UNIVERSIDADE FEDERAL DE MINAS GERAIS
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Avaliação de marcadores de metabolismo ósseo em pacientes transplantados renais

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FOLHA DE APROVAÇÃO

AVALIAÇÃO DOS MARCADORES DE METABOLISMO ÓSSEO EM PACIENTES TRANSPLANTADOS RENAIS

FLÁVIA MARIA BORGES VIGIL

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RESUMO

Introdução: O distúrbio mineral ósseo (DMO) após o transplante renal sofre influências dos imunossupressores, da osteodistrofia renal pré-existente, do hiperparatireoidismo *de novo* e fatores tradicionais relacionados ao risco de fraturas, osteoporose e osteopenia. Na doença renal crônica (DRC), o ambiente inflamatório, urêmico e o aumento do produto cálcio e fósforo predispõem às calcificações extra-ósseas, como a calcificação vascular. Ocorre um desequilíbrio entre os fatores inibitórios da calcificação e os indutores. Após o transplante renal, pouco se sabe sobre o efeito nos marcadores do metabolismo ósseo. Este estudo teve como objetivo dosar as concentrações séricas das moléculas do metabolismo ósseo nos pacientes transplantados renais e compará-las com as dosagens em pacientes dialíticos e indivíduos saudáveis.

Metodologia: Trata-se de um estudo transversal com três grupos de pacientes, o primeiro grupo formado por pacientes transplantados renais, o segundo por pacientes dialíticos e o terceiro por voluntários saudáveis. Foram dosadas as concentrações séricas de proteína 1 relacionada ao Dickkopf (DKK-1), esclerostina (SOST), fator de crescimento de fibroblastos 23 (FGF-23), osteocalcina (OC), osteopontina (OPN), osteoprogesterina (OPG) nos três grupos. Posteriormente avaliou-se as associações entre as medidas destas moléculas com variáveis clínicas, demográficas e laboratoriais (cálcio, fósforo, 25 OH vitamina D, fosfatase alcalina, PTH e creatinina).

Resultados: Participaram do estudo 114 pacientes (57 transplantados renais, 36 portadores de DRC em hemodiálise e 31 controles saudáveis).Os transplantados renais apresentaram menores níveis de DKK1 ($p < 0.001$), OPG ($p < 0.001$), OC ($p < 0.001$), OPN ($p = 0.001$), SOST ($p < 0.001$), FGF-23 ($p < 0.001$) quando comparados aos pacientes em hemodiálise. Na comparação com indivíduos saudáveis, os transplantados renais também apresentaram menores níveis séricos de DKK1 ($p = 0.019$), OPG ($p < 0.001$), OC ($p = 0.027$), SOST ($p < 0.001$) e FGF-23 ($p = 0.043$). No grupo de dialíticos, verificou-se que as mulheres apresentavam menor concentração de SOST quando comparados aos homens ($p=0.012$). Não foi encontrada nenhuma outra associação significativa entre os níveis das moléculas, dados demográficos e clínicos.

Conclusão: Nossos resultados mostraram redução significativa nos marcadores de metabolismo ósseo, DKK1, OPG, OC, OPN e SOST, após o transplante renal. Os

primeiros anos após o transplante renal modulam marcadores de DMO, sugerindo uma melhora significativa em relação à DRC em estágio terminal**.**

Palavras chaves: transplante renal, metabolismo ósseo, calcificação vascular, doença renal crônica

ABSTRACT

Introduction: Mineral and Bone Disorder (MBD) after kidney transplantation is influenced by immunosuppressive therapies, pre-existing renal osteodystrophy, *de novo* hyperparathyroidism and traditional risk factors of fractures, osteoporosis and osteopenia. In chronic kidney disease (CKD), the uremic and inflammatory environment and the increased product of calcium and phosphorus predispose to extraosseous calcifications such as vascular calcification. There is an imbalance between the inhibiting and inducing factors of calcification. After kidney transplantation, little is known about the effect on bone metabolism markers. This study aimed to measure the serum concentrations of bone metabolism molecules in kidney transplant patients and to compare with levels found in patients on hemodialysis and healthy individuals.

Methods: This is a cross-sectional study with three groups: kidney transplantation patients, patients on hemodialysis, and healthy controls. The plasma concentrations of Dickkopf -related protein 1 (DKK1), osteoprotegerin (OPG), osteocalcin (OC), osteopontin (OPN), sclerostin (SOST), and fibroblast growth factor 23 (FGF-23) were measured in these three groups. The associations between the measurements of these molecules with clinical, demographic and laboratory variables (calcium, phosphorous, 25 OH vitamin D, alkaline phosphatase, PTH and creatinine) were evaluated.

Results: A total of 114 patients were included in the study. Transplant recipients showed significantly lower levels of DKK1 ($p < 0.001$) OPG ($p < 0.001$), OC ($p < 0.001$), OPN ($p = 0.001$), OST ($p < 0.001$), and FGF-23 ($p < 0.001$) when compared to patients on hemodialysis. In comparison to healthy controls, transplant recipients also presented lower levels of DKK1 ($p = 0.019$), OPG ($p < 0.001$), OC ($p = 0.027$), SOST ($p < 0.001$) and FGF-23 ($p = 0.043$). Regarding demographic data, women presented lower serum SOST levels when compared to men in the hemodialysis group ($p = 0.012$). No other significant associations were found between levels of molecules and baseline demographic and clinical data.

Conclusion: Our findings showed a reduction in bone metabolism markers, DKK1, OPG, OC, OPN and SOST, after kidney transplantation. The first years after kidney transplantation modulate MBD markers, suggesting a significant improvement in relation to end-stage kidney disease.

Key words: kidney transplantation, bone metabolism, vascular calcification, chronic kidney disease.

LISTA DE ABREVIATURAS E SIGLAS

DRC – Doença Renal Crônica

DMO – Distúrbio Mineral Óssea

PTH – Paratormônio

HPTH - hiperparatireoidismo

RFG – Ritmo de Filtração Glomerular

FGF-23 – Fibroblast growth factor 23

SOST – esclerostina/sclerostin

DKK-1 – Dickkopf WNT Signaling Pathway Inhibitor 1

OC – osteocalcina/osteocalcin

OPN- osteopontina/osteopontin

OGP – osteoprotegerina/osteoprotegerin

MPG – matrix gla protein

CKD-EPI – Chronic Kidney Disease Epidemiology Collaboration

AVC – Acidente Vascular Cerebral

Ca – cálcio/calcium

P - Fósforo / fosfate

SRAA – Sistema Renina Angiotensina Aldosterona

KDIGO - Kidney Disease: Improving Global Outcomes

MBD – Mineral and Bone Disorder

CKD – Chronic Kidney Disease

eGFR – glomerular filtration rate

ALP – alkaline phosphatase

RANKL/RANK/OPG - receptor activator of NF-KB ligand/receptor activator of nuclear factor-kappa B/osteoprotegerin

KTx – kidney transplantation

CNIs – calcineurin inhibitors

GCs – glucococorticoids

TG – triglycerides

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Sumário

INTRODUÇÃO

Os pacientes com doença renal crônica (DRC) evoluem com distúrbio mineral ósseo (DMO) à medida que a função renal se reduz. O DMO consiste em alterações clínicas, bioquímicas, ósseas e calcificações extra-ósseas. (Sociedade Brasileira de Nefrologia., n.d.). As alterações bioquímicas geralmente encontradas são a hipo/hipercalcemia, hiperfosfatemia, redução da vitamina D e aumento do PTH (hiperparatireoidismo). Desta maneira, o tecido ósseo sofre modificações na remodelação, mineralização e volume, além do aparecimento de calcificações extra-ósseas, tais como as calcificações vasculares. (Stevens, 2004).

O transplante renal é considerado a melhor terapia renal substitutiva, proporcionando melhora significativa no ambiente urêmico e inflamatório do paciente com DRC. Entretanto, após o transplante, o DMO pode não se recuperar totalmente além de ser influenciado por fatores específicos relacionados ao retorno da função renal e imunossupressão.

Nos pacientes transplantados renais, os distúrbios minerais ósseos podem se manter devido ao uso de imunossupressores, ao hiperparatireoidismo persistente ou *de novo* e aos fatores tradicionais relacionados à perda de massa óssea. Uma série de alterações podem ser encontradas nos transplantados renais, tais como os distúrbios de fósforo e cálcio, osteoporose, osteopenia, fraturas ósseas, osteonecrose, deficiência de vitamina D, calcificação vascular e hiperparatireoidismo secundário ou terciário. (Vangala et al., 2018).

Após o transplante, ocorre uma redução da massa óssea nos primeiros 6 meses, permanecendo estável após um ano, mas ainda em níveis inferiores àqueles encontrados nos indivíduos saudáveis (Bouquegneau et al., 2016). Poucos estudos na literatura avaliaram marcadores do metabolismo ósseo após o transplante renal. Tais estudos são necessários para monitoramento do DMO e definição de estratégias para seu manejo, objetivando melhor qualidade de vida para os pacientes.

Doença mineral óssea na DRC

Uma das manifestações clássicas do DMO é o hiperparatireoidismo secundário à DRC. A produção do paratormônio (PTH) é influenciada pelo cálcio, fósforo e vitamina D. À medida que o ritmo de filtração glomerular (RFG) reduz, ocorre aumento do

fósforo sérico que estimula a liberação de PTH. No túbulo proximal, o PTH e o fator de crescimento de fibroblastos 23 (FGF-23) inibem o cotransportador NaP1-IIa, NaP1-IIc, ocasionando hiperfosfatúria, com o objetivo de manter os níveis séricos de fósforo dentro dos limites de referência (Vangala et al., 2018). No rim, o PTH ativa a enzima 1–alfa hidroxilase, que é responsável por catalisar a conversão da forma inativa da 25-hidroxi (25-OH) vitamina D na forma ativa 1,25 hidroxi (1,25 – OH) vitamina D. A vitamina D ativa aumenta a absorção de fósforo e cálcio no intestino, mantendo o cálcio em concentrações dentro dos limites de normalidade (Yuen, 2016) .

Quando o RFG reduz para valores menores do que 60 ml/min, os osteócitos e osteoblastos aumentam a liberação do FGF-23, que, além de promover fosfatúria, inibe a enzima 1-alfa hidroxilase. Desta maneira, ocorre uma menor produção da forma ativa da vitamina D pela ação do FGF-23 e também devido à redução da massa de néfrons funcionantes. A queda das concentrações de 1,25 OH vitamina D reduz a absorção intestinal de cálcio e fósforo, resultando consequentemente em hipocalcemia. Com a redução ainda mais acentuada do RFG, a hiperfosfatemia se instala apesar dos mecanismos compensatórios. Na paratireóide, os receptores sensíveis ao cálcio, a vitamina D e ao fósforo são estimulados pela hipocalcemia, hiperfosfatemia e pela hipovitaminose D, ocasionando maior liberação de PTH (Vangala et al., 2018).

As duas consequências mais importantes do hiperparatireoidismo secundário são a osteodistrofia renal e a calcificação vascular. O PTH se liga a receptores no osteoblasto estimulando a formação do osteoclasto que, por sua vez, ocasiona um aumento da reabsorção óssea, levando à osteodistrofia renal (Yuen, 2016).

Os pacientes com DRC possuem maior chance de apresentarem alterações relacionadas ao sistema cardiovascular, como calcificação das artérias coronarianas, quando comparados à população saudável (Leopold, 2015). O hiperparatireoidismo acelera a calcificação vascular, calcificação do miocárdio e valvas cardíacas (Yuen, 2016). A calcificação vascular pode ocorrer na camada íntima, geralmente associada à aterosclerose, mas também na camada média, principalmente associada a anormalidades no metabolismo mineral-ósseo (Goodman et al., 2004) O processo de calcificação tem sido relacionado a múltiplos fatores, tais como distúrbios do metabolismo ósseo (hipercalcemia e hiperfosfatemia), uremia, inflamação, bem como aos fatores de risco tradicionais para doença cardiovascular, incluindo idade mais avançada, tabagismo, diabetes mellitus, hipertensão arterial e dislipidemia (Moe & Chen, 2008).

No ambiente urêmico, característico do paciente em DRC, a hipercalcemia, a hiperfosfatemia e a presença de citocinas inflamatórias induzem as células musculares vasculares a se transformarem em células condrócito/osteoblasto-like. Estas células expressam proteínas da matriz óssea que se depositam nas camadas íntimas ou médias das artérias. O balanço positivo de cálcio e fósforo presente nos pacientes portadores de DRC leva à formação de vesículas de matriz óssea, contendo cálcio e fósforo, que também se depositam nestas artérias, contribuindo para o processo de mineralização (Moe & Chen, 2004) (Schlieper et al., 2016). Outro fator contribuinte é a deficiência na circulação de inibidores da calcificação, tais como osteopontina, fetuin A, matrix gla e pirofosfato (Schlieper et al., 2016).

Doença mineral óssea após o transplante renal

Após o transplante renal, os níveis de PTH reduzem nos primeiros meses, porém podem persistir elevados após o primeiro ano em 20 a 40% dos casos, refletindo pior sobrevida do enxerto (Alshayeb et al., 2013; Bouquegneau et al., 2016). Os níveis de PTH se reduzem devido à resolução da hiperfosfatemia e ao retorno da produção do calcitriol pelos néfrons funcionantes. Os níveis de FGF-23 e PTH ainda elevados agem nos túbulos que recuperaram sua função normal, aumentando a excreção renal de fósforo e absorção de cálcio. Nos primeiros 6 meses após o transplante, pode ocorrer hipofosfatemia e hipercalcemia que levam a calcificação do interstício renal e alterações no turnover ósseo. Entretanto, estas alterações no metabolismo do cálcio e fósforo podem persistir, mesmo após este período (Alshayeb et al., 2013; Bouquegneau et al., 2016). O FGF-23 reduz drasticamente ao longo do primeiro ano do transplante. Após o primeiro ano, as concentrações de FGF-23 dependem da função do enxerto renal (Alshayeb et al., 2013) Os pacientes transplantados com frequência apresentam hipovitaminose D devido à baixa exposição solar. A vitamina D exerce efeitos no sistema imune. Alguns estudos observacionais mostraram relação das concentrações reduzidas de vitamina D com o risco aumentado de rejeição, assim como seus efeitos na redução da função do enxerto renal (McGregor et al., 2014).

Diante do exposto, a qualidade e a densidade óssea se alteram em pacientes transplantados renais, aumentando o risco de fraturas, morbidade e mortalidade. Em comparação aos pacientes dialíticos, este risco aumenta nos primeiros anos após o transplante, sendo que, após este período, o risco de fratura se reduz (Palmer et al.,

2019). Fatores que contribuem para o aumento do risco de fratura após o transplante são sexo feminino, longo período em diálise, presença de hiperparatireoidismo antes de realizar o transplante, idade maior do que 50 anos, história prévia de fratura, desnutrição, alcoolismo, tabagismo, deficiência de vitamina D, hipomagnesemia e dose acumulada de corticoide (Bouquegneau et al., 2016) .

Tanto o osso trabecular quanto o cortical são afetados. O osso trabecular é afetado devido à ação dos corticóides, que inibem a proliferação e diferenciação dos osteoblastos, estimulam a apoptose dos osteoblastos e osteoclastos, além de aumentarem a excreção de cálcio. O osso cortical é afetado antes do transplante renal devido ao hiperparatireoidismo secundário. Entretanto, à medida que a função do enxerto renal se deteriora ao longo dos anos, o hiperparatireoidismo pode retornar ou se desenvolver (Palmer et al., 2019; Bouquegneau et al., 2016).

Nesse contexto, o presente estudo pretende investigar as concentrações plasmáticas de marcadores não tradicionais relacionados ao metabolismo ósseo em pacientes transplantados renais com função estável do enxerto e comparar com as mesmas moléculas dosadas nos pacientes em hemodiálise e controles saudáveis. Estes marcadores do metabolismo ósseo podem atuar como inibidores da calcificação vascular (osteoprotegerin (OPG), fetuín-A, proteína da matrix gla (MPG) e osteopontina (OPN)) ou indutores da calcificação (osteocalcina (OCN) esclerostina (SOST) e Dickkopf WNT 1 sinalizador da via de inibição (DKK1)). Por se tratar ainda de uma área pouco investigada e na qual ainda persistem dúvidas em relação ao manuseio, monitoração e prognóstico, pretendeu-se, com este estudo, ampliar a compreensão acerca das moléculas potencialmente relacionadas a DMO.

Marcadores não tradicionais do metabolismo ósseo

O DKK1 e SOST inibem a via de sinalização canônica Wnt, responsável pela osteoblastogenesis (diferenciação e proliferação dos osteoblastos), desta maneira agem reduzindo a formação óssea.(Cejka et al., 2012) SOST é um produto do gene SOST, sintetizado pelos osteócitos, enquanto DKK1 é expressado por diversos tecidos durante a embriogênese (Cejka et al., 2011) . Em contrapartida, a osteocalcina (OC) é uma proteína produzida pelos osteoblastos sendo um marcador de sua atividade e alto turnover ósseo (Bouquegneau et al., 2016). Da mesma forma, osteoprotegerin (OPG) também é produzida pelos osteoblastos, como parte do sistema RANKL/RANK/OPG.

Neste sistema, o RANKL se liga ao RANK presente nos osteoclastos os ativando. A OPG age como um receptor competitivo do RANK pois se liga ao RANKL, impedindo a ativação do RANK e desta maneira reduzindo a atividade do osteoclasto (Cianciolo et al., 2014). O osteoblasto também sintetiza a osteopontina (OPN), uma glicoproteína cujo papel na DMO ainda não está bem definido (Si et al., 2020). A OPN parece interferir na ativação do osteoclasto e proliferação dos osteoblastos. Nos pacientes com calcificações ectópicas, como naqueles com DRC, a OPN foi encontrada na superfície das placas ateromatosas calcificadas e valvas cardíacas calcificadas (Wada et al., 1999). Apesar de não estar diretamente relacionada às calcificações, o FGF-23 é crucial no entendimento da DMO, pois possui um papel importante na hipofosfatemia pós transplante e promove a supressão da atividade da 1 alfa hidroxilase no rim (Rao et al., 2012).

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OBJETIVO GERAL

Avaliar as concentrações sanguíneas de moléculas relacionadas ao metabolismo ósseo em pacientes transplantados renais, comparando-as com tais medidas em indivíduos saudáveis e pacientes em tratamento dialítico.

Objetivos específicos

1. Medir as concentrações sanguíneas de marcadores do metabolismo ósseo a saber: Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK-1), esclerostina (SOST), FGF-23, osteocalcina (OC), osteopontina (OPN), osteoprotegerina (OGP) em pacientes adultos submetidos a transplante renal;
2. Comparar as mensurações dessas moléculas nos pacientes transplantados renais com suas concentrações em portadores de DRC em hemodiálise e indivíduos saudáveis,
3. Verificar associações e/ou correlações entre as medidas das moléculas com variáveis clínicas e laboratoriais, incluindo tempo de diálise, tipo de imunossupressores utilizados, níveis séricos de cálcio, fósforo, 1,25-OH vitamina D, PTH e fosfatase alcalina tanto nos pacientes transplantados renais quanto nos pacientes em tratamento dialítico.

METODOLOGIA

Desenho do estudo

Trata-se de um estudo observacional transversal.

Local e Período

Realizou-se o estudo no ambulatório de transplante renal do Hospital Evangélico de Belo Horizonte e no Centro de Nefrologia do Hospital Evangélico – Unidade Contagem. A coleta das amostras de sangue foi realizada entre o período de agosto/2020 a julho/2021.

População/amostra

A população total escolhida para o estudo foi formada por 114 pacientes, dividida entre os três grupos a saber:

Grupo 1: Pacientes transplantados renais no período de 2010 até segundo semestre de 2018, cuja etiologia da DRC tenha sido nefroesclerose hipertensiva, diabetes mellitus, indeterminada ou glomerulonefrite crônica. Excluíram-se os pacientes transplantados com ritmo de filtração glomerular menor do que 30 ml/ min/1,73 m^2 e cujo tempo de transplante era inferior a um ano.

Grupo 2: Pacientes com DRC em hemodiálise (controle positivo). Estes pacientes foram pareados ao grupo de transplantados em relação a sexo, idade e etiologia da DRC.

Grupo 3: Indivíduos saudáveis, pareados por idade e sexo com os pacientes portadores de DRC e com o grupo de transplantados renais. Os indivíduos do grupo 3 não apresentavam fatores de risco detectáveis clínica e laboratorialmente para doenças renais e cardiovasculares (controle negativo).

Ressalta-se a importância do grupo de indivíduos saudáveis para avaliar se o transplante renal foi capaz de restaurar as concentrações das moléculas do metabolismo ósseo para valores similares aos de indivíduos saudáveis.

Critérios de inclusão

Aceitar participar do estudo e se enquadrar nas características de cada grupo, como mencionado acima.

Critérios de exclusão

Presença de outras comorbidades sistêmicas, além das mencionadas acima. Presença de fraturas, traumas ósteo-articulares ou com intercorrências clínicas/metabólicas agudas no momento da coleta.

Protocolo do Estudo

Os pacientes transplantados, os controles positivos (pacientes em hemodiálise) e negativos (indivíduos saudáveis) foram submetidos à coleta de sangue em uma única ocasião, respeitando-se os critérios de exclusão e inclusão. A coleta das amostras de sangue foi realizada por meio de punção venosa periférica, respeitando-se todos os critérios de assepsia. As amostras foram submetidas a centrifugação a 5000 rpm, por 10 minutos, a 4ºC. As amostras de plasma então obtidas foram transferidas para microtubos de 1,5 mL, transportadas para o Laboratório Interdisciplinar de Investigação Médica da Faculdade de Medicina- UFMG e armazenadas a -80ºC até o momento das análises.

No referido laboratório, as amostras de plasma foram então analisadas pelo método Luminex-based microbead assay (HBNMAG-51K, Millipore, Billerica, MA), sendo mensuradas as concentrações sanguíneas das moléculas relacionadas ao metabolismo ósseo: DKK-1, SOST, FGF-23, osteocalcina, osteopontina e osteoprotegerina. Tal técnica consiste na detecção de microesferas revestidas com anticorpos monoclonais específicos para cada molécula a ser analisada nas amostras dos pacientes. Dessa foram, foram adicionadas aos tubos microesferas revestidas com anticorpos monoclonais específicos junto com amostras de concentrações conhecidas (padrões) e amostras de plasma em estudo. Após a incubação e lavagem, adicionou-se aos tubos uma mistura de anticorpos secundários revestidos por biotina. Em seguida, proteínas fluorescentes conjugadas com estreptavidina foram incubadas por um curto período. Após nova lavagem, o sobrenadante foi descartado e o precipitado contendo as microesferas foi ressuspendido em uma solução tampão. As amostras padrão e do estudo foram avaliadas no MAGPIX® microsphere analyzer (Luminex Corporation, Texas, USA) e os resultados foram analisados pelo Milliplex Analyst program (MilliporeSigma), representados em pg/ml. Todas as medições foram realizadas em ensaios únicos para evitar variações entre os ensaios, sendo nossa variação intra-ensaio inferior a 3%. As concentrações mínimas detectáveis para cada molécula foram: 2,2 pg/mL para DKK1;

1,8 pg/mL para OPG; 15,6 pg/mL para OC; 33 pg/mL para SOST e 7,7 pg/mL para FGF-23.

Além das amostras para dosagem das moléculas relacionadas ao metabolismo ósseo, tanto nos grupos de transplantados como nos dialíticos foram realizadas coletas simultâneas dos exames laboratoriais para mensuração de cálcio, fósforo, 1,25-OH vitamina D, PTH, fosfatase alcalina, creatinina, ureia, utilizando metodologia rotineiramente empregadas no laboratório do Hospital Evangélico.

Nos pacientes transplantados e em diálise, foram pesquisadas as variáveis clínicas e demográficas de interesse, que incluíram sexo, idade, tempo de início da hemodiálise ou tempo da realização do transplante renal, tipos de imunossupressores utilizados, uso de anti-hipertensivos, estatinas, hipoglicemiantes orais, insulina, medicamentos para DMO, aspirina, presença de fatores de risco para doença cardiovascular (dislipidemia, diabetes, hipertensão, obesidade e tabagismo), presença de doenças cardiovasculares (acidente vascular cerebral, doença arterial coronariana crônica, doença vascular periférica), insuficiência cardíaca, osteoporose, osteopenia, fraturas, tipo de doador (vivo ou falecido).

O ritmo de filtração glomerular (RFG) foi calculado utilizando a fórmula, Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (Levey et al., 2009). Os diagnósticos de nefropatia hipertensiva, nefropatia diabética e glomerulonefrite crônica foram realizados de acordo com os critérios adotados pelo KDIGO(“KDIGO 2020 Clinical Practice Guideline for Diabetes Management in Chronic Kidney Disease,” 2020; Rovin et al., 2021).

Aspectos éticos

O projeto foi aprovado pelo Comitê de Ética em Pesquisa do Hospital Evangélico, número de registro: CAAE-31405120.3.0000.8787 (vide anexo A). Todos os pacientes assinaram o termo de consentimento livre e esclarecido no qual foi detalhadamente explicado o protocolo do estudo, assegurado o direito de recusa em participar do estudo e ressaltado o sigilo em relação à identificação dos participantes da pesquisa. Ressaltou-se também que os dados obtidos no estudo serão utilizados única e exclusivamente para fins de pesquisa científica.

Análise estatística

Os dados foram analisados pelos softwares GraphPad Prism versão 8.0 e SPSS versão 22.0. Os resultados obtidos foram expressos em média e erro padrão da média (EPM), mediana e intervalo interquartil ou porcentagens, quando apropriado. As variáveis categóricas foram comparadas pelo teste do qui-quadrado. O teste de Kolmogorov-Smirnov verificou a distribuição gaussiana. Para variáveis sem distribuição gaussiana, o teste de Mann-Whitney foi usado para comparar dois grupos e o teste de Kruskal-Wallis para comparações entre mais de dois grupos. Para variáveis com distribuição normal, as comparações entre dois grupos foram feitas pelo teste t de Student não pareado e para mais de dois grupos por análise de variância seguida pelo pós-teste de Bartlett. Os testes de Pearson ou Spearman foram utilizados para avaliar as correlações de acordo com a distribuição das variáveis. Todos os testes estatísticos foram bicaudais com nível de significância de $p < 0,05$.

REFERÊNCIAS BIBLIOGRÁFICAS DA METODOLOGIA

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RESULTADOS

Os resultados desse estudo serão apresentados por meio de dois artigos científicos. O primeiro deles trata-se de uma revisão narrativa da literatura intitulada *Chronic Kidney Disease-Mineral Bone Disease biomarkers in kidney transplant patients,* que se encontra aceita para publicação no periódico *Current Medicinal Chemistry* (fator de impacto = 4.53). O segundo consiste em um artigo original que apresenta os dados obtidos no presente estudo e se intitula *Evaluation of bone metabolism markers in kidney transplant recipients*

Artigo de revisão: *Chronic Kidney Disease-Mineral Bone Disease biomarkers in kidney transplant patients*

Chronic Kidney Disease-Mineral Bone Disease biomarkers in kidney transplant patients

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ABSTRACT

Background: Chronic Kidney Disease associated with Mineral Bone Disease (CKD-MBD) is frequent in kidney transplant patients. Post-transplantation bone disease is complex, especially in patients with pre-existing metabolic bone disorders that are further affected by immunosuppressive medications and changes in renal allograft function. Main biochemical abnormalities of mineral metabolism in kidney transplantation (KTx) include hypophosphatemia, hyperparathyroidism (HPTH), insufficiency or deficiency of vitamin D, and hypercalcemia. **Objective:** This review aimed to summarize the pathophysiology and main biomarkers of CKD-MBD in KTx. **Methods:** A comprehensive and non-systematic search in PubMed was independently made with an emphasis on biomarkers in mineral bone disease in KTx. **Results:** CKD-MBD can be associated with numerous factors including secondary HPTH, metabolic dysregulations before KTx, and glucocorticoids therapy in post-transplant subjects. Fibroblast growth factor 23 (FGF23) reaches normal levels after KTx with good allograft function, while calcium, vitamin D and phosphorus, ultimately, result in hypercalcemia, persistent vitamin D insufficiency, and hypophosphatemia respectively.

As for PTH levels, there is an initial tendency of a significant decrease, followed by a raise due to secondary or tertiary HPTH. In regard to sclerostin levels, there is no consensus in the literature. **Conclusion:** KTx patients should be continuously evaluated for mineral homeostasis and bone status, both cases with successful kidney transplantation and those with reduced functionality. Additional research on CKD-MBD pathophysiology, diagnosis, and management is essential to guarantee long-term graft function, better prognosis, good quality of life, and reduced mortality for KTx patients.

Key words: bone mineral disease, kidney transplant, chronic kidney disease, vitamin D, calcium, phosphate, hyperparathyroidism, fibroblast growth factor 23

1. INTRODUCTION

Kidney transplantation (KTx) is commonly used to effectively treat the end-stage failure of the organ and is one of the most frequently transplanted organs throughout the world[1]. Successful transplantation is able to restore organ function and improve patients' survival and quality of life.

However, kidney transplant patients frequently present Chronic Kidney Disease associated with Mineral Bone Disease (CKD-MBD), which are the systemic alterations in mineral and bone metabolism, cardiovascular system, and graft function that occur post-KTx. CKD-MBD includes abnormalities in phosphorus (P), calcium (Ca), parathormone (PTH), fibroblast growth factor 23 (FGF-23), Vitamin D, and changes in bone mineralization, strength, volume, density, and composition[2–6]. In CKD-MBD pre-transplant patients, the commonest alterations observed are hypocalcemia, hyperphosphatemia, higher levels of PTH or hyperparathyroidism (HPTH) and FGF-23, and vitamin D deficiency or insufficiency[2–4, 7]. In KTx patients, bone and general metabolism are influenced by the restored kidney function, nonetheless, many disturbances of ion balance, hormones, and other regulatory molecules still remain[2–4, 7]. Patients with CKD-MBD post KTx are at greater risk of vascular and soft tissue calcification and cardiovascular diseases, besides having a higher incidence of osteoporosis/osteopenia and other bone disorders[8]. Therefore, early recognition of bone metabolism alterations and how to predict, prevent and treat those changes can be of utmost importance. CKD-MBD post KTx may compromise short and long-term graft function. The adequate control of this disorder may provide a good quality of life and may reduce mortality in this population[4, 7, 9, 10]. The aim of this study is to review the pathophysiology of CKD-MBD and the main biomarkers altered in KTx patients.

2. METHODS

Data was obtained independently by the authors, who carried out a comprehensive and non-systematic search in the PubMed database. Search strategies included Medical Subject Heading terms as: "FGF-23", "Vitamin D", "DKK1", "IL-1beta", "IL-6", "insulin", "leptin", "osteocalcin", "osteopontin", "osteoprotegerin", "PTH", "iPTH", "SOST", "TNF", "sclerostin", separately searched from each other, but altogether with "mineral bone disease", "renal transplantation" and "kidney transplantation". The search emphasized biomarkers in mineral bone disease in KTx patients. Articles focusing on pediatric patients, non-transplant patients, treatments and interventions in CKD-MBD were excluded.

3. CHRONIC KIDNEY DISEASE-MINERAL BONE DISEASE (CKD-MBD) PATHOPHYSIOLOGY

The normal kidney function involves a series of feedback mechanisms that aim to keep serum concentrations of calcium and phosphate at a normal range and to maintain bone health[2–4, 7]. Some hormones and growth factors are key in this process, including calcium, phosphate, vitamin D, PTH, and bone-derived FGF23[2–4, 7].

PTH levels are influenced by the serum concentrations of phosphate, vitamin D, and, mainly, calcium[11]. This hormone is responsible for increasing calcium reabsorption from the bones, to compensate for its low serum levels. PTH also stimulates vitamin D production by means of the activation of 1-α-hydroxylase. PTH production and secretion are also increased by hyperphosphatemia, acting as phosphaturic hormone synergistically with FGF23[12, 13]. After binding with its co-receptor Klotho, FGF23 reduces phosphate tubular reabsorption in the kidneys, increasing urinary phosphate excretion. Vitamin D synthesis starts in the liver forming 25-hydroxyvitamin D ($25(OH)D_3$ or 25D). Then, 25D is converted in the kidney into $1,25(OH)_2D_3$ (1,25D) by the enzyme 1-α-hydroxylase. The 1,25D is the active form of the vitamin, which exerts endocrine and systemic effects through vitamin D Receptor (VDR) or can be degraded by 24-α-hydroxylase[9]. Active vitamin D stimulates intestinal absorption of calcium and phosphate, and PTH downregulation[14]. Vitamin D levels may be increased by PTH, through the upregulation of the 1-α-hydroxylase or decreased by high FGF23, via inhibition of the same enzyme. Low levels of active vitamin D may lead to hypocalcemia. Additionally, high FGF23 levels also decrease phosphate levels by interfering with a part of the phosphate intestinal absorption that depends on the vitamin D effect [15].

Regarding bone metabolism, the homeostasis between osteoclast and osteoblast genesis and functionality is closely related to calcium, vitamin D, PTH, and FGF23 pathways. Osteoclast activity is stimulated by PTH and receptor activator of nuclear factor Kappa-B ligand/osteoprotegerin (RANKL/OG), being responsible for bone demineralization[4, 16]. Alternatively, osteoblast is responsible for bone remineralization and is also reduced by the RANKL/OG system. Osteoblasts also produce FGF23, together with osteocytes. FGF23 interferes in bone metabolism through vitamin D, decreasing 1,25D and consequently, VDR[6, 9, 17]. **Figure 1** shows calcium and phosphate homeostasis under physiological conditions.

Patients with Chronic Kidney Disease (CKD) have a low glomerular filtration rate (GFR), which, in turn, leads to decreased ion excretion and higher serum levels of phosphate. To compensate for hyperphosphatemia, an increase in FGF23 production is observed at the early stages of the disease. This results in normal serum phosphate for most CKD patients[18]. FGF23 augmentation is also stimulated by the reduction of GFR and of the 1,25D[19–22]. FGF23 decreases renal phosphate reabsorption and also inhibits renal synthesis of active vitamin D, further reducing intestinal phosphate absorption[23]. In addition, low Klotho levels induce FGF23 resistance, resulting in a greater increase of FGF23 levels, and in the progressive loss of the ability to prevent hyperphosphatemia, vascular calcification, and cardiovascular disease[23–27].

As CKD advances and GFR falls further, the impaired kidney function leads to low 1-α-hydroxylase activity, with a consequent reduction in active vitamin D levels and hypocalcemia[18]. These changes result in an increase in PTH levels, which is further aggravated by the worsening of kidney function and the resultant hyperphosphatemia, perpetuating a maladaptive response. It is not elucidated whether high PTH levels are harmful to bone mineralization, once patients with CKD and skeletal resistance to PTH have been identified. However, PTH contributes to greater

calcium reabsorption from the bone[28, 29]. The overall result of this process is the development of hyperparathyroidism, hypovitaminosis D, and hypocalcemia. Hyperparathyroidism is associated with high bone turnover disease and vascular calcification[4, 23]. The elevated PTH and FGF23 combined with the low vitamin D and hypocalcemia leads to a systemic and local decompensation, termed CKD-MBD. Severe complications of CKD-MBD include renal osteodystrophy, fractures, vascular calcification, and increased cardiovascular mortality. **Figure 2** shows the alterations in calcium and phosphate homeostasis due to CKD.

Some studies have shown that vascular calcification can be accelerated by the use of warfarin[30, 31]. Patients with CKD that have atrial fibrillation can be anticoagulated with vitamin K´s antagonist, warfarin, or direct oral anticoagulants (DOACS). Matrix Gla Protein (MGP) is an inhibitor of vascular calcification that is produced by vascular smooth muscle cells (VSMCs) and chondrocytes[32]. Vitamin K is a cofactor necessary for the carboxylation of the inactive form, uncarboxylated MGP to the active form, carboxylated MGP[30]. The activated form of MGP inhibits bone morphogenic proteins (BMP-2 and BMP-4), reducing vascular calcification. Therefore, the use of warfarin or vitamin K deficiency can reduce the carboxylation of MGP, leading to vascular calcifications[32].

Several physiological processes start after KTx that alter bone, cardiovascular and general metabolism due to the recovery of kidney function. Immediately post-transplant, circulating levels of FGF-23 and PTH are still high, which can lead to phosphaturia and hypophosphatemia[2]. With a functional graft, GFR increases, and FGF-23 levels decrease significantly, contributing to higher levels of active vitamin D and intestinal absorption of calcium and phosphorus[33]. Additionally, serum Klotho levels increase significantly after KTx, which may improve phosphate homeostasis. The combination of increased GFR and higher Klotho expression results in good allograft function[25, 34, 35].

However, some key factors post-transplant may lead to bone loss and reduced graft function, including the use of immunosuppressants and persistent or *de novo* HPTH. Frequently, post kidney transplant patients develop mineral and bone disorders, characterized by the loss of bone volume and mineralization abnormalities, which compose different types of renal osteodystrophy, mainly low turnover bone disease[4].

Glucocorticoids (GCs) increase osteoclastic and reduce osteoblastic activity, leading to augmented bone reabsorption. In addition, GCs also increase phosphorus and calcium loss, resulting in reduced bone density and formation, and less active vitamin D. GCs also act indirectly in bone metabolism through inhibition of testosterone, estrogen, adrenal androgens, and IGF-1 synthesis[3, 16, 36–40]. Additionally, GCs alter the RANKL/OG system, inducing bone remodeling through decreased osteoblast proliferation and differentiation, while promoting osteoclastogenesis, ultimately reducing bone mineral density[37, 41, 42]. Calcineurin inhibitors (CNIs) have been shown to increase PTH and decrease magnesium, VDR synthesis, and osteoprotegerin, resulting in increased osteoclastic activity and further augmenting the risk of osteoporosis[43, 44]. mTOR inhibitors have been shown to negatively impact bone structure and formation, but there are still very few studies regarding the effect of these

medications on bone mineralization[45, 46]. Although not directly associated with FGF23 levels, the use of immunosuppressive agents has been also directly related to alterations in lipid parameters, including triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels. These findings support the idea that post-transplant immunosuppressive therapy might be associated with cardiovascular complications in KTx recipients.

Persistent secondary HPTH 12 months post-transplant is observed in over 60% of patients, most likely due to severe secondary HPTH or tertiary HPTH prior to KTx[16, 47–49]. HPTH also contributes to hypercalcemia post-transplant, which has been reported in 5-15% of KTx patients, mostly 3-6 months after the procedure[50]. Hypercalcemia and high PTH levels are associated with interstitial microcalcification and poorer long-term graft outcomes[43]. High serum PTH levels in HPTH also increase bone reabsorption and decrease density, besides inducing phosphaturia and hypophosphatemia, which, in turn, impair the bone formation and mineralization[3]. HPTH is associated with unfavorable outcomes, including worse graft function[4]. This impairment is also influenced by a progressive graft dysfunction, resulting in increased FGF23 concentrations and decreased vitamin D levels. All these mechanisms contribute to increased fracture risk, vascular calcification, and loss of graft function[9, 10, 51–54].

In addition, vitamin D levels tend to decrease, remaining lower than in the general population, even in the case of successful kidney engraftment. This is due to the HPTH, the reduced renal mass after transplantation, and low 1-α-hydroxylase activity[47, 55]. As vitamin D is an immune modulator with anti-proliferative effects on B cells, its deficiency may also be related to allograft rejection and may contribute to hypocalcemia and impaired bone mineralization. Immunosuppressive therapy, PTH levels and residual renal function are important predictors of vitamin D deficiency[56].

Hypophosphatemia is a common electrolyte disturbance post KTx, found in up to 34% of patients. This alteration is likely due to decreased phosphorus reabsorption in the proximal tubule, which is related to persistently elevated PTH and/or FGF-23 levels[4, 33, 48, 55, 57]. Hypophosphatemia is associated with a decrease in osteoblast activity, leading to rickets and osteomalacia and is also related to anemia and increased mortality risk in KTx patients[58–61].

Another frequent alteration is hypomagnesemia, observed mainly during the first few weeks after kidney transplantation. Hypomagnesemia is most likely due to the use of immunosuppressive medications, mostly CNIs, which may interfere in magnesium metabolism decreasing its reabsorption and promoting urinary magnesium wasting[62, 63]. Magnesium is an integral component of the hydroxyapatite structure of the bone and it may also impair hydrogenase-potassium ATPase magnesium-dependent pumps in the bone. Its deficiency contributes to bone demineralization, reduced PTH secretion and increased PTH resistance, resulting in persistent secondary HPTH[64, 65]. Additionally, hypomagnesemia is also an independent predictor of new-onset or *de novo* diabetes mellitus in KTx patients and is associated with faster decline of graft function[63, 66].

As in pre-transplant patients, when graft function is reduced in KTx recipients, FGF23 levels start to increase at first, followed by elevated PTH and reduced GFR. GCs and HPTH lead to trabecular and cortical bone loss, respectively, finally resulting in

reduction in bone density, increased risk of fractures and overall high risk of mortality post-transplant, characterizing the post-transplant CKD-MBD[3]. Additionally, pre-transplant adults with CKD normally present cardiovascular complications of end-stage kidney disease, such as coronary artery calcification. Even after a successful KTx, with normal levels of calcium, phosphate and PTH, these patients may not completely recover from the complications of pre-transplant CKD[67, 68]. **Figure 3** shows the alterations in calcium and phosphate homeostasis after KTx.

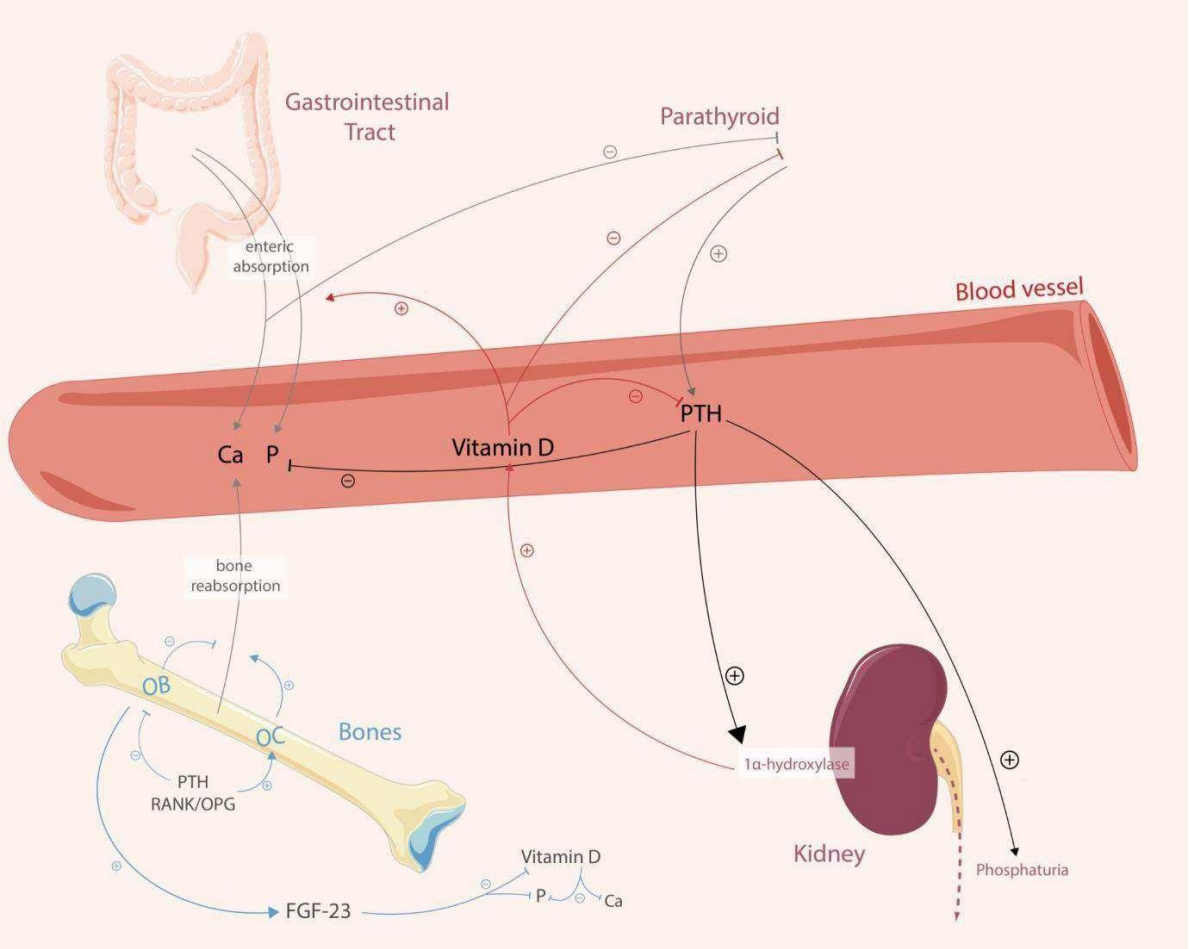

Figure 1. Calcium and phosphate homeostasis under physiological conditions. The main molecules involved in kidney and bone metabolism are shown in the bloodstream. PTH (Parathyroid hormone) is produced by the parathyroid glands and regulates the serum levels of Calcium (Ca) by activating the Osteoclast (OC) and inhibiting the Osteoblast (OB), resulting in bone reabsorption. This mechanism is reinforced by the RANK/OPG system (osteoprotegerin). OB stimulates fibroblast growth factor 23 (FGF-23) production, which inhibits vitamin D absorption through 1-α-hydroxylase inactivation and, ultimately, reduces phosphorus (P) and calcium (Ca) enteric absorption. In the kidneys, the PTH stimulates, by enhancing the 1-α-hydroxylase activity, the synthesis of vitamin D (Vit D), which, in turn, downregulates PTH levels through a negative-feedback mechanism. PTH also produces phosphaturia, synergically with FGF-23 and its coreceptor Klotho. Finally, vitamin D acts stimulating Ca and P absorption in the intestines.

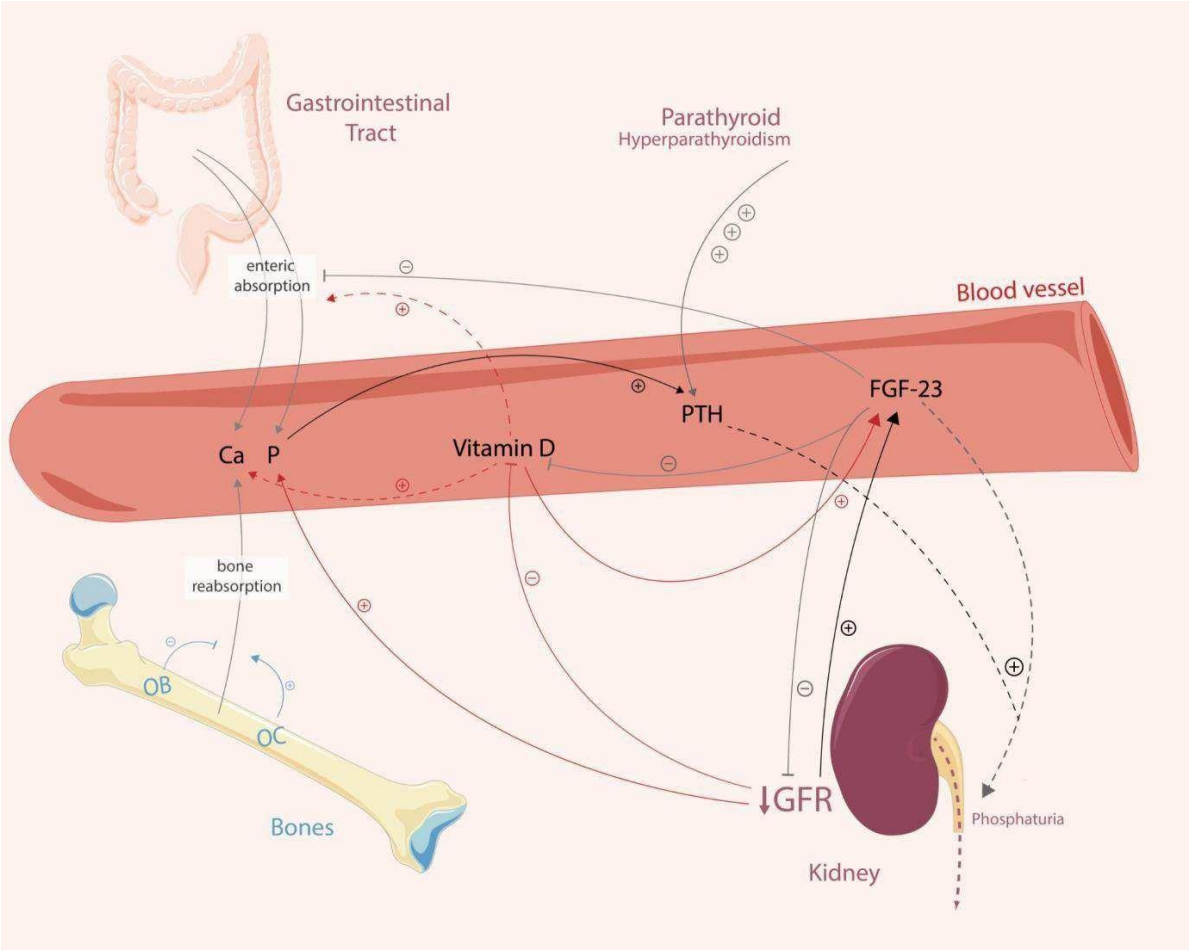

Figure 2. Altered Calcium (Ca) and Phosphate (P) homeostasis in Chronic Kidney Disease (CKD). In CKD patients, Glomerular Filtration Rate (GFR) is reduced leading to a decrease in the metabolites excretion, creating feedback loop between GFR and fibroblast growth factor 23 (FGF-23), in which the low GFR stimulates the FGF-23 activity that decreases the GFR. Additionally, FGF-23 causes an inhibition of enteric absorption of P and Ca as a consequence of the inhibition of vitamin D synthesis. Reduced vitamin D, high P serum levels and low Ca levels cause higher levels of PTH. As GFR progressively decreases, levels of vitamin D reduce resulting in diminished intestinal Ca absorption. Finally, hyperparathyroidism is reinforced by higher levels of P. These factors all together cause hypocalcemia, hyperphosphatemia, insufficiency/deficiency of vitamin D, higher FGF-23 and PTH levels.

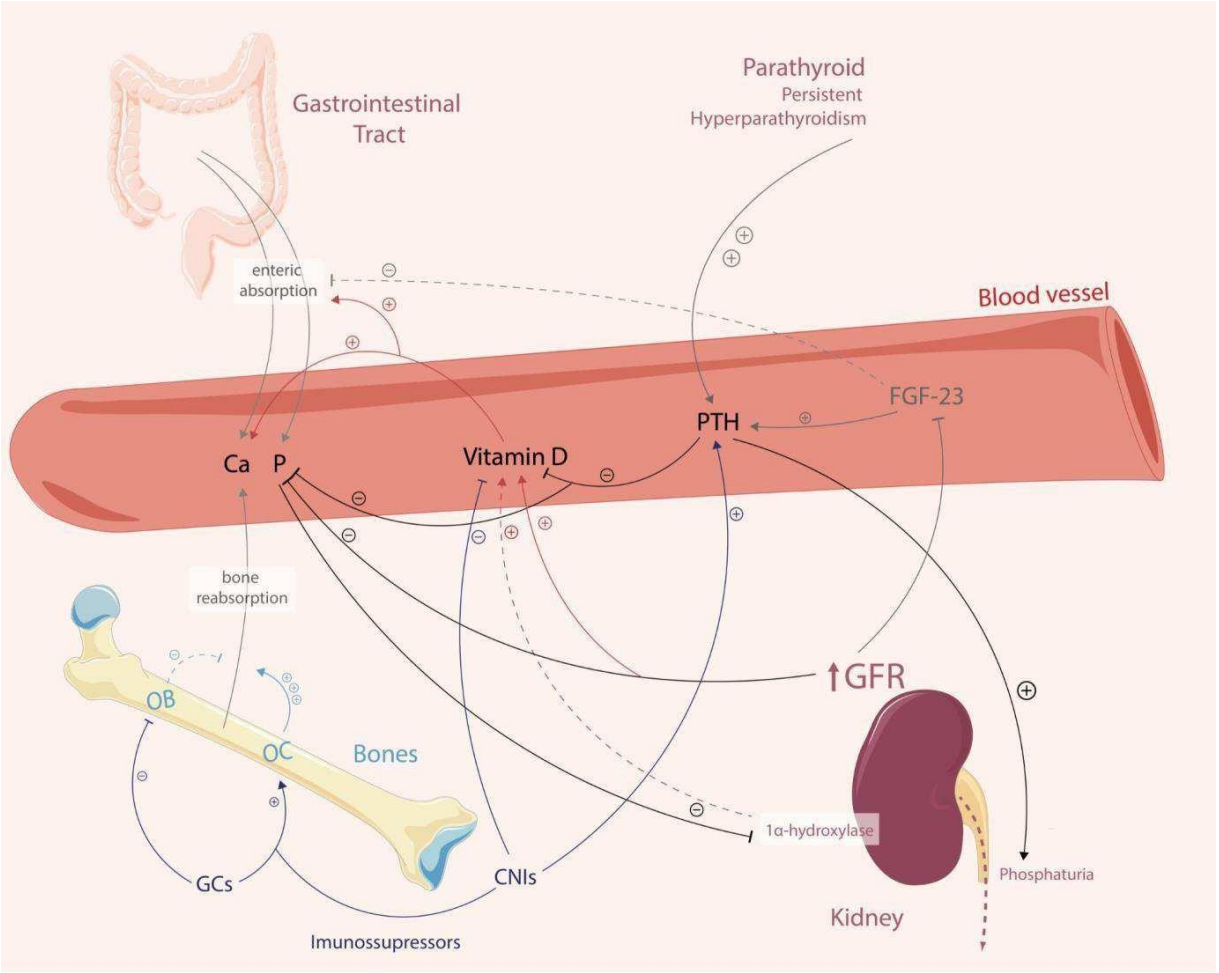

Figure 3. Altered Calcium (Ca) and Phosphate (P) homeostasis after kidney transplantation (KTx), leading to mineral bone disease (MBD). As GFR is restored after KTx, FGF-23 levels and serum levels of P start to decrease. Lower FGF-23 levels stimulate PTH production, synergically with a persistent HPTH and with calcineurin inhibitors (CNIs). CNIs also reduce vitamin D production, strengthened by high levels of PTH and antagonized by improved kidney function. Increased PTH levels and improved GFR result in lower P levels and increased phosphaturia. Regarding bone metabolism, both glucocorticoids (GCs) and CNIs act inhibiting osteoblast (OB) and stimulating osteoclast (OC), resulting in higher bone reabsorption.

4. BIOMARKERS

4.1 Fibroblast Growth Factor-23 (FGF-23) and Klotho

FGF23 is a phosphaturic hormone that alters NaPi-IIa and IIc tubular cotransporters, reducing phosphate reabsorption[12, 13, 69]. It also inhibits 1-α-hydroxylase activity and reduces serum levels of active vitamin D, leading to less calcium and phosphorus enteric absorption[15]. Besides, FGF-23 increases PTH levels and inhibits Klotho, its coreceptor, through feedback mechanisms. Additionally, FGF23 activates the Renin Angiotensin System (RAS), reduces angiotensin converting enzyme 2 (ACE2) and increases blood pressure. FGF-23 has been related to cardiovascular complications[70–73].

In CKD patients, FGF23 levels are elevated due to phosphorus retention, caused by the reduced GFR[19–22]. These high levels are found in up to 72.5% of CKD patients pre-transplant and contribute to reduce even further 1-α-hydroxylase activity, active vitamin D levels and calcium absorption, aggravating hypocalcemia[18, 55].

In kidney transplant patients with good graft function, as GFR starts to increase, FGF23 levels reduce. A significant reduction of FGF23 levels was found in KTx patients at 3, 6 and 12 months after the transplant. At 1 year post-transplant, FGF23 levels were similar to those detected in CKD patients with comparable GFR[33, 74]. As FGF23 levels decrease, 1-α-hydroxylase activity and active vitamin D levels increase, leading to higher calcium and phosphorus absorption. FGF23 levels post-transplant correlated negatively with GFR and vitamin D levels and positively with the post-transplant period[8]. Additionally, high FGF23 levels in KTx patients was associated with higher levels of serum phosphate and PTH[48]. The authors did not find any relation between FGF23 and MBD incidence in KTx patients.

High FGF23 levels are closely linked with cardiovascular alterations in patients with and without kidney disease. Increased concentrations of FGF23 were associated with left ventricular hypertrophy, diastolic dysfunction, hypertension through RAS activation, ACE2 reduction and arrhythmias[70–73, 75–84]. Besides, myocardial ischemia and aterosclerose were related to high FGF23 transcription. Ferrum deficiency, inflammation, obesity and hyperaldosteronism were also associated with higher FGF23 serum levels[85–92]. These studies support the idea that FGF23 is closely related to the pathophysiology of cardiovascular complications and mortality in CKD patients.

α-Klotho, an obligate FGF23 coreceptor, is an antifibrotic, antioxidant and anti-aging protein, acting through the inhibition of the insulin/IGF-1 signaling pathway. Klotho also protects the renal tubular cells from oxidative damage. It is an important marker of the relation between the bone and the kidney in patients with renal diseases. Klotho also decreases renal fibrosis by the inhibition of TGF-β1 and the Wnt pathway-associated β-catenin activation[93, 94]. It is produced by the distal convoluted tubule in the kidney and the chief cells of the parathyroid gland[95, 96]. Additionally, Klotho increases phosphaturia. Klotho expressed in the parathyroid gland decreases PTH production and increases calcium-sensing and vitamin D receptors[95, 97]. After KTx, Klotho levels are increased when compared to baseline levels before transplant, but the concentrations still remain lower than reference values[25, 98–100]. *Blekestad et al.* found comparable Klotho levels between kidney recipients and GFR-matched controls[101]. *Tartaglione et al.* described a positive association between serum levels of sclerostin and Klotho in patients ≤1 year post-transplant[100]. Finally, Klotho was also found to be a prognostic marker for good allograft function within the first year post-transplant in KTx from deceased donors[102, 103]. Further research on Klotho as a novel biomarker that reflects the relationship between bone metabolism and renal alterations post-transplant is necessary to comprehend its interactions with FGF23, sclerostin, and vitamin D in CKD-MBD post-transplant patients.

4.2. Vitamin D

Physiologically, vitamin D is essential for controlling intestinal and renal handling of calcium and phosphate, PTH levels and bone turnover with a direct effect on osteoblast and osteoclast activity[5, 104]. Vitamin D is also an endocrine inhibitor of the RAS via the downregulation of renin expression. Vitamin D inhibits pro-inflammatory pathways, ultimately having a cardiovascular protection effect[105, 106].

Human vitamin D metabolism starts by the sun exposure and the conversion of provitamin D into cholecalciferol. Following, cholecalciferol is converted into 25D by hepatic enzyme 25-α-hydroxylase, which is, then, converted into 1,25D by renal enzyme 1-α-hydroxylase. 1,25D has endocrine and systemic effects through VDR and is degraded by 24-α-hydroxylase[9]. PTH increases vitamin D activation through upregulation of 1-α-hydroxylase, while FGF23 decreases 1-α-hydroxylase and enhances 24-α-hydroxylase, counteracting vitamin D effects[9].

In CKD-MBD patients, the production of 1,25D is reduced due to high FGF23 levels and the lack of functioning renal tissue[107]. Vitamin D has an important fallout in KTx patients, mostly regarding graft outcome and cardiovascular complications, which are related to FGF23-PTH-vitamin D axis, immunosuppressive therapy and previous bone status. After renal transplant, recovery of graft function, high PTH levels and hypophosphatemia accelerate 25D conversion into 1,25D[7]. Alternatively, high levels of FGF23 post-transplant can inhibit 1-α-hydroxylase and enhance 24-α-hydroxylase, reducing both 25D and 1,25D[108]. FGF23 and PTH act in a negative feedback loop through the Klotho-FGFR1 complex in the parathyroid gland and via calcineurin-dependent pathways.

Immunosuppressive therapy, GCs mostly, alters vitamin D metabolism, increasing PTH, FGF23 and vitamin D catabolism. GCs activate the genes involved in the expression of enzymes that catabolize vitamin D, further reducing its levels[109]. Steroids also impair osteoblasts, enhancing apoptosis and decreasing their genesis and functionality. On the other hand, GCs enhance osteoclast activity, through regulation of the RANK/OPG (receptor activator of nuclear factor-kappa B/osteoprotegerin) ratio[7, 110–112]. In addition, CNIs suppress VDR, increasing calcium wasting[113].

Vitamin D insufficiency or deficiency was observed in up to 43% of KTx recipients 3 months post-transplant and 48.1% at 6 months[33, 55, 114]. *Balcázar-Hernández et al.* found, in a retrospective study with 74 KTx patients, that at 1 year post-transplant, all patients had hypovitaminosis D (91% deficiency and 9% insufficiency)[115]. Low vitamin D post-transplant leads to hypocalcemia and abnormal bone mineralization and is also associated with poor graft outcomes, including acute cellular rejection, lower GFR and higher risk for interstitial fibrosis[17, 114, 116]. *Falkiewicz et al.* observed that the vitamin D levels, in post-KTx patients, had a gradual increase over the period of 2 years, even though never reaching physiological levels measured in the control group[117]. The same study also suggests that vitamin D deficiency may predict delayed graft function and possible loss of the graft[117]. Extrarenal production of calcitriol controls, directly or indirectly, over 200 genes, being involved in the regulation of cellular proliferation and differentiation, angiogenesis, insulin and renin secretion. Vitamin D deficiency is also related to various diseases, such as breast, prostate and colon cancer, as well as diabetes mellitus[118, 119].

Active vitamin D supplementation reduced PTH levels and improved MBD after KTx[120–122]. Although very few randomized controlled trials (RCTs) were conducted investigating the effect of vitamin D supplementation, the Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend supplementation to correct vitamin D levels for the same values as in the general population, even if KTx patients may need a different dosage[123, 124]. Further investigation on vitamin D supplementation for KTx patients is necessary.

4.3 Parathyroid hormone (PTH)

Parathyroid hormone (PTH) is a 84-amino acid peptide, produced by the parathyroid gland that is secreted entirely or as the fragment (7-84)-PTH[125]. PTH levels are influenced mainly by the serum concentration of calcium, but also by serum phosphate levels and vitamin D[11]. PTH is responsible for increasing calcium reabsorption from the bones, for stimulating phosphate excretion and vitamin D production via the activation of 1-α-hydroxylase. PTH production and secretion is increased by hypocalcemia and by hyperphosphatemia, acting as phosphaturic hormone synergistically with FGF23[12, 13].

In CKD patients, hypocalcemia, hyperparathyroidism, hyperphosphatemia are frequently observed. *Balcázar-Hernández et al.* found that before KTx, all CKD patients evaluated had secondary HPTH (n = 74), 40% hypocalcemia and 86% hyperphosphatemia[115]. In a larger study (n = 861), *Evenepoel et al.* reported that 66.3% of patients had secondary HPTH at the time of KTx[49]. Augmented levels of PTH result in an increase in calcium reabsorption from the bone and enhanced osteoclastic activity, with an overall higher risk of osteoporosis and reduced bone mineralization[3, 28, 29]. HPTH is also associated with vascular calcification and high bone turnover disease[4, 23].

At the early post transplant period, there is a significant decrease in PTH levels due to improved kidney function, reduction in FGF23 and an adjustment in vitamin D, calcium and phosphate levels. *Pérez et al.* attested a progressive fall in PTH levels, more expressive during the initial 6-month period post-KTx, but continuously, tending to a plateau by the 6 and 12 month[126]. However, this decrease is not kept after one year post KTx. *Roodnat et al.* found that PTH values were similar to those before KTx[127]. Persistent secondary HPTH is observed in over 60% of patients 1 year post transplant, closely related to severe secondary HPTH or tertiary HPTH prior to KTx[16, 47–49]. HPTH also contributes to hypercalcemia post-transplant, which is frequently observed in KTx patients. Hypercalcemia associated with high PTH levels is accompanied by interstitial microcalcification and poorer long-term graft outcomes[4, 43, 50]. Increased PTH levels also contribute to increased bone reabsorption and decreased density, besides inducing phosphaturia and aggravating hypophosphatemia, which can impair bone formation and mineralization[3]. This impairment is also further influenced by a progressive graft dysfunction resulting in increased FGF23 levels and decreased vitamin D. All these changes increase fracture risk, vascular calcification and loss of graft function[9, 10, 51–54]. *Rao et al.* reported high levels of PTH (>65pg/mL) in 66% of 106 KTx patients[48]. Among those, roughly half were between 1 and 5 years post-transplant and males appeared to have significantly higher levels of PTH than women, which were also

associated with lower levels of serum phosphate[48]. *Araujo et al.* considered a higher cutoff point for PTH (PTH >100pg/mL, meaning >1.5 times the upper normal limit) and found that 62% of patients with HPTH. If the cutoff was set as >65pg/mL, the authors would report an even higher HTPH prevalence of 77%[128]. Similarly, in patients with good graft function and more than 1 year post transplant, *Blekestad et al.* detected HPTH in over 50% of patients and 7% had severe HPTH (increased levels >2.5 times the upper normal limits)[57].

Evenepoel et al. reported that elevated serum levels of PTH, Ca and phosphorus at the time of KTx are associated with higher incidence of HPTH. The authors also observed that hypercalcemia and hypophosphatemia post-transplant are higher in moderate to severe HPTH at the time of KTx[49]. In a cohort study of 11,776 patients, no association was found between pre-transplant serum PTH levels and post-transplant outcomes nor pretransplant iPTH levels and mortality[129]. In contrast, another study with fewer patients (n=407) reported that high levels of pretransplant serum PTH levels have an independent influence on the risk for graft failure death-censored[127]. However, neither in univariable nor in multivariable analysis, the influence of PTH concentrations on the death risk was confirmed[127].

In summary, KTx does not normalize PTH levels in most patients. The effects of PTH on bone metabolism continue despite improvements in other biomarkers, such as vitamin D and FGF-23[130]. Persistent HPTH causes hypercalcemia, is also linked to hypophosphatemia and closely associated with negative outcomes in KTx patients, including low bone density, fractures, vascular calcification, cardiovascular disease, nephrocalcinosis, allograft dysfunction and loss, and all-cause mortality[131–133]. The relation between the PTH mechanism and graft dysfunction is still not fully uncovered, but studies suggest that it can be due to vasoconstriction and tubulointerstitial calcification[134, 135]. For patients at the first year post KTx with an estimated GFR (eGRF) greater than 30mL/min/1.73m^2 and low BMD, the KDIGO guidelines suggest that treatment with vitamin D, calcitriol/alfacalcidol, and/or antiresorptive agents should be considered with low level of evidence (2D). However, as HPTH has a considerable impact on long-term graft function, and persists in a high number of patients, parathyroidectomy at early stages post KTx may be an option. As *Araujo et al.* described, 6 months post KTx values of PTH (>150pg/mL) are predictive of HPTH at 1 year post KTx with 92% of specificity. This finding should be considered to recommend early parathyroidectomy, in order to prevent persistent HPTH, low graft functioning, low bone density and other complications[128].

4.4 Phosphorus

Phosphorus homeostasis is thoroughly relevant in CKD-MBD context once its metabolism is mainly controlled by the kidney, bone and intestine. It is estimated that 85% of inorganic phosphorus (Pi) is contained in the skeleton, with approximately 15% in softs tissues and 1% in extracellular fluid[136]. Phosphorus and phosphate enteric absorption is stimulated by vitamin D, which may be impaired by FGF23 and PTH. Additionally, phosphorus reabsorption in the proximal renal tubule is inhibited by high

levels of PTH and FGF23, through the inhibition of NaPi cotransporters, leading to phosphaturia[137–139].

In CKD patients, lower GFR leads to a reduced ion excretion and higher serum levels of phosphate. In order to compensate for this, FGF23 levels increase and as GFR levels fall further, PTH levels also rise, resulting in a hyperphosphatemia.

In KTx patients, hypophosphatemia is commonly observed, found in up to 34% of patients and likely due to decreased phosphorus reabsorption in the proximal tubule, which is related to persistently elevated phosphaturic hormones PTH or FGF-23 levels[4, 33, 48, 55, 57]. Another contributing factor to hypophosphatemia is lower levels of vitamin D, which reduces intestinal absorption of phosphate and calcium and PTH downregulation[14]. Hypophosphatemia is associated with a decrease in osteoblast activity, leading to rickets and osteomalacia and is also related to anemia and mortality risk in KTx patients[58–61]. Finally, increased renal phosphorus wasting is also due to immunosuppressive drugs[140–142]. *Araujo et al.* reported that phosphorus levels were persistently lower in patients at 1 year post KTx with HPTH ($p<0.001$)[128]. Accordingly, *Evenepoel et al.* found that there was a prevalence of 39.7% of hypophosphatemia at the 1st year post KTx patients and, at the 4th year, the incidence decreased to 10.7%[49]. Furthermore, *Pérez et al.* observed the same tendency of hypophosphatemia. There was a significant decrease in phosphorus levels after the first month, followed by a slight increase until it reached a plateau below normal levels that persisted for months[126].

4.5 Calcium

Calcium balance and metabolism are closely related to phosphorus in the human body. Calcium is stored mainly in the bones and teeth through calcium-phosphate complex, which determines bone strength and constitutes its mineral content. Calcium metabolism occurs in the intestine, kidney and bone, and is mediated by different molecules, including PTH, FGF-23 and vitamin D. PTH stimulates renal calcium reabsorption, bone reabsorption through osteoclast activity, and enteric calcium absorption via the stimulation of 1-α-hydroxylase, which increases active vitamin D. Intestinal calcium absorption is mediated by vitamin D receptor (VDR) and the amount of calcium in the diet. On the other hand, FGF23 decreases calcium levels by inhibiting the enteric absorption and vitamin D activation. In cases of deficiency, calcium is regularly reabsorbed from the bone through osteoclastic activity. In contrast, calcium can be removed from circulation by osteoblasts and deposited in the bone, favoring bone mineralization. Low levels of calcium stimulate PTH action and the activation of vitamin D through negative feedback, while high levels of calcium suppress PTH and vitamin D activation.

In CKD patients, hypocalcemia is frequently observed due to reduced calcium absorption, heterogeneous bone disease, excessive vascular and soft tissue calcification[143]. Calcium levels are lower in those patients due to FGF-23 effects on vitamin D, which may be compensated through high calcium intake[143–145].

After KTx, serum levels of calcium are expected to decrease progressively. Hypercalcemia is frequently observed due to HPTH, which leads to higher bone and

renal calcium reabsorption and enteric absorption. *Evenepoel et al.* found that at the 1st year post KTx, 30.9% of patients had hypercalcemia, defined as total serum calcium >10.5 mg/dL, although this percentage reduces to 12.4% in patients at the 4th year post KTx[49]. In contrast, *Pérez et al.* found that more than half of the patients had a slight increase in Ca levels, remaining at physiological levels during the first year post-KTx[126]. Some studies also suggest that calcium follows a biphasic tendency, with a decrease in serum levels at the initial weeks, followed by an increase due to better allograft function and vitamin D production, associated with persistent secondary hyperparathyroidism[3]. These high levels of calcium, mostly associated with high levels of PHT, are a well known risk factor for the occurrence of instestial microcalcifications and further impairment of the renal allograft[3]. Finally, calcium levels pre-transplant seem to be a marker of graft failure in KTx patients[146].

4.6 Alkaline phosphatase

Alkaline phosphatase (AP) mediates bone turnover and is a serum marker of osteoblast activity. AP removes phosphate groups from several molecules and their levels must be regularly monitored as a biochemical parameter of CKD-MBD after KTx[23]. The high concentration of FGF-23, common in CKD patients, is associated with an elevation of AP, hypophosphatemia, hyperphosphaturia and reduction of 1-α-hydroxylase[12].

AP levels increase significantly at the 1st year post KTx, and are closely related to HPTH post transplant[48, 128]. *Reinhardt et al.* found significantly elevated levels of AP and of bone specific alkaline phosphatase (BAP) from 2 to 5 months post KTx[147]. After 12 months post KTx, both AP and BAP levels normalized[147]. AP levels pre-transplant were found to be related with graft failure in KTx patients[129, 136]. *Withold et al.* study suggests that AP and BAP levels are positively associated with PTH concentration[148]. However, BAP and AP levels measured in different studies show variable results and are not consistent with the aforementioned. Although AP is associated with bone remodeling, the serum level of this molecule is not sufficient to diagnose alterations of bone turnover in individual patients[149]. Further studies are necessary to determine the role of AP and BAP as biomarkers of CKD-MBD in KTx patients[150].

4.7 Bone molecules

Osteocalcin (OC), a marker of osteoblast activity and bone turnover, decreases significantly in the first year post-transplant[151]. *Sharma et al.* found that OC correlated, at 12 months post KTx, to both tibia and radius cortical thickness and area, which are suggestive of net bone formation, possibly due to a decline in bone reabsorption[151]. However, the post KTx treatment includes the use of GCs, which promote bone loss by reducing osteoblast proliferation and life span. These alterations are accompanied by reduced levels of OC, type 1 collagen and IGF-1[43]. *Reinhardt et al.* found that from 2 to 5 months post KTx, OC levels increased from 20.2±1.5 to 26.7±1.9 ng/ml (normal 4–12), and remained high 12 months post KTx[147]. In experimental studies, rats treated with the combination of cyclosporine and azathioprine had higher OC levels and

histomorphometric osteopenia when compared to animals receiving azathioprine alone[152]. Despite these findings, serum levels of OC have shown limited specificity and sensitivity in predicting MBD outcomes[3].

Osteoprotegerin (OPG) acts as a competitive receptor for RANK. By binding to RANKL, OPG decreases the activity of osteoclasts. Immunosuppressive medications also interfere with OPG effects by modifying the molecule itself and the receptor activator of nuclear factor-κB ligand, both important pieces of the bone remodeling process[2]. The use of GCs increases the levels of RANKL and decreases levels of OPG with consequent reduction of osteoblast differentiation and proliferation and higher osteoclastogenesis[2, 43].

4.8 Sclerostin

Sclerostin relation with CKD pre and post-transplant can be assessed either through serum levels of sclerostin or by its bone expression. Sclerostin is the gene product of SOST, located on chromosome 17q12-21[153]. Sclerostin is a bone anti-anabolic protein, which regulates bone formation by inhibiting the Wnt pathway via β-catenin stabilization. The Wnt pathway activation leads to osteoblast and osteoclast differentiation, ultimately promoting bone formation. Thereby, sclerostin produces β-catenin phosphorylation and degradation, finally decreasing transcription of genes responsible for bone formation. The final effect is osteoblast and osteocyte apoptosis and less osteoblastogenesis. In parallel, sclerostin induces RANKL synthesis, leading to osteoclastogenesis[100, 154]. Lastly, sclerostin also enhances FGF-23 through the inhibition of PHEX, a protein encoded by the Phosphate regulating genes with Homologies to Endopeptidases on the X chromosome, which stimulates FGF-23 degradation[154, 155].

At early stages of CKD, FGF-23, dentix matrix protein 1 (DMP-1) and sclerostin levels are elevated before the abnormalities in mineral iron, vitamin D or PTH levels[156, 157]. In CKD, sclerostin expression in bone increases early and, as CKD progresses, the levels remain high, even though PTH increases the downregulation of sclerostin[156, 158]. At this point, sclerostin levels correlate positively with serum phosphate and FGF23 and negatively with PTH, AP and bone turnover[159–164].

After KTx, GFR and 1,25D levels naturally increase due to improved kidney function. Simultaneously, serum phosphate, FGF-23 and PTH levels decrease. The studies showed different results regarding serum sclerostin levels post-transplant. *Araujo et al.* found a reduction in the serum levels of sclerostin to below pre-transplant levels at the first year post-transplant[165]. *Bonani et al.* also described reduced levels 15 days post-transplant, but they found a progressive rise in their levels 6 and 12 months post-transplant[153, 165]. Even though the serum levels of sclerostin were still altered, post-transplant patients had lower serum levels of sclerostin than pre-transplant CKD cases[165]. *Tartaglione et al.* found no alteration of sclerostin levels in patients with one year or less post-transplant[100]. Pre-transplant sclerostin levels, the interval from the KTx, 25D and serum Klotho were positive predictors of higher post-transplant sclerostin levels, while AP and 1,25D were negative predictors[100, 153]. Both *Tartaglione et al.* and *Bonani et al.* did not find correlation between serum sclerostin and estimated

GFR, nor between sclerostin and bone mineral density[100, 153]. In addition, *Tartaglione et al.* reported a negative correlation between sclerostin and AP, and positive correlation between sclerostin and FGF23, 25OH-vitamin D and Klotho[100].

Regarding the expression of sclerostin in bone tissue, two studies found elevated levels in KTx recipients, even though in one of the studies those levels were still lower than in CKD pre-transplant patients [157, 165]. *Araújo et al.* also found increased levels of phosphorylated β-catenin, supporting the role of augmented bone expression of sclerostin [165]. The authors raise the hypothesis that serum and bone expressed levels of sclerostin are unrelated.

Laster et al. pointed out some important factors to consider when interpreting serum sclerostin levels, which were the lack of a standardized assay between studies and the possibility that the molecule mostly reflects GFR rather than bone disease[166]. As sclerostin depends on renal excretion, it is natural that, with post-transplant improvement of the GFR, circulating levels of the molecule reduce and the contrary occurs in case of GFR decrease. Based on this assumption, serum levels of sclerostin simply reflect the changes in the molecule clearance without relation to bone disease. The measurement of urine sclerostin levels might be useful in evaluating the molecule excretion. In addition, sclerostin expression in the bone microenvironment may probably provide more accurate information on bone formation/degradation. Sclerostin expression is altered by a number of factors. One example is lower PTH levels, which regulate sclerostin through the activation Wnt pathway, resulting in higher osteocyte sclerostin expression. If PTH levels increase, the activation of the Wnt pathway leads to a downregulation of sclerostin gene expression.

It is still unclear how the use of immunosuppressors alter sclerostin levels. *Bonani et al.* found no correlation between the use of tacrolimus, cyclosporine and drugs that improve bone mineral metabolism and sclerostin levels[153, 165]. Studies *in vitro* and with mice models have shown different results on how immunosuppressants, mostly GCs, alter sclerostin levels[167]. The acute administration of GCs (96-hour period) decreased serum sclerostin, while long term administration (12 months) increased in a dose-dependent manner[168]. Further assessment on how GCs affect sclerostin expression is necessary.

Sclerostin has been recently linked to reduction in vascular calcification, cardiovascular disease and mortality due to its relation with mineral homeostasis and possibly to extra-skeletal calcification in non transplant CKD patients[161, 169, 170]. *Evenepoel et al.* found a negative relation between vascular calcification and serum levels of sclerostin in KTx patients[169]. Further research is necessary to better evaluate sclerostin relation to bone mineral homeostasis and its role as a biomarker of CKD-MBD. The comprehension of sclerostin role in CKD-MBD may support the therapeutic use of anti-sclerostin antibodies, which has been proven effective in postmenopausal women with low bone mass[166].

4.9 Other biomarkers

Inflammatory markers, including interleukin 6 (IL-6), C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α) and other molecules involved in the inflammatory process, have a role in CKD-MBD. TNF-α, CRP and IL-6 have been linked to episodes of acute graft rejection and are markers of tubular damage in the kidney[171–179].

IL-6 is a known marker of inflammation and has been related to different mechanisms in cardiovascular and systemic diseases[150]. IL-6 is related to higher CRP, oxidative stress, state of hypercoagulability, accelerated atherosclerosis and loss of renal function[180–184]. *Mota et al.* evaluated the relation between IL-6 and hypercoagulability through the measurement of thrombomodulin (TM) and von Willebrand factor (vWF), molecules involved in the coagulation cascade[185]. The authors found that levels of IL-6 correlated positively with both TM and vWF in KTx patients[185]. IL-6 is significantly elevated post KTx and is closely related to graft rejection and other ongoing systemic diseases in KTx patients[186–189]. *Cueto-Manzano et al.* detected that from 6 months post KTx both IL-6 and TNF-α increased progressively up to the end of follow-up at 18 months[186]. *Waiser et al.* reported that urine IL-6 levels were associated with infection, necrosis and antirejection treatments, recommending caution for using this molecule as a marker of rejection in KTx patients[172]. Other inflammatory molecules are being further investigated as possible markers for CKD-MBD and for systemic diseases in KTx patients.

Studies suggest that obesity, higher body mass index (BMI) and greater adipose tissue cause higher PTH levels through enhancing the degradation of vitamin D. In turn, adiposity may also act directly on bone tissue, through leptin secretion[147, 148, 190]. Leptin is a hormone secreted by the adipose tissue that exerts its effect on bone metabolism through beta-adrenergic receptors in bone cells, modulating osteoblast differentiation[149, 152]. Studies with dialysis patients detected an inverse relation between leptin levels and bone turnover[191, 192]. *Kovesdy et al.* measured biomarkers of bone turnover and bone metabolism in 978 KTx patients to analyse how leptin would interact with other markers and with bone and kidney biochemical alterations[193]. Higher serum leptin levels were more likely in patients with higher BMI and larger abdominal circumference. In addition, patients with increased leptin concentrations had lower estimated GFR, vitamin D, collagen type 1 cross-linked C-telopeptide (CTx), and OC levels. These patients also had higher PTH, CRP and IL-6 levels[193]. Importantly, CTx and OC, known markers of bone metabolism, had a negative association with leptin, independent of PTH or vitamin D. This finding suggests that leptin may exert a suppressive effect on bone turnover[193].

Other molecules have currently been assessed as potential markers of bone turnover activity, graft function and general metabolism effects in KTx patients. These potential markers include insulin, Dickkopf-1 (DKK-1) and adrenocorticotropic hormone (ACTH), TM, vWF, resistin, growth differentiation factor 15 (GDF15), hepcidin, neutrophil gelatinase-associated lipocalin (NGAL), TNF-α, CPR, interleukin 2 and its receptor[185, 186, 194–200]. However, data are very preliminary and further investigation is necessary to establish whether or not these molecules are markers of CKD-MBD (Table 1).

Table 1. Characteristics of the main studies that analysed the concentrations of biomarkers for CKD-MBD.

Author/Year	Sample size (n)	Type of study	Biomarkers assessed	Main Findings
Savaj, Ghods et al., (2012)[6]	113	Cross-sectional	**Vitamin D, PTH, Creatine**, Ca, P.	45% had vitamin D deficiency, 76,2% had hyperparathyroidism. A correlation between vitamin D deficiency and serum creatinine levels was described (p=0.001).
Rao et al. (2012)[48]	106	Cross-sectional	FGF23, **PTH**, **Phosphate**, **Ca**.	34% had hypophosphatemia, 3% had hypercalcemia in 3% and 66% had high levels of PTH.
Evenepoel et al. (2004)[49]	861	Cross-sectional	**PTH, Ca, P, AP,** Creatinine	63.3% had HPTH at the time of transplantation, and 17% 4 years post KTx. 30,9% had hypercalcemia at the 1st year and 12,4% at the 4th year post KTx, respectively. 39,7% had hypophosphatemia at the 1st year and 10.7% in the 4th year post KTx. An increase in AP levels was detected during the 1st year, followed by a progressive decline afterwards. Higher levels of PTH, Ca, P and longer dialysis at the time of KTx were associated with higher incidence of HPTH at 1 year post KTx.
Bleskestad et al. (2011)[57]	360	Cross-sectional	**iPTH**, eGFR, **P, Ca**.	52% had higher levels of iPTH in 52%, from which 7% was severe HPTH. Hypercalcemia in 13%, hypophosphatemia in 29% and hyperphosphatemia in 3%.
Tartaglione and Pasquali et al. (2017)[100]	80	Cross-sectional	**FGF23**, **Klotho**, Ca, P, PTH, AP, **25D**, 1,25D, FGF23, CTx.	Increased levels of FGF-23 and Klotho, and mildly reduced concentrations of 25D.
Balcázar-Hernández et al. (2020)[115]	74	Retrospective-cohort	**PTH, Ca, P, AP, Vitamin D**	Persistent HPTH in 60%, 50%, 47.2% and 40% at 1, 3, 6 and 12 months post KTx, respectively. Hypercalcemia in 13%, 9%, 5% and 4% at 1, 3, 6 and 12 months post KTx, respectively. Hypophosphatemia in 31%, 34%, 5% and 0 patients at 1, 3, 6 and 12 months post KTx, respectively. Hyperphosphatasemia in 5%, 7%, 1% and 0 patients at 1, 3, 6 and 12 months post KTx, respectively. Hypovitaminosis D in 100% of KTx patients.
Falkiewicz et al., (2009)[117]	90	Prospective-cohort	**Creatinine**, Ca, P, AP, albumin, PTH, **1,25D**.	83% had severe 1,25D deficiency immediately post KTx. 1,25D increased in 1st year and stabilized until 24 months post KTx. High incidence of 1,25D deficiency occurred in delayed graft function patients. Negative correlation was detected between 1,25D and creatinine immediately after KTx and during 1st year.
Pérez et al. (2020)[126]	1009	Retrospective-cohort	**PTH, Ca, P**	Significant reduction in Ca and P levels 1 year post KTx. Reduction in PTH levels, compared to pre transplant, but remaining above upper normal limit.
Araujo et al. (2018)[128]	911	Prospective-cohort	**PTH, Ca, AP, 25D, P**	62% had HPTH associated with higher levels of Ca, P, PTH and AP before transplant. Higher levels of Ca, AP and PTH levels, and lower of P, eGFR and 25D were found in patients with HPTH at 1 year post KTx, in comparison to those without HPTH (p<0.001).
Reinhardt et al. (1998)[147]	129	Prospective-cohort	**Creatinine, P, Ca, AP, BAP, OC, PTH**	From 2-5 months post KTx, AP and BAP levels increased, normalizing within 12 months. OC levels increased significantly and remained above normal throughout the 12 months post KTx. Calcium levels were increased 3 months post KTx and decreased afterwards. Two years post KTx, P increased, but remaining within the lower normal range. PTH levels were higher in patients with impaired graft function and associated with lower vitamin D levels.
Sharma et al. (2018)[151]	11	Prospective-cohort	**Creatinine**, Ca, **Phosphate**, **iPTH**, AP, 25D,	KTx patients have increased eGFR and 1,25VD, while decreased serum creatinine, phosphate, iPTH, OC and CTx.

			1,25D, **OC**, **CTx**, P1NP	
Bonani et al. (2014)[153]	42	Retrospective-cohort	**Sclerostin**, BMD, **PTH, P, Vitamin D, Ca**	Sclerostin levels were higher before KTx in 100% of patients, decreasing 15 days post KTx. Sclerostin levels raised progressively, but still remained lower than pre-KTx. Pre-KTx sclerostin and time after transplantation were found to be predictors for post KTx sclerostin elevation. Patients presented persistent HPTH, low vitamin D and hypophosphatemia. During the 1st year post KTx, PTH decreased and P, Ca and vitamin D increased.
Cueto-Manzano et al. (2005)[186]	37	Retrospective-cohort	**CRP, IL-6, TNF-α**	CRP post KTx levels decreased. TNF-α and IL-6 increased progressively up to the end of the 18-months follow-up.
Kovesdy et al. (2010)[193]	978	Cross-sectional	**Leptin, PTH, CTx, OC, Vitamin D**, AP, **CRP, IL-6, BMI**.	Higher serum leptin levels were associated with higher PTH, BMI, abdominal circumference, CRP, IL-6 and lower vitamin D. Higher leptin was associated with lower levels of bone turnover markers (OC and CTx),.
Tomei et al. (2014)[196]	19	Cross-sectional	**Sclerostin**, DKK-1, CTx, BAP, P	Sclerostin positively correlated to P and negatively with renal function.

Ca = Calcium; P = Phosphorus; iPTH = intact parathyroid hormone; AP = alkaline phosphatase; 25D = 25-hydroxyvitamin D; 1,25VD = serum 1,25-dihydroxyvitamin D; OC = osteocalcin; CTx = collagen type 1 cross-linked C-telopeptide; P1NP = procollagen type 1 N-terminal peptide; HTPH = hyperparathyroidism; KTx = kidney transplantation; BAP = bone specific alkaline phosphatase; BMD = bone mineral density; CRP = C-reactive protein; BMI = body mass index; DKK-1 = Dickkopf-1.

5. CONCLUSION

In summary, in KTx patients with good allograft function, FGF23 levels decrease significantly and remain within normal limits. PTH levels reduce rapidly in the first months post-transplant, but the hormone may remain high due to secondary or tertiary HPTH in up to 25% of KTx patients, despite the normalization of kidney function[49, 201]. Calcium and vitamin D levels tend to increase rapidly post-transplant. Although, as post-transplant period advances, hypercalcemia and persistent vitamin D insufficiency are frequently observed. Phosphorus tends to decrease and hypophosphatemia is also commonly reported. There is still no consensus regarding serum sclerostin levels. However, in general, low serum levels of sclerostin are detected, while bone-expressed sclerostin is found higher than expected.

Before transplantation, bone loss occurs preferentially in cortical bone and is highly associated with secondary HPTH and CKD metabolic alterations, including hypocalcemia, hyperphosphatemia and low levels of vitamin D. Alternatively, in post-transplant subjects, a decreased bone formation in the trabecular bone can occur likely due to glucocorticoids therapy[16]. This finding is also possibly associated with other post-transplant abnormalities, such as persistent HPTH, hypercalcemia, hypophosphatemia and vitamin D deficiency.

All the alterations in mineral homeostasis and bone remodeling that occur in KTx patients result in MBD. KTx patients should be continuously evaluated for MBD. Bone status and ongoing bone loss should also be monitored both patients with successful kidney transplantation and those with reduced functionality. The management of post-transplant MBD is complex and involves the diagnosis of biochemical abnormalities, through periodic monitoring of serum calcium, phosphate,

vitamin D, PTH and other biomarkers. Some preventive measures should be taken such as reducing GCs exposure, increasing calcium and vitamin D supplementation and the control of HPTH. Other important factors to consider are vascular calcification and increased risk of mortality due to cardiovascular disease. Reduced GFR is an independent risk factor for coronary disease. In turn, how mineral bone disorders alter cardiovascular functioning should be further investigated in order to reduce mortality in CKD-MBD post-transplant patients. Additional research on CKD-MBD pathophysiology, diagnosis and management is essential to guarantee long term graft function, better prognosis, good quality of life and reduced mortality for KTx patients.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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***Artigo original**: Evaluation of novel bone metabolism markers in kidney recipients*

Evaluation of novel bone metabolism markers in kidney transplant recipients

Running title: Bone metabolism markers in kidney transplant recipients

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ABSTRACT

Aim: Immunosuppressive therapies, *de novo* or persistent hyperparathyroidism, and other risk factors influence mineral and bone disorder (MBD) after kidney transplantation. However, little is known about their effect on bone metabolism biomarkers. Therefore, we aimed to understand how kidney transplant affects these markers in comparison to patients on hemodialysis and healthy individuals.

Methods: This is a cross-sectional study with three groups: kidney transplantation patients, patients on hemodialysis, and healthy controls. Plasma concentrations of Dickkopf-related protein 1 (DKK1), osteoprotegerin (OPG), osteocalcin (OC), osteopontin (OPN), sclerostin (SOST), and fibroblast growth factor 23 (FGF-23) were measured in these three groups. Associations between the measurements of these molecules with clinical, demographic, and laboratory data were evaluated.

Results: A total of 114 patients were included in the study. Transplant recipients had significantly lower levels of DKK1 ($p < 0.001$), OPG ($p < 0.001$), OC ($p < 0.001$), OPN ($p = 0.001$), OST ($p < 0.001$), and FGF-23 ($p < 0.001$) when compared to patients on hemodialysis. In comparison to healthy controls, transplant recipients also presented lower levels of DKK1 ($p = 0.019$), OPG ($p < 0.001$), OC ($p = 0.027$), SOST ($p < 0.001$) and FGF-23 ($p = 0.043$). Regarding demographic data, women presented lower plasma SOST levels when compared to men in the hemodialysis group ($p = 0.012$).

Conclusion: Our findings showed a reduction in bone metabolism markers, DKK1, OPG, OC, OPN, and SOST after kidney transplantation. Kidney transplantation modulates MBD markers, suggesting a significant improvement of MBD associated with end-stage kidney disease.

Keywords: kidney transplantation, hemodialysis, bone metabolism, renal osteodystrophy, chronic kidney disease.

INTRODUCTION

Defined as a systemic disorder of mineral and bone metabolism due to chronic kidney disease (CKD), mineral and bone disorder (MBD-CKD) comprises either one or a combination of alterations of calcium, phosphorus, parathyroid hormone (PTH), or vitamin D metabolism; abnormalities in bone turnover, mineralization, volume, linear growth, or strength; or vascular or other soft tissue calcification[1]. In kidney transplant recipients, a new type of MBD-CKD can occur due to the use of immunosuppressive drugs, factors related to aging, the persistence of hyperparathyroidism secondary to CKD, or the development of new hyperparathyroidism due to kidney graft dysfunction. Kidney transplant patients have a high risk of fractures, osteopenia, and osteoporosis due to reduced bone quality and mineral density, even years after the transplantation [2]. Hypophosphatemia, hypercalcemia, vitamin D deficiency, reduction of fibroblast growth factor 23 (FGF-23), and decrease or maintenance of PTH levels can occur in this population, especially in the first year after the transplant[2]. Moreover, the uremic and inflammatory environment of CKD contributes to the transformation of vascular smooth muscle cells into osteoblasts/chondrocytes-like cells, resulting in vascular calcification. The calcification is also secondary to an imbalance between calcification inhibitors and an increased supply of calcium and phosphorus[3].

Kidney transplantation improves the uremic environment, hyperphosphatemia, oxidative stress, and inflammation. Nevertheless, cardiovascular diseases persist as the first cause of mortality of renal transplant recipients in the United States[4], and the increased risk of fractures continues as a major cause of morbidity in this population[2]. There are few studies that measure the novel bone metabolism markers, and the ones published mostly compare kidney transplant recipients to dialysis patients[4]. These bone metabolism markers can act as vascular calcification inhibitors (osteoprotegerin (OPG), fetuin-A, matrix gla protein (MGP), and osteopontin (OPN)), calcification inducers (osteocalcin (OC), sclerostin (SOST), and Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1))[5]. Each marker has a different role in the pathogenesis of MBD.

The canonical Wnt signaling pathway, responsible for osteoblastogenesis (differentiation and proliferation of osteoblasts), is hindered by the DKK1 and SOST, which are inhibitors of bone formation[6]. SOST is a product of the SOST gene, synthesized by osteocytes, while DKK1 is expressed in several tissues during embryogenesis[7]. On the other hand, OC is a protein produced by osteoblasts, a marker of osteoblast activity and high bone turnover[2]. Similarly, OPG, a protein produced by osteoblasts, is part of the RANKL/RANK/OPG (receptor activator of NF-KB ligand/receptor activator of nuclear factor-kappa B/osteoprotegerin) system, acting as a competitive receptor for RANK, by binding with RANKL and decreasing osteoclasts' activity[8]. Osteoblasts also synthesize OPN, a glycoprotein with an uncertain role in MBD. OPN seems to interfere with osteoclast activation and osteoblast proliferation[9]. In patients with ectopic calcifications, such as in CKD, OPN was found on the surface of calcification deposits of atherosclerotic plaques and calcified aortic valves[9,10]. Hydroxyapatite crystals are responsible for the mineralization of smooth muscle cells in blood vessels[10]. OPN seems to inhibit the formation and growth of these crystals, avoiding vessel calcification[9,10]. Although not directly related to calcification, the FGF-23 is also crucial for the understanding of MBD, as it plays a major role in post transplant hypophosphatemia and promotes the suppression of 1α-hydroxylase activity in the kidney[11].

Bearing in mind the roles played by each marker, this study aimed to evaluate plasma concentrations of molecules related to bone metabolism in kidney transplant recipients and compare them with the same measurements in healthy individuals and patients undergoing hemodialysis. The molecules assessed were DKK1, SOST, FGF-23, OC, OPN, and OPG. We also verified associations between the measurements of these molecules with clinical and laboratory variables.

METHODS

Study design

This is a cross-sectional study that included kidney transplant recipients, patients on hemodialysis, and healthy subjects (controls) from the Hospital Evangélico of Belo

Horizonte, Minas Gerais, Brazil. All patients were recruited from August 2020 to July 2021.

Ethical aspects

The Ethics Committee of the hospital approved the study under the registration number CAAE-31405120.3.0000.8787. Informed consent was obtained from all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions.

Transplant recipients

Kidney transplant patients whose transplant was performed between 2010 and 2018 were included. The etiologies of the underlying disease that led to the transplant were classified as hypertensive nephrosclerosis, diabetes mellitus, chronic glomerulonephritis, or indeterminate. Transplant patients with glomerular filtration rate (GFR) lower than 30 ml/min/1.73m² and whose transplant time was less than one year were excluded.

Patients in hemodialysis

Patients with end-stage kidney disease (GFR < 15 ml/min/1.73 m²) on hemodialysis at the same institution were included in the study. Hemodialysis patients were matched to the transplant recipients according to gender, age, and CKD etiology.

Control Group

The control group included 31 healthy sex and age-matched subjects from the same institution. Health status was determined by subjects' medical history or self-report to rule out the presence of chronic or acute diseases.

Exclusion criteria for patients

Hospitalized patients, patients with fractures, osteoarticular traumas, or acute clinical/metabolic complications at the time of blood collection were excluded from the study.

Data collection and definitions

The following baseline demographic and clinical characteristics of the patients were collected: age, sex, comorbidities, risk factors for cardiovascular disease (dyslipidemia, diabetes, hypertension, obesity, and smoking), CKD etiology, presence of cardiovascular diseases (stroke, chronic coronary artery disease, peripheral vascular disease, and heart failure), history of osteoporosis, osteopenia and/or fractures, time since kidney transplant surgery and/or the start of hemodialysis, use of medications (immunosuppressive therapies, antihypertensive agents, statins and/or other therapies for dyslipidemia, antidiabetic medications, medications for MBD-CKD, and others), and whether the kidney was from a living donor or not.

Laboratory data included serum levels of electrolytes, creatinine, blood urea nitrogen (BUN), alkaline phosphatase (ALP), 25-hydroxy vitamin D (25(OH)D), and PTH. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula estimated the GFR of the patients. Hypercalcemia was defined as serum levels of calcium above 10.2 mg/dL. The upper limit for serum levels of PTH was considered 65 pg/ml, as previously described in Brazilian CKD patients[12].

Plasma samples

Blood samples were collected by peripheral venous puncture in individualized tubes with heparin. The collected samples were centrifuged at 5000 rpm for 10 minutes at 4ºC. The obtained plasma samples were transferred to 1.5 mL polyethylene microtubes and stored at -80ºC until the time of analysis.

Biomarkers measurement

The Luminex-based microbead assay (HBNMAG-51K, Millipore, Billerica, MA) measured plasma concentrations of DKK1, OPG, OC, OPN, SOST, and FGF-23, following the manufacturer's protocols.

Briefly, capture microspheres coated with specific monoclonal antibodies for each molecule were added to the wells, along with standards and plasma samples. After incubation and washing, a mixture of secondary biotinylated antibodies was added. Then, streptavidin conjugated to the fluorescent protein was incubated for a brief period. After washing, the supernatant was discarded and the precipitate containing the

microspheres was resuspended in a buffer solution. The standards and samples were acquired in the MAGPIX® microsphere analyzer (Luminex Corporation, Texas, USA). The results were analyzed using the Milliplex Analyst program (MilliporeSigma) and expressed as pg/ml.

All measurements were performed in a single assay to avoid interassay variations. Our intra-assay variation was below 5%. The minimum detectable concentrations for each molecule were: 2.2 pg/mL for DKK1; 1.8 pg/mL for OPG; 15.6 pg/mL for OC; 33 pg/mL for SOST, and 7.7 pg/mL for FGF-23.

Statistical analysis

The software SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. The results obtained were expressed as means and standard error of the mean (SEM), medians and percentages, when appropriate. Categorical variables were compared by the Qui-square test. The Kolmogorov-Smirnov test checked the Gaussian distribution. For variables without Gaussian distribution, Mann–Whitney test was used to compare two groups and the Kruskal-Wallis test for comparisons between more than two groups. For variables with normal distribution, comparisons between two groups were made by unpaired Student's t-test and for more than two groups by analysis of variance followed by Bartlett's post-test. Pearson or Spearman tests were adopted to evaluate correlations according to the variables' distribution. All statistical tests were two-tailed with a significance level of $p < 0.05$.

RESULTS

A total of 114 patients were included in the study, being 57 (50.0%) transplant recipients, 26 (22.8%) patients on hemodialysis, and 31 (27.2%) healthy controls. The main clinical and demographic features of the study population are summarized in Table 1. In the transplanted group, 33 (57.9%) patients were using mycophenolate sodium, 51 (89.5%) used tacrolimus and 25 (43.8%) used m-TOR inhibitors. All transplant recipients were using 5 mg of prednisone. The mean creatinine levels in the transplanted group were 1.29±0.05 mg/dl and mean estimate GFR was 66.8±20.5 ml/min/1.73m². In

52% of the transplant recipients, the time since transplant procedure was between 5 and 10 years, and the mean transplantation age was 6.71±3.07 years.

Regarding clinical data, patients on hemodialysis used more antihypertensive drugs when compared to transplant recipients, especially calcium channel blockers, diuretics, and β-blockers. On the other hand, transplant recipients were more diagnosed with dyslipidemia, although they did not differ statistically regarding the use of statins (Table 1). As expected, patients on hemodialysis presented higher serum levels of phosphate, PTH, ALP, and 25(OH)D levels when compared to transplant recipients (Table 2). In total, 17 (29.8%) transplant recipients had hypercalcemia, while 64.9% still had PTH levels above 65 pg/mL. There was no significant difference in hypercalcemia occurrence between patients with and without PTH above 65 pg/mL (32.4% and 21.0% respectively, $p = 0.372$).

The comparisons between the plasma levels of bone metabolism molecules of kidney transplant recipients and patients on hemodialysis showed that transplant recipients had significantly lower levels of DKK1 ($p < 0.001$, Figure 1A), OPG ($p < 0.001$, Figure 1B), OC ($p < 0.001$, Figure 1C), OPN ($p = 0.001$, Figure 1D), SOST ($p < 0.001$, Figure 1E), FGF-23 ($p < 0.001$, Figure 1F) when compared to patients on hemodialysis. In comparison to healthy controls, transplant recipients also exhibited lower levels of DKK1 ($p = 0.019$, Figure 1A), OPG ($p < 0.001$, Figure 1B), OC ($p = 0.027$, Figure 1C), SOST ($p < 0.001$, Figure 1E) and FGF-23 ($p = 0.043$, Figure 1F). No significant association was found between levels of the studied molecules and baseline demographic and clinical data. The same pattern for all molecules was observed regardless of gender.

Different correlations between the human bone metabolism components were detected in transplant recipients and hemodialysis patients. In the transplant group, DKK1 was positively correlated with SOST ($r = 0.484$, $p = 0.001$, Figure 2A), OPN ($r = 0.420$, $p = 0.005$, Figure 2B) and OPG ($r = 0.521$, $p < 0.001$, Figure 2C). In the same group, SOST levels were positively correlated with FGF-23 ($r = 0.424$, $p = 0.027$, Figure 2D) and OPG ($r = 0.703$, $p < 0.001$, Figure 2E), while OC only positively correlated with OPN ($r = 0.572$, $p < 0.001$). By contrast, patients on hemodialysis only showed positive correlations between OPN and DKK1 ($r = 0.420$, $p = 0.005$, Figure 3A)

and between SOST and OPG ($r = 0.757$, $p < 0.001$, Figure 3B). Transplant age and GFR did not correlate with any of the studied markers.

Concerning laboratory parameters and their correlations with human bone metabolism molecules, both transplant recipients and hemodialysis patients showed a negative correlation between 25(OH)D levels and FGF-23 (Table 3). On the other hand, ALP levels were positively correlated with different molecules in the two groups. In transplant patients, ALP levels positively correlated with OPN ($r = 0.572$, $p < 0.001$), while, in hemodialysis patients, the positive correlation was with OPG ($r = 0.548$, $p = 0.012$).

DISCUSSION

In this study, kidney transplant recipients showed an improvement in traditional laboratory markers of bone metabolism and significant lower levels of non-traditional molecules when compared to hemodialysis patients. After kidney transplantation, calcium levels were higher, while phosphorus, ALP, 25(OH)D, and PTH were lower when compared to hemodialysis patients, similar to what has been previously reported[2]. Hypercalcemia was found in 29.8% of transplant patients. It is noteworthy that all patients had over one year of transplantation. Similarly, Evenepoel et al. found that at the 1st year post kidney transplantation, 30.9% of patients had hypercalcemia, although this percentage decreased to 12.4% at the 4th year post-surgery[13].

All non-traditional markers of bone metabolism were significantly reduced in our group of transplant recipients when compared to hemodialysis patients and healthy subjects. On one hand, this may represent an improvement of MBD after transplantation in comparison to patients on hemodialysis. On the other hand, this may also indicate a general reduction of bone turnover when compared to healthy individuals. This latter difference could be attributed no only to immunosuppression[14], but also to an impairment of bone homeostasis after transplantation, as MBD after transplantation is usually characterized by low remodeling[15].

Plasma levels of FGF-23 were significantly lower in the transplant recipients when compared to patients on hemodialysis. In the early post-transplant period, FGF-23

levels decrease dramatically as a consequence of the resolution of hyperphosphatemia and the recovery of renal function[16]. Nevertheless, even after the successful transplantation, plasma levels of FGF-23 can remain higher for a certain period, leading to hyperphosphaturia[11]. Although this study has a cross-sectional design and, therefore, cannot address temporal relationships, it is noteworthy that the lower levels of FGF-23 found in the transplant recipients of this study may reflect the improvement of the 25(OH)D metabolism after transplantation.

Plasma levels of OC are elevated in patients on hemodialysis due to uremia and hyperparathyroidism, acting as a calcification inducer[17]. The levels are expected to decrease post-transplant, as a consequence of the action of glucocorticoids, improvement of kidney function, and bone metabolism[18]. Glucocorticoids, by inducing apoptosis, reduce osteoblast proliferation and lifespan, consequently leading to bone loss and reduction of OC[19]. As expected, we found significant lower levels of OC in transplant recipients when compared to hemodialysis patients.

Regarding SOST, Cejka et al. found high levels in dialysis patients, which were associated with low bone turnover and a reduced number of osteoblasts, contributing to osteoporosis[7]. Previous studies have shown increased SOST levels in dialysis patients and non-dialysis CKD when GFR decreases, suggesting an increased production by osteocytes[6]. Araujo et al. found a reduction in plasma levels of SOST to below pre-transplant levels at the first year post-transplan[20]. Bonani et al. also reported reduced SOST levels 15 days post-transplant, but the levels progressively rose in 6 and 12 months after transplantation[21]. The explanation for the reduction of SOST after kidney transplantation is still uncertain, but the use of glucocorticoids and the improvement of GFR with greater clearance of the molecule may contribute. PTH inhibits the expression of SOST and DKK1[7]. After transplantation, significantly lower levels of PTH and increased levels of SOST are expected. However, steroids, both endogenous and exogenous, seem to reduce plasma SOST production[22]. This reduction could also be explained by the increase in SOST clearance after recovery of renal function. Conversely, a recent study with CKD patients showed that the urinary excretion of SOST increases as the GFR decreases[23]. Therefore, the increase in SOST in CKD patients seems not to be due to a reduction in its clearance as there will be an increase in its tubular excretion[23]. Another recent study, however, showed no correspondence between the GFR and the plasma SOST levels in kidney transplant patients, making it

less likely that the reduction in SOST after kidney transplantation is secondary to its increased clearance[24]. Similarly, we found no association between SOST levels and GFR in trasnplant recipients. These conflicting results call upon the necessity of more studies to comprehend the role that SOST plays in the disease.

Patients in hemodialysis presented higher plasma levels of OPG when compared to kidney transplant recipients, as shown in previous studies[5]. Although OPG is an inhibitor of vascular calcification, its increased levels have been associated with increased coronary artery and aorta calcification and cardiovascular events in CKD and kidney transplant patients[25]. Higher plasma levels of OPG could reflect a low bone turnover, leading to more calcium availability in the circulation and, consequently, increasing calcification[18]. Bone vascular smooth muscle cells, which are calcified in patients with CKD, could also act as osteoblast-like cells, releasing OPG. The OPG binds to the RANKL and avoids the formation of osteoclast-like cells, causing the progression of vascular calcification[5]. OPG levels increase as kidney function decreases and can be altered by post-transplantation medications, including calcineurin inhibitors and glucocorticoids. These medications decreased OPG levels, increased osteoclastogenesis, osteoclastic activity, and diminished osteoblast differentiation and proliferation[26]. Accordingly, our post-transplant patients had significantly lowered OPG levels when compared to hemodialysis patients, reaching values similar to healthy controls.

Transplant recipients presented lower levels of DKK1 when compared to hemodialysis patients and healthy controls. It is known that DKK1 and SOST are inhibitors of osteoblastogenesis, while OPG inhibits osteoclastogenesis[27]. Thus, these molecules reduce bone turnover[6]. After kidney transplantation, the decrease in DKK1, SOST, and OPG levels could reflect improvement of bone mineral density. However, bone densitometry and/or bone biopsy are required to establish the relationship between these molecules and the bone structure.

Hemodialysis patients showed higher OPN levels, possibly related to the inflammatory environment and the presence of vascular calcification. On the other hand, OPN levels were reduced in healthy controls and transplant patients, suggesting an improvement of the inflammatory setting after kidney transplantation. As inhibitors of vascular calcification, OPN and OPG are usually higher in patients with vascular

calcification, possibly as a negative feedback to counteract this process[10]. Furthermore, it is important to note that higher levels of OPN and OPG have been previously associated with all-cause mortality in patients with CKD stage 5 in hemodialysis without diabetes[28]. In the present study, significant lower levels of OC, OPN, and OPG levels were found in transplant patients, which suggest an improvement in extraosseous calcifications.

Regarding correlations between traditional markers and molecules related to bone metabolism, we found that levels of 25(OH)D and FGF-23 levels negatively correlated in transplant recipients and hemodialysis patients. This can be explained by the inhibitory action of FGF-23 on 1α-hydroxylase, reducing the levels of active vitamin D[29]. Similarly, OPN levels and ALP were positively correlated in the transplant group. As expected, levels of both molecules were lower in kidney transplant patients, possibly reflecting low bone turnover. There was a positive correlation between OPG and ALP in hemodialysis patients, similar to what was reported previously by Morena et al. in CKD patients before dialysis[25]. In patients with CKD, PTH stimulates the release of OPG by osteoblasts [25]. OPG prevents the binding of RANKL to RANK in osteoclasts, inhibiting osteoclastogenesis and thereby reducing bone reabsorption[30].

The present study has limitations, including the cross-sectional design, the performance in a single center, and the lack of investigation on mechanisms. Due to the cross-sectional design, it is not possible to establish the cause for the changes found in bone metabolism markers in kidney transplant recipients, hemodialysis patients, and healthy controls. This study was carried out in patients more than one year after kidney transplantation, in whom the dose of prednisone was only 5 mg/day. Therefore, it was not possible to investigate the influence of high doses of corticosteroids on the levels of bone markers. Serial measurements in the same patient before and after kidney transplant would allow understanding dynamic changes of these molecules. Nevertheless, the fact that the differences in the studied biomarkers persisted regardless of the gender and transplant time, two possible confounding factors, reinforces the hypothesis that there is an improvement of MBD after kidney transplantation.

CONCLUSION

In conclusion, bone metabolism biomarkers levels in kidney transplant recipients were lower, possibly due to the amelioration in inflammation and uremia obtained after recovery in GFR post-transplantation. Diagnosis and management of MBD in kidney transplant recipients are still difficult due to limited diagnostic tools and few studies with this population. Longitudinal studies with additional methods to evaluate bone structure are needed to understand the role of these molecules on bone metabolism after kidney transplant.

Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

Author's Contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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TABLES

Table 1. Baseline characteristics of the kidney transplant recipients, patients on hemodialysis, and healthy subjects (controls).

Parameters	Transplant recipients (n = 57) (%)	Patients on dialysis (n = 26) (%)	Controls (n = 31) (%)	P-value
Age (years)				0.486
20 to 30	7 (12.3)	2 (7.7)	4 (12.9)	
31 to 40	14 (24.6)	12 (46.1)	13 (41.9)	
41 to 50	20 (35.1)	5 (19.2)	8 (25.8)	
51 to 60	9 (15.8)	4 (15.4)	2 (6.4)	
61 to 70	6 (10.5)	1 (3.8)	3 (9.7)	
> 70	1 (1.7)	2 (7.7)	1 (3.2)	
Sex (male/female)	31 / 26	15 / 11	15 / 16	0.768
Obesity				0.059
Grade 1	14 (24.6)	3 (11.5)	1 (3.2)	
Grade 2	6 (10.5)	2 (7.7)	1 (3.2)	
Smoker	4 (7.0)	4 (15.4)	5 (16.1)	0.276
Underlying kidney disease				0.298
Glomerulopathy	23 (40.3)	9 (34.6)	-	
Hypertensive nephrosclerosis	5 (8.8)	2 (7.7)	-	
Diabetic nephropathy	6 (10.5)	7 (26.9)	-	
Undetermined	23 (40.3)	8 (30.8)	-	
Time since dialysis start				
Less than 5 years	-	18 (69.2)	-	
Between 5 and 10 years	-	6 (23.1)	-	
More than 10 years	-	2 (7.7)	-	
Time since transplant				
Less than 5 years	16 (28.1)	-	-	
Between 5 and 10 years	30 (52.6)	-	-	
More than 10 years	11 (19.3)	-	-	

Diabetes mellitus	19 (33.3)	9 (34.6)	-	0.909
Insulin therapy	7 (12.3)	7 (26.9)	-	0.098
Hypertension	45 (78.9)	23 (88.5)	-	0.296
Use of antihypertensive drugs	48 (84.2)	24 (92.3)	-	0.313
ACEI/ARB	41 (71.9)	14 (53.8)	-	0.106
Calcium channel blockers	13 (22.8)	12 (46.1)	-	**0.032**
Diuretics	11 (19.3)	19 (73.1)	-	**<0.001**
β-blockers	14 (24.6)	12 (46.1)	-	**0.049**
Other	10 (17.5)	9 (34.6)	-	0.086
The total amount of antihypertensive drugs	1.56 ± 1.12	2.65 ± 1.13	-	**<0.001**
Dyslipidemia	32 (56.1)	8 (30.8)	-	**0.032**
Use of statin	25 (43.8)	6 (23.1)	-	0.069
Use of ciprofibrate	2 (3.5)	-	-	
In treatment for MBD	8 (14.0)	26 (100)	-	**<0.001**
Use of alendronate	1 (1.8)	26 (100)	-	**<0.001**
Use of cinacalcet	3 (5.3)	16 (61.5)	-	**<0.001**
Use of cholecalciferol	4 (7.0)	6 (23.1)	-	**0.037**
Use of phosphorus chelating agents	-	22 (84.6)	-	
Use of calcitriol/paracalcitriol	-	6 (23.1)	-	

Values are expressed as mean and standard deviation. ACEI: angiotensin-converting enzyme inhibitors; ARB: angiotensin-receptor blockers.

MBD: bone mineral disorder.

Table 2. Baseline laboratory measurements of transplant recipients and patients on hemodialysis.

Laboratory parameters	**Transplant recipients**	**Patients on dialysis**	**P-value**
	n = 57	**n = 26**	
Serum Calcium (mg/dL)	11.52 (±1.54)	8.75 (±0.13)	0.080
Serum Phosphorus (mg/dL)	3.79 (±0.57)	5.62 (±0.32)	**0.007**
Serum Urea (mg/dL)	40.25 (±2.38)	-	-
	n = 56	**n = 26**	
Serum PTH (pg/mL)	105.69 (±10.97)	753.23 (±125.39)	**<0.001**
Serum ALP (IU/L)	87.69 (±5.00)	297.60 (±58.36)	**0.009**
Serum 25(OH)D (ng/mL)	24.89 (±1.55)	30.92 (±2.47)	**0.045**
Serum Creatinine (mg/dL)	1.29 (±0.05)	-	-

Values are expressed as mean and standard deviation. PTH: parathyroid hormone; ALP: alkaline phosphatase; 25(OH)D: 25-hydroxyvitamin D.

Table 3. Correlation between laboratory parameters and human bone metabolism biomarkers in kidney transplant recipients and patients on hemodialysis.

Laboratory parameters	**Transplant recipients (n = 56)**		**Patients on hemodialysis (n = 26)**	
	FGF-23 (pg/mL)	**OPN** (pg/mL)	**FGF-23** (pg/mL)	**OPG** (pg/mL)
25(OH)D (ng/mL)				
r	-0.531	-0.212	-0.430	-0.366
p	**0.019**	0.237	**0.040**	0.112
ALP (IU/L)				
r	0.213	0.572	0.375	0.548
p	0.868	**<0.001**	0.078	**0.012**

Pearson or Spearman tests were adopted to evaluate correlations according to the variables' distribution. ALP: alkaline phosphatase; OPG: osteoprotegerin; OPN: osteopontin; 25(OH)D: 25-hydroxyvitamin D.

FIGURES LEGENDS

Figure 1. Levels of human bone metabolism molecules in plasma samples of kidney transplant recipients, patients on hemodialysis, and healthy sex, and age-matched subjects (controls). Results are expressed as bar graphs with mean values and standard error of the mean (SEM). Differences were significant at $p < 0.05$ (Mann–Whitney U test).
DKK1: Dickkopf WNT Signaling Pathway Inhibitor 1; OPG: osteoprotegerin; OC: osteocalcin; OPN: osteopontin; SOST: sclerostin; FGF-23: fibroblast growth factor 23.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 2. Correlations between human bone metabolism components in plasma samples of kidney transplant recipients. Statistical significance was considered when $p < 0.05$.
DKK1: Dickkopf WNT Signaling Pathway Inhibitor 1; OPG: osteoprotegerin; OPN: osteopontin; SOST: sclerostin; FGF-23: fibroblast growth factor 23.

Figure 3. Correlations between human bone metabolism components in plasma samples of patients on hemodialysis. Statistical significance was considered when $p < 0.05$.
DKK1: Dickkopf WNT Signaling Pathway Inhibitor 1; OPG: osteoprotegerin; OPN: osteopontin; SOST: sclerostin; FGF-23: fibroblast growth factor 23.

CONCLUSÃO FINAL

Apesar de não ser possível a completa resolução do DMO secundária a DRC, o transplante renal promove efeitos benéficos. No entanto, fatores como o uso dos imunossupressores e as condições prévias ao transplante renal podem resultar no ressurgimento ou piora do DMO. Conforme mostrado neste estudo, houve redução significativa do PTH, fósforo e fosfatase alcalina nos pacientes transplantados renais quando comparados aos dialíticos. Em relação aos biomarcadores do metabolismo ósseo, todas as moléculas (OPG, OC, OPN, FGF-23, SOST, DKK1) apresentaram redução significativa em relação ao grupo de pacientes com DRC em hemodiálise. Além disso, algumas dessas moléculas (OPG, OC, SOST, FGF-23, DKK1) apresentaram níveis ainda menores do que em indivíduos saudáveis.

Nos pacientes com DRC, o nível elevado de FGF-23 parece estar relacionado a alterações cardiovasculares como hipertrofia do ventrículo esquerdo, disfunção diastólica e hipertensão arterial via ativação do SRAA (de Borst et al., 2011). Após o transplante renal, observa-se uma redução no FGF-23 o que poderia implicar em uma melhora nestas alterações cardiovasculares, além do seu efeito no metabolismo ósseo. Os marcadores ósseos, DKK1 e SOST, são responsáveis por inibir a osteoblastogênese, enquanto a OPG inibe a osteoclastogênese. Desta maneira, essas três moléculas reduzem o turnover ósseo (Cejka et al., 2012; Tobeiha et al., 2020). Após o transplante renal, a redução de seus níveis pode implicar em uma melhora na densidade mineral óssea, sendo necessário o estudo da densidade por meio de densitometria óssea e/ou biópsia óssea para estabelecer a relação entre estas moléculas e a estrutura óssea.

A osteocalcina, produzida pelos osteoblastos, é um marcador de alto turnover ósseo e indutor da calcificação (Komori, 2020). Osteopontina e OPG apesar de inibidores da calcificação vascular são encontradas em altos níveis nos pacientes com calcificação vascular, ou seja, parecem ser liberadas para se contraporem ao aumento da calcificação (Moe et al., 2005; Si et al., 2020). No presente estudo, foi encontrado uma redução nos níveis de OC, OPN e OPG, o que pode refletir uma melhora nas calcificações extraósseas. A realização da tomografia mutislice das artérias coronárias e aorta permite estimar a calcificação destes vasos, sendo um método não invasivo que poderia ser utilizado em conjunto com a dosagem destes marcadores ósseos para avaliar o risco de eventos cardiovasculares nos transplantados renais.

Este estudo foi realizado em pacientes com mais de um ano de transplante renal, nos quais a dose de prednisona é de apenas 5mg/dia. Sendo assim, não foi possível avaliar a influência de altas doses dos corticoides nos níveis sanguíneos dos marcadores ósseos dosados. Como o risco de fraturas, no primeiro ano, é maior nos pacientes transplantados renais em relação aos dialíticos, seria interessante realizar um estudo comparando os mesmos pacientes antes e após o transplante renal.

O diagnóstico e manejo do DMO nos pacientes transplantados renais ainda é objeto de amplo debate. Há na literatura poucos estudos que abordam o problema em transplantados renais. Novos métodos para diagnóstico e monitoração, como biomarcadores e exames de imagens, ainda são limitados, não sendo possível utilizá-los para predizer o risco de fraturas e eventos cardiovasculares. Com o intuito de melhorar a qualidade de vida do paciente transplantado renal, a sobrevida do enxerto e a redução da mortalidade faz-se necessário estudos mais robustos que investiguem o diagnóstico e o tratamento do DMO neste grupo.

REFERÊNCIAS BIBLIOGRÁFICAS DA CONCLUSÃO

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Komori, T. (2020). Functions of Osteocalcin in Bone, Pancreas, Testis, and Muscle. *International Journal of Molecular Sciences*, *21*(20), 7513. https://doi.org/10.3390/ijms21207513

Moe, S. M., Reslerova, M., Ketteler, M., O'Neill, K., Duan, D., Koczman, J., Westenfeld, R., Jahnen-Dechent, W., & Chen, N. X. (2005). Role of calcification inhibitors in the pathogenesis of vascular calcification in chronic kidney disease (CKD). *Kidney International*, *67*(6), 2295–2304. https://doi.org/10.1111/j.1523-1755.2005.00333.x

Si, J., Wang, C., Zhang, D., Wang, B., Hou, W., & Zhou, Y. (2020). Osteopontin in Bone Metabolism and Bone Diseases. *Medical Science Monitor*, *26*. https://doi.org/10.12659/MSM.919159

Tobeiha, M., Moghadasian, M. H., Amin, N., & Jafarnejad, S. (2020). RANKL/RANK/OPG Pathway: A Mechanism Involved in Exercise-Induced Bone Remodeling. *BioMed Research International*, *2020*, 1–11. https://doi.org/10.1155/2020/6910312

ANEXO A

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação de marcadores do metabolismo ósseo em pacientes transplantados renais

Pesquisador: FLAVIA MARIA BORGES VIGIL **Área Temática:**

Versão: 1

CAAE: 31405120.3.0000.8787
Instituição Proponente: ASSOCIACAO EVANGELICA BENEFICENTE DE MINAS GERAIS
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.015.859

Apresentação do Projeto:
Avaliação dos marcadores de metabolismo ósseo em pacientes transplantados renais.

Objetivo da Pesquisa:
Objetivo Geral:
Avaliar as concentrações sanguíneas e urinárias de moléculas relacionadas ao metabolismo ósseo em pacientes transplantados renais, comparando-as com tais medidas em indivíduos saudáveis e pacientes em tratamento dialítico.

Objetivo Especifico:
- Medir as concentrações sanguíneas de marcadores do metabolismo ósseo a saber: ACTH, DKK-1,insulina, leptina, FGF-23, interleucina (IL)-1beta, IL-6, osteocalcina, osteopontina, osteoprogerina (OGP), PTH, TNF alfa, 25 OH vitamina D e fosfatase alcalina. em pacientes adultos submetidos a transplante renal; - Comparar as mensurações dessas moléculas em pacientes transplantados renais com as mesmas dosagens realizadas em indivíduos saudáveis, pareados por idade e sexo (grupo controle negativo) e em pacientes portadores de DRC em tratamento dialítico também pareados por idade e sexo com o grupo de transplantados renais (grupo controle positivo);

- Verificar associações e/ou correlações entre as medidas das moléculas com variáveis clínicas e

laboratoriais incluindo tempo de diálise, tipo de imunossupressores utilizados, níveis séricos de cálcio, fósforo, 1,25-OH vitamina D, PTH e fosfatase alcalina óssea tanto nos pacientes transplantados renais quanto nos pacientes em tratamento dialítico.

Avaliação dos Riscos e Benefícios:

Riscos:
A tipificação do risco é classificada como moderada.
A coleta das amostras de sangue será realizada por meio de punção venosa periférica cujo riscos durante a coleta - Garroteamento Excessivo; Flebite;Transfixação do cateter; Extravasamento de líquido e hematoma. Perda da amostra devido erro na coleta ou problemas durante o processamento, ou transporte irregular ou identificação errada; Informações confidenciais reveladas quando se sabe que essas pessoas fazem parte do conjunto de dados anônimos;

Benefícios:
Esta pesquisa tem como objetivo beneficiar os pacientes transplantados renais em relação a prevenção das doenças cardiovasculares, como o infarto agudo do miocárido e o acidente vascular cerebral. A princípio a pesquisa não trará nenhum benefício aos pacientes doentes renais crônicos em hemodiálise e aos voluntários, exceto se algum deles for submetido a um transplante renal ao longo de sua vida. A pesquisa é relevante pois permitirá maior conhecimento de eventos que reduzem a qualidade de vida e aumentam a mortalidade dos pacientes transplantados renais.

Comentários e Considerações sobre a Pesquisa:

O pesquisador responsável fundamenta a pesquisa nas diretrizes da Comissão Nacional de Ética em Pesquisa e demais normativas das boas práticas clínicas, agências regulatórias e dos órgãos competentes das esferas municipais, estaduais e federais.

Documentos consultados:

Procedimentos Operacionais Padrão – POP,
Documento de Boas Praticas Clínicas: Documento das Américas,
Portaria 2201 de 14 de setembro de 2011,

Resolução nº 466, de 12 de dezembro de 2012,
Resolução nº 441, de 12 de ano

de 2011, Norma Operacional nº

001/2013.

Considerações sobre os Termos de apresentação obrigatória:

Folha de Rosto;
Brochura do Projeto de Pesquisa;
Termo de Consentimento de Uso de Dados - TCUD;
Termo de Responsabilidade;
Declaração Institucional;
Cronograma;
Orçamento;
Termo de Consentimento Livre e Esclarecido/TCLE;
Documento de Cessão;
Declaração Inter institucional do Laboratório e Biorrepositório: Interdisciplinar de Investigação Médica da
Faculdade de Medicina da UFMG;
Termo de guarda do material biológico em biorrepositório;
Termo de descarte de material biológico em biorrepositório;
Procedimento Operacional Padrão para transporte de amostras biológica;
Procedimento Operacional Padrão para coleta de amostra biológica;
Declaração Comprometimento do Laboratório de Análises Clínicas do Hospital

Evangélico de Belo Horizonte.

Recomendações:

O pesquisador responsável deverá emitir ao Comitê de Ética em Pesquisa o relatório semestral, conforme Norma Operacional 01/2013.

Qualquer procedimento referente a amostra biológica, novo projeto de pesquisa, biorrepositório e demais, deverão ser encaminhadas para o Comitê de Ética em

Pesquisa e ou Comissão Nacional de Ética em Pesquisa, para análise e parecer.

Consultar as diretrizes éticas.

Conclusões ou Pendências e Lista de Inadequações:

O projeto de pesquisa está adequado conforme as normativas da Comissão Nacional de Ética em Pesquisa - CONEP. Projeto de Pesquisa aprovado.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_P ROJETO_1344796.pdf	04/05/2020 19:45:53		Aceito
Folha de Rosto	FolhaRosto.pdf	04/05/2020 19:45:02	FLAVIA MARIA BORGES VIGIL	Aceito
Projeto Detalhado / Brochura Investigador	MestradoDMO.docx	04/05/2020 16:44:13	FLAVIA MARIA BORGES VIGIL	Aceito
Outros	PRSLACN05TRANSPORTE.pdf	04/05/2020 16:42:29	FLAVIA MARIA BORGES VIGIL	Aceito
Outros	POPTEC05COLETA.pdf	04/05/2020 16:41:30	FLAVIA MARIA BORGES VIGIL	Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoLaboratorioUFMG.docx	04/05/2020 16:40:18	FLAVIA MARIA BORGES VIGIL	Aceito

Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Terrmodeguardadematerial.pdf	04/05/2020 16:35:42	FLAVIA MARIA BORGES VIGIL	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	DescarteMaterialBiologico.pdf	04/05/2020 16:34:04	FLAVIA MARIA BORGES VIGIL	Aceito
Declaração de Pesquisadores	Termousodedados.pdf	04/05/2020 16:33:08	FLAVIA MARIA BORGES VIGIL	Aceito
Declaração de Pesquisadores	Termodepesquisador.pdf	04/05/2020 16:31:43	FLAVIA MARIA BORGES VIGIL	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	DocumentoCessao.docx	04/05/2020 16:30:12	FLAVIA MARIA BORGES VIGIL	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.docx	04/05/2020 16:29:22	FLAVIA MARIA BORGES VIGIL	Aceito
Orçamento	Orcamento.docx	04/05/2020 16:28:56	FLAVIA MARIA BORGES VIGIL	Aceito
Declaração de Instituição e	TERMOLABORATORIoHE.pdf	04/05/2020 16:27:19	FLAVIA MARIA BORGES VIGIL	Aceito

Infraestrutura	TERMOLABORATORIoHE.pdf	04/05/2020 16:27:19	FLAVIA MARIA BORGES VIGIL	Aceito
Declaração de Instituição e Infraestrutura	Declaracaoeuler.pdf	04/05/2020 16:25:54	FLAVIA MARIA BORGES VIGIL	Aceito

Cronograma	cronograma.docx	04/05/2020 16:21:55	FLAVIA MARIA BORGES VIGIL	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BELO HORIZONTE, 08 de Maio de 2020

Assinado por:
BIANCA REGINA ARANTES
(Coordenador(a))