BONE PATHOLOGY AND CARDIOVASCULAR DISEASE IN CHRONIC KIDNEY DISEASE: NEW BIOMARKERS AND MEDIATORS OF THE BONE-KIDNEY-VESSEL AXIS

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“I HAVE NO SPECIAL TALENTS.
I AM ONLY PASSIONATELY CURIOUS.”

Albert Einstein

PREFACE

In 2003 I began my Nephrology residency at the Department of Nephrology of Hospital Santa Cruz - Centro Hospitalar de Lisboa Ocidental. This is a department reputable for its clinical quality and its contribution to clinical investigation, allowing me to develop my skills in all areas of Nephrology and scientific activity.

Since the beginning of my residency, I was fortunate to have had the opportunity to work closely with Prof. Doutor Aníbal Ferreira, who trusted and inspired me to develop my research skills. The study of the mechanisms and biomarkers of renal osteodystrophy and the link between bone and cardiovascular disease in uremic patients soon brought us together. I was very privileged to find an exceptional mentor for my scientific studies and thesis who challenged and encouraged me daily and provided a supportive work environment.

Firstly, we measured for the first time in Portugal the levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in our prevalent hemodialysis patients and recognized that the great majority had deficient / insufficient levels. Lower levels of these vitamins were associated with cardiovascular risk factors, like pulse pressure, left ventricular hypertrophy and vascular calcifications, and with morbidity and mortality. So, it became inevitable to perform a prospective long-term study with native vitamin D (cholecalciferol) supplementation in our patients in order to evaluate its impact on cardiovascular risk markers. The supplementation was safe, well-tolerated and enabled an improvement in mineral metabolism, inflammation, and cardiac parameters.

A positive association between 25-hydroxyvitamin D levels and serum magnesium has raised our attention, particularly because patients whose levels of vitamin D did not increase with supplementation were all hypomagnesemic. Consequently, we studied magnesium levels in our hemodialysis patients which showed an inverse association with cardiovascular risk factors and mortality. Calcium acetate / magnesium carbonate, used as a phosphate binder, was associated in our population with an increase in magnesium levels, an improvement in left ventricular hypertrophy and stabilization in cardiac valve calcifications.

A study characterizing the incidence, main localizations, and risk factors of pathological bone fractures in Portuguese hemodialysis patients was lacking. In a large follow-up study, we demonstrated that lower and higher levels of parathormone (PTH) were associated with fragility fractures, which is in accordance with the PTH levels defined by the Kidney Disease: Improving Global Outcomes (KDIGO). Higher bone alkaline phosphatase values, particularly those evaluated just before the fracture, also emerged as a significant risk factor for fragility fractures.

As we explain deeply in the discussion and conclusions of this thesis, despite the limitations, we are very pleased that our studies have gained international attention and have been included in editorials, reviews, and several meta-analyses. However, we are even more pleased that our results have led to a change in clinical practice, especially in the implementation of native vitamin D supplementation for all prevalent dialysis patients in Portugal. I still have some hope that the same could happen with magnesium, often forgotten in hemodialysis patients, and that it would be possible to increase its concentration in dialysate to reduce bone and cardiovascular disease, in particular vascular calcifications.

Like all things in life, a doctoral dissertation takes time and maturation. My PhD experience was intellectually challenging. I was able to develop my research skills, mature my scientific reasoning and critical thinking, and improve my ability to develop and present scientific projects. Moreover, I collected important information relevant to clinical practice which made this experience extremely rewarding.

I aim to maintain an active role in the investigation of bone and cardiovascular disease in uremic patients to improve the medical care of my patients by connecting my activities as a physician and as a scientist.

ACKNOWLEDGMENTS

We thank NephroCare Portugal - Dialverca and Vila Franca de Xira units - where the studies were conducted. We thank the Portuguese Society of Nephrology for awarding three publications included in this thesis. The first of our studies also received a prize for one of the best abstracts submitted by young nephrologists to the European Renal Association - European Dialysis and Transplantation Association Congress.

We thank all the patients who consented to participate in the studies.

I would also like to thank my co-authors, for their collaboration in the several projects, and their simultaneous passion in clinical practice and research.

A special thanks to my supervisor Prof. Doutor Aníbal Ferreira for providing me with an example of mastership in excellence in all he does, how to combine medicine and research, and above all for his friendship and belief. I would like to thank Prof. Doutor Fernando Nolasco for continuous positive input and for believing in my work.

I also would like to thank Dra. Margarida Gonçalves, Dra. Cristina Jorge, Prof. Doutora Ana Carina Ferreira, Dr. Ivo Laranjinha, Dr. Gonçalo Ávila, and Dra. Susan Marum for all the help, ideas, and constructive criticism.

Finally, to my beloved family and my guardian angel, for their love, continuous support, trust, and most of all, for their precious and most appreciated patience throughout this work.

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SUMMARY

The studies included in this thesis contributed to clarify the role of some non-traditional cardiovascular risk factors, such as native vitamin D and serum magnesium, in hemodialysis patients. These biomarkers / mediators consolidate the existence of a strong interconnection between bone and cardiovascular disease in chronic kidney disease.
Magnesium also appears to have an important role, until now underestimated, in the synthesis and metabolism of vitamin D. In some clinical situations, magnesium supplementation can reverse the resistance to therapy with native vitamin D, ensuring adequate levels of vitamin D, with a particularly positive impact on cardiovascular mortality. It is therefore essential to ensure adequate levels of magnesium in order to optimize the benefits of vitamin D supplementation in chronic kidney disease patients.
We also proposed, for the first time, to evaluate the incidence of bone fractures in a large population of prevalent hemodialysis patients in Portugal and study their possible association with markers of bone disease and cardiovascular risk factors.

Main results of the studies included in this thesis:

- **Deficiency of native vitamin D (calcidiol) and chronic kidney disease**

  This study included 223 patients on hemodialysis who underwent two determinations, separated by 6 months of 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. We found deficient levels (< 15 ng/mL) of 25(OH)D in 33.6% of the patients and insufficient levels (15 - 30 ng/mL) in 45.8% of the patients. We observed a positive association between 25(OH)D and 1,25$(OH)_2$D levels and a negative association between 25(OH)D levels and age, diabetes mellitus, and markers of inflammation such as C-reactive protein.
  We also identified, for the first time, cardiovascular risk factors associated with insufficiency / deficiency in native vitamin D in hemodialysis patients. In the univariable analysis, serum 25(OH)D levels were negatively associated with brain natriuretic peptide (BNP) levels, with an elevated pulse pressure (≥ 65 mmHg) and with the presence of more vascular calcifications. In the multivariable analysis, low serum 25(OH)D levels were also independently associated with high BNP values, a pulse pressure ≥ 65 mmHg and a vascular calcification score ≥ 3. We demonstrated that baseline serum levels of 25(OH)D appear to be an excellent marker of global and cardiovascular morbidity and mortality

in hemodialysis patients. In a 48-month prospective evaluation, we found that baseline serum levels of 25(OH)D were significantly lower in patients who died of all causes and in those who died of cardiovascular causes. Baseline serum 25(OH)D levels were also lower in patients who required hospitalization during the study.

Considering these apparent protective effects of 25(OH)D on cardiovascular system dysfunction, we carried out a prospective study with a 5-year follow-up, on 97 hemodialysis patients, in which they were supplemented with oral cholecalciferol during hemodialysis sessions. Given the lack of specific recommendations regarding supplementation doses to be used in hemodialysis patients, we decided to transpose the proposals of the KDOQI guidelines for the pre-dialysis stage of chronic kidney disease to our population.

We demonstrated a significant increase in serum 25(OH)D levels after 6 months (23.1 ± 13.2 *vs.* 39.9 ± 11.1 ng/mL; $p < 0.001$) and after 60 months (23.1 ± 1 3.2 *vs.* 44.1 ± 11.9 ng/mL; $p < 0.001$) of supplementation compared to baseline values. In comparison to the values obtained after 6 and 60 months of cholecalciferol supplementation, there was a slight, but not significant, increase at the end of the study. In 94% of the patients, 25(OH)D levels became normal (> 30 ng/mL) at the end of the study, compared to only 18% at the beginning of the study.

Patients whose 25(OH)D levels did not increase were all diabetic and had hypomagnesemia (serum magnesium < 0.7 mmol/L). In clinical practice, we emphasize the importance of excluding magnesium deficiency in patients who, despite high doses, do not respond to native vitamin D supplementation.

Serum levels of calcium, phosphorus and intact parathormone (iPTH) were significantly reduced with supplementation. On the contrary, there was an increase in serum magnesium. We also demonstrated a reduction in the number of patients treated with active vitamin D, a decrease in doses of erythropoiesis-stimulating agent, a reduction in C-reactive protein, and an increase in albuminemia. Finally, we found that supplementation with cholecalciferol was associated with a significant reduction in plasma BNP levels, pulse pressure, and left ventricular mass index.

The supplementation was well tolerated, with no documented adverse effects (namely hypercalcemia greater than 10.5 mg/dL), and none of the patients achieved serum 25(OH)D levels considered toxic (> 100 ng/mL). These results demonstrated that long-term cholecalciferol supplementation in hemodialysis patients was safe, allowed correction of 25(OH)D deficiency, improved mineral metabolism control, attenuated the inflammatory state, and improved cardiac dysfunction.

- **Serum magnesium and chronic kidney disease**

In a 48-month prospective study, performed on 206 prevalent hemodialysis patients, we observed a mean pre-dialysis serum magnesium concentration of 1.36 ± 0.18 mmol/L and that none of the patients presented hypomagnesemia (magnesium < 0.7 mmol/L). In univariable analysis, pre-dialysis serum magnesium levels were negatively associated with age, diabetes mellitus, pulse pressure, left ventricular mass index and vascular calcifications. There was a positive association between serum magnesium and albumin. In multivariable analysis, lower magnesium concentrations were predictors of increased pulse pressure (≥ 65 mmHg) and left ventricular mass index (≥ 140 g/m^2), and of a higher vascular calcification score (≥ 3). We also found that patients with serum magnesium < 1.15 mmol/L had lower survival at the end of the 48-month follow-up.

Based on these results, we studied the effects of a phosphate binder with magnesium and low calcium concentration (calcium acetate / magnesium carbonate), for the treatment of hyperphosphatemia, whose effects on the cardiovascular system remain to be determined. We evaluated, in a 48-month prospective study, the relationship between calcium acetate / magnesium carbonate therapy and markers of cardiovascular risk, already validated in this population of hemodialysis patients, such as pulse pressure, left ventricular mass index and cardiac valve calcifications.

Forty patients were on calcium acetate / magnesium carbonate therapy, 52 patients were on sevelamer and 114 were not taking phosphate binders. Patients taking calcium acetate / magnesium carbonate had higher serum magnesium levels compared to the other two groups. At the end of the study, we found that patients who were on calcium acetate / magnesium carbonate showed a reduction in pulse pressure, left ventricular mass index, and aortic and mitral valve calcifications compared to patients who were not taking phosphate binders. Compared to patients on sevelamer, patients on calcium acetate / magnesium carbonate had a reduction in pulse pressure, left ventricular mass index, and aortic valve calcifications. In multivariable analysis, therapy with calcium acetate / magnesium carbonate was inversely associated with the progression of left ventricular hypertrophy and aortic valve calcifications. In this 48-month prospective study, we observed for the first time that the use of calcium acetate / magnesium carbonate in the control of hyperphosphatemia in hemodialysis patients was associated with a reduction in pulse pressure and left ventricular mass index and stabilization of cardiac valvular calcifications.

- **Bone fractures: incidence and risk factors in chronic kidney disease**

We performed a retrospective analysis on 341 prevalent patients on hemodialysis since the beginning of the dialysis technique (median of 51 months). Fifty-seven episodes of fragility fractures were identified, with a median time on hemodialysis of 47 months, which corresponded to an incidence rate of 31 per 1,000 person-years.

In univariable analysis, compared to patients without fractures, those who presented fractures were more frequently female and older patients, with longer hemodialysis vintage, diabetics, had a lower body mass index and lower albumin values. Patients who had fractures were less frequently receiving active vitamin D therapy and had a higher vascular calcification score. Therapy with phosphate binders or calcimimetics was not associated with the development of fractures.

A significantly higher risk of fractures was also associated with higher bone alkaline phosphatase values and iPTH levels < 300 or > 800 pg/mL, compared to levels between 300 - 800 pg/mL. Analysis of long-term fracture-free survival demonstrated that patients with iPTH < 300 (HR: 2.88) or iPTH > 800 pg/mL (HR: 3.48) had a significantly higher risk of fragility fractures.

In multivariable analysis, age, female gender, longer time on dialysis, lower serum albumin levels and the presence of more vascular calcifications were independently associated with a higher risk of bone fractures. On the other hand, active vitamin D therapy was associated with a decreased risk.

In this study, with a long follow-up, we observed that the incidence of fragility bone fractures in prevalent hemodialysis patients was high, and their risk appears to increase with age, female gender, presence of inflammation and more vascular calcifications. Bone disease associated with uremia also appeared to be an important factor in the development of fractures, as patients with lower or higher iPTH levels and increased bone alkaline phosphatase had a higher risk of fracture. On the other hand, active vitamin D therapy appeared to have a protective effect.

RESUMO

Com os estudos incluídos nesta tese propusemo-nos contribuir para o esclarecimento do papel de fatores de risco cardiovascular não tradicionais, tais como a vitamina D nativa e o magnésio sérico, nos doentes hemodialisados. Estes biomarcadores / mediadores consolidam a existência duma forte interligação entre doença óssea e cardiovascular na doença renal crónica.

O magnésio parece também ter um importante papel, até agora subestimado, na síntese e metabolismo da vitamina D. Em algumas situações clínicas a suplementação com magnésio pode reverter a resistência à terapêutica com vitamina D nativa, assegurando níveis adequados desta, com impacto positivo particularmente na mortalidade cardiovascular. Torna-se, por isso, essencial assegurar níveis adequados de magnésio de modo a otimizar os benefícios da suplementação com vitamina D nos doentes renais crónicos.

Pretendemos também avaliar, pela primeira vez em Portugal, a incidência de fraturas ósseas de fragilidade em doentes hemodialisados, assim como os fatores que podem predispor à sua ocorrência e a sua relação com marcadores de risco cardiovascular.

Principais resultados dos estudos incluídos nesta tese:

- **Carência de vitamina D nativa (calcidiol) e doença renal crónica**

  Este estudo incluiu 223 doentes em hemodiálise submetidos a duas determinações, separadas por 6 meses, de 25-hidroxivitamina D [25(OH)D] e 1,25-dihidroxivitamina D [1,25$(OH)_2$D]. Encontrámos níveis de deficiência (< 15 ng/mL) de 25(OH)D em 33.6% dos doentes e níveis insuficientes (15 - 30 ng/mL) em 45.8% dos doentes. Observámos uma associação positiva entre os níveis de 25(OH)D e 1,25$(OH)_2$D e uma associação negativa entre os níveis de 25(OH)D e a idade, a diabetes *mellitus* e marcadores de inflamação como a proteína C-reativa.

  Identificámos ainda, pela primeira vez, fatores de risco cardiovascular associados a insuficiência / deficiência em vitamina D nativa em hemodialisados. Na análise univariável, os níveis séricos de 25(OH)D associaram-se negativamente com os níveis de *brain natriuretic peptide* (BNP), com uma pressão de pulso elevada (≥ 65 mmHg) e com a presença de mais calcificações vasculares. Na análise multivariável, os níveis séricos de 25(OH)D associaram-se também, de forma independente, a valores elevados de BNP, a

uma pressão de pulso ≥ 65 mmHg e a um índice de calcificação vascular ≥ 3.

Demonstrámos que os níveis séricos basais de 25(OH)D parecem ser um excelente marcador de morbilidade e de mortalidade global e cardiovascular nos hemodialisados. Numa avaliação prospetiva de 48 meses verificámos que os níveis séricos basais de 25(OH)D eram significativamente menores nos doentes que faleceram de qualquer causa e de causa cardiovascular. Os níveis séricos basais de 25(OH)D eram também menores nos doentes que necessitaram de hospitalização durante o estudo.

Tendo em conta os aparentes efeitos protetores da 25(OH)D na disfunção do sistema cardiovascular, efetuámos um estudo prospetivo com duração de 5 anos, em 97 doentes hemodialisados, no qual procedemos à suplementação com colecalciferol oral durante as sessões de hemodiálise. Face à ausência de recomendações específicas acerca das doses de suplementação a utilizar nos doentes em hemodiálise, resolvemos transpor para esta população as propostas das KDOQI *guidelines* para a fase pré-diálise da doença renal crónica.

Demonstrámos um aumento significativo dos níveis séricos de 25(OH)D após 6 meses (23.1 ± 13.2 *vs.* 39.9 ± 11.1 ng/mL; p < 0.001) e após 60 meses (23.1 ± 1 3.2 *vs.* 44.1 ± 11.9 ng/mL; p < 0.001) de suplementação comparativamente com os valores basais. Em comparação com os valores obtidos após 6 e 60 meses de suplementação com colecalciferol, verificou-se um aumento ligeiro, mas não significativo, no final do estudo. Em 94% dos doentes os níveis de, 25(OH)D tornaram-se normais (> 30 ng/mL) no final do estudo, comparando com apenas 18% no início do estudo.

Os doentes cujos níveis de 25(OH)D não aumentaram eram todos diabéticos e apresentavam hipomagnesémia (magnésio sérico < 0.7 mmol/L). Salientamos na prática clínica, a importância de excluir deficiência de magnésio em doentes que não respondem a suplementação com vitamina D nativa, apesar de doses elevadas.

Os níveis séricos de cálcio, fósforo e de paratormona intacta (iPTH) reduziram-se significativamente com a suplementação. Pelo contrário, verificou-se um aumento do magnésio sérico. Demonstrámos também uma redução do número de doentes tratados com vitamina D ativa, uma diminuição das doses de estimulador da eritropoiese e uma redução da proteína C-reativa, acompanhada de um aumento da albumina sérica. Finalmente, verificámos que a suplementação com colecalciferol, se acompanhou de uma significativa redução dos níveis plasmáticos de BNP, da pressão de pulso e do índice de massa ventricular esquerdo.

A suplementação foi bem tolerada, sem efeitos adversos documentados (nomeadamente

hipercalcemia superior a 10.5 mg/dL), não havendo também nenhum doente que tivesse atingido níveis séricos de 25(OH)D considerados tóxicos (> 100 ng/mL). Estes resultados parecem demonstrar que a suplementação a longo-prazo com colecalciferol em doentes hemodialisados é segura, permite uma correção da deficiência de 25(OH)D, melhora o controlo do metabolismo mineral, reduz o estado inflamatório e melhora a disfunção cardíaca.

- **Magnésio sérico e doença renal crónica**

Num estudo prospetivo com duração de 48 meses, efetuado em 206 doentes prevalentes em hemodiálise, observámos uma concentração sérica média de magnésio pré-diálise de 1.36 ± 0.18 mmol/L e que nenhum dos doentes apresentou hipomagnesemia (magnésio < 0.7 mmol/L). Na análise univariável, os níveis séricos de magnésio pré-diálise associaram-se negativamente com a idade, a diabetes *mellitus*, a pressão de pulso, o índice de massa ventricular esquerdo e as calcificações vasculares. Verificou-se uma associação positiva do magnésio sérico com a albumina.

Na análise multivariável, as concentrações mais baixas de magnésio foram preditoras de uma pressão de pulso mais elevada (≥ 65 mmHg), de um maior índice de massa ventricular esquerdo (≥ 140 g/m^2) e de um maior score de calcificação vascular (≥ 3). Verificámos também que os doentes com magnésio sérico < 1.15 mmol/L apresentaram uma sobrevida menor no final dos 48 meses.

Com base nestes resultados, fomos avaliar os efeitos de um captador de fósforo com magnésio e baixa concentração de cálcio (o acetato de cálcio / carbonato de magnésio) para tratamento da hiperfosfatémia, cujos efeitos no sistema cardiovascular permanecem ainda por determinar. Num estudo prospetivo com duração de 48 meses, avaliámos a relação entre a terapêutica com acetato de cálcio / carbonato de magnésio e marcadores de risco cardiovascular, já validados nesta população de hemodialisados, como a pressão de pulso, o índice de massa ventricular esquerdo e as calcificações valvulares cardíacas.

Quarenta doentes estiveram sob terapêutica com acetato de cálcio / carbonato de magnésio, 52 doentes sob sevelamer e 114 não tomavam captadores de fósforo. Os doentes a tomar acetato de cálcio / carbonato de magnésio apresentaram níveis mais elevados de magnésio sérico, em comparação com os outros dois grupos.

No final do estudo, verificámos que os doentes que estavam sob acetato de cálcio / carbonato de magnésio apresentaram uma redução da pressão de pulso, do índice de

massa ventricular esquerdo e das calcificações valvulares aórticas e mitrais comparativamente com os doentes que não efetuavam captadores de fósforo. Em comparação com os doentes sob sevelamer, os doentes a efetuar acetato de cálcio / carbonato de magnésio apresentaram uma redução da pressão de pulso, do índice de massa ventricular esquerdo e das calcificações valvulares aórticas. Na análise multivariável, a terapêutica com acetato de cálcio / carbonato de magnésio associou-se de forma inversa com a progressão da hipertrofia ventricular esquerda e das calcificações valvulares aórticas.

Observámos pela primeira vez, neste estudo prospetivo de 48 meses, que o uso de acetato de cálcio / carbonato de magnésio no controlo da hiperfosfatémia em doentes hemodialisados se associou a uma redução da pressão de pulso, do índice de massa ventricular esquerdo e a uma estabilização das calcificações valvulares cardíacas.

- **Fraturas ósseas: incidência e fatores de risco na doença renal crónica**

Realizámos uma análise retrospetiva em 341 doentes prevalentes em hemodiálise desde o início da técnica dialítica (mediana de 51 meses). Foram identificados 57 episódios de fraturas de fragilidade, com uma mediana de tempo em hemodialise de 47 meses, o que correspondeu a uma taxa de incidência de 31 por 1,000 pessoas-ano.

Na análise univariável, comparativamente com os doentes sem fraturas, os doentes que apresentaram fraturas eram mais frequentemente do género feminino, mais idosos, com maior tempo em hemodiálise, diabéticos, com menor índice de massa corporal e com valores mais baixos de albumina. Nos doentes em que se identificaram fraturas o uso de terapêutica com vitamina D ativa foi menos frequente e estes apresentavam um score de calcificação vascular mais elevado. A terapêutica com quelantes do fósforo ou calcimimético não se associou com o desenvolvimento de fraturas.

Um risco significativamente maior de fraturas também se associou a valores mais elevados de fosfatase alcalina óssea e níveis de iPTH < 300 ou > 800 pg/mL, comparativamente com níveis entre 300 - 800 pg/mL. A análise de sobrevida a longo-prazo sem fraturas demonstrou que os doentes com iPTH < 300 (HR: 2.88) ou iPTH > 800 pg/mL (HR: 3.48) apresentavam um risco significativamente superior de fraturas de fragilidade.

Na análise multivariável, a idade, o género feminino, maior tempo em diálise, níveis mais baixos de albumina sérica e a presença de mais calcificações vasculares associaram-se de forma independente com um maior risco de fraturas ósseas. Por outro lado, a

terapêutica com vitamina D ativa associou-se a um risco diminuído.

Neste estudo, com um longo follow-up, observámos que a incidência de fraturas ósseas de fragilidade em doentes prevalentes em hemodiálise é elevada e o seu risco parece aumentar com a idade, género feminino, presença de inflamação e mais calcificações vasculares. A doença óssea associada à uremia também pareceu ser um fator importante no desenvolvimento de fraturas, uma vez que doentes com níveis de iPTH mais baixos ou mais elevados e com aumento da fosfatase alcalina óssea apresentaram maior risco de fratura. Por outro lado, a terapêutica com vitamina D ativa pareceu ter um efeito protetor.

GLOSSARY

25(OH)D: 25-hydroxyvitamin D
1,25(OH)$_2$D: 1,25-dihydroxyvitamin D
ACE: Angiotensin - converting enzyme
ALP: Alkaline phosphatase
ARBs: Angiotensin II receptor blockers
AUC: Area under the curve
bALP: Bone alkaline phosphatase
BMD: Bone mineral density
BMI: Body mass index
BMP-2: Bone morphogenetic protein 2
BNP: Brain natriuretic peptide
CaSR: Calcium-sensing receptor
CI: Confidence interval
CKD: Chronic kidney disease
CKD-MBD: Chronic kidney disease–mineral and bone disorder
CRP: C-reactive protein
CYP: Cytochrome
DBP: Diastolic blood pressure
DEXA: Dual-energy X-ray absorptiometry
DOPPS: Dialysis Outcomes and Practice Patterns Study
EDTA: Ethylenediaminetetraacetic acid
ESRD: End-stage renal disease
FCM: Faculdade de Ciências Médicas
FGF-23: Fibroblast growth factor 23
FMD: Flow-mediated dilation
GFR: Glomerular filtration ratio
HD: Hemodialysis
HR: Hazard ratio
IL: Interleukin
iPTH: Intact parathyroid hormone
IV: Intravenous

KDIGO: Kidney Disease: Improving Global Outcomes
KDOQI: Kidney Disease Outcomes Quality Initiative
LVMI: Left ventricular mass index
MAGT 1: Magnesium transporter 1
Mg: Magnesium
Na/K - ATPase: Sodium / potassium adenosine triphosphatase
NHANES: National Health and Nutrition Examination Survey
NMS: NOVA Medical School
NS: Non - significant
PP: Pulse pressure
PTH: Parathyroid hormone
RANKL: Receptor activator of NF-kB ligand
RCT: Randomized controlled trial
ROC: Receiver operating characteristic
RUNX 2: Runt - related transcription factor 2
SBP: Systolic blood pressure
SD: Standard deviation
TAL: Thick ascending limb
TNF - α: Tumor necrosis factor α
TRPM: Transient receptor potential melastatin
VDBP: Vitamin D - binding protein
VDR: Vitamin D receptor
VSMCs: Vascular smooth muscle cells

1. RELATED PUBLISHED ARTICLES

Published original articles based on the results obtained by our investigations:

- As first author:

1. **Matias PJ**, Ferreira C, Jorge C, Borges M, Aires I, Amaral T, Gil C, Cortez J, Ferreira A. 25 hydroxyvitamin D_3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. *Nephrol Dial Transplant* 2009;24:611-16 doi: 10.1093/ndt/gfn502

2. **Matias PJ**, Jorge C, Ferreira C, Borges M, Aires I, Amaral T, Gil C, Cortez J, Ferreira A. Cholecalciferol supplementation in hemodialysis patients: effects on mineral metabolism, inflammation, and cardiac dimension parameters. *Clin J Am Soc Nephrol* 2010;5:905-911 doi: 10.2215/CJN.06510909

3. **Matias P**, Ferreira A. 25-Hydroxyvitamin D and chronic kidney disease. *Port J Nephrol Hypert* 2011;25:253-61

4. **Matias PJ**, Laranjinha I, Ávila G, Azevedo A, Jorge C, Ferreira C, Aires I, Amaral T, Gil C, Ferreira A. Long-term cholecalciferol supplementation in hemodialysis patients: Effects on mineral metabolism, inflammation, and cardiac parameters. *Semin Dial* 2023;36:29-36 doi: 10.1111/sdi.13066

5. **Matias PJ**, Azevedo A, Laranjinha I, Navarro D, Mendes M, Ferreira C, Amaral T, Jorge C, Aires I, Gil C, Ferreira A. Lower serum magnesium is associated with cardiovascular risk factors and mortality in haemodialysis patients. *Blood Purif* 2014;38:244-52 doi: 10.1159/000366124

6. **Matias PJ**, Jorge C, Azevedo A, Laranjinha I, Navarro D, Mendes M, Amaral T, Ferreira C, Aires I, Gil C, Stuard S, Ferreira A. Calcium Acetate/ Magnesium Carbonate and Cardiovascular Risk Factors in Chronic Hemodialysis Patients. *Nephron* 2016;132:317-26 doi: 10.1159/000444421

7. **Matias PJ**, Laranjinha I, Azevedo A, Raimundo A, Navarro D, Jorge C, Aires I, Mendes M, Ferreira C, Amaral T Gil C, Ferreira A. Bone fracture risk factors in prevalent hemodialysis patients. *J Bone Miner Metab* 2020;38:205-12 doi: 10.1007/s00774-019-01041-9

8. **Matias P**, Ávila G, Ferreira AC, Laranjinha I, Ferreira A. Hypomagnesemia: a potential underlooked cause of persistent vitamin D deficiency in chronic kidney disease. *Clin Kidney J* 2023;16:1776-85 doi: 10.1093/ckj/sfad123

- As co-author:

9. Ferreira C, **Matias P**, Jorge C, Borges M, Aires I, Amaral T, Gil C, Cortez J, Ferreira A. Vitamin D, inflammation, and malnutrition in prevalent haemodialysis patients - is there a link? Port J Nephrol Hypert 2008;22(4):305-12.

Table 1 describes the type of article, the author's contribution, as well as the Quartile and Impact Factor of the Journal on the date of each publication. Table 2, the prizes and awards obtained by the author with several of the studies included in this doctoral thesis.

N°	Type of Article	1st Author	Co-Author	Quartile	Impact Factor	
					Journal	NMS PhD*
1	Original - Observational, cross-sectional	X		Q1	2.492	4.984
2	Original - Interventional, prospective	X		Q1	5.056	10.112
3	Review - X	X		_	_	_
4	Original - Interventional, prospective	X		Q2	2.886	5.772
5	Original - Observational, prospective	X		Q2	1.513	3.026
6	Original - Observational, prospective	X		Q1	2.085	4.170
7	Original - Observational, retrospective	X		Q2	2.344	4.688
8	Review - X	X		Q1	4.600	9.200
9	Original - Observational, cross-sectional		X	_	_	_
	Impact Factor of Original Investigations				16.376	32.752
	Total Impact Factor				20.976	41.952

* Impact Factor according to the regulations of PhD program in NMS I FCM

Table 1 - Contributions of the author in all the manuscripts included in the thesis, with the quantification of Quartile and Impact Factor according to the Journal and NMS I FCM regulations

Year	Name of the Prize or Award	Name of the Granting Entity
2008	Roche Prize (Best article not yet published in nephrology)	Portuguese Society of Nephrology
2008	Best abstract presented by young authors in the XLV ERA-EDTA Congress	European Dialysis and Transplant Association
2009	Best oral presentation in hemodialysis at the Portuguese Nephrology Congress	Portuguese Society of Nephrology
2010	Best oral presentation in hemodialysis at the Portuguese Nephrology Congress	Portuguese Society of Nephrology
2014	Roche Prize (Best article not yet published in nephrology)	Portuguese Society of Nephrology
2018	Best oral presentation in nephrology - rheumatology at the Portuguese Nephrology Congress	Portuguese Society of Nephrology
2020	DaVita Prize (Best article published in the previous year in hemodialysis)	Portuguese Society of Nephrology

Table 2 - Prizes and awards related to the studies included in the thesis

2. ETHICS AND INFORMED CONSENT

The institutions' local ethics committees (from NOVA Medical School and NephroCare Portugal) approved all the studies included in this thesis. The studies were also performed following the principles of the Helsinki Declaration.

Each study's characteristics were explained to the potential participants and written informed consent was obtained from all participants.

Patients included in the study had an ID number to ensure their anonymity. The principal investigator was the person with the key between the ID number and the name of the patient.

3. INTRODUCTION

CHRONIC KIDNEY DISEASE - MINERAL AND BONE DISORDER (CKD-MBD) SYNDROME IN CHRONIC KIDNEY DISEASE STAGE 5

Chronic kidney disease (CKD) is an increasing problem that affects millions of people worldwide, and cardiovascular (CV) disease is the most common cause of mortality in these patients [1]. CV disease is 10 to 20-fold greater than in the general population [2]. CV events are responsible for more than 50% of the mortality in CKD stage 5 patients, and a relative excess mortality is observed mainly in younger patients [3].

Progressive accumulation of uremic toxins, and disturbed mineral metabolism, including calcium, phosphorus, magnesium (Mg), parathyroid hormone (PTH), vitamin D, and fibroblast growth factor (FGF-23), among others, occurs during the course of CKD. As a consequence, bone disorders related to bone turnover, mineralization, and volume, are associated with the appearance of extra-skeletal calcifications [4]. This cross-talk between bone and vessels constitutes a systemic syndrome known as CKD-mineral and bone disorder (CKD-MBD) that is considered a major non-traditional CV risk factor that contributes to the high CV mortality observed in CKD patients [5]. CKD-MBD exists in almost all end-stage renal disease (ESRD) patients (Figure 1).

As a consequence of altered mineral and bone metabolism, CKD patients are at a higher risk of fragility fractures, which also contribute to the increased morbidity and mortality in this population. Bone fractures also occur at an earlier age than in the general population [6-8] and the risk of fracture-related mortality increases with the severity of CKD [9].

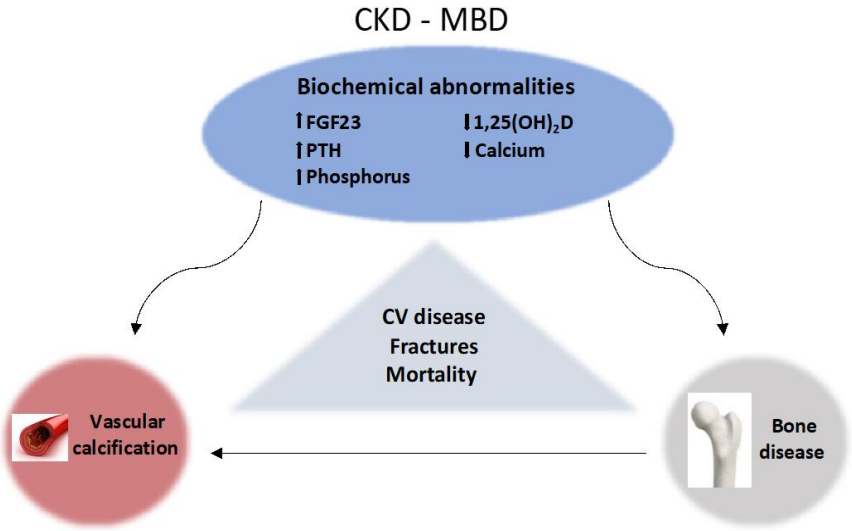


Figure 1 - CKD - mineral and bone disorder syndrome (adapted)[10]

Consistent results from both pre-clinical and clinical research showed that CV susceptibility in CKD is strongly associated with a considerable acceleration of vascular aging in the context of the CKD-MBD syndrome, in which new biomarkers are needed to improve the understanding of further intervention strategies. Vitamin D and Mg are some of the least studied key players of the CKD-MBD syndrome. Vitamin D and Mg supplementation in CKD is still a matter of debate. The close biological relationship between Mg and vitamin D is also frequently forgotten by physicians and the potential clinical impact of supplementing both, when necessary, while maintaining optimal levels must be explored.

3.1. VITAMIN D AND CHRONIC KIDNEY DISEASE

Vitamin D is a fat-soluble secosteroid hormone. It exerts rapid non-genomic cellular effects via membrane-bound receptors and, as the main pathway, genomic cellular effects via cytosolic receptors. The binding of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] induces a conformational change in the vitamin D receptor (VDR), leading to hetero-dimerization with the retinoid X receptor and ultimately to translocation of this complex into the nucleus, where it binds to vitamin D response elements in the promoter region of target genes [11].

This hormonal system is involved in the regulation of nearly 3% of the human genome. It was first known to play a central role in calcium and phosphate metabolism. However, more recently, vitamin D deficiency has been associated with numerous clinical events and conditions in the general population such as falls, fractures, diabetes, autoimmune disorders, CV and renal diseases, tuberculosis, depression, neurodegenerative diseases, and cancer [12-14].

The substrates for vitamin D, cholecalciferol (or vitamin D_3, synthesized mainly by our skin from 7 - dehydrocholesterol in response to UV light, but also obtained from some animal foods) and, ergocalciferol (or vitamin D_2, obtained by nutrition derived from plants) are transformed into calcidiol or 25-hydroxyvitamin D [25(OH)D] in the liver, by the action of 25-α-hydroxylase. In its turn, calcidiol is converted into the active form of vitamin D, calcitriol or 1,25$(OH)_2$D, by 1-α-hydroxylase present in proximal tubule cells and in extra-renal sites. For vitamin D to perform its actions, VDRs present in numerous cells and tissues of the body must be activated [15].

Patients with CKD frequently have low serum 25(OH)D [12-14]. There are several reasons for 25(OH)D deficiency or insufficiency in these patients: they are inactive and have decreased exposure to sunlight, have reduced ingestion of foods that are natural sources of vitamin D, and endogenous synthesis of vitamin D in the skin is also compromised in uremic patients [12, 13]. There is also an increased urinary loss of protein-bound vitamin D metabolites in proteinuric patients [14]. As CKD progresses, this tendency to vitamin D substrate insufficiency, coupled with the demonstrated loss of the renal 1α-hydroxylase activity, leads to progressive calcitriol deficiency [16, 17].

An additional factor that contributes to a reduction of the levels of 1,25$(OH)_2$D in the course of kidney disease is the progressive increase in the levels of FGF-23 [18, 19]. FGF-23 has been shown to directly suppress the activity and expression of 1α-hydroxylase [19] and thus can be an important factor that limits the ability of the failing kidney to maintain 1,25$(OH)_2$D production during the progression of kidney disease. FGF-23 also induces the expression of 24-hydroxylase [18], the enzyme responsible for the degradation of both 1,25$(OH)_2$D and 25(OH)D. Thus, increases in FGF-23 during CKD may be a major mechanism contributing to the decreased net production of 1,25$(OH)_2$D (Figure 2). Metabolic acidosis and calcitriol also decrease the activity of 1-α-hydroxylase. Unlike, PTH, calcitonin, growth hormone, estrogens, prolactin, hypocalcemia, and hypophosphatemia stimulate 1-α-hydroxylase. PTH stimulates

vitamin D activity, and simultaneously increases bone turnover, releasing calcium and phosphorus from the bone. For this reason, low levels of vitamin D are one of the main factors for the development of secondary hyperparathyroidism [20].

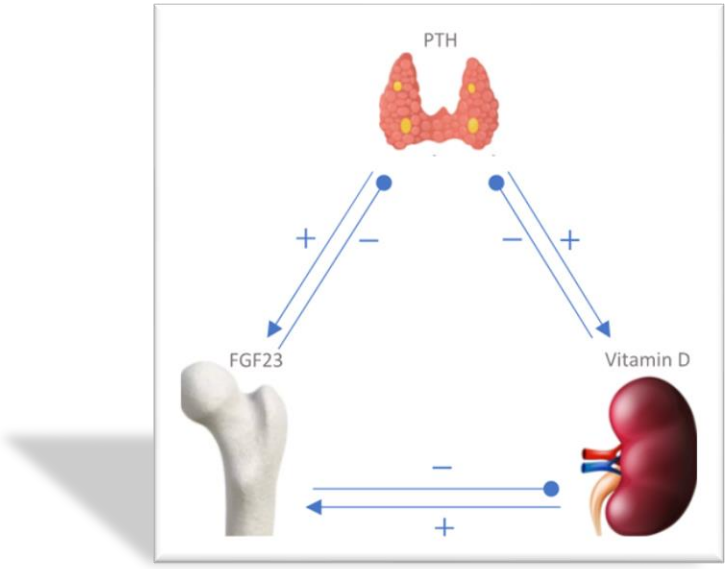


Figure 2 - Interactions between PTH, FGF-23 and active vitamin D (adapted) [18]

VDR levels are also reduced and 24-hydroxylase is increased in CKD patients [21]. In addition, vitamin D-binding protein (VDBP) seems to increase and is not involved in 25(OH)D deficiency in CKD patients [22]. Takemoto *et al*. reported that 25(OH)D tubular reabsorption is impaired due to decreased renal megalin [23]. As a compensatory mechanism, vitamin D catabolism, measured by 24, 25-dihydroxyvitamin D, is decreased in CKD patients and especially in dialysis patients [24]. Michaud *et al.* suggested that uremia decreases 25(OH)D synthesis secondary to PTH-mediated reduction in liver cytochrome (CYP) 450 isoforms [25]. Therefore, it could speculate on a vicious cycle involving vitamin D and secondary hyperparathyroidism that requires progressive amounts of vitamin D compounds to be reversed.

Table 3 summarizes the main factors associated with decreased levels of $1,25(OH)_2D$ in CKD.

Factors	Effects
Decreased synthesis of vitamin D in the skin	↓ Circulating 25(OH)D
Decreased circulating 25(OH)D	↓ Substrate for 1α-hydroxylase
Increased FGF-23	↓ 1α-hydroxylase and ↑24-hydroxylase
Decreased GFR / renal mass	↓ Delivery of 25(OH)D to 1α-hydroxylase
Decreased renal tissue	↓ $1,25(OH)_2D$ production
Decreased renal megalin	↓ Reabsorption of VDBP - bound to 25(OH)D into proximal tubules for 1α-hydroxylation
Accumulation of uremic toxins	↓ Production and action of $1,25(OH)_2D$

Table 3 - Main factors leading to decreased circulating $1,25(OH)_2D$ levels in CKD

Vitamin D is a hormone vital for mineral homeostasis, as it regulates renal and intestinal calcium and phosphorus handling; regulates bone turnover, inhibits PTH levels, and has a direct effect on osteoblast and osteoclast activity (Figure 3).

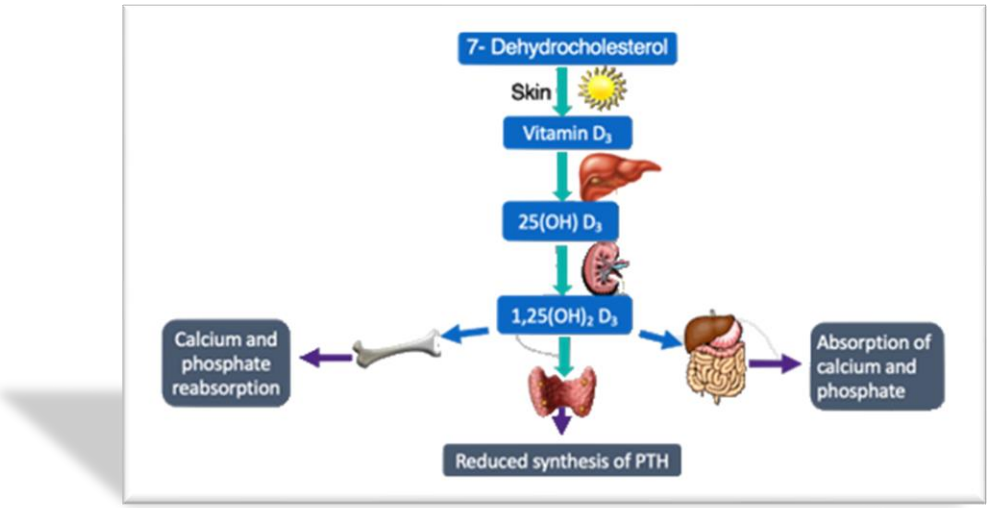


Figure 3 - Classical effects of active vitamin D (adapted) [26]

Levels of 25(OH)D are the best indicator of vitamin D status due to its longer half-life (3 weeks), as opposed to calcitriol's half-life, estimated at 4 to 6 hours, and whose serum levels are 1000 times inferior to calcidiol's [27]. The importance of measuring 25(OH)D levels is also supported by the emerging concept that an extra-renal 1α-hydroxylase is expressed in many sites outside the kidney and as peripheral production of $1,25(OH)_2D$ takes place, it seems that local concentration of 25(OH)D influences its production [28]. The extra-renal pool of 1α-hydroxylase (unlike its renal pool) seems to remain intact in kidney disease.

This locally produced $1,25(OH)_2D$ seems to have autocrine or paracrine effects, thus promoting additional roles for vitamin D, beyond its classical functions in mineral metabolism. Indeed, studies have demonstrated that $1,25(OH)_2D$ acts as a cell differentiating factor and anti-proliferative agent on a variety of tissues, plays a part in immune function (in innate and adaptive immunity), has endocrine effects (in inflammation, insulin-resistance, muscular function, and in the renin-angiotensin-aldosterone axis affecting blood pressure and CV disease), and may be involved in the pathogenesis of certain types of cancer [29-31]. Classical and non-classical actions of vitamin D are listed in Table 4.

Classical	
Intestine	Stimulates calcium absorption Stimulates phosphate absorption
Bone	Stimulates FGF-23 production Stimulates bone remodeling Improves bone mineralization in rickets / osteomalacia Increases bone release to normalize serum calcium levels
Parathyroid	Inhibits PTH synthesis and secretion
Non classical	
Muscles	Maintains integrity and improves muscle strength
Kidney	Reduces proteinuria Decreases magnesium absorption Stimulates calcium and phosphate reabsorption Inhibits renin-angiotensin-aldosterone system Increases nephrin expression Reduces megalin and cubulin shedding
Cardiovascular	Inhibits renin-angiotensin-aldosterone system Decreases the risk of hypertension Decreased the risk of cardiovascular diseases
Immune system	Stimulates innate immunity Inhibits acquired and modulatory immunity Decreases inflammatory markers such as C-reactive protein Decreases liver hepcidin expression
Cancer	May reduce the risk of certain cancers through its anti-proliferating and pro-differentiating properties
Pregnancy	Prevents against hypocalcemia and rickets May protect against preeclampsia, gestational diabetes, and premature birth
Diabetes	Improves insulin secretion and sensitivity May protect against diabetes

Table 4 - Classical and non-classical biological effects of vitamin D

▪ 3.1.1. NATIVE VITAMIN D SUPPLEMENTATION IN CHRONIC KIDNEY DISEASE

Vitamin D insufficiency [25(OH)D < 30 ng/mL] and deficiency [25(OH)D < 15 ng/mL] are common features in CKD patients [13]. Vitamin D insufficiency is present in 71-89% of the patients with CKD stages 3 to 5, increasing its prevalence throughout CKD stages [32]. A target of > 30 ng/mL is still suggested for optimal health [27, 33]. However, this remains controversial because of the lack of a consensus regarding the optimal range for serum 25(OH)D [34, 35]. Nevertheless, there is a common understanding that low serum 25(OH)D levels cause a negative calcium balance, secondary hyperparathyroidism, and bone disease.

In a meta-analysis of 10 prospective studies, Pilz *et al*. confirmed that the risk of death from any cause in patients with CKD increases by 14% for every 10 ng/mL reduction in 25(OH)D levels [36]. Therefore, the guidelines from Kidney Disease: Improving Global Outcomes (KDIGO) recommend evaluating calcidiol serum levels in CKD and dialysis patients and supplementing it with native vitamin D using strategies similar to the general population, if necessary [37]. Serum calcium and phosphate must also be monitored in CKD patients and supplementation dose should be adjusted when required. Active vitamin D or its analogs must only be used in patients with CKD stage 4 - 5 with severe and progressive secondary hyperparathyroidism and CKD-MBD after the correction of 25(OH)D deficient levels [37].

The Kidney Disease Outcomes Quality Initiative (KDOQI), proposed a more specific CKD-MBD guideline (Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease), published in 2003 [38]. This guideline includes recommendations for the management of vitamin D deficiency and secondary hyperparathyroidism, calcium, and phosphate metabolism. Also, the effects of vitamin D deficiency and supplementation on bone metabolism and disease were considered. These guidelines followed those for the general population and were predominantly based on evidence on the prevention and management of vitamin D deficiency and the progressive increase of PTH. Part of this guidance was revised in 2016 [39] focusing on vitamin D deficiency and secondary hyperparathyroidism in CKD stages 3 - 4. In this updated guidance, targets for iPTH thresholds by CKD stage, as defined in the 2003 guideline were removed, due to lack of evidence of benefit. In 2017, a position paper was also published by the KDOQI CKD-MBD working group, which was mostly in agreement with the updated KDIGO guidelines [40].

In conclusion, there are no specific guidelines for CKD patients. The US Endocrine Society,

the UK National Institute for Health and Care Excellence, and the UK Royal Osteoporosis Society offer guidelines for the prevention and treatment of vitamin D deficiency that can be used in the CKD population [35, 41, 42] (Table 5).

Dosage schemes for the correction of vitamin D deficiency		
US Endocrine Society	NICE	ROS
Sufficient: > 30 ng/mL Insufficient: 20 - 30 ng/mL Deficiency: < 20 ng/mL	Sufficient: > 20 ng/mL Insufficient: 10 - 20 ng/mL Deficiency: < 10 ng/mL	Sufficient: > 30 ng/mL Insufficient: 10 - 30 ng/mL Deficiency: < 20 ng/mL
No vitamin D preferred.	Vitamin D_3 is the preferred form of supplementation.	Vitamin D_3 is recommended for treating vitamin D deficiency.
Dietary intake for patients at risk: - 19 -70 years: 600 IU/day; - > 70 years: 800 IU/day. Treating vitamin D deficiency: - loading dose: 50,000 IU/week for 8 weeks or 6,000 IU/day; - maintenance therapy: 1,500 - 2,000 IU/day.	Vitamin D deficiency treatment: - loading dose: up to 300,000 IU, split dose either weekly or daily; - maintenance therapy: lifelong treatment with 800 IU/day.	Vitamin D deficiency treatment: - loading dose: up to 300,000 IU given either as weekly or daily split doses; - maintenance therapy: started one month after loading dose with 800 - 2,000 IU/day (maximum 4,000 IU/day) given either daily or intermittently.

No specific guidelines for CKD patients are included. Endocrine society and ROS are led by clinical experts and NICE is a government led committee. NICE, UK National Institute for Health and Care Excellence; ROS, UK Royal Osteoporosis Society.

Table 5 - Definitions of vitamin D insufficiency and deficiency and recommendations for its correction by some of the most important medical societies [35, 41, 42]

A meta-analysis assessing the effectiveness of ergocalciferol and cholecalciferol in the raising of serum 25(OH)D concentrations demonstrated that when given, as a bolus dose, cholecalciferol was 3-times more efficient than ergocalciferol, but the difference disappeared with daily administration [43].

While daily dosages seem more physiologic, spaced-out dosages are thought to favor adherence to supplementation. If intermittent dosages are chosen (most frequently in our practice), doses and intervals between doses should not be too large. Indeed, we currently have the demonstration that daily doses and the same cumulative doses administered weekly or monthly (i.e. 1,500 IU daily, 10,000 IU weekly, or 45,000 IU monthly) induce the same increase in the circulating 25(OH)D concentration [44, 45], while this is less obvious for

larger intervals. Although native vitamin D supplementation led to increased levels of 25(OH)D, a 3-year randomized control trial (RCT) of 500,000 IU vitamin D_3 *versus* a placebo administered once a year to elderly women, showed more fractures and falls in the vitamin D group than in the placebo group [45]. Interestingly, this excess in the number of falls and fractures was only observed during the 3 months following each yearly administration of this large dose of vitamin D. Meanwhile, meta-analyses have demonstrated that daily doses of 700 - 1,000 IU/day reduce the risk of falls [46] and fractures [47] in the elderly.

Nevertheless, it is generally accepted that a daily oral administration of 1,000 IU of cholecalciferol or ergocalciferol increases serum 25(OH)D levels by 5 - 10 ng/mL and is generally sufficient to keep 25(OH)D values above 20 ng/mL [48]. However, higher dosages are required in many patients if one targets a serum 25(OH)D level above 30 ng/mL and a significant reduction in PTH [35, 49]. Although this threshold is not fully established, toxicity studies showed no cases of hypercalcemia below 150 ng/mL of 25(OH)D [49].

Calcifediol is a product already hydroxylated at C25, serving as the direct substrate for 1-α-hydroxylase. Calcifediol has been suggested as an effective therapy to increase 25(OH)D levels in the general population, particularly in patients with chronic hepatic disease and obesity, and its application in CKD is relatively recent. There are two formulations of this compound: a simple one, which causes a rapid increase in 25(OH)D concentration and a rapid catabolism by stimulated expression of FGF-23, with a high risk of hypercalcemia; and an extended-release formulation which leads to a more progressive and effective increase in circulating 25(OH)D levels with better control of secondary hyperparathyroidism in CKD patients [50].

Sprague *et al.* published a multicenter study composed of a 26-week randomized, double-blind, placebo-controlled trial with a subsequent 26-week extension, enrolling 429 subjects with CKD stage 3 or 4, secondary hyperparathyroidism, and vitamin D insufficiency. Subjects were randomized 2:1 to receive oral extended-release calcifediol (30 or 60 μg) or placebo once daily. After treatment, more than 95% of patients receiving calcifediol achieved serum 25(OH)D concentration > 30 ng/mL. The extended-release calcifediol replacement also reduced plasma PTH by at least 10% in 72% of the patients, and reductions ≥ 30% increased progressively with treatment extension, achieving more than 50% at 52 weeks. The authors concluded that lowering PTH with calcifediol was independent of the CKD stage and not associated with adverse events, like hypercalcemia or

hyperphosphatemia, making extended-release calcifediol a safe and effective treatment [51].

▪ 3.1.2. VITAMIN D AND SECONDARY HYPERPARATHYROIDISM

The presence of the VDR and the calcium-sensing receptor (CaSR) in parathyroid chief cells enables the parathyroid gland to respond to vitamin D and calcium, two of the main regulators of the parathyroid function. Calcium and vitamin D have both independent and cooperative effects on parathyroid function inhibition. Besides modulating its own receptor, vitamin D also regulates the CaSR, which makes the parathyroid gland more sensitive to the suppressive action of calcium. Vitamin D upregulates VDR only if calcium is normal or high. Conversely, VDR is downregulated in hypocalcemia and upregulated by activation of the CaSR. Inhibition of parathyroid function by vitamin D is impaired in the presence of hypocalcemia [52].

In CKD, the prolonged stimulation of parathyroid glands promotes parathyroid hyperplasia. If treatment is not applied, severe secondary hyperparathyroidism develops, that may become resistant to medical treatment; such is the case of nodular hyperplasia of the parathyroids. Hyperplasia is accompanied by a decrease in the expression of parathyroid receptors, including FGF receptor-1 and klotho [52] (Figure 4). Although the exact mechanisms whereby parathyroid hyperplasia is developed are not completely understood, several factors such as hypocalcemia, phosphorus retention, and the deficiency in vitamin D have been directly associated with an increase in cell proliferation [52].

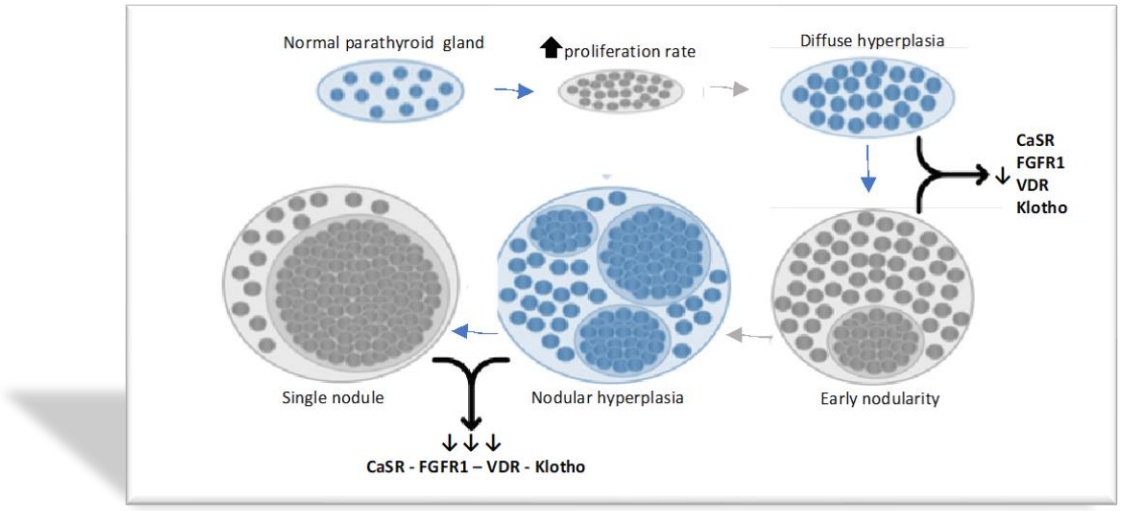


Figure 4 - Progression of parathyroid hyperplasia in chronic kidney disease - adapted from Komaba *et al.* [52]

One of the main expected effects of native vitamin D supplementation is the lowering of serum PTH levels. Hence, the results are not always positive. However, in a meta-analysis, in which our published results were included, Kandula *et al.* reported that nutritional vitamin D leads to increased 25(OH)D levels (mean of + 24 ng/mL), without any hypercalcemia or hyperphosphatemia, and with a decrease in serum PTH level (41% decrease), mostly in dialysis patients [53]. The mean dosage was 50,000 IU weekly during the first month and a lower dosage was usually used thereafter.

A decrease in secondary hyperparathyroidism in dialysis patients was also reported after systematic vitamin D supplementation during the predialysis period [54]. Some studies have shown an increased serum 1,25$(OH)_2$D level after cholecalciferol supplementation [55, 56]. Seibeirt *et al.* confirmed these data and additionally did not find an increase in FGF-23 concentration after vitamin D supplementation [57]. In an RCT, cholecalciferol supplementation has shown a tendency to a decrease in FGF-23 and a significant increase in α-klotho levels, unlike vitamin D analogs that cause an increase in FGF-23 [58].

▪ 3.1.3. VITAMIN D AND INFLAMMATION

Vitamin D is increasingly recognized as an important factor in immune regulation because virtually all immune cells have VDRs and can activate 25(OH)D. Vitamin D deficiency leads

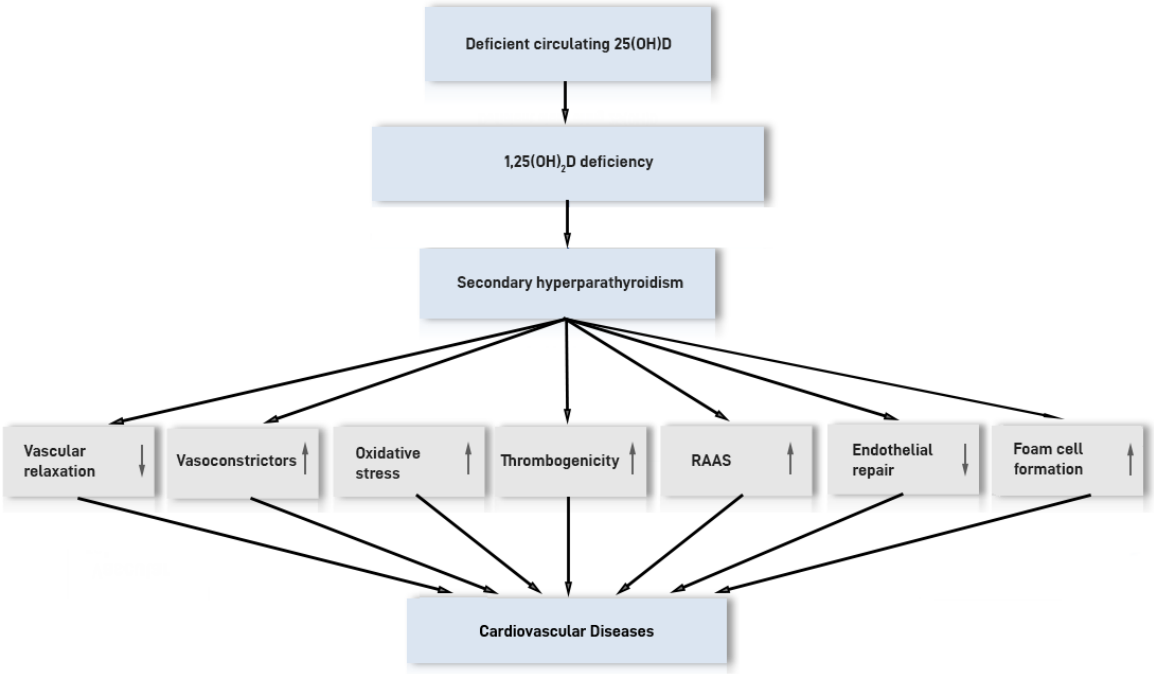
to an impaired immune system at several levels: decreased innate and adaptative immunity and increased inflammation. Moreover, low circulating 25(OH)D levels in CKD may also contribute to decreased innate immunity and increased inflammation or immune cell activation by modulating the microbiome and intestinal permeability [59].

Patients requiring hemodialysis (HD) are chronically inflamed, and its presence is associated with poor outcomes including CV events [60]. In this population, low serum levels of 25(OH)D have been associated with elevated levels of C-reactive protein (CRP) and interleukin (IL) - 6 [61]. Vitamin D has been identified as a potential modifier of inflammation where it has been proposed that $1,25(OH)_2D$, synthesized in monocytes, may inhibit the production of pro-inflammatory cytokines [62]. Recent meta-analyses have demonstrated that native vitamin D supplementation improved levels of CRP in the general population [63], in patients with diabetes [64], as well as in patients with diabetic kidney disease [65]. Two RCT studies in dialysis patients demonstrated a significant difference in CRP levels with supplementation [66, 67].
In dialysis patients, vitamin D deficiency has been independently associated with erythropoietin hyporesponsiveness and anemia [68]. Vitamin D has also been involved in the improvement of erythropoiesis by a direct effect on erythroid precursor proliferation, downregulation of hepcidin gene expression [69] and/or, marginally, by controlling secondary hyperparathyroidism [70].

▪ 3.1.4. VITAMIN D AND CARDIOVASCULAR DISEASE

Vitamin D effects on CV health may be mediated either by effects on classic and emerging CV risk factors or by direct effects on the CV system [71, 72]. Focusing on the latter, both the VDR and the 1α-hydroxylase are present in vascular tissues such as endothelial cells and vascular smooth muscle cells (VSMCs), and also in cardiomyocytes [73]. In the vascular wall, $1,25(OH)_2D$ has several beneficial genomic effects, including a reduction in thrombogenicity, a decrease in vasoconstrictors, an inhibition of oxidative stress and atherogenesis, an improvement of endothelial repair, a reduction in foam cell formation, and vascular relaxation and dilatation. However, $1,25(OH)_2D$ can also induce the trans-differentiation of VSMCs into osteoblast-like cells, which may lead to vascular calcifications [72, 73].
In cardiomyocytes, $1,25(OH)_2D$ can induce several genomic and non-genomic effects, which

regulate intracellular calcium metabolism [74]. There is evidence that, like the regulation of circulating $1,25(OH)_2D$, the synthesis of $1,25(OH)_2D$ in the heart and the vasculature is regulated by PTH and FGF-23 [75, 76]. In cardiomyocytes, FGF-23 action results in hypertrophic growth [77]. Although vitamin D signaling in cardiomyocytes and the vascular wall is not completely clarified, the available data indicate that $1,25(OH)_2D$ plays a pivotal role in adequate cardiac and vascular function. Various effects of vitamin D on the CV system may, in addition to VDR activation, also be mediated by PTH (Figure 5).



25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; RAAS, renin-angiotensin-aldosterone system.

Figure 5 - Suggested effects of vitamin D deficiency on the risk of CV disease (adapted) [78]

A review of numerous studies in the general population found evidence that vitamin D is important for reducing the risk of a variety of chronic illnesses including CV disease [79]. Giovannucci *et al*. in the Health Professionals Follow-up Study [80] found a nearly 2.5-fold increased risk of myocardial infarction for those with vitamin D deficiency, and Acharya *et al*. showed that patients with no vitamin D deficiency and no history of myocardial infarction or atrial fibrillation who were treated with native vitamin D to obtain 25(OH)D levels of > 20 ng/mL and > 30 ng/mL had significantly lower mortality risk and lower risk of

atrial fibrillation [81, 82].

Additionally, an analysis of individuals with hypertension found a significant association between resistant hypertension and vitamin D deficiency [83], and a meta-analysis by Mirhosseini *et al.* [84] showed that vitamin D supplementation was associated with lower blood pressure, and with an anti-inflammatory impact. Further, a recent examination of stroke risk by Wang *et al*. in 8,523 participants of the National Health and Nutrition Examination Survey (NHANES) found 25(OH)D deficiency a significant risk factor for stroke [85]. Another research has shown associations between vitamin D deficiency and left ventricular hypertrophy, endothelial dysfunction, hypertension, and arterial stiffness [86].

However, vitamin D supplementation does not appear to reduce CV events [87]. Most of the studies in the general population were primary prevention trials where vitamin D deficiency is uncommon, and the CV event rate was typically a secondary outcome. The duration of follow-up may also not have been sufficient to capture events related to chronic disease in healthier people. A recent 5-year follow-up study with 21,315 patients indicates that vitamin D supplementation might reduce the incidence of major CV events, particularly myocardial infarction, and the need for coronary revascularization. This protective effect could be more marked in those taking statins or other CV drugs at baseline [88].

CV disease has a higher incidence and is the most common cause of mortality in patients with CKD and has been associated with vitamin D deficiency. In a meta-analysis, Duranton *et al*. suggested that vitamin D replacement reduces CV mortality risk by 27% when administered to CKD patients [89]. Endothelial dysfunction happens early in patients with CKD and is associated with atherosclerosis acceleration and future CV events [90]. Left ventricular hypertrophy and vascular calcifications are common complications in CKD and promote an increase in CV morbidity and mortality rates.

Chitalia *et al.* [90] investigated the relationship between vitamin D levels and endothelial function in nondiabetic patients with mild to moderate CKD. Endothelial function was evaluated by endothelium-dependent brachial artery flow-mediated dilation (FMD). The authors showed that patients with serum 25(OH)D level ≤ 15 ng/mL had a significantly lower FMD compared to patients with 25(OH)D level > 15 ng/mL. A multivariable regression analysis indicated an independent association between low vitamin D levels and low FMD, even, after adjustments for traditional CV risk factors, such as age, gender, smoking,

hypertension, and hyperlipidemia [90]. Also, Capusa *et al.* in a cross-sectional study that included 87 clinically stable CKD patients, concluded that hypovitaminosis D is associated with subclinical peripheral arterial disease, evaluated by aortic calcification score and ankle-brachial index, independently of other traditional or non-traditional risk factors for atherosclerosis [91].

Lishmanov *et al.* conducted a study to analyze if vitamin D replacement can decrease the incidence of CV events [92]. The study enrolled 126 men, with CKD stages 3 - 4 with serum 25(OH)D level < 30 ng/mL. After 6 months of ergocalciferol supplementation, according to KDOQI guidelines, the patients whose serum 25(OH)D level was increased by 25% from baseline were included in the treatment group (n = 90). Other patients who did not respond to treatment were considered as controls (n = 36). At 27.2 months, the treatment group had fewer CV events compared to the control group (21% *vs.* 44%; p = 0.001). Both overall survival and CV disease-specific survival were higher in the treatment group compared to the control. Although there were some limitations, as a retrospective study, a relatively small group of patients, the possibility of unmeasured confounders and the fact that the treatment group had a lower history of diabetes compared to controls (53% *vs.* 73%; p = 0.02), the authors concluded that vitamin D supplementation with ergocalciferol seems to be associated with a significant reduction in CV events in patients with moderate CKD [92].

The studies by Kumar *et al.* [93], Kendrick *et al.* [94], and Marckmann *et al.* [95], all used cholecalciferol supplementation as one of their study interventions in investigating the effect of vitamin D on endothelial dysfunction and/or vascular stiffness. The total dose of vitamin D over the study period used by Kumar *et al.*, was the highest among the three (600,000 IU), with 25(OH)D levels increasing by 25 ng/mL. This study was the only one that demonstrated improvements in all parameters measured (increased FMD and pulse wave velocity). Importantly, no patient developed severe hypercalcemia. In contrast with the studies by Kendrick *et al.* and Marckmann *et al.*, who administered less total dose of cholecalciferol over the study duration, 420,000 IU and 320,000 IU, respectively, showed no change in the surrogate markers investigated. The intervention group in this last study achieved the highest levels of 25(OH)D at a median of 62 ng/mL, but there was no improvement in vascular stiffness. Their intervention lasted only 8 weeks, and prolonged vitamin D therapy could have improved certain markers.

Persisting uncertainty about the effectiveness of vitamin D replacement on CV disease in the general population and CKD patients still exists. The results of RCTs in CKD patients

have also been contradictory and inconclusive. This may be because of the heterogeneity in patient populations and dosing regimens. Doses have been kept low in studies, presumably to avoid hypercalcemia, which was uncommon in all studies. Because of this, vitamin D levels did not rise to biologically significant levels. In addition, some of the studies were of short duration. These factors may explain the lack of improvement in surrogate markers seen in certain studies.

■ 3.1.5. VITAMIN D AND MORTALITY

Vitamin D deficiency has been associated with mortality. Observational studies and meta-analyses of observational studies have persistently shown an association between low serum levels of 25(OH)D and increased overall mortality risk, sometimes with CV death or cancer-related death [96, 97]. In 2017, Gaksch *et al.* [98] published a large meta-analysis of individual participant data, comprising a total of 26,916 people that were followed for a median period of 10.5 years. They found an increasing mortality risk for 25(OH)D levels less than 20 ng/mL.

Despite an inverse association between vitamin D and mortality being consensual, the causality cannot be defined with these studies. Naturally, sicker individuals may have lower vitamin D levels as a consequence of their disease. Mendelian randomization studies try to find a genetically defined characteristic with a specific outcome and therefore can be useful. In the search for causality, Afzal *et al.* reported a Mendelian randomization study on alleles of genes implicated in the synthesis of 25(OH)D (in the skin, from 7 dehydrocholesterol-DHCR7 and in the first hydroxylation in the liver-CYP2R1) in 95,766 individuals of Denmark [99]. They concluded that genetically determined low levels of vitamin D were associated with increased overall and cancer mortality. They did not find a causal relationship with CV mortality and hypothesized that this association in previous studies could be due to confounding factors.

More recently a second Mendelian randomization study analyzed single nucleotide polymorphisms on the pathway of vitamin D synthesis and mortality in 10,501 individuals from Iceland, Germany, and Norway [100]. The authors of this study concluded that there was an increase in mortality for 25(OH) vitamin D levels less than 16 ng/mL. Lastly, a meta-

analysis, which analyzed 84 articles (from 2006 until 2018) and included 57 studies comprising mainly elderly people and with a follow-up that varied from 1.7 to 37 years, concluded that the majority of the studies found an inverse relationship between vitamin D levels and mortality. In fact, there was a progressively lower mortality with higher levels of vitamin D up to a certain threshold beyond which there was no survival benefit. They considered that this relationship was mainly with mortality due to cancer and respiratory diseases and was much weaker with CV disease.

Low $1,25(OH)_2D$ [101] and low 25(OH)D [102] have also been associated with mortality in CKD and dialysis patients. In some dialysis cohorts, a clear association between low serum 25(OH)D levels and mortality has been reported. For example, in a French cohort, mortality risk was increased by 30% when the serum 25(OH)D level was below 18 ng/mL [102].

A meta-analysis in the general population [103] and in CKD patients [67] failed to show that vitamin D supplementation is associated with a reduced risk of all-cause mortality risk. However, we think that in CKD the relationship between outcomes and serum 25(OH)D level should be interpreted together with serum phosphorus, PTH and FGF-23.

3.2. MAGNESIUM AND CHRONIC KIDNEY DISEASE

Magnesium is the second most abundant intracellular cation and seems to play a role in CKD-MBD, particularly in the synthesis and metabolism of PTH and vitamin D [104, 105].

Only about 30% of the total dietary Mg is absorbed in the small intestine, but in deficient states, higher absorption can exist, either by a saturable transport system or by passive diffusion [106]. There are two processes responsible for intestinal Mg absorption. The first is active transport, which depends on a transient receptor potential melastatin (TRPM) 6 and 7 members of the long transient receptor potential channel family, which also play a central role in intestinal calcium transport. The second and most important is a passive process that occurs by a paracellular pathway [106].

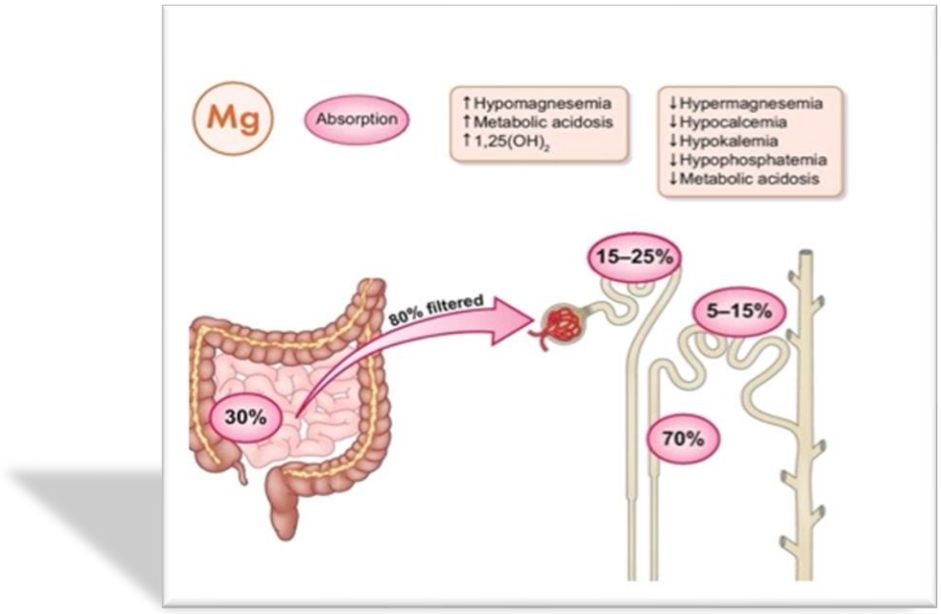
The kidney mainly regulates Mg excretion, where 80% of total plasma Mg is filtered by the glomerulus. The filtered Mg is reabsorbed by the renal tubules: 10 - 25% in the proximal

tubule, 60 - 70% in the thick ascending limb (TAL) of the Henle loop, and 5% in the distal tubule. Transport of Mg in TAL is primarily passive (moving from the lumen to the interstitium) via the paracellular channels, due to a transepithelial gradient generated by the apical NaK2Cl cotransporter. The selectivity of the paracellular pathway is determined by claudins, which form a cation-selective tight junction whereby the Mg paracellular transport is done [107, 108]. Three claudin proteins, claudin-14, claudin-16, and claudin-19, make the cation-selective paracellular pathway for calcium and Mg. Claudin-16 and claudin-19 form the pores and claudin-14 inhibits the cation selectivity of that pore [109].

The extracellular fluid volume modulates tubular Mg transport, as does CaSR located in the basal pole of the tubular cells of the TAL, which are sensitive to serum calcium and Mg [110]. The activation of CaSR inhibits the NaK2Cl cotransporter, dissipating the positive transepithelial gradient and decreasing the passive calcium and Mg reabsorption, which leads to the increase of calcium and Mg urinary losses [107, 108]. CaSR also inhibits the phosphorylation of claudins (and unphosphorylated claudins are not expressed in tight junctions) reducing the tight junction permeability to calcium and Mg [110].

A small percentage of Mg is reabsorbed in the distal tubule through active transport via the apical TRPM6 / TRPM7 pathway. Concerning the active mechanism of Mg transport, TRPM6 and TRPM7 channels are located in the apical membrane of the cells in the TAL of the Henle loop. However, some evidence suggests the participation of a sodium-dependent exchange mechanism where the sodium - potassium adenosine triphosphatase (Na/K - ATPase) pump participates together with low concentrations of Na [106].

Hypomagnesemia stimulates loop Mg transport. On the contrary, hypermagnesemia inhibits loop transport, as well as hypercalcemia, hypokalemia, hypophosphatemia, and metabolic acidosis (Figure 6). Furthermore, intestinal absorption of Mg can also be influenced by calcium and vice versa. High intestinal calcium concentrations have been reported to reduce the absorption of Mg [110]. In addition, vitamin D may influence the intestinal absorption of Mg. High doses of $1,25(OH)_2D$ increase the absorption of Mg [111].



$1,25(OH)_2D$, 1,25-dihydroxyvitamin D

Figure 6 - Magnesium absorption through intestine and kidney [112]

Recommended daily allowance for men is 5 - 6 mg/kg of body weight and for women is 4 - 5 mg/kg of body weight [113]. Dietary intake of Mg is inadequate in most adults because the majority of the Mg is lost during food processing [114]. Although drinking water accounts for 10% of daily Mg intake, food (spinach, nuts, and seeds) remains the richest source of Mg.

The adult human body contains approximately 24 g of Mg, mostly (99%) contained in the bone, muscles, and soft tissues. Serum Mg concentration does not correlate with tissue pools, except for interstitial fluid and bone. Only 1% of total body Mg is present in extracellular fluids, and only 0.3% of total body Mg is found in serum, so serum Mg concentrations are poor predictors of intracellular / total body Mg content [114].

What is considered the 'normal level' might actually be slightly too low, representing a mild Mg deficit present in the normal population [115]. In addition, there are individuals, in particular those with a subtle chronic Mg deficiency, whose serum Mg levels are within the reference range but who still may have a deficit in total body Mg [115]. In conclusion, a 'normal' serum Mg level (1.5 - 2.5 mEq/L) may be associated with a moderate to severe Mg deficiency [115, 116], which is known as normomagnesemic Mg depletion. In the literature, patients with serum Mg concentrations < 0.61 mmol/L (1.5 mEq/L) are considered hypomagnesemic, and those with Mg > 1.1 mmol/L (2.5 mEq/L) are considered hypermagnesemic [116].

The kidney has a vital role in Mg homeostasis: regulation of Mg excretion is determined by filtration and reabsorption. The renal handling of Mg depends to a great extent on the plasma Mg concentration: in hypermagnesemia, the fractional excretion of Mg is high, while during hypomagnesemia it is low [117]. Because the renal excretion of Mg is so adaptable, impairment of renal function has long been recognized as a frequent prerequisite for the development of hypermagnesemia. However, in moderate CKD, the increase in the fractional excretion of Mg compensates for the loss of renal function, such that serum levels are maintained within the normal range. Interestingly, there seem to be differences between diabetics and non-diabetics. When patients with and without diabetes (with creatinine clearance > 30 mL/min) and not treated with diuretics were investigated, serum total and ionized Mg levels were significantly lower in diabetics, but still in the normal range in both groups [118].

As renal function progresses to CKD stages 4 and 5, the excretion of Mg tends to decrease and cannot be compensated any longer by an increased fractional excretion of Mg [117]. This first becomes apparent as creatinine clearance reduces to < 30 mL/min and particularly to < 10 - 15 mL/min [118]. Thus, overt hypermagnesemia develops frequently in patients with creatinine clearance < 10 mL/min [117, 119]. However, studies have investigated ionized serum Mg in dialysis patients in comparison with healthy controls to assess the utility of ionized and total Mg assay in detecting Mg overload. A lower ionized fraction (that is biologically active) is frequently present in these patients and could be due to a higher fraction of complexed Mg (with phosphates, citrate, sulphates) in dialysis patients than in healthy individuals [120].

In uremic patients on HD, the serum Mg concentration parallels the dialysate Mg level as Mg readily crosses the dialysis membrane with its movement determined by the gradient between the concentration of diffusible Mg in blood and the level of Mg in the dialysate. In patients of our unit dialyzed with a standard dialysate Mg (0.75 mmol/L) the mean serum Mg concentration was 1.15 mmol/L; 68% of subjects had hypermagnesemia [121]. This is not surprising as the dialysate level of 0.75 mmol/L exceeds the normal diffusible Mg level in blood. Several authors have reported that a reduction in the Mg dialysate produces a significant decrease in the serum Mg concentration as early as the first month [122, 123]. In contrast, in another study, changing the dialysate Mg from 0.75 to 1.5 mmol/L the mean serum Mg concentration increased from 1.25 to 1.7 mmol/L [124]. These data demonstrate that dialysate Mg plays a critical role in maintaining Mg homeostasis in ESRD patients on HD [125].

Certain CKD-specific risk factors could also predispose to hypomagnesemia, including long-term use of certain medications (diuretics, proton pump inhibitors, calcineurin inhibitors, and others), the presence of diabetes, proteinuria, hyperaldosteronism, volume expansion, and metabolic acidosis [126].

▪ 3.2.1. MAGNESIUM AND SECONDARY HYPERPARATHYROIDISM

In normal parathyroid glands under conditions of moderate hypocalcemia, Mg decreases PTH levels by activating the CaSR and upregulating the VDR and the FGF receptor 1 / klotho co-receptor. However, hypomagnesemia impairs PTH secretion and lowers $1,25(OH)_2D$ levels. In addition, certain enzymes on the PTH - vitamin D axis are also Mg-dependent and have decreased activity in hypomagnesemia [127].

In CKD patients, severe hypomagnesemia has been associated with profound hypocalcemia, which has been commonly attributed to impaired PTH release and peripheral resistance to its action. In this situation, Mg replacement was associated in most cases with increased PTH levels, despite a concomitant increase in serum calcium [128]. Important information has been obtained from dialysis patients, as well as uremic patients treated with Mg salts.

Several trials on the successful use of Mg salts to control hyperphosphatemia and hyperparathyroidism support the hypothesis that hypermagnesemia may have a suppressive effect on PTH secretion in dialysis patients [129-132]. O'Donovan *et al.* [129] compared the evolution of patients treated with Mg carbonate with respect to a control group on therapy with aluminum hydroxide. After 2 years, the authors observed a significant reduction in the mean serum PTH concentration in patients receiving Mg salts, but not in the controls. Morinière *et al.* [130] evaluated the effects of the administration of Mg hydroxide in association with moderate doses of calcium carbonate. They found that plasma concentration of Mg progressively increased concomitantly with a progressive reduction in PTH. Delmez *et al.* [131] observed that the administration of Mg carbonate allowed a reduction in the doses of calcium carbonate without any increase in the serum PTH level.

Furthermore, these findings are concordant with the variation of PTH after modifications of

dialysate Mg content. Parsons *et al.* [132] and Nilsson *et al.* [122] reported that the correction of raised serum Mg concentration in patients dialyzed against a low dialysate Mg fluid resulted in a significant increase in serum PTH. In contrast, McGonigle *et al.* [133] observed that in hypermagnesemic patients a further rise in serum Mg caused a subsequent fall in PTH.

Navarro *et al.* [134] analyzed serum Mg concentrations in dialysis patients classified according to their PTH level. Patients with low PTH (< 120 pg⁄mL) had a serum Mg concentration significantly higher than patients with moderate (120 - 250 pg⁄mL) or higher (> 250 pg⁄mL) PTH. Moreover, we found a significant negative linear relationship between serum concentrations of PTH and Mg. Similar findings have been reported by Saha *et al.* [135], who also found a significant inverse correlation between iPTH and serum Mg. Finally, this relationship between Mg and PTH occurs independently of other factors that can affect the PTH synthesis or secretion. Thus, plasma Mg has been demonstrated to be a significant determinant of serum PTH concentration, independently of serum calcium and phosphorus [121, 136].

▪ 3.2.2. MAGNESIUM AND INFLAMMATION

Mg interferes in the immunoregulation of the body, and it is critical to natural and adaptive immunity, partly by influencing the activity of vitamin D metabolites [107, 137]. The close relationship between Mg and vitamin D and the necessity of an optimal Mg status for the synthesis, transport, and activation of vitamin D suggest that the higher incidence of infectious diseases associated with vitamin D deficiency can be at least in part explained by a deficit of Mg. Even if most studies regarding the direct association between poor Mg status and poor immune system function are derived from animal models, human studies have shown that Mg deficiency seems to be associated with a higher rate of infectious diseases, particularly when considering older people [138].

Mg transporter 1 (MAGT 1) is an evolutionally conserved Mg2+-specific ion transport facilitator found in all animals and has been shown to participate in the multienzyme complex responsible for enzymatic coupling of N-glycans onto peptide substrate. Recent studies demonstrate that humans lacking functional MAGT 1 have a selective deficiency in both immune and nonimmune glycoproteins, and several critical glycosylation defects

were also identified in important immune-response proteins and in the expression of genes involved in immunity, particularly CD28 [139].

A meta-analysis on the effect of Mg supplements on CRP in non-CKD populations demonstrated a robust and significant reduction in CRP levels [140]. A cross-sectional, multiple-adjusted analysis also confirmed an inverse association between serum Mg levels and CRP in people with CKD [136]. However, only one RCT in patients with diabetes treated with HD observed a significant decrease in CRP with Mg administration [141]. A post-hoc analysis in HD patients with a focus on different inflammatory markers observed significant reduction in both IL-6 and tumor necrosis factor α (TNF-α) in those treated with higher Mg in the dialysate (1.00 mmol/L) compared to lower Mg (0.50 mmol/L) after 28 days [142].

▪ 3.2.3. MAGNESIUM AND CARDIOVASCULAR DISEASE

Mg modulates the activity of both cardiac myocytes and pacemaker cells by regulating several potassium and calcium channels (i.e., Na/K - ATPase, inwardly rectifying potassium channels, and L-type calcium channels) [143]. Mg depletion could cause depolarization of the transmembrane potential at rest, as well as prolong the depolarization time during the action potential (phase 3), making the heart more vulnerable to arrhythmia and sudden cardiac death. Mg influences cardiomyocyte contractility by acting as a natural antagonist to calcium (competes for binding to troponin C and calmodulin) and also limits intracellular calcium overload during myocardial ischemia [144]. Furthermore, Mg can favorably affect cardiac remodeling through its inhibitory effect on the production of matrix metalloproteinase-2 in cardiac fibroblasts [145].

In addition to being proarrhythmogenic, Mg deficiency promotes a proatherogenic phenotype by increasing oxidative stress and inflammation in cardiac tissue and VSMCs [146]. The relationship between low Mg levels and oxidative stress is bidirectional, and oxidative stress may also exacerbate Mg deficiency [147].

Low Mg level is associated with high blood pressure. Mg has a substantial vasodilatory effect by being a natural calcium channel antagonist, inhibiting endothelin 1 expression, and increasing nitric oxide production and prostacyclin 2 levels. Mg also decreases directly

aldosterone production, decreasing sodium reabsorption and limiting volume overload [148].

Observational and small intervention studies have shown that Mg may reduce CV events by improving vascular tone and endothelial function, reducing platelet aggregation, increasing high-density lipoprotein cholesterol levels, improving glucose homeostasis, and favorably affecting metabolic syndrome parameters [149, 150]. An RCT in patients with stage 3 - 4 CKD and risk factors for coronary artery calcification showed a significantly smaller change in the median coronary artery calcification score for individuals taking Mg oxide versus controls (11.3% *vs.* 39.5%) at 2 years [151]. In dialysis patients, Mg supplementation has been reported to reduce carotid intima-media thickness [152].

A meta-analysis of serum Mg, including 20 studies and 200,934 participants with CKD and ESRD, in which our results were included, showed that compared with individuals with normal or higher Mg levels, those with lower levels had significantly increased risk for CV and all-cause mortality [153].

In conclusion, possible underlying mechanisms that may drive the inverse association between Mg and all-cause mortality, CV events and mortality include the protective effects of Mg on the development of vascular arterial calcification, cardiac arrhythmia, heart failure, hypertension, endothelial dysfunction, and low-grade inflammation (Figure 7).

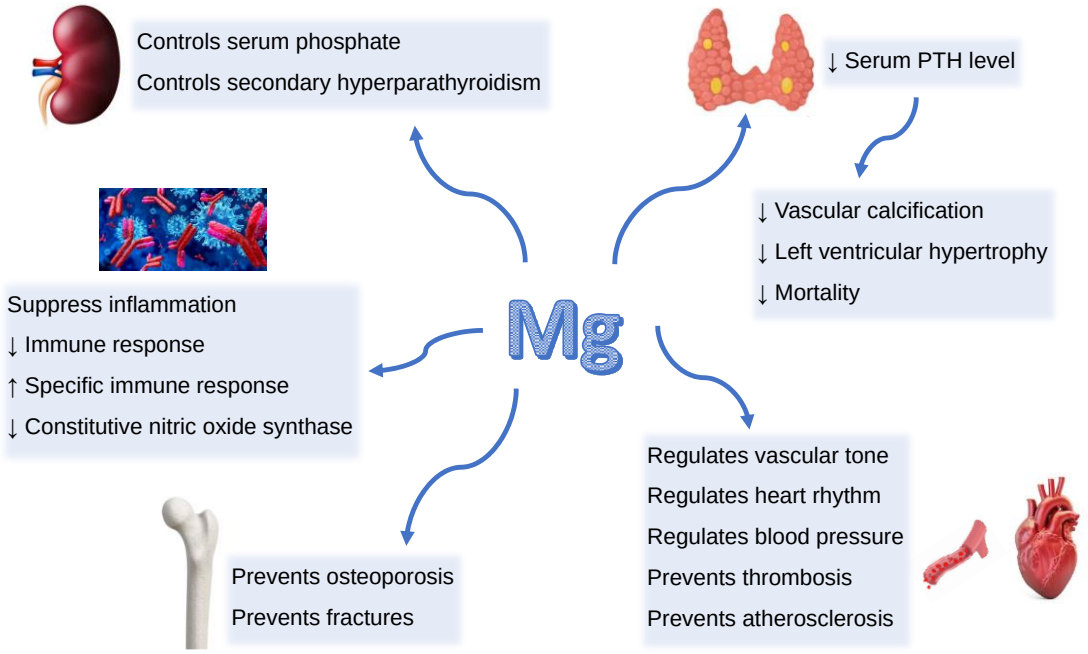


Figure 7 - Possible mechanisms of interplay between magnesium and cardiovascular disease in chronic kidney disease (adapted) [152]

▪ 3.2.4. MAGNESIUM AND MORTALITY

In a cohort study of 142,555 HD patients from the Japanese Society for Dialysis Therapy - Renal Data Registry, lower pre-dialysis serum Mg levels were associated with a higher risk of 1-year mortality [154]. Those with mild hypermagnesemia, i.e., serum Mg levels of 1.11 - 1.23 mmol/L, showed the best survival. Unexpectedly, serum Mg levels of ≥ 1.27 mmol/L were associated with higher mortality although the causality of this association is unknown.

A recent meta-analysis involving 348,059 CKD patients in 33 studies, that included our study, showed that Mg concentration is inversely associated with all-cause mortality and CV mortality and events [155].

Given that Mg counteracts the detrimental effect of phosphate, a close interaction between serum Mg and phosphate levels can be assumed with respect to the risk of CV outcomes. In fact, the association between serum phosphate levels and CV deaths was significantly modified by serum Mg levels. Among individuals with lower serum Mg levels, hyperphosphatemia was remarkably associated with an increased risk of CV mortality,

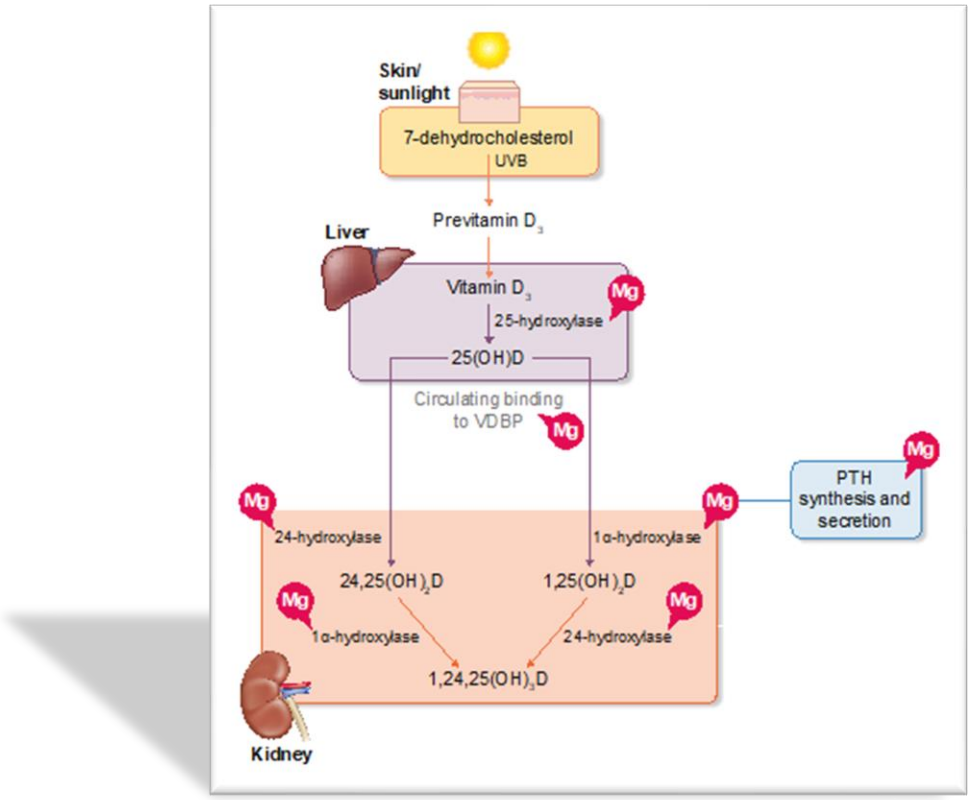
whereas there was no significant association between serum phosphate levels and CV mortality among those with higher serum Mg levels [156]. This finding further supports the notion that Mg supplementation would benefit especially individuals with hyperphosphatemia.

Also, in a mice model of CKD fed a high-phosphorus diet, a low-Mg diet was associated with higher FGF-23 levels than the normal Mg diet group. In a recent clinical study, subgroup analyses by FGF-23 categories showed that participants in the lower Mg tertile had significantly increased risk for mortality compared with participants in the mid tertile, suggesting that high FGF-23 levels have an additive negative effect in hypomagnesemic states [157]. A high-Mg diet can bind dietary phosphorus, resulting in less hyperphosphatemia in CKD. In a large cohort of HD patients, increasing serum Mg largely decreased the mortality risk associated with hyperphosphatemia [156].

3.3. INTERACTIONS BETWEEN MAGNESIUM AND VITAMIN D METABOLISM

Several steps in the vitamin D metabolism seem to depend on Mg as a cofactor, such as vitamin D binding to VDBP, 25(OH)D synthesis, $1,25(OH)_2D$ synthesis, 25-hydroxylase synthesis, and VDR expression for cellular effects [158]. Mg deficiency can also decrease PTH synthesis and secretion and can also decrease the number of available VDRs in target cells (Figure 8) [159].

Some studies indicate that Mg status affects concentrations of CYP P450 enzymes[140]. CYP enzymes include not only the vitamin D - activating enzymes [25-hydroxylase (i.e. CYP2R1) and 1α-hydroxylase (i.e. CYP27B1)], but also vitamin D - deactivating enzymes [24-hydroxylase (i.e. CYP24A1 and CYP3A4)]. These enzymes display similar properties including a requirement for Mg ions, molecular oxygen, and a source of reduced pyridine nucleotides[140].



Mg, magnesium; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D (biologically active form); 1,24,25(OH)$_3$D, 1,24,25-trihydroxyvitamin D; 24,25(OH)$_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; UVB, ultraviolet B; DBP, vitamin D - binding protein.

Figure 8 - Potential interactions of magnesium in vitamin D metabolism - magnesium is involved in both activation and inactivation of vitamin D [112]

On the other hand, it has been reported that 1,25(OH)$_2$D can stimulate intestinal Mg absorption, by up-regulating intestinal VDR [111]. However, most Mg is also absorbed independently of vitamin D and VDR. Claudins 2 and 12, which are involved in paracellular calcium transport are regulated by 1,25(OH)$_2$D [109]. Studies in animals showed that low and high dietary Mg affects calcium balance via the kidney (increased reabsorption and elimination, respectively). The mechanisms responsible for this fact are unknown, but some authors suggest that a regulatory role for the CaSR could explain this interaction between Mg and calcium and, its impact on vitamin D metabolism [160]. In conclusion, Mg seems to be an essential cofactor for vitamin synthesis, nevertheless activated vitamin D, in turn, can increase intestinal absorption of Mg, and therefore can form a feed-forward loop to maintain its homeostasis.

The effects of vitamin D supplementation on Mg circulating levels were investigated in 126 patients with type 2 diabetes. A significant increase in Mg levels was found after supplementation with native vitamin D (cholecalciferol) (2,000 IU/day) for 6 months [161].

NHANES data analysis showed epidemiological evidence of this interaction between 25(OH)D and Mg [162]. High Mg intake was associated with a reduced risk of vitamin D deficit or insufficiency. Data also indicated an inverse association between circulating 25(OH)D and mortality, particularly CV mortality, among those with Mg intake above the median level. However, in this study, only Mg intake was evaluated, and Mg serum levels were not measured and no confirmation of Mg deficiency in patients with low intake could be directly assumed. Further studies of vitamin D will likely need to account for Mg levels and Mg intake.

3.4. POTENTIAL EVIDENCE THAT CORRECTION OF MAGNESIUM DEFICIENCY CAN RESTORE VITAMIN D LEVELS

Hypomagnesemia has been implicated in the development of vitamin D-resistant rickets [163, 164]. For patients with Mg-dependent vitamin D-resistant rickets, characterized by reduced $1,25(OH)_2D$ and impaired parathyroid response, intramuscular infusion with ≤ 600,000 IU vitamin D alone did not lead to any improvements in biochemical measures of vitamin D deficiency. However, Mg supplementation did substantially reverse the resistance to vitamin D treatment [164], as serum $1,25(OH)_2D$ levels were substantially increased by supplementation with Mg and vitamin D compared to that of vitamin D or Mg alone [163]. Rickets thought to be secondary to vitamin D resistance, could also be treated with Mg therapy [165].

High levels of Mg increase $1,25(OH)_2D$ by increasing the levels of 25-hydroxylase and vitamin D catabolites, but also by facilitating the transfer of vitamin D to target tissues, such as bone, through VDBP. However, it should be noted that while Mg intake is associated with higher levels of vitamin D [161], Mg supplementation alone cannot fully rescue vitamin D deficiency [163]. Similarly, the serum levels of $1,25(OH)_2D$ in critically Mg-deficient patients were unchanged after 5 - 13 days of parenteral Mg therapy alone [160]. Together, these studies suggest that Mg and vitamin D can interact to influence the levels of vitamin D, but more

studies are still needed.

Furthermore, Mg deficiency is more common in women, obese, CKD patients, and those with higher levels of PTH, and all these situations are also more common in individuals at high risk for vitamin D insufficiency [163]. These findings suggest an interaction between vitamin D and Mg levels, particularly in the elderly and those with osteoporosis, which could have a major impact on human health.

A cross-sectional study, performed in the US general population (NHANES 2001 - 2006), showed that Mg intake alone or its interaction with vitamin D intake may contribute to vitamin D status [162]. This study demonstrated associations between serum 25(OH)D and the risk of CV and possibly colorectal cancer mortality, which may be also modified by the intake level of Mg [162]. A Finnish cohort study also revealed that low serum 25(OH)D concentration was associated with a higher risk of death, and this association was most significant among men with a lower intake of Mg [166].

A recent randomized controlled trial in 180 patients found that Mg supplementation significantly affects vitamin D metabolism, dependent on the vitamin D status at baseline. Serum concentrations of both 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 increased with Mg supplementation only when baseline 25(OH)D was < 30 ng/mL but decreased when baseline 25(OH)D was higher (from 30 to 50 ng/mL) [167]. Thus, emerging evidence suggests that adequate Mg levels seem to be necessary for adequate vitamin D function, particularly in patients with vitamin D deficiency and that giving Mg itself can increase 25(OH)D in patients with low levels.

Two small clinical trials of Mg-deficient patients [160, 168] found that Mg infusion alone led to a non-significant increase in $1,25(OH)_2D$ and 25(OH)D [160] whereas Mg infusion plus oral vitamin D significantly increased both serum 25(OH)D and $1,25(OH)_2D$ [168]. These findings suggest a potential interaction between vitamin D and Mg supplementation and a possible moderate effect of Mg on 25(OH)D status.
In animal studies, although vitamin D supplementation improves both calcium and Mg absorption, it also increases Mg excretion and reduces Mg retention [169]. In rats, the combined vitamin D and Mg deficiencies impair the calcemic response to vitamin D, because of a diminished vitamin D-mediated intestinal calcium absorption in the Mg-deficient rats [164, 170].

3.5. ROLE OF MAGNESIUM AND VITAMIN D IN BONE AND EXTRA-OSSEOUS CALCIFICATIONS

Bone abnormalities, with decreased bone mass and an increased risk of bone fractures, become more common with age and in CKD patients. This condition is often associated with low bone volume and/or mineralization and is caused by an imbalance of bone reabsorption and new bone formation. Decreased calcium intake, impaired absorption of calcium from aging, CKD, or vitamin D deficiency (which is common in elderly and CKD patients) can cause an increased secretion of PTH to increase serum calcium levels. As the active form of vitamin D is necessary for optimal absorption of calcium, CKD patients can have persistent high levels of PTH [171]. Secondary hyperparathyroidism accelerates bone loss, increases fragility, and impairs neuromuscular function, which increases the risk of falls [171]. The relationship between serum PTH and bone fractures appears to be U-shaped in HD patients: lower PTH levels are also associated with higher fracture risk, possibly because of its association with poorer nutritional status and inflammation [172].

PTH is the most frequently used biomarker for bone turnover in CKD. It is, however, important to realize, that, while PTH is a regulator of bone turnover, it does not reflect turnover *per se*. PTH may also reflect parathyroid disease, PTH molecules may be biologically inert, and PTH resistance at bone level can exist, all conditions in which PTH value fails as an indicator of bone turnover. In contrast, circulating bone alkaline phosphatase (bALP) is mainly derived from active osteoblasts and reflects bone turnover more directly. In the absence of liver disease, bALP comprises approximately 50% of total circulating alkaline phosphatase (ALP) activity; thus, total ALP could be commonly used as a biomarker for routine control of bone formation in CKD [172].

In a recent study in a large HD population, in addition to well-known classical risk factors for fragility fractures, high serum phosphate was consistently associated with increased risk for bone fractures, regardless of other factors such as previous fractures, age, gender, time on HD, serum calcium and PTH [173].

Many vitamin D-responsive genes are expressed in bone-forming osteoblast cells, and bone-reabsorbing osteoclast cells including the tumor necrosis factor ligand family gene of the receptor activator of NF-kB ligand (RANKL), which is involved in osteoclastogenesis, and is modulated by the $1,25(OH)_2D$ [174]. In osteoporosis patients, there is a higher rate of Mg

deficiency in those with vitamin D deficiency compared to those with normal levels of vitamin D [175]. Interestingly, a study conducted among osteoporotic patients showed much higher prevalence rates of Mg deficiency or insufficiency among people with insufficient 25(OH)D than those with sufficient 25(OH)D serum levels [176]. Mg deficiency might affect bone directly (by reducing bone stiffness, increasing osteoclasts, and decreasing osteoblasts) and indirectly (by interfering with PTH and vitamin D, promoting inflammation / oxidative stress, and subsequent bone loss) [177].

Extra-skeletal calcifications are one of the features of CKD-MBD. Vascular calcifications are the inappropriate and pathological deposition of minerals in the form of several calcium and phosphate salts into vascular tissues. There are two pathophysiological distinct forms of vascular calcifications, one occurring within atherosclerotic plaques in the intima of blood vessels and the other occurring within the media of blood vessels. Although patients with CKD are prone to both types of calcifications, the latter is more specific to CKD [178]. Vascular calcifications are independent predictors of CV events and mortality, and their prevalence increases with decreasing renal function and generally follows a progressive course [178].

Identification of vascular calcifications in CKD patients has an important impact on CV risk stratification and therapeutic guidance [179]. Although more sophisticated methods exist, a plain X-ray of the pelvis and hands for evaluation of vascular calcifications is a very simple and inexpensive method to evaluate vascular calcifications (Figure 9). The Adragão score, a Portuguese CKD-validated calcification score [179, 180], has been proven to be an independent predictor of vascular disease, CV hospitalizations, and mortality in dialysis patients [179].

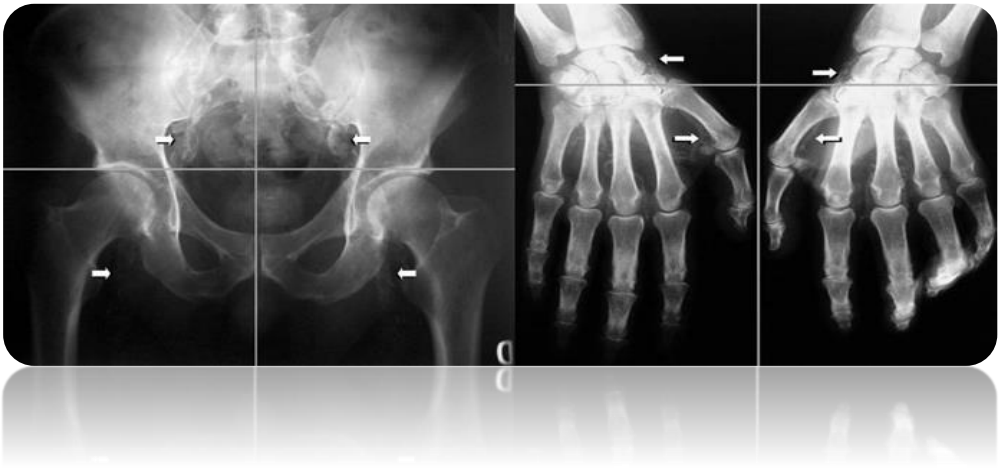


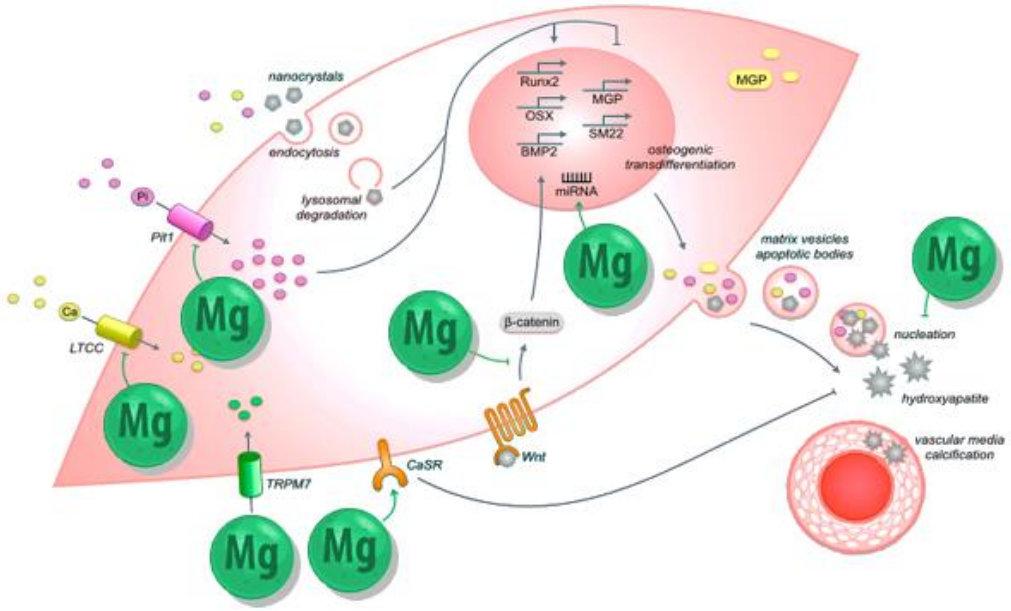
Figure 9 - Simple vascular calcification score - Adragão score [180]

Abnormalities of mineral and bone metabolism are also important risk factors for heart valve calcifications, especially in aortic and mitral valves. These calcifications could also be evaluated in CKD patients, as they are eight times more prevalent in dialysis patients than in the general population [181], and occur 10 to 20 years earlier in these patients [182].

Several studies have shown that Mg inhibits vascular calcifications [151, 183]. Mg may counteract vascular calcification by inhibition of intestinal phosphate uptake as a result of phosphate binding, by systemic effects on both promoting and inhibiting factors of calcification or by local effects at the vascular tissue level. Regarding the tissue level, Mg counteracts calcification of VSMCs and aortic tissue *in vitro* and inhibits expression of osteogenic transcription factors, including runt-related transcription factor 2 (RUNX 2), osterix, and bone morphogenetic protein-2 (BMP-2); and genes associated with matrix mineralization, including ALP [184-187].

The local inhibiting effects of Mg on vascular calcification may be mediated in multiple ways (Figure 10). First, Mg passively interferes with the maturation of amorphous calcium / phosphate particles, thereby preventing the formation of stable hydroxyapatite [187]. Second, Mg inhibits the influx of calcium via L-type calcium channels in VSMCs as outlined above, and this affects vascular tone [188]. It is plausible that inhibition of calcium influx channels limits the intracellular rise of calcium concentration that is associated with vascular calcification, which may inhibit possible stimulating effects of intracellular calcium on the calcification process [189]. Third, Mg acts on the CaSR that is expressed on VSMCs. This expression is decreased in calcifying conditions, and stimulation of the CaSR by calcimimetics inhibits VSMCs calcification [190].

In vitro studies also demonstrated that Mg supplementation reduces phosphate-induced calcification of VSMCs through the inhibition of osteogenic transdifferentiation by the Wnt / β-catenin signaling pathway [191]. Mg deficiency may accelerate vascular calcifications and atherosclerosis, thus causing CV disease [192]. In uremic rats, Mg supplementation prevents aortic calcification together with an improvement in mineral metabolism and renal function [191]. Theoretically, Mg may be useful to prevent the progression of coronary artery calcification, which predicts CV events and mortality among CKD patients. In several epidemiological studies, circulating Mg is inversely associated with coronary artery calcifications [192]. In fact, an RCT showed that oral Mg oxide delayed coronary artery calcification progression in patients with CKD stage 3 - 4 [151].



LTCC: L-type calcium channel, Pit1: phosphate transporter Pit1; TRPM7: transient receptor potential cation channel subfamily M member 7; CaSR: calcium sensing receptor; Wnt: Wnt protein; Runx2: runt-related transcription factor 2; OSX: osterix; BMP-2: bone morphogenetic protein-2; MGP: matrix gla protein; SM22: transgelin (smooth muscle specific protein); miRNA: micro-RNA.

Figure 10 - Possible actions of magnesium on vascular smooth muscle cells (adapted)[143]

A study using an animal model showed a potential interaction between vitamin D and Mg [193]. This study revealed that combining Mg with calcitriol treatment can reduce hypercalcemia and similarly suppress PTH while protecting, at least in part, the vasculature from calcium and phosphate deposition. These results demonstrate that calcitriol can increase vascular calcifications under certain circumstances, an effect that is attenuated in the presence of increased Mg. Importantly, the calcitriol-induced reduction in vascular TRPM7 protein expression was abrogated, at least in part, by Mg co-treatment [193]. Taken together, these data suggest that the benefit of this combined treatment likely involves preventing reductions in TRPM7 expression and increasing the relative entry and availability of Mg (reducing calcium / Mg ratio) in the vascular calcification-susceptible microenvironment.

4. DOUBTS AND AIMS

The high incidence of CV disease and mortality in CKD patients is not entirely explained by traditional risk factors. The KDIGO has defended, since 2006, the existence of a link between mineral and bone disorder and CV disease in CKD patients [10]. Vitamin D and Mg remain the least studied of the key mineral biomarkers in advanced CKD. Its effects on CV and bone disease, particularly associated with the role of supplementation, have been investigated in recent years but are still a matter of debate.

There were no studies in Portugal measuring calcitriol and calcidiol levels in dialysis patients, nor with native vitamin D supplementation and their potential pleiotropic effects. Studies with Mg levels evaluation and its impact on ESRD were lacking. The close biological relationship between Mg and vitamin D is frequently forgotten by physicians and the potential clinical impact of supplementing both, when necessary while maintaining optimal levels must be explored.

Bone fractures are associated with important morbidity and mortality in CKD patients. The incidence of fragility fractures in prevalent HD patients and in large follow-up studies were required and the risk factors for fractures in this population are not yet clear.

These questions are still unresolved and could help to develop new therapeutic options in order to improve bone health and reduce CV burden and mortality in CKD patients.

The primary aim of the author's studies was to contribute to further clarification of the role of non-traditional CV risk factors, such as native vitamin D and serum Mg, in HD patients. These biomarkers consolidate the existence of a strong interaction between bone and CV disease in CKD.

A secondary aim was to study in a large population of prevalent HD patients in Portugal, for the first time, the incidence of bone fractures and evaluate their possible association with markers of bone disease in CKD and with CV risk factors.

To achieve these aims, three topics were studied, as exposed below. Each topic had specific objectives which were analyzed in several studies. All studies were performed in Portuguese prevalent HD patients.

NATIVE VITAMIN D DEFICIENCY IN HEMODIALYSIS PATIENTS

- Characterization of serum calcidiol levels and identification of patients with sufficiency, insufficiency, and deficiency levels of this vitamin.
- Association between serum calcidiol levels and CV risk factors, namely pulse pressure, vascular calcifications, heart failure, left ventricular hypertrophy, and inflammation.
- Association between native vitamin D deficiency and 48-month CV and overall mortality.
- In a long-term prospective study with therapeutic intervention, to correct the insufficiency or deficiency in 25(OH)D and assess whether this correction was associated with a reduction of CV risk factors.

MAGNESIUM SERUM LEVELS IN HEMODIALYSIS PATIENTS

- Association between serum Mg levels and the presence of CV risk markers, namely pulse pressure, vascular calcifications, and left ventricular hypertrophy.
- Association between lower serum Mg levels and 48-month CV and overall mortality.
- In a prospective study, to compare the impact on CV risk factors of using Mg-based phosphate binders *versus* those without Mg, to control hyperphosphatemia in HD patients.

BONE FRAGILITY FRACTURES: INCIDENCE AND RISK FACTORS IN PREVALENT HEMODIALYSIS PATIENTS

- Quantification of the incidence of fragility fractures.
- Association between the presence of fractures and biomarkers of bone disease.
- Association of bone fractures with CV risk factors, particularly with the presence of vascular calcifications and inflammation.

5. METHODS AND RESULTS

NATIVE VITAMIN D DEFICIENCY IN HEMODIALYSIS PATIENTS

5.1. LEVELS OF 25-HYDROXYVITAMIN D, INFLAMMATION, CARDIOVASCULAR RISK MARKERS, AND MORTALITY IN PREVALENT HEMODIALYSIS PATIENTS

PATIENTS AND METHODS

- **Study design**

Firstly, a cross-sectional, single-center study was performed in a cohort of prevalent HD patients to determine serum calcidiol and calcitriol levels. Secondly, the same population was followed prospectively for 48 months to evaluate hospitalizations and mortality.

- **Eligibility criteria**

All prevalent HD patients of our center were included. The exclusion criteria were age below 18 years old and incapacity to give informed written consent.

- **Population**

All patients were submitted to post-dilution online hemodiafiltration, with high-flux membranes and ultrapure water (evaluated monthly by a kinetics chromogenic test) used. The following data was collected: age, gender, presence of diabetes, hypertension, coronary artery disease and active medication with doses (with emphasis on active vitamin D or its

analogs, and phosphate binders).
Pulse pressure was evaluated in the two mid-week HD sessions in which blood chemistry analyses were obtained, based on blood pressure measurement before HD. Pulse pressure was calculated by the formula PP = SBP - DBP (PP, pulse pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure).

- **Biochemical analysis**

Serum 25(OH)D and 1,25$(OH)_2$D were measured on two occasions (after summer, in November and after winter, in June), with a radioimmunoassay provided by IDS (Boldon, UK). Following an extraction procedure, the assay was carried out with the anti-25-hydroxyvitamin D or the anti-1,25-dihydroxyvitamin D ovine antibody and phase separation was performed with anti-ovine IgG antiserum. The assay measured 25$(OH)D_3$ and 25$(OH)D_2$ or 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$. Intra-assay and inter-assay variability were 5% and 8%, respectively. The normal range for 25(OH)D was 10 - 60 ng/mL and for 1,25$(OH)_2$D was 20 - 46 pg/mL.

Definitions of deficiency (< 15 ng/mL), insufficiency (< 30 ng/mL) and normal values (> 30 ng/mL) for 25(OH)D were based on KDOQI guidelines for predialysis [38], whereas the definitions used for 1,25$(OH)_2$D were based on biochemical normality of the assay (deficiency < 20 pg/mL and normal values > 20 pg/mL).

Serum calcium, serum phosphorus, calcium x phosphorus product, iPTH, bALP, hemoglobin, albumin and CRP were measured simultaneously with 25(OH)D and 1,25$(OH)_2$D. Calcium was corrected for hypoalbuminemia by the addition of 0.8 mg/dL to the Ca concentration for each 1 g/dL decrease in albumin concentration from 4.0 g/dL. Total iPTH was evaluated by immunochemiluminescence using a second-generation assay, and the normal range of values is 10 - 65 pg/mL. Albumin was measured using a colorimetric assay and CRP was evaluated by an immunoturbidimetric assay.

The levels of BNP were determined in June, in pre-HD collected ethylenediaminetetraacetic acid (EDTA) plasma samples, using the AxSYM BNP Assay on the AxSYM 2 Immunochemical Analyzer (Abbott Laboratories, Chicago IL, USA). In a previous study [194], we found no significant difference in BNP-measured levels before or after dialysis. All blood samples were collected before HD in midweek sessions.

- **Echocardiographic evaluation**

During the study, each patient underwent an echocardiographic examination (M mode and 2D), and left ventricular mass index (LVMI) was calculated using the Devereux formula [195], indexed to body surface area. The presence of left ventricular hypertrophy was defined based on an LVMI > 125 g/m^2 for both men and women. This exam was performed on a midweek nondialysis day and in the same cardiologic center.

- **Vascular calcification score**

Scoring of vascular calcifications through radiography of the pelvis and hands by Adragão score [180] was also performed for all patients.

- **Statistical analysis**

For statistical analysis, the arithmetic media of the two laboratory measurements (after summer and after winter) was used. Variables were expressed as frequencies for categorical variables. Mean values with standard deviation (SD) for normally distributed variables and median (interquartile range) for non-normally distributed variables, in continuous variables.

Comparison between groups was performed using Mann-Whitney U and chi-square tests. Spearman correlation was used for univariable analysis and linear regression for multivariable analysis (confidence interval - CI of 95%). Variables entered in multivariable analysis were diabetes, albumin, BNP, pulse pressure, and vascular calcification score. For multiple correlations, Bonferroni adjustment was performed.

Survival curves were estimated by Kaplan-Meier analysis and compared by log-rank test. An adjusted Cox regression model was used to identify predictors of mortality. Statistical analysis was performed with the SPSS 16.0 (SPSS Inc., Chicago, IL, USA). For all comparisons, a $p < 0.05$ was considered statistically significant.

RESULTS

The study included 223 patients, 116 (52%) males, with a mean age of 62.7 ± 15.3 years and a median vintage on HD of 44 months.

The demographic, biochemical and vascular characteristics of the population are shown in Table 6.

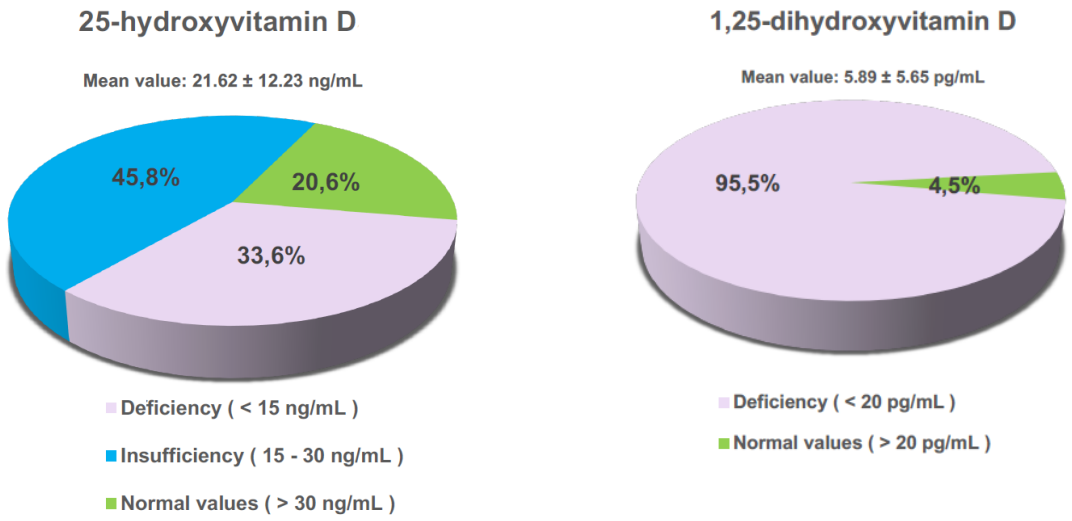
Variable	Patients (n = 223)
Age (years)	62.7 ± 15.3
Male gender (n, [%])	116 (52%)
HD duration (months)	42.9 ± 39.3
Diabetes (n, [%])	60 (27%)
Hypertension (n, [%])	71 (32%)
Coronary disease (n, [%])	65 (29%)
Active vitamin D therapy (n, [%])	104 (47%)
Calcium carbonate therapy (n, [%])	14 (6%)
Sevelamer therapy (n, [%])	160 (72%)
Calcium (mg/dL)	8.5 ± 0.6 (6.9 - 10.5)
Phosphorus (mg/dL)	4.6 ± 1.5 (1.7 - 8.7)
Calcium x phosphorus $(mg/dL)^2$	48.6 ± 15.5 (16.5 - 83.2)
iPTH (pg/mL)	277 ± 333 (3 - 4,461)
bALP (µg/L)	20.3 ± 14.3 (2.2 - 119.5)
Hemoglobin (g/dL)	12.3 ± 1.3 (8.2 - 15.4)
C-reactive protein (mg/dL)	0.9 ± 1.6 (0.1 - 17.4)
Albumin (g/dL)	4.1 ± 0.4 (3.1 - 5.1)
25(OH)D (ng/mL)	21.6 ± 12.2 (5.6 - 52.8)
$1,25(OH)_2D$ (pg/mL)	5.9 ± 5.7 (0.1 - 49.1)
BNP (pg/mL)	596 ± 689 (11 - 3851)
PP (mmHg)	69 ± 18 (33 - 116)
LVMI (g/m^2)	133 ± 34 (64 - 224)
SVCS > 0 (n, [%])	130 (58%)
SVCS ≥ 3 (n, [%])	80 (36%)

bALP, bone alkaline phosphatase; BNP, brain natriuretic peptide; PP, pulse pressure; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

Table 6 - Clinical, biochemical, and vascular parameters of the studied population

Characterization of serum calcidiol levels and identification of patients with sufficiency, insufficiency, and deficiency levels of this vitamin.

In this study, serum 25(OH)D was below sufficiency values (< 30 ng/mL) in almost 80% of the patients, and levels of $1,25(OH)_2D$ were considered deficient (< 20 pg/mL) in more than 95% of the patients (Graphic 1).



Graphic 1 - Serum 25(OH)D and $1,25(OH)_2D$ distribution in the studied population

Comparing serum levels obtained in June (after winter) with those of November (after summer), 25(OH)D serum values were higher following summer, but not statistically significant (22 ± 16.0 *vs*. 20.8 ± 12.3 ng/mL; p NS). Serum levels of $1,25(OH)_2D$ were similar on the two occasions (6.4 ± 7.6 *vs*. 6.4 ± 7.7 pg/mL; p NS).

A comparison of studied variables according to levels of 25(OH)D is shown in Table 7 and according to $1,25(OH)_2D$ levels is shown in Table 8.

25(OH)D	< 30 ng/mL (n = 177)	> 30 ng/mL (n = 46)	p
Age (years)	63.8 ± 15.4	58.5 ± 14.7	<0.001
Male gender (n, [%])	85 (48%)	24 (52%)	NS
HD vintage (months)	43.9 ± 41.3	39.2 ± 30.6	0.03
Diabetes (n, [%])	65 (37%)	10 (21%)	0.01
Hypertension (n, [%])	60 (34%)	14 (31%)	NS
Coronary disease (n, [%])	57 (32%)	13 (28%)	NS
Active vitamin D therapy (n, [%])	73 (41%)	24 (52%)	NS
Calcium carbonate therapy (g/day)	1.5 ± 0.6	1.7 ± 0.8	NS
Sevelamer therapy (g/day)	3.6 ± 1.8	3.9 ± 1.7	NS
Calcium (mg/dL)	8.6 ± 0.6	8.7 ± 0,7	NS
Phosphorus (mg/dL)	4.5 ± 1.5	4.7 ± 1.6	NS
Calcium x phosphorus $(mg/dL)^2$	49.3 ± 13.8	51.9 ± 15.1	NS
iPTH (pg/mL)	312 ± 284	337 ± 271	NS
bALP (µg/L)	20.3 ± 18.1	20.2 ± 12.3	NS
Hemoglobin (g/dL)	12.3 ± 1.3	12.6 ± 1.5	NS
C-reactive protein (mg/dL)	1.0 ± 1.8	0.5 ± 0.7	0.002
Albumin (g/dL)	4.1 ± 0.4	4.4 ± 0.2	0.004
$1,25(OH)_2D$ (pg/mL)	4.5 ± 5.2	6.0 ± 5.7	0.01

Comparison between means: Mann-Whitney U; comparison between frequencies: chi-square.

Table 7 - Comparison of studied variables according to 25(OH)D serum levels

Older patients, with higher HD vintage and diabetics presented lower 25(OH)D serum values. We found no differences regarding active vitamin D prescription in 25(OH)D levels (Table 7). Diabetics also show decreased values of $1,25(OH)_2D$. Patients taking active vitamin D had significantly lower $1,25(OH)_2D$ levels, but with the same 25(OH)D values (Table 8).

$1,25(OH)_2D$	< 20 pg/mL (n = 213)	> 20 pg/mL (n = 10)	p
Age (years)	62.4 ± 15.6	65.7 ± 12.8	NS
Male gender (n, [%])	109 (52%)	6 (60%)	NS
HD vintage (months)	42.5 ± 38.0	47.4 ± 52.3	NS
Diabetes (n, [%])	60 (28%)	1 (10%)	0.03
Hypertension (n, [%])	83 (39%)	4 (40%)	NS
Coronary disease (n, [%])	70 (33%)	3 (30%)	NS
Active vitamin D therapy (n, [%])	109 (51%)	3 (30%)	<0.001
Calcium carbonate therapy (g/day)	1.8 ± 0.9	1.7 ± 1.1	NS
Sevelamer therapy (g/day)	3.9 ± 1.9	3.3 ± 2.1	NS
Calcium (mg/dL)	8.7 ± 0.7	8.8 ± 0.6	NS
Phosphorus (mg/dL)	4.5 ± 0.7	4.0 ± 1.4	NS
Calcium x phosphorus $(mg/dL)^2$	49.3 ± 14.6	47.8 ± 15.3	NS
iPTH (pg/mL)	261 ± 352	245 ± 121	NS
bALP (µg/L)	17.0 ± 13.5	15.1 ± 9.7	NS
Hemoglobin (g/dL)	12.5 ± 1.3	12.3 ± 1.3	NS
C-reactive protein (mg/dL)	0.8 ± 1.7	0.6 ± 0.7	NS
Albumin (g/dL)	4.2 ± 0.4	4.1 ± 0.2	NS
25(OH)D (ng/mL)	22.6 ± 16.5	22.5 ± 8.9	NS

Comparison between means: Mann-Whitney U; comparison between frequencies: chi-square.

Table 8 - Comparison of studied variables according to $1,25(OH)_2D$ serum levels

Deficiency of 25-hydroxyvitamin D and inflammation.

In our population, serum 25(OH)D was negatively associated ($r = -0.25$; $p < 0.001$) with CRP levels. The potential role of 25(OH)D as an anti-inflammatory mediator (or at least as a marker) was reinforced by a coexistent positive association with albumin ($r = 0.23$; $p = 0.001$) (Table 9). These associations were confirmed in the multivariable analysis (Table 10).

Associations between 1,25(OH)$_2$D, CRP and albumin were not observed.

Deficiency of 25-hydroxyvitamin D and cardiovascular risk markers.

Plasma BNP values were high (574 ± 696 pg/mL), pulse pressure was elevated (69 ± 18 mmHg), and 127 (57%) patients had echocardiographic criteria of left ventricular hypertrophy (LVMI > 125 g/m^2). Vascular calcifications were identified in 130 (58%) patients. A simple vascular calcification score ≥ 3 was observed in 80 (36%) patients. There was no difference in serum calcium, phosphorus, calcium x phosphorus product and iPTH between patients with or without vascular calcifications.

In univariable analysis, serum levels of 25(OH)D were negatively associated with BNP (r = - 0.18; p = 0.009), pulse pressure > 65 mmHg (r = - 0.19; p = 0.003), and simple vascular calcification score (r = - 0.26; p < 0.001), after Bonferroni adjustment (Table 9).

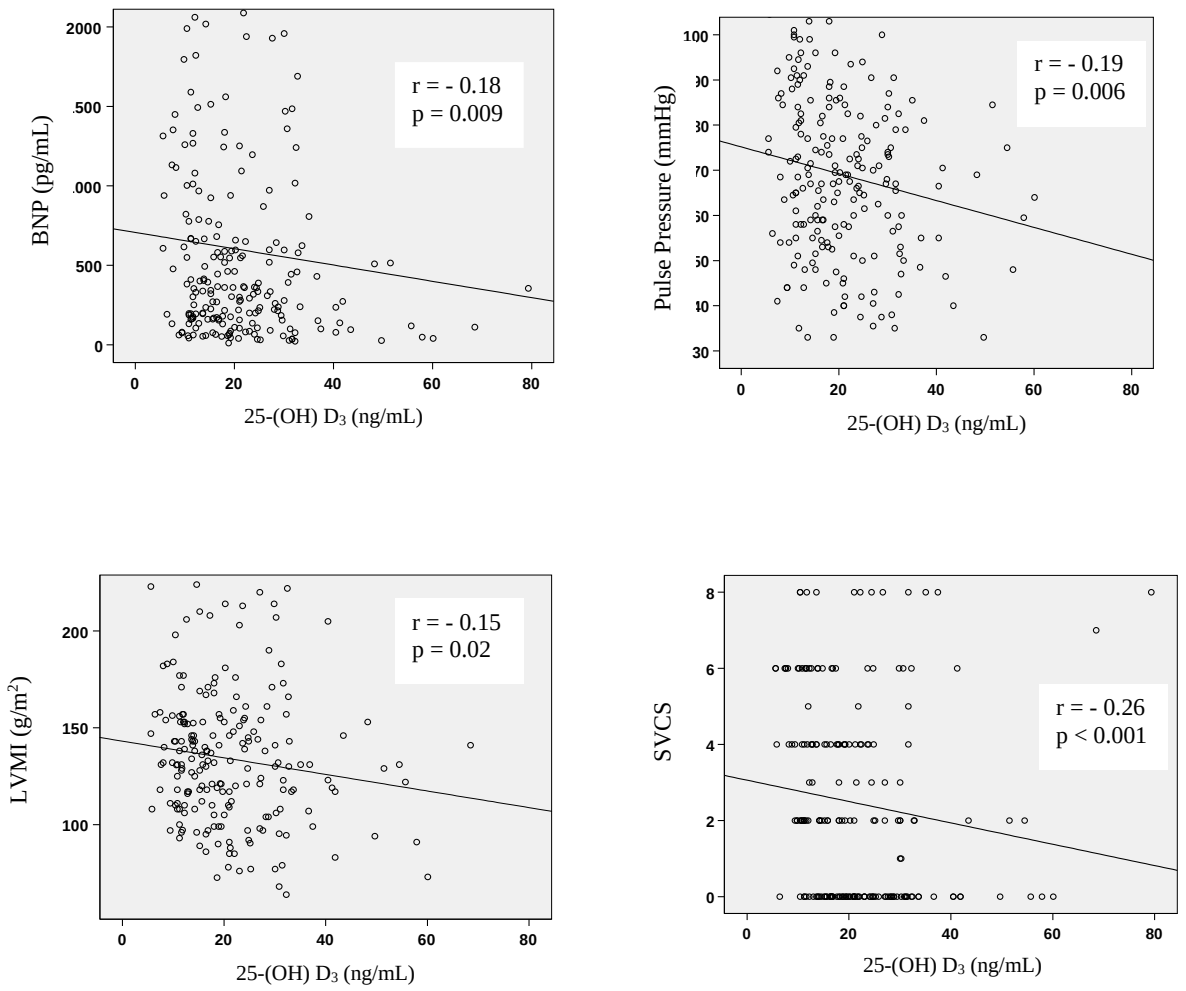
	25(OH)D	
	r	p
Age	- 0.31	< 0.001*
Time on HD	- 0.14	0.04
Diabetes	- 0.20	0.004*
C- reactive protein	- 0.25	< 0.001*
Albumin	0.23	0.001*
BNP	- 0.18	0.009*
PP > 65 mmHg	- 0.19	0.003*
LVMI	- 0.15	0.02
SVCS	- 0.26	< 0.001*

BNP, brain natriuretic peptide; PP, pulse pressure; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

* Statistically significant after Bonferroni adjustment.

Table 9 - Univariable associations between 25-hydroxyvitamin D and other studied parameters (Spearman correlation)

The inverse associations between 25(OH)D levels and plasma BNP, pulse pressure, left ventricular mass index, and simple vascular calcification score are shown in Figure 11.



BNP, brain natriuretic peptide; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

Figure 11 - Inverse associations between 25-hydroxyvitamin D levels and BNP, pulse pressure, left ventricular mass index, and simple vascular calcification score

Serum levels of $1,25(OH)_2D$ did not show any association with BNP, pulse pressure, LVMI or vascular calcifications.

On multivariable analysis, serum levels of 25(OH)D were independently associated with diabetes ($p < 0.001$), lower albumin levels ($p = 0.003$), higher $\log_{10}$ BNP values ($p = 0.005$), PP > 65 mmHg ($p = 0.006$) and a higher simple vascular calcification score (≥ 3) ($p = 0.002$) (Table

10). Active vitamin therapy was associated with a lower simple vascular calcification score (< 3) (p = 0.03).

Dependent variable	Independent variables	β	95% CI	p	R^2
25(OH)D	Diabetes mellitus	- 0.44	- 0.23 to - 0.01	<0.001	0.532
	Albumin	0.41	1.24 to 2.68	0.003	
	Log_{10} BNP	- 0.37	- 0.24 to - 0.02	0.005	
	PP > 65 mmHg	- 0.36	- 0.41 to - 0.05	0.006	
	SVCS ≥ 3	- 0.39	- 0.28 to - 0.04	0.002	

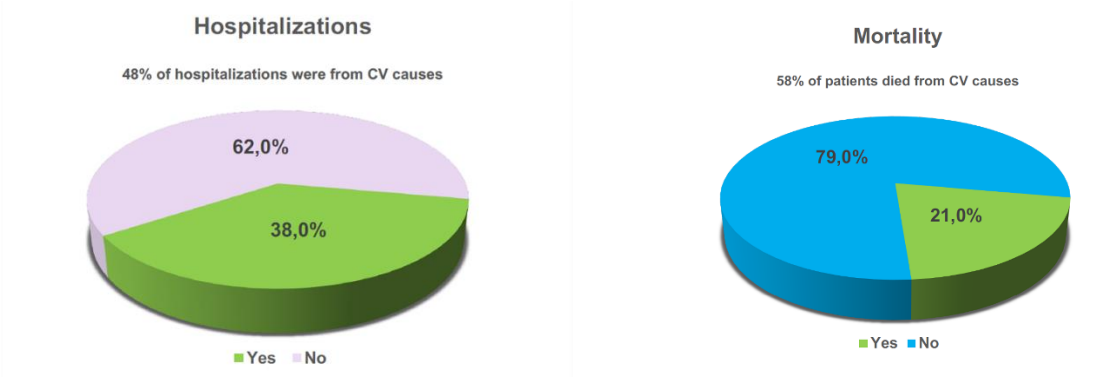
BNP, brain natriuretic peptide; PP, pulse pressure; SVCS, simple vascular calcification score.

Table 10 - Associations between 25-hydroxyvitamin D and cardiovascular risk factors (multivariable analysis - linear regression)

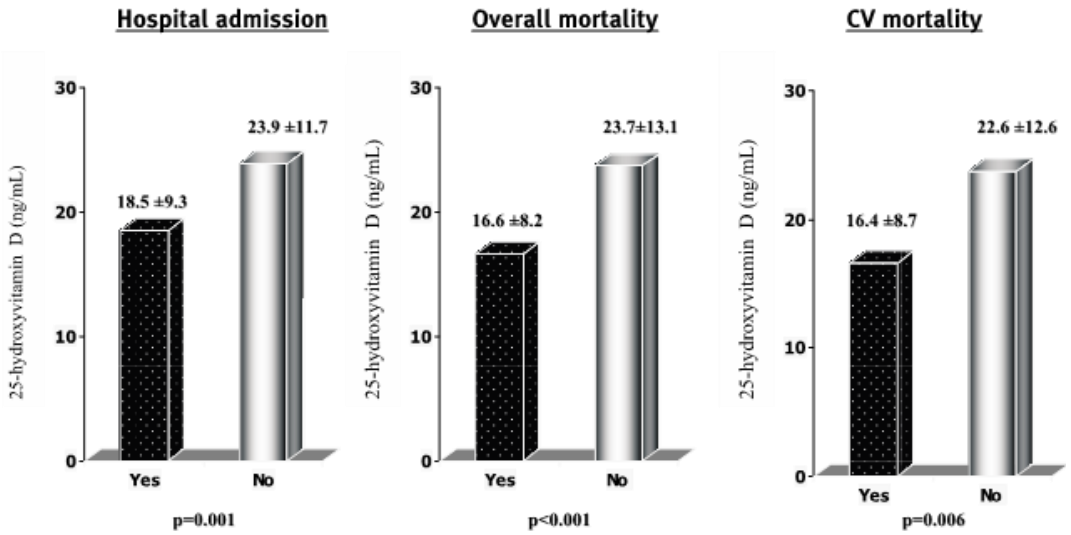
Deficiency of 25-hydroxyvitamin D, hospitalizations, and mortality.

During the study, 38% of the patients were admitted to the hospital at least once and 21% of the patients died (mainly from CV causes) (Graphic 2). The median hospitalization episodes per patient was 1 and the days of hospitalization per patient was 11.

Levels of 25(OH)D were significantly lower in the patients that died from all causes (16.6 ± 8.2 *vs*. 23.7 ± 1 3.1 ng/mL; p < 0.001) and in patients that died from CV causes (16.4 ± 8.7 *vs*. 22.6 ± 12.6 ng/mL; p = 0.006). Levels of 25(OH)D were also lower in patients admitted to the hospital during the study (18.5 ± 9.3 *vs*. 23.9 ± 11.7 ng/mL; p = 0.001) (Graphic 3). Levels of $1,25(OH)_2D$ were similar in all groups.



Graphic 2 - Hospitalizations and mortality in the studied population



Graphic 3 - Levels of 25-hydroxyvitamin D according to hospital admissions, overall, and cardiovascular mortality

In multivariable analysis, lower levels of 25(OH)D were predictors of hospitalizations (p = 0.007), death from all causes (p = 0.002) and from CV causes (p = 0.03), as shown in Table 11.

Dependent variable	Independent variables	OR	95% CI	p	R^2
Hospitalizations	Age	1.8	1.05 to 3.12	0.008	0.436
	Time on HD	3.3	2.03 to 3.73	< 0.001	
	Diabetes mellitus	1.5	1.10 to 1.57	0.01	
	25(OH)D	0.8	0.78 to 0.93	0.007	
Overall mortality	Age	1.4	1.08 to 1.53	0.02	0.471
	Time on HD	5.2	1.70 to 13.52	< 0.001	
	Diabetes mellitus	7.3	2.81 to 18.83	< 0.001	
	25(OH)D	0.9	0.88 to 0.99	0.002	
CV mortality	Age	1.7	1.12 to 2.98	0.01	0.418
	Time on HD	5.7	1.94 to 14.37	0.005	
	Coronary disease	1.8	1.23 to 3.34	0.02	
	Diabetes mellitus	2.9	1.29 to 7.37	0.008	
	25(OH)D	0.7	0.62 to 0.85	0.03	

Table 11 - Lower 25(OH)D levels were predictors of hospitalization and mortality (overall and CV) - Cox regression adjusted for age, vintage on HD and diabetes for hospitalizations and overall mortality, and also for coronary disease in CV mortality

Patients with 25(OH)D deficiency (< 15 ng/mL) had a significantly lower survival at the end of the 48 months (p < 0.001) (Figure 12).

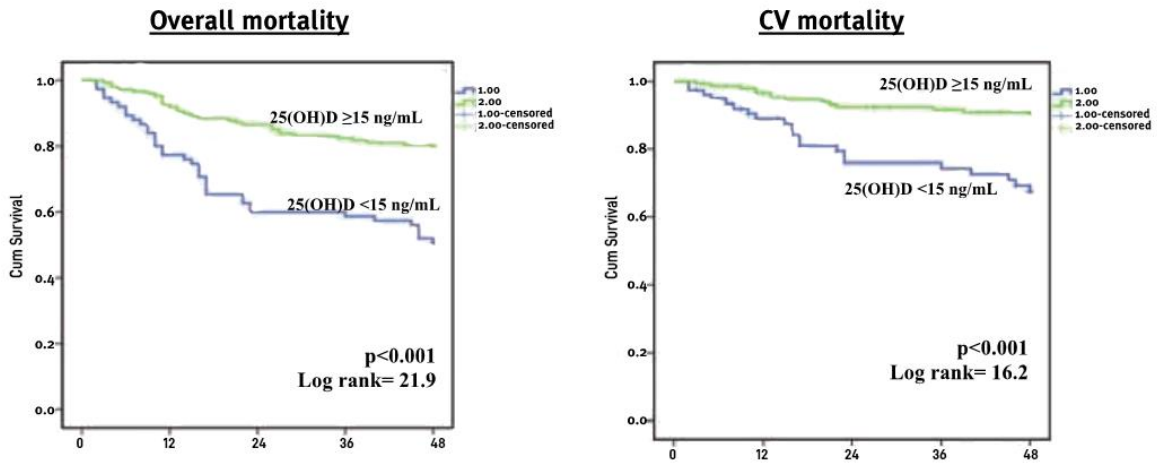


Figure 12 - Lower survival in relation to overall and cardiovascular mortality in patients with 25-hydroxyvitamin D < 15 ng/mL (Kaplan-Meier analysis)

5.2. LONG-TERM CHOLECALCIFEROL SUPPLEMENTATION IN HEMODIALYSIS PATIENTS: EFFECTS ON MINERAL METABOLISM, INFLAMMATION, AND CARDIAC PARAMETERS

PATIENTS AND METHODS

- **Study design**

This was a 5-year follow-up prospective study of a cohort of prevalent HD patients from a single-center. Baseline serum 25(OH)D levels were measured on two occasions with a 6-month interval (end of winter and of summer, respectively) and after 6 and 60 months of cholecalciferol supplementation. Its effects on mineral metabolism, inflammation, and cardiovascular risk markers were evaluated during the study.

- **Eligibility criteria**

The inclusion criteria were age above 18 years old and the capability to give informed written consent. The exclusion criteria were previous parathyroidectomy and current therapy with calcimimetics or calcium carbonate.

- **Population**

All the patients were submitted to online hemodiafiltration with a dialysate calcium concentration of 1.25 or 1.5 mmol/L. The following data were collected: age, gender, presence of diabetes, hypertension, coronary artery disease, and active medication with doses (with emphasis on active vitamin D or its analogs, phosphate binders, darbepoetin, antihypertensive medication, and statins). The target for ferritin was between 200 and 800 ng/mL for all patients, and when needed, only intravenous iron saccharate was used.

SBP and DBP were measured in all HD sessions, and for this study, its mean values in the month before the beginning of supplementation and in the last month of cholecalciferol supplementation were considered. Pulse pressure was also calculated.
The percentage of patients on antihypertensive therapy, namely angiotensin-converting

enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) and treated with statins was not different at the beginning and at the end of the study. Interdialytic weight gain in the last 3 months before cholecalciferol supplementation, and in the last 3 months of supplementation was similar.

Oral cholecalciferol (Vigantol®) was prescribed once or thrice weekly, after each HD session, ensuring 100% adhesion. Supplementation was started in all patients at the same time and was maintained for 60 months. In the first 6 months, it was based on baseline calcidiol serum levels: 50,000 IU (2.5 mL or 75 drops) once a week for patients with 25(OH)D levels < 15 ng/mL, 10,000 IU (0.5 mL or 15 drops) once a week when 25(OH)D was between 16 and 30 ng/mL, and 2,700 IU (4 drops) three times per week when levels were > 30 ng/mL. The median dose of cholecalciferol used in the first 6 months was 21,000 IU/week. Afterward and until the end of the study, all patients were medicated with the same dose of 8,000 IU/week.

- **Biochemical analysis**

Serum 25(OH)D was measured on four occasions, at baseline (after summer, in November and after winter, in June), and after 6 and 48 months of supplementation. Serum $1,25(OH)_2D$ was measured only at baseline and after 6 months of cholecalciferol supplementation.

Serum calcium, serum phosphorus, calcium x phosphorus product, Mg, iPTH, bALP, hemoglobin, ferritin, albumin, and CRP were evaluated before the beginning of supplementation, quarterly during the studied period, and at the end of the study. The levels of BNP were determined before the start and at the end of supplementation.

- **Echocardiographic evaluation**

At the beginning and at the end of the study, each patient underwent an echocardiographic examination, and LVMI was calculated as described before.

- **Statistical analysis**

For statistical analysis, the arithmetic mean of the two measurements of 25(OH)D at baseline (end of winter and summer, respectively) was used.
Demographic and laboratory data were compared at baseline and after 60 months of

cholecalciferol supplementation.

Studied variables were analyzed using paired t-test or Wilcoxon rank sum test, as appropriate. Spearman's rank correlations were used for univariable analysis. A $p < 0.05$ was considered statistically significant.

RESULTS

The final study population was composed of 97 patients, 57 (59%) males, with a mean age of 63.2 ± 14.1 years and a median HD vintage at baseline of 25 months Patients who were lost to follow-up were excluded from the analysis (patients that died, were transferred, and received a transplant) as shown in the flow chart of the study (Figure 13).

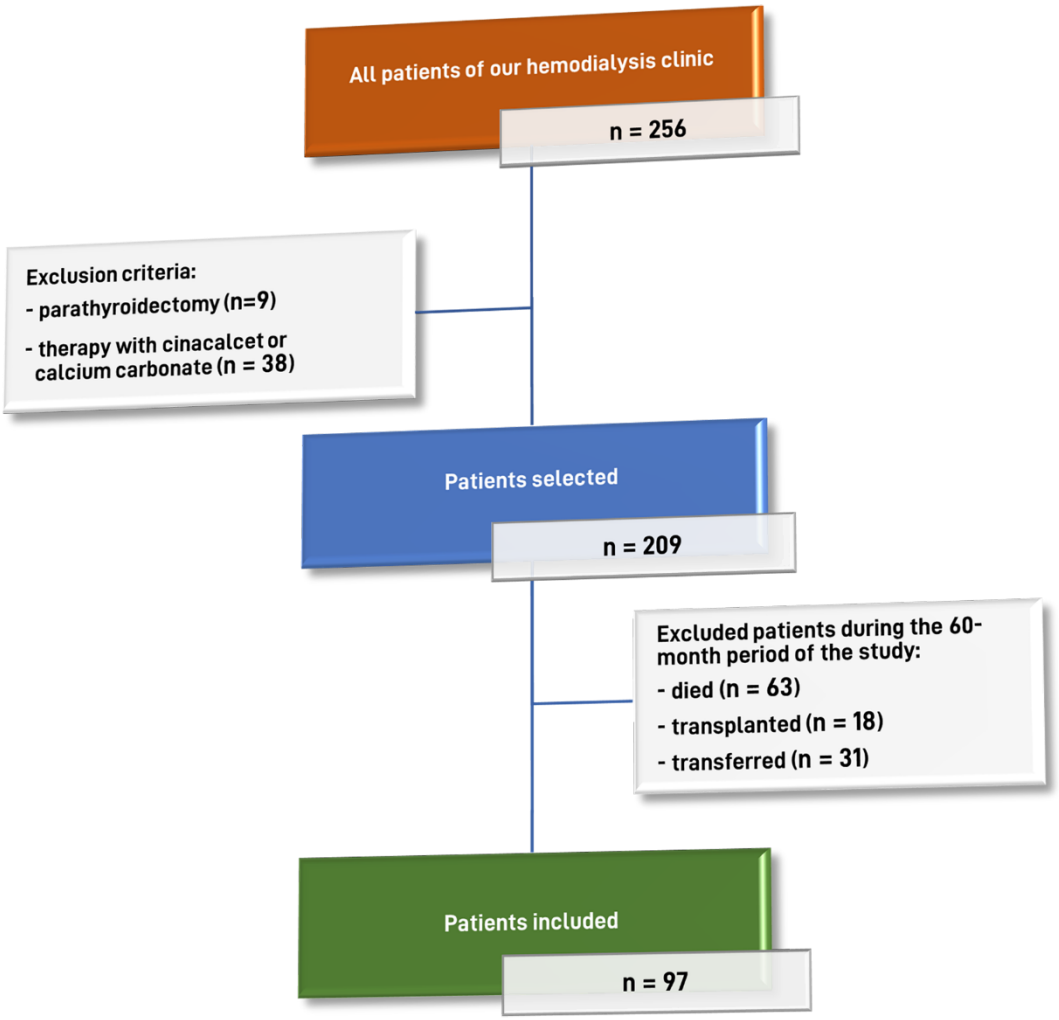


Figure 13 - Flow chart of the study

Twenty-six (27%) patients had diabetes, 37 (38%) had hypertension, and 28 (29%) had coronary artery disease. At baseline, 37 (38%) patients were taking intravenous (IV) paricalcitol, with a mean dosage of 6.2 ± 2.8 (2.5 - 22.5) µg/week. For economic reasons, after 6 months of cholecalciferol supplementation, the active vitamin D taken by all the patients was changed to oral alfacalcidol, with a mean dosage of 3.2 ± 1.7 (1 - 9) µg/week).

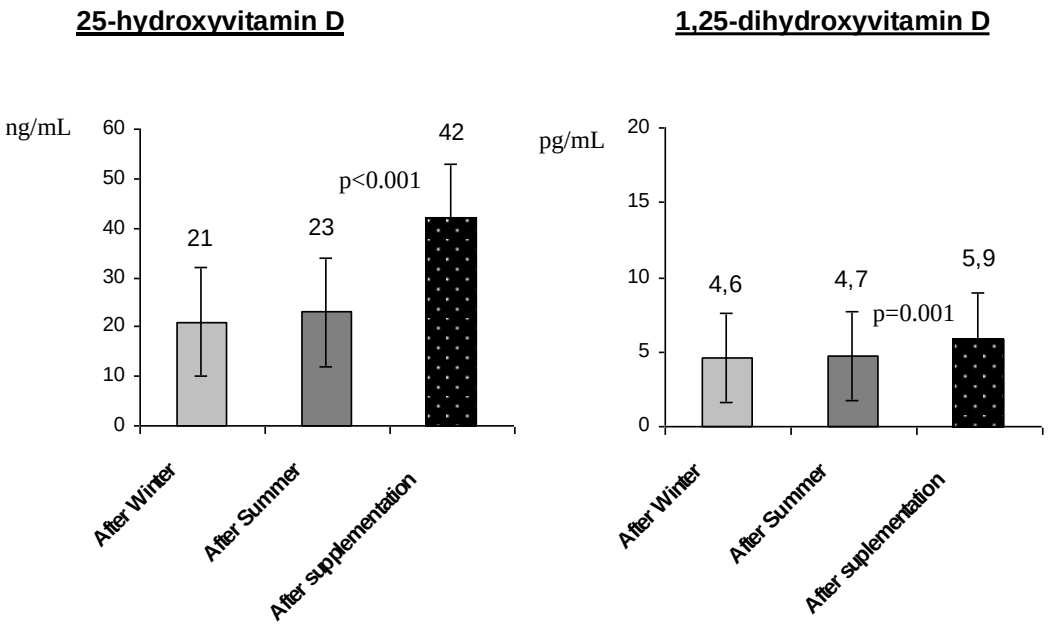
At the beginning of the study, 50 patients (52%) were under phosphate binders, and all the patients were taking sevelamer with a median dosage of 3,200 (1,600 - 4,800) mg/day. After 6 months of supplementation, about half of the patients taking sevelamer were changed to calcium acetate / Mg carbonate with a median dosage of 1,675 (1,340 - 6,030) mg/day.

A total of 91 (94%) of the patients were on darbepoetin therapy, with a median dosage of 0.038 µg/kg/week per g/dL. The target for ferritin was between 200 and 800 ng/mL for all patients, and if needed, only IV iron saccharate was used.

Mg dietary intake was quantified, yearly, by the dietician of the dialysis center and remained stable during the studied period.

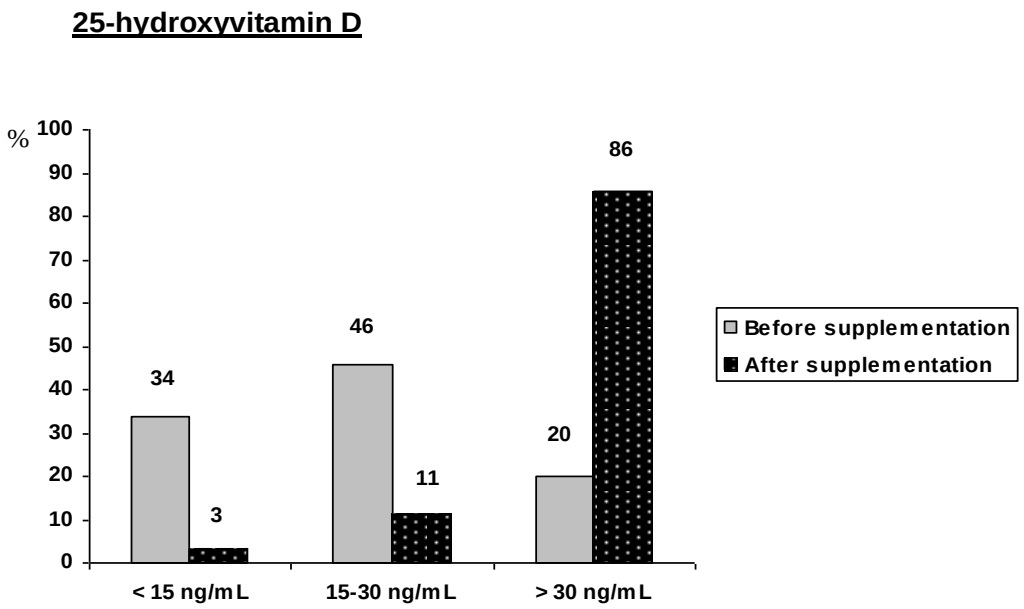
Levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D after 6 months of cholecalciferol supplementation.

The mean of post-winter and post-summer baseline measurements of 25(OH)D and $1,25(OH)_2D$ were compared with the values obtained after 6 months of oral cholecalciferol supplementation in 158 patients. There was a significant increase in 25(OH)D (22.3 ± 12.0 *vs.* 42.0 ± 12.1 ng/mL; $p < 0.001$), and $1,25(OH)_2D$ (4.6 *vs.* 5.9 pg/mL; $p = 0.001$) levels (Graphic 4).



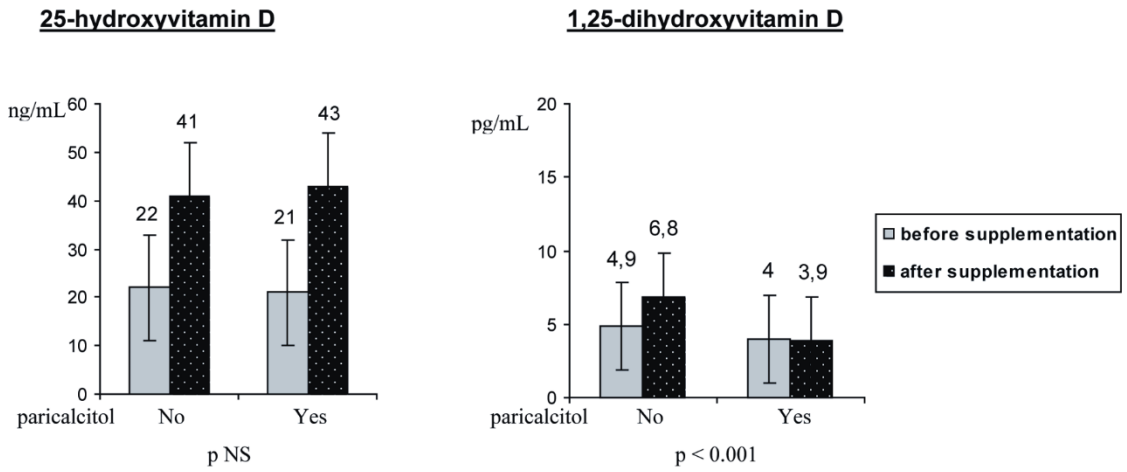
Graphic 4 - Serum levels of 25(OH)D and $1,25(OH)_2D$ after winter, after summer, and after 6 months of cholecalciferol supplementation

In 86% of the patients, 25(OH)D levels became normal (> 30 ng/mL), in comparison with only 20% at baseline (Graphic 5).



Graphic 5 - Serum 25(OH)D distribution before and after 6 months of cholecalciferol supplementation

Patients who were taking active vitamin D (paricalcitol) during the first 6 months of the study showed significantly lower 1,25(OH)$_2$D levels (3.9 *vs.* 6.8 pg/mL; p < 0.001) despite a similar increase in 25(OH)D values (Graphic 6).

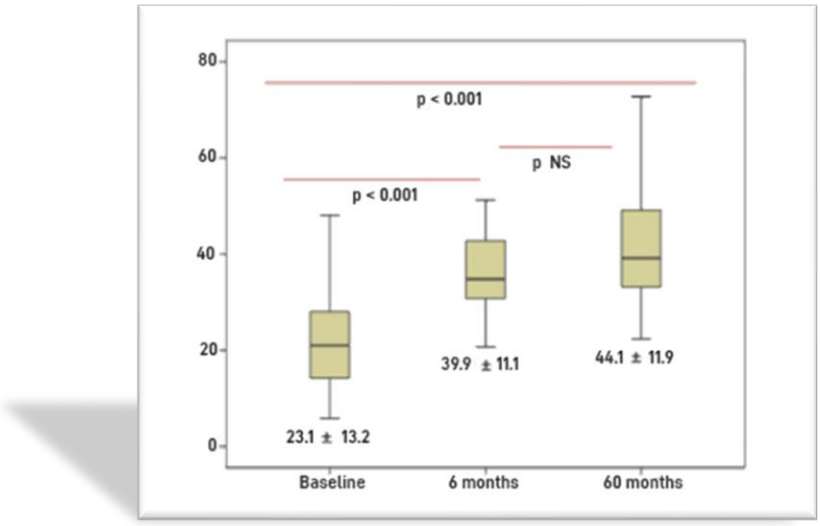


Graphic 6 - Serum levels of 25(OH)D and 1,25(OH)$_2$D before and after 6 months of cholecalciferol supplementation in patients without and with paricalcitol therapy

Levels of 25-hydroxyvitamin D after 60 months of cholecalciferol supplementation.

In the 97 patients that reached the end of follow-up, there was a significant increase in 25(OH)D levels after 60 months (23.1 ± 13.2 *vs.* 44.1 ± 11.9 ng/mL; p < 0.001) of supplementation compared with mean baseline values (Graphic 7).

Comparing 25(OH)D values obtained after 6 and 60 months of cholecalciferol supplementation, there was a slight, but not significant, increase at the end of the study.



Graphic 7 - Mean 25-hydroxyvitamin D serum levels at baseline and after 6 and 60 months of cholecalciferol supplementation

In 91 (94%) patients, 25(OH)D levels became normal (> 30 ng/mL) at the end of the study, which was slightly above the 86% obtained after the first 6 months. This was statistically different from the numbers in baseline where only 20% of the patients had normal values. Patients whose 25(OH)D levels did not increase all had diabetes and hypomagnesemia (Mg < 0.7 mmol/L). None of the patients achieved a toxic level of 25(OH)D > 100 ng/mL and there were no episodes of hypercalcemia > 10.5 mg/dL during the study.

Clinical and biochemical parameters at the beginning and at the end of cholecalciferol supplementation are reported in Table 12.

	Before supplementation	After supplementation	P
25(OH)D (ng/mL)	23.1 ± 13.2	44.1 ± 11.9	<0.001
Calcium (mg/dL)	8.6 ± 0.5	8.4 ± 0.2	0.02
Phosphorus (mg/dL)	4.7 ± 0.9	4.5 ± 0.8	0.018
Magnesium (mmol/L)	1.34 ± 0.16	1.38 ± 0.18	0.03
iPTH (pg/mL)	267 (311)	227 (243)	0.03
bALP (µg/L)	18.1 (11.4)	18.5 (9.9)	NS
Hemoglobin (g/dL)	11.6 ± 1.2	11.5 ± 1.3	NS
Ferritin (ng/mL)	441 ± 167	437 ± 181	NS
Albumin (g/dL)	3.8 ± 0.7	4.1 ± 0.4	<0.001
CRP (mg/dL)	0.9 (1.7)	0.7 (1.3)	0.01
BNP (pg/mL)	343 (211)	336 (188)	<0.001
eKt/V	1.5 ± 0.3	1.6 ± 0.5	NS
nPCR (g/kg/day)	1.26 ± 0.6	1.28 ± 0.7	NS
Active vitamin D (%)	38	29	<0.001
Phosphate binders (%)	52	43	0.02
Darbepoetin (%)	92	90	NS
Darbepoetin dose (µg/kg/week)	0.033 (0.027)	0.026 (0.019)	0.02
Antihypertensive therapy (%)	72	69	NS
ACE inhibitors (%)	44	42	NS
ARBs (%)	11	9	NS
Statins (%)	22	24	NS
PP (mmHg)	71 ± 14	64 ± 16	0.007
Interdialytic weight gain (mL)	1,618 ± 958	1,648 ± 899	NS
Dry weight (Kg)	67.1 ± 14.1	66.8 ± 13.2	NS
LVMI (g/m^2)	135 ± 29	122 ± 31	0.02

ACE, angiotensin-converting enzyme; ARBs, angiotensin II receptor blockers; BNP, brain natriuretic peptide; bALP, bone alkaline phosphatase; CRP, C-reactive protein; LVMI, left ventricular mass index; nPCR, normalized protein catabolic rate; PP, pulse pressure; 25(OH)D, 25-hydroxyvitamin D. Percentage, mean ± SD or median (interquartile range), used as appropriate.

Table 12 - Clinical, biochemical, and echocardiographic parameters before and after 60 months of cholecalciferol supplementation

Effects of long-term cholecalciferol supplementation on mineral metabolism.

Serum calcium (p = 0.02) and phosphorus (p = 0.018) showed a significant reduction during supplementation. This fact was independent of active vitamin D therapy since patients who were not administered active vitamin D during the studied period also presented this reduction.

Mg serum levels increased significantly during the study (p = 0.03). Levels of iPTH (p = 0.03) presented a significant decrease with supplementation. Only five patients (5%) of those who had iPTH levels between 150 and 300 pg/mL at the beginning of the study showed values below 150 pg/mL after supplementation.

There was a significant reduction in the number of patients treated with active vitamin D (p < 0.001) with cholecalciferol supplementation. Paricalcitol dose (7.2 ± 4.5 vs. 6.0 ± 4.1 µg/week; p < 0.001) was decreased during the first 6 months, and the dose of alfacalcidol at the end of the study was also reduced compared with the dose at 6 months (3.2 ± 1.7 *vs.* 2.1 ± 1.5 µg/week; p < 0.001). Patients taking active vitamin D during the study showed a similar increase in 25(OH)D values compared with patients not taking this medication. There was also a significant decrease in the number of patients treated with phosphate binders after supplementation (p = 0.02).

Cholecalciferol supplementation and inflammation.

Serum albumin increased (p < 0.001), and CRP decreased (p = 0.01) with supplementation. Darbepoetin dose also decreased during the study (p = 0.02), with no modification of hemoglobin and ferritin values.

Long-term cholecalciferol supplementation and cardiac parameters.

Although BNP plasma levels were high, compared with the general population with no cardiac disease, they showed a significant reduction during the study (p < 0.001). Pulse pressure (p = 0.007) and LVMI (p = 0.02) also had a significant decrease after

supplementation.

There was a significant negative association between the percentage of change in 25(OH)D *versus* the percentage of change in BNP levels ($r = -0.45$; $p = 0.001$), pulse pressure ($r = -0.41$; $p = 0.001$), and LVMI ($r = -0.81$; $p < 0.001$) (Figure 14). The percentage of change in BNP was positively correlated with the percentage of change in LVMI ($r = 0.39$; $p = 0.008$).

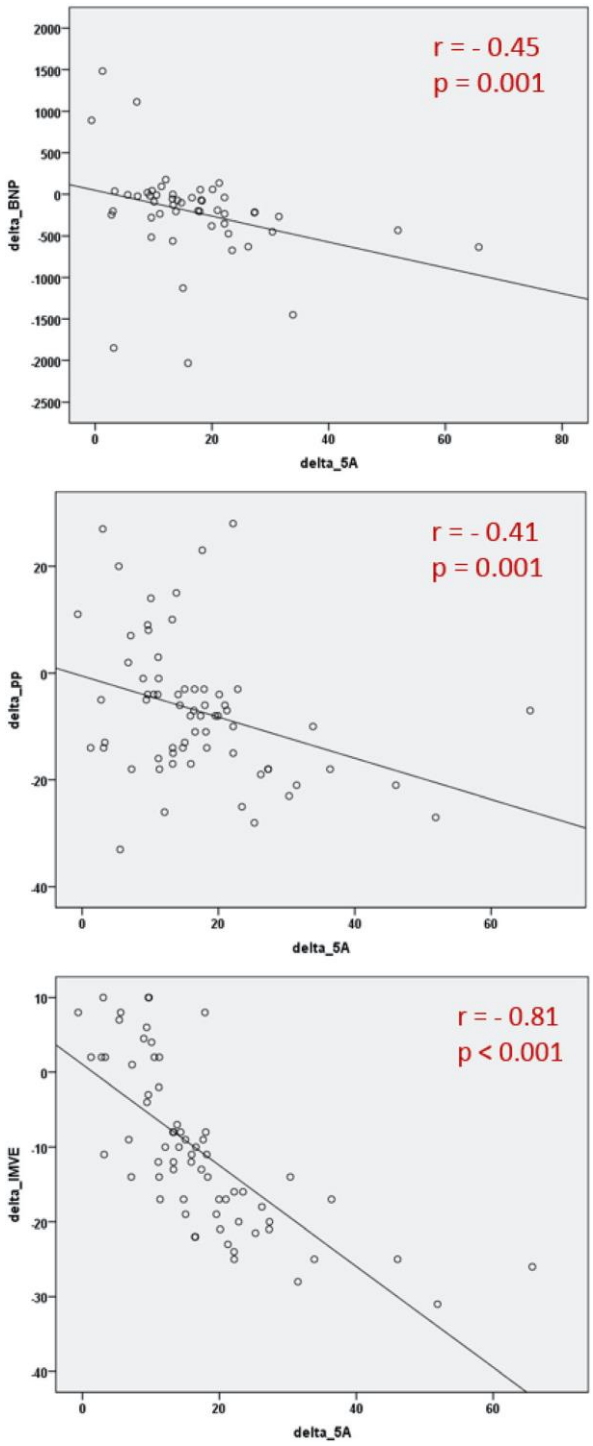


Figure 14 - Associations between the percentage of change in 25-hydroxyvitamin D and the percentage of change in brain natriuretic peptide (BNP), pulse pressure (PP), and left ventricular mass index (LVMI) with cholecalciferol supplementation

MAGNESIUM SERUM LEVELS IN HEMODIALYSIS PATIENTS

5.3. LOWER SERUM MAGNESIUM, CARDIOVASCULAR RISK FACTORS AND MORTALITY IN PREVALENT HEMODIALYSIS PATIENTS

PATIENTS AND METHODS

- **Study design**

An observational, 48-month prospective, single-center study of a cohort of prevalent HD patients was performed.

- **Eligibility criteria**

The inclusion criteria were age above 18 years old and the capability to give informed written consent. Patients taking medications with Mg were excluded from the study.

- **Population**

All the patients were submitted to online hemodiafiltration with a dialysate Mg concentration of 1.0 mmol/L and a dialysate calcium concentration of 1.25 or 1.5 mmol/L. Native vitamin D supplementation with cholecalciferol 2,700 IU three times per week after HD was given to all patients.

The following data were analyzed: age, gender, presence of diabetes, hypertension, coronary artery disease and active medication with doses (with emphasis on active vitamin D or its analogs, phosphate binders, diuretics, and proton pump inhibitors).

Pulse pressure was evaluated, as described before, at baseline in the mid-week HD session in which blood chemistry analyses were obtained.

- **Biochemical analysis**

Serum Mg levels were measured predialysis in a mid-week HD session using a xylidyl blue colorimetric method on a Roche / Hitachi cobas c 701 analyzer (Roche Diagnostics, Mannheim, Germany) and the normal range of values is 0.67 to 1.07 mmol/L.

Serum calcium, serum phosphorus, calcium x phosphorus product, iPTH, hemoglobin, albumin, and CRP were also evaluated simultaneously with Mg.

- **Echocardiographic evaluation**

At the beginning of the study, each patient underwent an echocardiographic examination, and LVMI was calculated with the Devereux formula [195], indexed to body surface area.

- **Vascular calcification score**

Scoring of vascular calcifications through radiography of the pelvis and hands by Adragão score [180] was also performed for all patients.

- **Statistical analysis**

Comparison between groups was performed using a t-test for normally distributed variables and a Wilcoxon test for non-normally distributed variables. Spearman correlation was used for univariable analysis and linear regression was used for multivariable analysis (CI of 95%). Variables entered in multivariable analysis were age, diabetes, albumin, pulse pressure, LVMI, and vascular calcification score.

Survival curves were estimated by Kaplan-Meier analysis and compared by the log-rank test. Cox regression model was used to identify predictors of mortality. The Mg cut-off value used in survival curves was determined via a receiver operating characteristic (ROC) curve. Statistical analysis was performed with SPSS system 19.0. For all comparisons, a $p < 0.05$ was considered statistically significant.

RESULTS

The study included 206 patients, 113 (55%) males, with a mean age of 63.6 ± 14.3 years, and a median HD vintage at baseline of 44 months. Baseline demographic, clinical, biochemical, and vascular characteristics are reported in Table 13.

Fifty-four patients (26%) were diabetics and seventy (34%) had hypertension. Coronary artery disease was diagnosed in 58 (28%) patients.

At the beginning of the study, ninety-four (46%) patients were taking active forms of vitamin D: 12% (n = 11) oral calcitriol, with a mean dose of 1.1 ± 0.5 (0.25 - 1.75) µg/week, and 88% (n = 83) IV paricalcitol, with a mean dose of 7.0 ± 4.5 (2.5 - 30) µg/week.

One hundred and forty-eight patients (72%) were under therapy with phosphate binders: 13 (9%) were taking calcium carbonate, with a mean dose of 1.5 ± 0.8 (1 - 4) g/day, and 135 (91%) were taking sevelamer, with a mean dose of 3.8 ± 1.7 (0.8 - 7.2) g/day. None of the patients were under calcium acetate / Mg carbonate treatment, as those were excluded from the study.

Only 8 patients (4%) were taking diuretics. On the other hand, the use of proton pump inhibitors was very common with 152 patients (74%) under that therapy.

Serum magnesium levels in prevalent hemodialysis patients.

Mean serum Mg was 1.36 ± 0.18 (0.82 - 1.81) mmol/L and only 5 patients (4.5%) had normal values, with the remaining 95.5% (n = 197) showing hypermagnesemia. None of the patients presented hypo (< 0.7 mmol/L) or severe hypermagnesemia (> 2 mmol/L).

Diabetic patients presented lower Mg levels (p = 0.03). Patients taking active vitamin D or phosphate binders presented similar Mg values. The use of proton pump inhibitors was not associated with Mg levels.

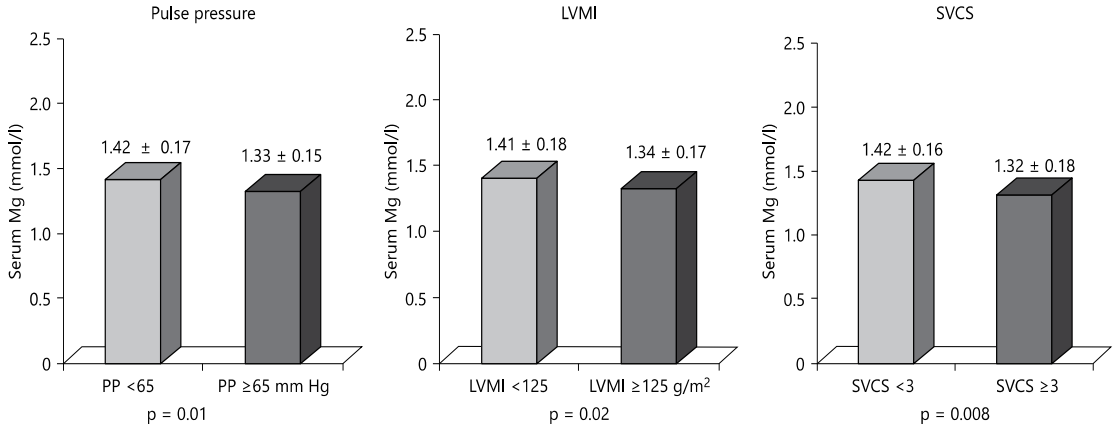
Variables	Patients (n = 206)
Age (years)	63.6 ± 14.3
Male gender (n, [%])	113 (55%)
HD vintage (months)	42.3 ± 38.6
Diabetes (n, [%])	54 (26%)
Hypertension (n, [%])	70 (34%)
Coronary disease (n, [%])	58 (28%)
Active vitamin D (n, [%])	94 (46%)
Phosphate binders (n, [%])	148 (72%)
Diuretics (n, [%])	8 (4%)
Proton pump inhibitors (n, [%])	152 (74%)
Calcium (mg/dL)	8.4 ± 0.5 (7.3 - 10.5)
Phosphorus (mg/dL)	4.6 ± 1.5 (1.8 - 9.7)
Calcium x phosphorus $(mg/dL)^2$	48.5 ± 15.7 (16.6 - 83.4)
iPTH (pg/mL)	289 ± 248 (3 - 1,873)
Magnesium (mmol/L)	1.36 ± 0.18 (0.82 - 1.81)
Hemoglobin (g/dL)	12.4 ± 1.3 (8.6 - 15.2)
C-reactive protein (mg/dL)	0.8 ± 1.6 (0.1 - 16.2)
Albumin (g/dL)	4.0 ± 0.3 (3.1 - 5.1)
eKt/V	1.5 ± 0.3 (0.9 - 2.6)
nPCR (g/Kg/day)	1.2 ± 0.3 (0.7 - 1.6)
PP (mmHg)	69 ± 19 (38 - 114)
LVMI (g/m^2)	129 ± 34 (63 - 213)
SVCS > 0 (n, [%])	122 (59%)
SVCS ≥ 3 (n, [%])	78 (38%)

nPCR, normalized protein catabolic rate; PP, pulse pressure; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

Table 13 - Clinical, biochemical, and vascular parameters of the studied population

Serum magnesium and cardiovascular risk markers.

Patients with higher pulse pressure (≥ 65 mmHg) (p = 0.01), left ventricular hypertrophy (LVMI ≥ 125 g/m^2) (p = 0.02) and more vascular calcifications (simple vascular calcification score ≥ 3) (p = 0.008) had significantly lower serum Mg concentrations (Graphic 8).



PP, pulse pressure; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

Graphic 8 - Patients with higher pulse pressure (≥ 65 mmHg), left ventricular hypertrophy (LVMI ≥ 125 g/m^2) and more vascular calcifications (simple vascular calcification score ≥ 3) had significantly lower serum magnesium concentrations

In univariable analysis, Mg levels were negatively associated with age (r = - 0.44; p = 0.006), diabetes (r = - 0.42; p = 0.007), iPTH (r = - 0.33; p = 0.02), pulse pressure (r = - 0.36; p = 0.01), LVMI (r = - 0.37; p = 0.01) and simple vascular calcification score (r = - 0.40; p = 0.008). Serum Mg concentrations were positively associated with albumin (r = 0.57; p < 0.001) (Table 14).

	Magnesium	
	r	p
Age	- 0.44	0.006
Diabetes mellitus	- 0.42	0.007
Albumin	0.57	<0.001
iPTH	- 0.33	0.02
PP	- 0.36	0.01
LVMI	- 0.37	0.01
SVCS	- 0.40	0.008

PP, pulse pressure; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

Table 14 - Univariable associations between serum Mg and other studied parameters (Spearman correlation)

In patients with CRP ≤ 5 mg/dL (n = 194), there was a negative association (r = - 0.41; p = 0.007) between Mg and CRP serum levels.

In multivariable analysis, lower Mg concentrations were independently associated with an increased pulse pressure (≥ 65 mmHg) (p=0.002) and LVMI (≥ 140 g/m^2) (p=0.03) and a higher simple vascular calcification score (≥ 3) (p=0.01) (Table 15).

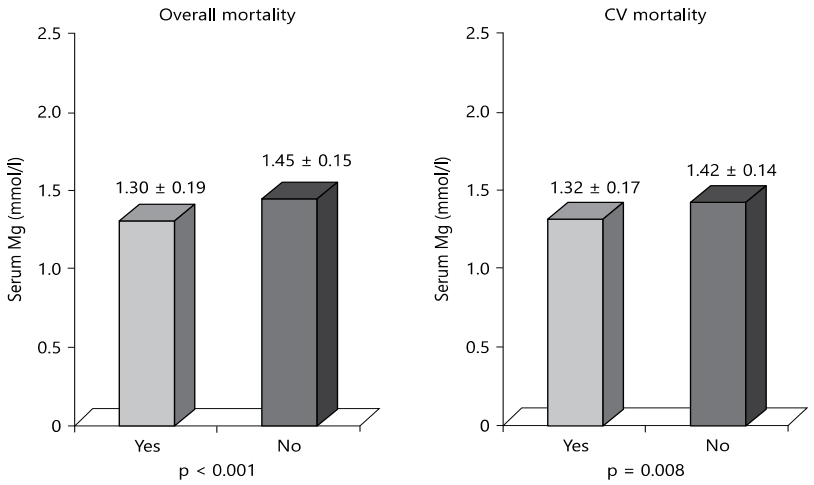
Dependent variable	Independent variables	ß	95% CI	p	R^2
Magnesium	Age	0.19	0.05 to 0.33	< 0.001	0.436
	Diabetes mellitus	0.16	0.09 to 0.29	0.006	
	Albumin	3.3	2.03 to 3.73	0.01	
	PP ≥ 65 mmHg	0.18	0.07 to 0.29	0.002	
	LVMI ≥ 140 g/m^2	0.15	0.10 to 0.24	0.03	
	SVCS ≥ 3	0.17	0.08 to 0.30	0.01	

PP, pulse pressure; LVMI, left ventricular mass index; SVCS, simple vascular calcification score.

Table 15 - Associations between magnesium and CV risk markers (multivariable analysis - linear regression)

Serum magnesium and mortality (overall and cardiovascular).

Seventy-six (37%) patients died during the 48 months of the study, forty-three (57%) from CV causes. Patients who died from overall causes had lower Mg values (1.30 ± 0.19 *vs.* 1.45 ± 0.15 mmol/L; p < 0.001). Also, patients who died from CV causes had lower levels of serum Mg (1.32 ± 0.17 *vs.* 1.42 ± 0.14 mmol/L; p = 0.008) (Graphic 9).



Graphic 9 - Patients who died from overall and cardiovascular causes had significantly lower Mg levels

The cut-off value of serum Mg determined by ROC curve analysis was 1.15 mmol/L (area under de curve - AUC: 0.74; 95% CI: 0.66-0.83; 73% sensitivity; 71% specificity; 86% negative predictive value, and 2.27 positive likelihood ratio). Kaplan-Meier analysis showed that patients with Mg levels ≤ 1.15 mmol/L had higher all-cause (log-rank = 15.37; p < 0.001) and CV (log-rank = 11.74; p = 0.001) 48-month mortality (Figure 15).

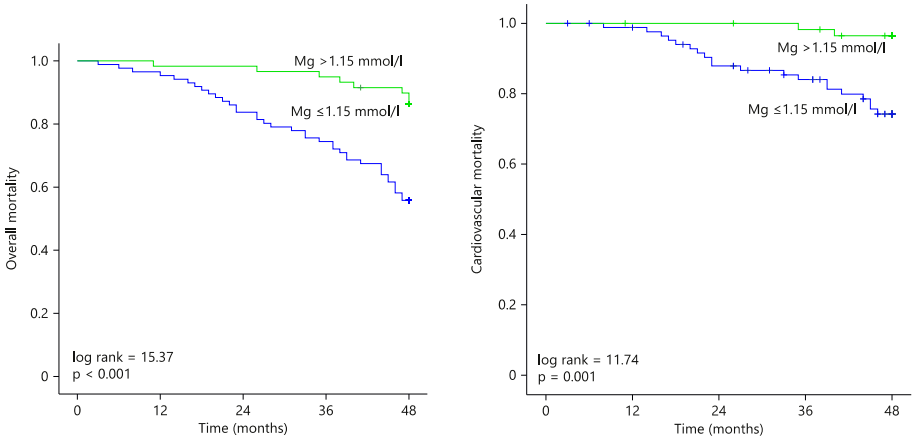


Figure 15 - A serum magnesium ≤ 1.15 mmol/L was associated with a lower overall and cardiovascular survival (Kaplan-Meier analysis)

Cox regression analysis showed that serum Mg is an independent negative predictor of all-cause (hazard ratio - HR: 0.87; p = 0.01) and CV mortality (HR: 0.82; p = 0.02), using models adjusted for age, diabetes mellitus, time on HD, CRP, pulse pressure, LVMI, and simple vascular calcification score (Table 16).

Dependent variable	Independent variables	HR	95% CI	p	R^2
Overall mortality	Age	1.42	1.08 to 1.53	0.02	0.473
	Time on HD	5.21	1.70 to 13.52	<0.001	
	Diabetes mellitus	7.34	2.81 to 18.83	<0.001	
	Albumin	0.81	0.72 to 0.97	0.01	
	C-reactive protein	1.93	1.07 to 3.16	0.007	
	Magnesium	0.87	0.78 to 0.99	0.01	
	PP ≥ 65 mmHg	2.17	1.13 to 3.24	0.004	
	LVMI ≥ 140 g/m^2	1.98	1.09 to 3.20	0.003	
	SVCS ≥ 3	3.26	2.11 to 5.08	0.001	
Cardiovascular mortality	Age	1.76	1.12 to 2.98	0.01	0.418
	Time on HD	5.77	1.94 to 14.37	0.005	
	Diabetes mellitus	2.93	1.29 to 7.37	0.008	
	Coronary disease	1.87	1.23 to 2.34	0.02	
	C-reactive protein	1.90	1.25 to 2.87	0.01	
	Magnesium	0.82	0.72 to 0.95	0.02	
	PP ≥ 65 mmHg	1.95	1.13 to 3.19	0.006	
	LVMI ≥ 140 g/m^2	2.19	1.16 to 3.22	0.004	
	SVCS ≥ 3	3.12	2.17 to 6.14	0.002	

Table 16 - Predictors of all-cause and cardiovascular mortality (Cox regression)

5.4. CALCIUM ACETATE / MAGNESIUM CARBONATE AND CARDIOVASCULAR RISK MARKERS IN HEMODIALYSIS PATIENTS

PATIENTS AND METHODS

- **Study design**

This was an observational, 48-month prospective, single-center study of a cohort of prevalent HD patients.

- **Eligibility criteria**

The inclusion criteria were age above 18 years old, capability to give informed written consent, and being under calcium acetate / Mg carbonate, sevelamer or no phosphate binder therapy for at least 36 months.

Patients taking calcium carbonate or calcimimetics, and those who had undergone parathyroidectomy were excluded from the study, as well as patients who did not complete 36 months of calcium acetate / Mg carbonate, sevelamer or no phosphate binder therapy.

- **Population**

All the patients were submitted to online hemodiafiltration with a dialysate Mg concentration of 0.5 mmol/L, and a dialysate calcium concentration of 1.25 or 1.5 mmol/L. Native vitamin D supplementation with cholecalciferol 2,700 IU three times per week after HD was given to all patients.

The following data were analyzed: age, gender, presence of diabetes, hypertension, coronary artery disease and active medication with doses (active vitamin D or its analogs, phosphate binders, darbepoetin, hypotensive therapy, statins, and proton pump inhibitors). Pulse pressure was evaluated at baseline in the mid-week HD session in which blood chemistry analyses were obtained.

- **Biochemical analysis**

Biochemical parameters were evaluated at the beginning of the study and repeated on a quarterly basis. Serum Mg was measured predialysis in a mid-week HD session using a xylidyl blue colorimetric method on a Roche / Hitachi cobas c 701 analyzer (Roche Diagnostics, Mannheim, Germany) and the normal range of values is 0.67 to 1.07 mmol/L.

Serum calcium, serum phosphorus, calcium x phosphorus product, iPTH, hemoglobin, albumin, and CRP were also evaluated simultaneously with Mg.

- **Echocardiographic evaluation**

At the beginning of the study, each patient underwent an echocardiographic examination, and LVMI was calculated. The presence of aortic and mitral valve calcifications was also evaluated at baseline. Cardiac valve calcifications were classified as mild, moderate, and severe. This examination was performed on a mid-week non-dialysis day and in the same cardiologic center. Echocardiographic evaluation was repeated after 48 months.

- **Statistical analysis**

Comparisons between 2 groups were performed using a t-test for normally distributed variables and the Mann-Whitney test for non-normally distributed variables. Comparisons within the same group were performed with the paired t-test for normally distributed variables and the Wilcoxon test for non-normally distributed variables. Multiple comparisons were performed with analysis of variance with posthoc analysis for normally distributed variables or Kruskal-Wallis test for non-normally distributed variables.

Binary regression was used for multivariable analysis (95% CI). Variables entered in multivariable analysis were age, time on HD, diabetes mellitus, albumin, CRP, serum phosphorus, serum Mg, pulse pressure, interdialytic weight gain, and therapy with vitamin D and calcium acetate / Mg carbonate. Progression of LVMI and aortic valve calcification was considered when there was an increase of 5% from the baseline value.

ROC curve analysis allowed the identification of the best cut-off values for LVMI progression (AUC: 0.656; 95% CI: 0.581 - 0.726) and aortic valve calcification progression (AUC: 0.763; 95% CI: 0.693 - 0.823).

Statistical analysis was performed with SPSS system 19.0 and the MedCalc program version 6.0. For all comparisons, a $p < 0.05$ was considered statistically significant. For multiple comparisons, Bonferroni adjustment was performed.

RESULTS

This study enrolled 138 chronic HD patients receiving either calcium acetate / Mg carbonate or sevelamer for at least 36 months or no phosphate binder therapy. Sixty-six (48%) patients were under therapy with phosphate binders: 30 (22%) patients were taking sevelamer with a mean dose of 3,578 ± 1,741 (800 - 7,200) mg/day and 36 (26%) were taking calcium acetate / Mg carbonate with a mean dose of 1,858 ± 796 (670 - 4,020) mg/day. Seventy-two (52%) patients did not take phosphate binders.

Patients who were lost to follow-up were excluded from the analysis (41 died, 16 received a transplant and 11 were transferred), as shown in Figure 16.

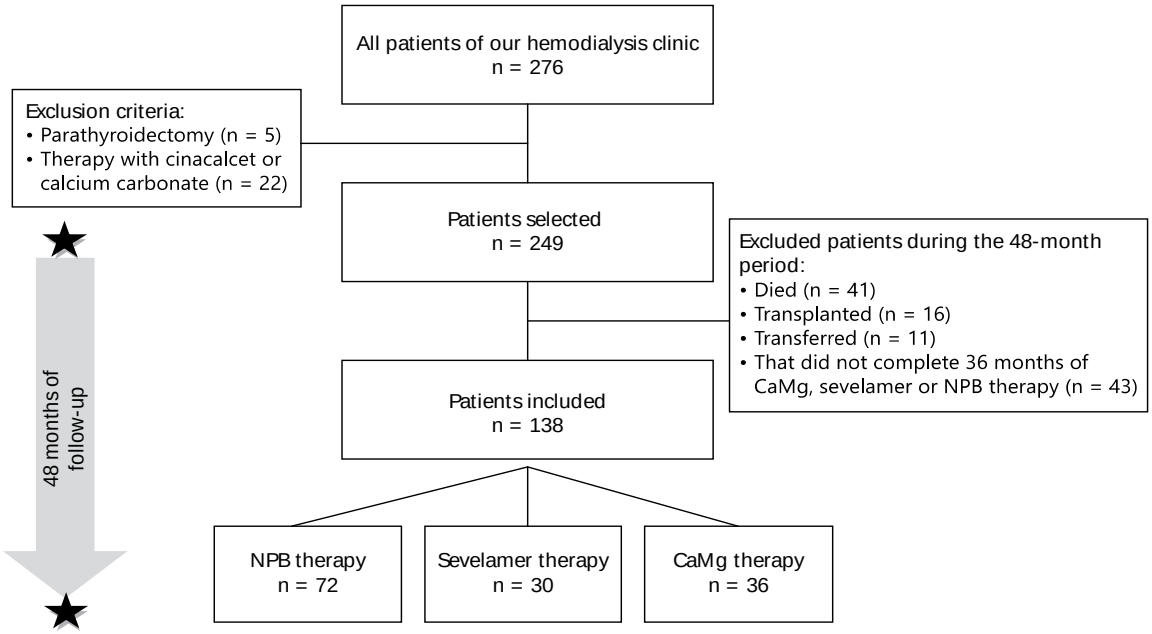


Figure 16 - Flow chart of the study. ★ Evaluation of the endpoints (pulse pressure, left ventricular mass index and valvular calcifications) at 0 and 48 months

Seventy-seven (56%) of the patients were men, with a mean age of 62.7 ± 14.2 years. The median time on HD was 43 months. Thirty-nine (28%) patients were diabetics and 48 (35%) had hypertension. Coronary artery disease was diagnosed in 37 (27%) patients.

At the beginning of the study, 57 (41%) patients were taking active forms of vitamin D: 44 (32%) patients under oral alfacalcidol, with a mean dose of 2.7 ± 1.8 (1 - 9) µg/week, and 13 (9%) under IV paricalcitol, with a mean dose of 8.1 ± 4.6 (2.5 - 30) µg/week. A total of 128 (93%) patients were under darbepoetin therapy, with a mean dosage of 0.042 ± 0.011 µg/kg/week per g/dL.

The demographic, clinical and biochemical characteristics of patients taking calcium acetate / Mg carbonate, sevelamer or no phosphate binders, respectively, as well as a comparison between the groups, are reported in Table 17.

	No phosphate binders (n=72)	CaMg (n=36)	Sevelamer (n=30)	p value (CaMg vs. no phosphate binder)	p value (CaMg vs. sevelamer)
Age (years)	68.5 ± 13.9	68.9 ± 15.4	55.9 ± 15.2	NS	< 0.001
Time on HD (months)	49.3 ± 39.5	48.5 ± 39.9	49.9 ± 39.1	NS	NS
Diabetes mellitus (%)	42.1	40.2	14.9	NS	< 0.001
Hypertension (%)	34.3	35.3	36.2	NS	NS
Coronary disease (%)	31	28	26	NS	NS
Hypotensive therapy (%)	66	69	67	NS	NS
ACEIs (%)	46	44	41	NS	NS
ARBs (%)	9	7	6	NS	NS
Statins (%)	22	19	18	NS	NS
PPIs (%)	75	72	71	NS	NS
Interdialytic weight gain (g)	1,858 ± 964	1,732 ± 953	1,915 ± 868	NS	NS
Active vitamin D therapy (%)	29.4	43.2	46.1	< 0.001	NS
Darbepoetin (%)	95	93	91	NS	NS
Hemoglobin (g/dL)	11.1 ± 1.2	11.3 ± 1.1	11.4 ± 1.2	NS	NS
CRP (mg/dL)	1.1 ± 0.4	0.8 ± 0.5	0.6 ± 0.4	0.02	NS
Albumin (g/dL)	3.2 ± 0.4	3.6 ± 0.2	3.8 ± 0.4	0.02	NS
Calcium (mg/dL)	8.6 ± 0.5	8.8 ± 0.3	8.9 ± 0.6	NS	NS
Phosphorus (mg/dL)	3.7 ± 0.9	4.8 ± 1.3	5.0 ± 1.1	0.01	NS
iPTH (pg/mL)	267 ± 142	309 ± 221	423 ± 307	0.04	0.01

ACEIs, angiotensin-converting enzyme inhibitors; ARBs, angiotensin II receptor blockers; CRP, C-reactive protein; PPIs, proton pump inhibitors; NS, not significant.

Table 17 - Demographic, clinical, and biochemical parameters of the studied population

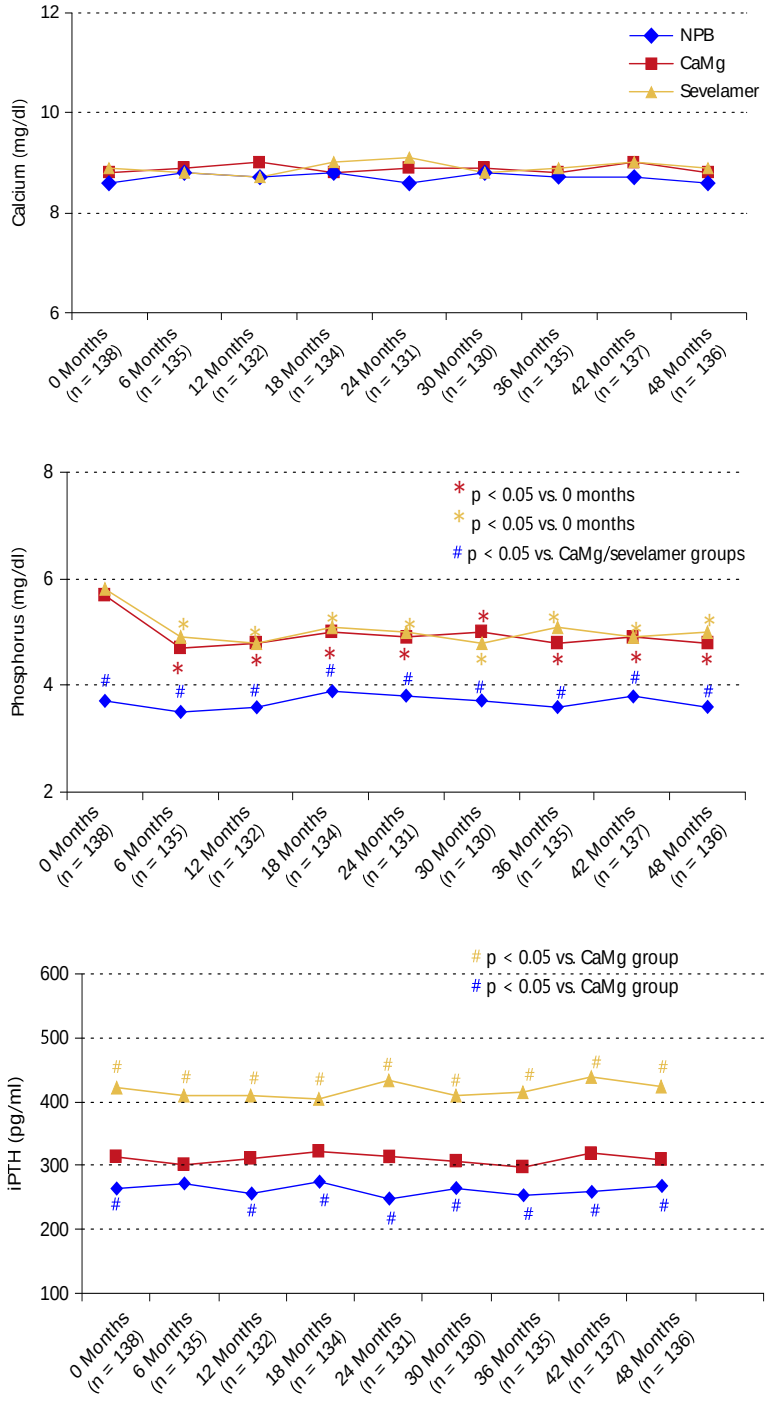
Comparison of mineral metabolism between patients taking calcium acetate / magnesium carbonate, sevelamer or no phosphate binders.

Patients in the calcium acetate / Mg carbonate group were older ($p < 0.001$) and this group had a higher proportion of diabetic patients ($p < 0.001$) compared to the sevelamer group.

More patients taking calcium acetate / Mg carbonate were under active vitamin D compared with those not taking phosphate binders ($p < 0.001$) and presented lower CRP ($p = 0.02$) and higher albumin ($p = 0.02$) levels compared to the same group. As expected, the group not taking phosphate binders showed lower phosphorus ($p = 0.01$) and iPTH ($p = 0.04$) compared with those under this therapy (Table 17).

During the study (Figure 17), calcium serum levels were similar between the 3 groups and remained stable. Compared with the calcium acetate / Mg carbonate group, the sevelamer group showed significantly higher values of iPTH. Phosphorus serum levels remained significantly different between the 3 groups throughout the study, with higher values in patients taking either calcium acetate / Mg carbonate or sevelamer. There was a significant reduction in phosphorus levels in calcium acetate / Mg carbonate and sevelamer groups only in the first 6 months, thereafter values were maintained stable during the rest of the follow-up in each group.

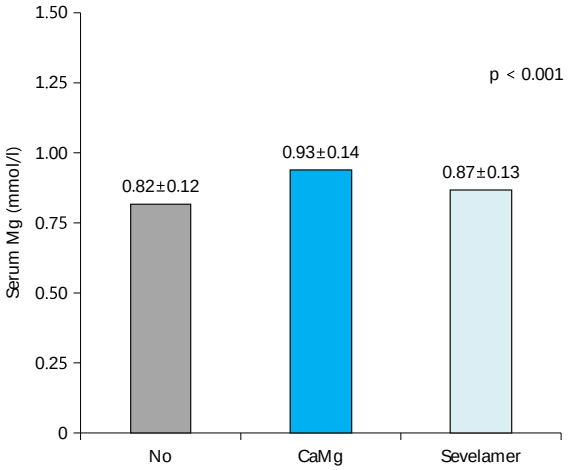
Levels of iPTH remained significantly higher in calcium acetate / Mg carbonate and sevelamer groups during the study but stayed unchanged in each group during the follow-up time.



Calcium: p = NS between the 3 groups during the study and p = NS in each group during the 48 months of the follow-up. iPTH: p = NS in each group during the 48 months of the follow-up. NS = Not significant.

Figure 17 - Serum calcium, phosphorus and iPTH levels during the study

Patients who had taken calcium acetate / Mg carbonate showed higher serum Mg levels (0.93 ± 0.14 mmol/L) compared to patients under sevelamer (0.87 ± 0.13 mmol/L) or patients without phosphate binder therapy (0.82 ± 0.12 mmol/L; p < 0.001), as shown in Graphic 10.



CaMg, calcium acetate / magnesium carbonate.

Graphic 10 - Magnesium serum levels and type of phosphate binder

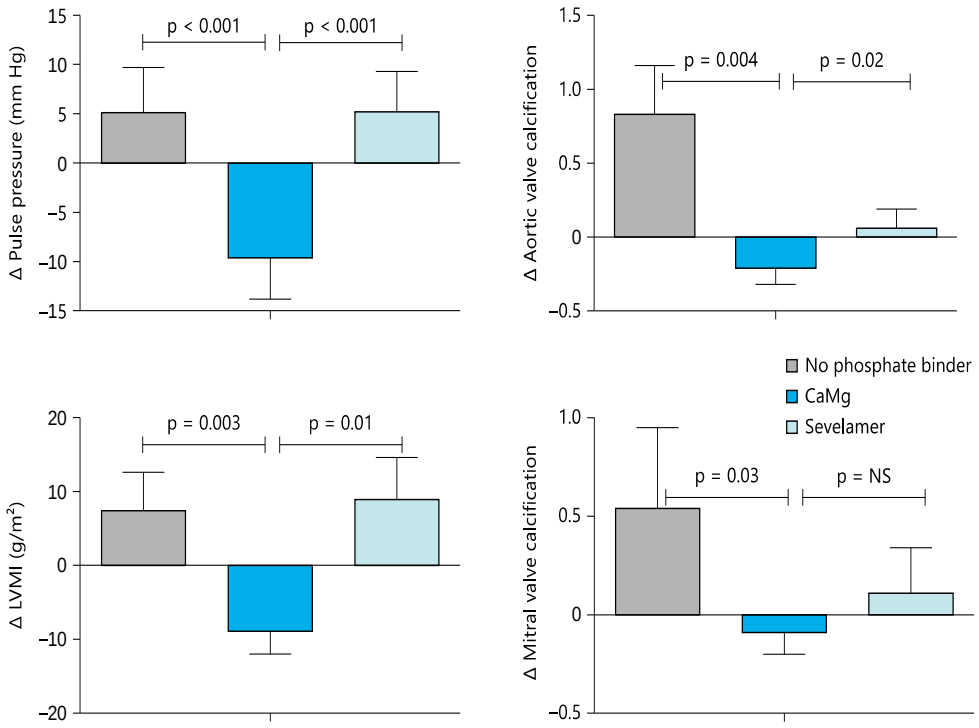
The use of proton pump inhibitors was very common with > 70% of the patients under that therapy, but proton pump inhibitor use was not associated with Mg levels.

At baseline, there was no significant difference in pulse pressure, LVMI and mitral or aortic valve calcifications between the 3 groups. Mortality was also not different between the groups during the 48 months of the study.

Relationship between phosphate binder therapy and cardiovascular risk markers.

At the end of the study, patients who had taken calcium acetate / Mg carbonate showed a significant reduction in pulse pressure (p < 0.001), LVMI (p = 0.003), aortic valve calcifications (p = 0.004) and mitral valve calcifications (p = 0.03) compared with patients who did not take phosphate binders.

In comparison to patients who had taken sevelamer, patients under calcium acetate / Mg carbonate showed a significant reduction of pulse pressure ($p < 0.001$), LVMI ($p = 0.01$) and aortic valve calcifications ($p = 0.02$), but not of mitral valve calcifications at the end of the study (Figure 18).



CaMg, calcium acetate / magnesium carbonate; PP, pulse pressure.

Figure 18 - Influence of treatment with magnesium-based phosphate binder on pulse pressure, left ventricular mass index, aortic and mitral valve calcification compared with no phosphate binder and sevelamer (univariable analysis)

Calcium acetate / magnesium carbonate therapy and progression of left ventricular mass index and aortic valve calcification.

In multivariable analysis, calcium acetate / Mg carbonate was negatively associated with LVMI progression ($p = 0.02$) and aortic valve calcification progression ($p = 0.01$), after adjustment for multiple variables including serum Mg (as shown in Table 18).

In a separate adjusted multivariable analysis, sevelamer was not significantly associated with either LVMI or aortic valve calcification progression.

Variables	OR	95% CI	p value	R^2 value
LVMI progression				
Age	2.21	1.09 to 3.67	0.003	0.417
Time on dialysis	2.18	1.26 to 3.43	0.002	
Diabetes mellitus	1.18	0.97 to 2.16	0.34	
Albumin	0.19	0.08 to 0.24	< 0.001	
CRP	1.92	0.81 to 3.31	0.29	
Serum phosphorus	1.19	1.11 to 2.09	0.006	
Serum magnesium	0.18	0.03 to 0.25	0.003	
PP	2.35	1.21 to 2.93	< 0.001	
Interdialytic weight gain	1.38	1.09 to 2.95	0.007	
Vitamin D therapy	0.48	0.19 to 1.23	0.13	
CaMg therapy	0.17	0.02 to 0.28	0.02	
Aortic valve calcification progression				
Age	1.77	1.11 to 3.39	0.01	0.423
Time on dialysis	2.12	1.18 to 2.41	0.003	
Diabetes mellitus	1.21	1.01 to 3.32	0.02	
Albumin	0.19	0.05 to 0.28	< 0.001	
CRP	1.24	0.87 to 1.85	0.36	
Serum phosphorus	1.27	1.09 to 1.94	0.02	
Serum magnesium	0.18	0.04 to 0.29	0.02	
Vitamin D therapy	0.98	0.44 to 2.19	0.61	
CaMg therapy	0.15	0.02 to 0.23	0.01	

CaMg, calcium acetate / magnesium carbonate; CRP, C-reactive protein; LVMI, left ventricular mass index; PP, pulse pressure.

Table 18 - Predictors of LVMI and aortic valve calcification progression (multivariable analysis - binary logistic regression). Calcium acetate / magnesium carbonate therapy was associated with a lower progression of LVMI and aortic valve calcification

BONE FRAGILITY FRACTURES: INCIDENCE AND RISK FACTORS IN HEMODIALYSIS PATIENTS

5.5. BONE FRACTURE RISK FACTORS IN PREVALENT HEMODIALYSIS PATIENTS

PATIENTS AND METHODS

- **Study design**

An observational, retrospective, single-center study of a cohort of prevalent HD patients was performed.

- **Eligibility criteria**

The inclusion criteria were age above 18 years old and the capability to give informed written consent.

- **Population**

All the patients were submitted to online hemodiafiltration with a dialysate calcium concentration of 1.25 or 1.5 mmol/L, and all received native vitamin D supplementation with cholecalciferol 2,700 IU three times per week after HD during the study.

The following data were analyzed: age, gender, prior kidney transplantation, presence of diabetes, hypertension, coronary artery disease, cerebrovascular disease, and peripheral vascular disease. Mean body mass index (BMI) at the start of HD was evaluated. Medication with doses (active vitamin D or its analogs, phosphate binders, and darbepoetin) was also registered.

- **Fracture status**

Fractures that occurred during the follow-up of the study were determined by patient interview and chart review and classified as fragility fractures if resulting from trauma equivalent to a fall from standing height, or less [196]. High trauma fractures and fractures of fingers, toes, face, and skull were excluded. All fractures were confirmed by radiographs or radiology reports.

- **Biochemical analysis**

Serum calcium, serum phosphorus, magnesium, iPTH, bALP, hemoglobin, albumin, and CRP were recorded quarterly during the study follow-up. All blood samples were collected before dialysis in midweek sessions.

- **Vascular calcification score**

Simple vascular calcification score was evaluated through radiography of the pelvis and hands by Adragão score [180] for all patients at the beginning of HD.

- **Statistical analysis**

Follow-up begins at the start of HD for each patient and continues until the date of the last recorded serum laboratory results in November of 2017. The incidence of fracture was expressed as the total number of fractures per 1000 patient-years of follow-up.

For statistical analysis, the arithmetic means of the quarterly laboratory results obtained during follow-up were used. Variables were expressed as frequencies for categorical variables, mean values with SD for normally distributed variables, and median (interquartile ranges) values for nonnormally distributed variables. Differences in mean values between patients with and without fractures were evaluated using the unpaired Student's t-test for parametric data and the Mann-Whitney U test for nonparametric data.

Kaplan-Meier survival analysis was used to calculate the HR for fractures associated with the three iPTH groups; log-rank tests were calculated and compared with the survival curves between the groups. Binary regression was used for multivariable analysis (95% CI). Variables entered in multivariable analysis were age, female gender, time on HD, diabetes,

BMI, serum albumin, bALP, iPTH < 300 or > 800 pg/mL, therapy with active vitamin D and vascular calcification score ≥ 3.

Statistical analysis was performed with SPSS system 19.0. A $p < 0.05$ was considered statistically significant.

RESULTS

The study included 341 patients that were evaluated retrospectively, since they started HD, with a median follow-up of 51 (4 - 378) months, 206 (60%) males and 135 (40%) females, with a mean age of 68.7 ± 13.6 years at the beginning of HD.

One hundred and fifty-six (46%) patients had diabetes, and 116 (34%) had hypertension. Coronary artery disease was diagnosed in 109 (32%) patients, cerebrovascular disease in 85 (25%) and peripheral vascular disease in 89 (26%). The mean BMI at the start of HD was 27.1 ± 4.8 kg/m^2.

During the study, 99 (29%) patients were taking active forms of vitamin D: 44 (13%) patients under oral alfacalcidol, with a median dose of 2 (1 - 9) µg/week, and 55 (16%) under IV paricalcitol, with a median dose of 5 (2.5 - 15) µg/week. Forty-five (13%) were under calcimimetic (oral cinacalcet) with a median dose of 30 (15 - 90) mg/day, and ten (3%) of the patients were submitted to parathyroidectomy. None of the patients was taking another anti-osteoporotic treatment.

A total of 314 (92%) patients were under epoetin beta therapy, with a mean dosage of 77 ± 19 IU/kg/week per g/dL. The target for ferritin was between 200 and 800 ng/mL for all patients, and if needed, only IV iron saccharate was used.

During the studied period, 116 (34%) patients were under therapy with phosphate binders: 69 (20%) patients were taking calcium acetate / Mg carbonate with a median dose of 1,675 (1,340 - 6,030) mg/day, 27 (8%) patients were under sevelamer with a median dose of 3,200 (1,600 - 4,800) mg/day, and 20 (6%) were taking sucroferric oxyhydroxide with a median dose of 1,000 (500 - 1,500) mg/day. Two hundred and twenty-five (66%) patients did not take

phosphate binders.

Characterization of the incidence of fragility fractures for the first time in a Portuguese prevalent hemodialysis population.

There were 57 episodes of fragility fractures during the study follow-up (median of 51 months) which corresponds to an overall incidence rate of 31 per 1000 person-years.

The sites of fracture were forearm - 23% (n = 13), leg - 23%, (n = 13), hip - 17% (n = 10), arm - 14% (n = 8), spine - 13% (n = 7), and ribs - 10% (n = 6). Three (5%) patients presented more than one episode of fracture and 7 (12%) patients who had a fracture during the study had already presented a fragility fracture before the beginning of HD. Prior kidney transplantation was not associated with more fractures. The median HD time to the first fracture was 47 months.

Identification of risk factors for fragility fractures in hemodialysis patients.

Compared with patients without fractures, patients who presented episodes of fracture were more frequently female ($p < 0.001$), older ($p = 0.01$), diabetic ($p < 0.001$), with lower BMI ($p = 0.02$), longer HD vintage ($p < 0.001$), lower albumin ($p < 0.001$) and Mg levels ($p < 0.001$), and higher bALP values ($p = 0.01$) (Table 19).

These patients were also under less active vitamin D therapy ($p = 0.002$) and presented a higher vascular calcification score ($p < 0.001$). Therapy with phosphate binders or calcimimetic was not associated with fractures.

	Without fractures (n=287)	With fractures (n=54)	p
Age (years)	67.3 ± 13.6	71.2 ± 13.7	0.01
Female gender (%)	36.6	54.4	< 0.001
HD vintage (months)	44	93	< 0.001
Prior kidney transplantation (%)	9	10	NS
Diabetes mellitus (%)	40.4	52.6	< 0.001
Hypertension (%)	32.3	35.1	NS
Coronary disease (%)	31.3	34.1	NS
Peripheral vascular disease (%)	24.7	27.2	NS
Cerebrovascular disease (%)	22.8	26.6	NS
BMI (Kg/m^2)	27.7 ± 5.3	25.1 ± 3.7	0.02
Phosphate binder therapy (%)	36.6	33.2	NS
Active vitamin D therapy (%)	36.1	19.8	0.002
Calcimimetic therapy (%)	14.2	12.3	NS
Parathyroidectomy (%)	2.5	4.5	NS
Hemoglobin (g/dL)	11.4 ± 0.9	10.9 ± 0.8	NS
CRP (mg/dL)	0.8 ± 0.4	1.1 ± 0.5	NS
Albumin (g/dL)	4.0 ± 0.3	3.7 ± 0.4	< 0.001
Calcium (mg/dL)	9.0 ± 0.7	9.1 ± 0.6	NS
Phosphorus (mg/dL)	4.5 ± 1.3	4.3 ± 1.2	NS
Magnesium (mmol/L)	1.15 ± 0.2	0.95 ± 0.3	< 0.001
bALP (ug/L)	24	33	0.01
iPTH (pg/mL)	343	329	NS
Vascular calcification score (0 - 8)	4	6	< 0.001

BMI, body mass index; CRP, C-reactive protein; bALP, bone alkaline phosphatase; iPTH, intact parathyroid hormone.

Table 19 - Clinical, biochemical, and vascular calcification parameters in patients with and without fragility fractures (univariable analysis)

Since a U-shaped curve association between fracture risk and iPTH values was observed in our population (Figure 19), patients were stratified by different iPTH levels in three groups (iPTH < 300, 300 < iPTH < 800, iPTH > 800 pg/mL).

There were 33 episodes of fracture in the group with iPTH < 300 pg/mL (n = 172) - fracture incidence rate of 39 per 1000 person-years. Only 9 episodes of fracture occurred in the group with iPTH between 300 and 800 pg/mL (n = 136) - fracture incidence rate of 17 per 1000 person-years. In the group with iPTH > 800 pg/mL (n = 33), there were 15 episodes of fracture - incidence rate of 78 per 1000 person-years.

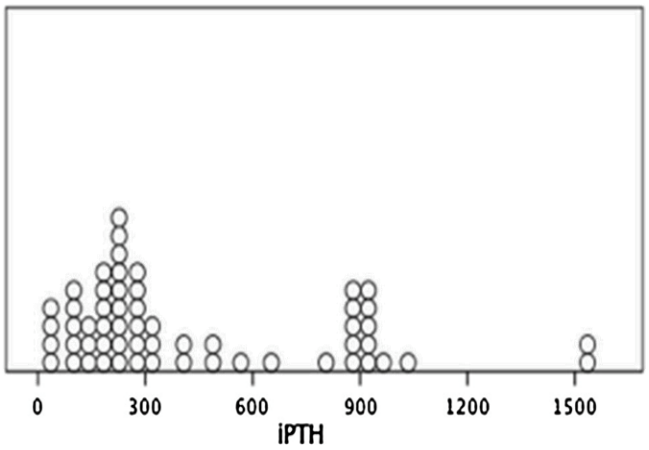


Figure 19 - U-shaped distribution of the population regarding iPTH levels (each circle represents one patient)

Kaplan-Meier fracture-free survival analysis also demonstrated that patients with mean iPTH levels < 300 (HR: 2.88; p = 0.01) or > 800 pg/mL (HR: 3.48; p < 0.001) throughout the follow-up presented a significantly higher fracture risk compared to those with iPTH between 300 and 800 pg/mL (Figure 20).

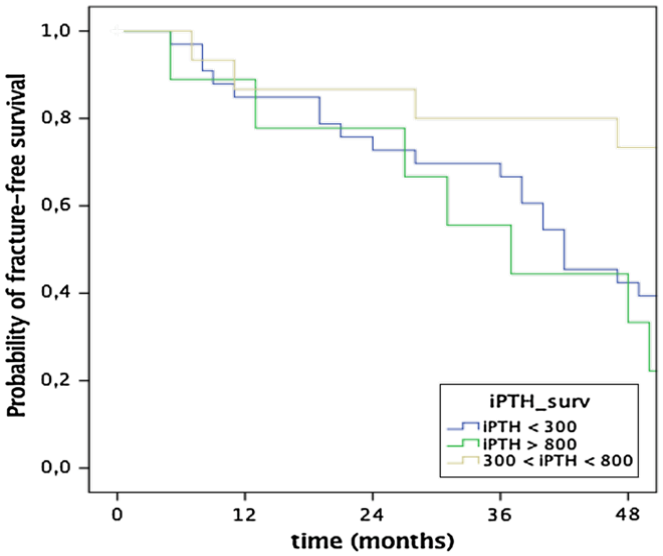


Figure 20 - Patients fracture-free survival according to iPTH levels (Kaplan-Meier analysis)

In a multivariable analysis, age ($p < 0.001$), female gender ($p < 0.001$), time on HD ($p < 0.001$), lower levels of albumin ($p = 0.02$), reduced serum magnesium ($p = 0.02$), higher values of bALP ($p = 0.01$), iPTH < 300 or > 800 pg/mL ($p = 0.006$), and the presence of a higher vascular calcification score (≥ 3) ($p < 0.001$) were associated with the presence of fragility fractures. On the contrary, active vitamin D therapy was associated with less fracture risk ($p = 0.03$) (Table 20).

Dependent variable	Independent variables	HR	95% CI	p	R^2
Fractures	Age	3.17	2.05 to 3.89	< 0.001	0.638
	Female gender	1.82	1.32 to 2.26	< 0.001	
	HD vintage	2.21	1.87 to 2.55	< 0.001	
	Diabetes mellitus	1.16	0.89 to 2.37	0.07	
	BMI	1.11	0.81 to 3.11	0.08	
	Serum albumin	0.87	0.77 to 0.91	0.02	
	Serum magnesium	0.85	0.78 to 0.87	0.02	
	bALP	1.21	1.16 to 1.33	0.01	
	iPTH < 300 or > 800 pg/mL	1.24	1.18 to 1.29	0.006	
	Active vitamin D therapy	0.89	0.86 to 0.95	0.03	
	Vascular calcification score ≥ 3	1.52	1.43 to 1.61	< 0.001	

BMI, body mass index; bALP, bone alkaline phosphatase; iPTH, intact parathyroid hormone.

Table 20 - Independent predictors of fragility fractures (multivariable analysis)

6. DISCUSSION

To achieve the three goals of this thesis, it was divided, presented and discussed into five main sections, according to the published articles: **1)** characterization for the first time of a large Portuguese prevalent HD population regarding serum calcidiol levels and its association with CV risk factors, and mortality; **2)** prospective evaluation of a therapeutic intervention (cholecalciferol supplementation) during 60 months of follow-up regarding the correction of the insufficiency or deficiency of 25(OH)D levels and assessment of bone and mineral improvement and CV risk markers; **3)** evaluation of pre-HD serum levels of Mg and the association of those with the presence of CV risk factors and overall and CV mortality; **4)** prospective 48-month follow-up study evaluating the CV impact of using Mg-based phosphate binders to control hyperphosphatemia in prevalent HD patients; **5)** quantification, for the first time, of the incidence of fragility fractures in a Portuguese HD population and the association between the development of fractures and markers of bone and CV disease.

1) Calcidiol levels, inflammation, cardiovascular risk markers, and mortality in 223 hemodialysis patients

In the first section of the thesis, the authors described, for the first time in our country, that in a large prevalent HD population from a "sunny country", 25(OH)D insufficiency was much more frequent than suspected, most probably contributing for the $1,25(OH)_2D$ deficiency (observed in > 95% of the patients). Levels of 25(OH)D as expected were higher, but not significantly, after summer because of increased sun exposure. On the contrary, $1,25(OH)_2D$ levels did not show any seasonal variation.

In our study, as already described in other studies [197], older patients and diabetics showed lower levels of 25(OH)D. This observation may be explained due to the fact that elderly patients have reduced sun exposure, due to poor health and immobility, and have reduced ingestion of foods that are natural sources of vitamin D, and dermal synthesis of vitamin D is reduced with increasing age [197]. Also, diabetes may predispose to an even greater risk of vitamin D deficiency because of bowel motility disturbances, fat malabsorption, and an association with coeliac disease [198]. In this study, lower levels of $1,25(OH)_2D$ were also present

in diabetics, probably reflecting lower production of the substrate.

Plasma levels of BNP have been shown to be a good CV risk marker in the general population and in patients with renal failure [199, 200]. Plasma BNP is synthesized mainly in cardiomyocytes in response to ventricular stretch and pressure overload [201]. Elevated BNP concentrations are seen in patients with cardiac insufficiency and renal failure [200]. Increased BNP levels in these patients are thought to be the result of water retention and ventricular volume increase [199-201], but they may also be a result of concomitant coronary artery disease or left ventricular dysfunction [202]. Studies have also indicated that the degree of BNP elevation in patients with CKD may predict left ventricular function and future cardiac events [200-202]. We have also previously found an increase in CV morbidity and mortality in HD patients with higher BNP plasma levels [194]. In our study, as reported in other studies, mean levels of BNP were high compared with the general population, because of renal failure and in some patients' simultaneous cardiac disease.

CKD patients have stiffer vessels compared to the general population, contributing to reduced arterial compliance. In CKD, there is a similar magnitude of atherosclerotic plaque burden and intimal thickness, but there is a markedly increased medial calcification. This phenomenon is a major determinant of left ventricular pressure overload and abnormal coronary perfusion [203]. Left ventricular hypertrophy is found early in the course of uremia and progresses with further impairment of renal function [204]. LVMI is an independent predictor of survival in patients with CKD [204]. Our patients presented increased pulse pressure and more than 50% showed echocardiographic criteria of left ventricular hypertrophy, both strong predictors of increased CV risk.

Predisposition to develop vascular calcifications is increased in CKD patients and the presence of hyperphosphatemia, hypercalcemia and increased calcium x phosphorus product have been considered major pathogenic factors leading to vascular and soft tissue calcification in uremic patients [205, 206]. In our study, we found no correlation between serum calcium, serum phosphorus, calcium x phosphorus product and iPTH and vascular calcifications, probably because mean values were low compared with other studies.

The role of vitamin D in vascular calcifications is still controversial. In experimental models, very high doses of some vitamin D metabolites have been associated with an increase in vascular calcifications [207, 208]. In contrast, epidemiological observational studies have shown

that the currently used doses of different vitamin D metabolites and analogs have been associated with an improvement in vascular function and survival [209-213]. In our study, patients taking active vitamin D (mainly paricalcitol) showed less vascular calcifications, probably because the used doses were not high, and the serum levels of calcium, phosphorus, calcium x phosphorus product and iPTH were well controlled. On the other hand, studies have shown that serum 25(OH)D levels were negatively associated with vascular calcifications [210] and both 25(OH)D and $1,25(OH)_2D$ serum levels were negatively associated with aortic pulse wave velocity and positively correlated with brachial artery distensibility [214], suggesting a protective role for vitamin D in vascular function. An observational study by Wolf *et al.* [215] found that, in incident HD patients, deficient values of vitamin D were associated with early mortality and that treatment with active vitamin D could probably reverse this situation.

Our results demonstrate that high 25(OH)D serum levels could have a protective role in CV disease, since deficient 25(OH)D levels were associated with higher BNP plasma values, increased pulse pressure (> 65 mmHg) and higher vascular calcification score (≥ 3). These results were in accordance with the study published by London *et al.* [214]. A similar correlation could not be demonstrated for $1,25(OH)_2D$, which can be explained by the fact that most of our patients showed very low levels of $1,25(OH)_2D$ and were treated mainly with a vitamin D receptor activator (paricalcitol), which does not interfere / is not measured by the $1,25(OH)_2D$ assay. Patients treated with paricalcitol also showed lower $1,25(OH)_2D$ levels, which could be explained by a possible negative feedback mechanism. This effect was not observed in the 14 patients treated with calcitriol, probably due to the low doses that were used.

The negative association observed between 25(OH)D and CRP and the positive association with albumin may only illustrate 25(OH)D as another marker of inflammation or represent the already described immunomodulator and anti-inflammatory actions of this hormone, which can also contribute to the CV protection.

As shown in a meta-analysis in CKD patients, we also described in a large population of prevalent HD patients that lower levels of 25(OH)D were predictors of hospitalization, death from all causes and death from CV causes. Patients with 25(OH)D deficiency (< 15 ng/mL) had a significantly lower survival at the end of the 48-month studied period.

2) Long-term cholecalciferol supplementation in 97 hemodialysis patients: effects on mineral metabolism, inflammation, and cardiac parameters

The authors described the first Portuguese report of native vitamin D supplementation in HD patients and one of the studies with the longest follow-up ever published on this topic. Unlike other studies, most of the supplemented population had low levels of 25(OH)D and native vitamin D was given after each HD session ensuring 100% therapeutic adherence.

Stage 5 CKD patients lack the capacity of the kidney to perform 1α-hydroxylation, thereby making it impossible to produce the necessary amount of $1,25(OH)_2D$. However, even anephric patients exhibit some degree of circulating $1,25(OH)_2D$ levels. The peripheral tissue production of $1,25(OH)_2D$ does not seem to be regulated by calciotropic hormones (PTH, calcitonin, and FGF-23) and depends probably on the local 25(OH)D concentration in these tissues [216]. Likewise, we described that calcitriol levels in HD patients increased after a 6-month supplementation with cholecalciferol.

In vitro studies reported that 25(OH)D may directly activate the VDR in the parathyroid gland, independent of $1,25(OH)_2D$, contributing to the control of secondary hyperparathyroidism [217]. Kandula *et al.* [53] meta-analysis of vitamin D supplementation in CKD patients, in which our preliminary 6-month results were included, showed a decline in PTH levels (mean decrease of 41.7 pg/mL in observational studies and 31.5 pg/mL in randomized trials) with vitamin D supplements. This decrease was higher in dialysis patients compared with nondialysis-dependent CKD patients.

Our study showed a decrease in calcium and phosphorus levels and an improvement in secondary hyperparathyroidism control with cholecalciferol supplementation. This reduction in serum calcium and phosphorus was more significant than the reduction seen in iPTH and was independent of active vitamin D therapy since patients that have never used this medication also showed this reduction. Active vitamin D dose was also significantly reduced during the study and might contribute to a less calcemic and phosphoremic action seen at the end of the study.

Relatively to Mg levels, serum Mg was positively associated with 25(OH)D values and patients whose 25(OH)D levels did not increase during the study all had hypomagnesemia. Meanwhile, there was a significant increase in Mg serum levels after cholecalciferol supplementation, although there was no change in Mg intake during the study. Studies

have shown that the activities of three major enzymes that determine 25(OH)D level and VDBP are Mg-dependent [105]. Mg intake has been associated with a reduced risk of vitamin D deficit or insufficiency [160]. On the other hand, it has been reported that 1,25$(OH)_2$D can stimulate intestinal Mg absorption [170].

In conclusion, when we have a patient who, despite high doses of native vitamin D supplementation, remains unresponsive, it is mandatory to exclude Mg deficiency. The effects of vitamin D supplementation on Mg circulating levels were investigated in 126 patients with type 2 diabetes mellitus, and a significant increase in Mg levels was found after they were supplemented with cholecalciferol for 6 months [161].

In uremic patients, vitamin D deficiency has been independently associated with erythropoietin hyporesponsiveness and anemia [68]. Vitamin D has also been involved in the improvement of erythropoiesis by a direct effect on erythroid precursor proliferation, downregulation of hepcidin gene expression [69], and/or, marginally, by controlling secondary hyperparathyroidism [70]. In our study, supplementation with cholecalciferol allowed an erythroid-stimulating agent dose reduction, with no modification of hemoglobin values. Our results also indicate a reduction in the inflammatory parameters with long-term supplementation, with an increase in albumin and a reduction in CRP.

As already described, lower 25(OH)D levels have been associated with increased blood pressure and LVMI, endothelial dysfunction, and CV events [86]. Our study has shown that the correction of vitamin D deficiency with prolonged therapy with native vitamin D, namely, cholecalciferol, could decrease LVMI. The reduction in BNP plasma levels and LVMI seen with supplementation was independent of pulse pressure and of the use of antihypertensive therapy, namely, ACE inhibitors, angiotensin II receptor blockers, and statins.

Based on our results, we corroborate long-term native vitamin D supplementation in CKD stage 5D patients, as it is a simple and safe measure with a possible positive impact on mineral and CV condition, although the effects in morbidity and mortality still need to be confirmed in RCTs.

3) Lower serum magnesium, cardiovascular risk factors, and mortality in 206 hemodialysis patients

Hypomagnesemia or, even more strikingly, lower Mg levels may be actively detrimental for CKD-MBD in HD patients, contributing to low bone turnover, poor insulin sensitivity and lower protection against inflammation and oxidative stress in arteries leading to higher vascular calcification, CV events and mortality. According to our results, from a large prevalent HD population with a long follow-up, lower Mg levels are a good CV risk marker, associated with higher pulse pressure, LVMI and simple vascular calcification score, and a good predictor of all-cause and CV mortality.

In our study, Mg levels are negatively associated with the presence of diabetes. In the general population, hypomagnesemia has also been linked to the development of type 2 diabetes mellitus [218, 219]. It has been suggested that Mg regulates cellular glucose metabolism directly because it serves as an important cofactor for various enzymes and acts as a second messenger for insulin [220]. It was also observed that insulin enhances intracellular Mg uptake and this in turn mediates diverse effects ascribed to insulin [221]. Furthermore, hypomagnesemia may induce altered cellular glucose transport, reduced pancreatic insulin secretion, defective post-receptor insulin signaling and/or altered insulin receptor interactions and thus aggravate insulin resistance [222].

Patients with lower serum Mg were significantly older, and serum albumin was lower, compared with those with higher serum Mg. These results are in accordance with others [223, 224] and may indicate that lower Mg is related to malnutrition in uremic patients. Unlike what we observed in our study, proton pump inhibitors have been described as a frequent cause of hypomagnesemia in the general population [225].

Hypomagnesemia has also been linked to increased inflammation. Experimental Mg deficiency induces an inflammatory syndrome in animal models, characterized by macrophage and white blood cell activation, the release of proinflammatory cytokines, activation of an acute-phase response and excessive production of oxygen-free radicals [226]. In our study, in patients with CRP ≤ 5 mg/dL, there was a negative association between Mg and inflammation (measured by CRP serum levels).

One other important role of Mg is the effect on PTH lowering through the binding to CaSR [227]. This receptor is expressed in both parathyroid and kidney, and it has distinct binding

sites for both calcium and Mg [227]. Furthermore, it seems that in the parathyroid gland, sensitivity to Mg is 2 - 3 times less than for calcium. In situations of high serum Mg, the cation is thought to bind the CaSR in the parathyroid gland and might cause reduced PTH release [228]. As in other studies, we demonstrated that Mg concentrations were inversely associated with iPTH levels.

Over the years, studies have reported an inverse relationship between Mg and blood pressure [219]. Despite considerable research, the exact underlying causes for altered Mg metabolism in hypertension remain unclear. It is assumed that inadequate dietary Mg intake or a malfunction in Mg metabolism can lead to vasospasm and endothelial damage [229, 230]. Mg deficiency, when combined with stress and catecholamine secretion, might lead to enhanced entry of calcium into VSMCs, which in turn can result in increased arteriolar tone and coronary spasm. Hypertension and its complications may also be the final consequence of increased calcium influx and contraction of VSMCs [188]. Moreover, it was observed that Mg acts on most types of calcium channels in VSMCs exerting substantial arterial blood pressure-lowering properties, resulting in a reduction of peripheral and cerebral vascular resistance [221].

In vivo and *in vitro* studies in animals (pregnant rats) have demonstrated Mg-induced relaxation of smooth muscle. Such findings suggest the vasodilatory potential of Mg in large arteries such as the aorta [231], in smaller resistance vessels such as the mesenteric arteries and the cerebral arteries [232]. These and similar data indicate that vascular tone can be modified by decreasing blood pressure via minor changes in Mg levels [233]. Our study also showed, in a large HD population, an inverse association between Mg concentrations and pulse pressure.

A large epidemiological study revealed that low Mg levels, regardless of other CV risk factors, were associated with the long-term gain of left ventricular mass [234], a significant predictor of adverse CV events. In line with these results, we revealed that lower Mg concentrations were predictors of an increased LVMI. One possible explanation for this association is the relationship between hypomagnesemia and high aldosterone levels [235], responsible for the development of left ventricular hypertrophy and myocardial fibrosis. As well as in the general population [235] and CKD patients [236-238], our study demonstrated that lower Mg levels were associated with the presence of more vascular calcifications (as showed by a higher X-ray simple vascular calcification score).

Hypomagnesemia has been associated in CKD [223, 239] and non-CKD [221] patients with overall and CV mortality. Accordingly, we found that lower Mg levels are an independent predictor of all-cause and CV mortality in dialysis patients. The reason for this association is still unknown but may be because lower Mg concentrations are related to decreased immunity [240], increased inflammation [241], higher susceptibility to malignant neoplasia [242], and CV disease [221, 235]. Our results were included in a large meta-analysis in CKD patients that concluded that lower Mg concentration is associated with CV events and CV and overall mortality [153, 155, 243].

While the mean Mg serum concentration of our HD patients (1.36 mmol/L) would be considered indicative of mild hypermagnesemia in healthy population, serum Mg concentrations in HD patients may be optimal at a higher concentration, because of a lower ionized fraction and, given a better survival under uremic conditions, without causing severe and symptomatic hypermagnesemia. Further, dialysate Mg concentrations may be optimal at a concentration higher than the current concentrations to achieve better patient' outcomes, since the serum Mg concentration in HD patients parallels the Mg level in the dialysate [135]. In hemodiafiltration, we must also consider the fact that convection generates a negative balance of this cation, which is independent of its concentration in the dialysate [239].

Alternatively, the use of calcium acetate / Mg carbonate as a phosphate binder could also amplify these protective effects of Mg on CV dysfunction. More studies are required to establish an optimal level of Mg in HD patients and an optimal concentration of Mg in the dialysate.

4) Calcium acetate / magnesium carbonate therapy and cardiovascular risk markers in 138 hemodialysis patients

The authors demonstrated, for the first time, that in prevalent HD patients, calcium acetate / Mg carbonate, used as phosphate binder therapy, is associated with a significant reduction in LVMI and pulse pressure as well as in aortic valve calcifications and mitral valve calcifications, compared with patients who did not take phosphate binders. Furthermore, calcium acetate / Mg carbonate turned out to be superior to sevelamer therapy in terms of reduction of pulse pressure, LVMI, and aortic valve calcifications at the end of the study

while there was no significant difference between these 2 groups of patients in terms of mitral valve calcifications.

In conclusion, patients on calcium acetate / Mg carbonate showed a significant reduction of pulse pressure and aortic valve calcifications while calcifications progressed in patients on sevelamer and in patients without any phosphate binder. Furthermore, in a multivariable analysis, calcium acetate / Mg carbonate therapy was negatively associated with the progression of LVMI and aortic valve calcification. These data indicate that this therapy, in contrast to calcium-based phosphate binders without Mg, might be able to benefit aortic calcification in HD patients and positively influence CV risk markers, and in this regard, to some extent, even surpasses the calcium-free phosphate binder sevelamer in terms of vascular protection.

Supplementation with Mg in CKD patients is still a matter of debate since total Mg is frequently increased in these patients. In a small interventional study with 47 HD patients, Mg supplementation was associated with a significant improvement in bilateral carotid intima-media thickness [152]. In a recent randomized double-blinded, placebo-controlled study, Mg supplementation over 6 months also resulted in a decrease of carotid intima thickness, which is possibly due to an inhibition of calcification [244]. Mg seems to influence molecular processes associated with the inhibition of vascular calcification such as a decrease of the calcium entry into the cells of the media, prevention of cell damage (apoptosis) [184] and inhibition of Wnt / β-catenin signaling pathway [186].

The improvement of LVMI under the influence of calcium acetate / Mg carbonate therapy (- 8.7 ± 3.6 g/m^2) in our study was surprisingly pronounced compared to data from the literature. In the population-based longitudinal 'Study of Health in Pomerania' with 1,348 subjects, Reffelmann *et al.* [234] found a decrease of LVMI by 0.5 ± 2.8 g/m^2 in subjects with the highest Mg serum levels in the whole study population (highest quintile 0.85 mmol/L). A possible explanation might be that our data refers to HD patients while the data of Reffelmann *et al.* [234] refers to the general population. CKD patients with their increased CV morbidity and mortality might be more susceptible to Mg supplementation, using Mg-containing phosphate binders, possibly leading to more pronounced effects on the CV system. This assumption is in line with data from the literature, indicating that increased serum Mg levels in HD patients are associated with beneficial effects, such as decreased vascular calcification [223, 237, 245].

In general, our data are in accordance with other publications underlining the efficacy of calcium acetate / Mg carbonate in terms of lowering serum phosphate levels without promoting a continuously positive calcium balance. In several smaller studies in HD patients, the addition of Mg to calcium-based phosphate binders resulted in an effective reduction of serum phosphorus levels that were at least as good as with the calcium salt alone, but without increasing calcium levels [246]. In a 3-year parallel-group study in HD patients, treatment with calcium acetate / Mg carbonate significantly lowered both serum phosphate and serum calcium concentrations more effectively than treatment with calcium carbonate alone [247]. This information from the literature indicates that the use of combined Mg- and calcium-based phosphate binder regimens, besides showing a good tolerability profile, allows a reduction in the calcium load.

The important question of Mg intake via Mg-containing phosphate binders can invert the negative effect of calcium-based phosphate binders on vascular calcification in HD patients can only be answered by the means of prospective clinical RCTs but there is some first evidence for this assumption [127]. This outcome was confirmed by determining the effect of calcium acetate / Mg carbonate and sevelamer on the vascular media calcifications in a rat model of uremia with both phosphate binders being equally effective in reducing aortic calcifications [248].

A large clinical trial was published in 2010, comparing the efficacy and tolerability of calcium acetate / Mg carbonate with that of sevelamer [249]. In this randomized, controlled parallel-group, investigator-blinded multicenter study, 255 HD patients were randomized to receive either calcium acetate / Mg carbonate (n = 126) or sevelamer (n = 129). The study aimed to show the non-inferiority of calcium acetate / Mg carbonate in lowering serum phosphorus levels into the KDOQI target level range after 24 weeks. This primary efficacy measure was fulfilled. With both drugs, serum phosphorus levels decreased significantly at week 25 and calcium acetate / Mg carbonate turned out to be non-inferior to sevelamer in this respect.

Additionally, as in our study, serum calcium did not differ between the 2 groups and there was only a minimal increase in ionized serum calcium in the calcium acetate / Mg carbonate group (treatment difference of 0.048 mmol/L), which was not associated with a higher risk of hypercalcemia. Both treatments turned out to be comparably effective and well tolerated. This outcome was confirmed by investigating the effects of both phosphate binders on FGF-23, an important marker for outcome and severity of CV disease in CKD [250]

that is inversely associated with serum Mg concentration in HD patients [251]. Here, again, calcium acetate / Mg carbonate and sevelamer turned out to have comparable efficacy.

The data of our investigation even went beyond that and showed some advantages of calcium acetate / Mg carbonate *versus* sevelamer, namely in terms of reduction of pulse pressure, LVMI and aortic valve calcifications at the end of the study. In summary, our data, together with information from the literature, confirm that calcium acetate / Mg carbonate is at least as effective as sevelamer in reducing serum phosphorus and in preventing a continuously positive calcium balance, which is associated with an increased risk of vascular calcifications.

Our additional finding is that patients who had taken calcium acetate / Mg carbonate showed significantly higher serum Mg levels compared to patients under sevelamer or patients without phosphate binder therapy. The question remains unanswered if the differences in serum Mg between the 3 patient groups, though statistically significant, are of clinical relevance in terms of outcomes. However, serum Mg is a less-than-ideal marker of body Mg status, as 99% of body Mg is located intracellularly [252].

The recommended dietary allowance for Mg in the European Union is presently 375 mg/day [253], and dialysis patients who follow a renal diet can be expected to ingest much less than this amount [254]. Thus, the average supplementation of 168 mg/day of elemental Mg may have considerably contributed to an adequate Mg supply in our patients taking calcium acetate / Mg carbonate, with a consequent reduction in CV risk markers.

5) Bone fragility fracture risk factors in 341 prevalent hemodialysis patients

In this retrospective study with a large population and a long follow-up, the authors determined for the first time, the incidence of fragility fractures in a Portuguese HD population. Besides the classical features that are also present in the general population, features specific to CKD-MBD, such as hyperphosphatemia, diminished activation of vitamin D, secondary hyperparathyroidism, and elevated FGF-23, associated with exposure to medications altering bone metabolism in patients with CKD are likely to contribute to fracture risk [7].

Our study found an incidence of fragility fracture in prevalent HD patients of 31 per 1000 person-years, which is nearly three times greater than that reported for elderly patients without CKD [255]. This incidence rate is similar to that presented in the Dialysis Outcomes and Practice Patterns Study (DOPPS)[256], and the US Renal Data System [257]; however, in our study, we analyzed all radiologically proven fractures, whether hospitalized or not, which may have overestimated the incidence rate.

Incident vertebral fractures were identified in 13% of the patients of our HD cohort, compared with a prevalence of 55.3% in a study of 387 patients by Fusaro *et al.* [258]. The authors in that study determined the prevalence of historical vertebral fractures in HD patients, irrespective of symptoms, identified radiologically using specialized, quantitative vertebral morphology software (MorphoX-Press), whereas we identified the incidence of only new symptomatic fractures.

Like other studies, demographic risk factors, including female gender, older age, diabetes, low BMI, longer dialysis vintage, and a previous history of fracture, were also highly associated with an increased risk of fracture [259]. Our data are also consistent with the previous reports that have shown an association between the risk of fractures in the HD population and lower serum albumin [260] and no significant fracture association with serum calcium and phosphorus [261].

On the contrary, like other studies lower Mg serum levels were associated with a higher incidence of fractures [176, 177]. Although mild hypermagnesemia may be preferable for HD patients in terms of survival, there are some concerns about its potential harmfulness to bone metabolism. While Mg is an essential bone mineral, excess Mg can disturb bone mineralization.

According to the data from the Japanese Society for Dialysis Therapy - Renal Data Registry, however, mild hypermagnesemia was not associated with an increased risk of hip fracture in HD patients [262]. Rather, lower serum Mg levels were significantly associated with an increased risk of hip fracture. Notably, the population-attributable fraction of serum Mg levels for incident hip fracture was higher than that of serum calcium, serum phosphate, and iPTH levels, suggesting that low Mg may contribute to the fracture burden more deeply than the other factors related to mineral and bone disorder in CKD. Further studies are needed to ensure whether intervention for Mg with supplements prevents the occurrence

of fracture.

Blayney *et al.* [263] reported that a higher serum total ALP was associated with a higher incidence of hospitalization for fracture in the analysis of the DOPPS study. Coco *et al.* [264] analyzed 56 hip fracture patients on dialysis, and they found that age, albumin, PTH, and ALP were independent predictors. In addition, Kaji *et al.* [265] investigated 183 HD patients and found that serum ALP was significantly higher in HD patients with hip fractures than in those without fractures.

ALP is mainly a biochemical marker of bone turnover, and it is usually used to monitor metabolic bone disease, particularly the management of CKD-MBD. A higher ALP might be associated with fracture through high bone turnover [266]. Indeed, Park *et al.* [267] found that serum ALP was negatively associated with bone mineral density (BMD) assessed by dual-energy X-ray absorptiometry (DEXA) in HD patients. However, ALP is primarily secreted by the liver and bone, and a small amount is also secreted by the intestine, kidneys, and leukocytes. Thus, monitoring of bALP is preferred in the assessment of bone mineral metabolism [268]. In a single-center cohort study, Iimori *et al.* [269] investigated 485 HD patients with a median follow-up time of 39.9 months, and they found that serum bALP was associated with any type of incident fracture. Our results also showed that higher values of serum bALP were predictors of fragility fractures in prevalent HD patients.

Our study demonstrated that fracture risk was U-shaped and associated with serum iPTH levels when patients were stratified by different iPTH levels in three groups (iPTH < 300, 300 < iPTH < 800, iPTH > 800 pg/mL). It is known that the level of PTH in CKD patients may suggest the histologic change associated with bone fracture. When serum PTH level is < 150 pg/mL, the fracture is most likely due to adynamic bone disease or osteomalacia. However, in patients with higher PTH (> 600 pg/mL), the main cause of fractures is most likely to be due to osteitis fibrosa, which is prone to developing fractures, despite frequently increased trabecular bone mass [270].

Although within the PTH range of 150 - 300 pg/mL, many patients may exhibit either one or the other forms of renal osteodystrophy [271]. Unfortunately, we lack evidence of an association between fracture incidence and the histologic type of bone disease. Despite definitive proof, low or high bone turnover seems to favor fractures as both increase bone fragility. This might be explained in part by the absence of analysis of cortical bone, which

is predominantly affected in CKD [272].

Both high and low circulating PTH levels can be associated with a high fracture rate and mortality risk [256, 260, 261]. Interestingly, serum PTH levels, just before the occurrence of a new fracture, are associated with the increased risk of fracture, in contrast to baseline or time-averaged serum PTH levels [269]. Long-term exposure to high PTH could induce a preferential loss of cortical bone, which could be even more pronounced in female than in male patients with CKD stage 5D [273].

Our study showed a possible protective role of active vitamin D in preventing fractures in HD patients. Active vitamin D administration is associated with increased bone strength in animal models [274] and a prospective, randomized, placebo-controlled, double-blind study showed improved BMD assessed by DEXA with lowering of plasma PTH levels [275]. Poor performance on tests of neuromuscular function also may identify those at higher risk of fracture in CKD. This is likely to reflect, in part, their higher risk of falls due to impaired muscle strength. The mechanism underlying this association is not clear, but a reduction in active vitamin D seems to be important [276, 277].

We believe that instead of restricting active vitamin D therapy for the control of secondary hyperparathyroidism, physiologically low doses of VDR agonists should be given to all CKD patients, even those with normal or low PTH, to add all vitamin D beneficial effects [278]. In addition, native vitamin D supplementation should be performed on all patients to assure 25(OH)D levels above 30 ng/mL [278]. In non-CKD populations, pooled data from 11 double-blind RCTs of oral native vitamin D supplementation demonstrated an association, although not statistically significant, of vitamin D (≥ 800 IU / daily) and reduced hip fracture and any nonvertebral fracture in participants with 65 years of age or older [279]. Nevertheless, in CKD patients, we still have insufficient evidence correlating the correction of serum 25(OH)D level with reduced fracture risk [47, 280].

Even though the pathogenic factors linking vascular calcifications and bone fragility are not clear, low bone volume, evaluated using bone biopsy, was found to be a significant risk factor for vascular calcifications in uremic patients [214, 281]. In addition, low bone volume has been recognized as an important risk factor for the occurrence of fractures in both the general population [282] and dialysis patients [279]. Schulz *et al.* [283] also demonstrated that patients with the highest degree of aortic calcification had the lowest BMD. In the same

cohort followed for 2 years, bone loss was greater in patients with progressive vascular calcifications [283].

In agreement with these results, another study has shown that after 4 years of follow-up, subjects with the most severe aortic calcification had not only a lower bone mass but also a higher incidence of new osteoporotic fractures [284]. Therefore, it is reasonable to assume that dialysis patients with evident vascular calcifications are more likely to experience a fracture. Our results showed that prevalent HD patients with a higher vascular calcification score presented an increased risk of fractures.

Older CKD patients are also prone to develop osteoporosis. The features of osteoporosis in CKD patients are low trabecular bone volume and disrupted micro-architecture, even without significant abnormalities in mineralization and bone turnover [285]. Bone loss is mostly from the cortical bone in subjects with CKD-MBD, and their iPTH, bALP, klotho, sclerostin, and fetuin-A levels are pronouncedly altered [279]. Due to the high prevalence of osteoporosis and CKD-MBD in CKD subjects, both conditions commonly exist simultaneously.

However, CKD-MBD is more complex than osteoporosis and CKD-MBD influences bone quality, contributes to high rates of fracture, and, most importantly, may facilitate the appearance of vascular calcifications in CKD patients. Although they both result in bone fragility, they do have different pathophysiology to destroy the bone. Osteoporosis is induced by excessive osteoclastic bone resorption, deficient osteoblastic activity, and bone formation in postmenopausal women and older subjects. However, CKD-MBD is related to altered mineral metabolism and the imbalance of pro- and anti-calcification factors which induce either high or low-turnover bone disease in CKD patients [273].

7. LIMITATIONS

We acknowledge that we performed mostly observational, unicentric studies, and consequently, associations may not mean causality.

In the first study included in this thesis, regarding the observed association between calcidiol levels, inflammation, CV risk markers, and mortality in 223 HD patients, the main limitation was being cross-sectional and observational. However, it showed for the first time in a Portuguese HD population a significant association between 25(OH)D insufficiency, CV risk factors, and mortality. Large randomized prospective studies are still needed to clarify the role of 25(OH)D as a prognostic marker or risk factor on the increased CV morbidity and mortality observed in CKD patients. Nevertheless, based on some meta-analyses and guidelines [103, 286-289] that included our study, it seems relevant that serum levels of 25(OH)D should be evaluated regularly in patients with CKD and insufficiency / deficiency treated.

The following study, a prospective and interventional study, about long-term (60 months) cholecalciferol supplementation in 97 HD patients: effects on mineral metabolism, inflammation, and cardiac parameters, the main limitation was the absence of a control group. As the general care and therapeutic approach of these patients did not change during the study, the results could be "assumed" to be due to cholecalciferol supplementation. Other limitations were the small number of patients studied and some drawbacks of echocardiography in evaluating LVMI throughout the study. However, this study was one of the first with native vitamin supplementation and with the longest follow-up in HD patients still yet reported, so it has also been included in some meta-analyses, reviews, and guidelines of CKD patients [36, 53, 67, 273, 286, 287, 290-306].

The 48-month prospective study on Mg, CV risk factors, and mortality in 206 HD patients also had some limitations since it was an observational study and because serum Mg levels were measured once at the beginning of the study. Nevertheless, this study showed a significant relationship between lower Mg concentrations and CV risk markers, like pulse pressure, LVMI and simple vascular calcification score, and with all-cause and CV mortality in a large HD population. This study was one of the longest follow-up studies regarding Mg levels in HD patients and was included in a large meta-analysis performed in HD patients [153, 155, 243, 307].

The main limitation of the prospective study about calcium acetate / Mg carbonate used as a phosphate binder in HD patients and its association with CV risk factors was the fact of not being randomized, so a selection bias was introduced into the study population. The patients who were treated with sevelamer were significantly younger and were less diabetic than the patients in the calcium acetate / Mg carbonate group and patients in the group without phosphate binders. This is remarkable, as it would render the sevelamer group much less prone to progression of calcifications and seems to underline the findings of our study instead of questioning them. A randomized study is warranted, which might find even stronger differences in calcification progression between the treatment groups.

Another possible positive aspect of our study was its long follow-up (48 months) so that the CV modifications could be observed in our patients. The study was also a single-center study with all HD patients of our dialysis center included, without any pre-selection.

The last study included in this thesis, regarding the incidence of bone fragility fractures and its risk factors in a large HD population (341 patients), with a long follow-up, had also some limitations. First, the retrospective and observational nature of this study precludes the conclusions of a causal relationship. Second, we did not evaluate the bone mineral density of the participants, although the ability of bone mineral density, as measured by DEXA in dialysis patients to predict the risk of fractures, is still dubious. Third, this was a single-center study, so the results cannot be generalized. However, all the participants were treated by the same physicians and underwent uniform laboratory measurements during the observation period, which might guarantee the accuracy of our results.

8. CONCLUSIONS

Patients on dialysis have a mortality rate higher than the general population, particularly CV mortality. Disturbed mineral and bone metabolism are a feature of these patients that predisposes them to CV disease and to a high risk of fragility fractures and extra-osseous calcifications, which also contribute to the increased morbidity and mortality of this population. Several mechanisms and biomarkers, not fully clarified, may be implicated in this CKD-MBD syndrome.

We demonstrated that deficiency of 25(OH)D and 1,25$(OH)_2$D is very prevalent in HD patients. Our results suggest that lower levels of 25(OH)D are a CV risk marker in prevalent HD patients since they are independently associated with higher BNP plasma levels, increased pulse pressure and the presence of higher vascular calcifications. Deficiency of 25(OH)D was also associated with CV and overall mortality.

Long-term cholecalciferol supplementation in prevalent HD patients corrected vitamin D deficiency without evident toxicity. It improved mineral metabolism control with less use of active vitamin D, decreased inflammatory parameters with reduction of erythropoiesis-stimulating agent consumption, and improved cardiac dysfunction (reflected by lower BNP levels and decreased LVMI). These effects may be related to the direct action of 25(OH)D on target cells and/or to persistent renal or extra-renal 1α-hydroxylation. If 25(OH)D levels are not corrected with supplementation, the concomitant existence of Mg deficiency should be excluded.

This supplementation was simple, apparently safe and cost-effective. It seems to be a treatment that positively affects surrogate markers and may potentially improve clinical outcomes. Based on this study, we corroborate long-term native vitamin D supplementation in CKD stage 5D patients, although the effects in morbidity and mortality still need to be confirmed in large randomized controlled studies.

In HD patients, lower serum Mg was associated with CV risk markers, such as higher pulse pressure, LVMI and simple vascular calcification score. Lower Mg levels also seemed to be independent predictors of overall and CV mortality in this population of prevalent HD patients. Randomized controlled trials examining the question of whether increased serum

Mg or higher Mg intake is beneficial or not, in terms of outcomes, in CKD patients are needed.

In prevalent HD patients, the use of calcium acetate / Mg carbonate as a phosphate binder, over 48 months was associated with a reduction of pulse pressure and LVMI and with a stabilization of aortic valve calcifications. In this regard, calcium acetate / Mg carbonate turned out to be at least as effective as or even superior to sevelamer. This phosphate binder was found to be a promising alternative for the treatment of hyperphosphatemia in HD patients with higher CV risk. These results need to be confirmed in randomized controlled studies.

The incidence of bone fragility fractures in HD patients is high and its risk increases with age, female gender, low serum albumin and the presence of more vascular calcifications. Prevalent HD patients with low or high iPTH levels or increased bALP also had a higher fracture risk. Active vitamin D therapy seems to have a protective role in the development of both fractures and vascular calcifications. Assessment of fracture risk and management in dialysis patients at the greatest risk requires further study.

This new knowledge changed / could change our clinical practice:

1) All HD patients must have regular measurements of 25(OH)D levels and deficiency / insufficiency should be supplemented with native vitamin D. This strategy is safe and effective. It reduces iPTH and could also have pleiotropic effects decreasing CV events in these patients. We should try to achieve target levels of 25(OH)D from 40 to 60 ng/mL to have maximum benefits.

2) Adding a low dose of active vitamin D to native vitamin D and/or calcimimetic could protect patients from bone fractures and vascular calcifications.

3) Despite apparently high Mg levels in HD patients, levels of ionized Mg (the biologically active form) are reduced in these patients. Lower levels of Mg in dialysis patients prove to be associated with CV risk markers and mortality. Increasing the Mg of dialysate or using medications that could increase Mg levels should be considered.

4) Normal levels of Mg seem to be necessary for adequate vitamin D function and giving Mg itself can increase 25(OH)D in patients with 25(OH)D deficiency. In clinical practice, in a patient who, despite high doses of vitamin D supplementation, remains unresponsive,

it is mandatory to exclude Mg deficiency. A combined Mg and vitamin D treatment may be more effective in increasing 25(OH)D levels compared with native vitamin D supplementation alone, particularly in patients with lower 25(OH)D concentrations such as obese and CKD patients.

5) The value of bALP is highlighted in our bone fracture study as a risk marker for fractures in HD patients, and not only low and high levels of PTH should be considered as risk factors for fragility fractures.

6) The follow-up of CKD patients must now include other available biomarkers and/or mediators of bone-kidney-vessel axis in our daily practice besides PTH, as regular measurements of 25(OH)D, Mg and bALP.

Further prospective randomized multicentric studies with more patients are needed to confirm our observations.

Patrícia Matias

November 2023

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