both single nucleotide polymorphisms, respectively (underlined are mismatched bases). The fragments of 176 and 209 bp were digested with *Bgl* II and *Sau* 96I, respectively, followed by electrophoresis on 3% agarose gels, and ethidium bromide staining to visualize the fragments.

Statistical analysis

The SPSS Statistical Package (v.12.0; SPSS Inc., Chicago, Illinois, USA) was used for the standard statistical comparisons. Continuous baseline characteristics were compared between the two groups using a Student's t-test, and categorical data with a χ^2 test, which was also used to compare allele and genotype frequencies for each polymorphism and the deviation from Hardy–Weinberg equilibrium. Linkage disequilibrium coefficients (D') between polymorphisms were calculated with the SHEsis online program [18], which was also used to estimate haplotype frequencies in both groups.

Results and discussion

Several studies suggested that MMP-1 prevents or delays the progression of atherosclerotic plaques. Null mice for TIMP-1 + ApoE and overexpressing human MMP-1 have

less atherosclerotic lesions than expected, suggesting that MMP-1 could play a protective role in the progression of lesions [4]; but the role of MMP-1 in the subsequent events of CAD, such as MI, or in carotid artery intima-media thickness, remains unclear [12].

Our case–control study included 455 individuals, among which 261 were unrelated patients and 194 were healthy controls, sex and smoking status matched, with no differences in levels of total cholesterol, low-density lipoprotein-cholesterol, and triglycerides. However, we found statistically significant differences in some conventional risk factors such as age, number of hypertensives and hypercholesterolemics, and high-density lipoprotein-cholesterol levels (Table 1). All the participants were genotyped for the −1607 1G/2G, −519 A/G, and −340 T/C *MMP-1* polymorphisms, which were selected because they have been described to affect the transcription of the gene. The three polymorphisms were in Hardy–Weinberg equilibrium in patients and controls, and we found a weak linkage disequilibrium between −1607 1G/2G and −519 A/G ($D' = 0.57$), but not with the −340 C/T polymorphism.

We did not find significant differences in allelic or genotypic frequencies between both groups, for the three polymorphisms taken separately (Table 2). These results are in agreement with earlier studies on 1G/2G polymorphism and MI risk, which reported no association of any genotype of this polymorphism and MI [10], although some groups have found a reduced risk of coronary heart disease for carriers of the $2G_{-1607}$ allele [7] or a increased risk of CAD for the $1G_{-1607}$ allele [8]. Another study have found an association between the single presence of the A allele of the −519 A/G polymorphism and the risk of acute coronary syndrome (ACS) [11], but we did not find the same result.

There are also described haplotypic effects of the −519 A/G and −340 T/C polymorphisms on MI risk, with the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes being protective for MI in Caucasian population [10], but A_{-519}-C_{-340} being of risk for ACS in Chinese population [11]. In our study, we could not find any effect for the G_{-519}-T_{-340} haplotype (Table 3), but we found a borderline protective effect of the A_{-519}-C_{-340} haplotype [odds ratio (OR) = 0.73; 95% confidence interval (95% CI) = 0.53–0.99; $P = 0.045$], supporting the earlier data on the Caucasian population and, although we found a trend to increase the risk of MI for the G_{-519}-C_{-340} haplotype as is described in British and Swedish patients [10] (OR = 1.45; 95% CI = 0.81–2.58), it does not reach statistical significance (Table 3). In addition, Han *et al.* [11] reported that the A_{-519}-T_{-340} haplotype significantly increased the risk of ACS, but we do not found any statistically significant protective effect for this combination (Table 3).

The −1607 1G/2G is the most studied polymorphism in the *MMP-1* gene, in part, because the $2G_{-1607}$ allele has over 20-fold higher transcriptional activity than $1G_{-1607}$ [6]. In our population, the G_{-519}-T_{-340} haplotype that has been described as protective for MI [10] is also protective, but only if it is forming a haplotype with the $1G_{-1607}$ allele ($1G_{-1607}$-G_{-519}-T_{-340}: OR = 0.68; 95% CI = 0.50–0.94; $P = 0.02$) (Table 4). If the haplotype is with the $2G_{-1607}$ variant (the more active), it confers a significant risk of suffering MI (OR = 2.40; 95% CI = 1.27–4.55; $P < 0.005$). This effect is not only because of the −1607 1G/2G polymorphism, as neither the allelic nor the genotypic frequencies are statistically different in patients and controls (Table 2). Therefore, it is necessary a specific haplotypic background with the

Table 2 Allelic and genotypic frequencies for the −1607 1G/2G, −519 A/G, and −340 T/C *MMP-1* individual polymorphisms in cases and controls

Position	Allele	MI (N=522)	Controls (N=388)	Genotype	MI (N=261)	Controls (N=194)
−1607	2G	0.56	0.54	2G2G	0.33	0.28
	1G	0.44	0.46	2G1G	0.46	0.51
				1G1G	0.21	0.21
−519	A	0.67	0.68	AA	0.47	0.45
	G	0.33	0.32	AG	0.41	0.45
				GG	0.12	0.10
−340	T	0.73	0.70	TT	0.54	0.48
	C	0.27	0.30	TC	0.39	0.43
				CC	0.07	0.09

MI, myocardial infarction.

Table 3 Comparison of haplotype frequencies of the −519 A/G and −340 T/C polymorphisms in cases and controls

	MI (frequency)	Controls (frequency)	χ^2	Pearson's P value	Odds ratio (95% CI)
A C	104.46 (0.20)	99.36 (0.26)	4.010	0.045	0.73 (0.53–0.99)
A T	246.54 (0.47)	162.64 (0.42)	2.538	0.11	1.24 (0.95–1.62)
G C	35.54 (0.07)	18.64 (0.05)	1.596	0.21	1.45 (0.81–2.58)
G T	135.46 (0.26)	107.36 (0.28)	0.336	0.56	0.92 (0.68–1.23)

CI, confidence interval; MI, myocardial infarction.

Table 4 Comparison of haplotype frequencies of the −1607 1G/2G, −519 A/G, and −340 T/C polymorphisms in cases and controls

	MI (frequency)	Control (frequency)	χ^2	Pearson's P value	Odds ratio (95% CI)
1G A C	36.19 (0.07)	34.24 (0.09)	1.169	0.28	0.76 (0.47–1.24)
1G A T	73.54 (0.14)	40.97 (0.11)	2.433	0.12	1.38 (0.92–2.08)
1G G C	27.61 (0.05)	10.59 (0.03)	3.568	0.058	1.98 (0.96–4.07)
1G G T	94.67 (0.18)	94.20 (0.24)	5.327	0.02	0.68 (0.50–0.94)
2G A C	68.05 (0.13)	65.05 (0.17)	2.595	0.11	0.74 (0.51–1.07)
2G A T	173.22 (0.33)	121.74 (0.31)	0.273	0.6	1.08 (0.81–1.43)
2G G C	8.15 (0.02)	8.12 (0.02)	NA	NA	NA
2G G T	40.57 (0.08)	13.09 (0.03)	7.667	0.006	2.40 (1.27–4.55)

CI, confidence interval; MI, myocardial infarction; NA, no analyzed.

−519 A/G and −340 T/C polymorphisms to have an effect on MI risk.

Different studies showed that alleles linked to higher MMP-1 transcriptional activities would be associated with increased risk of MI and alleles with less activity would confer a protective effect [7,10]. In our study, the protective effect of the haplotype is given when the three alleles with lower transcriptional activity are together, but the presence of a more active allele ($2G_{-1607}$) and the less active haplotype G_{-519}-T_{-340} confers an increased risk of MI, suggesting that the associations are mainly driven by the −1607 1G/2G polymorphism and the −519 A/G and −340 T/C polymorphisms should be modifiers of the relationship. We cannot determine the effect of the haplotype with the more active alleles ($2G_{-1607}$-G_{-519}-C_{-340}) because of its little frequency in our population (2%; Table 4), which does not allow the program to analyze it.

Finally, our study has some limitations. First, it was based on a limited number of cases and controls, although we analyzed a homogeneous population: Caucasian, male, smoker, nondiabetic, and below 60 years of age, younger than populations of other studies. Compared with other studies, hypercholesterolemics and hypertensives were underrepresented in our patients, and even these variables did not match in our groups, therefore we cannot exclude that this mask the effect of the *MMP-1* polymorphisms on MI risk. In addition, the control group did not include individuals angiographically evaluated to confirm the absence of diseased vessels, although this could act underestimating our findings.

In conclusion, our data showed that concrete haplotypes of these three *MMP-1* promoter polymorphisms have significant associations with early MI in smoking males, in a Northern Spanish population, where MI is strongly linked to this habit. The results suggest that this association is mainly driven by the –1607 1G/2G polymorphism, although with a background of a specific haplotype of –519 A/G and –340 T/C polymorphisms.

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References

1 Lusis AJ. Atherosclerosis. *Nature* 2000; **407**:233.
2 Katsuda S, Kaji T. Atherosclerosis and extracellular matrix. *J Atheroscler Thromb* 2003; **10**:267.
3 Sukhova GK, Schonbeck U, Rabkin E, Schoen FJ, Poole AR, Billinghurst RC, *et al.* Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation* 1999; **99**:2503.
4 Lemaitre V, O'Byrne TK, Borczuk AC, Okada Y, Tall AR, D'Armiento J. ApoE knockout mice expressing human matrix metalloproteinase-1 in macrophages have less advanced atherosclerosis. *J Clin Invest* 2001; **107**:1227.
5 Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev* 2005; **85**:1.
6 Rutter JL, Mitchell TI, Buttice G, Meyers J, Gusella JF, Ozelius LJ, *et al.* A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. *Cancer Res* 1998; **58**:5321.
7 Ye S, Gale CR, Martyn CN. Variation in the matrix metalloproteinase-1 gene and risk of coronary heart disease. *Eur Heart J* 2003; **24**:1668.
8 Horne BD, Camp NJ, Carlquist JF, Muhlestein JB, Kolek MJ, Nicholas ZP, *et al.* Multiple-polymorphism associations of 7 matrix metalloproteinase and tissue inhibitor metalloproteinase genes with myocardial infarction and angiographic coronary artery disease. *Am Heart J* 2007; **154**:751.
9 Nojiri T, Morita H, Imai Y, Maemura K, Ohno M, Ogasawara K, *et al.* Genetic variations of matrix metalloproteinase-1 and -3 promoter regions and their associations with susceptibility to myocardial infarction in Japanese. *Int J Cardiol* 2003; **92**:181.
10 Pearce E, Tregouet DA, Samnegard A, Morgan AR, Cox C, Hamsten A, *et al.* Haplotype effect of the matrix metalloproteinase-1 gene on risk of myocardial infarction. *Circ Res* 2005; **97**:1070.
11 Han Y, Wu Z, Zhang X, Yan C, Xi S, Yang Y, *et al.* Impact of matrix metalloproteinase-1 gene variations on risk of acute coronary syndrome. *Coron Artery Dis* 2008; **19**:227.
12 Armstrong C, Abilleira S, Sitzer M, Markus HS, Bevan S. Polymorphisms in MMP family and TIMP genes and carotid artery intima-media thickness. *Stroke* 2007; **38**:2895.
13 Tunstall-Pedoe H, Kuulasmaa K, Amouyel P, Arveiler D, Rajakangas AM, Pajak A. Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation* 1994; **90**:583.
14 Rodriguez I, Coto E, Reguero JR, Gonzalez P, Andres V, Lozano I, *et al.* Role of the CDKN1A/p21, CDKN1C/p57, and CDKN2A/p16 genes in the risk of atherosclerosis and myocardial infarction. *Cell Cycle* 2007; **6**:620.
15 Gonzalez P, Diez-Juan A, Coto E, Alvarez V, Reguero JR, Batalla A, *et al.* A single-nucleotide polymorphism in the human p27kip1 gene (−838C>A) affects basal promoter activity and the risk of myocardial infarction. *BMC Biol* 2004; **2**:5.
16 Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18**:499.
17 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**:1215.
18 Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005; **15**:97.