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ROZPRAWA DOKTORSKA

Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek

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Spis treści

Zestawienie publikacji

Publikacje/ osiągnięcia	**Liczba**	**Impact Factor**	**Punktacja MNiSW**
Prace oryginalne włączone do rozprawy	1	6.706	140
Prace poglądowe włączone do rozprawy	1	6.706	140
Współautor prac niewłączonych do rozprawy	1	5.924	140
Streszczenia zjazdowe	3	-	-
Razem	6	19.336	420

Rozprawa doktorska

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1. Artykuły stanowiące cykl prac włączonych do rozprawy doktorskiej

Rodzaj publikacji	Tytuł artykułu	Nazwa czasopisma	Data publikacji	Punkty MNiSW	Impact factor
Publikacja nr 1 – praca poglądowa	*Vitamin K and D supplementation and bone health in chronic kidney disease–apart or together?*	Nutrients	01.03.2021	140	6.706
Publikacja nr 2 – praca oryginalna	*Vitamin K-dependent carboxylation of osteocalcin in bone–ally or adversary of bone mineral status in rats with experimental chronic kidney disease?*	Nutrients	01.10.2021	140	6.706

2. Wykaz stosowanych skrótów i oznaczeń

ALP (ang. *Alkaline Phosphatase*) – fosfataza alkaliczna

ATF4 (ang. *Activating Transcription Factor 4*) – czynnik transkrypcyjny 4

BALP (ang. *Bone Alkaline Phosphatase*) – fosfataza zasadowa izoenzym kostny

BGLAP (ang. *Bone Gamma-Carboxyglutamate Protein*) – białko gamma-karboksyglutaminianowe kości/ osteokalcyna

BMA (ang. *Bone Mineral Area*) – obszar mineralny kości

BMC (ang. *Bone Mineral Content*) – zawartość mineralna kości

BMD (ang. *Bone Mineral Density*) – gęstość mineralna kości

CKD (ang. *Chronic Kidney Disease*) – przewlekła choroba nerek

CKD-MBD (ang. *Chronic Kidney Disease – Mineral Bone Disorders*) – zaburzenia mineralno-kostne w przewlekłej chorobie nerek

CON (ang. *Control Group*) – grupa kontrolna

dp-ucMGP (ang. *Desphospho-Uncarboxylated Matrix Gla Protein*) – defosfonieukarboksylowane białko macierzy Gla

DEXA (ang. *Dual Energy X-ray Absorptiometry*) – absorpcjometria rentgenowska o podwójnej energii

ELISA (ang. *Enzyme-Linked Immunosorbent Assay*) – test immunoenzymatyczny

FGF-23 (ang. *Fibroblast Growth Factor*) – czynnik wzrostu fibroblastów-23

FOXO1 (ang. *Forkhead Box Transcription Factor 1*) – czynnik transkrypcyjny należący do rodziny forkhead box 1

GAPDH (ang. *Glyceraldehyde-3-Phosphate Dehydrogenase*) – dehydrogenaza aldehydu 3-fosforanowego gliceryny

GFR (ang. *Glomerular Filtration Rate*) – współczynnik filtracji kłębuszkowej

GGCX (ang. *γ-Glutamyl Carboxylase*) – γ-glutamylokarboksylaza

HD (ang. *Hemodialysis*) – hemodializa

HPLC (ang. *High-Performance Liquid Chromatography)* – wysokosprawna chromatografia cieczowa

IL1α (ang. *Interleukin 1α*) – interleukina 1α

LC-MS/MS (ang. *Liquid Chromatography – Mass Spectrometry*) – chromatografia cieczowa z tandemową spektrometrią mas

M-CSF (ang. *Macrophage Colony-Stimulating Factor*) – czynnik stymulujący tworzenie kolonii makrofagów

MGP (ang. *Matrix Gla Protein*) – białko macierzy Gla

MKs (ang. *Menaquinones*) – menachinony/ witamina K2

MRM (ang. *Multiple Reactions Monitoring*) – monitorowanie reakcji wielokrotnych

MSCs (ang. *Multipotent, Mesenchymal Stem Cells*) – multipotencjalne, mezenchymalne komórki macierzyste

OC (ang. *Osteocalcin*) – osteokalcyna

OPG (ang. *Osteoprotegerin*) – osteoprotegryna

OPN (ang. *Osteopontin*) – osteopontyna

PGE2 (ang. *Prostaglandin E2*) – prostaglandyna E2

PIVKA-II (ang. *Protein Induced by VK Absence/Antagonism* II) – białko indukowane przez brak witaminy K lub antagonistę II

pQCT (ang. *Peripheral Quantitative Computed Tomography*) – obwodowa ilościowa tomografia komputerowa

PTH (ang. *Parathyroid Hormone*) – parathormon

R1 (ang. *Metaphyseal Area of Femur*) – obszar przynasadowy kości udowej

R2 (ang. *Diaphyseal Area of Femur*) – obszar trzonu kości udowej

RANKL (ang. *Receptor Activator for Nuclear Factor κB*) – aktywator receptora dla ligandu czynnika jądrowego κB na osteoblastach

RCT (ang. *Randomized Control Trials*) – randomizowane kontrolowane badania kliniczne

RT-PCR (ang. *Real-Time Polymerase Chain Reaction*) – ilościowa reakcja łańcuchowej polimerazy w czasie rzeczywistym

RUNX2 (ang. *Runt-Related Transcription Factors 2*) – czynnik transkrypcyjny związany z Runt 2

SXR (ang. *Steroid and Xenobiotic Receptor*) – receptor steroidowy i ksenobiotyczny

TRAP (ang. *Tartrate-Resistant Acid Phosphatase*) – winianooporna kwaśna fosfataza

UBIAD1 (ang. *UbiA Prenyltransferase Domain Containing 1*) – enzym zawierający domenę transferazy prenylowej UbiA

ucOC (ang. *Uncarboxylated Osteocalcin)* – nieukarboksylowana osteokalcyna

vBMD (ang. *Volumetric Bone Mineral Density*) – objętościowa gęstość mineralna kości

VK (ang. *Vitamin K*) – witamina K

VKDPs (ang. *VK-Dependent Proteins*) – białka zależne od witaminy K

VKOR (ang. *Vitamin K Epoxide Reductase*) – reduktaza epoksydowa witaminy K

VKORC1 (ang. *Vitamin K Epoxide Reductase Complex Subunit 1*) – podjednostka 1 kompleksu reduktazy epoksydu witaminy K

VSMC (ang. *Vascular Smooth Muscle Cell*) – komórki mięśni gładkich naczyń

3. Wstęp

Na przełomie ostatnich dekad znacząco wzrosła zapadalność na choroby nerek, stając się głównym problemem zdrowia publicznego. Rozpowszechnienie przewlekłej choroby nerek (CKD, *Chronic Kidney Disease*) w populacji sięga 13% [1]. Ponadto prognozuje się, że do 2040 roku CKD stanie się piątą na świecie najczęstszą przyczyną przedwczesnej umieralności [2].

Z uwagi na szereg funkcji pełnionych przez nerki oraz ich wpływ na homeostazę całego organizmu [3] zaburzenia zarówno strukturalne, jak i czynnościowe nerek wiążą się z wystąpieniem powikłań CKD, które znacząco wpływają na jakość życia chorych [4].

3.1. Zaburzenia mineralno-kostne w przewlekłej chorobie nerek

Upośledzona czynność nerek prowadzi do rozwoju zaburzeń gospodarki kostnej i mineralnej, co wiąże się z nieprawidłową mineralizacją kości, zwapnieniem naczyń krwionośnych oraz tkanek miękkich [5,6]. Powyższe powikłania w przewlekłej chorobie nerek określone są terminem zaburzeń mineralno-kostnych (CKD-MBD, *Chronic Kidney Disease – Mineral Bone Disorders*) [7] i charakteryzują się wystąpieniem jednej lub więcej nieprawidłowości dotyczących stężenia wapnia, fosforu, układu czynnika wzrostu fibroblastów-23 (FGF-23)/Klotho, parathormonu (PTH) czy metabolizmu witaminy D. Powyższe nieprawidłowości przekładają się na zaburzenia obrotu kostnego, mineralizacji lub wytrzymałości kości oraz występowanie zwapnień w układzie naczyniowym lub innych tkankach miękkich [5-9]. CKD-MBD istotnie przyczynia się do zwiększonego ryzyka złamań oraz wzrostu śmiertelności wśród pacjentów nefrologicznych [10]. Wysokie ryzyko złamań w tej populacji wynika z połączenia zmian w metabolizmie kostnym i mineralnym wywołanych przez CKD oraz klasycznych czynników ryzyka złamań obserwowanych w populacji osób bez dysfunkcji nerek [10,11]. Patogeneza CKD-MBD oraz skłonność do złamań jest złożona, dodatkowo leczenie skupiające się na wyrównaniu zaburzeń mineralnych w surowicy nie jest wystarczające, by ograniczyć wystąpienie i rozwój powikłań CKD-MBD.

Hiperfosfatemia, będąca skutkiem retencji fosforu, jest zjawiskiem stosunkowo późnym w naturalnym przebiegu CKD, ujawniającym się klinicznie zwykle w stadium 4-5. Wzrost wydalania fosforu przez pozostałe nefrony przywraca jego homeostazę kosztem wyższych poziomów FGF-23 i PTH w surowicy. W sytuacji, gdy GFR (*Glomerular Filtration Rate*, współczynnik filtracji kłębuszkowej) osiągnie poziom poniżej 30% normy, hiperfosfatemia utrwala się z powodu niedostatecznego wydalania przez nerki [12]. W trzecim stadium CKD, wskutek zatrzymywania fosforanów lub jako efekt wtórny poprzez stymulację FGF-23, zmniejsza się stężenie krążącej we krwi 1,25-dihydroksywitaminy D (1,25$(OH)_2$D). Obniżony

poziom kalcytriolu w organizmie prowadzi do zmniejszenia wchłaniania wapnia z jelita i kanalików proksymalnych, powodując skłonność do hipokalcemii, czemu przeciwdziała zwiększona produkcja i sekrecja PTH. Hiperfosfatemia również bezpośrednio zwiększa ekspresję genu PTH, a efektem końcowym powyższych zaburzeń jest wtórna nadczynność przytarczyc [6].

Kluczowymi badaniami stosowanymi w diagnostyce CKD-MBD są zatem: stężenie wapnia, fosforanów i fosfatazy alkalicznej (ALP, *Alkaline Phosphatase*) w surowicy. W wytycznych KDIGO zalecane jest, aby były one rutynowo monitorowane od 3-go stadium CKD [7]. Parathormon powinien być kontrolowany w stadium 4 CKD, natomiast można również uwzględnić jego oznaczanie już w stadium 3. Pacjentom należy także przeprowadzić badania pod kątem niedoboru witaminy D, a stężenie 25-hydroksywitaminy D powinno być mierzone co najmniej raz w roku [7,9]. Pomiary FGF-23 i białka Klotho nie odgrywają istotnej roli w diagnostyce oraz w rutynowym monitorowaniu CKD-MBD, a przydatność kliniczna tych markerów do oceny ryzyka złamań u pacjentów z CKD wymaga dalszych badań.

W przebiegu CKD-MBD dochodzi do upośledzenia zarówno jakościowego, jak i ilościowego tkanki kostnej, co prowadzi do zmniejszenia wytrzymałości kości. Najwcześniejsze nieprawidłowości immunohistochemiczne kości u chorych z CKD-MBD obserwuje się już przy stosunkowo łagodnym zmniejszeniu wskaźnika przesączania kłębuszkowego (między 60 a 90 ml/min/1,73 m^2). Masa kostna jest zwykle oceniana za pomocą absorpcjometrii rentgenowskiej o podwójnej energii (DEXA, *Dual Energy X-ray Absorptiometry*), która pozwala ocenić powierzchniową gęstość mineralną kości (BMD [g/cm^2], *Bone Mineral Density*). Jednakże badania obrazowe, takie jak zdjęcia rentgenowskie oraz obliczenie BMD nie są rutynowo wykonywane w przebiegu CKD, ponieważ nie pomagają przewidzieć ryzyka złamań kości ani rozpoznać rodzaju osteodystrofii [9]. Natomiast obwodowa ilościowa tomografia komputerowa (pQCT, *Peripheral Quantitative Computed Tomography*) jest techniką obrazowania, która mierzy rzeczywistą objętościową gęstość mineralną kości (vBMD [g/cm^3], *Volumetric Bone Mineral Density*) [13]. W zwierzęcych modelach CKD istnieje możliwość wykonania biopsji kości do analizy histomorfometrycznej lub przeprowadzenia badań właściwości biomechanicznych i geometrycznych kości [14-17].

Obecne zalecenia dotyczące terapii CKD-MBD skupiają się na hamowaniu wysokiego obrotu kostnego poprzez zastosowanie suplementacji aktywną formą witaminy D i/lub farmakoterapię kalcymimetykami [5,6,18]. Rekomenduje się, aby leczenie głównych nieprawidłowości związanych z CKD-MBD, takich jak niedobór witaminy D, hiperfosfatemia i nadczynność przytarczyc, miało miejsce przed rozpoczęciem leczenia środkami

antyresorpcyjnymi lub anabolicznymi [5,7-9,18]. Ze względu na fakt, że dla pacjentów z powikłaniami CKD-MBD nie opracowano specjalistycznego leczenia przeciwdziałającego złamaniom, farmakologiczne postępowanie w stadium 1-3 CKD nie różni się od postępowania w terapii osteoporozy u chorych bez CKD. Jednakże terapia zaburzeń kostnych w stadium 4-5 CKD pozostaje wyzwaniem, ponieważ większość leków przeciwdziałających złamaniom kości jest przeciwwskazana u pacjentów z eGFR <30 ml/min [10,19].

3.2. Proces przebudowy tkanki kostnej

Kość jest wysoce dynamiczną tkanką, która przez całe życie podlega procesom przebudowy mającym na celu utrzymanie odpowiedniej objętości, mikrostruktury, wytrzymałości kości oraz homeostazy mineralnej. W przebudowie tkanki kostnej odgrywają rolę dwie antagonistycznie działające populacje komórkowe – osteoklasty, które mają zdolność do resorpcji zmineralizowanej tkanki kostnej oraz osteoblasty kontrolujące tworzenie kości [20]. W zdrowym dorosłym szkielecie ilość zresorbowanej kości jest na ogół równa ilości nowo powstałej tkanki kostnej, co pozwala zachować integralność szkieletu i prawidłową wytrzymałość mechaniczną kości. Zaburzenie równowagi homeostazy kostnej prowadzi do rozwoju metabolicznych chorób kości, takich jak osteoporoza i osteoliza, gdzie występuje nadmierna osteoklastogeneza lub osteopetroza, w której dochodzi do nadmiernego kościotworzenia [21,22].

Osteoklasty są to wielojądrzaste komórki olbrzymie utworzone z komórek jednojądrzastych linii monocytów/makrofagów. Ich przeżywalność, ekspansja i różnicowanie prekursorów osteoklastów podlega stymulacji przez dwa główne czynniki – czynnik stymulujący tworzenie kolonii makrofagów (M-CSF, *Macrophage Colony-Stimulating Factor*) oraz aktywator receptora ligandu jądrowego czynnika kappa-B (RANKL, *Receptor Activator Of Nuclear Factor Kappa-B Ligand*) [23]. Osteoblasty pochodzą z multipotencjalnych, mezenchymalnych komórek macierzystych (MSCs, *Multipotent, Mesenchymal Stem Cells*), a ich różnicowanie jest regulowane przez główny czynnik transkrypcyjny RUNX2 (*Runt-Related Transcription Factors 2*), który odgrywa zasadniczą rolę w ekspresji genów związanych z osteoblastogenezą, takich jak fosfataza alkaliczna, osteokalcyna (BGLAP, *Bone Gamma-Carboxyglutamate Protein*) i osteopontyna (OPN, *Osteopontin*) [20,24].

Podczas przebudowy kości dochodzi do rekrutacji osteoklastów (faza aktywacji), których zadaniem jest niszczenie nieorganicznych składników macierzy zewnątrzkomórkowej (faza resorpcji). Następnie osteoklasty ulegają apoptozie, a w ich miejsce rekrutowane są osteoblasty (faza odwrócenia). W ostatnim etapie (faza formowania) osteoblasty odkładają

niezmineralizowaną macierz osteoidową, która wkrótce potem jest mineralizowana [22]. Proces przebudowy tkanki kostnej jest zatem kluczowym elementem zapewniającym odnowę uszkodzonej kości, w związku z czym podlega ścisłej kontroli przez hormony – głównie PTH i kalcytoninę, a także witaminę K, 1,25-dihydroksywitaminę D oraz czynniki wzrostu [20,22].

3.3. Witamina K

Witamina K (VK, *Vitamin K*) to grupa rozpuszczalnych w tłuszczach związków chemicznych, które występują w postaci witaminy K1 (filochinon) oraz K2 (MKs, ang. *Menaquinones*, menachinony). U ludzi najczęstszymi MKs są krótkołańcuchowe MK-4, które jako jedyne wytwarzane są przez ogólnoustrojową konwersję filochinonu do menachinonów przy udziale enzymu zawierającego domenę transferazy prenylowej UbiA (UBIAD1, *UbiA Prenyltransferase Domain Containing 1*). Wszystkie warianty VK są wchłaniane przez wątrobę, gdzie zachodzi ich katabolizm, natomiast metabolity są wydalane w około 40% z żółcią oraz w 20% z moczem [25].

Witamina K jest kofaktorem enzymu – γ-glutamylokarboksylazy (GGCX, *γ-Glutamyl Carboxylase*), który jest niezbędnym elementem w procesie powstawania białek zależnych od witaminy K (VKDPs, *VK-Dependent Proteins*). W reakcji gamma-karboksylacji do reszt Glu w nieukarboksylowanych białkach dodawane są grupy karboksylowe, co pozwala na ich przekształcenie w domeny Gla. W konsekwencji powstają aktywne VKDPs, takie jak osteokalcyna (OC, *Osteocalcin*) oraz białko macierzy Gla (MGP, *Matrix Gla Protein*), które poprzez wiązanie wapnia uczestniczą w procesach krzepnięcia krwi, zwapnienia naczyń oraz mineralizacji kości [26].

Aby witamina K mogła prawidłowo pełnić swoją fizjologiczną rolę, jest poddawana regeneracji określonej mianem „cyklu witaminy K". Są to reakcje redoks, których zadaniem jest przekształcenie epoksydu VK pod wpływem reduktazy epoksydowej (VKOR, *Vitamin K Epoxide Reductase)* do postaci chinonu, a następnie przy udziale reduktazy chinonowej do postaci hydrochinonu VK. Postać zredukowana witaminy K może zostać ponownie wykorzystana do γ-karboksylacji białek [27].

3.3.1. Rola witaminy K w przebudowie kości

Osteokalcyna (OC) jest jednym z głównych niekolagenowych białek występujących w kościach, wydzielanym przez osteoblasty. Po karboksylacji zależnej od witaminy K, OC rozwija wysokie powinowactwo do jonów wapnia, które włącza do kryształów hydroksyapatytu w macierzy kostnej [28]. Transkrypcja i translacja genu OC są regulowane

przez 1,25$(OH)_2$D [29] oraz PTH [30], a w ich wyniku powstaje niedojrzała forma – ucOC (*Uncarboxylated Osteocalcin,* nieukarboksylowana osteokalcyna). Krążąca OC jest wykorzystywana jako dobry biomarker tworzenia kości, jednakże gdy kość ulega resorpcji, fragmenty OC są uwalniane do krążenia, w wyniku czego stężenie w osoczu może odzwierciedlać obrót kostny. W badaniu przeprowadzonym u kobiet po menopauzie oraz u osób zdrowych wykazano ujemną korelację między stężeniem OC w surowicy a BMD [31]. U zdrowych dziewcząt zauważono, że filochinon w osoczu był odwrotnie skorelowany z krążącą OC, co wskazuje, że lepszy status witaminy K był związany ze zmniejszonym obrotem kostnym [32].

Białko Matrix Gla (MGP) jest syntetyzowane w komórkach mięśni gładkich naczyń (VSMC, *Vascular Smooth Muscle Cell*), chondrocytach i osteoklastach. Zgodnie z wynikami badań zarówno na zwierzętach, jak i na ludziach, MGP hamuje zwapnienie naczyń tętniczych i chrząstek, jednocześnie ułatwiając prawidłowy metabolizm kości [28,33]. Zhang i in. [34] wykazali, że nadekspresja MGP jest związana z zahamowaniem osteoklastogenezy i wzrostem masy kostnej, podczas gdy niedobór MGP stymuluje powstawanie i funkcjonowanie osteoklastów, przyspieszając resorpcję kości.

Jak ilustrują wyniki badań *in vitro*, witamina K (szczególnie K2) poprawia funkcję osteoblastów poprzez indukcję ich proliferacji i różnicowania [35,36] oraz hamowanie apoptozy osteoblastów za pośrednictwem białka Fas [37]. W obecności witaminy K2 hodowle komórkowe osteoblastów zwiększają zarówno aktywność ALP, jak i poziom OC [36]. Wyższa aktywność ALP związana jest z lepszym tworzeniem macierzy organicznej i części mineralnej kości oraz z odkładaniem się OC i hydroksyapatytu w kości. Witamina K2 aktywuje receptor steroidowy i ksenobiotyczny (SXR, *Steroid and Xenobiotic Receptor*) [38,39] oraz działa jako regulator transkrypcji licznych genów, będących biomarkerami linii osteoblastów i genów związanych z macierzą zewnątrzkomórkową [39,40]. Ponadto witamina K2 wspomaga kościotworzenie i hamuje resorpcję kości poprzez stymulację ekspresji osteoprotegeryny (OPG, *Osteoprotegerin*) oraz hamuje ekspresję RANKL na osteoblastach [36]. Yamagushi i wsp. [41] zaobserwowali, że witamina K2 indukowała spadek podstawowej i indukowanej przez cytokiny ekspresji NF-κB w osteoblastach, jak również w prekursorach osteoklastów, co wyjaśnia jej podwójne działanie proanaboliczne oraz antykataboliczne.

Z drugiej strony obecne dowody sugerują, że witamina K2 zmniejsza aktywność osteoklastów. Dla przykładu, VK zapobiega powstawaniu osteoklastów bezpośrednio lub pośrednio poprzez ingerencję w układ RANKL/OPG [36]. Witamina K zmniejsza zarówno proliferację komórek TRAP-dodatnich, jak i aktywność winianoopornej kwaśnej fosfatazy

(TRAP, *Tartrate-Resistant Acid Phosphatase*) w osteogennej pożywce hodowlanej [36,42]. Ponadto witamina K2 hamuje resorpcję kości indukowaną przez czynniki resorpcyjne kości, takie jak: prostaglandyna E2 (PGE2, *Prostaglandin E2*), interleukina 1α (IL1α, *Interleukin 1α*) i 1,25$(OH)_2$D [43]. Badanie Kameda i in. [42] wykazało potencjał witaminy K2 do indukowania apoptozy osteoklastów. W celu zbadania wpływu witaminy K na metabolizm kości wykorzystano kilka modeli zwierzęcych osteoporozy. W badaniach wykazano pozytywny wpływ suplementacji witaminą K na zdrowie kości, wynikający z hamowania utraty masy kostnej, poprawienia funkcji osteoblastów oraz architektury kości [44-47]. Kim i in. [48] zaobserwowali, że podawanie witaminy K myszom na diecie wysokotłuszczowej powodowało wzrost tworzenia tkanki kostnej oraz zmniejszenie resorpcji kości.

Większość badań obserwacyjnych u ludzi wykazała, że niskie stężenie VK1 i wysokie poziomy ucOC w surowicy oraz niskie spożycie witaminy K w diecie są związane z wyższym ryzykiem złamań [49-53], ale uzyskano również odmienne wyniki [54,55]. Ponadto sprawdzano związki występujące między witaminą K a biomarkerami kości. W badaniu Framingham Offspring Study stężenie VK1 w osoczu było odwrotnie proporcjonalne do OPG [56]. U starszych mieszkańców północnej Europy poziomy VK1 były odwrotnie skorelowane z BALP (*Bone Alkaline Phosphatase*, fosfataza zasadowa izoenzym kostny) – markerem tworzenia kości [49]. W kilku badaniach przekrojowych wykazano, że poziom witaminy K lub jej odpowiednia podaż poprawia wartości BMD [50,57,58]. Natomiast kilka badań obserwacyjnych nie potwierdziło takiego związku [53,55], dlatego na podstawie badań obserwacyjnych trudno stwierdzić, czy witamina K jest czynnikiem ochronnym dla zdrowia kości.

Istnieją kontrowersyjne wyniki dotyczące wpływu suplementacji witaminy K2 na zdrowie kości. Randomizowane kontrolowane badania kliniczne (RCT, *Randomized Control Trials*) o małej liczebności próby wykazały ochronny wpływ MK-4 na BMD oraz zmniejszone ryzyko złamań szyjki kości udowej, złamań kręgowych i pozakręgowych [59-62]. Trzyletnie badanie z udziałem zdrowych kobiet po menopauzie wykazało, że suplementy MK-7 poprawiły wytrzymałość kości w porównaniu z placebo [63]. Jednak wyniki największego i najdłuższego badania z udziałem MK-4 nie wykazały tak korzystnego wpływu na złamania kręgów, z wyjątkiem kobiet z zaawansowaną osteoporozą [64]. Co więcej, mechanizmy, dzięki którym witamina K może chronić przed złamaniami kości, pozostają nieokreślone. Sugeruje się, że VK może zachować zarówno ilość, jak i jakość kości poprzez hamowanie obrotu kostnego, ale dostępne dowody są niejasne [65].

3.3.2. Status VK u pacjentów z przewlekłą chorobą nerek

W populacji pacjentów z CKD leczonych zachowawczo, hemodializami (HD, *Hemodialysis*) lub dializą otrzewnową zaobserwowano niedobór witaminy K [66-68], który może być spowodowany ograniczeniami dietetycznymi, zaburzonym recyklingiem VK oraz stosowaniem leków, takich jak: statyny, inhibitory pompy protonowej, środki wiążące fosforany, sterydy, leki hipotensyjne oraz suplementacja witaminy D. Ponadto antybiotyki o szerokim spektrum działania przyjmowane przez pacjentów z CKD mogą upośledzać ich mikroflorę jelitową, przyczyniając się do zmniejszenia syntezy witaminy K [69-70]. Biorąc pod uwagę, że witamina K jest niezbędna do aktywacji ważnych dla metabolizmu kości VKDP, takich jak osteokalcyna czy MGP, funkcjonalny niedobór witaminy K może prowadzić do zaburzeń przebudowy kości. W badaniach przeprowadzonych na szczurach z mocznicą wykazano, że przewlekła choroba nerek może wpływać na metabolizm witaminy K z uwagi na spadek ekspresji VKOR i GGCX w aorcie oraz obniżenie poziomu UBIAD1 w nerkach [69]. W badaniu wpływu VK2 na masę i wytrzymałość kości korowej u szczurów z niewydolnością nerek wykazano, że podawanie witaminy K2 zwiększyło wytrzymałość kości korowej bez zmiany BMD [14]. W związku z powyższym istnieje możliwość, że VK może wpływać na integralność kości bez zmiany gęstości mineralnej kości. Podsumowując, dowody z badań *in vitro* oraz badań na zwierzętach potwierdziły ochronną rolę witaminy K2 w odniesieniu do zdrowia kości, sugerując potencjalne korzyści z jej stosowania u pacjentów z osteoporozą.

W dostępnej literaturze istnieje niewiele doniesień dokumentujących związek między niedoborem witaminy K a spadkiem BMD lub ryzykiem złamań u pacjentów z przewlekłą chorobą nerek. Badanie przeprowadzone na pacjentach z CKD w stadium 3-5 wykazało, że wysoki poziom ucOC w surowicy był dodatnio skorelowany z fosforanami i PTH, natomiast odwrotnie skorelowany z poziomami 25(OH)D, co sugeruje związek z przebudową kości [67]. Po raz pierwszy wykazano niezależny związek między niedoborem witaminy K1 a ryzykiem złamań kości u pacjentów hemodializowanych w badaniu przeprowadzonym przez Kohlmeier i wsp. [71]. Ponadto autorzy zaobserwowali, że pacjenci bez historii złamań kości mieli około trzykrotnie wyższe stężenia VK1 w surowicy. Fusaro i wsp. w swoim badaniu zaobserwowali wyższe poziomy całkowitej OC i ucOC u pacjentów z CKD w porównaniu z osobami zdrowymi, a niedobór witaminy K1 był niezależnym predyktorem złamań kręgów u pacjentów z HD [72]. Klirens OC odbywa się poprzez przesączanie kłębuszkowe, stąd stężenie OC w surowicy jest wyższe u pacjentów z przewlekłą chorobą nerek w porównaniu z grupą kontrolną dobraną pod względem wieku [22]. Evenepoel i wsp. wykazali, że wysoki poziom

defosfonieukarboksylowanego MGP (dp-ucMGP, *Desphospho-Uncarboxylated Matrix Gla Protein*) był niezależnie skorelowany z niską gęstością mineralną kości oraz z występowaniem złamań. Jednakże u pacjentów ze schyłkową niewydolnością nerek autorzy nie zaobserwowali związku między statusem witaminy K a markerami obrotu kostnego [73]. Roczna suplementacja MK-4 u pacjentów z kłębuszkowym zapaleniem nerek zapobiegała utracie masy kostnej wywołanej sterydami [74]. Zmniejszenie dp-ucMGP, ucOC oraz białek indukowanych przez brak VK lub antagonistę II (PIVKA-II, *Protein Induced By VK Absence/Antagonism* II) zaobserwowano u pacjentów HD poddanych suplementacji MK-7, co wskazuje, że powyższa forma witaminy K poprawia status VK w wątrobie, kościach oraz naczyniach krwionośnych [75-77]. Obecnie w bibliografii brakuje randomizowanych badań dotyczących suplementacji witaminy K oraz złamań kości u pacjentów z CKD.

4. Cele pracy

CKD-MBD jest powszechnym powikłaniem występującym u pacjentów z przewlekłą chorobą nerek, a częstość wystąpienia złamań kości jest czterokrotnie wyższa niż w populacji ogólnej. Nieprawidłowości w mikroarchitekturze kości w przebiegu CKD-MBD są związane z obniżeniem wytrzymałości kości, wynikającej z uszkodzenia zarówno jakości, jak i ilości tkanki kostnej. Predyspozycja do rozwoju osteoporozy znacząco wpływa na jakość życia pacjentów poprzez zwiększanie ryzyka złamań, podwyższanie kosztów hospitalizacji i przedwczesną śmierć. Farmakoterapia osteoporozy koncentruje się głównie na przeciwdziałaniu zaburzeniom gospodarki mineralnej. Ponadto u pacjentów z CKD, zwłaszcza w zaawansowanym stadium, leczenie jest ograniczone i kosztowne, a niektóre opcje farmakoterapii są trudno dostępne, bądź nawet niemożliwe do zastosowania. Ze względu na brak postępu w metodyce leczenia CKD-MBD w ciągu ostatnich kilku lat, konieczne jest opracowanie nowych, alternatywnych i efektywnych strategii hamowania rozwoju CKD-MBD.

Dotychczas przeprowadzone zostało tylko jedno badanie dotyczące wpływu suplementacji witaminy K2 na gęstość mineralną kości oraz siłę kości udowych u szczurów z eksperymentalną CKD, natomiast brakuje randomizowanych kontrolowanych badań klinicznych oceniających wpływ suplementacji witaminy K na zdrowie kości. W związku z powyższym wpływ witaminy K na metabolizm kości nie został dotąd określony i nadal pozostaje nieznany. Wyjaśnienie roli endogennej witaminy K w przebudowie kości może pomóc w stworzeniu podstaw, które w przyszłości mogą odegrać kluczową rolę w profilaktyce zaburzeń mineralno-kostnych lub pozwolą doprowadzić do opracowania nowych strategii terapeutycznych w leczeniu tego powikłania wśród pacjentów z CKD. Tak więc istotne jest odkrycie dokładnego mechanizmu działania endogennej witaminy K w kościach oraz jej udziału w przebudowie kości, ze szczególnym uwzględnieniem procesu mineralizacji.

Zarówno u pacjentów z przewlekłą chorobą nerek, jak i u zwierząt z eksperymentalną CKD zaobserwowano niedobór witaminy K. Dlatego zasadne jest oznaczenie poziomu witaminy K1, która jest główną dietetyczną postacią VK oraz najczęstszych form menachinonów, tj. MK-4 i MK-7, a także ustalenie stosunków MK-7/VK1 i MK-4/VK1, które stanowią wskaźnik skuteczności konwersji witaminy K1 do K2. Pośrednim wskaźnikiem niedoboru VK są również białka zależne od witaminy K, takie jak osteokalcyna czy MGP, w związku z czym istotnym wydaje się oznaczenie poziomu VKDP oraz ich nieukarboksylowanych form – ucOC oraz ucMGP zarówno w surowicy, jak i w tkance kostnej. Do prawidłowego funkcjonowania witaminy K w procesie γ-karboksylacji białek konieczny

jest jej recykling, w który zaangażowane są enzymy, takie jak VKOR i GGCX. Tak więc wartościowym badaniem będzie ocena ekspresji genów związanych z cyklem witaminy K w kościach oraz zestawienie uzyskanych wyników z parametrami stanu mineralnego kości udowych szczurów z eksperymentalną CKD. Przeprowadzone badania pozwolą zrozumieć skomplikowaną rolę endogennej witaminy K w procesie mineralizacji kości w przebiegu przewlekłej choroby nerek.

Mając na uwadze powyższe, celem zrealizowanych badań była:

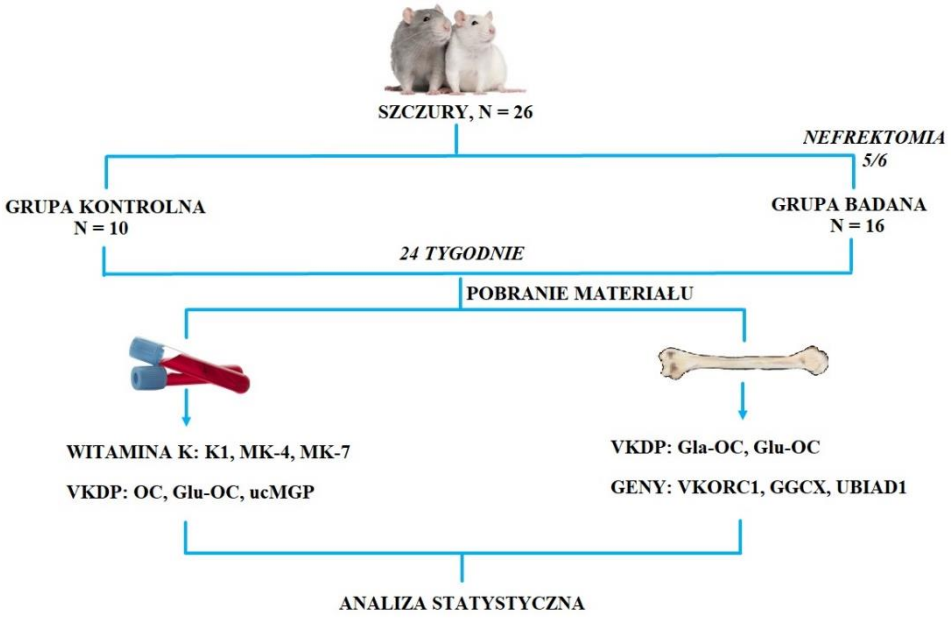
1. Kompleksowa ocena metabolizmu endogennej witaminy K w eksperymentalnym, szczurzym modelu CKD,
2. Ocena białek zależnych od witaminy K na poziomie surowicy oraz kości,
3. Ocena recyklingu witaminy K w kości,
4. Ustalenie związku występującego między VKDP a stanem mineralnym kości u szczurów z CKD.

5. Materiały i metody

Szczegółowe informacje dotyczące zastosowanego modelu doświadczalnego oraz metodyki badań znajdują się w niżej wymienionej pracy włączonej do rozprawy:

Publikacja nr 2 – Ziemińska, M.; Pawlak, D.; Sieklucka, B.; Chilkiewicz, K.; Pawlak, K. **Vitamin K-dependent carboxylation of osteocalcin in bone–ally or adversary of bone mineral status in rats with experimental chronic kidney disease?** *Nutrients*. 14 (2022) 4082.

Ogólny zarys zastosowanej metodyki pracy przedstawiono na Rycinie 1.



Skróty: N – liczba zwierząt; K1 – witamina K1 (filochinon); MK-4 – menachinon 4; MK-7 – menachinon 7; VKDP – białka zależne od witaminy K; OC – osteokalcyna; Glu-OC – nieukarboksylowana osteokalcyna; Gla-OC – karboksylowana osteokalcyna; uc-MGP – nieukarboksylowane białko macierzy Gla; VKORC1 – podjednostka 1 kompleksu reduktazy epoksydu witaminy K; GGCX – γ-glutamylokarboksylaza; UBIAD1 – enzym zawierający domenę transferazy prenylowej UbiA.

Rycina 1. Schematyczna prezentacja metodyki zastosowanej w niniejszej rozprawie doktorskiej.

5.1. Model zwierzęcy

W niniejszej rozprawie wykorzystano model doświadczalny oraz materiał biologiczny, który był przedmiotem wcześniejszych badań dotyczących zaburzeń kostnych w eksperymentalnym modelu przewlekłej choroby nerek [78]. W części doświadczalnej wykorzystano 26 czterotygodniowych samców szczurów rasy Wistar, które na czas

eksperymentu trzymano w konwencjonalnych klatkach z nieograniczonym dostępem do sterylizowanej wody wodociągowej. Zwierzętom zapewniono wilgotność powietrza wynoszącą 50%, stałą temperaturę (24°C), cykl światło/ciemność 12h/12h oraz standardową karmę (Ssniff R/MH) składającą się z 19% białka, 1% wapnia, 0,70% fosforu, 1000 IU witaminy D3 na kg i 5 mg/kg witaminy K w postaci menadionu. Badania wykonano zgodnie z dyrektywą UE 2010/63/UE, a zgodę na przeprowadzenie doświadczeń na zwierzętach uzyskano od Lokalnej Komisji Etycznej (24 kwiecień 2013; numer zgody: 29/2013).

Szczury zostały losowo podzielone na dwie grupy (kontrolną i badaną). Grupa kontrolna (CON, n = 10) została poddana „pozorowanej operacji", natomiast u zwierząt z grupy badanej (CKD, n = 16) przeprowadzono dwuetapową chirurgiczną nefrektomię 5/6, prowadzącą do przewlekłej choroby nerek. W celu obserwacji rozwoju choroby eksperyment prowadzono przez 24 tygodnie i zakończono pobraniem próbek krwi przez nakłucie serca oraz pobraniem kości udowych. Po odwirowaniu krwi uzyskano surowicę, którą zamrożono w temperaturze -80°C, natomiast kości po uprzednim oczyszczeniu przechowywano do dalszych badań w temperaturze -20°C.

5.2. Metody badawcze

5.2.1. Parametry oznaczane w surowicy

W niniejszej rozprawie doktorskiej zbadano stężenia witaminy K1 (filochinon), witaminy K2 (menachinony – MK-4 i MK-7), a także białek zależnych od VK, tj. całkowitej osteokalcyny i nieukarboksylowanych form: Glu-OC oraz ucMGP. Dodatkowo wykorzystano wyniki wcześniejszych badań, w których oznaczono poziomy markerów czynności nerek (mocznik, kreatynina), PTH oraz 1,25-dihydroksywitaminy D_3 u szczurów z eksperymentalną mocznicą [78].

Pomiary stężenia witaminy K (VK1, MK-4 i MK-7) zostały wykonane przez Laboratorium Perlan Technologies Polska z siedzibą w Gdyni. Do oznaczenia VK wykorzystano chromatografię cieczową z tandemową spektrometrią mas (LC-MS/MS, *Liquid Chromatography – Mass Spectrometry*) i wysokosprawny chromatograf cieczowy (1260 Infinity II LC System, Agilent Technologies, Santa Clara, CA, USA). Do próbek surowicy dodano znakowany deuterem wzorzec witaminy K1-[d7], acetonitryl i heksan. Górną warstwę organiczną przeniesiono do nowej probówki i wysuszono w atmosferze azotu w temperaturze

pokojowej. Końcowy suchy ekstrakt rozpuszczono w acetonitrylu, a następnie automatycznie wstrzyknięto do systemu HPLC (*High-Performance Liquid Chromatography,* wysokosprawna chromatografia cieczowa). Rozdzielanie próbek przeprowadzono w stałej temperaturze 30°C na kolumnie z odwróconą fazą Zorbax (SB-C8 RRHT, 3.0 x 50 mm, 1,8 μm, 600 bar). Zastosowano dwuskładnikowy układ gradientowy, składający się z 0,1% kwasu mrówkowego i 5 mM mrówczanu amonu w wodnej fazie ruchomej (eluent A) oraz metanolu zakwaszonego 0,1% kwasem mrówkowym (eluent B). Jako gaz rozpylający zastosowano azot, natomiast gazem kolizyjnym był azot o ultrawysokiej czystości. Identyfikację i ocenę ilościową oparto na monitorowaniu reakcji wielokrotnych (MRM, *Multiple Reactions Monitoring*) z wykorzystaniem spektrometrii mas.

Oznaczenie stężenia kreatyniny i mocznika wykonano z użyciem analizatora biochemicznego Minidray BS-120 (USA) oraz komercyjnego zestawu CORMAY (Lublin, Polska). Za pomocą testu immunoenzymatycznego ELISA (*Enzyme-Linked Immunosorbent Assay)* zmierzono PTH (Rat Intact PTH ELISA kit, Immunotopic, San Clemente, Kalifornia, USA) oraz 1,25 dihydroksywitaminę D (Rat (DHVD3) ELISA kit, Shanghai Sunred Biological Technology Co., Ltd, Chiny).

Ilościowe oznaczenie VKDP w surowicy zostało wykonane za pomocą zestawów ELISA, odpowiednio: stężenie całkowitej osteokalcyny (Rat Osteocalcin ELISA firmy Immutopics, Inc., San Clemente, CA, USA), Glu-OC (Rat Glu-Osteocalcin High Sensitive EIA, Takara Bio Inc., Shiga, Japonia) oraz ucMGP (Rat Undercarboxylated matrix Gla protein firmy MyBioSource, Inc., San Diego, CA, USA).

5.2.2. Badania przeprowadzone w homogenatach kości

W celu otrzymania homogenatów tkanki kostnej pobrano fragmenty trzonu kości udowej (obszar kości korowej) oraz dalszej nasady kości udowej (obszar kości beleczkowej), które następnie zostały precyzyjnie zważone i wypłukane. Homogenizację przeprowadzono w zimnym buforze fosforanu potasu (50 mM, pH = 7,4; POCh) za pomocą wysokosprawnego homogenizatora (Ultra-Turrax T25; IKA, Staufen, Niemcy) wyposażonego w element dyspergujący ze stali nierdzewnej (S25N-8G; IKA). Otrzymane w powyższy sposób 10% homogenaty kości wirowano (10 min, 700 x g, 4°C), a zebrany supernatant był przechowywany w temperaturze -80°C do czasu wykonania badań.

Zarówno w części beleczkowej, jak i korowej kości udowej dokonano pomiarów karboksylowanej i nieukarboksylowanej osteokalcyny, wykorzystując do tego celu testy ELISA firmy Takara Bio Inc. (Shiga, Japonia). Stężenie Gla-OC określono za pomocą zestawu

Rat Gla-Osteocalcin High Sensitive EIA, natomiast stężenie Glu-OC oznaczono z użyciem Rat Glu-Osteocalcin High Sensitive EIA. Otrzymane wyniki pozwoliły na obliczenie stosunku Gla-OC/Glu-OC, który jest wskaźnikiem dostępności witaminy K na poziomie kości.

Ekspresję genów zaangażowanych w recykling witaminy K (VKORC1, GGXC i UBIAD1) na poziomie kości zmierzono metodą ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym (RT-PCR, *Real-Time Polymerase Chain Reaction*) z wykorzystaniem spektrofotometru Thermo Scientific NanoDrop 2000 (Waltham, MA, USA). Za pomocą zestawu do oczyszczania RNA (Thermo Scientific GeneJET RNA, Thermo Scientific, Wilno, Litwa) wyizolowano całkowity RNA z tkanki kości udowej w celu ilościowej oceny i kontroli jakości RNA, a odwrotną transkrypcję przeprowadzono z użyciem zestawu do syntezy cDNA RevertAid™ First Stand (Thermo Fisher Scientific, Waltham, MA, USA). Do wykonania RT-PCR użyto SYBR Green Master Mix (EURx, Gdańsk, Polska), natomiast do względnej ilościowej oceny ekspresji genów zastosowano metodę porównawczą CT (ΔΔCT). Normalizację poziomu ekspresji osiągnięto dzięki użyciu dehydrogenazy aldehydu 3-fosforanowego gliceryny (GAPDH, *Glyceraldehyde-3-Phosphate Dehydrogenase*), którą wykorzystano jako gen metabolizmu podstawowego. Sekwencje zastosowanych starterów ujęto w pracy oryginalnej, będącej częścią niniejszej rozprawy (Publikacja nr 2).

W celu wykrycia zależności między ekspresją enzymów cyklu witaminy K, a kluczowymi genami zaangażowanymi w etapy osteoblastogenezy wykorzystano wyniki wcześniejszych badań [79], gdzie oznaczona została ekspresja genu FOXO1 (*Forkhead Box Transcription Factor 1*), ATF4 (*Activating Transcription Factor 4*), RUNX2, ALP oraz BGLAP.

5.2.3. Analiza stanu mineralnego kości udowej

Przeanalizowanie zależności między obiema formami osteokalcyny – Glu-OC i Gla-OC oraz stosunkiem Gla-OC/Glu-OC w kości a stanem mineralnym kości udowych szczurów z CKD było możliwe dzięki wykorzystaniu skanów densytometrycznych wykonanych w ramach wcześniej opublikowanej pracy naszego zespołu [78]. Badania zostały przeprowadzone na densytometrze rentgenowskim Horizon QDR Series (Hologic Inc., Bedford, MA, USA) przy użyciu specjalnego oprogramowania dla małych zwierząt. Wszystkim szczurom wykonano pomiary całej kości udowej – obszar mineralny kości (BMA, *Bone Mineral Area*; cm^2), gęstość mineralną kości (BMD; mg/cm^2) oraz zawartość mineralną kości (BMC, *Bone Mineral Content;* mg). Dodatkowo określono ilościowo subregionalne BMC

i BMD małych jednolitych obszarów w dystalnej przynasadzie (region R1, mieszana kość beleczkowa i korowa) oraz trzonie środkowym (region R2, kość korowa).

5.2.4. Analiza statystyczna

W celu określenia normalności rozkładu zmiennych ciągłych zastosowano test Shapiro-Wilka. Wyniki badań o rozkładzie normalnym zostały wyrażone jako średnia ± odchylenie standardowe (SD), natomiast dane nie mające rozkładu Gaussa przedstawiono jako medianę (od 25 do 75 percentyla). Grupa kontrolna i grupa badana zostały porównane za pomocą niesparowanego testu t-Studenta z poprawką Welcha lub nieparametrycznego testu Manna-Whitneya. Za poziom istotności statystycznej uznano $p < 0,05$. Korelacje między badanymi zmiennymi obliczono za pomocą analizy korelacji rang Spearmana. Analizę statystyczną dla wszystkich oznaczeń wykonano z użyciem programu Statistica ver.13 (StatSoft, Tulsa, OK, USA), natomiast do graficznej prezentacji wyników wykorzystano oprogramowanie GraphPad Prism 6.0 (San Diego, USA).

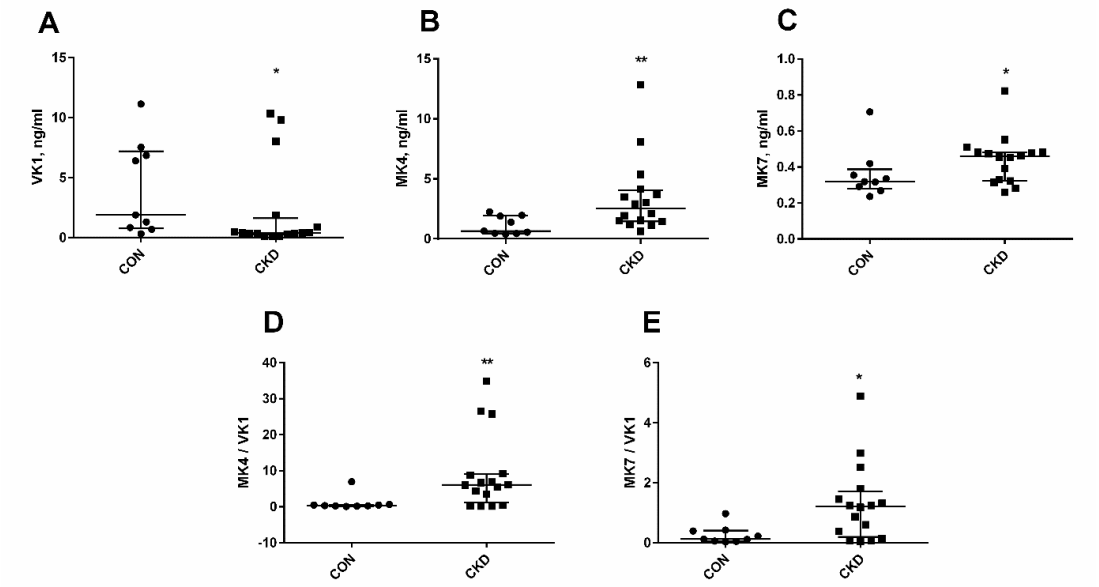
6. Omówienie wyników badań

Przedstawione wyniki badań wraz z szczegółowym opisem zostały opublikowane w niżej wymienionej pracy włączonej do rozprawy:

Publikacja nr 2 – Ziemińska, M.; Pawlak, D.; Sieklucka, B.; Chilkiewicz, K.; Pawlak, K. **Vitamin K-dependent carboxylation of osteocalcin in bone–ally or adversary of bone mineral status in rats with experimental chronic kidney disease?** *Nutrients*. 14 (2022) 4082.

Materiał badawczy użyty w niniejszej rozprawie doktorskiej był przedmiotem wcześniejszych badań [78,79], a uzyskane wyniki zostały wykorzystane w celu kontynuacji badań dotyczących zaburzeń metabolizmu kości w przewlekłej chorobie nerek.

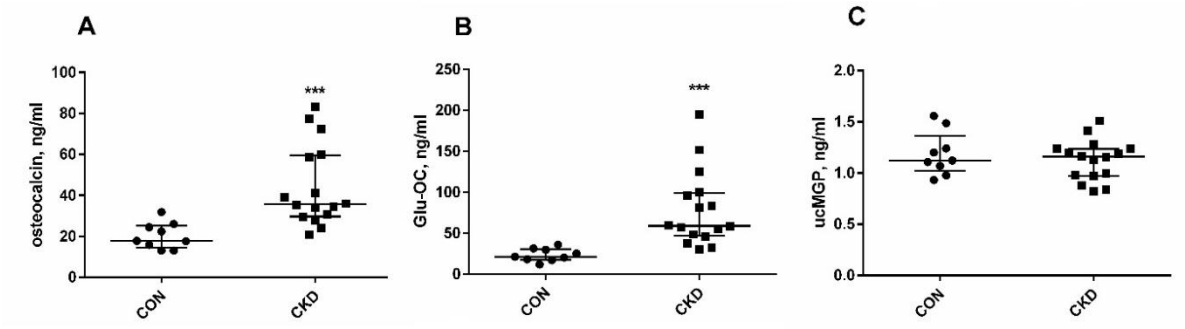
W niniejszym badaniu statusu witaminy K wykazano znaczny spadek stężenia VK1, natomiast istotny wzrost VK2 (MK-7, a zwłaszcza MK-4) u zwierząt z eksperymentalną niewydolnością nerek w porównaniu z grupą kontrolną (Rycina 2, A-C). W badaniach dotyczących CKD, zarówno u pacjentów, jak i u zwierząt doświadczalnych zaobserwowano niedobór witaminy K1 [67,69], co jest zgodne z uzyskanymi przez nasz zespół wynikami. Na Rycinie 2 (D-E) przedstawiono wyliczone stosunki: MK7/VK1 oraz MK4/VK1, które były znacząco wyższe u zwierząt z CKD w stosunku do zwierząt zdrowych. Powyższe współczynniki są uznawane za wskaźnik skuteczności konwersji witaminy K1 do menachinonów. VK1 jest pierwotną postacią witaminy K w krążeniu i może być, podobnie jak MK-7 [80], prekursorem do syntezy tkankowej MK-4 [81]. W związku z powyższym obserwowane przez nas obniżone stężenia filochinonu, któremu towarzyszył wzrost stężenia MK-4 i MK-7 mogą wynikać ze zwiększonego wykorzystania VK1 przez tkanki w grupie badanej, co prawdopodobnie jest odpowiedzią na wzrost zapotrzebowania organizmu na witaminę K podczas niewydolności nerek.



Linie odpowiadają 25. i 75. percentylowi oraz medianie; * p <0.05, ** p <0.01; CON w porównaniu ze szczurami z CKD; Skróty: VK1 – filochinon; MK4 – menachinon 4; MK7 – menachinon 7.

Rycina 2. Stężenia witaminy K1, MK-4 i MK-7 oraz stosunek MK4/VK1 i MK7/VK1 w surowicy szczurów z przewlekłą chorobą nerek (CKD) oraz w grupie kontrolnej (CON).

W niniejszej rozprawie dokonano pomiaru białek zależnych od witaminy K, które są uznawane za markery subklinicznego niedoboru VK [82]. Stężenia całkowitej osteokalcyny oraz nieukarboksylowanych form – Glu-OC i ucMGP w surowicy przedstawiono na Rycinie 3. W grupie z eksperymentalną CKD wykazano istotny wzrost stężenia osteokalcyny (3A) oraz Glu-OC (3B) w stosunku do zdrowych zwierząt, a uzyskane wyniki miały odzwierciedlenie w literaturze [67,77]. Mimo doniesień naukowych dotyczących podwyższonego poziomu uc-MGP w przebiegu CKD [76], nie zaobserwowano istotnych różnic w stężeniu tego parametru między grupą kontrolną a szczurami z eksperymentalną CKD (Rycina 3C).



Linie odpowiadają 25. i 75. percentylowi oraz medianie; *** $p < 0.001$; CON w porównaniu do szczurów z CKD; Skróty: osteocalcin – osteokalcyna; Glu-OC – nieukarboksylowana osteokalcyna; ucMGP – nieukarboksylowane białko Matrix Gla.

Rycina 3. Stężenia VKDP – osteokalcyny, Glu-OC i ucMGP w surowicy szczurów z przewlekłą chorobą nerek (CKD) oraz w grupie kontrolnej (CON).

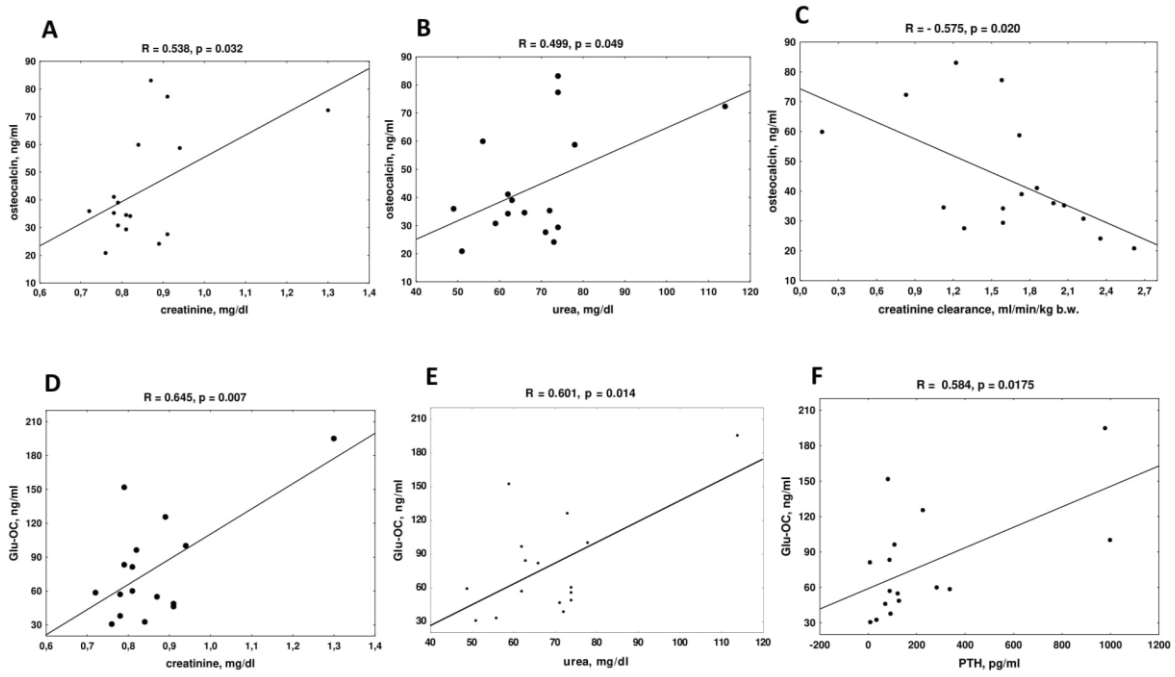
W Tabeli 1. przedstawiono opublikowane wcześniej wyniki badań biochemicznych [78]. Podsumowując, w grupie badanej wykazano podwyższone stężenie kreatyniny i mocznika, co odpowiadało stopniowi łagodnej do umiarkowanej niewydolności nerek oraz znacznie wyższy poziom PTH w porównaniu do grupy kontrolnej. W badaniu poziomu 1,25-dihydroksywitaminy D3 nie zaobserwowano istotnych różnic między szczurami z CKD a zwierzętami zdrowymi. Wzrost markerów niewydolności nerek stanowił pozytywną weryfikację poprawności wywołania eksperymentalnej mocznicy. Ponadto uzyskane wcześniej wyniki pozwoliły na odkrycie powiązań między VKDP a powyższymi parametrami.

	CON (n = 10)	**CKD (n = 16)**
kreatynina [mg/dl]	0.47 ± 0.08	0.86 ± 0.13***
klirens kreatyniny [ml/min]	1.16 ± 0.22	0.65 ± 0.25*
mocznik [mg/dl]	32.33 ± 7.38	68.63 ± 14.93***
PTH [pg/ml]	45.89 (12.79–84.17)	99.84 (33.44–997.6)**
$1,25(OH)_2$ D3 [nM]	7.74 ± 1.48	8.05 ± 1.86

Dane zostały wyrażone jako średnia ± SD (odchylenie standardowe) lub mediana (pełny zakres), w zależności od ich rozkładu; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Skróty: CON – grupa kontrolna; CKD – grupa badana; n – liczebność grupy; PTH – parathormon; $1,25(OH)_2$ D3 – dihydroksywitamina D3.

Tabela 1. Badania biochemiczne w surowicy zwierząt zdrowych (CON) i szczurów z przewlekłą chorobą nerek (CKD) [78].

W niniejszym badaniu wykazano dodatnią korelację między stężeniem kreatyniny oraz mocznika a poziomem OC (Rycina 4A-B) i Glu-OC w surowicy (4D-E), natomiast stężenie całkowitej osteokalcyny było ujemnie skorelowane z klirensem kreatyniny (4C). Powyższe obserwacje wskazują, że OC, niezależnie od uwalniania z kości, może kumulować się we krwi szczurów z eksperymentalną mocznicą, co zostało wcześniej przedstawione w badaniu przeprowadzonym przez Price'a i wsp. [83]. Na rycinie 4F przedstawiono dodatnią korelację między surowiczym stężeniem Glu-OC i parathormonem. Dane literaturowe wskazują na możliwość pobudzenia przez PTH syntezy Glu-OC w wyniku stymulacji osteoblastogenezy [84,85]. W związku z powyższym wzrost stężenia Glu-OC w surowicy niezupełnie odzwierciedla niedobór VK w przebiegu niewydolności nerek ze współistniejącą nadczynnością przytarczyc. Podsumowując, uzyskane przez nas dane sugerują wystarczający poziom witaminy K u szczurów z CKD.

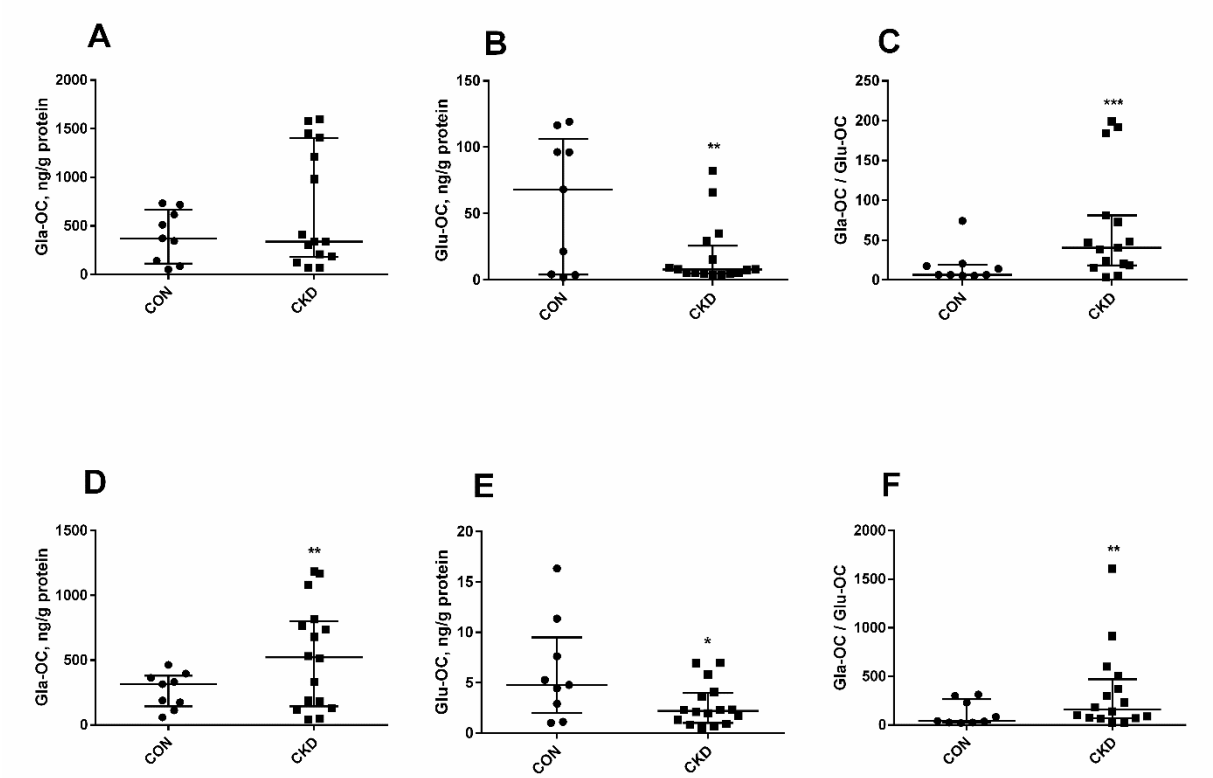


Skróty: osteocalcin – osteokalcyna; creatinine – kreatynina; urea – mocznik; creatinine clearence – klirens kreatyniny; Glu-OC – nieukarboksylowana osteokalcyna.

Rycina 4. Korelacje pomiędzy stężeniem osteokalcyny i Glu-OC w surowicy a markerami czynności nerek oraz stężeniem parathormonu (PTH) u szczurów z przewlekłą chorobą nerek.

Przedmiotem badań niniejszej rozprawy były również homogenaty tkanki kostnej, które wykorzystano do oceny stężenia Gla-OC i Glu-OC. Ponadto otrzymane wyniki pozwoliły na wyliczenie stosunku Gla-OC/Glu-OC, który jest wskaźnikiem dostępności witaminy K na poziomie kości. Powyższe parametry przedstawiono na Rycinie 5. W tkance kostnej

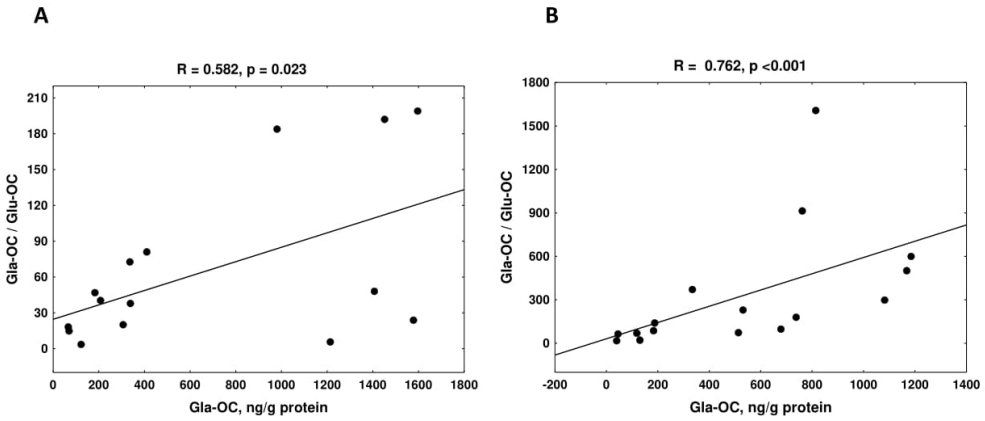
beleczkowej (5A-C) nie zaobserwowano różnic w stężeniu Gla-OC między grupą badaną a kontrolną (5A), natomiast stężenie Glu-OC było znacznie niższe u szczurów z CKD w porównaniu ze zwierzętami zdrowymi (5B). W kości korowej (5D-F) w grupie eksperymentalnej wykazano istotny wzrost stężenia Gla-OC (5D) oraz znacznie zmniejszone stężenie Glu-OC (5E) w porównaniu z grupą kontrolną. Dodatkowo, zarówno w tkance kostnej beleczkowej (5C) oraz korowej (5F), zaobserwowano znamienny wzrost stosunków Gla-OC/Glu-OC w grupie z eksperymentalną mocznicą w stosunku do szczurów bez chirurgicznej nefrektomii 5/6. Nieukarboksylowana postać osteokalcyny (Glu-OC) jest przekształcana do postaci karboksylowanej (Gla-OC) za pośrednictwem witaminy K. W związku z powyższym uzyskane przez nas wyniki wskazują na przyspieszoną transformację Glu-OC do Gla-OC w kościach szczurów z eksperymentalną CKD, odzwierciedlając tym samym wystarczający lub nawet podwyższony poziom VK w ich kościach.



Linie odpowiadają 25. i 75. percentylowi oraz medianie; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; CON w porównaniu ze szczurami z CKD; Skróty: Gla-OC – karboksylowana osteokalcyna; Glu-OC — nieukarboksylowana osteokalcyna.

Rycina 5. Stężenie karboksylowanej osteokalcyny (Gla-OC), nieukarboksylowanej osteokalcyny (Glu-OC) oraz stosunek Gla-OC/Glu-OC w tkance beleczkowej (A-C) i korowej (D-F) kości udowej szczurów z przewlekłą chorobą nerek (CKD) oraz w grupie kontrolnej (CON).

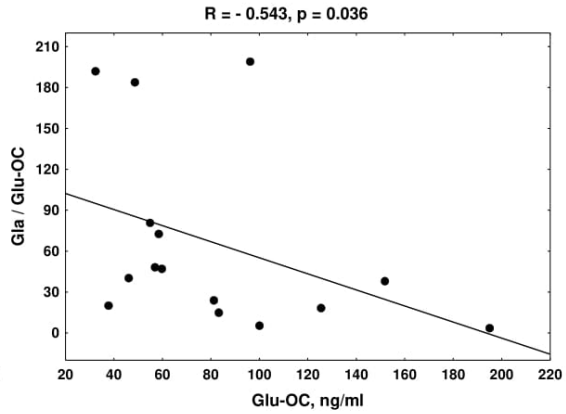
Analizie poddano również relacje między stężeniem Gla-OC a stosunkiem Gla-OC/Glu-OC w beleczkowej i korowej kości udowej szczurów z CKD. Wykazano pozytywne korelacje, które przedstawiono na Rycinie 6. Silny związek Gla-OC ze stosunkiem Gla-OC/Glu-OC (6B) uzyskany w tkance korowej wskazuje, że powstawanie Gla-OC w tej części kości zachodzi skuteczniej niż w tkance beleczkowej, która charakteryzuje się szybszym obrotem kostnym. Analizowano także związek między stężeniem Glu-OC a Gla-OC w kości szczurów z mocznicą, jednakże zauważono jedynie słabą korelację między powyższymi parametrami w tkance beleczkowej (R = 0.512, p = 0.045).



Skróty: Gla-OC – karboksylowana osteokalcyna; Glu-OC – nieukarboksylowana osteokalcyna.

Rycina 6. Związek pomiędzy karboksylowaną osteokalcyną (Gla-OC) a stosunkiem Gla-OC/Glu-OC w beleczkowej (A) i korowej (B) tkance kostnej u szczurów z przewlekłą chorobą nerek (CKD).

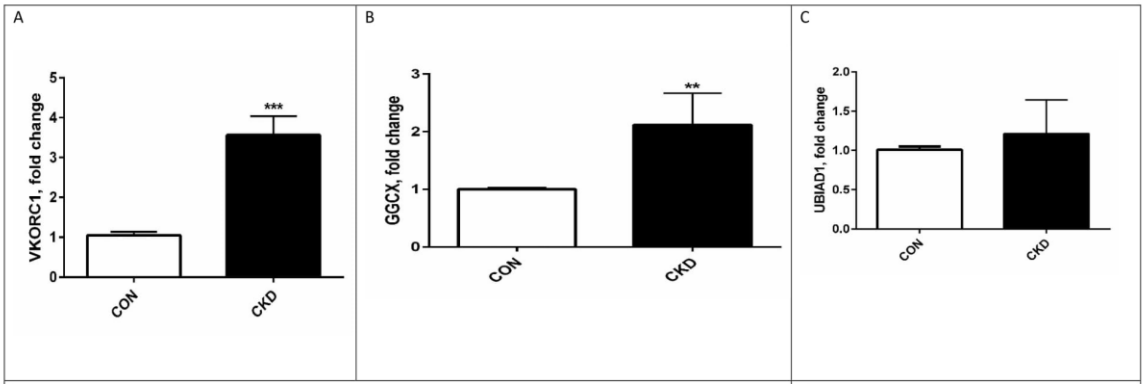
Dodatkowo, w niniejszej rozprawie ocenie poddano związek parametrów: Glu-OC, Gla-OC i Gla-OC/Glu-OC w tkance kości udowej ze stężeniem Glu-OC w surowicy, wykazując jedynie ujemną jej korelację ze stosunkiem Gla-OC/Glu-OC w beleczkowej tkance kostnej (Rycina 7). Powyższa zależność wskazuje, że proces powstawania Gla-OC w obszarze kości beleczkowej jest ważnym czynnikiem wpływającym na poziom Glu-OC w surowicy.



Skróty: Glu-OC – nieukarboksylowana osteokalcyna; Gla-OC – karboksylowana osteokalcyna.

Rycina 7. Związek między stosunkiem Gla-OC/Glu-OC w beleczce a poziomami nieukarboksylowanej osteokalcyny (Glu-OC) w surowicy szczurów z przewlekłą chorobą nerek (CKD).

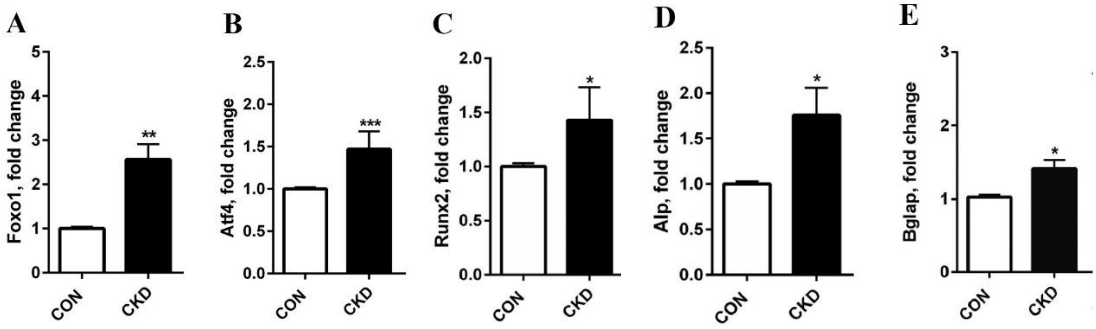
Reakcje redukcji zwane „cyklem witaminy K" zachodzą przy udziale enzymów VKOR oraz GGCX [27], natomiast UBIAD1 odpowiada za konwersję VK1 do MK4 w tkankach pozawątrobowych [86]. W związku z powyższym w naszym badaniu dokonano pomiarów ekspresji genów zaangażowanych w recykling witaminy K na poziomie kości, a uzyskane wyniki przedstawiono na Rycinie 8. Ekspresja genów VKORC1 i GGCX była znamiennie wyższa w grupie CKD w porównaniu z grupą kontrolną (8A-B), podczas gdy wzrost ekspresji genu UBIAD1 nie osiągnął poziomu istotnego statystycznie (8C). Z otrzymanych danych wynika, że u szczurów z niewydolnością nerek recykling witaminy K, konwersja VK1 do MK4 i późniejsze wykorzystanie menachinonu 4 w kościach może zachodzić wydajniej niż u zdrowych zwierząt.



** p < 0.01, *** p < 0.001; CON w porównaniu do szczurów z CKD; Skróty: VKORC1 – kompleks reduktazy epoksydowej witaminy K podjednostka 1; GGCX – karboksylaza gamma-glutamylowa; UBIAD1 – białko zawierające domenę prenylotransferazy UbiA 1.

Rycina 8. Ekspresja genów enzymów cyklu witaminy K w kościach udowych szczurów z przewlekłą chorobą nerek (CKD) oraz w grupie kontrolnej (CON).

Na Rycinie 9. przedstawiono opublikowane wcześniej wyniki dotyczące ekspresji genów osteoblastogenezy w kościach szczurów z mocznicą [79]. Pawlak i wsp. wykazali znaczny wzrost ekspresji genów wczesnego rozwoju osteoblastów – FOXO1 (9A), ATF4 (9B), RUNX2 (9C) i ALP (9D) w kościach szczurów z CKD w porównaniu ze zdrowymi zwierzętami. Ekspresja genu BGLAP (9E), który jest markerem późnego stadium różnicowania osteoblastów, była tylko nieznacznie wyższa w grupie badanej niż w grupie kontrolnej. Powyższe wyniki wskazywały na upośledzenie dojrzewania osteoblastów, co skutkowało obniżeniem stanu mineralnego kości [79].



* p < 0.05, ** p < 0.01, *** p < 0.001; CON w porównaniu do szczurów z CKD; Skróty: FOXO1 – czynnik transkrypcyjny należący do rodziny forkhead box O1; ATF4 – czynnik transkrypcyjny 4; RUNX2 – czynnik transkrypcyjny związany z Runt 2; ALP – fosfataza alkaliczna; BGLAP – białko gamma-karboksyglutaminianowe kości/ osteokalcyna.

Rycina 9. Ekspresja genów osteoblastogenezy w kościach szczurów z przewlekłą chorobą nerek (CKD) oraz w grupie kontrolnej (CON) [79].

Następnie oceniono powiązania pomiędzy ekspresją genów osteoblastogenezy i genów cyklu witaminy K oraz stężeniem Glu-OC w tkance beleczkowej kości udowej szczurów z CKD, a uzyskane wyniki przedstawiono w Tabeli 2. Wykazano silną dodatnią korelację ekspresji genów uczestniczących w cyklu witaminy K, w szczególności VKORC1 i UBIAD1, z ekspresją genów wczesnych etapów rozwoju osteoblastów (FOXO1, ATF4). Zauważono również tendencję do dodatniej korelacji z markerami różnicowania osteoblastów (RUNX2, ALP) oraz brak zależności między ekspresją genów cyklu VK a ekspresją BGLAP.

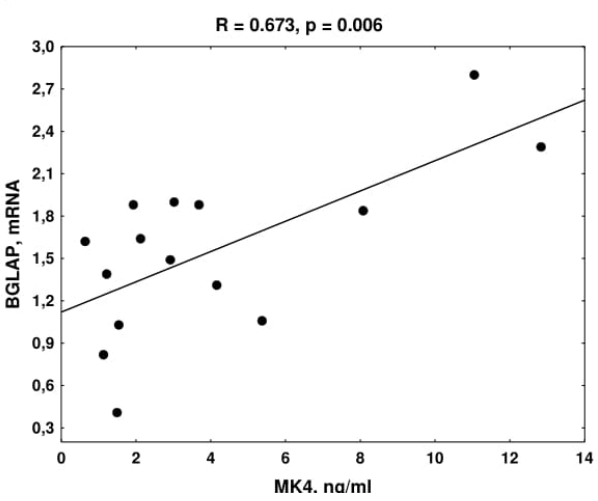
	FOXO1	ATF4	RUNX2	ALP	BGLAP
VKORC1	**R = 0.632 p = 0.009**	**R = 0.649 p = 0.006**	R = 0.430 p = 0.096	R = 0.462 p = 0.071	R = 0.195 NS
GGCX	**R = 0.538 p = 0.031**	**R = 0.568 p = 0.022**	R = 0.259 NS	R = 0.361 NS	R = 0.340 NS
UBIAD1	**R = 0.839 p < 0.0001**	**R = 0.556 p = 0.031**	R = 0.445 p = 0.087	R = 0.440 NS	R = 0.154 NS
Glu-OC	R = 0.361 NS	**R = 0.628 p = 0.012**	**R = 0.552 p = 0.033**	R = 0.464 p = 0.081	R = 0.013 NS

Kolor niebieski obrazuje korelacje istotne statystycznie, natomiast kolor żółty wskazuje na tendencję do korelacji; Skróty: R – współczynnik korelacji; p – poziom prawdopodobieństwa; NS – wynik nieistotny statystycznie; VKORC1 – podjednostka 1 kompleksu reduktazy epoksydu witaminy K; GGCX – karboksylaza gamma-glutamylowa; UBIAD1 – białko zawierające domenę prenylotransferazy UbiA 1; FOXO1 – czynnik transkrypcyjny Forkhead Box 1; ATF4 – aktywujący czynnik transkrypcyjny 4; RUNX2 – czynnik transkrypcyjny związany z Runt 2; ALP – fosfataza alkaliczna; BGLAP – białko gamma-karboksyglutaminianu kości/ osteokalcyna; Glu-OC – nieukarboksylowana osteokalcyna.

Tabela 2. Zależności między ekspresją enzymów cyklu witaminy K a ekspresją genów osteoblastogenezy i stężeniem Glu-OC w tkance beleczkowej kości udowej szczurów z przewlekłą chorobą nerek (CKD).

Przeanalizowano również zależności między ekspresją genów osteoblastogenezy a stężeniem Glu-OC w tkance beleczkowej kości udowej szczurów z mocznicą. Zaobserwowano silny związek między poziomem Glu-OC a ekspresją ATF4 i RUNX2 oraz tendencję do korelacji z ALP, natomiast brak zależności z ekspresją FOXO1 i BGLAP. Z otrzymanych danych wynika, że zarówno ekspresja enzymów cyklu witaminy K, jak i tworzenie Glu-OC w beleczkowej tkance kostnej miały miejsce we wczesnych stadiach rozwoju osteoblastów. Brak zależności między ekspresją BGLAP a ekspresją enzymów cyklu VK i poziomem Glu-OC w beleczkowej tkance kostnej może wynikać z faktu, że wzrost ekspresji BGLAP zachodzi dopiero na późnym etapie różnicowania osteoblastów. Zaobserwowano natomiast, że ekspresja genu BGLAP

wykazywała silny, pozytywny związek ze stężeniem MK-4 w surowicy (Rycina 10), co wskazuje na istotny wpływ tej formy VK na proces dojrzewania osteoblastów.

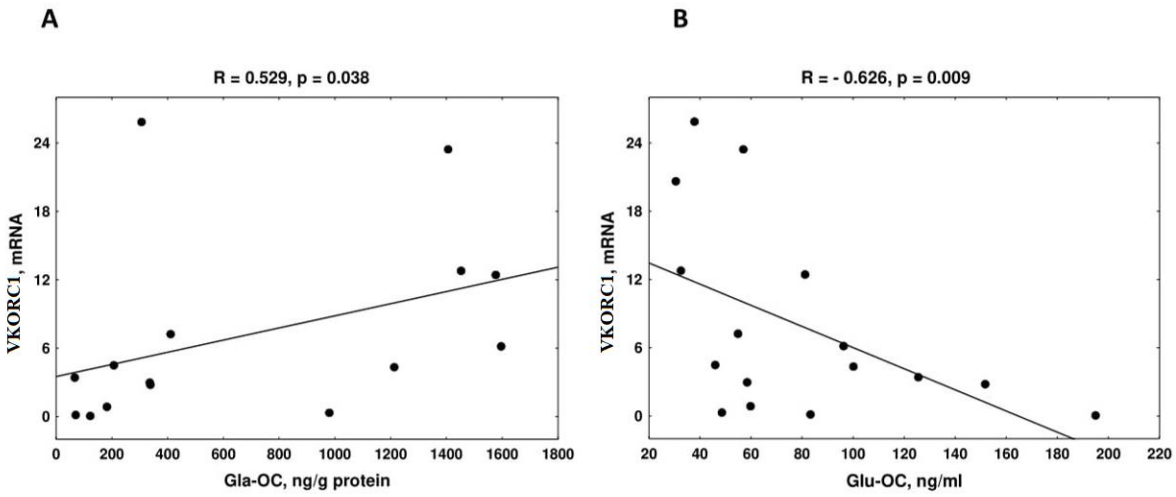


Skróty: BGLAP – białko gamma-karboksyglutaminianu kości/ osteokalcyna; MK4 – menachinon 4.

Rycina 10. Związek między poziomem MK4 w surowicy a ekspresją BGLAP w kościach szczurów z przewlekłą chorobą nerek (CKD).

Ponieważ we wcześniejszym badaniu zaobserwowano wpływ endogennego PTH na nasilenie osteoblastogenezy [87], ścisły związek istniejący pomiędzy genami wczesnych etapów osteoblastogenezy, enzymami recyklingu witaminy K i tworzeniem Glu-OC obserwowany w niniejszej pracy sugeruje pośredni wpływ endogennego PTH na tworzenie VKDPs w kościach szczurów z CKD.

Następne analizy miały na celu wykrycie zależności między powstawaniem osteokalcyny a ekspresją enzymów cyklu witaminy K. W tym celu skorelowano ekspresję genu VKORC1 i genu GGCX z pomiarami obu form osteokalcyny w kości udowej szczurów z mocznicą, a także ze stężeniem postaci Glu-OC w surowicy. Ekspresja VKORC1 była dodatnio skorelowana ze stężeniem Gla-OC w obszarze beleczkowym kości udowej oraz ujemnie z poziomem Glu-OC w surowicy (Rycina 11), natomiast w przypadku genu GGCX nie wykazano żadnych zależności w stosunku do osteokalcyny. Uzyskane wyniki, szczególnie odwrotna zależność między VKORC1 w kości a Glu-OC w surowicy (11B), potwierdzają znaczenie enzymów cyklu VK, a zwłaszcza VKORC1 w procesie powstawania aktywnej formy Gla-OC w kości zwierząt z przewlekłą chorobą nerek.



Skróty: VKORC1 – podjednostka 1 kompleksu reduktazy epoksydowej witaminy K; Gla-OC – karboksylowana osteokalcyna; Glu-OC – nieukarboksylowana osteokalcyna.

Rycina 11. Związek między ekspresją VKORC1 i poziomami Gla-OC w kości beleczkowej (A) oraz ekspresją VKORC1 i poziomami Glu-OC (B) w surowicy szczurów z przewlekłą chorobą nerek (CKD).

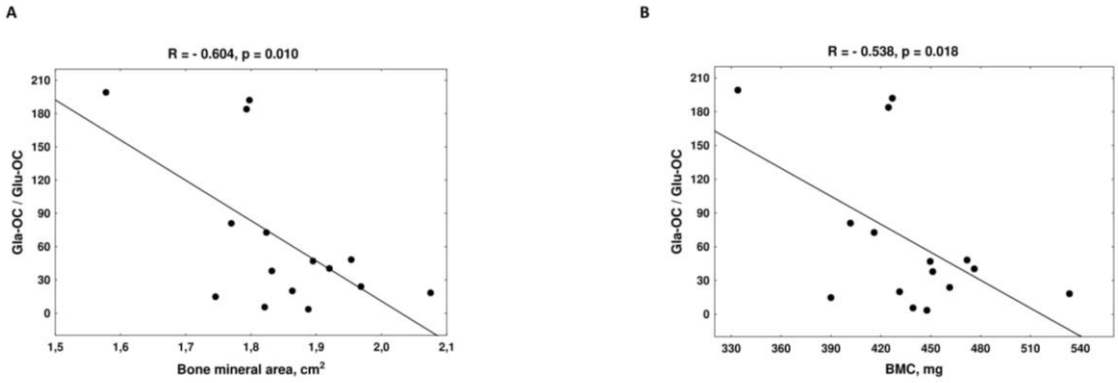
Ostatnim etapem niniejszej rozprawy było ustalenie roli, jaką pełnią białka zależne od endogennej witaminy K w mineralizacji kości zwierząt z eksperymentalną CKD. W celu określenia statusu mineralnego kości zwierząt z eksperymentalną CKD i zdrowej grupy kontrolnej wykorzystano skany densytometryczne kości udowych wykonane we wcześniejszych badaniach [78], a uzyskane wyniki przedstawiono w Tabeli 3. Zaobserwowano istotne obniżenie parametrów densytometrycznych – BMA ($p < 0.05$), BMC ($p < 0.01$) i BMD ($p < 0.01$) u szczurów z mocznicą w porównaniu do zdrowych zwierząt, zwłaszcza w obszarze R1, odpowiadającemu okolicy przynasadowej kości udowej, w którym pomiary BMC i BMD były znamiennie niższe w grupie badanej w porównaniu do grupy kontrolnej (oba $p < 0.001$). W okolicy trzonu kości udowej (R2) wartości BMC były niższe u szczurów z CKD niż u zwierząt kontrolnych ($p < 0.01$), natomiast w pomiarze BMD nie zaobserwowano różnic między badanymi grupami.

		CON	CKD
BMA (cm^2)		1,92 ± 0,06	1,84 ± 0,11 *
BMC (mg)		473,52 ± 21,08	435,13 ± 43,77 **
BMD (mg/cm^2)		247,00 ± 6,05	235,68 ± 10,57 **
R1	BMC (mg)	47,98 ± 2,69	43,23 ± 2,93 ***
	BMD (mg/cm^2)	217,96 ± 8,32	203,19 ± 10,09 ***
R2	BMC (mg)	70,47 ± 3,94	64,14 ± 5,95 **
	BMD (mg/cm^2)	245,18 ± 7,93	237,40 ± 14,19

Dane przedstawiono jako średnią ± odchylenie standardowe (SD); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; CON w porównaniu z CKD; Skróty: BMA – obszar mineralny kości; BMC – zawartość mineralna kości; BMD – gęstość mineralna kości; R1 – obszar przynasadowy kości; R2 – obszar trzonu kości.

Tabela 3. Status mineralny kości udowej zwierząt zdrowych (CON) oraz szczurów z przewlekłą chorobą nerek (CKD) [78].

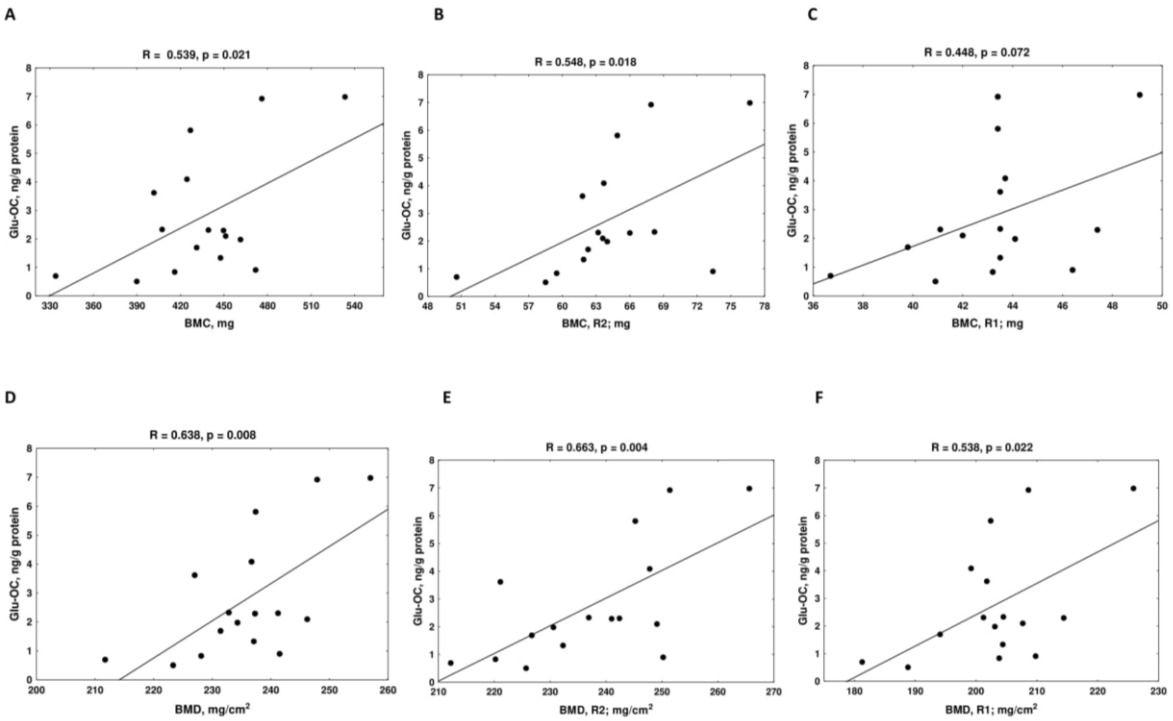
Następnie dokonano analizy zależności pomiędzy wyżej opisanymi parametrami densytometrycznymi a obiema formami osteokalcyny i stosunkami Gla-OC/Glu-OC oznaczonymi w beleczkowej i korowej tkance kostnej. W beleczkowej tkance kostnej szczurów z eksperymentalną CKD zaobserwowano jedynie silną, ujemną zależność między stosunkiem Gla-OC/Glu-OC a BMA oraz BMC (Rycina 12).



Skróty: Gla-OC – karboksylowana osteokalcyna; Glu-OC – nieukarboksylowana osteokalcyna; BMC – zawartość mineralna kości; Bone mineral area – obszar mineralny kości (BMA).

Rycina 12. Zależności pomiędzy stosunkiem Gla-OC/Glu-OC w beleczkowej tkance kostnej a parametrami stanu mineralnego: powierzchnią mineralną kości (A) i BMC (B) kości udowej szczurów z przewlekłą chorobą nerek (CKD).

Natomiast w warstwie korowej kości udowej wykazano istotne relacje między parametrami densytometrycznymi a stężeniem Glu-OC, zilustrowane na Rycinie 13. Zaobserwowano, że Glu-OC korelowało dodatnio z wartościami BMC (13A) i BMD (13D), szczególnie w obszarze R2, odpowiadającemu okolicy trzonu kości udowej (13B,E). Zauważono również, że poziom Glu-OC w warstwie korowej kości jest w niewielkim stopniu powiązany z wartościami BMC i BMD w okolicy R1.



Skróty: Glu-OC – nieukarboksylowana osteokalcyna; BMD – gęstość mineralna kości; BMC – zawartość mineralna kości; R1 – okolica przynasadowa kości; R2 – obszar trzonu kości udowej.

Rycina 13. Zależności pomiędzy stężeniem nieukarboksylowanej osteokalcyny (Glu-OC) w korowej tkance kostnej a pomiarami BMC (A) i BMD (D) w całej kości udowej, BMC (B) i BMD (E) w obszarze trzonu kości (R2) oraz BMC (C) i BMD (F) w okolicy przynasadowej kości (R1) u szczurów z przewlekłą chorobą nerek (CKD).

Uzyskane wyniki sugerują, że zależne od PTH przyspieszenie procesu osteoblastogenezy w obszarze kości beleczkowej spowodowało powstawanie niedojrzałych osteoblastów z niewystarczającą produkcją Gla-OC, co w konsekwencji u szczurów z mocznicą mogło doprowadzić do obniżenia stanu mineralnego kości. W korowej tkance kostnej, gdzie poziom Gla-OC był wyższy niż w kości beleczkowej, osteoblasty powinny być bardziej dojrzałe. Jednakże w tym obszarze status mineralny kości był bezpośrednio związany z nieukarboksylowaną formą osteokalcyny (Glu-OC), co może wskazywać na niekorzystną rolę Gla-OC w mineralizacji kości długich w przebiegu przewlekłej choroby nerek.

7. Wnioski

1. Obniżony poziom witaminy K1 obserwowany w surowicy szczurów z eksperymentalną przewlekłą chorobą nerek jest wynikiem przyspieszonej konwersji filochinonu do postaci menachinonów.
2. W tkance kostnej *in situ* znajduje się zestaw enzymów umożliwiający recykling witaminy K oraz biokonwersję witaminy K1 do postaci menachinonów.
3. W kościach szczurów z CKD i współistniejącą nadczynnością przytarczyc, mimo odpowiednich warunków do powstawania aktywnych form witaminy K, dochodzi do nasilenia procesu osteoblastogenezy i powstawania niedojrzałych osteoblastów, produkujących niewystarczające ilości aktywnej formy osteokalcyny, co w konsekwencji zaburza proces mineralizacji, prowadząc do obniżenia stanu mineralnego kości.
4. Badanie stężenia całkowitej osteokalcyny i jej formy nieukarboksylowanej – Glu-OC w surowicy, które są powszechnie wykorzystywane jako markery niedoboru witaminy K, wydaje się mieć niewielkie zastosowanie w przewlekłej chorobie nerek, szczególnie przy współistnieniu nadczynności przytarczyc.

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9. Streszczenie w języku polskim

Przewlekła choroba nerek (CKD) stanowi poważny problem zdrowotny, podobnie jak powszechne powikłanie, jakim są zaburzenia mineralne i kostne określane mianem CKD-MBD. Upośledzona mineralizacja i nieprawidłowości w mikroarchitekturze kości wiążą się z obniżeniem jej wytrzymałości, co przekłada się na wzrost częstości złamań u chorych z CKD. Zaburzenia jakościowe i ilościowe tkanki kostnej mają negatywny wpływ na życie pacjentów, a ich leczenie, szczególnie w zaawansowanych stadiach CKD, jest ograniczone.

Celem niniejszej rozprawy była kompleksowa ocena metabolizmu endogennej witaminy K (VK) poprzez pomiar stężenia VK1 (filochinon) oraz MK-4 i MK-7 (menachinony) w surowicy zdrowych zwierząt oraz w modelu CKD indukowanym chirurgiczną nefrektomią 5/6. W surowicy dokonano oceny stężenia białek zależnych od VK (VKDP), takich jak osteokalcyna (OC), jej forma nieukarboksylowana (Glu-OC) oraz nieukarboksylowane białko macierzy Gla (ucMGP). Na poziomie kości oznaczono obie formy OC: karboksylowaną (Gla-OC) i Glu-OC, a następnie dokonano oceny recyklingu VK poprzez analizę ekspresji genów – VKORC1, GGCX i UBIAD1. Uzyskane wyniki zestawiono z pomiarami densytometrycznymi w celu ustalenia związku między VKDP a stanem mineralnym kości.

Analiza metabolizmu endogennej witaminy K wykazała obniżone stężenie VK1, któremu towarzyszył wzrost MK-4 i MK-7 w surowicy szczurów z CKD, co świadczy o wzmożonej konwersji VK1 do postaci menachinonów. Wzrost stężenia całkowitej OC jest spowodowany jej kumulacją we krwi szczurów z CKD, natomiast zwiększone stężenie Glu-OC może być konsekwencją pobudzania jej syntezy przez parathormon (PTH). Przeprowadzone badania udowodniły, że tkanka kostna posiada zestaw enzymów umożliwiający recykling witaminy K oraz konwersję VK1 do postaci menachinonów. Pomimo tego nie dochodzi do produkcji odpowiedniej ilości aktywnej formy osteokalcyny, co powoduje nieprawidłową mineralizację tkanki kostnej w przebiegu CKD. Analiza zależności występujących pomiędzy parametrami stanu mineralnego kości, ekspresją enzymów cyklu witaminy K i zależnych od niej białek uczestniczących w procesie mineralizacji wykazała, że przyczyną powyższych zaburzeń może być nadczynność przytarczyc i nasilanie przez PTH procesu osteoblastogenezy, w wyniku którego powstają niedojrzałe osteoblasty, niezdolne do produkcji odpowiedniej ilości aktywnej formy OC. Praktycznym aspektem powyższego badania jest obserwacja, że pomiar stężenia całkowitej OC i jej formy nieukarboksylowanej – Glu-OC w surowicy, które są powszechnie wykorzystywane jako markery niedoboru witaminy K, wydaje się mieć niewielkie zastosowanie w warunkach CKD, szczególnie przy współistnieniu nadczynności przytarczyc.

10. Streszczenie w języku angielskim

Chronic kidney disease (CKD) and its main complication, called mineral and bone disorders (CKD-MBD), are leading health problems. Impaired mineralization, abnormalities in bone microarchitecture, and reduce bone strength, results in increased risk of fractures in patients with CKD. Disturbed bone quality and quantity negatively affect patient's life, and treatment, especially in advanced stages of CKD.

The aim of this doctoral dissertation was comprehensive assessment of the endogenous vitamin K (VK) metabolism by measuring the concentrations of vitamin K1 (phylloquinone), MK-4 and MK-7 (menaquinones) in the serum of healthy and 5/6 nephrectomy-induced CKD animals. Similarly, the concentrations of VK-dependent proteins (VKDP), such as osteocalcin (OC), non-carboxylated form (Glu-OC), and non-carboxylated matrix Gla protein (ucMGP) were assessed in serum. Additionally, both forms of OC: carboxylated (Gla-OC) and Glu-OC were evaluated at the bone level. To assess VK recycling, the expression of VKORC1, GGCX, and UBIAD1 genes in the bone tissue was determined. In order to assess the relationships between VKDP and bone mineral status, the obtained results were compared with densitometric measurements.

The analysis of vitamin K metabolism showed simultaneous decrease of VK1 levels and increase MK-4 and MK-7 concentrations in CKD rats. This may results from increased VK1 conversion to menaquinones. Progressive loss of kidney function results in increased concentration of total OC, which is associated with accumulation of this protein in the blood of uremic rats. On the other hand, the enhanced Glu-OC levels may be related to stimulatory effect of PTH. Despite the fact that bone tissue has required set of enzymes that enables VK recycling and its conversion to menaquinones, the production of sufficient amounts of active form of osteocalcin is impaired. In a consequence, in the course of CKD occur disturbed bone mineralization process. The analysis of the relationships between the parameters of bone mineral status, the VK cycle enzymes and VKDPs expression, showed that the above mentioned disorders may be associated with hyperparathyroidism and accelerated process of osteoblastogenesis stimulated by PTH. As a result, immature osteoblasts are unable to produce an adequate amount of active form of OC. The practical, and clinical significance of this research is that the measurement of the serum concentrations of total OC and Glu-OC, which are commonly used markers of VK deficiency, appear to have a limited importance in the course of CKD, especially in the presence of hyperparathyroidism.

11. Publikacja nr 1

Marta Ziemińska, Beata Sieklucka, Krystyna Pawlak

Vitamin K and D Supplementation and Bone Health in Chronic Kidney Disease—Apart or Together?

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Review

Vitamin K and D Supplementation and Bone Health in Chronic Kidney Disease—Apart or Together?

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Abstract: Vitamin K (VK) and vitamin D (VD) deficiency/insufficiency is a common feature of chronic kidney disease (CKD), leading to impaired bone quality and a higher risk of fractures. CKD patients, with disturbances in VK and VD metabolism, do not have sufficient levels of these vitamins for maintaining normal bone formation and mineralization. So far, there has been no consensus on what serum VK and VD levels can be considered sufficient in this particular population. Moreover, there are no clear guidelines how supplementation of these vitamins should be carried out in the course of CKD. Based on the existing results of preclinical studies and clinical evidence, this review intends to discuss the effect of VK and VD on bone remodeling in CKD. Although the mechanisms of action and the effects of these vitamins on bone are distinct, we try to find evidence for synergy between them in relation to bone metabolism, to answer the question of whether combined supplementation of VK and VD will be more beneficial for bone health in the CKD population than administering each of these vitamins separately.

Keywords: vitamin K; vitamin D; chronic kidney disease; bone remodeling; vitamin K and D supplementation

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1. Introduction

Chronic kidney disease (CKD) represents a global health issue involving about 13% of the general population, of which about 11% are patients in the 3–5 stage of CKD [1]. Impaired kidney function impacts the quality of bone tissue and results in the development of disorders in bone and mineral metabolism, which are defined as Chronic Kidney Disease-Mineral Bone Disorders (CKD-MBD) [2]. Abnormalities in mineral and bone metabolism contribute, in part, to severity of vascular calcification (VC). In this context, CKD-specific risk factors are believed to drive substantially to VC and cardiovascular disease. It is also established that patients with CKD stage 3–5 will die due to cardiovascular events before the need of renal replacement therapy [3,4].

CKD-MBD impacts bone remodeling (Figure 1)—the dynamic process mediated mainly by the two antagonistically acting cellular populations: osteoblasts (OBs) that control the formation of bone and osteoclasts (OCs), with the ability to resorb mineralized bone [5]. This process is tightly regulated by local and systemic hormones, such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (1,25D), and vitamin K (VK) [5,6]. The process of bone remodeling is composed of four phases: the activation phase (the recruitment of OCs); the resorption phase (the resorption of bone by OCs); the reversal phase (the apoptosis of OCs and the recruitment of OBs); and the formation phase (the OBs lay down new organic bone matrix that subsequently mineralize) [6]. Bone remodeling together with bone size, geometry, structure, and volume determines bone's biomechanical properties, integrity, and strength, providing renewal of damaged bone. An imbalance between the amount of resorbed bone and the quantity of new bone formation substantially

contributes to the increased risk of fractures, which is associated with higher mortality in patients with CKD [7–9].

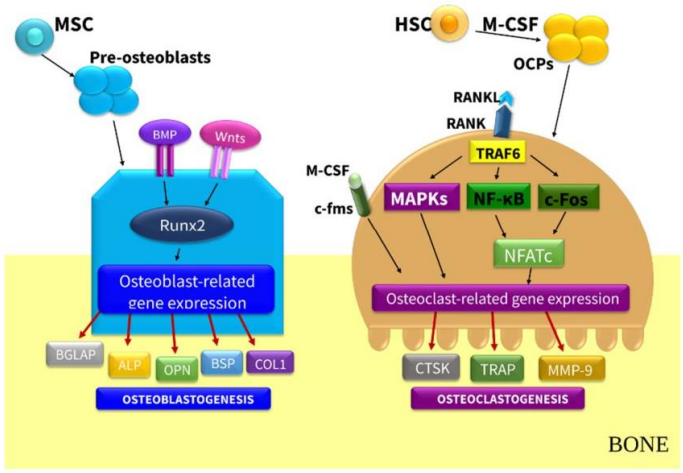

Figure 1. Bone remodeling. Abbreviations: ALP, alkaline phosphatase; BGLAP, bone-Gla-protein; BMP, bone morphogenic protein; BSP, bone sialoprotein; c-fms, colony-stimulating factor-1 receptor; COL1, collagen type 1; CTSK, cathepsin K; HSC, hemapoietic stem cells; MAPKs, mitogen-activated protein kinases; M-CSF, macrophage colony-stimulating factor; MMP-9, matric metalloproteinase 9; Msc, mesenchymal stem cell; NFATc, nuclear factor of activated T-cells; NFkB, nuclear factor-kappa B; OPN, osteopontin; OCPs, osteoclast precursors; RANK, receptor activator of nuclear factor kappa B; RANKL, receptor activator of nuclear factor kappa B ligand; Runx2, runt-related transcription factor 2; TRAF6, Tumor necrosis factor receptor associated factor 6; TRAP, tartare-resistant acid phosphatase.

Many clinical studies reported on VK and vitamin D (VD) deficiency in patients with CKD or undergoing dialysis [3,10–12]. These vitamin deficiencies could result from both dietary and nondietary determinants. Dietary recommendations for CKD patients, such as diets low in potassium (fewer leafy green vegetables rich in vitamin K1, VK1) and low in phosphate (fewer dairy products rich in vitamin K2, VK2) could promote VK deficiency. Holden et al. [3] showed that patients with stage 3–5 CKD have higher VK1 levels than those on maintenance dialysis. They concluded that patients who were clinically better nourished have also better vitamin K status. Nutritional factors may also affect the deficiency of VD status in CKD. The low food intake was frequently noticed in this population, due to numerous reasons, such as reduced appetite, dietary restrictions, i.e., low protein and phosphate diets, uremic-related gastrointestinal symptoms, and impaired gastrointestinal absorption of VD [13]. The nondietary determinants of VD in a cohort of patients with CKD included age, gender, low physical activity, less sunlight exposure, blunted the response of plasma VD to ultraviolet (UVB) irradiation, and hyperpigmentation, which may play a role in the impaired endogenous VD synthesis [14]. Additionally, with an increased loss of renal tissue, the availability and functionality of 1-α hydroxylase decreases, thereby reducing 1,25D [15]. Proteinuria has also been described as a contributing factor in the pathogenesis of VD deficiency [3,13,14]. Vitamin D binding protein (VDBP) carries about 85% of the circulating 25-hydroxyvitamin D (25D), VDBP–25D complexes are filtered in the glomerulus. Patients with proteinuria usually present the increased urinary VDBP excretion, but they might also show impaired megalin and cubilin-mediated protein reuptake in the proximal tubules, which may contribute to VD deficiency in the setting of CKD and proteinuria, especially in diabetic chronic kidney disease (DCKD) [16,17]. The peritoneally dialyzed patients are at particularly high risk of VD deficiency due to increased loss of 25D

and VDBP through the peritoneal effluent [18]. Moreover, the chronic inflammatory state, which is a common feature of CKD, can affect VD status [3].

Nondietary determinants of VK status in CKD include taking drugs prescribed to patients with CKD, such as warfarin, statins, proton-pump inhibitors, phosphate binders, steroids, or antihypertensives drugs [12,19,20]. The genetic variability can contribute to the large interindividual variation in VK biomarkers. Holden et al. [3] showed that Apolipoprotein E4 (ApoE4) carriers may be at risk for undercarboxylated VK-dependent proteins (VKDPs) due to rapid clearance of VK1 in the liver. Thus, the apoE4 allele, carried by 34% of this CKD population, may potentially represent a nonmodifiable risk factor influencing VK status.

Regarding the general population, a recent report recommended that serum 25D concentrations should be maintained at 20–50 ng/mL, and values >30 ng/mL should be considered normal [21]. However, there is some doubt as to whether the values considered "normal" in the general population could be applied to CKD patients. The Kidney Disease Improvement Global Outcomes (KDIGO) guidelines, published in 2017, do not consider any reference value for 25D level in CKD, but they recommended its evaluation when PTH progressively increases or stays above normal at stages of CKD above 3 [2]. A more recent study performed on stage 1–5 CKD patients showed no evidence of a decreasing effect of 25D on PTH lowering until 25D levels of 42–48 ng/mL [22], suggesting a higher VD target in CKD without any additional risk of hypercalcemia and hyperphosphatemia.

Establishment of the reference value of VK in patients with CKD is a challenge, because there is no gold standard for the measurement of VK levels and there is a lack of standardization. Instead, a functional deficiency of VK is commonly used as a surrogate of VK status in these patients. Measurements of uncarboxylated prothrombin (known as protein induced by VK absence/antagonism II (PIVKA-II), uncarboxylated OC (ucOC), and desphospho-uncarboxylated matrix Gla protein (dp-ucMGP) MGP are indicative of VK deficiency [3,11,23].

This review focuses on the contribution of VD and VK to skeletal health in CKD, discussing their effects on bone remodeling, derived from in vitro, in vivo, and clinical studies. In particular, we tried to find a functional synergy between these vitamins in relation to bone health in CKD and answer the question of whether simultaneous supplementation with VD and VK may be more beneficial in counteracting the effects of CKD-MBD than supplementing the deficiency of a particular vitamin.

2. Vitamin D and Vitamin K in CKD

2.1. General Characteristics of Vitamin K—Chemical Structure, Metabolism, and Laboratory Evaluation

VK constitutes a group of fat-soluble chemical compounds, whose common property is a structure containing 2-methyl-1,4-naphthoquinone. Naturally, VK occurs in two forms—VK1 (phylloquinone) and VK2 (including different menaquinones, MKs). VK1 is the main source of dietary VK and is mainly found in green leafy vegetables and plant oils. MKs are derived from intestinal bacteria (*Lactococcus* or *Bacteroides*) and fermented food [24]. The most common MKs in humans are the short-chain MK-4; it is the only MK produced by systemic conversion of phylloquinone to menaquinones. MK-4 can be endogenously produced from phylloquinone in some tissues, which is probably due to local biosynthesis [25]. The recently identified MK-4 biosynthetic enzyme, UbiA prenyltransferase containing 1 (UBIAD1), is widely expressed, but the mechanisms regulating its expression are not currently known [26]. The main sources of VK are illustrated in Figure 2.

The main physiologic role of VK is to act as cofactor for the γ-glutamyl carboxylase (GGCX) enzyme in the gamma-carboxylation reaction that add carboxyl groups to glutamic acid (Glu) residues in proteins. GGCX oxidizes VK into VK epoxide and then adds CO2. The newly carboxylated residues in such proteins are referred to as gamma-carboxyglutamic Gla domains. This process transforms inactive (uncarboxylated) proteins into active carboxylated VKDPs, enabling them to bind to calcium. Adequate calcium binding is a critical physiological step in blood coagulation, bone mineralization, and

vascular calcification. The most acknowledged extrahepatic VKDPs are MGP, osteocalcin (OC), growth arrest specific protein 6 (Gas6), and Gla-rich protein (GRP) [27].

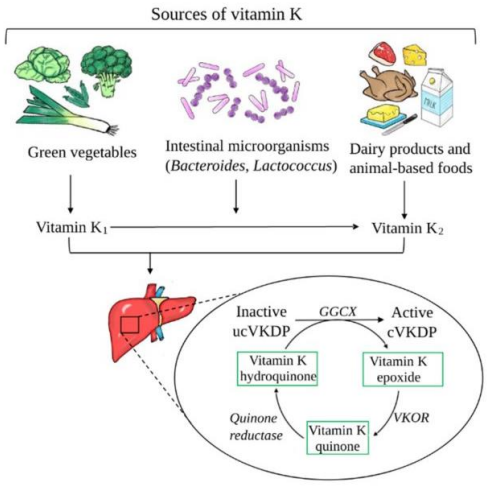

Figure 2. Vitamin K sources, metabolism, and mechanism of action. Abbreviations: ucVKDP, uncarboxylated vitamin K-dependent proteins; cVKDP, carboxylated vitamin K-dependent proteins; GGCX, enzyme γ-glutamyl carboxylase; VKOR, vitamin K epoxide reductase.

In addition to protein modification, a novel mechanism was uncovered in the signaling that regulates the transcription of target genes by VK through the activation of a nuclear receptor, the steroid and xenobiotic receptor (SXR; also known as nuclear receptor subfamily 1 group I member 2 (NR1I2) and pregnane X receptor (PXR), which is the mouse and rat ortholog of SXR) [28]. VK2 was shown to bind to and activate the SXR, which could induce expression of osteoblastic marker genes, such as alkaline phosphatase (ALP) and osteoprotegerin (OPG), extracellular matrix-related genes, and collagen accumulation in osteoblastic cells [29]. When we compare both forms of VK, VK1 is predominant form of VK in the human diet due to its relatively high content in food [30], but VK2 is required for OC to become activated and bind calcium, which makes VK2 a vital player in case of supporting the osteoprotective effect and maintenance of bone health [31–33]. Additionally, VK2 plays an important role in promoting bone formation: it stimulates the differentiation of osteoblasts, upregulates the gene expression of bone markers, and inhibits osteoclastogenesis [33]. VK reserves in the body are limited, and it is efficiently recycled through a series of redox reactions, which are defined as the "VK cycle" (Figure 2). The transformation of VK epoxide to quinone form occurs through VK epoxide reductase (VKOR). Then, quinone is converted by quinone reductase to a VK hydroquinone form, which can be reused. This last stage of the VK regeneration cycle is necessary for proper γ-carboxylation, because only a reduced form of VK can act as a cofactor for GGCX [34].

The determination of VK levels is difficult because of its physicochemical properties and low levels of VK in circulation. Measurements can be done using direct and indirect methods. One of the most popular direct methods is the determination of VK using high-performance liquid chromatography (HPLC). A disadvantage of this method is the possibility of interaction between an HPLC column and lipoproteins transporting VK, which may affect the results [35]. The most common indirect method for determining VK status is measuring uncarboxylated VKDPs—ucOC and ucMGP—through the enzyme-linked immunosorbent assay (ELISA). The uncarboxylated forms (uc)VKDP appear when protein carboxylation is decreased and increased levels of ucOC, ucMGP or dp-ucMGP reflect a VK deficiency.

The ucOC level, the total OC level, and the ratio between the two (%ucOC) are frequently used to reflect VK status linked to bone health [36]. The diagnostics of VK deficiency are also based on measuring PIVKA. These proteins are formed in the liver as inactive, under-carboxylated precursors that cannot perform their biological functions. Long-term VK status can be shown by PIVKA II measurement that together with prothrombin time are markers of the hepatic concentration of VK [10].

2.2. Vitamin K Status in CKD Patients

Many observational and interventional studies reported that patients with CKD undergoing conservative treatment, peritoneal dialysis (PD), or hemodialysis (HD) suffer from subclinical VK deficiency [3,10,11,20,23,37]. The number of CKD patients with VK deficiency reaches 70%–90% of that population and is more pronounced than in the general population [38]. The alterations in the OC levels have been already observed in the early stages of CKD, as 60%–70% of pre-dialysis patients had a high percentage of serum ucOC [3,39]. Several studies demonstrated that HD patients had poor VK status [3,10,11,20,23,37,40,41], which is rather associated with the dietary regimen and overall poor nutrient intake. In addition to the fact that VK has a lipophilic character, it should not be absorbed or removed by the membrane during dialysis [42,43]. Westenfeld et al. [41] showed that HD patients had significantly higher levels of dp-ucMGP and ucOC as compared to a healthy group, pointing out that most HD patients suffer from a VK deficiency. A recently published study by Cranenburg et al. [23] demonstrated low intake (140 μg/day) of VK1 and VK2 by HD patients. Interestingly, low VK intake was observed on the weekends and days of dialysis in comparison to the control group. Additionally, dp-ucMGP and ucOC were significantly elevated in the majority of HD patients, confirming a subclinical hepatic VK deficiency, whereas high levels of non-carboxylated MGP in these patients pointed to a vascular VK deficiency. Voong et al. [37] showed that the majority of HD patients had high levels of ucOC, and almost 30% had low levels of phylloquinone, confirming a subclinical VK deficiency. A recent observational study by Fusaro et al. [40] showed that total OC and ucOC levels were higher in patients with CKD than in healthy controls.

There are a few studies showing that PD patients have a comparable degree of VK deficiency to HD patients. Stankowiak-Kulpa et al. [44] demonstrated that 46% of PD patients had a VK insufficiency, as measured by elevated PIVKA-II levels. Another cross-sectional study of PD patients [11] showed that almost 30% of them had a VK deficiency, as assessed by serum VK1 level, and all patients had a VK deficiency, as measured by the level of ucOC. Interestingly, Jansz et al. [45] demonstrated that patients after kidney transplantation had lower levels of dp-ucMGP compared to HD or PD patients, indicating that the restoration of kidney function may contribute to an improvement in VK status.

It is widely known that VK is crucial for the activation of OC, which is involved in bone metabolism. OC (also known as bone-Gla-protein, BGLAP) is one of the main noncollagenous proteins that is synthetized by OBs during bone formation. Modification by VK-dependent carboxylation converts ucOC to an active carboxylate form (cOC). The cOC binds calcium ions and incorporates them into hydroxyapatite crystals in the bone matrix to promote bone formation [46]. Transcription and translation of the OC gene is under the control of 1,25D [47] and PTH [48], creating immature ucOC. Circulating OC is used as a good biomarker of bone formation, whereas high ucOC levels are an expression of poor VK levels and intake [43]. However, when bone is resorbed, OC fragments are released into the circulation, and their serum concentrations may reflect bone turnover. In a healthy organism, the proportion of ucOC to total OC typically does not exceed 20% [49]. OC clearance is through glomerular filtration; hence, patients with CKD demonstrate significantly increased levels of total serum OC and ucOC compared with healthy controls [10,23,39,41,50].

2.3. General Characteristics of Vitamin D

VD is a prohormone that acts in a variety of paracrine and autocrine systems. VD exerts a pleiotropic effect in the body, plays an important role in calcium–phosphate homeostasis and the regulation of PTH, bone metabolism, immune system, and cardiovascular disease [51,52].

VD is a fat-soluble vitamin that exists in two distinct forms, ergocalciferol (VD_2) and cholecalciferol (VD_3) [12]. The sources of VD_2 are vegetables and "fortified" food, whereas VD_3 is derived from animal-based foods but is mainly synthetized in the skin [12,13]. VD_3 is produced through the action of ultraviolet (UV) sunlight in the skin by photolytic conversion of 7-dehydrocholesterol (pro-VD_3) to pre-VD_3 (precalciferol); then, it is subsequently is changed to VD_3 [53,54]. Due to the fact that both forms of vitamin D (VD_2 and VD_3) are biologically inactive, they need further metabolism to be activated. In the next step, they are transported by VDBP in the liver, where they are subjected to the hydroxylation process by 25-hydroxylase (CYP2R1) to create 25D (calcidiol) [55]. The final step of VD activation, a second hydroxylation, occurs in the kidneys, where 25D is transformed into a biologically active form of 1,25D (calcitriol) by 1α-hydroxylase (CYP27B1) [56]. The level of the circulating form of 25D is 1000-fold higher than the active form of VD—1,25D [54,57,58]. The main sources of VD and their metabolism are illustrated in Figure 3.

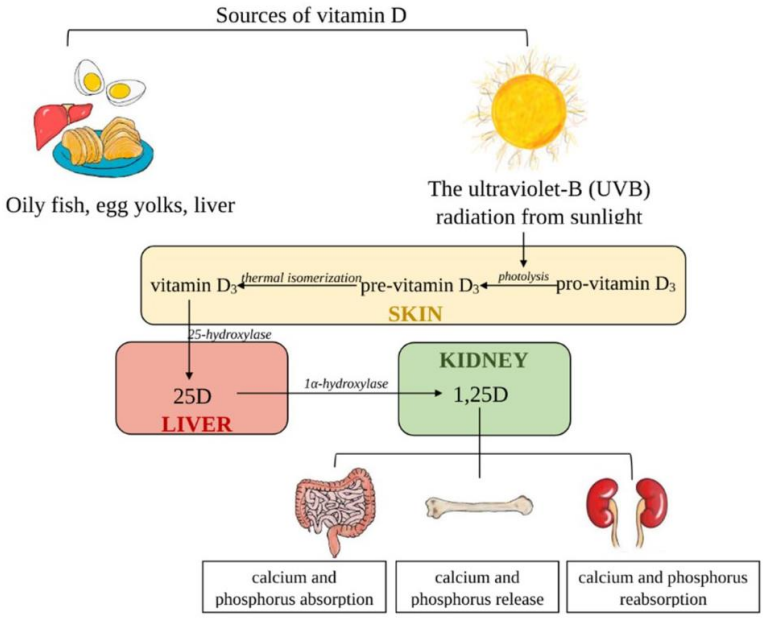

Figure 3. Vitamin D sources, metabolism, and role in calcium homeostasis. Abbreviations: 25D, 25-hydroxycholecalciferol; 1,25D, 1,25-dihydroxycholecalciferol.

In a healthy individual, the kidneys are the main place of 1,25D synthesis, but under specific conditions (CKD, rheumatoid arthritis, pregnancy), other cell types can also release it into circulation [55]. Interestingly, these extrarenal 1,25D products do not include the 1,25D pool [55]. Additionally, renal production of the active form of VD is strictly dependent on substrate availability, when 25D concentration is low [57,58]. A wide range of biological actions are mediated through binding with the VD receptor (VDR) and lead to changes in the expression of many genes e.g., Receptor Activator for Nuclear Factor κ B Ligand (RANKL), Low-density lipoprotein receptor-related protein 5 (LRP5), Cytochrome P450 family 24 subfamily A member 1 (CYP24A1), and Transient Receptor Potential Cation

Channel Subfamily V Member 6 (TRPV6) [59,60]. The circulating active forms of VD are highly regulated by many hormones, e.g., PTH, fibroblast growth factor 23 (FGF-23), low blood calcium, or phosphorus concentration [59]. VD and PTH interact in a tightly controlled feedback cycle and play a major role in the regulation of calcium and phosphate homeostasis [61]. VD deficiency with hypocalcemia and decreased calcium absorption from diet leads to enhanced PTH secretion, which results in increased renal calcium reabsorption and osteoclastic bone resorption [62–64]. PTH and hypocalcemia enhance CYP27B1 pathway-mediated hydroxylation of 25D to its active form: 1,25D. Therefore, 1,25D augments Ca^{2+} absorption in small intestines, increases PTH-dependent Ca^{2+} reabsorption in kidney, and mediates PTH-stimulated calcium release from bone [62]. Thus, PTH is a pivotal stimulator of VD synthesis, while on the other hand, VD has a negative influence on PTH secretion [61].

Serum 25D concentration is the most reliable biomarker for assessing VD status. To date, there is a lack of standardized methods for quantifying the level of 25D [65]. Although the gold standard for evaluating VD status is HPLC, it is not widely used due to high costs as well as the need for experience and special equipment. The second method that is extensively used to establish the reference range of serum VD is the DiaSorin Liaison assay, which is a quantitative chemiluminescent immunoassay (CLIA) [66,67]. Other common methods include ELISA and radioimmunoassay (RIA) [65,68]. Lately, more attention has been paid to liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is able to measure the various serum forms of VD and VD_3 [69].

2.4. Vitamin D Deficiency in CKD Patients

Low levels of 25D are common in CKD as well as in the general population, but the prevalence of 25D deficiency is much greater in the CKD population [70,71]. VD deficiency/insufficiency affects more than 80% of patients with CKD [14,65]. Moreover, many observational and interventional studies reported that kidney transplant recipients are susceptible to low levels of VD [72]. A VD deficiency increases with the progression of CKD, and it accounts for 20% in CKD stage 3 and 30% in CKD stages 4–5 [73]. Interestingly, Cankaya et al. demonstrated that PD and HD patients' VD levels were lower in comparison with CKD and renal transplant patients [74]. The low VD status has been related to increased progression of kidney and bone disease [75,76], cardiovascular events, metabolic syndrome, vascular calcification, ventricular hypertrophy, muscle weakness, insulin resistance, and overall mortality in this population [12,75,76].

Both the Kidney Disease Outcomes Quality Initiative (KDOQI) and KDIGO guidelines recommend checking and supplementing low serum 25D levels in CKD and dialysis patients [2,77]. Additionally, KDIGO experts suggest that VD concentrations in patients with CKD should be tested; thus, repeated measurements should be individualized as a result of the baseline values and interventions. However, there is no consensus on how frequently VD level should be measured and administered [2,77].

3. Role of Vitamin K and Vitamin D in Bone Remodeling in CKD: Pre-Clinical Evidence

3.1. Vitamin K and Bone Remodeling—In Vitro Studies

VK (in particular K2) improves the function of OBs by inducing their proliferation, differentiation [78,79], and inhibiting Fas-mediated OB apoptosis [80]. VK2 treatment of OBs can increase both ALP activity and the level of bone OC in the cell medium [78]. The higher ALP activity is associated with better formation of the organic matrix and the mineral part of the bone, and the deposition of OC and hydroxyapatite in the bone. VK2 activates SXR [28,29] and operates as a transcriptional regulator of a number of osteoblastic biomarker genes and extracellular matrix-related genes [29,81]. Moreover, VK2 supports bone formation and suppresses bone resorption by stimulating the expression of OPG and inhibiting the expression of RANKL on OBs [78].

Yamagushi et al. [82] observed that VK2 induced the downregulation of basal and cytokine-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) expression in OBs as well as in the OC precursors, explaining its dual pro-anabolic and anti-catabolic activities. Interestingly, the combined use of VK2 and 1,25D enhanced calcium deposition and OC expression in the OBs of obese diabetic mice [83], suggesting that this combined therapy may be more effective for the treatment of diabetes associated osteoporosis than the use of VK alone. On the other hand, the current evidence suggests that VK2 reduces osteoclastic activity via different strategies. It prevents OC formation either directly or indirectly (by interfering with the RANKL/OPG system [78]). VK decreases both the proliferation of tartrate-resistant acid phosphatase (TRAP) positive cells and TRAP activity in osteogenic culture medium [78,84]. Moreover, VK2 inhibits bone resorption induced by bone resorbing factors, such as Prostaglandin E2 (PGE2), Interleukin 1α (IL1α), and 1,25D [85]. The study of Kameda et al. [84] showed the potential of VK2 to induce OCs apoptosis. The characteristics of the main studies regarding VK in pre-clinical studies included in the review are shown in Table 1.

Table 1. Role of vitamin K and vitamin D in bone remodeling in chronic kidney disease (CKD): pre-clinical evidence.

Reference	Model	Dose	Results
		Vitamin K	
[79]	MC3T3-E1 osteoblasts cell line	VK2 (10^{-8}–10^{-3} M) for 1–5 days VK2 (10^{-5}, 10^{-6} and 10^{-7} M) for 24 h on days 1, 3, 5 and 7	VK2 promoted osteoblast differentiation and mineralization, induced autophagy in osteoblasts
[28]	The human cell lines HOS, MG-63, Saos-2, LS180, and HeLa	VK2	VK2 activates SXR and induces expression of the SXR target gene; VK2 treatment of osteosarcoma cells increased mRNA levels of OB: ALP, OPG, OPN, and MGP
[85]	Bone marrow cells were isolated from male Wistar rats (3 weeks old)	MK-7 (10^{-8}–10^{-5} M)	MK-7 can inhibit osteoclastic bone resorption; MK-7 has an inhibitory effect on the bone-resorbing factors-induced decrease in bone calcium content
		Vitamin D	
[78]	Bone marrow cells from the femur from elderly patients with type II osteoporosis	10 nM 1,25D and 0.5, 1.0, 2.5, 10 μM MK-4 or VK1	MK-4 and VK1 inhibited 1,25D-induced osteoclast formation and promoted the differentiation of bone marrow cells; MK-4 and VK1 decreased the RANKL and enhanced OPG
[86]	iliac crest bone biopsy samples from 11 paediatric dialysis patients	8 months of doxercalciferol therapy (an average of 19.3 ± 3.8 mcg of doxercalciferol per week)	1,25D increases the maturation of OBs lineage cells, stimulates osteocyte apoptosis and increases RANKL/OPG expression, increases the number of osteocytes
[87]	hMSCs from 53 subjects scheduled for hip arthroplasty	10 nM 1,25D	1,25D stimulated the differentiation of hMSCs to OBs; greater stimulation of in vitro osteoblast differentiation by 1,25D in hMSCs from younger subjects, and who had serum 25D ≤ 20 ng/mL
[88]	Primary OB cells, with a pre-osteoblastic phenotype from healthy male donors	1,25D (10^{-8} M)	1,25D increased differentiation, mineralization and survival of osteoblasts
[89]	Monocytes from blood of healthy adult volunteer donors	VD (25D-100 nM and 1,25D-5 nM)	1,25D inhibits osteoclastogenesis

Table 1. *Cont.*

Reference	Model	Dose	Results
[90]	The tibia from 4-week-old littermate C57BL/6J mice		1,25D can directly (in absence of RANKL) suppress OC precursor autophagy, which negatively regulates the proliferation of these cells; 1,25D can indirectly upregulate the autophagy response of OC precursors, thereby enhancing OC formation in the presence of RANKL
		Vitamin D and Vitamin K	
[83]	Primary osteoblasts harvested from the iliac crests of C57BL/KsJ lean (+/+) and obese/diabetic (db/db) mice	VK2 (10 nM) and 1,25D (10 nM) alone and in combination	The combined use of VK2 and 1,25D enhanced calcium deposits formation in OBs and increased the levels of bone anabolic markers and bone formation transcription factors

Abbreviations: VK2, vitamin K2; HOS, human osteosarcoma cell line; SXR, steroid and xenobiotic receptor; OB, osteoblast; ALP, alkaline phosphatase; OPG, osteoprotegerin; OPN, osteopontin; MGP, matrix Gla protein; MK-7, menaquinone-7; VK1, vitamin K1; 1,25D, 25-dihydroxyvitamin D; RANKL, Receptor Activator for Nuclear Factor κ B Ligand; hMSCs, human bone marrow stromal cells; 25D, 25-hydroxyvitamin D; VD, vitamin D; OC, osteocalcin.

3.2. Vitamin D and Bone Remodeling—In Vitro Studies

3.2.1. Impact of 1,25D on Osteoblast Function

In the available literature, there are few data on the in vitro effect of 1,25D on OBs from patients with CKD. Table 1 summarizes the main pre-clinical studies regarding VD. The first report by Zhou et al. [91] showed that different forms of VD, 25D and 1,25D, can stimulate in vitro OBs differentiation of marrow stromal cells from healthy controls and CKD subjects. Several years later, it was shown that primary OBs derived from CKD patients display a maturation defect in vitro [92]. A recent study of this team [86] documented that 1,25D markedly stimulated the expression of FGF-23, and the mature OB marker, BGLAP, in primary OBs derived from CKD patients. However, recombinant human FGF-23 countered VD-stimulated OBs differentiation of human bone marrow stromal cells (hMSCs) by reducing VDR and CYP27B1 expression as well as inhibiting 1,25D biosynthesis and signaling through bone morphogenic protein-7 (BMP-7) [93]. 1,25D in very high concentration (100 nM), which far exceeds the concentrations achieved in dialysis patients receiving high doses of calcitriol, improved in vitro OBs mineralization. On the other hand, VD stimulated the expression of the osteoclast differentiation factor, RANKL, in primary CKD OBs, and especially its high doses (10 nM and 100 nM) increased the ratio of RANKL/OPG expression. In contrast, VD sterols had no effect on the expression of the early osteoblastic marker, Runt-related transcription factor 2 (RUNX2), and they had very little effect on ALP expression in CKD cultures. These data suggest that 1,25D may play an important role in OBs maturation by regulating osteoclast–osteoblast coupling in the bone of CKD patients [86].

Over the course of the last decades, 1,25D has been studied extensively for its pleiotropic actions promoting bone remodeling in the general population, and numerous in vitro studies have implicated 1,25D in the regulation of both osteoblastic and osteoclastic activity [94,95]. Both OBs and OCs express VDR [96,97], which allows 1,25D to directly affect their biological activity. Moreover, both OBs and OCs can locally synthesize the active form of 1,25D as they express CYP27B1 [96,98]. However, data obtained from in vitro studies are very heterogenic with regard to the differentiation stage of the cells (mesenchymal stem cells vs. primary OBs vs cell line), time points of treatment (2–72 h after treatment), OB origin (human/rat vs. murine), and the 1,25D concentration that was used (1–100 nM) [99,100]. This makes it difficult to compare the different studies and to draw final conclusions. Herein, we focused on the in vitro impact of VD on human OBs (hOBs), hMSCs, and human OCs (hOCs).

3.2.2. Effect of 1,25D on hOBs and hMSCs

1,25D has been shown to stimulate bone formation and mineralization in all studies using hOBs, and it induced osteogenic differentiation from hMSCs. Ten nM of 1,25D stimulated the differentiation of hMSCs to OBs, and osteoblastogenesis was stimulated to a greater degree by 1,25D in hMSCs that were obtained from subjects with inadequate or deficient 25D levels than the people who were VD sufficient [87]. hMSCs, similar to hOBs and hOCs, express VDR and possess the molecular machinery for VD synthesis and metabolism, which makes them a producer and target of 1,25D [101]. Moreover, OBs express the VDBP receptors cubilin and megalin to uptake 25D [102]. In cultured primary hOBs, active VD increased the survival, differentiation, and function of these cells. Mechanisms explaining this effect include increased osteoblastogenesis [99] and inhibition of apoptosis [103,104], leading to the formation of bone nodules and bone mineralization. 1,25D has anti-apoptotic effects on primary OBs and osteoblastic cell lines by inhibiting Fas ligand-induced apoptosis and regulating components of both the Fas-related and mitochondrial apoptosis pathways [88,103]. The carefully regulated OB apoptosis plays a crucial role in healthy bone remodeling; if this process is excessive, osteocyte differentiation, bone deposition, and mineralization will all be reduced as well [105].

1,25D has been shown to increase RUNX2, small mother against decapentaplegic (SMAD) 1–3,5, osterix (OSX), ALP, and BGLAP expression in hOBs [86,88,106,107]. The other genes involved in OB proliferation and differentiation, whose expression has been shown to be increased by 1,25D, are bone morphogenetic protein-2 (BMP-2) [108] and insulin-like growth factor-binding proteins (IGFBPs): 2–4 [87,109].

The Wingless-type, Wnt-β-catenin pathway is an important regulator of OBs differentiation and function. Cytosolic β-catenin is translocated into the nucleus to stimulate osteoblastogenic gene transcription. The levels of β-catenin expression represent the functional status of the Wnt/β-catenin signaling pathway in OBs [110]. The impact of 1,25D on Wnt-β-catenin signaling in OBs was well recognized an in vitro study, where 1,25D stimulated Wnt signaling, increased β-catenin protein expression, or induced the Dickkopf-related protein 1 (DKK1) expression, leading to the intensification of calcified nodule formation in mineralized OBs [88,111,112].

Autophagy was recently recognized as an important regulator of OB survival and function. During autophagy, the toxic cytoplasmic components are removed, while nutrients are recycled to maintain cell functions and to protect against apoptosis. Impaired autophagy causes cellular dysfunction and cell death. Therefore, modulating the functional autophagy in bone cells is of therapeutic interest [113,114]. Recently, Al Saedi et al. [88] demonstrated that 1,25D may improve OB viability and function through the stimulation of functional autophagy. An additional benefit of 1,25D on the functioning of these cells could be through its effects on mitochondrial mass [88].

Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors involved in the regulation of new bone blood vessel formation. Human osteoblastic cells produce VEGF, and receptors for VEGF have been identified on these cells, allowing VEGF to directly regulate survival, chemotactic migration, and OB activity [115]. 1,25D treatment increased VEGF gene expression and protein levels in primary hOBs, indicating that this hormone can exert its anabolic effects on bone by inducing angiogenesis [116,117].

In vitro experiments with primary bone cells isolated from humans demonstrate that treatment with 1,25D inhibited OB proliferation and enhanced OB maturation and mineral deposition. The expression of many genes key to OB maturation and mineral deposition are modulated by 1,25D, as has been above described [86,88]. The activation of VDR by 1,25D can exert a catabolic effect on bone mineralization to ensure serum calcium homeostasis, or it may act as a mineralization enhancer through stimulating OB maturation and the expression of genes associated with mineralization. It has been proposed that the stage of OB differentiation is one of the possible factors determining which of these two effects predominates. The phenotypically immature OBs precursors respond to 1,25D through the stimulation of RANKL expression, whereas mature OBs predominately re-

spond through the stimulation of OC expression [118]. However, a later study by Woeckel et al. [119] demonstrated that 1,25D enhanced mineralization by the effects on hOBs in the pre-mineralization phase; it is involved in the appropriate preparation of the extracellular matrix (ECM) for mineralization. 1,25D stimulates the expression of the OB differentiation marker, ALP, and other ECM proteins, such as collagen type I (COL1A1). ALP-positive matrix vesicle production was significantly increased by 1,25D in this period of OB differentiation, and they can translocate ALP to the ECM, where ALP was incorporated to initiate mineralization [119].

In addition to the stimulation of bone formation and mineralization, 1,25D has a certain protective potential to avoid pathological over-mineralization. It may induce activin A and osteopontin (SPP1) gene expression—the recognized inhibitors of mineralization [120,121]. Moreover, a stimulator of mineralization, bone integrin-binding sialoprotein (IBSP), is inhibited by 1,25D [122].

3.2.3. Effect of 1,25D on hOCs and Human Peripheral Blood Mononuclear Cells (hPBMCs)

Current evidence suggests that endogenous 1,25D synthesis and the response to this vitamin in human bone is linked with coordinated functions in both the osteoclastic and osteoblastic cells, controlling bone remodeling [123,124].

The stimulation of osteoclastogenesis by 1,25D via the OB is one of better established effects of this vitamin on OC activation. With respect to gene regulation in OBs, the 1,25D–VDR complex induces the expression of RANKL that activates RANK on OCs and their hematopoietic precursors, stimulating bone resorption through osteoclastogenesis. OPG, the soluble decoy receptor for RANKL, is repressed by 1,25D in OBs, so that the biological effect of RANKL is reinforced [124,125]. The cell-to-cell contact in combination with macrophage colony-stimulating factor (m-CSF) induces the differentiation of precursors to OCs and promotes their activity. These data indicate that OBs are the key cell responding to 1,25D with respect to OC formation [126].

In vitro studies on the effect of 1,25D on osteoclastogenesis and hOC function are conflicting, showing both stimulatory as well as inhibitory effects of this vitamin on OC differentiation and resorptive activity [89,90,96,126–137]. In 1992, Suda et al. [128] suggested that 1,25D promotes bone resorption by increasing the number and activity of osteoclasts. These effects may be direct, if the osteoclast contains the VDR and CYP27B1, and 25D promotes their differentiation in the presence of macrophage colony-stimulating factor (m-CSF) and RANKL. Kogawa et al. [129] showed that OC formation from hPBMCs in the presence of physiological concentrations of 25D resulted in significant up-regulation of the key OC transcription factor, nuclear factor of activated T cells-c1 (NFATC1), and a number of key osteoclast marker genes. An interesting observation of this study was that the OCs generated in the presence of 1,25D, although more numerous, exhibit reduced resorptive activity on hydroxyapatite-coated slides when compared to OCs that matured simply in the presence of RANKL and m-CSF [129].

The study of Zarei et al. [96] showed that the treatment of hOCs with 1,25D significantly suppressed the expression of osteoclast fusion markers NFATC1 and transmembrane 7 superfamily member 4 (TM7SF4), reduced OC size, but increased OC number and resorptive activity. An increase in osteoclast resorption was due to less fusion, resulting in more small OCs in 1,25D-treated samples, as a few larger multinucleated OCs were observed in the control samples. Sakai et al. [130] also demonstrated that 1,25D treatment significantly inhibited the expression of NFATC1 in hOCs by upregulating the expression of interferon-beta, which is a strong inhibitor of osteoclastogenesis. However, the suppression of NFATC1 resulted in significantly inhibited hOC formation, which is opposite to the finding of Zarei et al. [96]. A similar effect of 1,25D treatment on mature multinucleated osteoclasts obtained from human monocytes was observed by Allard et al. [89], who demonstrated that 1,25D significantly inhibited osteoclastogenesis at early stages but had no effect on osteoclast-mediated bone resorption activity. Kudo et al. [131] also noticed that

1,25D did not stimulate resorptive activity of hOCs formed from cultures of hPBMCs. They suggested that it was more likely that 1,25D could influence OC activity indirectly.

Kim et al. [132] examined the direct effects of 1,25D on the osteoclastogenesis of human peripheral blood osteoclast precursors. They showed that 1,25D suppressed the expression of RANK in the hOC precursor and strongly inhibited OC differentiation. The mechanism responsible for the inhibition of RANK by this vitamin was a down-regulation of the c-Fms, the receptor of m-CSF, which is required for RANK expression. In line with the above observation, the treatment of PBMCs from healthy donors with 1,25D dose-dependently suppressed osteoclastogenesis in vitro, as has been shown by the reduced number of TRAP-positive OCs [133].

Wnt ligand 10b (Wnt-10b) is a key pathway for bone formation through increases in the number of OBs and the rate of mineral apposition [134]. A recent study by Lu et al. [135] demonstrated that in primary cell cultures of OCs, calcitriol increased Wnt-10b expression, but in parallel, it reduced the OCs fusion ability, the number of TRAP-positive OCs, as well as their bone-resorbing activity. This finding is compatible with higher Wnt-10b levels and lower TRAP-5b activity in HD patients receiving calcitriol compared with patients not taking this vitamin [135]. Although both hOBs and hOCs may be the source of Wnt-10b, the therapeutic dose of calcitriol enhanced Wnt-10b secretion only from OCs in this study. Taken together, the bone anabolic effect of a therapeutic dose calcitriol can promote OB function and it can inhibit OC maturation and resorbing capacity both in OC cultures in vitro and in hemodialyzed patients in vivo [135].

Autophagy has been reported to increase the number and function of OCs [135,137]. The recent study by Ji et al. [90] proved that 1,25D may be a strong regulator of autophagy in OCs, and it had a dual effect on osteoclastogenesis this way. 1,25D can directly (without RANKL) suppress OC precursor autophagy, which negatively regulates the proliferation of these cells. However, 1,25D can indirectly upregulate the autophagy response of OC precursors, thereby enhancing OC formation in the presence of RANKL.

Taken together, the in vitro studies revealed that 1,25D may function to optimize osteoclastogenesis, but on the other hand, it can mitigate hyperactive osteoclastic resorptive activity. The main effects of VK and VD on bone cells derived from in vitro studies are summarized in Figure 4.

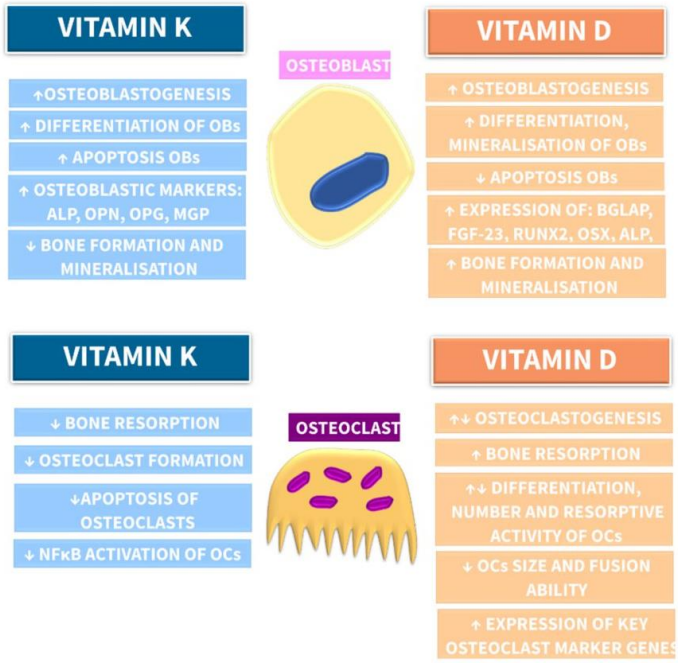

Figure 4. Impact of vitamin K and vitamin D on bone remodeling—evidence derived from in vitro studies. Abbreviations: OBs, osteoblasts; ALP, alkaline phosphatase; OPN, osteopontin; OPG, osteoprotegerin; MGP, matrix Gla proteins; BGLAP, bone-Gla-protein; FGF-23, fibroblast growth factor 23; RUNX2, Runt-related transcription factor 2; OSX, osterix; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OCs, osteoclasts; ↑, increase; ↓, decrease.

4. Vitamin K and Vitamin D in Bone Remodeling—In Vivo Studies

4.1. Vitamin K and Bone Remodeling—In Vivo Animal Models

According to our best knowledge, there is only one report examining the influence of VK2 on cortical bone mass and bone strength in rats with renal insufficiency. This study demonstrated that the administration of VK2 increased cortical bone strength without changing bone mineral density (BMD) in nephrectomized rats [138], suggesting that VK could affect bone integrity without altering BMD.

However, several animal models of osteoporosis have been used to study the effects of VK on bone metabolism. Table 2 reports the main in vivo studies with VK and its association with bone remodeling. The treatment of ovariectomized [139,140], unilaterally sciatic neurectomized [38], and tail suspended rats [141] with VK found positive effects on bone health. Histologic and microcomputed tomographic evaluations demonstrated that VK2 supplementation inhibited the loss of bone mass as well as improved OB function and bone architecture. Kim et al. [142] observed that VK administration in high-fat diet mice resulted in an increase in bone formation and a reduction in bone resorption. Some animal studies investigated the effect of the coadministration of VK2 and other bone acting drugs on osteoporosis. The coadministration of VK2 and Teriparatide improved OB function and increased Gla-OC serum levels [140]. The effect of the combined use of VK2 and bisphosphonate showed that VK2 could ameliorate the suppressive effect of bisphosphonates on bone turnover and increase bone volume as well as the bone formation parameters [143]. Combining VK2 with VD_3 showed an additional protective effect on osteoporosis versus VK2 treatment alone [144]. In addition, the combined effect of VK and antiresorptive drugs on bone mechanical strength were assessed, providing contrasting results. Otomo

et al. [145] did not observe any effects of K2 supplementation with Risendronate on bone quality, while Matsumoto et al. [146] showed that MK-4 treatment enhanced the positive effect of Risendronate on bone strength. These observations suggest that the combined administration of VK with other osteoprotective drugs may exert a more promising effect on bone health than VK alone.

Table 2. Vitamin K and vitamin D in bone remodeling—in vivo studies.

Reference	Model	Dose	Results
		Vitamin K	
[138]	*n* = 30, male Sprague–Dawley rats; assigned to three groups: sham operation (control), 5/6 nephrectomy and 5/6 nephrectomy + oral VK2	VK2 (menaquinone-4, menatetrenone): 30 mg/kg, 5 days/week	The administration of VK2 increased cortical bone strength without changing bone mineral density (BMD) and improved renal function.
[140]	*n* = 25, OVX female Sprague–Dawley rats assigned to five groups: the sham, ovariectomy (OVX), VK, TPTD and VK + TPTD	VK (menaquinone-4): 30 mg/kg/day TPTD: 30 µg/kg, 3 times/week	The coadministration of VK2 and TPTD improved OB function and the OB surface, and increased Gla-OC serum levels, improved the BMD and bone strength of the femur.
[142]	*n* = 42, male C57BL/6J mice divided into six groups: normal diet, normal diet + VK1, normal diet + VK2, 45% high-fat diet, 45% high-fat diet + VK1, a 45% high-fat diet + VK2	VK1 and VK2: 200 mg/1000 g	VK administration in high-fat diet mice resulted in an increase in bone formation and a reduction in bone resorption.
[143]	*n* = 30, male Sprague–Dawley rats assigned to five groups: nonsuspended group, tail-suspended group with vehicle alone, tail-suspended group with VK2, tail suspended group with bisphosphonate, tail-suspended group with combination of bisphosphonate and VK2	Bisphosphonate (incadronate): 0.1 mgP/kg body weight VK2: 24 mg/kg body weight/day	The effect of combined use of VK2 and bisphosphonate showed increased bone volume without supressing bone turnover.
[146]	*n* = 59, female ICR mice after sham-operated or ovariectomized; OVX divided into six groups: treated with risedronate (R), MK-4 (K), R+K, either the treatment was withdrawn or switched to K or R in the case of R and K	Risedronate: 0.25 mg/kg/day VK2: 100 µg MK-4/kg/day	Prior 8-week treatment with MK-4 followed by the 8-week risedronate significantly increased femur strength.
		Vitamin D	
[147]	*n* = 45, rats assigned to sham-operation or 5/6 nephrectomy surgery (NTX): divided into two groups: the untreated NTX and NTX + paricalcitol.	1500 IU/kg VD; for the 12-week: paricalcitol:100 ng/rat, 3 times per week	Paricalcitol efficiently ameliorates advanced renal insufficiency induced loss of mineral and mechanical competence of rat bones, prevented the renal impairment associated decrease in vBMD at the femoral neck and cBMD at the femoral midshaft, and restored bone strength at the femoral neck
[148]	*n* = 49, female Sprague–Dawley rats after 7/8 nephrectomy and CKD + OVX group; CKD + OVX were divided into 6 groups: placebo, E2 (10 µg/kg/day), E2 (30 µg/kg/day), calcitriol (10 ng/kg/day), E2 (10 µg/kg/day) + calcitriol, E2 (30 µg/kg/day) + calcitriol	Calcitriol:10 ng/kg BW, 5 times per week for 8 weeks	Calcitriol reduces bone loss but also improves trabecular connectivity; combined treatment with E2-30 + calcitriol was capable of achieving normal trabecular bone volume, trabecular remodeling, and connectivity

Table 2. *Cont.*

Reference	Model	Dose	Results
[149]	*n* = 36, 5/6 nephrectomized male Wistar rats divided into groups: control, Nx-Int D, Nx-Daily D, Ns-Phos	Calcium: 1.2%, phosphate: 1.2%, VD: 0.5 µg/kg 3 times per week	Calcitriol enhanced chondrocyte maturation and restoration of the growth plate architecture; calcitriol increased PTH/PTHrP receptor and d markers of chondrocyte differentiation; daily and intermittent calcitriol had similar effects on endochondral bone growth in phosphorus-loaded rats with renal failure
Vitamin K and Vitamin D			
[144]	*n* = 60, female Sprague–Dawley rats after OVX or sham operation; OVX rats were classified into three groups: a VK alone, a VD alone, and combination of VK and VD	VK (menaquinone): 48 mg/100 g diet VD: 0.16 mg/ 100 g diet	VK and VD may have a synergistic effect on reducing bone loss

Abbreviations: VK2, vitamin K2; BMD, bone mineral density; OVX, ovariectomy; TPTD, teriparatide; Gla-OC, γ-carboxylated osteocalcin; VK1, vitamin K1; NTX, 5/6 nephrectomy surgery; vBMD, volumetric bone mineral density; cBMD, cortical bone mineral density; CKD, chronic kidney disease; E2, 17β-estradiol; BW, body weight; Nx, nephrectomized animals; VD, vitamin D; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein.

In general, evidence from in vitro and animal studies supported the role of VK2 in bone health, suggesting a potential benefit for its use in patients with osteoporosis.

4.2. Vitamin D and Bone Remodeling—In Vivo CKD Animal Models

Despite widespread clinical use, there are limited studies on animal models of CKD that examine the bone tissue material and structural properties after VD (or its analogs) treatment, and the obtained results are inconclusive. Table 2 shows the main in vivo studies with VD and bone remodeling. One of the first studies, performed on female dogs with a 5/6 nephrectomy showed that the oral administration of 20 µg of 25D, three times a week, prevented secondary hyperparathyroidism and morphologic abnormalities associated with renal osteodystrophy during a two-year observation period [150]. Jablonski et al. [151] also demonstrated that rats that underwent a 5/6 nephrectomy, treated three times a week for 3 months with 0.17 µg/100 g body weight (BW) of calcitriol, exhibited higher trabecular volume, lower eroded surface and osteoid surface compared to untreated animals. A cross-sectional analysis showed that with 1,25D treatment, the inner femoral shaft diameter, femoral widths, bone stiffness, and time to fracture were normalized [151].

In the study by Jokihaara et al. [147], 5/6 nephrectomized rats obtained paricalcitol at a dose of 100 ng/rat, 3 times per week for 12 weeks. The femoral neck BMD and mechanical properties were higher than in untreated CKD animals, while no beneficial effects were observed in BMD or mechanical properties at the femoral diaphysis. The treatment of female rats subjected to a 7/8 nephrectomy with calcitriol at a dose of 10 ng/kg BW, 5 times per week for 8 weeks, proved to have a positive effect on bone microarchitecture, achieving normal trabecular connectivity [148]. Lu et al. [135] explored the effects of calcitriol on bone microarchitectures in CKD mice, using the 5/6 nephrectomy model, which were treated orally with 25 or 150 IU/kg/day of calcitriol. The bone volume fraction increased in mice treated one month with a higher dose of 1,25D; however, trabecular thickness was not significantly different in any group. The average cortical thickness was higher, whereas cortical porosity was lower in CKD animals treated with 150 IU/kg/day of calcitriol than in untreated CKD mice. Although there was no change in femoral BMD between the studied groups, the authors concluded that calcitriol, especially in the higher dose, can promote the growth of both trabecular and cortical bone in CKD.

The effect of daily or intermittent calcitriol administration in enhancing bone growth in CKD was studied by Sanchez et al. [149]. The animals were treated daily or thrice weekly with calcitriol for 4 weeks, but the total weekly dose of calcitriol was the same (350 ng/kg/week). Although calcitriol increased the serum calcium, it did not lower PTH or improve tibia and body length. However, calcitriol was effective in enhancing chondrocyte maturation and restoration of the growth plate architecture. Moreover, RANKL levels were improved with calcitriol treatment without changes in OPG, suggesting an enhancement of chondroclastogenesis and mineralization.

Newman et al. [152] used a rat model of progressive CKD (Cy/+), which is characterized by autosomal dominant cystic disease. Starting at 25 weeks of age, Cy/+ male rats were treated with 10 ng/kg BW of calcitriol, intraperitoneally, 3× weekly for 5 weeks. Apart from a significant suppression of PTH levels in animals with CKD, 1,25D had no impact on cortical or cancellous bone volume, bone turnover, OC number, or whole bone mechanical properties.

In contrast to the above data, some studies reported that 1,25D therapy can lead to bone turnover alteration and a reduction of cortical thickness in CKD rats. Male subtotally nephrectomized Sprague–Dawley rats were treated with 0.25 µg/kg/day of calcitriol starting 2 weeks after subtotal nephrectomy and continued for the next 14 weeks. In rats treated with 1,25D, a dramatically increased bone formation rate, an irregular osteoid deposition, and chaotic mineralization were observed. The dynamic bone histomorphometric parameters could not be measured in these animals due to the chaotic tetracycline incorporation. An excessive amount of osteoid, in combination with reduced bone resorption, led to a high bone area, which was improperly mineralized in rats treated with 1,25D. Moreover, kidney function was significantly more impaired, whereas aortic calcification was increased in rats treated with calcitriol compared to the CKD group [153].

Bisson et al. [154] treated rats with a 5/6 nephrectomy with 0.5 µg/kg BW of 1,25D, 3 times per week for 6 weeks using a high calcium and phosphate diet. Healthy rats on a standard diet, healthy rats with 1,25D on a high calcium and phosphate diet, as well as 5/6 nephrectomized rats on a standard diet were also included in this study. Cortical bone volume and area were significantly reduced in both CKD groups as compared to healthy controls; however, cortical bone thickness, the inner and outer cortical perimeter, and cortical bone mineral content were the most reduced in CKD rats treated with 1,25D on a high calcium and phosphate diet as compared to the other groups. The trabecular bone volume, trabecular thickness, trabecular number, osteoid volume, and osteoid thickness were significantly increased in these animals. Dynamic bone parameter analysis revealed a lower mineralization surface, bone formation rate, mineral apposition rate, and OC number in CKD treated with 1,25D on a high calcium and phosphate diet compared to the other groups. This study indicated that despite low PTH levels, treatment with calcitriol combined with a high calcium and phosphate diet induced low bone turnover and mineralization defects, which is likely explained by the high calcitriol dose [154].

Summarizing, the recently performed studies showed that the treatment of CKD animals with 1,25D may not improve bone quality [152], or even can be associated with mineralization defects [153,154]. These observations are consistent with the results of studies performed on non-CKD animals [155–157], which reported that treatment with calcitriol led to growth plate defects, an accumulation of osteoid and prolongation of mineralization lag time, reduction of cortical thickness, and suppression of bone matrix mineralization. Interestingly, VDR−/−, 1α-hydroxylase −/−, or double mutant mice on a rescue diet showed reduced bone formation, which is corrected by 1,25D, indicating a physiological anabolic role for the endogenous VD mediated by VDR in vivo [158,159]. Thus, it is possible that pharmacological, but not physiological, doses of 1,25D markedly increase RANKL expression by OBs, stimulating cortical osteoclastogenesis and bone resorption [94].

Such divergent data obtained during the treatment of renal osteodystrophy with 1,25D may result from the use of different animal species, doses, and treatment regimens,

a differential degree of PTH reduction, and possibly distinct effects of this vitamin in different skeletal sites. Nevertheless, data obtained from animal models indicated that 1,25D may have a direct effect on bone, independent of its effect on PTH suppression.

5. Vitamin K, Vitamin D, and Bone Health in Patients with CKD—Clinical Evidence

5.1. The Impact of Vitamin K on Bone Health in Patients with CKD

Studies regarding the association between poor VK status and bone metabolism, BMD, and the risk of fracture in CKD patients are limited. The main results obtained using VK supplementation and its association with bone health are summarized in Table 3. A small but growing number of recent studies have consistently suggested that there is an association between poor VK status in CKD patients and bone health [40,160–163].

Table 3. Vitamin K, vitamin D, and bone health in patients with CKD—clinical evidence.

Reference	Population	Outcome Measure	Main Findings
		Vitamin K	
[162]	*n* = 468, Adult patients with ESRD referred for single kidney transplant	VK, BMD, parameters of mineral metabolism	Poor vitamin K status is highly prevalent among patients with ESRD and associates with inflammation and low aBMD
[163]	*n* = 20, patients with chronic glomerulonephritis	VK, markers of bone metabolism	MK-4 supplementation suppressed bone loss
[37]	*n* = 141, patients with CKD stages 1–4	PIVKA-II	Subclinical VK deficiency is detectable at just the point in terms of loss of renal function with VC
[160]	*n* = 68, HD patients	VK1, OC, ucOC, iPTH	Suboptimal VK nutriture in HD patients is associated both with increased bone fracture risk and with a high prevalence of hyperparathyroidism
		Vitamin D	
[164]	*n* = 104, HD patients	VD, transiliac bone biopsy, ALP, iPTH	PTH serum levels are equally elevated in low and high 25D patients; calcitriol levels are constantly low; 25D deficiency resulted in mineralization and bone formation defect; the optimal level of 25D appears to be in the order of 20 to 40 ng/mL
[165]	*n* = 144, HD patients	VD, iPTH, bone densitometry	Increased bone fragility in HD patients is associated with VD deficiency and relative hypoparathyroidism in addition to reduced BMD at the radius
[166]	*n* = 610, elderly women	VD, BMD	Combined calcium and vitamin D_3 supplementation was effective in reducing the rate of BMD loss in women with moderate CKD
[167]	*n* = 120, patients with stages 1–4 CKD	VD, BMD, OC, NTx, FGF-23	Daily (2000 IU/d) and monthly (40,000 IU/month) VD supplementation for six months in adults with DM and CKD was safe, and it resulted in equivalent adherence and improvements in overall VD status, but only modest changes in markers of bone health
[168]	*n* = 47, CKD patients in stage 3 and 4	PTH, calcium, creatinine, VD	No statistically significant difference between the two treatments: cholecalciferol (4000 IU/d × 1 month, then 2000 IU/d) to doxercalciferol (1 µg/d) in lowering PTH

Table 3. *Cont.*

Reference	Population	Outcome Measure	Main Findings
		Vitamin K and Vitamin D	
[3]	n = 172, patients with stage 3 to 5 CKD	VK, VD, ucOC	Proteinuria was associated with both a suboptimal VD status as well as worse peripheral VK status; high serum ucOC levels were positively associated with phosphate and PTH, and inversely with 25D levels

Abbreviations: ESRD, end-stage renal disease; VK, vitamin K; BMD, bone mineral density; aBMD, areal bone mineral density; MK-4, menaquinone-4; CKD, chronic kidney disease, PIVKA-II, protein induced by VK absence/antagonism II; VC, vascular calcification; HD, hemodialysis; VK1, vitamin K1; OC, osteocalcin; ucOC, uncarboxylated osteocalcin; iPTH, intact parathyroid hormone; ALP, alkaline phosphatase; 25D, 25-hydroxyvitamin D; NTx, N-terminal telopeptide; FGF-23, fibroblast growth factor 23; VD, vitamin D; DM, diabetes mellitus.

Kohlmeier et al. [160] were the first to demonstrate an independent association between poor VK1 status and risk of bone fracture in HD patients. In the VIKI study, total OC and ucOC levels were higher in patients with CKD than in healthy controls, and over 50% of HD patents had vertebral fractures. Additionally, in this observational study, VK1 deficiency was the strongest independent predictor for vertebral fractures in these patients [56]. In another study by Fusaro et al. [161], HD patients treated with warfarin (an antagonist of VK) had an increased risk of vertebral fractures compared to those without warfarin treatment. These studies suggest that the VK axis is important in preserving bone mass. Evenepoel et al. [162] observed an independent association between VK status and bone health. Data from this study showed that the high dp-ucMGP levels were independently correlated with low BMD and incident of fractures, whereas no associations were observed between VK status and bone turnover markers in patients with end-stage renal disease (ESRD). Additionally, poor VK status at the time of renal transplantation can be considered as a risk factor for incident fractures.

Studies on the effect of clinical VK supplementation on BMD are also scarce. Sasaki at al. [163] showed that MK-4 supplementation for a year in steroid-treated patients with glomerulonephritis prevented steroid-induced bone loss. The patients on hemodialysis supplemented with MK-7 showed a decrease in dp-ucMGP, ucOC, and PIVKA-II, implicating that MK-7 improves VK status in the liver, bone, and vasculature [30,169,170].

To date, very little is known about the VK insufficiency and bone remodeling in CKD. Holden et al. [3] performed a study on patients with stage 3–5 CKD and found that high serum ucOC levels were positively associated with phosphate and PTH, whereas it was inversely associated with 25D levels, suggesting a relationship with bone remodeling [9]. Moreover, 6%, 60%, and more than 90% of patients in this study met the criteria for subclinical VK insufficiency, regarding VK1, ucOC, and PIVKA-II levels, respectively. Voong et al. [37] showed that subclinical VK deficiency is common in patients on dialysis, but it is also more frequent with worsening renal function in those CKD patients not yet on dialysis.

So far, VK supplementation and bone fractures in CKD patients have not been studied in a controlled, randomized clinical trial. Currently, there is one randomized controlled double-blind trial, RenaKvit, being performed in Denmark to address the effect on VK2 (MK7) on cardiovascular and bone disease in CKD patients. This study is evaluating the impact of VK2 supplementation on arterial stiffness and bone mineral density in HD patients. The RenaKvit trial is evaluating the impact of 360 µg of VK2 during a period of 2 × 12 months [171]. Increasing evidence that VK is also involved in vascular health is supported by controlled, randomized trials [14,172,173]. Oikonomaki et al. [174] investigated 1-year supplementation of 200 µg of VK (VK2/MK-7) in the prevention of VC progression among HD patients and found reduced serum uc-MPG levels, but they did not observe significant effects on the regression of VC. There are still ongoing trials evaluating the influence of oral VK supplementation on VC in HD patients—with 5 mg VK1 [172]

and 360 mcg VK2, 3 times weekly for 18 months. Other trials that are in process include Vitamin K supplementation in patients on hemodialysis (VISTA) in phase 2, with 400 mcg of VK1 three times a week, on dialysis days for four months [175]; Evaluation of Vitamin K Supplementation for Calcific Uremic Arteriolopathy (VitK-CUA) with administration of 10 mg of VK1 three times a week after dialysis for 12 weeks [176]; Comparative Study Evaluating the Effect of Vitamin K1 Versus Vitamin K2 on Vascular Calcification in Dialysis Patients in phase 2, with 10 mg of VK1 thrice a week for 3 months and phase 3 with 90 µg per day of VK2 [177].

5.2. Impact of Vitamin D on Bone Health in Patients with CKD

Together with the declining kidney function, many abnormalities concerning 1,25D, FGF-23, and PTH levels were observed. A disruption of the delicate balance between 1,25D, calcium, phosphorus, and PTH lead to secondary hyperparathyroidism and increased risk of bone disease. Several studies showed an inverse association between VD deficiency/insufficiency and PTH levels [178–182]. Metzger et al. [182] observed that serum PTH levels rise steeply when 25D values fall below 8 ng/mL; on the contrary, a mild decrease in this hormone concentration was seen when 25D levels exceeded 20 ng/mL. These observations are in line with other studies [183–185], suggesting that PTH increases significantly when 25D levels in CKD patients are below 30 ng/mL. Some authors noticed that VD deficiency was associated with lower values of serum calcium [179,186], which may be an additional cause of secondary hyperparathyroidism.

Studies showing an association between VD and BMD or bone fractures in the CKD population are limited. A summary of the key findings is presented in Table 3. A retrospective study conducted by Coen et al. [164] demonstrated that patients with low 25D levels (<15 ng/mL) had a lower bone formation rate and trabecular mineralization surface. Another retrospective study [165] showed that HD patients with fractures had a significantly lower VD concentration in comparison to patients without fractures, and low VD levels were associated with reduced BMD. Additionally, low levels of VD were independently related to increased fracture risk. Other studies showed that patients with lower 25D levels had increased subperiosteal resorption, reduced BMD, and increased skeletal fractures [187–189]. Interestingly, ESRD patients showed radiologic features of secondary hyperparathyroidism [188]. In line with these results is the Korean National Health and Nutrition Examination Survey [190], which reported that the BMD of CKD patients was lower in those with serum 25D < 50 nmol/L than in patients within serum 25D > 50 nmol/L. On the contrary, Brunerová et al. [191] did not demonstrate significant differences in trabecular bone and T-scores in HD patients with regard to their 25D levels. Based on these studies, it seems that low VD status is associated with an increased risk of fractures due to mineralization defects and lower BMD.

The optimal management of CKD-MBD is a daily challenge for nephrologists. VD supplementation is required for CKD patients to suppress PTH increases as well as to correct abnormalities of bone and mineral metabolism. In daily practice, VD (cholecalciferol or ergocalciferol) can be used in daily, weekly, or monthly doses. On the other hand, when VD supplementation is ineffective, therapy with VDRA (calcitriol, paricalcitol, doxercalciferol, alfacalcidiol) can be initiated [65]. Nevertheless, the issue of which form of VD should be used in patients with CKD is still a matter for debate. Current guidelines propose that CKD patients with VD deficiency should receive supplementation using the same recommendation as the general population [2,77,192]. The KDOQI recommend a dosage of 1000–2000 IU of VD_3 for VD repletion, but it confirmed that some patients with CKD may require a more aggressive therapeutic strategy [77]. However, irrespective of the chosen form of VD, it is worth emphasizing that when serum 25D levels are greater than 100 ng/mL, the risk of hypervitaminosis D toxicity can occur, including adverse effects such as hypercalcemia and hyperphosphatemia [65]. According to KDOQI 2017 recommendations, "mild and asymptomatic hypocalcemia can be tolerated in order to avoid inappropriate calcium loading in adults". The KDOQI work group holds the view that avoidance of

hypercalcemia will protect vascular and valvular calcifications, arrhythmia, and an increased risk for cardiovascular events in adults with CKD. In contrast to adults, the KDOQI work group endorses the recommendation to maintain serum calcium concentrations in children with CKD in the age-appropriate normal range, because the growing skeleton must be in positive calcium balance to achieve normal bone accrual. In this age group, the permissive mild hypocalcemia may have deleterious effects on skeletal integrity and should be avoided [193]. The excessive VC can also be caused by hyperphosphatemia (especially in the setting of persistent hypercalcemia) and a positive net phosphate balance. As has been recently summarized by Cozzolino et al. [194], hyperphosphatemia can cause damage in several cells and tissues, among others in the heart and blood vessels, where it is strongly associated with vascular and valvular calcification, arteriosclerosis, and an increased risk of cardiovascular death, especially in advanced CKD patients.

Vitamin D analogs (VDRAs), which are less calcemic and phosphatemic than the active form of vitamin D, are becoming the standard for the treatment of secondary hyperparathyroidism. The experimental models and clinical studies suggest that VDRAs can promote VC probably only at high doses if they induce or exacerbate hyperphosphatemia, while the use of these agents in more physiological doses (just enough to correct secondary hyperparathyroidism) might even be protective against VC [195–197].

In a study by Oksa et al. [198], a 12-month cholecalciferol supplementation of 5000 or 20,000 IU/week significantly improved VD deficiency, increased calcidiol, and less markedly, calcitriol levels, and decreased PTH levels without adverse effects on serum mineral parameters. Additionally, the number of hypercalciuric patients increased with a higher VD dose, although there was no sustained rise in calcuria. A similar decrease in PTH levels, following cholecalciferol supplementation, had also been presented in other studies [199–201]. Additionally, Yadav et al. [201] reported that cholecalciferol supplementation not only suppressed secondary hyperparathyroidism but also favorably changed the biochemical parameters of mineral metabolism in patients with CKD. On the contrary, supplementation with 50,000 IU cholecalciferol weekly [202] or 1000 IU cholecalciferol daily [203] found no difference in PTH levels in CKD patients. Post-hoc analysis of the Vitamin D, Calcium, Lyon Study II (DECALYOS II) study [166] reported that daily supplementation of 800 IU of cholecalciferol in combination with 1200 mg of calcium significantly increased serum 25D concentrations and radius BMD in an elderly woman with moderate CKD and severe VD deficiency. On the other hand, Mager et al. [167] found no significant differences in FGF-23, OC, N-terminal telopeptide (NTx), and BMD as measured by dual X-ray absorptiometry (DXA) after daily (2000 IU/day) and monthly (40,000 IU/month) VD_3 supplementation for six months in adults with diabetes mellitus and CKD. Summarizing, all the above-mentioned randomized studies demonstrated that a correction of VD deficiency with cholecalciferol supplementation led to the efficient achievement of a sufficient level of 25D in CKD patients.

In another study, Moe et al. [168] compared cholecalciferol at a dose of 4000 IU/d for a month, and then 2000 IU/d for two months and doxercalciferol at a dose of 1 μg/d for 12 weeks. The PTH levels decreased by 10% and 30% in the cholecalciferol and the doxercalciferol groups, respectively. However, there was no significant difference in the mean change between these two treatments. Additionally, there were no increases in serum calcium and urinary calcium/creatinine in the cholecalciferol group, whereas in the doxercalciferol-treated patients, there was a slight increase in the serum calcium level and urinary calcium exertion level. In the next study by Zelnick et al. [204], patients received either cholecalciferol (4000 IU daily for 1 month and then 2000 IU daily for 5 months) or calcitriol (0.25 μg daily for 1 month and then 0.5 μg daily for 5 month). There was no difference in PTH levels in both groups, and only the calcitriol-treated group showed a significant change in FGF-23 levels. Interestingly, both groups significantly increased circulating 24,25D concentrations and the ratio of 24,25D/25D.

Studies regarding VD supplementation, using ergocalciferol in CKD patients with VD insufficiency, have shown effective correction of 25D [205–210]. Treatment with ergocal-

ciferol among patients with stage 3 CKD resulted in a significant decrease of serum PTH concentrations [207,208]. Similarly, Zisman et al. [205] observed a decrease in PTH levels but only in CKD stage 3. On the contrary, Porter et al. [206] and Gravesen et al. [209] did not find any differences in PTH levels and bone/mineral parameters. Wetmore et al. [211] compared the efficacy of cholecalciferol and ergocalciferol in the CKD population, and they showed that therapy with cholecalciferol is more effective at raising serum 25D concentration, suggesting that cholecalciferol may be more effective. These results are in line with other studies conducted on CKD patients [212,213].

6. Impact of Combined Vitamin K and Vitamin D Supplementation on Bone Health in Patients with CKD

A growing body of evidence from in vitro [83] and in vivo studies [144], as well as clinical trials [214–217], showed that bone metabolism depends on the interaction between vitamins D and K, as has been schematically presented in Figure 5. However, the interplay between these vitamins in relation to bone health remains not fully elucidated, especially in CKD.

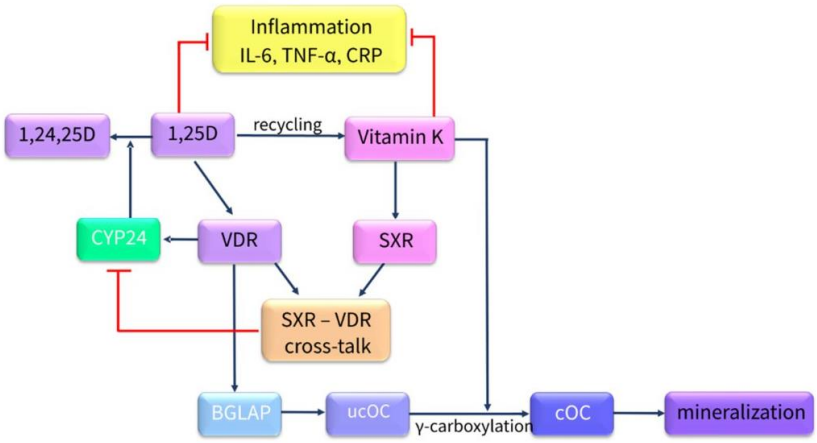

Figure 5. Potential synergy between vitamin K and vitamin D action in bone. Abbreviations: IL-6, interleukin 6; TNF-α, tumor necrosis factor α; CRP, C-reactive protein; 1,24,25D, 1,24,25-trihydroxyvitamin D; 1,25-dihydroxyvitamin D; CYP-24, 25-hydroxyvitamin D-24α-hydroxylase; VDR, vitamin D receptor; SXR, steroid and xenobiotic receptor; BGLAP, bone-Gla-protein; ucOC, uncarboxylated osteocalcin; cOC, carboxylated osteocalcin.

OC is produced by OBs during bone formation. It is one of the most abundant proteins in bone and is necessary for bone mineralization. The synthesis of fully functional OC and its expression are controlled by both VK and VD [218]. 1,25D is a known promoter for OC gene expression [219], whereas VK is acquired for proper γ-carboxylation of OC, thereby increasing its beneficial effect on bone formation [220,221]. Fully carboxylated OC is positioned into hydroxyapatite and strongly binds calcium, providing bone mineralization [222]. Moreover, 1,25D is able to regulate the γ-carboxylation of OC, decreasing ucOC secretion in human osteosarcoma cells [223].

VD can exert an anabolic effect in bone through increasing OB activity and reducing OC activity [224]. Koshihara et al. [225] demonstrated that VK2 promoted α25D-induced mineralization in human periosteal OBs. Similarly, the study by Poon et al. [83] showed that the coadministration of VK and VD caused an enhancement of calcium deposits and additionally increased the levels of bone anabolic markers of bone formation in the OBs of obese/diabetic mice. These findings suggest the synergistic effect of both vitamins in relation to bone formation and mineralization.

On the other hand, there are suggestions that excessive amounts of VD can increase VK requirements, inducing a relative VK deficiency by direct stimulation of the synthesis of VK-dependent proteins [226,227].

Another field of VD and VK cooperation is inflammation, which is casually implicated in osteoporosis [228]. VK is related to a decreased production of inflammatory markers: C-reactive protein, isoprostanes, and proinflammatory IL-6 [229–232]. VD exerts several immunomodulatory functions, such as the suppression of pro-inflammatory cytokine expression and regulation of immune cell activity [233]. VD supplementation reduced tumor necrosis factor α (TNF-α) levels in patients with osteoporosis [234].

VK and VD also overlap metabolically at the cellular level. The VK cycle is a source of electron transfer for antioxidant power in hOBs, and 1,25D can enhance the reductive recycling of MK4 [220]. SXR, which can be activated by VK2, was shown to be able to crosstalk with VDR, and this way deranging 1,25D metabolism. It was shown that the SXR-VDR crosstalk can inhibit VDR-mediated CYP24 promoter activity [235]. CYP24-mediated hydroxylation of 1,25D is a critical step in its catabolism, and it appears to be responsible for controlling systemic 1,25D levels. CYP24 is directly regulated by VDR, and it is expressed mainly in the kidney, where VDR is also abundant. However, recent studies [5–7] have shown that bone cells also have molecular machinery capable of producing and metabolizing 1,25D, suggesting that such an interaction may be present in bone as well. Therefore, VK and VD can mutually intensify each other's metabolism.

On the other hand, the activation of SXR in the liver can lead to the down-regulation of CYP2D25, which is an enzyme involved in 1,25D biosynthesis [236] that may be related to osteomalacia.

In vivo evidence also reported that combined VD and VK2 supplementation prevented bone loss by stimulating OC production in ovariectomized (OVX) rats [144,224,237,238], whereas no effect was observed when these vitamins were given separately [238]. A recently published study [239] demonstrated the beneficial effects of eggshell calcium, VD_3, and VK2 on the inhibition of OVX-induced bone loss in rats. The combination of these three elements increased cortical and trabecular bone quality as well as improved biochemical and densitometrical parameters. Meanwhile, Iwamoto et al. [240] found no synergistic effect of VK and VD on intestinal calcium absorption, renal calcium reabsorption, and cancellous and cortical bone mass in calcium-deficient rats. Therefore, it seems that the availability of calcium is an important factor in determining the synergistic effect of these vitamins in relation to bone mineralization.

The above presented data suggest that combined VD and VK supplementation may be beneficial for bone health in the course of CKD. Unfortunately, there have been no randomized, controlled trials that examined the effects of such a combination in this population. Many studies have investigated the combined effect of VK and VD supplementation on skeletal integrity in the general population, especially in postmenopausal women with osteoporosis [214–217,241]. Nevertheless, these studies did not provide consistent conclusions, and the effect of the coadministration of these vitamins is still poorly understood. A recently published meta-analysis [241] was based on eight selected randomized controlled trials, which evaluated the combined effect of supplementation with VK and VD on bone quality. This meta-analysis showed that the simultaneous administration of VD and VK can improve bone quality by increasing the total and third lumbar BMD and decreasing ucOC. However, in the remaining lumbar segments and femoral neck, the combined supplementation of these vitamins did not significantly increase BMD. Taken together, this report indicates that the coadministration of VD and VK can have a more favorable effect on bone health than giving each one separately. However, the authors emphasize that conclusions from this meta-analysis should be interpreted with caution due to potential publication bias.

Taken together, despite the growing body of evidence from in vitro and in vivo studies, as well as clinical trials, the synergy between VK and VD in relation to bone quality and quantity remains not fully elucidated. Therefore, further studies are needed to explain the

exact mechanisms of the combined effects of these vitamins on bone health, especially in the CKD population.

7. Conclusions

The current understanding is that patients with CKD are a clinical group at high risk for VD and VK deficiency. Therefore, these patients are prone to suffering from many consequences of VK and VD deficiency, such as poor bone health and a higher risk of fractures. Therefore, finding new solutions for the prevention/treatment of osteoporosis in this population is a particular challenge. The majority of randomized-controlled studies performed on osteoporotic patients without CKD suggest that combined VK and VD supplementation may be more beneficial for the prevention and treatment of bone loss. Moreover, supplementation with these vitamins is easily accessible, safe to use, and relatively inexpensive. However, there is still not enough evidence to recommend VK and VD supplementation. Based on studies in the general population, we can suspect that VD and VK supplementation in CKD patients may be a possible therapeutic target for improving bone health. Due to the lack of adequate clinical studies in this population, the question arises whether CKD patients might benefit from simultaneous VK and VD supplementation. This creates a need for further research, in which an investigation of the potential synergistic effect of combined supplementation of VD and VK on bone health in this population should receive more attention.

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Abbreviations

1,25D	1,25-dihydroxyvitamin D
25D	25-hydroxyvitamin D
ALP	alkaline phosphatase
ApoE4	apolipoprotein E4
BGLAP	bone-Gla-protein; osteocalcin
BMD	bone mineral density
BMP-2	bone morphogenetic protein-2
BMP-7	bone morphogenic protein-7
BW	body weight
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease–mineral bone disorders
CLIA	chemiluminescent immunoassay
cOC	carboxylated osteocalcin
COL1A1	collagen type I
CYP2R1	25-hydroxylase; cytochrome P450 family 2 subfamily R member 1
CYP24A1	cytochrome P450 family 24 subfamily A member 1
CYP27B1	1α-hydroxylase; cytochrome P450 family 27 subfamily B member 1

DCKD	diabetes chronic kidney disease
DECALYOS II	Vitamin D, Calcium, Lyon Study II
DKK1	Dickkopf-related protein 1
dp-ucMGP	desphospho-uncarboxylated matrix Gla protein
DXA	dual X-ray absorptiometry
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ESRD	end-stage renal disease
FGF-23	fibroblast growth factor 23
Gas6	growth arrest specific protein 6
GGCX	γ-glutamyl carboxylase
Gla	gamma carboxyglutamic acid
Glu	glutamic acid
GRP	Gla-rich protein
HD	hemodialysis
hMSCs	human bone marrow stromal cells
hOBs	human OBs
hOCs	human OCs
hPBMCs	human peripheral blood mononuclear cells
HPLC	high-performance liquid chromatography
IBSP	integrin-binding sialoprotein
IGFBPs	insulin-like growth factor-binding proteins
IL1α	interleukin 1α
KDIGO	Kidney Disease Improvement Global Outcomes
KDOQI	Kidney Disease Outcomes Quality Initiative
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LRP5	low-density lipoprotein receptor-related protein 5
m-CSF	colony-stimulating factor
MKs	menaquinones
NFATC1	nuclear factor of activated T cells-c1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NR1I2	nuclear receptor subfamily 1 group I member 2
NTx	N-terminal telopeptide
OBs	osteoblasts
OCs	osteoclasts
OPG	osteoprotegerin
OSX	osterix
OVX	ovariectomized
PD	peritoneal dialysis
PGE2	prostaglandin E2
PIVKA-II	protein induced by VK absence/antagonism II
PTH	parathyroid hormone
PXR	pregnane X receptor
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
RIA	radioimmunoassay
RUNX2	Runt-related transcription factor 2
SMAD	small mother against decapentaplegic
SPP1	osteopontin
SXR	steroid and xenobiotic receptor
TM7SF4	transmembrane 7 superfamily member 4
TNFα	tumor necrosis factor α
TRAP	tartrate-resistant acid phosphatase
TRPV6	Transient Receptor Potential Cation Channel Subfamily V Member 6
UBIAD1	UbiA prenyltransferase domain-containing protein
ucOC	uncarboxylated osteocalcin
UV	ultraviolet
VC	vascular calcification

VD	vitamin D
VDBP	VD binding protein
VDR	VD receptor
VEGF	Vascular endothelial growth factor
VK	vitamin K
VK1	vitamin K1
VK2	vitamin K2
VKDPs	VK-dependent proteins
VKOR	VK epoxide reductase
Wnt	Wingless-type
Wnt-10b	Wnt ligand 10b

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12. Publikacja nr 2

Marta Ziemińska, Dariusz Pawlak, Beata Sieklucka, Katarzyna Chilkiewicz, Krystyna Pawlak

Vitamin K-Dependent Carboxylation of Osteocalcin in Bone—Ally or Adversary of Bone Mineral Status in Rats with Experimental Chronic Kidney Disease?

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Vitamin K-Dependent Carboxylation of Osteocalcin in Bone—Ally or Adversary of Bone Mineral Status in Rats with Experimental Chronic Kidney Disease?

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Abstract: Chronic kidney disease (CKD) commonly occurs with vitamin K (VK) deficiency and impaired bone mineralization. However, there are no data explaining the metabolism of endogenous VK and its role in bone mineralization in CKD. In this study, we measured serum levels of phylloquinone (VK1), menaquinone 4 and 7 (MK4, MK7), and VK-dependent proteins: osteocalcin, undercarboxylated osteocalcin (Glu-OC), and undercarboxylated matrix Gla protein (ucMGP). The carboxylated osteocalcin (Gla-OC), Glu-OC, and the expression of genes involved in VK cycle were determined in bone. The obtained results were juxtaposed with the bone mineral status of rats with CKD. The obtained results suggest that the reduced VK1 level observed in CKD rats may be caused by the accelerated conversion of VK1 to the form of menaquinones. The bone tissue possesses all enzymes, enabling the conversion of VK1 to menaquinones and VK recycling. However, in the course of CKD with hyperparathyroidism, the intensified osteoblastogenesis causes the generation of immature osteoblasts with impaired mineralization. The particular clinical significance seems to have a finding that serum osteocalcin and Glu-OC, commonly used biomarkers of VK deficiency, could be inappropriate in CKD conditions, whereas Gla-OC synthesized in bone appears to have an adverse impact on bone mineral status in this model.

Keywords: bone mineral status; chronic kidney disease (CKD); genes of vitamin VK cycle; vitamin K (VK); VK-dependent proteins; 5/6 nephrectomy model

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1. Introduction

Chronic Kidney Disease Mineral and Bone Disorder (CKD-MBD) is a common complication of CKD, associated with abnormalities in bone metabolism and impaired bone mineralization [1–3]. In the course of CKD-MBD, both quality and quantity of bone tissue have been compromised, and the development of osteoporosis has an impact on a substantial increased risk of fractures and mortality in those patients [4,5].

Throughout life, bone undergoes a remodeling process in which the amount of resorbed bone should be equivalent to the amount of new bone formation and appropriate mineralization [6,7]. In the regulation of the mineralization process in CKD, many factors play a key role, such as parathormon (PTH), vitamin D, and vitamin K (VK) [8,9]. Vitamin K occurs in two forms—K1 (phylloquinone) and K2 (menaquinones, MKs). The most common MK in humans is the short-chain menaquinone 4 (MK4), which can be produced by endogenous conversion of phylloquinone to menaquinones with the enzyme UbiA prenyltransferase containing 1 (UBIAD1) [10–12]. The long chain MKs, such as menaquinone 7 (MK7), are found in fermented foods [13]. The pivotal role of vitamin K acts as a cofactor for the enzyme γ-glutamyl carboxylase (GGCX) in the gamma-carboxylation reaction, which in proper course is closely associated with its recycling, defined as the

"vitamin K cycle". The vitamin K epoxide is converted into the quinone form by vitamin K epoxide reductase complex subunit 1 (VKORC1). The transformation of the quinone to VK hydroquinone form occurs through quinone reductase. This form of VK can be reused as a cofactor for GGCX in the gamma-carboxylation reaction [14]. In the course of that reaction, carboxyl groups are added to Glu residues in proteins and transformed to Gla domains. This process transforms inactive (undercarboxylated) proteins into active (carboxylated) vitamin K-dependent proteins (cVKDPs), such as matrix Gla protein (MGP) and osteocalcin (OC) [15]. The increased circulating levels of undercarboxylated VKDPs—Glu-OC and ucMGP—reflect VK deficiency, and the measurement of these proteins is used to determine VK status [16,17].

Patients with CKD frequently suffer from subclinical VK deficiency, which may result from dietary restrictions, poor nutrient intake, and using drugs such as proton-pump inhibitors, steroids, statins, antihypertensives drugs, or broad-spectrum antibiotics, which decrease vitamin K synthesis by the impairment of the natural intestinal microflora [18–22]. Subclinical VK deficiency was confirmed in hemodialysis (HD) patients on the grounds of the high levels of ucOC, dephosphorylated-uncarboxylated MGP (dp-ucMGP), and low levels of VK1 [21,23]. In CKD, VK metabolism and recycling may also be impaired. McCabe et al. [24] assessed the genes expression of the VK cycle in a rat model of adenine-induced CKD and observed the reduced GGCX and VKORC1 expression in the thoracic aorta and a decreased level of UBIAD1 in the kidney.

Despite a small amount of research on the relationship between VK and bone health in CKD patients, it has been observed that low VK status has been linked to an increased risk of bone fracture [25,26] or reduced bone mineral density (BMD) [27–30]. Studies on the effect of VK supplementation on BMD are limited and inconclusive. In some research, an increase in BMD [31–36] or bone mineral content (BMC) [37] was observed under the influence of VK supplementation. However, in other studies, the above relationship was not shown [38,39]. MK7 supplementation reduced the levels of ucOC and dp-ucMGP in hemodialysis patients [40–42], whereas MK4 supplementation prevented bone loss in steroid-treated glomerulonephritis patients [43].

In the available literature, there is a lack of research explaining the role of endogenous VK in the bone mineralization process in the course of CKD. Therefore, the aim of the present study was the comprehensive assessment of endogenous VK metabolism in rats with CKD through determination of VK1, MK4, MK7, and undercarboxylated VKDPs—Glu-OC and ucMGP levels in serum, and the measurement of Gla-OC, Glu-OC levels, and their ratios in trabecular and cortical region of femurs. Then, the expression of genes related to VK recycling in bone were investigated. The obtained results were juxtaposed with the bone mineral status of uremic rats.

2. Materials and Methods

2.1. Animals

Twenty-six 4-week-old male Wistar rats were randomly assigned into two groups: the control group (CON, $n = 10$) subjected to sham-operation by renal decapsulation, and the experimental group (CKD, $n = 16$) after a two-step surgical 5/6 nephrectomy. During the experiment, animals were kept in conventional cages in vivarium conditions (humidity of 50%, 24 °C, and 12-h/12-h light/dark cycle) with unlimited access to sterilized tap water. The rats were fed a standard diet (Ssniff R/M-H) composed of 19% protein, 1% calcium, 0.70% phosphorus, 1000 IU of vitamin D3 per kg, and 5 mg of vitamin K as menadione per kg. The experiment's overall time course lasted 24 weeks in order to monitor the development and progression of CKD. Finally, blood samples and femurs were obtained from the anesthetized animals and were secured for further studies. In the presented experiment, we employed materials from our previous research, where we gave a detailed description of animals' characteristics, tissue collection, and applied procedures [44]. Briefly, markers of kidney function—serum urea and creatinine concentrations—were increased in CKD rats compared to CON (both $p < 0.001$). The animals with CKD had significantly

higher levels of PTH ($p < 0.01$), whereas 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] levels were comparable to healthy animals [44].

2.2. Measurement of Vitamin K Concentrations in Rat Serum

Vitamins K (i.e., phylloquinone (VK1), Menaquinone 4 (MK4), and Menaquinone 7 (MK7) were determined by the Laboratory of Perlan Technologies Polska, based in Gdynia, Poland. The following reagents and solvents from Merck KGaA were used: LC-MS grade water, LC-MS grade methanol, acetonitrile, hexane, bovine serum albumin (BSA), ammonium formate solution, formic acid, and standards of: vitamin K1 (phylloquinone), vitamins K2: MK4 (Menaquinone-4), and MK7 (Menaquinone-7) from Supelco Analytical Products(Merck Life Science Sp.z.o.o., Darmstadt, Germany), Deuterium-labelled internal standard of the vitamin K1-[d7] (phytonadione) were obtained from IsoSciences LLC (Ambler, PA, USA). Serum samples (100 µL) were spiked with Vitamin K1-[d7] ISTD, vortexed briefly, and acetonitrile was added to each tube, vortexed for 1 min, followed by hexane and again vortexed for 1 min. The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature. The final dry extract was dissolved in acetonitrile and transferred to an MS vial. Aliquots of 10 µL were automatically injected into the HPLC system.

The Agilent Technologies 1260 LC system(Agilent Technologies, Santa Clara, CA, USA). was used for vitamins K analysis, including an autosampler, binary pump, and thermostated column compartment with G6470A Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA, USA). The sample separation was carried out on a reversed phase column Zorbax SB-C8 RRHT, 3.0 × 50 mm, 1.8 µm, 600 bar (Agilent Technologies, Santa Clara, CA, USA). The column temperature was kept constant at 30 °C. A gradient elution system was used with a flow rate of 0.4 mL/min. The binary gradient system consisted of 0.1% formic acid and 5 mM ammonium formate in water mobile phase (eluent A) and methanol acidified with 0.1% formic acid (eluent B). Gradient elution was applied as follows: 0 min—70% A, 30% B; 2 min—10% A, 90% B; 3 min—0% A, 100% B; 15 min—0% A, 100% B; 20 min—70% A, 30% B. The AJS ESI ion source operated in positive ion mode with drying gas temperature of 330 °C and 8 L/min gas flow. The sheath gas temperature was set to 320 °C with a flow of 11 L/min. Nitrogen was used as a nebulizer gas (45 psi) and ultrahigh-purity nitrogen was used as collision gas. The capillary and nozzle voltages were 3000 V and 1000 V, respectively. Identification and quantification were based on MS/MS multiple reaction monitoring (MRM). An overview of the MRM transitions, collision energies, and retention time for the analytes is given in Table 1. All aspects of system operation and data acquisition were controlled using Agilent MassHunter Workstation Software (version B.10.00).

Table 1. LC-MS/MS parameters.

	Precursor ion (*m*/*z*)	Production (*m*/*z*)	Collision Energy (V)	Retention Time (min)
K1	451.4	187.2	26	6.02
K1-d7	458.4	194.3	26	6
MK4	445.3	187.3	18	5.42
MK7	649.5	187.2	38	7.35

K1—phylloquinone; K1-d7—deuterium-labelled internal standard of the vitamin K1; MK4—Menaquinone-4; MK7—Menaquinone-7.

2.3. Serum and Bone Levels of Vitamin K-Dependent Proteins

Undercarboxylated osteocalcin (Glu-OC), carboxylated osteocalcin (Gla-OC), and total osteocalcin were determined by ELISA kits: Rat Glu-Osteocalcin High Sensitive EIA, Rat Gla-Osteocalcin High Sensitive EIA (Takara Bio Inc., Shiga, Japan), and Rat Osteocalcin ELISA kit from Immutopics, Inc., San Clemente, CA, USA, respectively. Rat undercarboxylated Matrix Gla Protein (ucMGP) was quantified in serum by ELISA kit from

MyBioSource, Inc., San Diego, CA, USA, according to the manufacturer's recommendations. Trabecular and cortical concentrations of Gla-OC and Glu-OC were adjusted for protein concentration, and the ratio Gla-OC/Glu-OC was calculated.

2.4. Preparation of Bone Tissue Homogenates

The procedure of the preparation of 10% homogenates from bone tissue, using a high-performance homogenizer (Ultra-Turrax T25; IKA, Staufen, Germany) with a stainless-steel dispersing element (S25N-8G; IKA), has been described in detail previously [45]. Briefly, segments of bone tissue were taken from the femoral diaphysis and distal femoral epiphysis (from the cortical bone region and trabecular bone region, respectively). Collected materials were weighed, closely rinsed, and then homogenized in the cold potassium phosphate buffer (50 mM, pH = 7.4; POCh). The supernatant was obtained by the centrifugation of 10% homogenates for 10 min at 700× *g* at 4 °C and stored at −80 °C.

2.5. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from femoral tissue using Thermo Scientific GeneJET RNA Purification Kit (Thermo Scientific, Vilnius, Lithuania), and Quantitative RT-PCR was performed as previously described [46]. Briefly, using the Thermo Scientific NanoDrop 2000 spectrophotometer (Waltham, MA, USA), RNA was quantified, and its quality was confirmed. The RevertAid™ First Stand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to reverse-transcribe total RNA (1 µg). In order to perform quantitative real-time PCR, the SYBR Green Master Mix was used (EURx, Gdańsk, Poland), and relative quantification of gene expression was performed by the comparative CT method (ΔΔCT). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as a housekeeping gene to normalize expression level [46]. Primers were designed using Primer-BLAST software(Primer3 program). The primer sequences were (5′-3′ forward-reverse): VKORC1 (5′-TCCCGCGTCTTCTCCTCTC-3′; 5′-CCAACGTCCCCTCAAGCAAC-3′), GGCX (5′-GGATGCTGACTGGGTTGAGG-3′; 5′-GCTCCTCCGACAACACTAGC-3′) and UBIAD1 (5′-GCTGTGTGTGTGCTGCTTAC-5′; CCCAGTGCCACGTACTTGAA-3′).

2.6. Genes of Osteoblastogenesis

The expression of key genes involved in osteoblastogenesis, such as: Forkhead Box Transcription Factor 1 (FOXO1), Activating Transcription Factor 4 (ATF4), Runt-Related Transcription Factor 2 (RUNX2), Alkaline Phosphatase (ALP), and Bone Gamma-Carboxyglutamate Protein (BGLAP) were determined previously [46].

2.7. The Mineral Status of Femurs

Using the appropriate small animal software, bone densitometry scans were carried out on a Horizon QDR Series X-ray Bone Densitometer (Hologic Inc., Bedford, MA, USA). For all rats were conducted whole-femur measurements—bone mineral area, bone mineral density (BMD), and bone mineral content (BMC). Moreover, at the distal metaphysis (R1 region) and midshaft (R2 region), the subregional BMC and BMD of small uniform areas were quantified. The R1 region is constituted of mixed trabecular and cortical bone, whereas the R2 region represents cortical bone. The results of these densitometric measurements have been shown elsewhere [44].

2.8. Statistical Analysis

Shapiro–Wilk tests were performed to determine if continuous variables were normally distributed. Normally distributed data were expressed as mean ± SD. Non-Gaussian data were presented as median (25th to 75th percentiles). The two groups (CON and CKD) were compared by using an unpaired *t*-test with Welch correction or nonparametric Mann–Whitney Test. $p < 0.05$ was the accepted level of significance. The correlations between study variables were calculated using Spearman's rank correlation analysis. Computations were performed using Statistica ver.13 software (StatSoft, Tulsa, OK, USA), and the graphic

design presentation of the results was performed using GraphPad Prism 6.0 software (San Diego, USA).

3. Results

3.1. The Status of Endogenous Vitamin K in Rats with CKD

As has been shown in Figure 1, the levels of vitamin K were changed in chronic kidney disease. The concentration of VK1 in CKD was lower than in controls (Figure 1A). In contrast, we observed a considerable increase in MK7 (Figure 1B), especially in MK4 levels (Figure 1C) in uremic rats. The ratio of MK7/VK1 and MK4/VK1 indicates how efficiently phylloquinone is converted to menaquinone (VK2). In the present study, a significant rise in the above ratios was shown, particularly in MK4/VK1 ratio in CKD compared with the controls (Figure 1D,E).

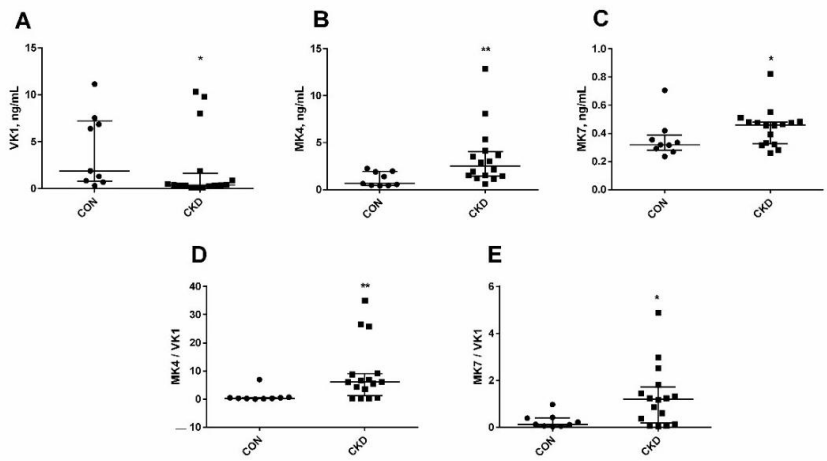


Figure 1. Vitamin K1 (**A**), MK4 (**B**), MK7 (**C**) concentrations, and the ratios of MK4/VK1 (**D**) and MK7/VK1 (**E**) in serum rats with chronic kidney disease (CKD) and healthy controls (CON) fed with a standard rodent diet. The lines correspond to the 25th and 75th percentiles and the median. * $p < 0.05$, ** $p < 0.01$ CON versus CKD rats; VK1—phylloquinone; MK4—Menaquinone 4; MK7—Menaquinone 7; the circles represent results in controls; the squares represent results in CKD.

3.2. Serum Levels of Vitamin K-Dependent Proteins in Rats with CKD, and the Impact of Kidney Function and PTH on Their Concentrations

The concentrations of total osteocalcin, its undercarboxylated form (Glu-OC), and uncarboxylated matrix Gla protein (ucMGP) are shown in Figure 2. These proteins reflect VK status and are used as markers of VK deficiency. A significant increase was observed in both total OC and Glu-OC in rats with CKD in comparison with healthy animals (Figure 2A,B). However, there was no difference in the ucMGP concentration between uremic rats and controls (Figure 2C).

Both total osteocalcin and its undercarboxylated form (Glu-OC) in serum were positively correlated with kidney function markers: creatinine and urea concentrations in CKD rats (Figure 3A,B,D,E). The inverse relationship was observed between total osteocalcin and creatinine clearance (Figure 3C), whereas circulating Glu-OC was related to PTH concentrations (Figure 3F).

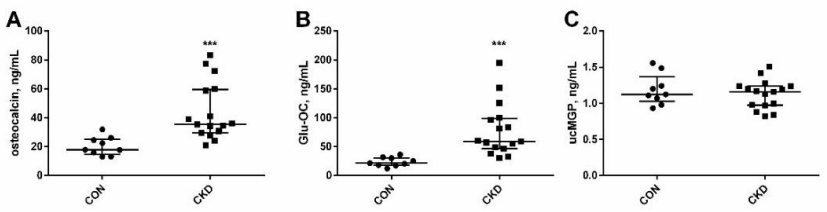


Figure 2. Serum levels of vitamin K-dependent proteins: osteocalcin (**A**), Glu-OC (**B**) and ucMGP (**C**) in rats with chronic kidney disease (CKD) and healthy controls (CON). The lines correspond to the 25th and 75th percentiles and the median. *** $p < 0.001$ CON versus CKD rats; Glu-OC—undercarboxylated osteocalcin; ucMGP—undercarboxylated Matrix Gla Protein.

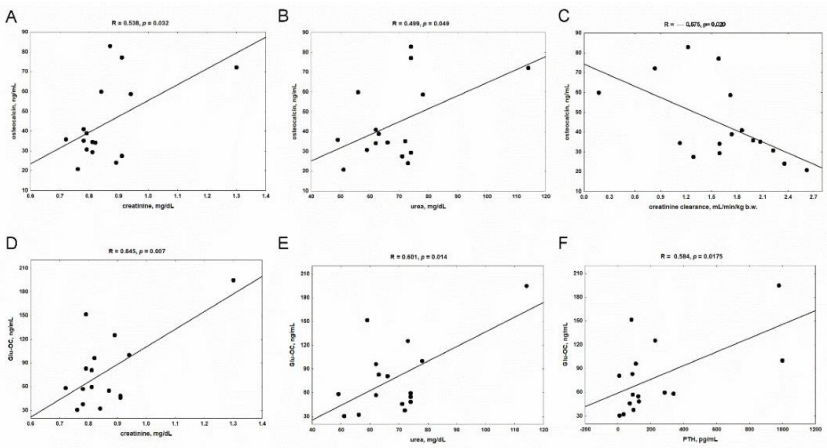


Figure 3. The associations between serum levels of osteocalcin and its undercarboxylated form (Glu-OC), and markers of kidney function: creatinine (**A**,**D**), urea (**B**,**E**), creatinine clearance (**C**) and parathyroid hormone (PTH) concentration (**F**) in rats with chronic kidney disease (CKD).

3.3. The Levels of Glu-OC, Gla-OC, and Gla-OC/Glu-OC Ratios in Femoral Bone Tissue of Rats with CKD, and Their Relations with Serum Glu-OC

In the current study, Gla-OC and Glu-OC concentrations and the Gla-OC/Glu-OC ratio were measured in trabecular (Figure 4A–C) and cortical (Figure 4D–F) bone tissue. Gla-OC levels in trabecular bone did not differ between the studied groups (Figure 4A), whereas Glu-OC level was considerably lower in CKD than in controls (Figure 4B). It is interesting to note that in CKD, the Gla-OC level in cortical bone tissue significantly increased (Figure 4D), while the Glu-OC concentration was reduced when compared to healthy animals (Figure 4E). Moreover, in both bone areas, the Gla-OC/Glu-OC ratios in uremic rats were considerably higher than in CON (Figure 4C,F).

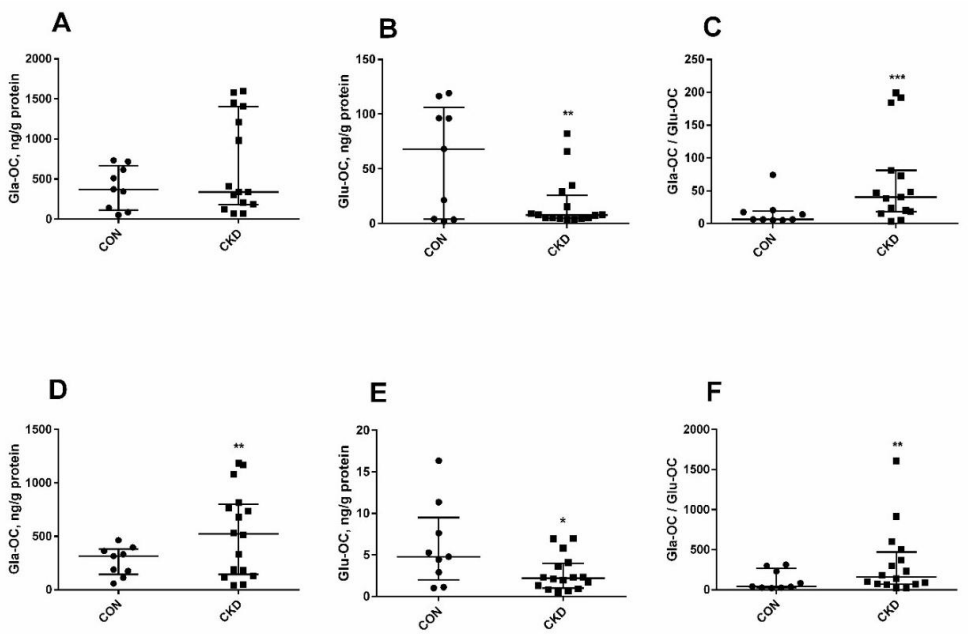


Figure 4. The levels of carboxylated osteocalcin (Gla-OC), undercarboxylated osteocalcin (Glu-OC) and their ratios (Gla-OC/Glu-OC) in trabecular (**A**–**C**) and cortical (**D**–**F**) femoral bone tissue. The lines correspond to the 25th and 75th percentiles and the median. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CON versus CKD rats; CKD—chronic kidney disease; CON—controls.

In CKD group, we observed a positive association between Gla-OC concentrations and Glu-OC/Gla-OC ratios in trabecular (Figure 5A) and in cortical (Figure 5B) bone regions. The weak correlation was also noticed between Glu-OC and Gla-OC levels in trabecular (R = 0.512, $p = 0.045$), but not in cortical bone tissue (R = 0.105, NS). Among the analyzed parameters, only the Gla-OC/Glu-OC ratio in the trabecular bone region was inversely correlated with the concentration of Glu-OC in the serum (Figure 5C).

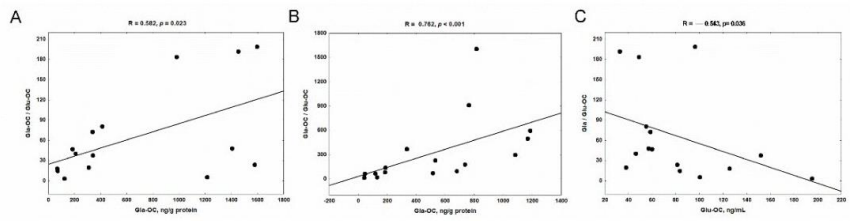
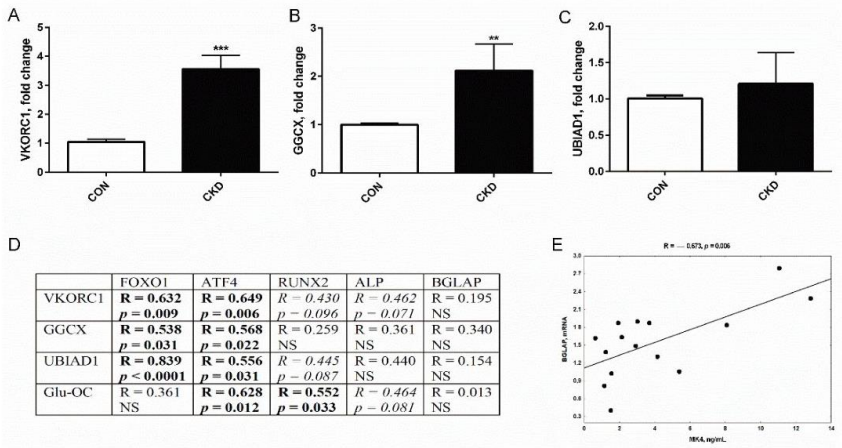


Figure 5. The associations between carboxylated osteocalcin (Gla-OC) and Gla-OC/Glu-OC ratio in trabecular (**A**) and cortical (**B**) bone tissue; the relations between trabecular Gla-OC/Glu-OC ratio and serum undercarboxylated osteocalcin (Glu-OC) levels; (**C**) in rats with chronic kidney disease (CKD).

3.4. The Expression of Genes Coding Vitamin K Cycle Enzymes in Femurs of Rats with CKD, and Their Associations with Genes Participating in Osteoblastogenesis

The expression of VKORC1 and GGCX gene was significantly higher in uremic rats compared to the control group (Figure 6A,B), whereas the increase in the UBIAD1 gene did not achieve a statistically significant level (Figure 6C).



	FOXO1	ATF4	RUNX2	ALP	BGLAP
VKORC1	**R = 0.632** **p = 0.009**	**R = 0.649** **p = 0.006**	*R = 0.430* *p = 0.096*	*R = 0.462* *p = 0.071*	R = 0.195 NS
GGCX	**R = 0.538** **p = 0.031**	**R = 0.568** **p = 0.022**	R = 0.259 NS	R = 0.361 NS	R = 0.340 NS
UBIAD1	**R = 0.839** **p < 0.0001**	**R = 0.556** **p = 0.031**	*R = 0.445* *p = 0.087*	R = 0.440 NS	R = 0.154 NS
Glu-OC	R = 0.361 NS	**R = 0.628** **p = 0.012**	**R = 0.552** **p = 0.033**	*R = 0.464* *p = 0.081*	R = 0.013 NS

Figure 6. The expression of genes of vitamin K cycle enzymes in femurs of rats with chronic kidney disease (CKD) (**A**–**C**), and their associations with the expression of genes of osteoblastogenesis and trabecular Glu-OC levels (**D**), as well as the relationship between serum MK4 level and BGLAP expression in bone of rats with CKD (**E**). ** $p < 0.01$, *** $p < 0.001$ CON versus CKD rats; CON—controls; VKORC1—vitamin K epoxide reductase complex subunit 1; GGCX—the gamma-glutamyl carboxylase; UBIAD1—UbiA prenyltransferase domain-containing protein 1; FOXO1—Forkhead box transcription factor 1; ATF4—activating transcription factor 4; RUNX2—Runt-related transcription factor 2; ALP—alkaline phosphatase; BGLAP—bone gamma-carboxyglutamate protein; Glu-OC—undercarboxylated osteocalcin; MK4—menaquinone 4; the bold text indicates statistically significant correlations; italic text indicates tendency to correlation.

Our previous research performed on the bones of rats with CKD established that the expression of FOXO1, ATF4, RUNX2, and ALP, which are the key genes responsible for the initial phases of osteoblast development, was significantly increased, whereas the expression of BGLAP, which is linked to the late stage of osteoblast differentiation, was only slightly elevated [46]. As has been shown in Figure 6D, the expression of the genes involving in VK cycle (especially VKORC1 and UBIAD1) was strongly and positively correlated with the expression of the genes of the early stages of osteoblast development. The tendency of these positive relationships was also seen between VK cycle genes expression and the markers of osteoblast differentiation, such as RUNX2 and ALP. Interestingly, we also observed the association between trabecular Glu-OC levels and the expression of genes reflecting the osteoblast differentiation (ATF4, RUNX2, ALP). Other interesting observations were the lack of the correlation between BGLAP expression and the genes belonging to VK cycle and trabecular Glu-OC levels (Figure 6D), as well as the strong positive association between BGLAP mRNA and circulating MK4 level (Figure 6E).

3.5. The Relationship between the Expression of VKORC1 and the Concentration of Gla-OC in Trabecular Bone Tissue and Serum Glu-OC

Next, we analyzed the relations between the expression of VK cycle enzymes and both forms of osteocalcin in femoral bone of rats with CKD. There was no correlation between GGCX and the levels of Gla-OC and Glu-OC. Surprisingly, we found a positive relationship between VKORC1 expression and Gla-OC concentration in trabecular bone tissue (Figure 7A), as well as a strong inverse relationship between VKORC1 expression and serum Glu-OC concentration (Figure 7B).

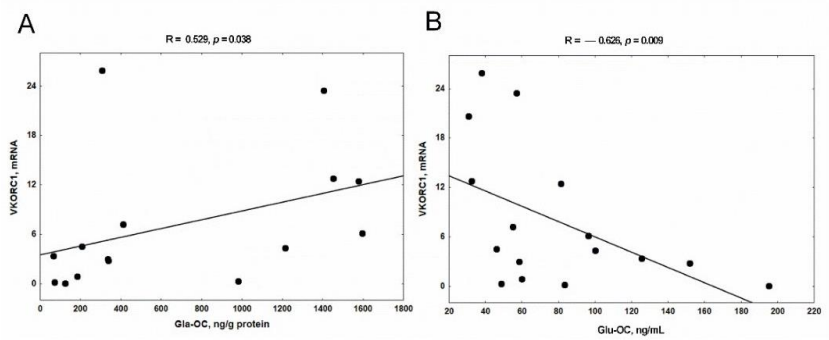


Figure 7. The association between bone VKORC1 expression and Gla-OC levels in trabecular bone (**A**), and VKORC1 expression and Glu-OC levels in serum of rats with CKD (**B**). MK4—menaquinone 4; BGLAP—bone gamma-carboxyglutamate protein; VKORC1—vitamin K epoxide reductase complex subunit 1; Gla-OC—carboxylated osteocalcin; Glu-OC—undercarboxylated osteocalcin; CKD—chronic kidney disease.

3.6. The Associations between Bone Glu-OC, Gla-OC, and Bone Mineral Status in Rats with CKD

Rats with CKD had significantly decreased densitometric parameters compared with controls in the whole-femur measurements, namely in the bone mineral area ($p < 0.05$), BMC, and BMD (both $p < 0.01$). These differences were particularly seen in the metaphyseal area (R1) of femurs, where the values of BMC and BMD were significantly decreased in rats with CKD compared to controls (both $p < 0.001$). In contrast, mineral status measured in the diaphyseal area (R2) was only slightly impaired—the values of BMC were lower in CKD than in controls ($p < 0.01$), whereas BMD did not differ between the studied groups (for details please see our previous study [44]).

In the present study, we analyzed the associations between both forms of osteocalcin and their ratios measured in trabecular and cortical bone tissue, with these densitometric parameters of femurs. At the level of the trabecular bone, we have demonstrated that only Gla-OC/Glu-OC ratio was strongly and inversely related to bone mineral area (Figure 8A) and BMC (Figure 8B) in uremic rats.

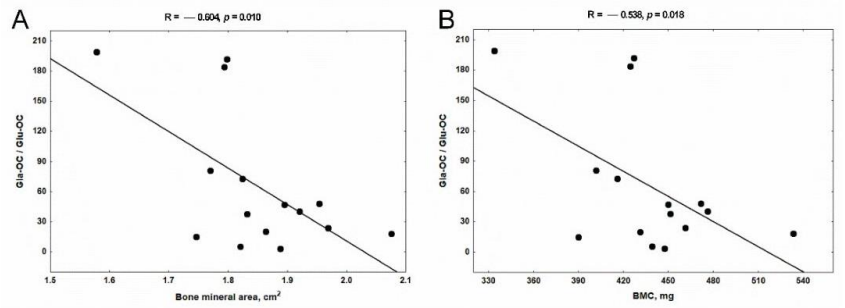


Figure 8. The associations between Gla-OC/Glu-OC ratio in trabecular bone tissue and the parameters of mineral status: bone mineral area (**A**) and BMC (**B**) of femoral bone of rats with chronic kidney disease (CKD). Gla-OC—carboxylated osteocalcin; Glu-OC—undercarboxylated osteocalcin; BMC—bone mineral content.

Surprisingly, at the level of the cortical bone, we observed that only Glu-OC values were positively associated with the whole-femur values of BMC (Figure 9A) and BMD (Figure 9D). These relations were particularly strong in the diaphyseal area (R2), which is

rich in cortical bone (Figure 9B,E), but they also were noticeable in the metaphyseal area (R1) of femurs (Figure 9C,F).

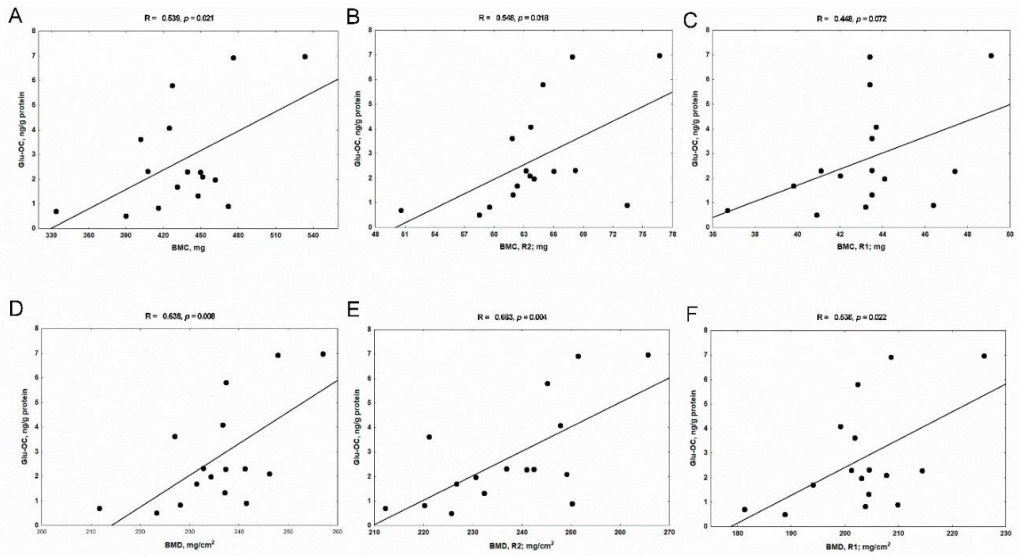


Figure 9. The associations between undercarboxylated osteocalcin (Glu-OC) levels in cortical bone tissue and whole BMC, BMD (**A**,**D**), diaphyseal BMC, BMD (**B**,**E**) and metaphyseal BMC, BMD (**C**,**F**) of femurs of rats with chronic kidney disease (CKD). BMC—bone mineral content; BMD—bone mineral density; R1—metaphyseal area; R2—diaphyseal area of femur.

4. Discussion

Although a high prevalence of subclinical vitamin K (VK) deficiency has been described in patients with CKD [18–22] and there are suggestions that this condition can impact bone quality [47,48], there are a lack of experimental data on this issue. To the best of our knowledge, this is the first comprehensive study in which we determined the endogenous VK metabolism, the bone levels of VK-dependent proteins (VKDPs), and the expression of VK cycle enzymes in femoral bone of rats with 5/6 nephrectomy. For determination of the impact of endogenous vitamins K on bone mineralization in this CKD model, the obtained results were juxtaposed with the parameters of the mineral status of femurs.

Among the K vitamins, the concentrations of phylloquinone (VK1), menaquinone 4 (MK4), and menaquinone 7 (MK7) were measured in rats' serum. Despite the fact that all rats have received the same standard diet supplemented with menadione, the VK status of uremic rats was altered, with a decrease in VK1 concentration and a significant increase in MK7, particularly in MK4 levels. VK1 is the major dietary form of VK, and the primary form found in circulation [49]. VK1 is endogenously converted to MK4 via a menadione intermediary [50–52]. Hirota et al. [52] were the first to show that the release of menadione from VK1 and its conversion to MK4 occurred in the intestine of rats, therefrom MK4 entered the blood circulation through the mesenteric lymphatic system. On the other hand, this same group proposed that menadione from intestine is delivered via the mesenteric lymphatics into the blood and then transported to peripheral tissues, where it is transformed into MK4 by UBIAD1. Details about MK7 distribution and metabolism in rats are still limited. Ikeda et al. [53] proposed that MK7 can also be a precursor of MK4 and may be converted to MK4 in the body. Regardless of how MK4 comes to be formed, it is believed that rodents consuming commercially available rodent chow, fortified with menadione (a precursor for MK4), have sufficient vitamin K for all tissue functions [54].

VK1 deficiency has been observed in CKD patients and animals with experimental CKD [24,48,55], and in this respect our results are in line with these earlier observations. However, the ratios of MK7/VK1 and MK4/VK1, which provide an index of the efficiency of conversion of VK1 to vitamins K2 [56], were increased in uremic animals compared to controls. This suggests that lower VK1 levels observed in CKD rats could be a result of greater tissue utilization of this vitamin, possibly reflecting an increased physiological demand, especially for MK4, during uremia. The similar phenomenon was previously described by Ferland et al. [57] in rats fed different VK1 diets during aging.

The measurement of serum levels of VKDPs, like total osteocalcin and its undercarboxylated form, Glu-OC, are sensitive indirect tests in detecting subclinical VK deficiency [16]. Both total osteocalcin and Glu-OC were markedly increased in the serum of CKD rats in this experiment, and similar findings have been consistently observed by others in human and experimental CKD [19,24,48]. It is generally believed that the high percentage of Glu-OC reflects insufficient VK intake, low VK status, and impaired bone mineralization [28–30]. In the present study, we observed the direct associations between kidney function markers and total osteocalcin, indicating that this parameter can be accumulated in the blood of CKD animals independently of its release from bone. This observation is in accordance with the study of Price et al. [58], who demonstrated that nephrectomy can block the serum clearance of osteocalcin. In turn, renal insufficiency combined with an increase in PTH levels seems to affect serum Glu-OC concentrations in our rats with CKD. It is possible that PTH may enhance the synthesis of Glu-OC by the stimulation of osteoblastogenesis, as has been reported previously [59–62]. Thus, the increased serum Glu-OC in the condition of uremia and hyperparathyroidism, observed in this study, does not necessarily reflect VK deficiency. A good alternative to determine VK status is measurement serum ucMGP, which is a recognized marker of VK status in nephrectomized rats [63]. In contrast to Glu-OC, ucMGP concentration did not differ between the studied groups in this study. All these data indicate that VK status was sufficient in CKD animals.

Osteocalcin is synthesized in osteoblasts, and its three-glutamate residues (Glu-OC) are posttranslationally converted into γ-carboxyglutamate (Gla-OC) by VK, which is a cofactor of GGCX [64,65]. Gla-OC strongly binds to hydroxyapatite in bone, whereas Glu-OC, which does not have this ability, is released into the blood [58]. We measured the Gla-OC, Glu-OC, and Gla-OC/Glu-OC ratio, being an index of the availability of VK at the bone level, in trabecular (Figure 4A–C) and cortical (Figure 4D–F) bone tissue. In both bone regions the Gla-OC/Glu-OC ratios were increased, and Glu-OC concentrations were reduced in CKD rats compared to controls. These results indicate the accelerated Glu-OC to Gla-OC transformation in bone of CKD rats, which increased Gla-OC while consuming Glu-OC [66], reflecting sufficient or even elevated levels of VK in their bones. However, in trabecular bone of uremic rats, Gla-OC level was comparable with that of controls, and the only weak relationship existed between Gla-OC and Gla-OC/Glu-OC ratio. In contrast, the Gla-OC level rose significantly, and it was strongly correlated with Gla-OC/Glu-OC ratio in the cortical bone region of these animals. These data support our new observation: that the generation of Gla-OC in trabecular bone, in which a more rapid bone turnover rate occurs, was not as effective as in cortical bone. Interestingly, serum Glu-OC levels were inversely related to trabecular Gla-OC/Glu-OC ratio, indicating that the Gla-OC generation process in trabecular bone region is an important factor influencing the serum Glu-OC level.

Vitamin K undergoes a cycle of oxidation followed by reduction through the "vitamin K cycle", which includes VKORC1, GGCX, and UBIAD1 enzymes [65,67]. During γ-carboxylation of Glu-OC, VK is converted into VK epoxide by GGCX [65], and VK epoxide can then be reduced by the enzyme VKORC1 to VK-hydroquinone, which can be used once again for the carboxylation reaction [65,67]. The UBIAD1 gene encodes an MK4 biosynthetic enzyme, which is responsible for VK1 to MK4 bioconversion in extrahepatic tissues [68].

The expressions of VK cycle enzymes were previously determined in different tissues of rats with adenine-induced CKD, and in general, the decreased recycling and utilization of VK was found [24,69,70]. To the best of our knowledge, this is the first study that measured the expression of genes involved in VK recycling at the bone level of uremic rats. There was a significant increase in the expression of VKORC1 and GGCX in the femurs of CKD rats compared to controls, whereas there was no difference in the expression of UBIAD1. These results indicated that VK1 to MK4 bioconversion, as well as MK4 recycling and utilization, can occur in the bones of CKD rats even more effectively than in healthy animals. Surprisingly, regarding Gla-OC levels in the studied bone regions, it seems that the enzymes of VK cycle should act better in a cortical, less metabolically active part of the bone than in the trabecular, which is characterized by more active bone turnover.

Previously, we observed the alterations in osteoblastogenesis process in bone of our rats with CKD, with the significantly increased expression of genes involved in the early stages of osteoblastogenesis, like FOXO1, ATF4, RUNX2, and ALP, whereas the expression of marker of the late stage of osteoblast differentiation, BGLAP, was only slightly increased. This phenomenon resulted in impaired osteoblast maturation and decreased bone mineral status [46]. Our findings were in line with clinical observation of Pereira et al. [71], who also showed that the increase of osteoblastic markers occurred simultaneously with low osteocytic gene expression in cells obtained from dialyzed patients. In the present study, we analyzed the associations between the expression of VK cycle enzymes and mentioned the above genes involved in the individual stages of osteoblastogenesis. As has been presented in Figure 6D, the expression of enzymes of VK cycles occurred in the early stages of osteoblastogenesis. What is more, Glu-OC levels in trabecular bone tissue were also associated with the early markers of osteoblast proliferation/differentiation, but not with BGLAP. Consistent with previous reports, PTH may accelerate differentiation of osteoprogenitor cells into osteoblasts [72]. Because PTH level was increased in serum of our CKD rats [44], and previously we showed that endogenous PTH may influence the expression of genes engaged in the early stage of osteoblastogenesis in young uremic rats [73], we speculate that this hormone could also contribute to the increased expression of VK cycle enzymes in this study. However, BGLAP expression increases significantly at the late stage of osteoblast differentiation [74], and this fact could explain the lack of association between BGLAP and the expression of VK cycle enzymes and bone Glu-OC generation. Interestingly, we noticed that circulating MK4 was positively associated with BGLAP expression in bone (Figure 6E). This is in accordance with the findings of Weng et al. [66], who evaluated the effect of MK4 on osteocalcin expression in calvarial bone defect repair in osteopenic rats. These and the above-presented results (Figure 6) suggest that in CKD conditions, the VK recycling, expression of BGLAP, and generation of different forms of osteocalcin may occur in the distinct stages of osteoblastogenesis under the control of endogenous PTH and MK4. Thus, the lack of difference in trabecular Gla-OC levels observed in the present study could be a result of the individual effects of PTH, which stimulates the synthesis of osteocalcin [60]; and MK4, which increased Gla-OC while consuming Glu-OC. In agreement with this hypothesis, the combined MK4 and PTH_{1-34} treatment increased serum Gla-OC/Glu-OC ratio in osteopenic rats, however the highest Gla-OC/Glu-OC ratio was observed not in the combined group, but in the MK4-treated group [66].

Another interesting finding of the present study is the association between VKORC1 mRNA levels and Gla-OC concentration in the trabecular bone region of uremic rats. In contrast, such relations were not noticed between GGCX and both forms of osteocalcin in the bone regions analyzed by us. The posttranslational modification of Glu-OC to Gla-OC inside osteoblasts generally involves two enzymes, GGCX and VKORC1, which together constitute the VK cycle. GGCX requires a reduced form of VK as an obligate cofactor, and γ-carboxylation is dependent on the reduction of VK epoxide by a VKORC1 [65]. Ferron et al. [75], using cell-specific gene inactivation models, demonstrated that VKORC1 is required for γ-carboxylation of osteocalcin in osteoblasts. They also showed that animals

lacking GGCX or VKORC1 presented almost no osteocalcin in their bones, providing the first in vivo evidence that the generation of Gla-OC is necessary for its accumulation in the mineral component of bone. This is consistent with the inverse relationship between bone VKORC1 gene expression and serum Glu-OC levels observed in our uremic rats (Figure 7B), confirming the significance of VKORC1 in the process of Gla-OC formation in conditions of CKD.

The second aim of the present study was to establish the potential consequence of VK-dependent mechanisms for the bone mineral status of uremic rats. Previously, we showed that CKD development resulted in significantly decreased densitometric parameters in our uremic animals compared with controls in whole-femur measurements, particularly in the metaphyseal area (R1). In contrast, mineral status measured in the diaphyseal area (R2) was damaged to a lesser extent [44]. In the present study, we juxtaposed the parameters of bone mineral status obtained in this previous study [44] with the concentrations of VK-dependent proteins: Gla-OC and Glu-OC in the corresponding regions of the femur. Unexpectedly, the Gla-OC/Glu-OC ratio in trabecular bone tissue was inversely associated with the mineral status of femurs (Figure 8). In turn, the positive relations were observed only between Glu-OC levels in cortical bone tissue and the parameters of bone mineral status, which were strongest in the diaphyseal area (R2) of femurs (Figure 9). Although Gla-OC, due to its specific interaction with hydroxyapatite, is thought to be associated with bone mineralization [76–78], there is also evidence showing that Gla-OC is not critical for bone mineralization or even that it functions as a "negative regulator of skeleton" [79]. Genetic osteocalcin depletion does not change the mineral content of bone matrix in mice [80]. Complete loss of osteocalcin resulted in bones with significantly increased trabecular thickness, density, and volume, whereas cortical bone volume and density were not increased in osteocalcin-null male rats [79]. The treatment with warfarin, despite significantly lowering Gla-OC levels, did not reduce the mineral content of fracture calluses [59], and is not directly linked with BMD of rat bone [81]. Amizuka et al. [82] demonstrated that osteocalcin is not related to mineral deposition but does participate in the growth and maturation of hydroxyapatite. A recent study by Simon et al. [83] demonstrates that osteocalcin takes on the functions of Ca-ion transport and suppression of hydroxyapatite expansion. An interesting study by Uchida et al. [84] revealed that the commensal microbiota prevents excessive bone mineralization by stimulating osteocalcin expression in osteoblasts, enhancing both osteoblast and osteoclast activity.

Assuming that Gla-OC is the marker of mature osteoblast [84], the relations observed in this study between bone mineral status and Glu-OC to Gla-OC transformation are compatible with the results of these teams, which demonstrated that Gla-OC may act as an inhibitor of bone mineralization [79–84]. We believe that in the trabecular bone region, PTH-dependent acceleration of osteoblastogenesis resulted in the generation of immature osteoblasts with insufficient Gla-OC production, which may lead to decreased bone mineral status [46]. In cortical bone, where Gla-OC level was approximately 1.5 times that in trabecular bone, the osteoblasts should be more mature. However, the mineral status in this bone region was directly associated with Glu-OC, representing the part of osteocalcin, which was not transformed to Gla-OC. The above results indicate an unfavorable role of Gla-OC in the mineralization of long bones in CKD conditions.

Some limitations should be considered in the interpretation our results. Firstly, the cross-sectional design of this study does not determine whether a causal relationship exists between VK metabolism in bone and the parameters of bone mineral status. Secondly, we cannot exclude the possibility that the observed associations could be partially attributed to other factors not considered in this study that may affect osteoblast proliferation/differentiation and bone mineral status, such as the metabolites of tryptophan [44,85]. Thirdly, gonadectomy was not performed in this study, so contributions to mineral and bone metabolism of sex hormones, especially androgen in the rats, were not examined.

A major strength of our study is the measurement of circulating vitamins K together with the expression of VK recycling enzyme in bone, and Glu-OC, Gla-OC directly in

the appropriate bone tissue, as the determination of these compounds in blood may not accurately represent the bone microenvironment [81]. Moreover, the studied groups were homogeneous with regard to age, sex, diet, and the absence of medication.

5. Conclusions

This study presents a comprehensive assessment of the metabolism of endogenous vitamin K, VK recycling in bone, VKDPs at serum and bone levels, and their impact on bone mineral status of rats with CKD. The obtained results indicate that the reduced level of VK1 observed in rats with CKD may be caused by the accelerated conversion of this vitamin to the form of menaquinones. The measurement of serum osteocalcin and Glu-OC, commonly used as the indicators of VK deficiency, seems to be ineffective in CKD conditions, especially in the presence of hyperparathyroidism. For the first time, we showed that bone tissue possesses a set of enzymes that allows for the conversion of VK1 to the form of menaquinone, as well as to recycling of VK. However, in the course of CKD with hyperparathyroidism, despite the appropriate conditions for the formation of active forms of VK, the intensified process of osteoblastogenesis causes the generation of immature osteoblasts with impaired mineralization capacity. Of particular clinical significance seems to be the fact that Gla-OC formed at the bone level turned out to be inversely correlated with bone mineral status in this model. This sheds new light on the metabolism of endogenous VK and its importance in the process of bone mineralization in CKD, particularly in patients with hyperparathyroidism.

Author Contributions: Conceptualization—M.Z., D.P. and K.P.; participation in animal experiments and data collection—B.S. and K.C.; statistical analysis—D.P. and K.P.; writing—original draft preparation, M.Z. and K.P.; visualization, B.S.; founding acquisition—D.P.; writing—review & editing, M.Z. and K.P. All authors have discussed the results. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the EU Directive 2010/63/EU for animal experiments and was approved by the local Bioethics Committee of Medical University of Bialystok No 29/2013.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is stored by corresponding author and may be share upon request.

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Abbreviations

1,25 $(OH)_2D$	1,2-dihydroxyvitamin D
ALP	alkaline phosphatase
ATF4	activating transcription factor 4
BGLAP	bone gamma carboxyglutamate protein; osteocalcin
BMC	bone mineral content
BMD	bone mineral density
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease–mineral bone disorders
CON	control group

dp-ucMGP	desphospho-uncarboxylated matrix Gla protein
FOXO1	forkhead box transcription factor 1
GGCX	γ-glutamyl carboxylase
Gla	gamma carboxyglutamic acid
Gla-OC	carboxylated osteocalcin
Glu	glutamic acid
Glu-OC	undercarboxylated osteocalcin
HD	hemodialysis
MGP	matrix gla protein
MKs	menaquinones
MK4	menaquinone 4
MK7	menaquinone 7
OC	osteocalcin
PTH	parathyroid hormone
R1	metaphyseal area of femur
R2	diaphyseal area of femur
RUNX2	Runt-related transcription factor 2
UBIAD1	UbiA prenyltransferase domain-containing 1 protein
ucMGP	uncarboxylated matrix gla protein
ucOC	uncarboxylated osteocalcin
VK	vitamin K
VK1	vitamin K1; phylloquinone
VK2	vitamin K2; menaquinones
VKDPs	vitamin K-dependent proteins
VKORC1	vitamin K epoxide reductase complex subunit 1

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13. Dorobek naukowy

Wykaz publikacji stanowiących cykl prac włączonych do rozprawy doktorskiej:

1) **Ziemińska, M**.; Sieklucka, B.; Pawlak, K. Vitamin K and D Supplementation and Bone Health in Chronic Kidney Disease–Apart or Together? *Nutrients*. 13 (**2021**) 809. https://doi.org/10.3390/nu13030809
   IF = **6.706**; MNiSW = **140**.

2) **Ziemińska, M**.; Pawlak, D.; Sieklucka, B.; Chilkiewicz, K.; Pawlak, K. Vitamin K-Dependent Carboxylation of Osteocalcin in Bone–Ally or Adversary of Bone Mineral Status in Rats with Experimental Chronic Kidney Disease? *Nutrients*. 14 (**2022**) 4082. https://doi.org/10.3390/nu14194082
   IF = **6.706**; MNiSW = **140**.

Wykaz publikacji niewłączonych do rozprawy doktorskiej:

1) Mor, A.; Pawlak, K.; Kałaska, B.; Domaniewski, T.; Sieklucka, B.; **Ziemińska, M.;** Cylwik, B.; Pawlak, D. Modulation of the Paracrine Kynurenic System in Bone as a New Regulator of Osteoblastogenesis and Bone Mineral Status in an Animal Model of Chronic Kidney Disease Treated with LP533401. *International journal of molecular sciences.* 21 (**2020**) 5979.
   https://doi.org/10.3390/ijms21175979
   IF = **5.924**; MNiSW = **140**.

Wykaz doniesień zjazdowych/konferencji naukowych:

1) **Ziemińska, M.;** Sieklucka, B.; Pawlak, K. Osteokalcyna a właściwości biomechaniczne i geometryczne kości udowej w eksperymentalnej niewydolności nerek u szczura. VII Ogólnopolskie Sympozjum Biomedyczne ESKULAP, on-line, 28-29.11.2020.

2) Sieklucka, B.; Domaniewski, T.; **Ziemińska, M**.; Gałażyn-Sidorczuk, M.; Pawlak, A.; Pawlak, D.; Pawlak, K. Correlations between OPG/RANKL/RANK axis, vitamin D status, PTH and vascular calcification in an adenine-induced model of chronic kidney disease. 57th ERA-EDTA Congress, on-line, 06-09.06.2020.

3) Pawlak, D.; Mor, A.; Kałaska, B.; Domaniewski, T.; Sieklucka, B.; Pawlak, A.; **Ziemińska, M**.; Pawlak, K. The activation of kynurenic system in bone tissue as a new regulator of osteoblastogenesis in rats with experimental chronic kidney disease during LP533401 therapy. 57th ERA-EDTA Congress, on-line, 06-09.06.2020.

4) Pawlak, K.; Domaniewski, T.; Sieklucka, B.; Pawlak, A.; **Ziemińska, M**.; Pawlak D. The impact of endogenous PTH/PTH1R/ATF4 axis on trabecular and cortical bone remodeling and bone growth of young rats with experimental chronic kidney diseases. 57th ERA-EDTA Congress, on-line, 06-09.06.2020.

14. Oświadczenie autora rozprawy doktorskiej

Informacja o charakterze udziału współautorów w publikacjach wraz z szacunkowym określeniem procentowego wkładu:

1. „Vitamin K and D supplementation and bone health in chronic kidney disease - apart or together?" autorów: Marty Ziemińskiej, Beaty Siekluckiej i Krystyny Pawlak opublikowanej w *Nutrients*, 2021; 13(3): 809.

Imię i nazwisko współautora	Charakter udziału w przygotowaniu publikacji	Procentowy wkład
doktorant – mgr Marta Ziemińska	Współtworzenie koncepcji pracy, zebranie i przegląd literatury, analiza i interpretacja zebranych danych literaturowych, pisanie manuskryptu	70%
prof. dr hab. Krystyna Pawlak	Opracowanie koncepcji pracy, nadzór merytoryczny i korekta manuskryptu	15%
dr n. med. Beata Sieklucka	Współtworzenie koncepcji pracy, opracowanie części manuskryptu, przygotowanie tabel i rycin	15%

2. „Vitamin K-dependent carboxylation of osteocalcin in bone - ally or adversary of bone mineral status in rats with experimental chronic kidney disease?" autorów: Marty Ziemińskiej, Dariusza Pawlaka, Beaty Siekluckiej, Katarzyny Chilkiewicz i Krystyny Pawlak opublikowanej w *Nutrients*, 2022; 14(19): 4082.

Imię i nazwisko współautora	Charakter udziału w przygotowaniu publikacji	Procentowy wkład
doktorant – mgr Marta Ziemińska	Współtworzenie koncepcji pracy, zebranie i przegląd literatury, wykonywanie części badań, analiza i interpretacja wyników, opracowanie manuskryptu	55%
prof. dr hab. Krystyna Pawlak	Opracowanie koncepcji pracy, nadzór merytoryczny i korekta manuskryptu	15%
dr n. med. Beata Sieklucka	Współtworzenie koncepcji pracy, współudział w wykonaniu badań, wykonanie rycin	15%
prof. dr hab. Dariusz Pawlak	Współtworzenie koncepcji pracy, ocena merytoryczna	10%
Mgr Katarzyna Chilkiewicz	Współuczestnictwo w wykonywaniu badań	5%

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższych publikacji w pracy doktorskiej mgr Marty Ziemińskiej.

Ziemińska Marta
.....
Podpis

15. Oświadczenia współautorów

Białystok, 17 kwietnia 2023 r.
miejscowość, data

mgr Marta Ziemińska
imię i nazwisko współautora

Zakład Farmakoterapii Monitorowanej
nazwa jednostki

Uniwersytet Medyczny w Białymstoku
ul. Jana Kilińskiego 1
15-089 Białystok

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. „Vitamin K and D Supplementation and Bone Health in Chronic Kidney Disease — Apart or Together?" autorów: Marty Ziemińskiej, Beaty Siekluckiej i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **70%** polegał na współtworzeniu koncepcji pracy, zebraniu i przeglądzie literatury, analizie i interpretacji zebranych danych literaturowych oraz napisaniu manuskryptu do publikacji.

2. „Vitamin K-dependent carboxylation of osteocalcin in bone - ally or adversary of bone mineral status in rats with experimental chronic kidney disease?" autorów: Marty Ziemińskiej, Dariusza Pawlaka, Beaty Siekluckiej, Katarzyny Chilkiewicz i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **55%** polegał na współtworzeniu koncepcji pracy, zebraniu i przeglądzie literatury, wykonywaniu części badań, analizie i interpretacji wyników oraz opracowaniu manuskryptu do publikacji.

Ziemińska Marta
Podpis

Białystok, 17 kwietnia 2023 r.
miejscowość, data

prof. dr hab. Krystyna Pawlak
imię i nazwisko współautora

Zakład Farmakoterapii Monitorowanej
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2. „Vitamin K-dependent carboxylation of osteocalcin in bone - ally or adversary of bone mineral status in rats with experimental chronic kidney disease?" autorów: Marty Ziemińskiej, Dariusza Pawlaka, Beaty Siekluckiej, Katarzyny Chilkiewicz i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **15%** polegał na opracowaniu koncepcji pracy, nadzorze merytorycznym i korekcie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie przez Panią mgr Martę Ziemińską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Krystyna Pawlak
Podpis

Białystok, 17 kwietnia 2023 r.
miejscowość, data

dr n. med. Beata Sieklucka
imię i nazwisko współautora

Zakład Farmakodynamiki
nazwa jednostki

Uniwersytet Medyczny w Białymstoku
ul. Jana Kilińskiego 1
15-089 Białystok

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. „Vitamin K and D Supplementation and Bone Health in Chronic Kidney Disease — Apart or Together?" autorów: Marty Ziemińskiej, Beaty Siekluckiej i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **15%** polegał na współtworzeniu koncepcji pracy, opracowaniu części manuskryptu oraz przygotowaniu tabel i rycin.

2. „Vitamin K-dependent carboxylation of osteocalcin in bone - ally or adversary of bone mineral status in rats with experimental chronic kidney disease?" autorów: Marty Ziemińskiej, Dariusza Pawlaka, Beaty Siekluckiej, Katarzyny Chilkiewicz i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **15%** polegał na współtworzeniu koncepcji pracy, współudziale w wykonywaniu badań oraz wykonaniu rycin.

Jednocześnie wyrażam zgodę na wykorzystanie przez Panią mgr Martę Ziemińską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Beata Sieklucka
Podpis

Białystok, 17 kwietnia 2023 r.
miejscowość, data

prof. dr hab. Dariusz Pawlak
imię i nazwisko współautora

Zakład Farmakodynamiki
nazwa jednostki

Uniwersytet Medyczny w Białymstoku
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15-089 Białystok

Oświadczenie

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„Vitamin K-dependent carboxylation of osteocalcin in bone - ally or adversary of bone mineral status in rats with experimental chronic kidney disease?" autorów autorów: Marty Ziemińskiej, Dariusza Pawlaka, Beaty Siekluckiej, Katarzyny Chilkiewicz i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **10%** polegał na współtworzeniu koncepcji pracy oraz ocenie merytorycznej manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie przez Panią mgr Martę Ziemińską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

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Podpis

Białystok, 17 kwietnia 2023 r.
miejscowość, data

mgr Katarzyna Chilkiewicz
imię i nazwisko współautora

Zakład Farmakoterapii Monitorowanej
nazwa jednostki

Uniwersytet Medyczny w Białymstoku
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15-089 Białystok

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

„Vitamin K-dependent carboxylation of osteocalcin in bone - ally or adversary of bone mineral status in rats with experimental chronic kidney disease?" autorów: Marty Ziemińskiej, Dariusza Pawlaka, Beaty Siekluckiej, Katarzyny Chilkiewicz i Krystyny Pawlak opublikowanej w *Nutrients*, wchodzącej w skład rozprawy doktorskiej „Rola endogennej witaminy K w procesie przebudowy kości szczurów z eksperymentalną przewlekłą chorobą nerek", wynoszący **5%** polegał na współuczestnictwie w wykonywaniu badań.

Jednocześnie wyrażam zgodę na wykorzystanie przez Panią mgr Martę Ziemińską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Katarzyna Chilkiewicz
Podpis