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Rozprawa doktorska

Analiza Czynników Modulujących Efekty Suplementacji Witaminą D: Perspektywa Literaturowa, Badania Transkryptomiczne i Epigenomiczne.

mgr inż. Alicja Wierzbicka

Praca wykonana pod kierunkiem
dr hab. Marii Oczkowicz prof. IZ
oraz Promotor pomocniczej
dr inż. Anny Koseniuk

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Spis treści

1. Streszczenie

1.1. Wersja polskojęzyczna

Witamina D_3 jest związkiem syntetyzowanym w naskórku zwierząt pod wpływem promieniowania słonecznego oraz ciepła. w wyniku nieenzymatycznej reakcji prowitamina D_3 (7-dehydrocholesterol) przekształca się w cholekalcyferol. Ograniczenia kontaktu skóry ze słońcem, powodowane współczesnym stylem życia i praktykami stosowanymi w hodowli zwierząt, prowadzi do niedoboru cholekalcyferolu i stanowi istotne zagrożenie dla zdrowia.

Ze względu na skalę problemu, witamina D stanowi obecnie jeden z najczęściej rekomendowanych suplementów diety. Mimo to, zalecenia dotyczące postaci i dawkowania tego środka podlegają licznym kontrowersjom. Przykład stanowić mogą wytyczne Uni Europiejskiej dotyczące żywienia świń. Rekomendacje te nie wskazują, bowiem minimalnej dawki witaminy D, podczas gdy maksymalna dzienna dawka wynosi 50 µg/kg paszy (2000 IU) niezależnie od tego, czy jest podawana w postaci cholekalcyferolu czy kalcydiolu. Tymczasem, wyniki badań sugerują, że efekt suplementacji tych metabolitów jest różny. Co więcej, obowiązujące w Europie zalecenia nie specyfikują dawek dla poszczególnych grup technologicznych. Jednakże liczne badania wskazują, że zapotrzebowanie na witaminę D związane jest ze stanem fizjologicznym zwierząt, a co więcej również z płcią.

Niniejsza praca doktorska obrała za cel analizę kilku niewyjaśnionych dotychczas kwestii związanych z suplementacją witaminą D, w tym:

→ Czy istnieją różnice w stężeniu i działaniu witaminy D u samic i samców oraz jakie są potencjalne przyczyny ich występowania?

→ Czy długotrwała suplementacja zwiększoną dawką cholekalcyferolu i użycie kalcydiolu w diecie powoduje zmiany ekspresji mRNA w tkance mięśniowej świń?

→ Czy długotrwała suplementacja zwiększoną dawką cholekalcyferolu i użycie kalcydiolu w diecie powoduje zmiany ekspresji miRNA w tkance płuc świń?

→ Jaki wpływ na poziomy metylacji oraz ekspresję mRNA tkanki płuc świń wywiera długotrwała suplementacja zwiększoną dawką cholekalcyferolu?

Na podstawie przeglądu literatury, ustalono, że istnieje szereg czynników mogących powodować międzypłciowe różnice w koncentracji i działaniu witaminy D. z kolei za pomocą badań z zakresu nutrigenomiki, wykazano, że tkanka mięśniowa, w przeciwieństwie do tkanki płuc, nie stanowi bezpośredniego celu dla

cholekalcyferolu i kalcydiolu. Stwierdzono również, że zwiększenie dawki cholekalcyferolu i zastosowanie kalcydiolu w żywieniu świń wpływa istotnie na profil miRNA tkanki płuc, a zwiększone spożycie cholekalcyferolu wpływa na metylacje i ekspresję mRNA w tej tkance.

1.2. Wersja anglojęzyczna

Vitamin D_3 is synthesised in the epidermis of animals under the influence of sunlight and heat. Provitamin D_3 (7-dehydrocholesterol) is converted into cholecalciferol in a non-enzymatic reaction. The limitation of skin exposure to the sun caused mainly by modern lifestyle and animal husbandry systems leads to a health-threatening cholecalciferol deficiency.

Due to the scale of the problem, vitamin D is now one of the most widely recommended dietary supplements. However, there is considerable controversy about the form and dosage of this supplement. The European Union guidelines for pigs do not specify a minimum dose of vitamin D, while the maximum daily dose is 50 µg/kg of feed (2000 IU), supplemented either as cholecalciferol or as calcidiol. Nevertheless, research suggests that the effects of the intake of these respective metabolites are different. In addition, current European recommendations do not specify doses for particular technology groups. Meanwhile, numerous studies show that the need for vitamin D is related to the physiological state of the animal, as well as to sex.

This PhD thesis aims to address several questions regarding vitamin D supplementation, including:

→ Are there sex differences in vitamin D concentration and effect?

→ Does long-term supplementation with an increased dose of cholecalciferol and the use of calcidiol in the diet cause changes in mRNA expression in porcine muscle tissue?

→ Does long-term supplementation with an increased dose of cholecalciferol and the use of calcidiol in the diet cause changes in miRNA expression in porcine lung tissue?

→ What is the effect of long-term supplementation with an increased dose of cholecalciferol on methylation levels and mRNA expression in porcine lung tissue?

Based on the literature review, there are factors that may cause intersex differences in vitamin D levels and effects. On the other hand, the results of the nutrigenomic study showed that muscle tissue, unlike lung tissue, is not a direct target for cholecalciferol and calcidiol. Furthermore, increased intake of cholecalciferol and the use of calcidiol in the pig diet appear to significantly affect the miRNA profile of lung tissue, and increased intake of cholecalciferol on the methylation and mRNA of this tissue.

2. Wstęp

Witamina D (kalcyferol) jest obecnie jednym z najczęściej rekomendowanych suplementów diety (Pludowski i wsp., 2022). Witamina ta może być pozyskiwana z produktów żywnościowych, takich jak tłuste ryby, jaja, nabiał i grzyby. Niemniej jednak, głównym, naturalnym regulatorem poziomu witaminy D w organizmie większości ssaków jest ekspozycja skóry na promieniowanie słoneczne.

Do niedawna działanie witaminy D łączone było przede wszystkim z regulacją równowagi wapniowo-fosforanowej organizmu. Jednak witamina D wykazuje znacznie szersze spektrum działania. Uważa się, że witamina ta może kontrolować pracę nie tylko układu odpornościowego, ale również sercowo-naczyniowego, oddechowego i rozrodczego czy mięśni (Pludowski i wsp., 2018). Ocenia się, że receptor witaminy D reguluje aż 5% genomu ssaków, a jego ekspresję potwierdzono w ponad 60 typach komórek (Pasing i wsp., 2017; Passeron i wsp., 2019). Co więcej, szacuje się, że suplementacja witaminą D może zmieniać ekspresję ponad 700 genów w ludzkich komórkach jednojądrzastych krwi obwodowej (Neme i wsp., 2019). Jednocześnie, rekomendacje dotyczące dawkowania witaminy D wydają się być niejasne, a zakres jej działania podlega licznym dyskusjom. Jedną z prawdopodobnych przyczyn, dla których analizy wpływu witaminy D dają niespójne rezultaty może być to, iż większość badań sprawdza efekt witaminy D w odniesieniu do leczenia lub łagodzenia istniejących już schorzeń. Być może zapobiegawcze działanie witaminy D posiada większy potencjał niż działanie lecznicze. Wydaje się, że rozszerzenie wachlarza badań prowadzonych wyłącznie na zdrowych osobnikach mogłoby pomóc w ustaleniu faktycznych właściwości prewencyjnych witaminy D.

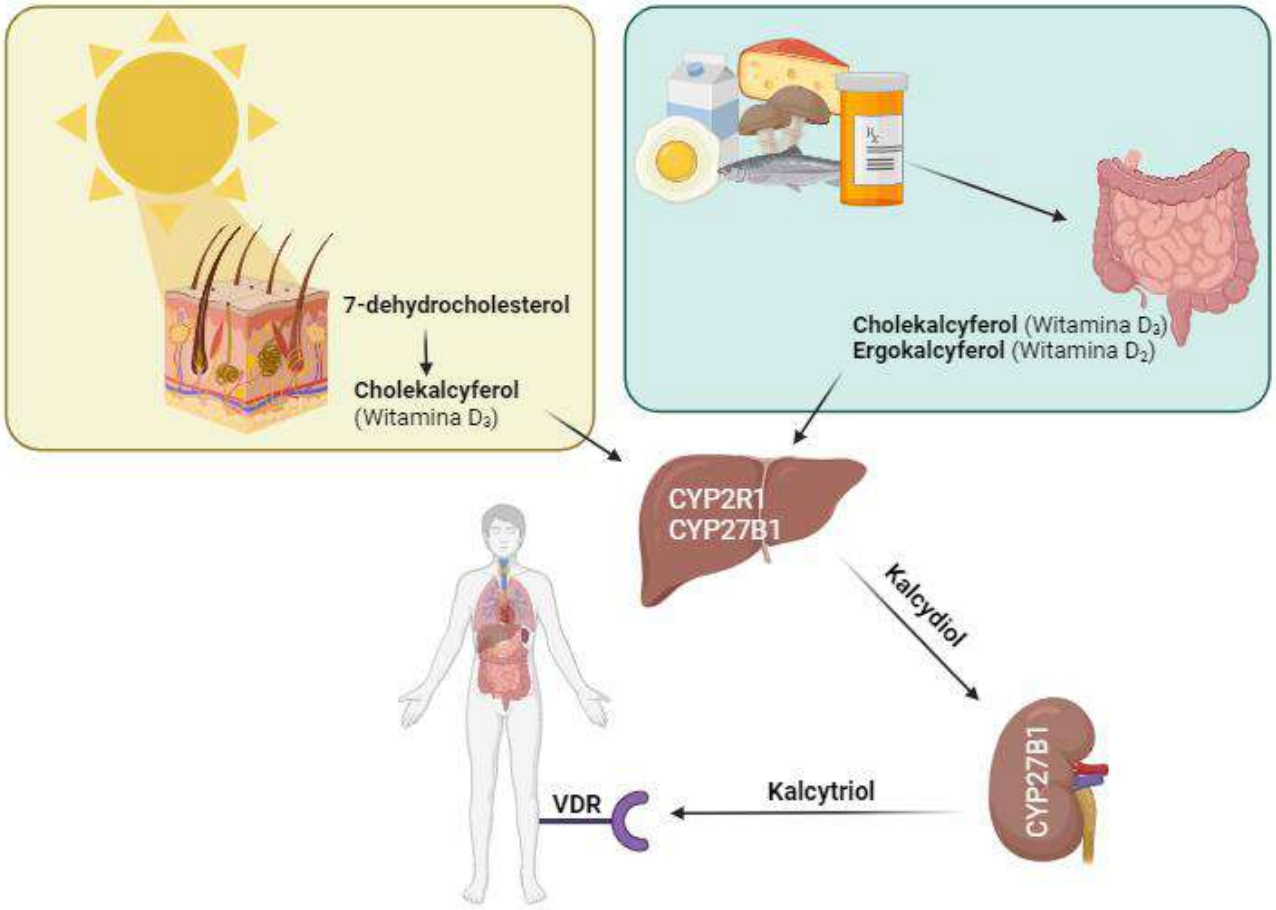
Należy zwrócić uwagę, że niedobór witaminy D towarzyszy wielu przewlekłym chorobom związanymi z ze stresem oksydacyjnym i stanem zapalnym (Bhattarai i wsp., 2020). Dużą część tych schorzeń stanowią choroby cywilizacyjne takie jak otyłość, cukrzyca typu II czy nowotwory (Turer i wsp., 2013; Lips i wsp., 2017). Występowanie niedoboru witaminy D i chorób cywilizacyjnych jest ze sobą silnie skorelowane ponieważ oba te problemy mają wspólne źródło- nienaturalny tryb życia, który często charakteryzuje kraje rozwinięte. Koncentracja witaminy D w organizmie uważana jest za wskaźnik dobrostanu i zdrowego stylu życia, jednak ocena, czy niedobór witaminy D jest przyczyną czy skutkiem wspomnianych chorób jest trudna. Współczesny styl życia ludzi jak i sposoby utrzymania zwierząt

charakteryzujące się brakiem ekspozycji na słońce, niewłaściwą dietą czy niewystarczającą aktywnością fizyczną, powodują niedobór witaminy D. Z drugiej strony, choroby cywilizacyjne, takie jak cukrzyca typu 2, choroby serca czy otyłość, mogą wpływać na metabolizm witaminy D. Pomimo licznych przesłanek świadczących o związku witaminy D z wieloma chorobami, jak również mimo powszechnej dostępności tego środka, istnieje szerokie pole do dalszych badań w tym zakresie.

Witamina D posiada strukturę sekosteroidową, która obejmuje pierścień steroidowy z charakterystycznym pęknięciem i boczną, łańcuchową grupą alifatyczną (Pérez-L´opez, 2007). Wyróżnia się dwie główne formy witaminy D: D_2 i D_3. Obie powstają w wyniku reakcji nieenzymatycznych z udziałem promieniowania słonecznego frakcji UV-B o długości fali 280-315 nanometrów (Gholami i wsp., 2019). Witamina D_2, zwana również ergokalcyferolem, powstaje w wyniku procesu fotolizy ergosterolu. Ergosterol, czyli prowitamina D_2 to sterol wchodzący w skład błon komórkowych grzybów. Z kolei witamina D_3, czyli cholekalcyferol, syntetyzowana jest w warstwie podstawowej naskórka zwierząt. Substratem niezbędnym do syntezy witaminy D_3 jest prowitamina D_3, czyli 7-dehydrocholesterol. Związek ten, pod wpływem promieniowania UV-B oraz ciepła, przekształca się w cholekalcyferol. W organizmach zwierząt witamina D, zarówno ta syntetyzowana w skórze (cholekalcyferol), jak i ta dostarczana drogą doustną (cholekalcyferol lub ergokalcyferol), ulega dwóm procesom metabolicznym w celu biologicznej aktywacji (Holick i wsp., 2011).

Cholekalcyferol, ergokalcyferol oraz wszystkie ich pochodne, transportowane są krwiobiegiem przyłączone do białka wiążącego witaminę D (DBP). Jednak w przypadku egzogennej witaminy D kluczową rolę w transporcie odgrywają również chylomikrony (Pludowski i wsp., 2018). Chylomikrony to lipoproteiny, które transportują trójglicerydy oraz witaminy rozpuszczalne w tłuszczach. W komórkach ścian jelita cienkiego witamina D włączana jest do struktury chylomikronów w procesie emulgacji tłuszczów. Następnie lipoproteiny wraz z witaminą D są transportowane przez naczynia limfatyczne do układu krwionośnego. W ten sposób chylomikrony rozprzestrzeniają witaminę D w różnych tkankach organizmu, gdzie może ona zostać uwolniona i wykorzystana w kolejnych procesach biologicznych. Jednakże, większość cholekalcyferolu i ergokalcyferolu trafia do wątroby związane z białkiem DBP. W wątrobie, za pośrednictwem hydroksylaz CYP2R1 oraz CYP27A1, odbywa się pierwszy etap aktywacji witaminy D. Wymienione enzymy przekształcają

cholekalcyferol i ergoklacyferol w kalcydiol (25(OH)D), który następnie transportowany jest do nerek (Jeon i Shin, 2018). Nerki są najważniejszym miejscem drugiej hydroksylacji witaminy D. W mitochondrialnych komórkach nerek, pod wpływem hydroksylazy CYP27B1, kalcydiol przekształcany jest w kalcytriol (1,25(OH)$_2$D) (Rycina 1.).



Rycina 1. Główna ścieżka biologicznej aktywacji i rozpoznawania endogennej oraz egzogennej witaminy D.

Kalcydiol stanowi główny metabolit witaminy D krążący w krwiobiegu, ponieważ białko DBP ma dwudziestokrotnie większe powinowactwo do kalcydiolu w porównaniu do kalcytriol (Fond i wsp., 2019). Co ciekawe, ekspresję enzymu aktywującego witaminę D (CYP27B1) zaobserwowano w wielu tkankach, miedzy innymi w: nabłonku prostaty, komórkach odpornościowych, komórkach tarczycy, wątroby, gonad czy trzustki (Adams i Hewison, 2012). Dlatego wydaje się, że kalcydiol może być aktywowany w różnych tkankach. Jednakże działalność

hydroksylazy CYP27B1 różni się w zależności od miejsca ekspresji. W nerkach głównymi regulatorami aktywności tego enzymu są zmiany w poziomie parathormonu (PTH) i czynnika wzrostu fibroblastów 23 (FGF23), podczas gdy w innych lokalizacjach, jego działanie jest kontrolowane przez dużą grupę cytokin (Kerschan-Schind, 2016; Oliveira i wsp., 2017).

Kalcytriol, czyli aktywna forma witaminy D, może pełnić swoją biologiczną funkcję dzięki połączeniu z receptorem witaminy D (VDR). VDR, należący do grupy receptorów jądrowych, znajduje się w większości tkanek ssaków. Niemniej jednak, poziom ekspresji tego genu różni się w zależności od tkanki i jest szczególnie zauważalny w wątrobie, jelitach, kościach, przytarczycach i nerkach, czyli w tkankach zaangażowanych w regulację homeostazy wapnia i fosforu (Pludowski i wsp., 2018). Co więcej, VDR posiada zdolność tworzenia heterodimeru z jądrowym receptorem retinoidowym RXR. Powstały w ten sposób kompleks VDR-RXR może oddziaływać z VDRE (ang. Vitamin D Response Element). Gdy aktywna forma witaminy D połączy się z VDR, kompleks VDR-RXR przyłącza się do sekwencji nukleotydowej VDRE w genie i w ten sposób reguluje jego ekspresje (Haussler i wsp., 2011). Ten proces odgrywa kluczową rolę w działaniu witaminy D, obejmując regulację jej poziomu w organizmie.

Ostatni etap metabolizmu, katabolizm witaminy D, zachodzi przy udziale enzymu CYP24A1, produkowanego w nerkach (Adams i Hewison, 2012). Działanie tego enzymu jest jednym ze sposobów zapobiegania akumulacji toksycznych stężeń witaminy D w organizmie. Hydroksylaza CYP24A1 metabolizuje zarówno kalcydiol jak i kalcytriol, chociaż wykazuje wyższe powinowactwo do kalcytriolu (Fond i wsp., 2019). Metabolity witaminy D są inaktywowane poprzez hydroksylacje do kwasu kalcytriolowego czyli 1,24,25$(OH)_3$D. Kontrola ekspresji enzymów CYP24A1 oraz CYP27B1 poprzez zmiany stężenia kalcytriolu jest głównym regulatorem poziomu witaminy D w organizmie (Jeon i Shin, 2018; Kerschan-Schind, 2016).

Mechanizm ujemnego sprzężenia zwrotnego witaminy D jest kontrolowany przez wspomniany już parathormon (PTH) oraz FGF-23 (fibroblast growth factor 23) (Adams i Hewison, 2012). PTH jest hormonem peptydowym, a FGF-23 to białko wytwarzane głównie przez osteocyty ale oba te czynniki pełnią kluczową rolę w regulacji homeostazy wapnia i fosforu poprzez wpływ na poziom witaminy D. PTH jest wydzielany przez przytarczyce w odpowiedzi na niski poziom Ca we krwi, w ten sposób stymuluje on aktywność CYP27B1 w nerkach. Zwiększona aktywność CYP27B1 powoduje wzrost stężenia kalcytriolu. Co z kolei może przyczyniać się do

zwiększenia wchłaniania wapnia w jelicie cienkim i resorpcji wapnia z nerek oraz wzrostu wchłaniania wapnia przez osteocyty (Suda i wsp., 2003). Z kolei FGF-23, jest produkowany przez osteoblasty i osteocyty w odpowiedzi na zbyt wysoki poziom fosforanów i kalcytriolu we krwi. FGF-23 hamuje nerkową ekspresję enzymu aktywującego witaminę D (CYP27B1) zwiększając jednocześnie aktywność enzymu katabolizującego tę witaminę (CYP24A1) (Passeron i wsp., 2019). Potwierdzają to również wyniki badań własnych przeprowadzone na szczurach, które wskakują, że wzrost stężenia witaminy D powoduje spadek ekspresji genu *Cyp27b1* w nerkach samic szczurów (Oczkowicz i wsp., 2021).

Wymienione wyżej, elementarne mechanizmy regulatorowe, kontrolowane są przez czynniki środowiskowe i nieśrodowiskowe. Wśród czynników środowiskowych wymienić należy przede wszystkim dietę oraz czas ekspozycji skóry na działanie słońca. Prawidłowe, zrównoważone żywienie pokrywa tylko kilka procent dziennego zapotrzebowania na witaminę D. Dlatego najważniejszym, naturalnym czynnikiem determinującym stężenie tej witaminy w organizmie jest czas ekspozycji skóry na promieniowanie UV-B (Gholami i wsp., 2019). Niestety, współczesne praktyki stosowane w masowej hodowli zwierząt sprawiają, że kontakt skóry ze słońcem jest silnie ograniczony, a czasami wręcz niemożliwy. Podobnie u ludzi, ekspozycja na słońce jest mocno limitowana poprzez częste przebywanie wewnątrz budynków oraz powszechne stosowanie kosmetyków z filtrem ochronnym (SPF - Sun Protective Filter). Warto wiedzieć, że kosmetyki z SPF ≥15 ograniczają syntezę cholekalcyferolu w skórze nawet do 95% (Matsuoka i wsp., 1987). Co więcej, na wielu obszarach, w tym w Polsce, czas i poziom nasłonecznienia zmieniają się radykalnie w ciągu roku. Efektem tych zmian są często diagnozowane, poważne, sezonowe deficyty witaminy D w osoczu (Sanghera i wsp., 2017). Dlatego suplementacja diety ludzi i zwierząt witaminą D jest tematem szczególnie istotnym dla krajów północnej Europy.

Druga kategoria czynników determinujących poziom witaminy D w organizmie obejmuje czynniki niezależne od środowiska takie jak wiek, stan fizjologiczny oraz kolor skóry. Wiek jest jednym z czynników najsilniej skorelowanych z poziomem witaminy D w organizmie (Bhattarai i wsp., 2020). Procesy metaboliczne u osób starszych są znacznie spowolnione, podobnie jak biochemiczny proces syntezy cholekalcyferolu w skórze. Seniorzy charakteryzują się również gorszym wchłanianiem witamin i minerałów z przewodu pokarmowego. Co więcej, udowodniono, że poziom ekspresji VDR zmniejsza się wraz z wiekiem

(Bischoff-Ferrari i wsp., 2004). Te czynniki powodują, że zalecenia dotyczące suplementacji witaminy D dla osób starszych powinny różnić się od tych rekomendowanych na przykład dla młodzieży. Wytyczne dotyczące suplementacji witaminy D powinny być również specyficzne dla kobiet i samic zwierząt w okresie ciąży i menopauzy. Przyczynami specjalnych rekomendacji dla tych grup są zmiany w koncentracji hormonów płciowych. Kolejnym ważnym czynnikiem regulującym syntezę cholekalcyferolu jest kolor skóry. Zwiększona ilość melaniny w skórze gwarantuje ochronę skóry przed słońcem. Stąd zależność: im ciemniejszy kolor skóry, tym słabsza synteza cholekalcyferolu (Passeron i wsp., 2019).

Wpływ wymienionych wcześniej czynników na funkcję i poziom witaminy D jest już solidnie udokumentowany, w przeciwieństwie do płci, w przypadku której istnieje wiele sprzecznych doniesień (Oczkowicz i wsp., 2021; Ning i wsp., 2016; Muhairi i wsp., 2013; Sanghera i wsp., 2017). Przeprowadzono szereg badań, w których koncentracja witaminy D różni się w zależności od płci (Ning i wsp., 2016; Kestenbaum i wsp., 2011; Hutchinson i wsp., 2010). Ponadto badania dowodzą, że suplementacja witaminy D może modulować funkcje wydzielnicze gruczołów płciowych oraz stężenie białka wiążącego hormony płciowe (SHBG) (Mehri i wsp., 2017). Wykazano również, że hormony płciowe, wpływają na poziom ekspresji enzymów CYP27B1 oraz CYP24A1, które ściśle regulują stężenie witaminy D (Donlon i wsp., 2018). Pomimo tych zależności, zalecenia dotyczące suplementacji witaminą D są bardzo ogólne i jednakowe dla obu płci. Dlatego uzasadnione wydaje się dokładne przeanalizowanie dostępnych danych literaturowych w celu uporządkowania zidentyfikowanych dotąd różnic w koncentracji i działu witaminy D u samic i samców.

Kolejnymi czynnikami, wywołującymi burzliwe dyskusje w środowisku badaczy jest optymalna dawka i forma witaminy D. Większość rekomendacji zakłada, że >30 nanogramów witaminy D w 1 mililitrze osocza krwi (>75 nmol/l) jest stężeniem prawidłowym (Pludowski i wsp., 2018; Holick i wsp., 2011). W przypadku niedoboru lub prewencyjnie, witamina D, zarówno u ludzi jak i zwierząt gospodarskich suplementowana jest najpowszechniej w formie cholekalcyferolu. Popularnie dostępne na rynku suplementy dla ludzi zawierają zazwyczaj 50 µg cholekalcyferolu (2000 j.m.). Podczas gdy dzienna dawka zalecana przez Europejski Urząd ds. Bezpieczeństwa Żywności (EFSA) wynosi 15 µg (600 j.m.) (Vitamin D: EFSA sets dietary reference values, 2016). Jednocześnie specjaliści EFSA uznali 250 µg/dzień (10 000 j.m.) za dawkę bezpieczną (Scientific Opinion on the Tolerable

Upper Intake Level of vitamin D, 2012). Co jeszcze bardziej zaskakujące, wyniki te zostały oparte na badaniach obejmującym wyłącznie grupę mężczyzn. Ponadto, jak napisano w raporcie- z powodu braku danych- panel UE określił 100 µg/dzień (4000 j.m.), jako dawkę najbezpieczniejszą dla dzieci, młodzieży, kobiet w ciąży i kobiet karmiących piersią (Scientific Opinion on the Tolerable Upper Intake Level of vitamin D, 2012). Podobne niejasności znaleźć można w wytycznych dotyczących żywienia świń. Brak jest konkretnych informacji dotyczących suplementacji witaminą D w poszczególnych grupach technologicznych. Aktualne zalecenia Uni Europiejskiej dotyczące świń, podobnie jak „innych gatunków zwierząt", nie wskazują minimalnej dawki witaminy D, podczas gdy maksymalna dzienna dawka wynosi 50 µg/kg paszy (2000 j.m.) niezależnie od tego, czy jest ona suplementowana w postaci cholekalcyferolu czy kalcydiolu (Rozporządzenie Wykonawcze Komisji (UE) 2019/849). Tymczasem wyniki porównania biodostępności i bioaktywności cholekalcyferolu i kalcydiolu wskazują, że suplementacja kalcydiolem może być skuteczniejsza w podnoszeniu poziomu witaminy D w osoczu (Duffy i wsp., 2018, Upadhaya i wsp., 2022). Z kolei według norm ustalonych w Stanach Zjednoczonych, dawka 800 j.m. (bez informacji o formie) jest wystarczająca do pokrycia zapotrzebowania na witaminę D u loch ciężarnych i karmiących (Nutrient Requirements of Swine, National Research Council, U.S., 2012). Co również zaskakujące, National Research Council, już w roku 1987 uznał, że dawka 2200 j.m. witaminy D w warunkach długotrwałego karmienia (powyżej 60 dni) jest dawką bezpieczną dla świń.

Praktycznie, w przemyśle trzody chlewnej, podobnie jak w żywieniu ludzi, najpowszechniej stosowana dzienna dawka cholekalcyferolu wynosi 2000 j.m. (Zhang i wsp., 2022). Biorąc pod uwagę istotność witaminy D dla prawidłowego funkcjonowania organizmu wymienione wyżej normy wydają się wymagać formalnego doprecyzowania i aktualizacji. Co więcej, wyniki badań prezentujące związek witaminy D z rozwojem chorób płuc ujawniają, że stosowanie alternatywnych form witaminy D, takich jak kalcydiol, budzi coraz większe zainteresowanie (Entrenas-Castillo i wsp., 2022). O ile wiadomo, że stosowanie kalcydiolu czyli częściowo aktywowanej formy witaminy D, skuteczniej zwiększa stężenie tego metabolitu w osoczu, nadal pozostaje niejasne, w jakim stopniu przekłada się to na korzyści zdrowotne.

Obecnie niewystarczająca koncentracja witaminy D w osoczu krwi ludzi jest globalnym problemem, który osiągnął status pandemii (Holick, 2017). Poziom

witaminy D w osoczu zwierząt nie podlega rutynowym kontrolom, dlatego nie sposób jest określić powszechność niedoboru tej witaminy u zwierząt gospodarskich, jaki i towarzyszących. Jednakże, ponieważ opisywane zjawisko ma charakter postępujący, konieczne jest opracowanie precyzyjnych strategii suplementacyjnych dla różnych grup fizjologicznych zarówno u ludzi jak i zwierząt. Wyłonienie wszystkich czynników regulujących efekt suplementacji witaminą D oraz dogłębne poznanie zależności występujących między nimi pomoże w określeniu zasady skutecznej i bezpiecznej suplementacji tą witaminą.

W ostatnich latach witaminie D przypisuje się szerokie spektrum działania, obejmujące przeciwdziałanie nowotworom, poprawę funkcjonowania mięśni czy poprawę kondycji psychicznej. Jednak badania kliniczne dostarczają sprzecznych rezultatów i jak dotąd, spośród chorób poza szkieletowych, najlepiej udowodnionym efektem witaminy D jest działanie w obrębie układu odpornościowego (Gombart, 2009). Wybuch pandemii wirusa SarCoV-2 dodatkowo zintensyfikował badania w tym zakresie w odniesieniu do tkanki płuc (Xiao i wsp., 2021). Zwierzęta hodowlane – zwłaszcza trzoda chlewna, narażone są na szkodliwe działanie pyłów pochodzących z pasz i ściółki, a także na infekcję drobnoustrojami takimi jak *Mycoplasma pneumoniae*. Wszystko to może prowadzić do chorób, spowalniać przyrosty, a nawet powodować upadki zwierząt. Dlatego porównanie efektów przyjmowania różnych dawek i form witaminy D na transkryptom i epigenom tkanki płucnej może pomóc w lepszym wykorzystaniu potencjału witaminy D. Z punktu widzenia hodowli zwierząt efekt działania witaminy D wart jest sprawdzenia także w tkance mięśniowej – głównym produkcie hodowli. Jednocześnie świnia domowa coraz częściej wykorzystywana jest jako zwierzę modelowe dla badań nad człowiekiem, dlatego uzyskane wyniki mogą pomóc w ocenie wpływu suplementacji witaminą D na zdrowie ludzi. Z kolei, zastosowanie nowoczesnych, wysokoprzepustowych technik sekwencjonowania umożliwi poznanie potencjalnych mechanizmów molekularnych odpowiadających za działanie witaminy D.

3. Cele pracy doktorskiej

Właściwości witaminy D podlegają badaniom od dziesiątek lat, jednakże dopiero od niedawna, dzięki wysokoprzepustowych metodom sekwencjonowania następnej generacji, możliwe jest dokładane poznanie mechanizmów molekularnego tego zagadnienia. Dlatego czerpiąc wiedzę z wniosków innych badaczy oraz poprzez analizę danych uzyskanych na drodze badań własnych podjęto próbę odpowiedzi na następujące pytania:

- Czy istnieją różnice w stężeniu i działaniu witaminy D u samic i samców oraz jakie są potencjalne przyczyny ich występowania?

- Czy długotrwała suplementacja zwiększoną dawką cholekalcyferolu i użycie kalcydiolu w diecie powoduje zmiany ekspresji mRNA w tkance mięśniowej świń?

- Czy długotrwała suplementacja zwiększoną dawką cholekalcyferolu i użycie kalcydiolu w diecie powoduje zmiany ekspresji miRNA w tkance płuc świń?

- Jaki wpływ na poziomy metylacji oraz ekspresję mRNA tkanki płuc świń wywiera długotrwała suplementacja zwiększoną dawką cholekalcyferolu?

4. Cykl publikacji naukowych

4.1. **Publikacja I**

Sex Differences in Vitamin D Metabolism, Serum Levels and Action

Obecność receptorów witaminy D oraz ekspresja enzymów metabolizujących witaminę D została potwierdzona w różnorodnych tkankach i komórkach ssaków (Wang i wsp., 2012). To rozpowszechnienie sugeruje, że witamina ta działa plejotropowo. Wskazują na to również wyniki badań, które donoszą o jej przeciwnowotworowych, kardioprotekcyjnych i przeciwcukrzycowych właściwościach (Manson i wsp., 2019). Jednak najlepiej udokumentowanymi funkcjami tej witaminy są: utrzymanie homeostazy wapniowo-fosforanowej oraz immunomodulacja (Gombart, 2009). Ze względu na działanie witaminy D w szerokim wachlarzu kluczowych dla organizmu funkcji, utrzymanie jej prawidłowej koncentracji jest niezbędne. Koncentracja witaminy D u ssaków regulowana jest przez wiele czynników, zarówno środowiskowych jak i nieśrodowiskowych. Płeć jest prawdopodobnie jednym z czynników nieśrodowiskowych, który może wpływać na koncentracje i działanie tej witaminy (Oczkowicz i wsp., 2021; Pasing i wsp., 2017; Ning i wsp., 2016; Muhairi i wsp., 2013; Sanghera i wsp., 2017).

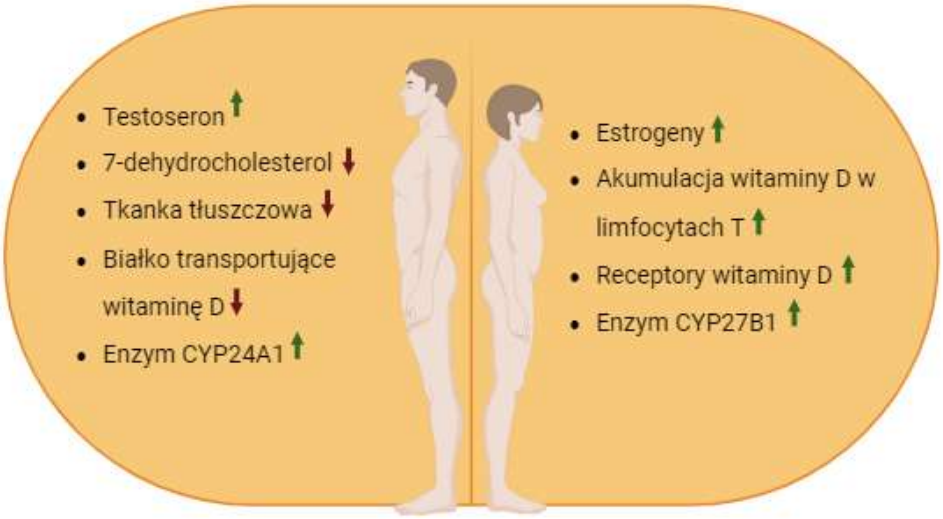
Ze względu na wielopoziomowy zakres aktywności oraz powszechność stosowania, poznanie różnic międzypłciowych w metabolizowaniu i działaniu witaminy D jest bardzo istotne. Poszerzenie wiedzy w tym obszarze może prowadzić do powstania rekomendacji dietetycznych dostosowanych do płci. Wydaje się to być ważne nie tylko z perspektywy zdrowia człowieka, ale również z punktu widzenia hodowli zwierząt. W dobie dynamicznego postępu w medycynie spersonalizowanej, różnice w procesach fizjologicznych pomiędzy pacjentami wymagają szczególnej uwagi (Carlberga i Haq, 2018). Co więcej nieprawidłowości związane z dystrybucją witaminy D mogą obniżać dobrostan zwierząt oraz zyski hodowców. Niniejszy przegląd literatury skupia wyniki ponad 150 prac naukowych, głównie z bazy PubMed, w celu identyfikacji różnic w koncentracji i działaniu witaminy D u samic i samców. Ponadto artykuł gromadzi wykryte dotychczas cechy płciowe mogące powodować różnice w syntezie i metabolizmie witaminy D.

Analizę zagadnienia rozpoczęto od ustalenia, czy istnieją dowody sugerujące, że niższa koncentracja witaminy D może być powiązana z którąś z płci. W tym celu stworzono zbiór artykułów opisujących wyniki pomiarów kalcydiolu w osoczu krwi kobiet i mężczyzn. Dokładna ocena umożliwiła wytypowanie 16 prac prezentujących najbardziej wiarygodne rezultaty. Podczas wyboru artykułów kierowano się jakością opisu badanej kohorty oraz jej liczebnością. Wykorzystane prace opisują wyniki badań ludzi w różnym wieku i z różnych stron świata. Najszerzej zakrojone, badanie VITAL (VITamin D and OmegA-3 TriaL) przeprowadzone w Stanach Zjednoczonych na grupie 15 804 osób w wieku ≤50 lat wykazało, że niedobór witaminy D dotyka znacznie częściej mężczyzn ($P < 0.001$) (Luttmann-Gibson i wsp., 2019). Jednakże, było to 1 z zaledwie 3 badań typujących płeć męską jako tą o niższym stężeniu witaminy D. Pozostałe 13 artykułów wykazało, że to płeć żeńska charakteryzuje się niższą koncentracją tej witaminy w osoczu.

Następnym etapem podjętego przeglądu było stworzenie zbioru prac raportujących o wynikach międzypłciowych porównań efektów witaminy D. Na podstawie niemal 100 artykułów przygotowano analizę związku między statusem witaminy D, a występowaniem i przebiegiem poszczególnych zaburzeń szkieletowych i nie szkieletowych. W przeglądzie omówiono różnice w działaniu witaminy D w obrębie układu mięśniowo-szkieletowego, sercowo-naczyniowego, nerwowego i odpornościowego, a także w zakresie procesu onkogenezy u samic i samców. Skrupulatna analiza zgormadzonych danych pozwoliła na sformułowanie potencjalnych różnic w odniesieniu do poszczególnych układów. i tak, w przypadku układu ruchu, ustalono, że to kobiety mogą być bardziej narażone na uszkodzenia i nieprawidłowości w funkcjonowaniu układu kostnego pod wpływem niskiego poziomu witaminy D. Wyniki badań sugerują również, że witamina D wykazuje mocniejszy efekt terapeutyczny u kobiet w porównaniu do mężczyzn w odniesieniu do chorób układu nerwowego. Z kolei związek niedoboru witaminy D z poziomem masy i siły mięśni oraz zdrowiem kardiometabolicznym wydaje się być bardziej istotny u mężczyzn niż u kobiet. Natomiast, wytypowanie płci, dla której związek koncentracji witaminy D z procesem powstawania nowotworu byłby większy jest bardzo trudne. Powodem tego jest ogrom możliwości dotyczący typów nowotworów i nieprzewidywalność samego procesu onkogenezy. Jednakże, na podstawie dokumentacji medycznej 217 244 osób, mieszkańców Dani, ustalono, że wyższe stężenie witaminy D nie jest związane z rozwojem raka piersi, jajnika i trzonu macicy, ale jest związane z częstszym występowaniem raka skóry, prostaty i raka hematologicznego (Vojdeman i wsp., 2019). Co więcej, ustalono również, że wyższe

stężenie witaminy D łączy się z mniejszym ryzykiem wystąpienia najczęściej diagnozowanego nowotworu, czyli raka płuc. Ustalenia te, typując nowotwory charakterystyczne dla płci, oraz wyniki innych badań mogą sugerować potencjalnie różny wpływ witaminy D na powstawanie nowotworów u kobiet i mężczyzn.

Na podstawie zgromadzonej literatury stworzono wykaz właściwości fizjologicznych samic i samców, które mogą prowadzić do potencjalnych różnic w koncentracji i działaniu witaminy D (Rycina 2.). Mimo to, zebrane wyniki badań nie umożliwiają postawienia jednoznacznych wniosków, ponieważ większość z nich to badania obserwacyjne o sprzecznych rezultatach. Niniejszy przegląd literatury ujawnia potrzebę przeprowadzenia starannie zaprojektowanych badania klinicznych i eksperymentów na modelach zwierzęcych w celu określenia roli czynników innych niż środowiskowe, w tym różnic międzypłciowych w działaniu witaminy D.



Rycina 2. Różnice płciowe, które mogą wpływać na syntezę i metabolizm witaminy D w organizmach samic i samców.

4.2. Publikacja II

Effect of Different Doses of Cholecalciferol and Calcidiol on Meat Quality Parameters and Skeletal Muscle Transcriptome Profiles in Swine

4.2.1. Materiały i metody:

- Zwierzęta i żywienie

W badaniu wykorzystano 34 próbki mięśni pobranych od świń potomstwa loch PBZ (Polska Biała Zwisłoucha) x WBP (Wielka Biała Polska) pokrytych knurem duroc x pietrain. Wszystkie procedury zawarte w tym badaniu, związane z wykorzystaniem żywych zwierząt zostały zatwierdzone przez lokalną Komisję Etyczną do spraw Doświadczeń na Zwierzętach w Krakowie (Uchwała nr 427/2020 z dnia 22.07.2020 r.). Zwierzęta biorące udział w eksperymencie były utrzymywane w Stacji Badawczej Instytutu Zootechniki Państwowego Instytutu Badawczego w Grodźcu Śląskim. Zwierzęta zostały losowo podzielone na cztery grupy żywieniowe. Każda z grup zawierała 12 osobników w tym 6 samic i 6 samców. Samce wykorzystane w eksperymencie zostały uprzednio wykastrowane. Mając na uwadze fakt, że płeć może wpływać na wyniki analiz, ilość zwierząt obu płci była taka sama w każdej z badanych grup. Dieta zwierząt różniła się jedynie dawką i formą witaminy D.

Dawkowanie witaminy D w grupach było następujące:

-1 grupa (grupa kontrolna) 2000 j.m. cholekalcyferolu (grower) i 1500 j.m. cholekalcyferolu/kg paszy (finiszer) -12 osobników

- 2 grupa 3000 j.m. cholekalcyferolu (grower) i 2500 j.m. cholekalcyferolu/kg paszy (finiszer) - 12 osobników

- 3 grupa 2000 j.m. cholekalcyferolu +1000 j.m. kalcydiolu (grower) i 1500 j.m. cholekalcyferolu +1000 j.m. kalcydiolu/kg paszy (finiszer) – 12 osobników

- 4 grupa 2000 j.m. kalcydiolu (grower) i 1500 j.m. kalcydiolu/kg paszy (finiszer) – 12 osobników

Wszystkie zwierzęta utrzymywane były w indywidualnych kojcach wyściełanych słomą. W kojcach panowały jednolite warunki środowiskowe. Masę ciała świń mierzono na początku eksperymentu, a następnie, co dwa tygodnie. W żywieniu zwierząt wykorzystano dwie mieszanki paszowe zgodnie z ich aktualnymi potrzebami (I okres tuczu - grower - 30-60 kilogramów, II okres tuczu - 60-110

kilogramów - finiszer). Dieta została opracowana tak, aby pokryć wszystkie potrzeby żywieniowe zwierząt (GROWER MIX: energia metaboliczna - 13,3 MJ, białko całkowite - 172 g/kg; FINISHER MIX: energia metaboliczna - 13,3 MJ, białko całkowite - 156 g/kg). Tucz doświadczalny trwał w przybliżeniu od 30 do 110 kilogramów żywej masy zwierząt. Pod koniec doświadczenia wszystkie świnie zostały poddane ubojowi przy użyciu kleszczy elektrycznych wysokiego napięcia (240-400 V). Natychmiast po uboju od wszystkich zwierząt pobrano próbki krwi. Krew do pomiaru stężenia kalcydiolu pobrano do probówek z antykoagulantem ACD (cytrynian kwaśnej dekstrozy/glukoza). Próbki krwi przechowywano w temperaturze +6°C do momentu pozyskania osocza. Osocze pozyskano poprzez wirowanie krwi w wirówce gradientowej (3000 RPM). Następnie przechowywano je w temperaturze -20°C.

Bezpośrednio po dokonaniu uboju, pobrano także próbki tkanki mięśniowej od 8 osobników z grupy 1, 3 i 4, oraz od 10 osobników z grupy 2. Tkankę mięśniową przeznaczoną do analizy genomowej pobrano z obszaru między ostatnim kręgiem piersiowym a pierwszym kręgiem lędźwiowym. Próbki te przechowywano w temperaturze -85 °C do czasu badania.

- Pomiar stężenia 25(OH)D w osoczu krwi .

Całkowite stężenie witaminy D w osoczu zostało oznaczone w 32 próbkach (8 próbek z każdej grupy) przez firmę zewnętrzną (ANCHEM Laboratorium, ul. Fredry 20, Katowice, Polska). Zamrożone próbki osocza zostały przesłane do laboratorium z zachowaniem równomiernej temperatury podczas transportu. Oznaczenie stężenia witaminy D w osoczu wykonano metodą RIA (oznaczenia radioimmunologiczne).

- Izolacja RNA, konstrukcja bibliotek mRNA i Next Generation Sequencing (NGS)

Izolację RNA z 34 próbek przeprowadzono przy użyciu odczynnika TRizol (Invitrogen) zgodnie z zaleceniami. Wyizolowany materiał genetyczny oczyszczono przy pomocy RNAClean XP (Beckman Coulter, Brea, Kalifornia, USA). Ocena jakości wyizolowanego RNA została przeprowadzona na Tapestation2200 (Agilent, Santa Clara, Kalifornia, USA), natomiast ocena ilości RNA za pomocą spektrofotometru NanoDrop™ 2000/2000c (Thermo Scientific™, Foster City, Kalifornia, USA) . Wynik RIN (ang. RNA Integrity Number) we wszystkich próbkach RNA był wyższy niż 7. Uzyskany materiał genetyczny wykorzystano do przygotowania bibliotek mRNA przy użyciu zestawu QuantSeq 3'mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen,

Wiedeń, Austria). Jakościową i ilościowa ocenę skonstruowanych bibliotek przeprowadzono wykorzystując urządzenia Qubit (Thermo Scientific™, Foster City, Kalifornia, USA) i Tapestation 2200 (Agilent, Santa Clara, Kalifornia, USA). Sekwencjonowanie puli bibliotek (pojedynczy odczyt 75 bp) przeprowadzono z wykorzystaniem urządzenia Nextseq 5500 (Illumina, San Diego, Kalifornia, USA) w Instytucie Zootechniki Państwowym Instytucie Badawczym w Balicach. Pula bibliotek została przygotowana zgodnie z standardową metodą normalizacji z protokołu NextSeq 500 i NextSeq 550 Sequencing Systems-Denature and Dilute Libraries Guide. Do sekwencjonowania użyto pulę bibliotek o stężeniu 2 nM i kontrolę PhiX.

- Walidacja wyników NGS przy użyciu metody PCR w czasie rzeczywistym (qPCR)

Walidacja wyników NGS została przeprowadzona z wykorzystaniem 18 próbek. RNA poddano odwrotnej transkrypcji wykorzystując zestaw High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Waltham, Massachusetts, Stany Zjednoczone). PCR w czasie rzeczywistym przeprowadzono przy użyciu RT PCR Mix SYBR® (A&A Biotechnology, Gdańsk, Polska) na QuantStudio ™ 7 Flex Real-Time PCR System (Applied Biosystems ™, Waltham, Massachusetts, Stany Zjednoczone). Ekspresję genów *VDR*, *MYH2*, *NDUFC2* i *SLC30A9* przebadano używając specyficzne startery.

- Analiza statystyczna
  - ★ Stężenie 25(OH)D w osoczu i ocena jakości mięsa

Analizy statystyczne danych dotyczących stężenia kalcydiolu w osoczu i jakości mięsa przeprowadzono za pomocą jednokierunkowej analizy wariancji ANOVA. Porównanie średnich przeprowadzono za pomocą testu wielokrotnego zakresu Duncana na poziomie istotności $P \leq 0{,}05$. Wszystkie analizy zostały przeprowadzone przy użyciu pakietu Statistica 12 (Copyright©StatSoft, Inc. 1984-2014).

  - ★ RNA-seq

Kontrolę jakości, przycinanie odczytów i mapowanie odczytów zdemultipleksowanych plików fastq pobranych z serwera przeprowadzono za pomocą oprogramowania FastQC 11.8, FLEXBAR 3.5.0 i TopHat 2.1.1. Do oceny statystyk mapowania i liczby odczytów wykorzystano oprogramowanie Samtools

1.9, RSeQC, HTSeq-count 0.11.1 i Gtf-Ensembl annotation 96. Do przeprowadzenia analizy ekspresji różnicowej wykorzystano oprogramowanie DEseq2. Geny ze współczynnikiem *Padj* < 0,05 (FDR-False Discovery Rate) z korektą Benjamini-Hochberg (BH) uznano za ulegające ekspresji różnicowej. Analizę funkcjonalną wyników RNA-seq przeprowadzono za pomocą oprogramowania BioMart i STRING, przy użyciu bazy danych *Sus scrofa* 11.1.

★ Walidacja qPCR

Względne dane ilościowe uzyskane z badania qPCR analizowano w Thermo Fisher Cloud (Thermo Scientific, USA). Istotność statystyczną oceniono za pomocą aplikacji Relative Quantification w ThermoFisher Connect. Porównanie danych qPCR oraz NGS wykonano za pomocą pakietu Statistica 12.

4.2.2. Wyniki i dyskusja

Celem badania było porównanie wpływu standardowej i zwiększonej dawki cholekalcyferolu, standardowej dawki kalcydiolu oraz zwiększonej dawki kombinacji cholekalcyferolu i kalcydiolu na tkankę mięśniową świń. Wyniki przeprowadzonych badań umożliwiły określenie efektu wymienionych wyżej dawek i form witaminy D na transkryptom tkanki mięśniowej. W publikacji przedstawiono także wyniki analizy jakości mięsa, przeprowadzonej wcześniej w ramach działalności statutowej Instytutu Zootechniki PIB.

Kluczowym etapem opisywanego eksperymentu była analiza ekspresji mRNA w tkance mięśni szkieletowych świń. Badanie to wykazało, że istotne statystycznie różnice w transkryptomie mięśni zwierząt suplementowanych zalecaną i zwiększoną dawką cholekalcyferolu oraz tych otrzymujących kalcydiol nie są duże. Co więcej, analiza PCA (Principal Component Analysis, Analiza Składowych Głównych) wykazała, że próbki nie różnicowały się pod względem płci. Uzyskane wyniki potwierdzają ustalenia Hangelbroek i wsp. wskazujące, że kalcydiol nie wpływa znacząco na transkryptom tkanki mięśniowej oraz, że mięśnie mogą nie być tkanką docelową dla witaminy D (Hangelbroek i wsp., 2019).

Porównanie transkryptomów zwierząt z grup otrzymujących standardową i zwiększoną dawkę cholekalcyferolu wykazało niewielkie różnice (log2FoldChange > -0,568 i < 0,509). Zidentyfikowano jedynie trzy geny o istotnie zróżnicowanej ekspresji: *ENSSSCG00000044439*, *ENSSSCG00000025403* i *SLC30A9*. Geny te

wykazały obniżoną ekspresję w grupie przyjmującej podwyższoną dawkę cholekalcyferolu. Gen *SLC30A9* funkcjonuje, jako transporter cynku i jest zaangażowany w wewnątrzkomórkową homeostazę tego pierwiastka. Aktywność *SLC30A9* jest także ściśle związana z zespołem Birk-Landau-Pereza, który powoduje regresję psychomotoryczną i zaburzenia ruchowe. Badanie przeprowadzone przez Perez i in. wskazuje, że *SLC30A9* ulega wysokiej ekspresji w mięśniach, *a* zmniejszenie aktywności tego genu skutkuje osłabieniem mięśni (Perez i wsp., 2017). Wyniki sekwencjonowania mRNA sugerują, że zwiększenie dawki cholekalcyferolu może wpływać na wewnątrzkomórkową homeostazę Zn poprzez obniżenie ekspresji *SLC30A9*, jednak wynik ten nie został potwierdzony metodą qPCR (real-time quantitative polymerase chain reaction) (r2 = 0,041).

Prezentując efekt zwiększonej dawki cholekalcyferolu należy wspomnieć również o genach, które zgodnie ze skorygowaną wartością *P (Padj)*, znajdują się na granicy istotności statystycznej (*Padj* = 0.052). Wśród nich na szczególną uwagę zasługują *RBFOX2* (log2FoldChange= -0.444) i *APOA5* (log2FoldChange= -0.546). Pierwszy z genów, *RBFOX2,* łączony jest szczególnie z rozwojem mięśni szkieletowych i wadami serca (Cao i wsp., 2021). W badaniu dotyczącym funkcji genu *RBFOX2* wykazano, że w komórki szczurów *Rbfox2* KO (knockout/dezaktywacja genu) występują znaczne zaburzenia alternatywnego poli(A) (APA) w genie *Slc25a4* (Cao i wsp., 2021). Co ciekawe, gen *Slc25a4* ma kluczowe znaczenie dla produkcji energii w mitochondriach. Badanie przeprowadzone na ludziach wskazuje, że mutacje w genie *SLC25A4* są związane z kardiomiopatią przerostową i miopatią mięśni szkieletowych (Cao i wsp., 2021). Zgodnie z tymi ustaleniami, prezentowane wyniki mogą sugerować potencjalnie niekorzystny wpływ zwiększenia dawki cholekalcyferolu na tkankę mięśniową, nie tylko ze względu na spadek ekspresji genu *SLC30A9*, ale również *RBFOX2*. z kolei gen *APOA5* jest kluczowym czynnikiem transferu tłuszczu w szlaku sygnalizacyjnym PPAR (ang. Peroxisome Proliferator-Activated Receptor), stanowi on regulator stężenia trójglicerydów i odpowiada za proces odkładania tłuszczu (Hui i wsp., 2013). Obniżenie ekspresji *APOA5* może skutkować zwiększeniem koncentracji trójglicerydów w mięśniach (Hui i wsp., 2013). Zatem podwyższona dawka cholekalcyferolu, może zwiększyć zawartość tłuszczu śródmięśniowego w mięsie wieprzowym (Perez i wsp., 2017). Zawartość tłuszczu śródmięśniowego zależy od stężenia trójglicerydów i jest jednym z wyznaczników jakości mięsa. Intensywna selekcja świń pod kątem tempa wzrostu przyczyniła się do spadku jakości wieprzowiny, szczególnie ze względu na obniżenie zawartości tłuszczu

śródmięśniowego (Fernandez i wsp., 1999; Zuo i wsp., 2003). Niemniej jednak przeprowadzona analiza chemiczna wieprzowiny nie potwierdza wzrostu procentowej zawartości tłuszczu w mięsie zwierząt otrzymujących zwiększoną dawkę cholekalcyferolu (1,52% vs 1,51%).

Porównania transkryptomów świń przyjmujących podwyższoną dawkę cholekalcyferolu i tych przyjmujących standardową dawkę kalcydiolu wykazało dwa geny o istotnie zróżnicowanej ekspresji. Oba te geny są jednak istotnie związane z funkcjonowaniem tkanki mięśniowej. Pierwszy z nich, gen *TMEM127* (log2FooldChange= -0.969), który działa jako inhibitor szlaku sygnalizacyjnego TOR (ang. Target of Rapamycin). Szlak sygnalizacyjny TOR aktywuje syntezę białek oraz sprzyja podziałowi komórek. Suplementacja kalcydiolem spowodowała znaczną redukcję *TMEM127* w porównaniu do cholekalcyferolu. Jeśli *TMEM127* powoduje inaktywację sygnalizacji TOR, a suplementacja kalcydiolem znacząco obniża ekspresję *TMEM127*, wydaje się, że wykorzystanie kalcydiolu w diecie świń może być korzystne dla procesu miogenezy. Potwierdzają to wyniki Gogulothu i wsp., 2020, wskazujące, że inaktywacja sygnalizacji TOR obserwowana u szczurów z niedoborem witaminy D jest szkodliwa dla tkanki mięśniowej tych zwierząt. Co więcej, wyniki badania przeprowadzonego przez Akagawa i wsp., 2018 z wykorzystaniem szczurów z cukrzycą typu 2 sugerują, że kalcydiol wykazuje korzystny wpływ na tkankę mięśniową poprzez wpływ na szlak TOR. Kolejnym genem istotnie różnicującym transkryptom świń suplementowanych podwyższona dawką cholekalcyferolu i standardową dawką kalcydiolu jest *NDUFC2* (log2FoldChange= -2.269). *NDUFC2* bierze udział w transporcie elektronów oddechowych, syntezie ATP i wytwarzaniu ciepła. Zgodnie z wynikami badań Nitert i wsp., 2012, ćwiczenia fizyczne zmniejszają metylację *NDUFC2* w mięśniach szkieletowych aktywując tym samym gen *NDUFC2*. Z kolei według Raffa i wsp., 2019, wyciszenie *NDUFC2* w ludzkich komórkach śródbłonka i mięśni gładkich powoduje znaczny wzrost stężenia ROS (ang. Reactive Oxygen Species), obniżenie poziomu ATP i wyższy stopień uszkodzenia struktury mitochondriów. Uzyskane wyniki wskazują, że stosowanie kalcydiolu w diecie świń może zmniejszać wydolność oddechową mięśni, poprzez potencjalne obniżenie ekspresji *NDUFC2* w tej tkance. Jednakże różnice zidentyfikowane w ekspresji opisywanych wyżej genów nie zostały potwierdzone metodą qPCR.

Przedstawione wyniki stanowią podstawę do dalszych badań nad zwiększeniem dawki witaminy D i zastąpieniem cholekalcyferolu kalcydiolem

w żywieniu świń. Wyniki sugerują, że zwiększenie dawki cholekalcyferolu i użycie kalcydiolu w diecie świń jest bezpieczne i nie wpływa znacząco na funkcjonowanie mięśni szkieletowych.

4.3. Publikacja III

Changes in miRNA Expression in the Lungs of Pigs Supplemented with Different Levels and Forms of Vitamin D

4.3.1. Materiały i metody

- Zwierzęta i żywienie

W badaniu wykorzystano 48 próbek płuc pochodzących od świń PBZ x WBP pokrytych knurem duroc x pietrain. Wszystkie procedury zawarte w tym badaniu, związane z wykorzystaniem żywych zwierząt zostały zatwierdzone przez lokalną Komisję Etyczną do spraw Doświadczeń na Zwierzętach w Krakowie (Uchwała nr 427/2020 z dnia 22.07.2020 r.). Zwierzęta biorące udział w eksperymencie były utrzymywane w Stacji Badawczej Instytutu Zootechniki Państwowego Instytutu Badawczego w Grodźcu Śląskim. Zwierzęta zostały losowo podzielone na cztery grupy żywieniowe. Każda z grup liczyła 12 zwierząt, 6 samic i 6 samców, jednakże samce wykorzystane w eksperymencie zostały uprzednio wykastrowane. Mając na uwadze fakt, że płeć może wpływać na wyniki analiz, ilość zwierząt obu płci była taka sama w każdej z badanych grup. Dieta zwierząt różniła się jedynie dawką i formą witaminy D.

Dawkowanie witaminy D w grupach było następujące:

1 grupa (grupa kontrolna) 2000 j.m. cholekalcyferolu (grower) i 1500 j.m. cholekalcyferolu /kg paszy (finiszer) -12 osobników

- 2 grupa 3000 j.m. cholekalcyferolu (grower) i 2500 j.m. cholekalcyferolu/kg paszy (finiszer) - 12 osobników

- 3 grupa 2000 j.m. cholekalcyferolu +1000 j.m. kalcydiolu (grower) i 1500 j.m. cholekalcyferolu +1000 j.m. kalcydiolu/kg paszy (finiszer) – 12 osobników

- 4 grupa 2000 j.m. kalcydiolu (grower) i 1500 j.m. kalcydiolu/kg paszy (finiszer) – 12 osobników

Wszystkie zwierzęta utrzymywane były w indywidualnych kojcach wyściełanych słomą. W kojcach panowały jednolite warunki środowiskowe. Masę ciała świń mierzono na początku eksperymentu, a następnie, co dwa tygodnie. W żywieniu zwierząt wykorzystano dwie mieszanki paszowe zgodnie z ich aktualnymi

potrzebami (I okres tuczu - grower - 30-60 kilogramów, II okres tuczu - 60-110 kilogramów - finiszer). Dieta została opracowana tak, aby pokryć wszystkie potrzeby żywieniowe zwierząt (GROWER MIX: energia metaboliczna - 13,3 MJ, białko całkowite - 172 g/kg; FINISHER MIX: energia metaboliczna - 13,3 MJ, białko całkowite - 156 g/kg). Tucz doświadczalny trwał w przybliżeniu od 30 do 110 kilogramów żywej masy zwierząt. Pod koniec doświadczenia wszystkie świnie zostały poddane ubojowi przy użyciu kleszczy elektrycznych wysokiego napięcia (240-400 V). Natychmiast po uboju od wszystkich 48 zwierząt pobrano próbki krwi i tkanki płuc. Próbki płuc pobrano ze środkowych części górnych płatów lewego płuca. Tkanki te przewieziono do laboratorium w ciekłym azocie, następnie przechowywano je w temperaturze -85 °C. Krew do pomiaru stężenia kalcydiolu pobrano do probówek z antykoagulantem ACD (cytrynian kwaśnej dekstrozy/glukoza). Próbki krwi przechowywano w temperaturze +6°C do momentu pozyskania osocza. Osocze pozyskano poprzez wirowanie krwi w wirówce gradientowej (3000 rpm). Następnie przechowywano je w temperaturze -20°C.

- Pomiar stężenia 25(OH)D

Całkowitego stężenia witaminy D w osoczu zostało oznaczone w 32 próbkach (8 próbek z każdej grupy) przez firmę zewnętrzną (ANCHEM Laboratorium, ul. Fredry 20, Katowice, Polska). Zamrożone próbki osocza zostały przesłane do laboratorium z zachowaniem równomiernej temperatury podczas transportu. Oznaczenie stężenia witaminy D w osoczu wykonano metodą RIA (oznaczenia radioimmunologiczne).

- Izolacja RNA, konstruowanie bibliotek miRNA i sekwencjonowanie NGS

Izolację RNA z 48 próbek płuc przeprowadzono przy użyciu komercyjnie dostępnego zestawu Direct-zol RNA Miniprep Kits (ZYMO Research, Orange, California). Wyizolowany materiał genetyczny oczyszczono przy użyciu zestawu Monarch® RNA Cleanup Kit (New England Lab, Woburn, USA). Jakość izolatów oceniono za pomocą urządzenia Tapestation 2200 (Agilent, Santa Clara, Kalifornia, USA). Za próbki odpowiedniej jakości uznawano te z RIN >/= 7. Ilość RNA została oceniona za pomocą spektrofotometru NanoDrop™ 2000/2000c (Thermo Scientific™, Foster City, Kalifornia, USA). Przygotowany i oceniony w ten sposób materiał genetyczny użyto do konstrukcji bibliotek miRNA wykorzystując zestaw NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Lab, Woburn, USA). Ocena ilościowa puli bibliotek została przeprowadzona przy użyciu

urządzenia Qubit (Ther-mo Scientific™, Foster City, Kalifornia, USA), podczas gdy wielkości uzyskanych fragmentów została oceniona na Tapestation 2200 (Agilent, Santa Clara, Kalifornia, USA). Sekwencjonowanie przeprowadzono w Instytucie Zootechniki Państwowym Instytucie Badawczym w Balicach wykorzystując urządzenie NextSeq 550 (Illumina, San Diego, Kalifornia, USA). Do sekwencjonowania użyto pulę bibliotek o stężenjiu 2 nM i kontrolę PhiX.

- Statystyka wyników sekwencjonowania miRNA

Uzyskane odczyty poddano demultipleksacji i kontroli jakości za pomocą oprogramowań bcl2fastq (Illumina) i FastQC. Przycinanie odczytów przeprowadzono za pomocą pakietu TrimGalore. Następnie, do identyfikacji znanych i potencjalnie nowych miRNA, zastosowano oprogramowanie miRDeep2 przy użyciu genomu referencyjnego *Sus scrofa* 11.1 z miRBase 22.1. Do przeprowadzenia analizy ekspresji różnicowej wykorzystano pakiet R i oprogramowanie DESeq2. miRNA z korektą *p-value* <0,05 Benjamini-Hochberg (BH) i bez progu zmiany krotności uznano za ulegające ekspresji różnicowej. Analizę funkcjonalną miRNA o zróżnicowanej ekspresji (DE) przeprowadzono za pomocą DIANA-miRPath v3.0.

- Badanie qPCR

W celu walidacji wyników miRNA-seq RNA z 28 próbek (7 próbek/grupę) poddano odwrotnej transkrypcji przy użyciu zestawu miRCURY LNA RT Kit (QIAGEN, Hilden, Niemcy). Do badania wybrano następujące miRNA: miR-215-5p, miR-96-5p i miR-381-3p. PCR w czasie rzeczywistym przeprowadzono wykorzystując miRCURY LNA SYBR Green PCR Kit (QIAGEN, Hilden, Niemcy) i miRCURY LNA miRNA PCR Assays (QIAGEN, Hilden, Niemcy) oraz urządzenie QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™, Waltham, Massachusetts, Stany Zjednoczone). Względne dane ilościowe analizowano w Thermo Fisher Cloud (Thermo Scientific). Do porównania wyników uzyskanych z NGS i qPCR wykorzystano współczynnik korelacji Pearsona (r2) w programie SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Na podstawie analizy funkcjonalnej wyników miRNA-seq, wybrano dwa geny, które są celami miRNA zmienionymi przez suplementację witaminą D. RNA wyizolowane z 28 próbek zostało dodatkowo poddane odwrotnej transkrypcji przy użyciu zestawu High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Waltham, Massachusetts, Stany Zjednoczone). Uzyskane cDNA wykorzystano do analizy

zmian w ekspresji genów *NEU1* (Neuraminidase 1) i *FUT1* (Fucosyltransferase 1) oraz kontroli endogennej *RPS29*. Analizę uzyskanych wyników przeprowadzono wykorzystując Thermo Fisher Cloud (Thermo Scientific). Poziom istotności różnic pomiędzy grupami sprawdzono za pomocą testu U Manna-Whitneya oraz testu T w programie SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

4.3.2. Wyniki i dyskusja

Celem badania było porównanie wpływu standardowej i zwiększonej dawki cholekalcyferolu, standardowej dawki kalcydiolu oraz zwiększonej dawki kombinacji cholekalcyferolu i kalcydiolu na ekspresję miRNA w tkance płuc świń. Warto podkreślić, że niniejsze badanie stanowi pierwszą próbę opisania wpływu kalcydiolu na ekspresję miRNA w tkance płuc.

Analizę wyników sekwencjonowania rozpoczęto od oceny efektu zwiększenie dawki cholekalcyferolu na profil miRNA zdrowych płuc świń. Istotne jest, że również w tym przypadku analiza PCA wykazała, że próbki nie różnicowały się pod względem płci. Ustalono, że zwiększenie dawki cholekalcyferolu z 2000 j.m. do 3000 j.m. wywołało istotny wzrost stężenia witaminy D w osoczu (39.67 ng/ml vs 63.96 ng/ml) i zmianę ekspresji tylko jednego miRNA - miR-215. Cząsteczka miR-215 zaangażowana jest w wiele podstawowych procesów, takich jak rozwój, proliferacja, przeżycie, migracja i metabolizm komórek (Vychytilova-Faltejskova i Slaby, 2019). Ze względu na swoje kluczowe funkcje, zmiany w ekspresji miR-215 zostały powiązane z patogenezą wielu chorób. Wykazano, że miR-215 może być zarówno onkogenem jak i genem supresorowym w zależności od typu nowotworu (Vychytilova-Faltejskova i Slaby, 2019). Co więcej jedno z badań ujawnia silny związek między poziomem ekspresji miR-215 a rozwojem zwłóknienia płuc (Huang i wsp., 2022). Badania *in vitro i in vivo* wykazały, że w tkance płuc objętej procesem włóknienia dochodzi do wzrostu ekspresji miR-215, a w konsekwencji do aktywacji szlaku TGF-β (ang. Transforming Growth Factor-Beta) poprzez hamowanie ekspresji genu docelowego *BMPR2* (Bone Morphogenetic Protein Receptor Type 2). Co więcej udowodniono, że spadek ekspresji miR-215 hamuje progresję nieodwracalnego zwłóknienia płuc (Huang i wsp., 2022). Zwiększenie dawki cholekalcyferolu spowodowało znaczący wzrost ekspresji miR-215 (log2FoldChange= 2, 65), co w świetle przytoczonych wyników wydaje się wywierać niekorzystny wpływ na tkankę płuc. Interesujące jest jednak to, iż kolejne analizy wykazały, że stosowanie

tej samej dawki kombinacji cholekalcyferolu i kalcydiolu nie wpłynęło istotnie na ekspresję miR-215.

Kolejnym celem badania było przetestowanie efektu zastąpienia powszechnie stosowanego cholekalcyferolu kalcydiolem. Kalcydiol jest znacznie bardziej efektywny w podnoszeniu stężenia witaminy D w osoczu w porównaniu do cholekalcyferolu. Uważa się, że wyższe stężenie witaminy D w osoczu może wykazywać korzystny wpływ na tkankę płuc (Sandhu i wsp., 2010; Herr i wsp., 2011). Przedstawione tutaj wyniki rzucają jednak nowe światło na te sugestie. Zaobserwowaliśmy, że wysokie stężenie witaminy D w osoczu, wywołane suplementacją kalcydiolem (39.67 ng/ml vs 133.5 ng/ml), nie wpływa znacząco na profil miRNA płuc. Jednakże, prawie równie wysokie stężenie witaminy D, wywołane suplementacją kombinacji cholekalcyferolu i kalcydiolu (39.67 ng/ml vs 124.93 ng/ml) spowodowało istotne zmiany w ekspresji miRNA tkanki płuc. Wynik ten sugeruje, że samo zwiększenie stężenia kalcydiolu w osoczu może być niewystarczające do wywołania zmian na poziomie regulacji genów. Konieczna wydaje się ku temu transformacja metaboliczna witaminy D.

Suplementacja kombinacją cholekalcyferolu i kalcydiolu wywołała największe zmiany w profilu miRNA badanych płuc w porównaniu do suplementacji cholekalcyferolem i kalcydiolem osobno. Podwyższona dawka kombinacji, w porównaniu do standardowej dawki cholekalcyferolu wpłynęła istotnie na 13 miRNA, 7 z nich wykazało wzrost, a 6 spadek ekspresji. Z kolei porównanie efektu suplementacji takiej samej dawki cholekalcyferolu oraz cholekalcyferolu w kombinacji z kalcydiolem wykazało różnice w ekspresji 12 miRNA. W tej puli 9 cząsteczek wykazało wzrost, a 3 spadek ekspresji pod wpływem kombinacji. Co więcej, porównanie profili miRNA zwierząt otrzymujących kombinacje oraz sam kalcydiol wykazało 17 miRNA o różnicowej ekspresji, 10 z nich charakteryzowało się niższą ekspresją w grupie przyjmującej kombinacje, a 7 wyższą. Warto wspomnieć, że tylko 5 z 17 zmienionych miRNA było charakterystycznych jedynie dla tego porównania.

Dalsza analiza wyodrębniała 3 miRNA łączące wyniki wszystkich trzech porównań z grupą przyjmująca kombinację cholekalcyferolu i kalcydiolu, są to miR-150, miR-193 i miR-574. We wszystkich zestawieniach wymienione miRNA prezentowały wzrost ekspresji pod wpływem zastosowanej kombinacji. Pierwszy z nich - miR-150 - reguluje ekspresję *TLR2* (Toll Like Receptor 2), czyli receptora zaangażowanego w podstawowy mechanizm odpowiedzi immunologicznej, który

chroni przed infekcjami bakteryjnymi i wirusowymi (Zhou i wsp., 2018). Zheng i wsp., 2015 udowodnili, że makrofagi pacjentów z gruźlicą charakteryzują się obniżoną ekspresją miR-150 oraz podwyższoną ekspresją miR-193 (Zheng i wsp., 2015). MiR-193a zaangażowany jest szczególnie w proces proliferacji komórek. Między innymi, dlatego uważa się, że ta cząsteczka może stanowić cenne narzędzie w prognozowaniu i diagnozowaniu raka płuc (Khordadmehr i wsp., 2019). Yu i i wsp., 2015 wykazali, że wysoka ekspresja miR-193 hamuje migrację komórek i tworzenie przerzutów w płucach chorych na niedrobnokomórkowego raka płuc (NSCLC). Sugeruje to, że miR-193a działa, jako supresor nowotworu, a suplementacja kombinacją cholekalcyferolu i kalcydiolu może mieć pod tym względem ochronny wpływ na tkankę płucną. Również wzrost ekspresji miR-574 sugeruje pozytywny efekt użycia kombinacji dwóch form witaminy D na tkankę płuc. Wskazują na to wyniki ujawniające związek między miR-574 a zespołem ostrej niewydolności płuc wywołanym sepsą. Wei i wsp., 2023 odkryli, że podwyższona ekspresja miR-574 powoduje obniżenie ekspresji genu *SOX6*, a w konsekwencji zmniejszenie uszkodzenia komórek (Hu i wsp., 2023). Wpływ zwiększonej ekspresji miR-574 na osłabienie uszkodzenia płuc wywołanego sepsą został również potwierdzony w innym badaniu (Sun i wsp., 2020). Jednak w tym przypadku efekt ten był prawdopodobnie związany ze obniżeniem poziomu *C3*, czyli przekaźnika zapalnego regulowanego przez miR-574 (Sun i wsp., 2020).

Analiza funkcjonalna miRNA zmienionych pod wpływem zastosowania kombinacji cholekalcyferolu i kalcydiolu wykazała, że zamiana standardowej dawki cholekalcyferolu na podwyższoną dawkę kombinacji może angażować 15 ścieżek biologicznych. Do ścieżek charakterystycznych dla tego porównania należą: szlak sygnalizacyjny oksytocyny, nieprawidłowa regulacja transkrypcji w raku oraz resorpcja wody regulowana przez wazopresynę. Z kolei analiza funkcji miRNA różnicujących zwierzęta przyjmujące tę sama dawkę cholekalcyferolu i kombinacji wykazała przypuszczalny wpływ na 5 ścieżek biologicznych. Co ciekawe 2 z nich ściśle łączą się z metabolizmem glikanów. Nie wykazano ścieżek wspólnych dla pierwszego i drugiego porównania. Jednakże analiza funkcjonalna miRNA różnicujących grupę przyjmującą kombinację i sam kalcydiol wskazała prawdopodobny wpływ na 13 ścieżek. 10 z tych ścieżek charakteryzuje również wyniki pierwszego porównania (podwyższona dawka kombinacji vs standardowa dawka cholekalcyferolu), a kolejne dwie występują w wynikach drugiego porównania (podwyższona dawka kombinacji vs podwyższona dawka cholekalcyferolu). Według wskaźnika FDR, wśród szlaków wspólnych dla przeprowadzonych porównań,

najbardziej istotnie zmienione zostały szlaki sygnałowe regulujące pluripotencję komórek macierzystych, szlak sygnałowy estrogenu, szlak sygnałowy TGF-β i proteoglikany w nowotworach. w grupie tej możemy wyróżnić również ścieżki charakterystyczne dla witaminy D (np. metabolizm ksenobiotyków przez cytochrom P450). Warto również dodać, że większość zidentyfikowanych ścieżek łączona jest bezpośrednio lub pośrednio z procesem powstawania nowotworów. Powodem tego może być fakt, że badania onkologiczne stanowią zdecydowaną większość wszystkich badań nad miRNA.

Wyniki analizy wskazują, że na szlak sygnałowy regulujący pluripotencję komórek macierzystych kombinacja cholekalcyferolu i kalcydiolu wpływa poprzez miR-340, miR-381 i miR-148. Jednym z celów miR-340 jest *Sox2*, a miR-148 gen *Klf4*. Oba geny należą do subsatelitarnej sieci transkrypcyjnej, która aktywuje przeprogramowanie komórek somatycznych z powrotem do stanu pluripotencjalnego. Ponadto uważa się, że oba geny mają kluczowe znaczenie dla mechanizmów proliferacji komórek nowotworowych (Hadjimichael i wsp., 2015). Innym interesującym pod tym względem szlakiem jest szlak sygnałowy TGF- β. Analiza funkcjonalna wykazała, że szlak ten był regulowany przez zmiany w ekspresji miR-381, miR-101, miR-148 i miR-340. Geny *SMAD* (*SMAD2, SMAD9, SMAD5, SMAD4*), które są genami regulowanymi przez wymienione miRNA, modulują proliferację, apoptozę, a także różnicowanie i migrację komórek (Sun i wsp., 2022). Jedno z badań dowiodło, że witamina D, poprzez wpływ na szlak TGF-β, zapobiega indukowanej przez komórki nowotworowe apoptozie komórek zapalnych (Moz i wsp., 2020). Co więcej wykazano, że witamina D hamuje indukowane przez komórki nowotworowe uwalnianie czynnika martwicy nowotworów alfa (TNF-α) i zmniejsza wewnątrzkomórkowy poziom transformującego czynnika wzrostu beta (TGF-β) (Moz i wsp., 2020).

Z kolei ścieżka sygnalizacji estrogenowej, została aktywowana w szczególności przez zmianę ekspresji miR-96, a następnie miR-181a, miR-181b i miR-193a. Działanie kalcydiolu związane z regulacją syntezy i sygnalizacji estrogenów może determinować przeciwnowotworowe działanie tego metabolitu (Bhattarai i wsp., 2020; Krishnan i wsp., 2010). Ponadto stwierdzono, że kalcydiol hamuje ekspresję *COX2* (Prostaglandin-Endoperoxide Synthase 2) i zwiększa ekspresję *15-PGDH* (15-Hydroxyprostaglandin Dehydrogenase), zmniejszając w ten sposób syntezę prostaglandyn, czyli mediatorów stanu zapalnego (Krishnan i wsp., 2010).

Wśród ścieżek, na które wpłynęła kombinacja cholekalcyferolu i kalcydiolu na szczególną uwagę zasługuje również szlak związany z proteoglikanami w nowotworach. Wykorzystana kombinacja wpłynęła na tę ścieżkę za pośrednictwem miR-381, miR-96, miR-148 oraz miR-340. Istotne jest to, iż porównanie efektów suplementacji tej samej dawki cholekalcyferolu i cholekalcyferolu w kombinacji z kalcydiolem wykazało wpływ na biosyntezę glikosfingolipidów oraz degradację glikanów. Znaczący wpływ na biosyntezę glikanów warunkowany jest zmianą ekspresji miR-205. z kolei zmiany w degradacji glikanów warunkowane są dodatkowo przez wpływ na ekspresję miR-125 i miR-148. Glikany biorą udział w angiogenezie, a także w proliferacji, adhezji i migracji komórek, tym samym znacząco wpływając na biologię różnych typów nowotworów (Zhou i wsp., 2020; Park i wsp., 2020; Lenh i wsp., 2018). Co ciekawe, potencjalny związek między witaminą D a proteoglikanami został również podkreślony w innym eksperymencie. Badanie przeprowadzone przez D'arrigo i wsp. wykazało, że aktywacja receptora witaminy D (VDR) poprzez zastosowanie parykalcytolu (syntetycznego analogu witaminy D) podnosi poziom jednego z proteoglikanów - trombomoduliny (TM), co z kolei poprawia funkcję śródbłonka (D'arrigo i wsp., 2019). Przytoczone wyniki sugerują, że suplementacja cholekalcyferolem i kalcydiolem może modulować mikrośrodowisko komórkowe poprzez wpływ na syntezę i metabolizm glikanów. Opierając się na powyższych wnioskach, postanowiono przetestować potencjał witaminy D w regulacji metabolizmu glikoprotein. w tym celu wybrano dwa geny - *NEU1* i *FUT1* - które są zaangażowane w metabolizm glikoprotein i które są celami miRNA zmienionych pod wpływem kombinacji cholekalcyferolu i kalcydiolu (miR-125b, miR-205). Pierwszy z wybranych genów - *NEU1* koduje neuraminidazę1. Enzym ten odgrywa ważną rolę w różnych procesach biologicznych, takich jak rozpoznawanie komórek, adhezja, sygnalizacja komórkowa, a także degradacja cząsteczek glikoprotein. Drugi gen - *FUT1* koduje enzym (fukozylotransferaze 1), który bierze udział w procesie dodawania cukrów fukozylowych do glikanów na powierzchni komórek. Przeprowadzone badanie qPCR nie wykazało jednak statystycznie istotnych różnic w ekspresji genów *NEU1* oraz *FUT1* pod wpływem kombinacji cholekalcyferolu i kalcydiolu.

Zidentyfikowane miRNA mogą potencjalnie wpływać na dziesiątki genów. Dlatego też przedstawione powyżej wyniki dostarczają cennych informacji i bodźca do dalszych badań, w tym wyników profilowania mRNA, analizy metylacji DNA i badań klinicznych. Na podstawie uzyskanych wyników można stwierdzić, że

suplementacja zwiększoną dawką kombinacji cholekalcyferolu i kalcydiolu powoduje wiele istotnych zmian w profilu miRNA płuc świń w porównaniu do standardowej suplementacji cholekalcyferolem. Rezultaty analizy funkcjonalnej sugerują, że kombinacja cholekalcyferol+kalcydiol może wpływać na procesy nowotworzenia między innymi poprzez modulację szlaku TGF- β oraz szlaków związanych z metabolizmem i syntezą glikanów. Jednocześnie ustalono, że zwiększenie poziomu witaminy D poprzez suplementację kalcydiolu, z pominięciem przemian enzymatycznych w wątrobie, ma znikomy wpływ na ekspresję miRNA w płucach.

4.4. Publikacja IV

Changes in DNA Methylation and mRNA Expression in Lung Tissue after Long-Term Supplementation with an Increased Dose of Cholecalciferol.

4.4.1. Materiały i metody

- Zwierzęta i żywienie

W badaniu wykorzystano próbki płuc pochodzących od świń PBZ x WBP pokrytych knurem duroc x pietrain. Wszystkie procedury zawarte w eksperymencie, związane z wykorzystaniem żywych zwierząt zostały zatwierdzone przez lokalną Komisję Etyczną do spraw Doświadczeń na Zwierzętach w Krakowie (Uchwała nr 427/2020 z dnia 22.07.2020 r.). Zwierzęta biorące udział w eksperymencie były utrzymywane w Stacji Badawczej Instytutu Zootechniki Państwowego Instytutu Badawczego w Grodźcu Śląskim. Zwierzęta zostały losowo podzielone na dwie grupy żywieniowe (n=12). Każda z grup zawierała taką samą liczbę samic (n=6) i samców (n=6), jednakże samce wykorzystane w eksperymencie zostały uprzednio wykastrowane. Dieta zwierząt różniła się jedynie dawką witaminy D.

Dawkowanie witaminy D oraz liczba osobników w grupach były następujące:

1 grupa (grupa kontrolna) 2000 j.m. cholekalcyferolu (grower) i 1500 j.m. cholekalcyferolu/kg paszy (finiszer)

- 2 grupa 3000 j.m. cholekalcyferolu (grower) i 2500 j.m. cholekalcyferolu/kg paszy (finiszer)

Wszystkie zwierzęta utrzymywane były w indywidualnych kojcach wyściełanych słomą. w kojcach panowały jednolite warunki środowiskowe. Masę ciała świń mierzono na początku eksperymentu, a następnie, co dwa tygodnie. w żywieniu zwierząt wykorzystano dwie mieszanki paszowe zgodnie z ich aktualnymi potrzebami (I okres tuczu - grower - 30-60 kilogramów, II okres tuczu - 60-110 kilogramów - finiszer). Dieta została opracowana tak, aby pokryć wszystkie potrzeby żywieniowe zwierząt (GROWER MIX: energia metaboliczna - 13,3 MJ, białko całkowite - 172 g/kg; FINISHER MIX: energia metaboliczna - 13,3 MJ, białko całkowite - 156 g/kg). Tucz doświadczalny trwał w przybliżeniu od 30 do 110 kilogramów żywej masy zwierząt. Pod koniec doświadczenia wszystkie świnie zostały poddane ubojowi przy użyciu kleszczy elektrycznych wysokiego napięcia (240-400 V). Natychmiast po uboju od wszystkich zwierząt pobrano próbki krwi i tkanki płuc.

Próbki płuc pobrano ze środkowych części górnych płatów lewego płuca. Tkanki te przewieziono do laboratorium w ciekłym azocie, następnie przechowywano je w temperaturze -85 °C. Krew do pomiaru stężenia kalcydiolu pobrano do probówek z antykoagulantem ACD (cytrynian kwaśnej dekstrozy/glukoza). Próbki krwi przechowywano w temperaturze +6°C do momentu pozyskania osocza. Osocze pozyskano poprzez wirowanie krwi w wirówce gradientowej (3000 rpm). Następnie przechowywano je w temperaturze -20°C.

- Pomiar stężenia 25(OH)D

Całkowitego stężenia witaminy D w osoczu zostało oznaczone w 16 próbkach (8 próbek z każdej grupy) przez firmę zewnętrzną (ANCHEM Laboratorium, ul. Fredry 20, Katowice, Polska). Zamrożone próbki osocza zostały przesłane do laboratorium z zachowaniem równomiernej temperatury podczas transportu. Oznaczenie stężenia witaminy D w osoczu wykonano metodą RIA (oznaczenia radioimmunologiczne).

- Izolacja DNA i RNA, konstrukcja bibliotek RRBS i mRNA oraz sekwencjonowanie

DNA z 16 próbek płuc (8 z każdej grupy, 4 samice i 4 samce) wyizolowano przy użyciu zestawu Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Ocenę ilościową wyizolowanego materiału określono przy użyciu spektrofotometrów NanoDrop™ 2000/2000c (Thermo Scientific™, Waltham, MA, USA). Biblioteki do RRBS (Reduced Representation Bisulfite Sequencing, methyl-seq) zostały przygotowane przy użyciu zestawu Ovation® RRBS Methyl-Seq System 1-16 (Tecan, San Jose, CA, USA). Ocenę ilościową uzyskanych bibliotek przeprowadzono wykorzystując urządzenie Qubit (Ther-mo Scientific™, Foster City, Kalifornia, USA). Sekwencjonowanie bibliotek wykonano przy pomocy zewnętrznej firmy Medical Research Foundation NGS Core (USA) wykorzystując urządzenie Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA). Długość odczytów wynosiła 150 bp. Podczas sekwencjonowania wykorzystano kontrolę PhiX.

RNA wyizolowano z 10 próbek płuc (5 próbek z grupy 1 i 5 próbek z grupy 2, wyłącznie samice) przy użyciu zestawu PureLink™ RNA Mini Kit (Invitrogen, Waltham, MA, USA). Wyizolowany materiał genetyczny poddano ocenie ilościowej i jakościowej przy użyciu NanoDrop™ 2000/2000c (Thermo Scientific™, Waltham, MA, USA) oraz Tapestation 2200 (Agilent, Santa Clara, CA, USA). Wysokiej jakości

próbki RNA (RNA Integrity Number >/= 7) zostały wykorzystane do przygotowania bibliotek przy użyciu zestawu QuantSeq 3′mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Wiedeń, Austria). Ocenę ilościową przygotowanych bibliotek przeprowadzono przy użyciu Qubit (Thermo Scientific™, Waltham, MA, USA), natomiast ocenę jakościową na urządzeniu Tapestation 2200 (Agilent, Santa Clara, CA, USA). Sekwencjonowanie mRNA (pojedynczy odczyt 75 bp) przeprowadzono wykorzystując Nextseq 550 (Illumina, San Diego, CA, USA) w Instytucie Zootechniki Państwowym Instytucie Badawczym. Pula bibliotek została przygotowana zgodnie z standardową metodą normalizacji z protokołu NextSeq 500 i NextSeq 550 Sequencing Systems-Denature and Dilute Libraries Guide. Do sekwencjonowania użyto pulę bibliotek o stężeniu 2 nM i kontrolę PhiX.

- Badnie qPCR

Do analizy qPCR wykorzystano 20 próbek (po 10 próbek z grupy 1 i 2, 5 samic i 5 samców w grupie). W celu rozszerzenia grupy badawczej wykonano izolację RNA z 10 dodatkowych próbek (5 z grupy 1 i 5 z grupy 2). Następnie RNA z wszystkich 20 próbek poddano odwrotnej transkrypcji przy użyciu zestawu High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Waltham, MA, USA). Przeprowadzono badanie qPCR genów *KLHL3*, *TTPA*, *UGT1A6* i *ITIH2* wykorzystując kontrole endogenną *RPS29*. Geny zostały wybrane na podstawie wyników sekwencjonowania RRBS i mRNA. PCR w czasie rzeczywistym przeprowadzono przy użyciu TaqMan ™ Fast Advanced Master Mix for qPCR (Applied Biosystems ™, Waltham, MA, USA) i testów TaqMan Real-Time PCR na QuantStudio ™ 7 Flex Real-Time PCR System (Applied Biosystems ™, Waltham, MA, USA) . Uzyskane dane analizowano w Thermo Fisher Cloud (Thermo Scientific ™, Waltham, MA, USA) oraz przy użyciu oprogramowania SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

- Analiza statystyczna wyników sekwencjonowani
  - ★ Methyl-Seq

Pierwszym etapem analizy danych była kontrola jakości surowych odczytów sekwencjonowania przy użyciu oprogramowania FastQC v. 0.12.1. Odczyty o niskiej jakości (poziom jakości <20 i długość odczytu <36) oraz fragmenty zawierające sekwencje adapterowe zostały odfiltrowane przy użyciu oprogramowania FlexBar v. 3.5.0. Dopasowanie do genomu referencyjnego świni (*Sscrofa*11.1) przeprowadzono przy użyciu oprogramowania bisulfitowego-BSMAP v. 2.9.0, z domyślnymi opcjami

zalecanymi dla danych RRBS. Oprogramowanie Methylation Caller dostarczone w pakiecie BSMAP v. 2.9.0 zostało użyte do określenia procentu metylacji w poszczególnych miejscach CpG. Analiza metylacji CpG obejmowała rozkład w chromosomach świń oraz rozkład w regionach upstream, 5′-UTR, 3′-UTR, eksonach, intronach i regionach międzygenowych. Następnie pliki zostały przetworzone przy użyciu pakietu R (wersja 4.3) W celu uzyskania danych wejściowych dla oprogramowania Methylkit. Oprogramowanie Methylkit v. 1.26.0 zostało użyte do identyfikacji miejsc różnie metylowanych (DMS- Differential Methylation Sites) z wartościami odcięcia, co najmniej 25% różnic metylacji między dwiema grupami i wartościami q <0,05. Adnotacje genów uzyskano z adnotacji Sscrofa11.1 Ensembl GTF.

★ mRNA-Seq

Zdemultipleksowane pliki fastq pobrane z serwera sekwencjonowania zostały poddane kontroli jakości, przycięte i zmapowane przy użyciu oprogramowań FastQC 11.8, FLEXBAR 3.5.0 i TopHat 2.1.1. Do oceny statystyk mapowania i liczby odczytów wykorzystano Samtools 1.9, RSeQC, oprogramowanie HTSeq-count 0.11.1 i adnotację Gtf-Ensembl 96. Następnie do przeprowadzenia analizy ekspresji różnicowej wykorzystano program R i pakiet oprogramowania DESeq 2. Za geny o różnicowej ekspresji uznano te o wartościach q <0,05 (FDR, ang. False Discovery Rate), korektach Benjamini-Hochberg (BH) i bez progów krotności zmian. Do dalszych analiz wykorzystano tylko te geny, które wykazywały base mean większy niż 20. Adnotacje genów uzyskano z adnotacji *Sscrofa11.1* Ensembl GTF.

★ Integracja wyników methyl-seq i mRNA-seq

W celu integracji wyników wykorzystano zbiory danych methyl-seq i RNA-seq, aby powiązać zmiany metylacji miejsc CpG ze zmianami poziomów ekspresji genów. Do tego porównania wykorzystano program Venny 2.1.

- Analiza funkcjonalna

Przeprowadzono analizę funkcjonalną wyników metyl-seq, wyników mRNA-seq i połączonych danych. Geny regulowane w górę/hipermetylowane i regulowane w dół/hipometylowane zostały wykorzystane do wspólnych (GSA- Gene Set Analysis) i oddzielnych analiz. Analizę funkcjonalną przeprowadzono przy użyciu oprogramowania STRING (wersja 12.0) wykorzystując bazy Gene Ontology (GO) i Kyoto Encyclopedia of Genes and Genomes (KEGG). Dodatkowo podczas analizy

funkcjonalnej wykorzystano programy BioMart (wydanie 110) i Venny 2.1. Najbardziej istotne wyniki analiz (top 10 zmienionych procesów) wybrano na podstawie wartości FDR (ang. False Discovery Rate) i sile zidentyfikowanych wzbogaceń (ang. enrichment score).

4.4.2. Wyniki i dyskusja

Celem badania było porównanie efektu standardowej i podwyższonej dawki cholekalcyferolu na transkryptom i metylom zdrowej tkanki płuc świń. Wyniki sugerują, że wyższa dawka cholekalcyferolu powoduje znaczące zmiany w transkryptomie i metylomie płuc. Wyniki sekwencjonowania mRNA (mRNA-seq) wskazują, bowiem, że zwiększone spożycie witaminy D wpłynęło na ekspresję 195 genów, a większość, bo 86.15% z nich uległo obniżeniu ekspresji. Ponadto, bazując na wynikach RRBS, zidentyfikowano 1116 różnie metylowanych miejsc związanych z genami i ich miejscami regulatorowymi (DMS), 56.27% z nich uległo hipermetylacji. Adnotacja DMS zgodna z cechami genu ujawniła, że większość, bo 47.17% miejsc o zmienionej metylacji znajdowało się w intronach. Z kolei DMS w kodujących częściach genu stanowiły 5.24%.

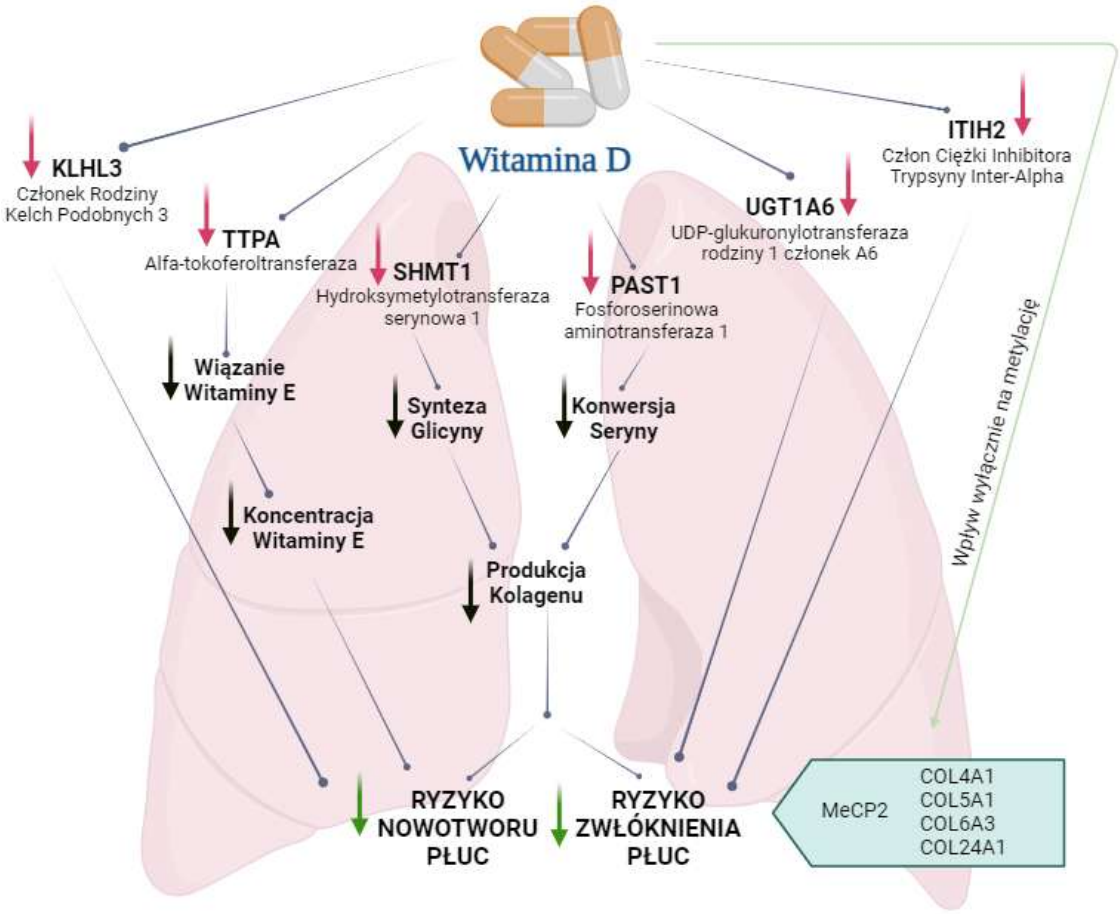
Analiza funkcjonalna wyników methyl-seq wykazała, że zwiększone spożycie witaminy D wpłynęło najistotniej na procesy związane z GTPazą, cytoszkieletem i aktyną. Co ciekawe, inni badacze ustalili, że spadek nasilenia wirusowej choroby płuc łączy się ze zmianami metylacji genów związanych właśnie z GTPazą i aktyną (Alkafaas i wsp., 2023). Z kolei analiza funkcjonalna wyników mRNA-seq wyszczególniła wpływ zwiększenia dawki cholekalcyferolu na ścieżki biologiczne związane z kaskadami dopełniacza i krzepnięcia oraz chemiczną kancerogenezą. Wykryto znaczące obniżenie ekspresji genów kodujących białka ostrej fazy (np. *FBG*, *FGA* i *FGG*) oraz genów związanych z nowotworami (np. *SERPINC1* i *F2*). Dla przykładu, *SERPINC1* jest uznawany za kluczowy gen w procesach proliferacji i migracji komórek nowotworowych, a obniżenie jego ekspresji może być skutecznym sposobem leczenia raka płuc (Zhang i wsp., 2022; Wang i wsp., 2019).

Integralna analiza wyników mRNA-seq oraz methyl-seq wyłoniła 11 genów o zróżnicowanej ekspresji oraz metylacji pod wpływem zwiększonej dawki cholekalcyferolu. w przypadku genów *HSD17B6*, *CYP3A22*, *TTPA*, *SHMT1*, *MIPEP*, *PSMA1*, *HDLBP*, *KLHL3*, *BHMT* i *UGT1A6* zmiana metylacji nastąpiła w intronach. Gen *ITIH2* charakteryzował się zmianą metylacji w kodującej części genu.

Przeprowadzone dodatkowo badanie qPCR potwierdziło rzetelność uzyskanych wyników w szerszej kohorcie obejmującej samice (n=10) i samce (n=10). W badaniu tym wykorzystano geny *ITIH2*, *KLHL3*, *TTPA* oraz *UGT1A6*. W przypadku wszystkich wymienionych zaobserwowano znaczny spadek ekspresji pod wpływem zwiększonej dawki witaminy D, co jest zgodne z wynikami sekwencjonowania. Jednakże spadek ekspresji genu *KLHL3* nie był statystycznie istotny *(p-value*=0,076).

Rezultaty analizy funkcjonalnej, ze szczególnym uwzględnieniem panelu 11 genów wspólnych dla wyników sekwencjonowania metylomu i mRNA wskazują na przeciwnowotworowe i przeciwzwłóknieniowe działanie zwiększonej dawki cholekalcyferolu (Rycina 3.). Świadczą o tym przede wszystkim zmiany w ekspresji genów *PSAT1* i *SHMT1*. Wyniki badania *in vitro* i *in vivo* przeprowadzonego na myszach wskazują, że *PSAT1* jest silnym promotorem zwłóknienia płuc, a *VDR* reguluje ekspresję tego genu (Zhu i wsp., 2021). Co więcej, badanie *in vitro* na ludzkich liniach komórkowych wykazało, że wyciszenie genu *PSAT1* skutkuje zahamowaniem proliferacji i wzrostu guza w niedrobnokomórkowym raku płuc (Yang i wsp., 2015). Gen *PSAT1* odgrywa ważną rolę w biosyntezie aminokwasów. Obniżona ekspresja *PSAT1* hamuje syntezę seryny, a w konsekwencji glicyny. Glicyna z kolei, jest kluczowym składnikiem kolagenu, a jej nieprawidłowa produkcja wiąże się zarówno zwłóknieniem płuc, jak i powstaniem zmian nowotworowych w tym narządzie. Zaobserwowano, że zwiększona dawki cholekalcyferolu powoduje znaczny spadek ekspresji *PSAT1.* Jednocześnie zaobserwowano wpływ zwiększonej dawki cholekalcyferolu na szlak glikolizy i syntezy aminokwasów, w tym glicyny i seryny. Co więcej, na przeciw zwłóknieniowe i przeciwnowotworowe działanie witaminy D wskazuje również obniżenie ekspresji i hipermetylacja w obrębie genu *SHMT1*. Gen *SHMT1* koduje hydroksymetylotransferazę serynową 1, czyli enzym niezbędny do przekształcania seryny w glicynę (Zhu i wsp., 2021). Bazując na tych odkryciach można stwierdzić, że wpływ witaminy D na hamowanie produkcji kolagenu w płucach jest dwukierunkowy: po pierwsze, poprzez hamowanie syntezy seryny, czyli materiału niezbędnego do tworzenia kolagenu, a po drugie, poprzez obniżenie ekspresji enzymu bezpośrednio zaangażowanego w syntezę tego białka. Co więcej, wyniki niniejszego badaniu sugerują, że zwiększona dawka cholekalcyferolu powoduje zmiany poziomu metylacji w intronie genu *MeCP2*. Gen ten uważany jest za kluczowy regulator procesu włóknienia (O'Reilly, 2017). Wykazano na przykład, że myszy *MeCP2* KO (knockout/dezaktywacja genu) są odporne na zwłóknienie płuc (Hu i wsp., 2011). Dodatkowo, rezultaty badania *in vitro* sugerują, że witamina D,

poprzez wpływ na TGFβ1, może hamować polimeryzację α-aktyny w mięśniach gładkich oraz produkcję fibronektyny i kolagenu w fibroblastach (Ramirez i wsp., 2010). Efekt ten potwierdzać mogą również istotne zmiany metylacji czterech genów kodujących kolagen (*COL4A1, COL5A1, COL6A3 i COL24A1*).



Rycina 3. Potencjalne cele cholekalcyferolu odpowiadające za przeciwnowotworowe i przeciwzwłóknieniowe właściwości związane z jego zwiększonym spożyciem.

Dodatkowo, wykazano, że gen UGT1A6, który prezentował zmiany w ekspresji i metylacji, również jest istotnie związany z rozwojem zwłóknienia płuc. Gen ten ulega zmianie ekspresji u pacjentów z idiopatycznym włóknieniem płuc w porównaniu do zdrowych osób (Qian i wsp., 2022). Co więcej, zwiększona ekspresja UGT1A6 jest również charakterystyczna dla pacjentów z nowotworami, w tym rakiem płuc (Li i wsp., 2020; Cengiz i wsp., 2015). UDP-glukuronozylotransferazy to grupa enzymów związanych z katabolizmem leków i ksenobiotyków. Dlatego uważa

się, że zmiana ekspresji genów UGT1A, w tym UGT1A6, może znacząco modulować odpowiedź na leczenie i progresję raka (Cengiz i wsp., 2015). Obniżenie ekspresji tego genu pod wpływem suplementacji witaminą D (kalcytriolem) zostało już zaobserwowany przez innych badaczy w tkankach wątroby szczurów (Doan i wsp., 2020).

Wpływ witaminy D na procesy związane ze zwłóknieniem płuc i rakiem sugeruje również obniżenie ekspresji i hipermetylacja w obrębie promotora genu *ITIH2* (*SHAP*). Gen *ITIH2* jest związany z grupą inhibitorów seryny. Gen ten może być zaangażowany w kontrolę stanu zapalnego i procesów immunologicznych (Hamm i wsp., 2008). Możliwe jest, że zdolność do regulacji angiogenezy może łączyć *ITIH2* z procesem włóknienia płuc. Badanie przeprowadzone przez Garantziotisa i i in. wykazało, że indukowane uszkodzenie płuc spowodowało ponad sześciokrotny wzrost ekspresji *ITIH2* w wątrobie i spadek jego ekspresji w płucach (Garantziotis i wsp., 2008). Podobnie, w nowotworach płuc, *ITIH2* wykazuje znaczną redukcję ekspresji w zmienionych tkankach (Hamm i wsp., 2008). ITIH2 jest częścią białka IaI, które jest syntetyzowane w wątrobie. IaI składa się z łańcucha lekkiego i dwóch łańcuchów ciężkich (ITIH1 i ITIH2). Uważa się, że IaI jest czynnikiem ogólnoustrojowym, który wzmacnia angiogenezę. Badania histologiczne wykazały silną kolokalizację białka inhibitora inter-α-trypsyny (IaI) w ogniskach chorobowych u pacjentów ze zwłóknieniem płuc. w przeciwieństwie do tego, płuca zdrowych osób były słabo wybarwione na obecność IaI (Garantziotis i wsp., 2008). Przytoczone wyniki wskazują na wyższa ekspresję białka IaI oraz obniżoną ekspresję genu *ITIH2* w płucach chorych na zwłóknienie. Ustalenia te oraz rezultaty niniejszej pracy sugerują inną, niezwiązaną z IaI, rolę genu *ITIH2* w tkance płuc.

Z kolei na przeciwnowotworowe właściwości zwiększonej dawki cholekalcyferolu wskazuje także obniżona ekspresja i hipermetylacja genu *KLHL3* (kelch-like family member 3). Wiele sugeruje, że gen *KLHL3* jest powiązany z rozwojem raka. Odkryto na przykład obniżenie ekspresji genu *KLHL3* w próbkach osocza pacjentów z rakiem płuc (Zhang i wsp., 2022). Brakuje jednak ustaleń dotyczących ekspresji i regulacji epigenetycznej tego genu w samej tkance płuc. Badania przedstawiające ekspresję genu *KLHL3* w tkankach nowotworowych wykazały, że może on ulegać zarówno nadekspresji, jak i obniżeniu ekspresji pod wpływem choroby (Lin i wsp., 2022). Niemniej jednak rola *KLHL3* w patogenezie nowotworów powstałych w wyniku interakcji wirusowej wydaje się być dobrze

udowodniona. Naukowcy odkryli, że *KLHL3* pośredniczy w infekcji i replikacji KSHV indukującej nowotworzenie (Qi i wsp., 2022).

Innym interesującym wynikiem niniejszego badania był również znaczący spadek ekspresji genu *TTPA* (białka przenoszącego alfa-tokoferol). TTPA wiąże biologicznie aktywną formę witaminy E (α-tokoferol) i odgrywa ważną rolę w regulacji poziomu tej witaminy w organizmie. Ze względu na silne właściwości przeciwutleniające, przeprowadzono liczne badania w celu ustalenia, czy suplementacja witaminą E może chronić przed rakiem. Co interesujące, pierwsze badanie kliniczne przeprowadzone na dużej grupie mężczyzn palących tytoń wykazało o 2% wyższą śmiertelność w grupie osób przyjmujących witaminę E w porównaniu do grupy osób niesuplementowanych (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994). Brak ochronnego wpływu suplementacji witaminą E w chorobach nowotworowych i sercowo-naczyniowych potwierdzono również w innym badaniu klinicznym przeprowadzonym na grupie zdrowych kobiet (Lee i wsp., 2005). Zaobserwowana zmiana ekspresji genu *TTPA* pod wpływem witaminy D wskazuje na możliwą interakcję pomiędzy jednoczesną suplementacją diety witaminami D i E, co wydaje się być ciekawym kierunkiem kolejnych badań.

Podsumowując, wyniki sugerują, że zwiększenie spożycia cholekalcyferolu może okazać się korzystane zarówno z punktu widzenia dobrostanu zwierząt jak i zdrowia ludzi. Wyniki wskazują, że zwiększone spożycie witaminy D może regulować produkcję kolagenu i hamować proces zwłóknienia płuc między innymi poprzez wpływ na ekspresję genów *SHMT1*, *UGT1A6* i *ITIH2*. Co więcej, zmiana ekspresji tych genów oraz genów *KLHL3* i *TTPA* wskazuje na przeciwnowotworowe właściwości zwiększonej dawki cholekalcyferolu. Uzyskane wyniki sugerują również, że witamina D może wpływać na poziom i działanie witaminy E. Prawdopodobnym mechanizmem epigenetycznym stającym za wszystkimi wymienionymi wyżej właściwościami zwiększonej dawki cholekalcyferolu jest metylacja DNA.

5. Wnioski

Analiza literatury oraz wyniki badań z zakresu transkryptomiki i epigenetyki umożliwiły sformułowanie następujących wniosków:

- Dane literaturowe pokazują, że niedobór witaminy D w osoczu dotyczy znacznie częściej płci żeńskiej, równocześnie to samice posiadają większy potencjał akumulacji witaminy D w komórkach. Ustalono również, że istnieje szereg czynników mogących powodować międzypłciowe różnice w poziomie i działaniu witaminy D. Wśród nich wymienić należy przede wszystkim różnice w ekspresji receptora witaminy D, genów metabolizujących witaminę D oraz białka transportującego witaminę D.
- Długotrwała suplementacja zwiększoną dawką cholekalcyferolu oraz zwiększoną dawką kombinacji cholekalcyferolu i kalcydiolu nie wpływa znacząco na ekspresję mRNA w tkance mięśniowej świń. Całkowite zastąpienie cholekalcyferolu kalcydiolem również wydaje się nie wywierać istotnego wpływu na ekspresję mRNA w tej tkance.
- Długotrwała suplementacja zwiększoną dawką cholekalcyferolu zmienia ekspresję miR-215, a zastąpienie cholekalcyferolu kalcydiolem nie wywołuje żadnych zmian w ekspresji miRNA w tkance płuc. Z kolei, zastosowanie podwyższonej dawki kombinacji cholekalcyferolu i kalcydiolu powoduje wiele istotnych zmian w profilu miRNA tej tkanki. Zidentyfikowane zmiany mogą wykazywać szerokie spektrum oddziaływań. Jednakże szczególnie istotny wydaje się wpływ kombinacji na szlak TGF-β oraz szlaki związane z metabolizmem i syntezą glikanów. Efekt ten sugeruje, że zwiększona dawka witaminy D w postaci cholekalcyferolu i kalcydiolu może regulować procesy związane z powstawaniem nowotworów.
- Długotrwała suplementacja podwyższoną dawką cholekalcyferolu wpływa znacząco na profil metylacji oraz mRNA tkanki płuc świń. Ustalono, że zwiększone spożycie cholekalcyferolu może regulować produkcje kolagenu oraz hamować proces zwłóknienia płuc. Ponadto wiele wskazuje, że tego typu suplementacja posiada właściwości przeciwnowotworowe. Co ciekawe, ustalono także, że zwiększona dawka cholekalcyferolu może regulować poziom tokoferolu, czyli witaminy E.

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Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr hab. Maria Oczkowicz prof. IZ
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., & Oczkowicz, M. (2022).* ***Sex differences in vitamin D metabolism, serum levels and action****. The British journal of nutrition, 128(11), 2115–2130.*, mój udział polegał na:
konceptualizacji oraz korekcie manuskryptu

Maria Oczkowicz
(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr hab. Maria Oczkowicz prof. IZ
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Świątkiewicz, M., Tyra, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Effect of different doses of cholecalciferol and calcidiol on meat quality parameters and skeletal muscle transcriptome profiles in swine****. Meat science, 197, 109071.*, mój udział polegał na:
konceptualizacji, nadzorze nad analizami
oraz korekcie manuskryptu

Maria Oczkowicz
(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr inż Tomasz Szmatoła
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Świątkiewicz, M., Tyra, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Effect of different doses of cholecalciferol and calcidiol on meat quality parameters and skeletal muscle transcriptome profiles in swine****. Meat science, 197, 109071.*, mój udział polegał na:
analizy bioinformatyczne, wizualizacja danych,
korekta manuskryptu

Tomasz Szmatoła

(czytelny podpis współautora)

Oświadczenie współautora

Bolice, 11 marca 2024
Miejscowość i data

Imię i nazwisko prof. Małgorzata Świątkiewicz
Afiliacja Zakład Żywienia Zwierząt i Pasiemnictwa
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Świątkiewicz, M., Tyra, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Effect of different doses of cholecalciferol and calcidiol on meat quality parameters and skeletal muscle transcriptome profiles in swine****. Meat science, 197, 109071.*, mój udział polegał na:
Koncepcja i opracowanie metodyki badań na zwierzętach.

Małgorzata Świątkiewicz
(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr hab. Mirosław Tyra prof. IZ
Afiliacja Zakład Hodowli Trady Chlewnej
Instytut Zootechniki PIB
ul. Krakowska 1
32-083 Balice

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Świątkiewicz, M., Tyra, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Effect of different doses of cholecalciferol and calcidiol on meat quality parameters and skeletal muscle transcriptome profiles in swine****. Meat science, 197, 109071.*, mój udział polegał na:
konsultacje odnośnie metod oceny jakości mięsa oraz korekta merytoryczna manuskryptu w tym zakresie

(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr hab. Maria Oczkowicz prof. IZ
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Pawlina-Tyszko, K., Świątkiewicz, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Changes in miRNA expression in the lungs of pigs supplemented with different levels and forms of vitamin D****. Molecular biology reports, 51(1), 8.*, mój udział polegał na:
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Maria Oczkowicz

(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr inż. Tomasz Szmatoła
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Pawlina-Tyszko, K., Świątkiewicz, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Changes in miRNA expression in the lungs of pigs supplemented with different levels and forms of vitamin D****. Molecular biology reports, 51(1), 8.*, mój udział polegał na:
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Tomasz Szmatoła
(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko prof. dr hab Małgorzata Świątkiewicz
Afiliacja Zakład Żywienia Zwierząt i Paszoznawstwa
Instytut Zootechniki PIB

Oświadczenie

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Małgorzata Świątkiewicz
(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 III 2024 r.
Miejscowość i data

Imię i nazwisko KLAUDIA PAWLINA-TYSZKO

Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Pawlina-Tyszko, K., Świątkiewicz, M., Szmatoła, T., & Oczkowicz, M. (2023).* ***Changes in miRNA expression in the lungs of pigs supplemented with different levels and forms of vitamin D****. Molecular biology reports, 51(1), 8.*, mój udział polegał na:
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Klaudia Pawlina-Tyszko
(czytelny podpis współautora)

Oświadczenie współautora

Bolice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr hab. Maria Oczkowicz prof. IZ
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Semik-Gurgul, E., Świątkiewicz, M., Szmatoła, T., Steg, A., & Oczkowicz, M. (2023).* ***Changes in DNA Methylation and mRNA Expression in Lung Tissue after Long-Term Supplementation with an Increased Dose of Cholecalciferol****. International journal of molecular sciences, 25(1), 464.*, mój udział polegał na:
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Maria Oczkowicz
(czytelny podpis współautora)

Oświadczenie współautora

Balice, 11 marca 2024
Miejscowość i data

Imię i nazwisko dr inż. Tomasz Szmatoła
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

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Tomasz Szmatoła
(czytelny podpis współautora)

Oświadczenie współautora

Bolice, 11 marca 2024
Miejscowość i data

Imię i nazwisko prof. dr hab. Małgorzata Świątkiewicz
Afiliacja Zakład Żywienia Zwierząt i Paszoznawstwa
Instytut Zootechniki PIB

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Małgorzata Świątkiewicz
(czytelny podpis współautora)

Oświadczenie współautora

Miejscowość i data
Balice, 11.03.2024r.

Imię i nazwisko Dr inż. Ewelina Semik-Gurgul
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

Oświadczenie

Oświadczam, że w pracy: *Wierzbicka, A., Semik-Gurgul, E., Świątkiewicz, M., Szmatoła, T., Steg, A., & Oczkowicz, M. (2023).* ***Changes in DNA Methylation and mRNA Expression in Lung Tissue after Long-Term Supplementation with an Increased Dose of Cholecalciferol****. International journal of molecular sciences, 25(1), 464.*, mój udział polegał na:
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Ewelina Semik-Gurgul
(czytelny podpis współautora)

Oświadczenie współautora

Miejscowość i data
Balice, 12.03.2024

Imię i nazwisko mgr Anna Steg
Afiliacja Zakład Biologii Molekularnej Zwierząt
Instytut Zootechniki PIB

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Anna Steg
(czytelny podpis współautora)

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Sex differences in vitamin D metabolism, serum levels and action

Alicja Wierzbicka* and Maria Oczkowicz

Department of Animal Molecular Biology, National Research Institute of Animal Production, Balice, Krakow, 32-083, Poland

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Abstract

The ubiquity of vitamin D metabolising enzymes and vitamin D receptors in mammalian organisms suggests that vitamin D has pleiotropic effects. There are quite a few studies indicating the anticancer, cardioprotective and antidiabetic effects of vitamin D; however, the best-documented actions of vitamin D are the regulation of Ca–phosphate balance and its effect on immune function.

Vitamin D levels in organisms are modulated by many environmental and non-environmental factors. One potential factor that may influence vitamin D levels and effects is the sex of the individuals studied. This review focuses on the scientific evidence indicating different synthesis and metabolism of vitamin D in females and males, mainly from PubMed database sources. The article verifies the sex differences in vitamin D levels reported around the world. Moreover, the different effects of vitamin D on the musculoskeletal, cardiovascular, nervous and immune systems, as well as cancer in males and females, were discussed.

Most studies addressing sex differences in vitamin D levels and effects are observational studies with conflicting results. Therefore, carefully designed clinical trials and experiments on animal models should be carried out to determine the role of non-environmental factors that may differentiate vitamin D levels in females and males.

Key words: **Vitamin D: Sex: Sex differences: Deficiency: Nutrigenomics**

Until recently, the effect of vitamin D has been primarily associated with the regulation of Ca and phosphate balance of the body. However, vitamin D shows a broader range of activities since it affects both the skeletal and immune, cardiovascular, and endocrine systems[1]. Nevertheless, the range and exact effect of vitamin D action are still debatable. Currently, vitamin D deficiency is associated with many chronic ailments related to oxidative stress, inflammation and ageing[2]. A significant part of these disorders is civilisation diseases, such as obesity or diabetes[3,4]. The occurrences of vitamin D deficiency and civilisation diseases are strongly correlated with each other because both problems originate from a common source. The reason for their existence is the presently preferred – but far from natural – human lifestyle. Vitamin D status is a proxy for a healthy lifestyle; thus, it is difficult to assess whether significant deficiency in vitamin D is the cause or effect of illnesses like those mentioned above. However, vitamin D deficiency is a global issue, and the primary source of this deficiency is the widespread isolation of the skin from exposure to sunlight[5,6]. Since this phenomenon shows a progressive character, it is necessary to uncover precisely all mechanisms influencing vitamin D levels in the body. The selection of all the factors involved and thorough knowledge of the relationships between them will help to create principles for the effective and safe supplementation of vitamin D.

Numerous studies that have examined plasma 25(OH) vitamin D concentrations show significant differences between sexes[5–9]. Additionally, analysis of the National Health and Nutrition Examination Survey (NHANES) dataset indicates that vitamin D supplementation should be sex-dependent[10]. Moreover, a transcriptome study investigating blood samples of persons supplemented with vitamin D revealed that vitamin D regulates 3·2 times more genes in women than in men[11]. In addition, many studies assessing the effects of vitamin D on disease units have demonstrated different outcomes in men and women. Despite this, many analyses relating to the impact of vitamin D have not included sex as an important factor. Further, experimental animal studies usually use only one sex to increase the standardisation of results. However, as many studies have indicated, sex can be important in determining the effectiveness of vitamin D supplementation.

Abbreviations: ACA, anterior cerebral arteries;; AD, Alzheimer's disease;; BC, breast cancer;; CRC, colorectal cancer;; EAE, encephalomyelitis;; FGF23, fibroblast growth factor 23;; MS, multiple sclerosis;; PTH, parathyroid hormone;; T2DM, type 2 diabetes mellitus;; VDR, vitamin D receptor.

* **Corresponding author:** Dr A. Wierzbicka, email alicja.wierzbicka@iz.edu.pl

The goal of this article is to review scientific reports that illustrate the occurrence of sex differences related to the effects and levels of vitamin D in mammals.

This article consists of four main parts. The first part explains how vitamin D is synthesised and metabolised, and the second part outlines which factors modulate vitamin D levels in the body. The third section characterises the differences between males and females in blood 25(OH) vitamin D. The last and largest part of the article is devoted to sex differences in the effect of vitamin D on various disease entities.

Synthesis and regulation of vitamin D levels

Vitamin D possesses a secosteroid structure similar to that of steroids[12]. In general, the term 'vitamin D' is most often used in reference to the different isoforms and metabolites of vitamin D.

There are two forms of vitamin D: D_2 and D_3. Both are the result of non-enzymatic reactions involving solar radiation[13]. Yeast, fungi and plants synthesise vitamin D_2 or ergocalciferol. In those mentioned above, the substrate for the production of vitamin D_2 is ergosterol (i.e. a compound that is part of cell membranes that performs the same function as cholesterol in animal cells). Under the influence of UVB radiation (280–315 nm), vitamin D_2 is synthesised from yeast, fungi and plants from ergosterol. Vitamin D_3, or cholecalciferol, is synthesised in animal tissues in a two-step process. The substrate needed to produce vitamin D_3 is 7-dehydrocholesterol (7-DHC), whose level is regulated by 7-DHC reductase (DHCR7)[13]. Under the influence of UVB radiation, 7-DHC is converted in the skin of animals into previtamin D_3, which isomerises to vitamin D_3 (cholecalciferol l) as a result of the heat of the skin. The process of synthesising vitamin D_3 in animal skin takes several hours. Initial products in the form of ergocalciferol and cholecalciferol are not biologically active (Fig. 1).

Vitamin D_3, produced in the skin, binds to vitamin D transporting protein (DBP) in the bloodstream. DBP transports all vitamin D metabolites. Cholecalciferol and ergocalciferol from food and dietary supplements, after absorption from the intestines, enter the circulatory system, initially on chylomicrons, and then it is slowly transferred to DBP[1,14]. As a result, cholecalciferol and ergocalciferol can be absorbed directly by peripheral tissues (e.g. muscle). However, cholecalciferol and ergocalciferol, bound to DBP, go mainly to the liver, where the first stage of its metabolism takes place. In the liver, 25-hydroxylases-CYP2R1 and CYP27A1 convert cholecalciferol and ergocalciferol into 25(OH) vitamin D (calcidiol – 25(OH)D)[15]. CYP2R1 in humans exhibits the highest levels of expression in the liver and testis microsomal fraction[16]. 25(OH)D, resulting from 25-hydroxylation, is the primary circulating form of vitamin D. DBP has twenty times greater affinity for this form of vitamin D than the active form[17].

The most important site for the next stage of vitamin D activation is the kidney. In mitochondrial kidney cells under the influence of 1α-hydroxylase-CYP27B1, 25(OH)D is hydroxylated to 1,25$(OH)_2$ vitamin D (calcitriol – 1,25$(OH)_2$D). The CYP27B1 enzyme is also active in the cells of other tissues. CYP27B1 expression has been observed, among others, in the liver, gonads, prostate epithelium, immune cells, thyroid cells and pancreas. However, CYP27B1 activity varies depending on the site of expression. In the kidney, the primary regulators of CYP27B1 action are changes in parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), while at other locations, its activity is regulated by a large group of cytokines[16,18].

1,25$(OH)_2$D is the biologically active form of vitamin D produced by many organisms including mammals and plants[19]. 1,25$(OH)_2$D enters the circulatory system, where after binding to DBP, it reaches the target tissues[20]. Calcitriol can perform its biological function thanks to its assembly with vitamin D receptor (VDR). VDR, belonging to the group of nuclear receptors, is located in most mammalian tissues. However, the level of its expression is tissue-dependent, being the most pronounced in the liver, intestines, bones, parathyroid glands and kidney (i.e. in tissues involved in maintaining the body's Ca-phosphate homoeostasis)[1,21]. VDR is dimerised together with the nuclear retinoid receptor RXR[16]. Calcitriol in the bound form with the VDR–RXR complex regulates the transcription of many genes, including *CYP27B1* and *CYP24A1*[15,16].

The CYP24A1 enzyme is produced mainly in the kidneys, and it is responsible for calcidiol and calcitriol catabolism. 24-Hydroxylase-CYP24A1 metabolises 1,25$(OH)_2$D and 25(OH)D, although it has a higher affinity for 1,25$(OH)_2$D[17]. CYP24A1 inactivates these vitamin D metabolites in the form of calcitroic acid. The regulation of CYP24A1 expression levels is a major factor in maintaining normal levels of vitamin D in the body. Calcitriol, acting as a hormone, uses a negative feedback mechanism to strictly regulate vitamin D metabolism. Thanks to calcitriol-dependent CYP24A1 activity in the kidneys, it is possible to control both 25(OH)D and 1,25$(OH)_2$D levels. Calcitriol also regulates the level of CYP27B1 in the kidneys, which is essential to prevent the accumulation of excessive levels of vitamin D in the body. In addition to using the negative feedback mechanism, vitamin D levels in the body are controlled by PTH, mentioned above, and FGF-23. The parathyroid glands secrete PTH in response to low blood Ca, and it stimulates CYP27B1 activity in the kidneys. FGF-23, produced by osteoblasts and osteocytes in response to too high levels of phosphates and calcitriol in the blood, inhibits the renal expression of CYP27B1 while enhancing CYP24A1 activity[20]. Moreover, calcitonin (i.e. a hormone produced mostly in thyroid C cells) affects the level of vitamin D in the body. Calcitonin is synthesised in a state of increased serum Ca. Lowering Ca levels due to calcitonin occurs by inhibiting osteoclasts formation[17].

Interestingly, according to our recent study in rats, the expression of metabolising genes differs between sexes. In the liver, the expression of *Vdr* and *Cyp2r1* genes is significantly higher in females compared with males, while the expression of *Cyp27b1* does not differ significantly between the sexes. Significant differences in the expression of these genes were also observed in kidney tissue. Again, *Cyp2r1* gene expression was higher in females, while the *Cyp27b1* gene, only under the influence of vitamin D supplementation, showed higher expression in males compared with females. Moreover, this study revealed higher expression of *Cyp2r1* and *Cyp27b1* in the rat brain tissue in females compared with males. Furthermore, females have a

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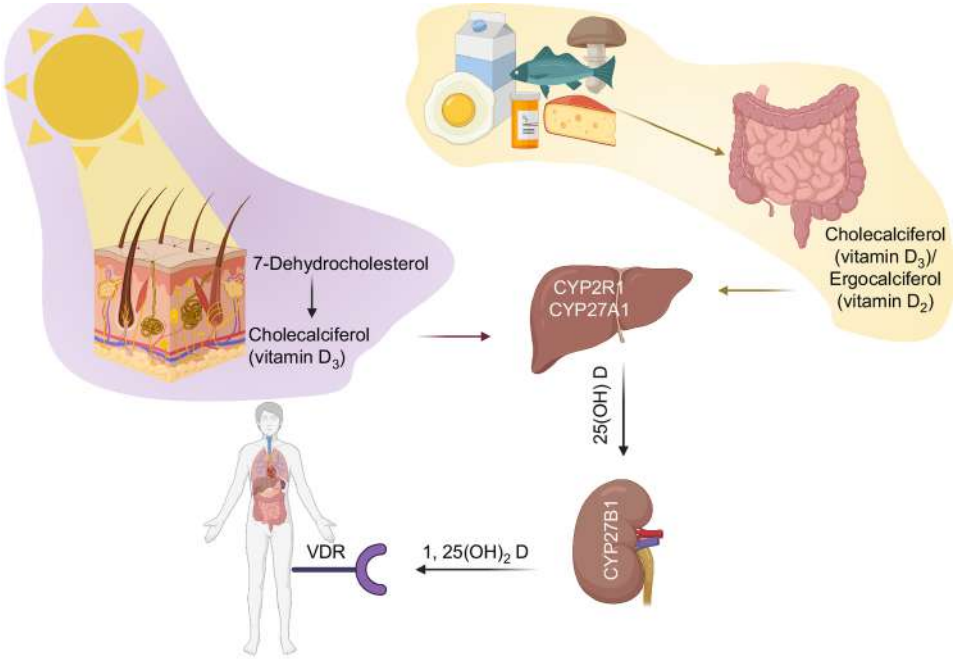

Fig. 1. The scheme shows the sources of vitamin D and its main activation pathway in the body. Vitamin D can be supplied to the body through dermal synthesis, during which 7-dehydrocholesterol is converted to cholecalciferol (D_3) under the influence of UVB radiation and heat generated by the body. Vitamin D can also be taken orally with food or in the form of dietary supplements. Vitamin D taken orally may be in the form of D_3 or D_2. Regardless of its source or form, vitamin D enters the liver where it undergoes its first hydroxylation to form 25(OH)D by CYP2R1 and CYP27A1 enzymes. The 25(OH)D then reaches the kidney where it is converted to 1,25(OH)D under the action of the enzyme CYP27B1. 1,25(OH)D is the active metabolite of vitamin D and is recognised by the vitamin D receptor (VDR). (Figure made with BioRender). VDR, vitamin D receptor.

higher expression of the *Cyp27b1* gene in adipose tissue compared with males[22].

An exogenous vitamin can be delivered to the body in both the D_2 and D_3 forms. Both forms undergo the same, two-stage biological activation process. However, the effect of vitamin D_2 diet supplementation shows a weaker effect[23]. Nevertheless, in the absence of skin exposure to UVB radiation, a varied diet cannot cover the daily requirement for calcitriol. Therefore, in many cases, vitamin D dietary supplementation is necessary. Despite the availability of many different dietary supplements with vitamin D, specific recommendations for their use remain controversial. Currently, the recommended doses refer to the general population and perhaps men and women should be treated differently in this regard. What is more, perhaps different recommendations should be applied depending on specific disease or condition. A routine way to determine the body's vitamin D levels is by testing 25(OH)D in blood plasma. In most recommendations, >30 ng/ml (>75 nmol/l) is considered an appropriate outcome[1,24].

Sources of vitamin D and factors affecting its synthesis

Vitamin D is called the sun's vitamin because the most efficient source is endogenous synthesis, starting in the skin under the influence of UV radiation. Therefore, a key element in maintaining proper levels of vitamin D in the body is regularly exposing the skin to the sun. Apart from recent limitations in exposure to UV rays related to the significant reduction in outdoor activities, attention should be paid to the popularity of cosmetics with UV protection filters. The use of cosmetics with a sun protection factor (SPF) 30 reduces the synthesis of vitamin D in the skin, as much as 99 %[25]. An additional obstacle to maintaining proper levels of vitamin D in plasma is the seasonal variability in the length and intensity of sun exposure. In many areas, the time and level of insolation change dramatically throughout the year, which leads to seasonal deficiency of vitamin D in humans[25]. Moreover, the range of seasonal variations in 25(OH)D in the blood depends on sex[26,27].

The amount of vitamin D contained in the Western diet is low. The most vitamin D-rich foods are fatty ocean fish such as salmon or eel. Apart from that, vitamin D is present in eggs, yellow cheese, or milk; however, the daily requirement (1000 IU) from food can only be covered by consuming fish. The impossibility of simultaneously maintaining a varied diet and supplementing the daily requirement of vitamin D, as well as the widespread and robust reduction of skin contact with the sun, contribute to the need for vitamin D supplementation.

When determining the appropriate daily dose of vitamin D, attention was given to the recipient's age, skin colour and physiological condition. Age is one of the best-proven factors correlated with vitamin D levels in the body[2]. Metabolic processes in the elderly are significantly slowed down. Older people are also characterised by lower absorption of vitamins and minerals from the digestive tract, as well as a slowdown in the synthesis of cholecalciferol through the skin. Moreover, recommendations for vitamin D supplementation are correlated with women's physiological status. The reason for these recommendations is fluctuations in oestrogenic concentrations associated especially with pregnancy and menopause.

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Another important factor defining the accurate level of vitamin D supplementation is genetic background. Recently, much attention has been given to the effects of polymorphisms of genes involved in the action and metabolism of vitamin D[28]. For example, a meta-analysis performed by Duan *et al.* proved that the rs10741657 polymorphism of the *CYP2R1* gene plays a vital role in determining the presence of vitamin D deficiency[29]. Nevertheless, establishing the exact relationship between the genetic profile and the ability to maintain adequate vitamin D levels requires many years of research, and it seems unattainable now.

Among significant aspects influencing the status of vitamin D in the body, we should also mention obesity, low levels of physical activity[30–32] and smoking cigarettes[33]. For example, it was shown that obese men have a higher vitamin D deficiency index than men with normal body weight[27]. Moreover, according to a Norwegian study, seasonal variations in vitamin D do not appear to be strictly related to BMI in women, while obese men have much smaller seasonal variations than non-obese men. In addition, the analysis of NHANES 2011–2014 data indicates that the most significant differences in vitamin D levels are observed between obese women and women with normal body weight[34].

One of the most important factors influencing the level of vitamin D synthesis in the body is skin colour. An increased amount of melanin in the skin guarantees its protection from the sun. Consequently, the darker one's skin colour is, the weaker the synthesis of vitamin D in the skin[20]. This draws attention to the problem of people with high pigment levels living in sun-poor areas. The incidence of vitamin D deficiency is more than three times higher among African Americans than among Caucasians[7].

There is a growing number of indications that sex is an additional, crucial non-environmental factor regulating the level and effect of vitamin D[5,8,25,27,35–38]. To date, a number of studies have been carried out in which vitamin D levels have differed between women and men, most of them defining the female sex as being more vulnerable to vitamin D deficiency[5,7,33].

Importantly, several studies confirm that sex hormones (androgens and oestrogens) affect the expression levels of genes related to vitamin D. The results of an experiment conducted on a human breast cancer (BC) cell line demonstrate that both dihydrotestosterone and estradiol-17 β (E2) increase the *VDR* gene expression[39]. The effect of E2 on *Vdr* expression was also confirmed in a study conducted on rat duodenal mucosa, which suggests that oestrogen up-regulates *Vdr* expression in this tissue and concurrently increases the responsiveness to endogenous $1,25(OH)_2D$[40]. Additionally, Schwartz *et al.* confirmed these results using several omics techniques. According to their findings, oestrogen increases *VDR* gene transcript level, protein expression and endogenous $1,25(OH)_2D$ bioactivity in colonic mucosa[41]. Central nervous system studies also confirm the effect of E2 on *Vdr* gene expression[42–43]. Analysis of the effect of E2 in T cells *in vitro* by Spanier *et al.* showed that E2 decreased *Cyp24a1* expression and increased *Vdr* expression. This result suggests that E2 may slow the rate of calcitriol degradation while increasing the reactivity to calcitriol in T cells[42]. Moreover, one of the more recent studies showed that testosterone may increase the catabolism of vitamin D. The result of this experiment indicates that testosterone induced *CYP24A1* while inhibiting *CYP27B1* gene expression in placental cell cultures[44]. Remarkably, not only do sex hormones affect vitamin D metabolism but also vitamin D affects sex hormone synthesis. According to one study, an active form of vitamin D stimulates the production of progesterone by 13 %, oestrogen by 21 % and estradiol by 9 % in cultured human ovarian cells[45].

Interestingly, the size of sex differences in plasma levels of vitamin D is correlated with ethnic origin. The most significant differences, regardless of age or health abnormalities, are between women and men from Latin American countries[46].

As shown above, serum vitamin D levels are determined by many non-environmental and environmental factors. Moreover, factors such as area of residence, exposure time to UVB radiation, age, skin colour, sex, genetic profile, physiological state and BMI interact with each other, making the search for the causes of insufficient vitamin D levels very individual. Therefore, the idea of creating a personal index has recently emerged, which would enable the calculation of one's own vitamin D requirement[47]. However, this may require a precise determination of the significance of all factors that considerably modulate vitamin D levels, including the role of sex.

Sex as a non-environmental factor may influence both overall vitamin D levels in the body and may also modulate the body's response to vitamin D supplementation in the prevention and treatment for various conditions. Both of these aspects are discussed below.

Sex differences in the serum levels of vitamin D

The results of numerous studies carried out in different countries regarding sex differences in the levels of vitamin D measured in plasma are inconsistent. A survey on a group of as many as 15 804 people revealed that in the USA, vitamin D deficiency is more common in men ($P < 0001$); however, an earlier study pointed out that women are more prone to vitamin D deficiency in this country[7,9]. The difference in the outcomes may be due to the fact that the first study was part of a clinical trial where the average age was 68 years, while the second study involved people over 65 years of age. On the other hand, Muhairi *et al.* conducted a study on a group of adolescents between 15 and 18 years of age in the United Arab Emirates. Their results showed that vitamin D deficits are much more prevalent in girls[8]. Similar findings were obtained by researchers in Brazilian adolescents and children aged 12–17 years. Additionally, in this case, 25(OH)D deficiency (**d** 20 ng/ml) was much more common in girls[48]. Similarly, an analysis of the Taiwanese middle-aged and elderly population indicated that as many as 72·3 % of people with vitamin D deficiency were women, and in the group of people with adequate vitamin D levels, women constituted only 23·9 %[49]. Lower vitamin D levels in females were also noted in a study performed on a group of people aged 5–101 years living in urban parts of China. According to the outcomes, the group most at risk of low levels, including severe plasma vitamin D deficits, consisted of females under 20 years of age and females over 80 years of age[5].

Sexual differences in vitamin D levels are most evident in overweight and obese people, especially children[3]. A cross-sectional survey with Brazilian children aged 12–17 years pointed to a significant relationship between weight category and hypovitaminosis D. According to the results, obese boys are particularly predisposed to vitamin D deficiency[48]. Moreover, insufficient levels of vitamin D are especially common in young obese men[36]. Similarly, Zhu *et al.* discovered that BMI and visceral obesity rates are significantly related to low serum concentrations of 25(OH)D in men[50]. Additionally, Lagunov *et al.* demonstrated that women with obesity (BMI ≥ 40) had higher concentrations of 25(OH)D than men from the same BMI category. Approximately 40 % of obese women and 75 % of obese men have vitamin D deficiency during winter and spring[27]. Further, an epidemiological study by McCullough *et al.* confirmed the link between low levels of vitamin D and obesity in men[51]. In contrast, the results of one of the latest cohort studies in Italy by Barrea *et al.* clearly signals that the level of vitamin D is significantly lower in women, regardless of the BMI class[52]. Moreover, the results of the analysis of patients before undergoing bariatric surgery indicate that as many as 72·5 % of them have insufficient levels of vitamin D. Interestingly, this deficiency was more often observed in women (77·5 % *v.* 69·2 %; $P = 0·019$)[53]. Recently, a correlation has also been found between vitamin D deficiency and the fat-free mass in the Italian population[54]. In addition, women deficient in vitamin D had higher levels of fat mass than men. The authors concluded that the lower 25(OH)D levels in women can be explained by the fact that generally women have more fat than men[54]. According to other research, the greater amount of body fat in women results in an ability to store more vitamin D from skin synthesis than in men, which also explains the fact that vitamin D levels are more variable over the year in men. Vitamin D accumulated in larger amounts of adipose tissue may be the reason for a more stable vitamin D level throughout the year in women than in men[27].

The molecular basis for differences in 25(OH)D serum level between the sexes is not fully understood. Recently, an increase in 25(OH)D with the use of oestrogen-containing contraceptives was observed[55], which is in line with a large multi-ethnic study of men and women aged 45–84 years suggesting that lower 25(OH)D concentrations were associated with the lower sex hormone-binding globulin and higher free testosterone in both men and women[56]. Moreover, it is hypothesised that females have a higher ability to synthesise vitamin D in the skin than males. In mice, it was shown that males exhibit lower levels of 25(OH)D after UV exposure than females because of a lower level of 7-DHC (the precursor to vitamin D) in male skin[57]. Furthermore, it was revealed that women have higher DBP (vitamin D-binding protein) concentration which is combined with oestrogen-dependent DBP synthesis[27]. Undoubtedly, the fact that sex hormones affect the expression levels of genes related to vitamin D is of great importance. Nevertheless, it may not be the only reason since the research results of different studies are inconsistent, indicating both men and women have lower average levels of vitamin D depending on a study. Therefore, there might be other mechanisms responsible for the occurrence of these variances.

One possible explanation for the discrepancy between the results of different experiments may be the cultural differences in the lifestyles of men and women around the world. For example, a cross-sectional study carried out in Saudi Arabia showed that women exhibit a lower general exposure to the sun, although their knowledge about vitamin D importance is greater than in men ($P < 0·002$)[58]. Moreover, they take vitamin D supplements more frequently than men[59]. Furthermore, baseline characteristics of participants in the Vitamin D and OmegA-3 TriaL (VITAL) in the USA suggest more frequent consumption of foods containing vitamin D by women than men[60]. Conversely, in several regions of Europe, men tended to have higher intakes of vitamin D than did women ($P < 0·01$)[61].

Generally, the findings of studies regarding different levels of vitamin D in males and females are inconsistent (Table 1). Overall, it appears that in some geographical regions, women are at higher risk of vitamin D deficiency[62]. This tendency may change with an increasing public awareness of the impact of vitamin D on health. There are some interesting suggestions attempting to explain sex differences in vitamin D status, but so far, no comprehensive research has been conducted to identify the causes of this imbalance unequivocally.

Sex differences observed in association studies examining the relationship between vitamin D status and the occurrence and course of skeletal and non-skeletal disorders

Recently, an increasing number of scientific articles have taken into account sex differences in the effectiveness of vitamin D in preventing and alleviating the course of human disorders. In this chapter, we aimed to systematise the most relevant articles on sex differences in the effect of vitamin D supplementation between females and males.

Skeletal and muscle disorders

Vitamin D deficiency in the body inhibits the absorption of Ca and P supplied from the diet. With significant deficits of vitamin D, only up to 15 % of Ca is absorbed from food. Low concentrations of Ca in the blood stimulate PTH synthesis. PTH increases Ca resorption in kidney tubules. Additionally, in the case of decreased Ca in blood plasma, PTH works similarly to the active form of vitamin D. Mammalian organisms are overly sensitive to fluctuations in Ca concentration in blood plasma. Therefore, when the plasma Ca level in plasma drops, $1,25(OH)_2D$ interacts with VDR in bone blasts to cause preosteoclasts to mature. Mature osteoclasts, releasing hydrochloric acid and collagenases, dissolve bones, thanks to which Ca and phosphorus are released into circulation[63]. For this reason, low levels of Ca and vitamin D in blood plasma cause abnormalities in the bone system. It is well known that a number of systemic hormones and transcription factors regulate bone turnover; however, the hormone whose action is best documented in this regard is oestrogen, which prevents bone loss via estrogen receptor (ER) α and induction of the Fas ligand (FasL) in osteoclasts[64]. Moreover, genetic variation at the *ER* locus, singly and in relation to the *VDR* locus, may influence bone mass and

Table 1. Sex differences in the level of vitamin D measured in the serum

Sex with lower serum vitamin D concentration	Age of participants (years)	Number of participants	Country of research	Years of measurement	References
Male	e 50	15 804	USA	2011–2014	4
Female	5–101	5531	China	2011–2013	3
Female	50	4814	UK	2012–2013	68
Female	18–75	2317	China	2018–2019	147
Female	e 65	2312	USA	1992–1993	1
Female	e 50	1839	Taiwan	2011–2013	47
Female	35–79	1600	Saudi Arabia	–	88
Female	e 65	1425	Portugal	2015–2016	77
Female	18 > 60	1347	Kazakhstan	2018	5
Male	18–89	1329	Chile	–	148
Female	80–112	1324	China	2011	73
Female	12–17	1152	Brazil	2013–2014	46
Female	>100	943	China	2014–2016	149
Female	15–18	315	United Arab Emirates	2010	2
Male	19–69	226	Pakistan	–	150
Female	41–60	120	India	–	151

provide insight into the biological nature of the variation in susceptibilities to osteoporosis[65]. Nevertheless, the exact mechanism of the interaction between oestrogens and vitamin D is not fully understood.

Several investigations indicate that older women are characterised by lower bone mineral density than older men[66–67]. Moreover, a body of evidence suggests that the risk of bone fractures depends on sex[28,68]. In the group of people between 65 and 70 years of age, women were 2·2 times more likely to have a non-vertebral fracture than men[28]. Another cross-sectional study revealed a correlation between the incidence of osteoporosis and Parkinson's disease. Osteoporosis and osteopenia were found in as many as 91 % of women with Parkinson's disease, while in the group of men with Parkinson's disease, the same diseases were present in 61 % of respondents[66]. Interesting information is provided by a case study that searched for differences in the type of hip fracture in relation to 25(OH)D and PTH serum concentrations. Researchers have found, among other outcomes, that severe vitamin D deficiency and being female are associated with a more frequent recurrence of falls.

Further, a UK cross-sectional study, using data from the English Longitudinal Study of Aging (ELSA) and conducted with people over 50 years old, demonstrated that a higher plasma concentration of 25(OH)D lowers the risk of disability in women, but not in men[69]. Moreover, the risk of basic activities of daily living disability is related to vitamin D deficiency in both sexes; however, in females, this risk is much higher (53 % *v.* 44 %)[69]. In addition, a greater risk of female skeletal abnormalities associated with vitamin D deficiency was confirmed by a study on so-called residual rickets. Residual rickets is diagnosed in adults who have suffered rickets in childhood due to severe vitamin D deficiency[70]. The survey, carried out on a group of 200 persons (100 women and 100 men), who were not diagnosed with osteomalacia, showed that there were as many as 21 women among 29 subjects with residual rickets[71].

In turn, a study of people with inflammatory bowel disease listed men as a group with a higher risk of reduced bone mineral density associated with low levels of vitamin D[72–73]. Additionally, multidimensional logistic regression models indicated a significant inverse relationship between the level of 25(OH)D and the risk of frailty and geriatric syndrome[74]. However, these studies have not proven that the relationship between 25(OH)D level and frailty risk is sex- or age-dependent. In contrast, an experimental study carried out on pregnant mice clearly underlines a different effect of vitamin D on offspring's bones according to sex. Further, vitamin D supplementation in pregnant C57BL/6J mice improves bone strength and structure, but only in male offspring[75]. Similar information is offered by a cross-sectional study that traced the relationship between 25(OH)D levels in two stages of pregnancy and bone measurements in offspring at the age of 11 years. This study signalled a positive correlation between 25(OH)D in the mother in early pregnancy and bone mineral content and density in boys. A similar relationship was not observed in girls[76].

Further information about the different effects of vitamin D on females and males is provided by research on sarcopenia (i.e. loss of muscle mass and strength). The study indicated that the sarcopenic condition was significantly related to total serum 25(OH)D concentration and cholesterol level in females only, and PTH level in males only[35]. Additionally, cross-sectional study demonstrated that vitamin D levels were positively correlated with relative skeletal muscle mass in males with sarcopenia[77]. Another study was conducted on a group of people aged 65 years to determine the relationship between vitamin D deficiency and the lowest values of gait speed and hand grip. The results revealed a relationship between vitamin D deficiency and gait speed and grip strength. This relationship was particularly important in the male group compared with the female group[78].

In sum, women are more likely to suffer damage and abnormalities in the functioning of the skeletal system associated with low levels of vitamin D than men. In turn, the relationship between vitamin D deficiency and muscle mass and strength seems to be stronger for men than for women.

Cardiovascular and cardiometabolic disorders

The importance and complexity of functions and the associated ubiquitous presence of blood vessels make CVD not only one of the most dangerous, but also one of the most common conditions in the world. Many studies have linked the functioning of the circulatory system with vitamin D[30,79,80].

Moreover, many indicate different effects of vitamin D in women and men. Further, studies have confirmed the positive impact of vitamin D supplementation on the cardiovascular system and connected its deficiency to an increased risk of CVD[46,79,80]. Low vitamin D content may lead to increased renal renin and angiotensin II production, consequently increasing blood pressure and leading to the development of hypertension and systemic and vascular inflammation[45,81]. In the USA, a survey was carried out on a group of over 15 000 people concerning the connection of vitamin D with the occurrence of CVD ailments. The results verified that low levels of vitamin D in plasma were significantly related to factors increasing the risk of CVD, such as inadequate blood pressure, diabetes mellitus, high BMI and high TAG levels[46]. A clinical study in rats also established that the occurrence of heart disease is associated with low levels of vitamin D, which suggests the usefulness of vitamin D supplementation in treating heart failure[82]. Moreover, a recent study showed that even short-term use of vitamin D affects the expression of genes associated with CVD[37].

One experiment examined how excess androgens, in combination with vitamin D deficiency, affect the thickness of the anterior cerebral arteries (ACA) in cerebrovascular disease in rats. The obtained results proved that females with vitamin D deficiency and excess androgens are characterised by increased ACA wall thickness, whereas among males, vitamin D deficiency alone is sufficient to increase ACA wall thickness. Moreover, immunohistochemical investigation indicated that vitamin D deficiency does not significantly affect the expression of androgen receptor (AR) protein in ACA walls in females, but in males, it is significantly higher than in females. This study proves the significance of vitamin D in correlation with androgens for ACA vasoconstriction, which reduces the cerebral circulation efficiency leading to stroke[83], implying that men are more likely to reduce cerebral circulation efficiency in the case of vitamin D deficiency than women.

Other evidence of sex differences in vitamin D action is provided by the results of a clinical study on total antioxidant capacity among candidates for coronary artery bypass grafting surgery. An appropriate total antioxidant capacity level in the body prevents CVD. According to the findings of the study, a higher serum vitamin D content was accompanied by a higher total antioxidant capacity, but only in males, suggesting that the appropriate level of vitamin D, especially in the male diet, can help to protect against cardiometabolic risk factors[84]. In contrast, a cross-sectional study on the relationship between cardiovascular mortality and vitamin D levels, based on data from the Swiss MONICA study, showed that serum 25(OH)D was inversely proportional to cardiovascular mortality in women, but not in men[85]. The outcomes of the coronary artery disease study by Verdoia *et al.* also provide interesting information. According to their findings, low levels of vitamin D are associated with a higher incidence and severity of coronary artery disease in women. In the case of men, the status of vitamin D is independently related to the incidence of coronary artery disease, but not to its severity[86], signalling that woman have a worse course of CVD due to vitamin D deficiency than men. Contrary, Jarrah *et al.* study did not confirm the relationship between vitamin D and coronary artery disease in either men or women[38].

Type 2 diabetes mellitus (T2DM) and metabolic syndrome increase the probability of death from cardiovascular causes. The leading mechanism proposed to explain the relationship between vitamin D deficiency and predisposition to metabolic syndrome and T2DM increased insulin resistance and dysfunction of pancreatic β cells[4,81]. Some investigations hint that vitamin D supplementation may prevent abnormalities in glucose metabolism and thus the development of T2DM and metabolic syndrome[87]. However, research in this area presents very inconsistent results[4,87].

Abudawood *et al.* indicated the relevance of the link between vitamin D deficiency and the occurrence of T2DM as a factor increasing the risk of CVD. This study compared glycaemic indexes, lipid profiles and vitamin D levels of healthy individuals and those with T2DM. The level of vitamin D in patients, regardless of sex, was significantly lower than in healthy people. Vitamin D and total cholesterol levels were positively correlated in both sexes; however, the significance of this correlation was demonstrated only in men[88]. In contrast, apparent intersex differences in vitamin D activity were found by analysing the relationship between vitamin D supplementation and apo expression[89]. The study revealed that under the influence of vitamin D supplementation, the production of apo C2 and C3 increased significantly, but only in men. Interestingly, the expression of apo C1 rose significantly, but only in women. At the same time, under the influence of vitamin D, the expression of apo B fell significantly in women only. These results led the authors to conclude that sexual dimorphism, in the modulatory action of lipoproteins in circulation, may explain the sex imbalance in cardiometabolic health[89]. Similarly, a cross-sectional study on MetS indicated potential differences in vitamin D activity between the sexes. The results of multifactorial logistic regression analysis, using data collected in this study, demonstrated that vitamin D deficiency (below 20 ng/ml) was associated with an increased risk of MetS in men only(90). Further, participants from the Asian Indian Diabetic Heart Study exhibited sex differences in the association of serum vitamin D deficiency with increased risk for cardiometabolic disorders in 3879 Asian Indians[25]. The authors observed that vitamin D deficiency and obesity may together have additive effects on cardiometabolic risk. However, the association of serum vitamin D with fasting blood glucose and homoeostasis model assessment (HOMA-B) in normoglycemic individuals was significant, but only in males[25].

Another study pointed out that women with vitamin D deficiency are characterised by a younger age and more frequent occurrence of dyslipidaemia than men. Further analysis based on the results of this study inferred a significant relationship between vitamin D deficiency and increased cardiometabolic risk (including high DBP, high total cholesterol, low-density lipoprotein and TAG) in men. However, the link between vitamin D deficiency and cardiometabolic risk in women was weaker[49]. The cross-sectional study conducted by Quaiz *et al.* signalled

that the risk of low HDL cholesterol associated with 25(OH)D deficiency was 2·1 times higher in men and 1·3 times higher in women than in their counterparts with normal vitamin D status. Simultaneously, an increased probability of having high TAG levels associated with 25(OH)D deficiency was observed in females, but not in males[91].

Recently, a quantitative, serum proteomics study in a cross-sectional cohort of non-diabetic adults with obesity and low *v.* high vitamin D status revealed sexual dimorphism in vitamin D action. According to the results, a high status of vitamin D correlates with high expression of serum insulin-like growth factor 2-binding protein (IGFBP-2), but only in men with obesity. Further, the association between vitamin D level and the expression of IGFBP-3 protein is positively regulated, but only in obese women with high vitamin D levels. Moreover, the authors observed that IGFBP-2 and IGFBP-3 may serve as markers of risk reduction in cardiometabolic disease[93].

Additionally, studies on non-alcoholic fatty liver disease have shown a different effect of vitamin D supplementation on alanine aminotransferase levels in women and men. The results of the cross-sectional analysis of the population cohort revealed that vitamin D deficiency was associated with elevated alanine aminotransferase levels only in men with normal body weight. A similar relationship was not observed in any of the BMI classes in women. Therefore, the authors stressed the need to conduct further studies on the relationship between vitamin D and non-alcoholic fatty liver disease, taking sex into account[92].

Maintaining a normal level of vitamin D in the body in the context of cardiometabolic health seems more important for men than for women. Importantly, a clinical study confirmed the therapeutic effect of vitamin D on the cardiovascular system of men only[83]. In addition, men are more frequently victims of CVD, but women have a worse course of events of this kind[94].

Nervous system disorders

The effect of vitamin D supplementation on the occurrence and course of multiple sclerosis (MS) is currently the best proven relationship among all non-skeletal diseases. MS is a chronic, progressive disease that leads to increasing disability in many individuals. The clinical status of people with MS is routinely determined using the Expanded Disability Status Scale (EDSS). Kragt *et al.* detected a negative correlation between EDSS scores and 25(OH)D levels[95]. Moreover, high levels of vitamin D were associated with a lower risk of MS, but only in women[95]. The prevalence of MS increases with latitude, which verifies the negative influence of low concentrations of vitamin D on the occurrence of this disease[96]. MS is diagnosed most frequently in women of reproductive age (female:male ratio 3:1), whereas the incidence of MS in pre-pubertal and postmenopausal women is equal compared with men[97]. Vitamin D_3 levels in the global population have dropped dramatically over the past few decades[98], correlating with growing female MS incidence. Therefore, decreased vitamin D status may be a reason for women's higher susceptibility to MS[42]. Several other studies indicate a different vitamin D metabolism in females and males with MS. Kragt *et al.* showed that a 10 nmol/l increase in serum 25(OH)D levels in women reduces the risk of MS by up to 19 %[95]. Moreover, Woolmore *et al.* confirmed that women with MS, with increased sensitivity to sunlight, have a lower risk of a severe course of disease[99].

A study conducted on a group of people with MS showed that the active form of vitamin D inhibits T cell proliferation in women much more strongly than in men[100]. Moreover, the increase in the number of regulatory T lymphocytes induced by $1,25(OH)_2D$ was higher in a group of females with MS. Additionally, exposure to vitamin D reduced the number of cells producing IFN-γ- and IL-17 and increased the number of cells producing IL-10. In this case, the effect of vitamin D was also stronger in females. Interestingly, exposure to the active form of vitamin D in healthy people and those suffering from MS caused a significant increase in CYP24A1 expression in T cells, but only in males. In this study, the amount of DBP associated with T cells in female patients with MS was 2·5 times higher than in male patients with MS. Moreover, male cells treated with 17-β estradiol showed a similar response to $1,25(OH)_2$ D as female cells[100]. These findings suggest that women have a better ability to accumulate the active form of vitamin D; thus, it has a stronger anti-inflammatory effect in women than in men.

The results of the study, aiming to compare the levels of vitamin D and FGF23 metabolites in subjects with MS and in healthy controls, indicated that the concentration of DBP was higher in patients with MS than in healthy subjects ($P = 0·02$). This difference was particularly significant in men. However, the authors suggested that the role of DBP in MS, as well as sex differences in the VD-FGF23 axis, require further research[101].

Furthermore, experiments on an animal model for investigating MS, mice with autoimmune encephalomyelitis (EAE), revealed that vitamin D_3-fed female mice had significantly less clinical, histopathological and immunological signs of EAE[102]. Moreover, intact female mice fed the feed with vitamin D had significantly more $1,25(OH)_2D$ and fewer *CYP24A1* transcripts in the spinal cord than ovariectomised females, intact or castrated males. The authors hypothesised that an ovarian hormone inhibited *CYP24A1* gene expression in the spinal cord, leading to the locally produced $1,25(OH)_2D$ and its accumulation, which may prevent the inflammation before severe EAE developed[102]. On the other hand, a study conducted by Correale *et al.* suggested that T cells in females and males synthesise similar amounts of $1,25(OH)_2D$, although females activate it slower, thus its accumulation in self-reactive T cells $1,25(OH)_2D$[100].

Additionally, Nashold *et al.* proved that female mice with ovarian hormone production disorder have lost the protective effect of vitamin D against EAE. This study also revealed that despite the administration of E2 (estradiol) to males, vitamin D did not inhibit EAE disease. The results indicate that vitamin D increases the synthesis of E2 and the expression of VDR only in females, which favours vitamin D's therapeutic effect on EAE disease[43]. These observations provide evidence there are synergies between the action of oestrogen and $1,25(OH)_2D$ in MS regulation, which suggests vitamin D supplementation has greater potential to prevent autoimmune-related nervous system diseases in women than in men.

Research on Alzheimer's disease (AD) has also detected sex differences in response to vitamin D supplementation. AD is one of the most common, incurable and progressive

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neurodegenerative ailments. Age and being female increase the risk of AD[103]. According to the results of one study, women are 2·6 times more likely to develop this disease than men[104]. Studies have confirmed the positive role of vitamin D in the treatment or preventing of AD[105]. Morello *et al.* established that vitamin D supplementation improves the working memory of mice with AD. In females with AD, chronic vitamin D supplementation was effective for working memory and amyloid loading only when taken during the symptomatic phase. The authors pointed to the need for additional preclinical studies to better understand the sex-specific mechanisms of vitamin D action in AD[106]. Additionally, Ouma *et al.* revealed sex differences in relation to vitamin D and mild cognitive impairment and various stages of AD. In the case of mild cognitive impairment, 25(OH)D and $1,25(OH)_2D$ were correlated with male sex, whereas in the case of AD, this correlation occurred in women[104].

Interesting results are also provided by another study on the therapeutic effect of vitamin D supplementation. This study involved a group of people with severe depressive disorders (major depressive disorder) who underwent 3 months of vitamin D supplementation. The effect of vitamin D in this study was determined using the Beck Depression Inventory (BDI) scale. Females with moderate, severe and extreme depression had significantly lower BDI results after vitamin D treatment ($P < 0·05$). In males, a significant improvement in the BDI score ($P < 0·05$) was diagnosed only in the group with severe depression[107]. The findings suggest that vitamin D therapy may be helpful in treating major depressive disorder, especially in women.

Further, Lowe *et al.* investigated whether vitamin D affects functional results in a rat model of infant hypoxia and ischemia[108]. According to the outcomes, treatment with HNAC and vitamin D (Hypothermia + NAC N-acetylcysteine $+1,25(OH)_2D$) significantly reduced the incidence of serious brain pathologies in males, while in females, there were no differences between the use of HNAC (NAC + hypothermia treatment) and HNAC + Vit. D therapy. In addition, HNAC + Vit. D improved motor skills compared with hypothermia (HYPO) treatment alone; however, there were some sex differences ($P = 0·01$). In general, a beneficial effect of vitamin D supplementation on asymmetry of the feet was observed ($P = 0·001$). However, males showed significantly greater asymmetry of the feet than females. Additionally, HNAC + Vit. D improved sensory motor adaptation after hypoxia and ischemia more in male rats than in females. Importantly, hypoxia and ischemia injury increased the CYP24A1 enzyme for vitamin D degradation, particularly in males, and males benefit more from $1,25(OH)_2D$ treatment in the long term[108]. A study on changes in fetal neurodevelopment with maternal vitamin D deficiency also provides interesting information[109]. Mice that were fed vitamin D or vitamin D-deficient feed before and during pregnancy were used as a research model. Here, too, the researchers indicated sex differences in response to supplementation or lack thereof. Morphological analysis of the brains obtained from fetuses signals that a deficiency of vitamin D during pregnancy leads to a reduction in crown-rump length, head size and lateral ventricle volume. Additionally, Foxp2-immunoreactive cells were reduced in the cerebral cortex of female fetuses with vitamin D deficiency. In the case of female fetuses, the expression of tyrosine hydroxylase was also reduced[109].

The outcomes of the quoted studies underscore both the protective role of vitamin D and sexual dimorphism in relation to vitamin D metabolism in the nervous system. The scientific evidence presented here implies that women are much more at risk from diseases of the nervous system. However, this research also suggests that vitamin D appears to have a stronger therapeutic effect in females with nervous system diseases than in males.

As in the case of CVD, there are many uncertainties about the link between vitamin D and the nervous system[110]. However, numerous studies stresses that vitamin D is involved in the pathophysiology and progression of many neurological illnesses[2,69]. In addition, low levels of vitamin D are diagnosed in patients with nervous system diseases such as MS and AD[43].

Cancer

Due to the contribution of vitamin D to the regulation of the survival, proliferation and differentiation of cells, its action is also often associated with the pathogenesis of cancer. It was shown that low levels of vitamin D in serum, regardless of age, sex or BMI, are correlated with advanced cancer[111]. Additionally, a number of studies revealing the link between latitude and the incidence of cancer have confirmed the protective role of vitamin D. For example, Cuomo *et al.* conducted a study in 172 countries. The incidence of leukaemia was inversely proportional to the intensity of UVB radiation in both women ($P < 0·01$) and men ($P < 0·01$)[112]. In turn, Tas *et al.* proved that the level of 25(OH)D in blood serum is not related to the duration and activity of the disease in patients with melanoma[113]. Additionally, a recent study (VITAL) carried out in the USA on a group of more than 25 000 people has not confirmed that vitamin D supplementation results in a lower incidence of invasive cancers[114]. Therefore, the role of vitamin D in the cancer pathogenesis is still debated.

However, there is also evidence of different effects of vitamin D in female and male organisms in cancer research. For example, a cross-sectional study carried out in the Swiss population revealed that an increase in 25(OH)D concentration of 10 ng/ml reduces overall mortality by as much as 20 %. In addition, the study pointed to an inverse relationship between 25(OH)D concentration and cancer mortality, but only in males[85]. Interesting information has been provided by a study on the expression of genes involved in vitamin D metabolism in individuals with colorectal cancer (CRC). In this study, the sex-specific analysis showed a significant difference in *CYP27B1* gene expression between rectal and CRC in women but not in men. Additionally, in tumours located in the distal part of the large intestine, the expression of the *CYP27B1* gene was more than twice as high in men as in women[115]. The association study between vitamin D, the VDR gene and the androgen receptor gene with colorectal and rectal cancer also provides interesting information. The *AR* gene partially regulates the transcriptional activity of the *VDR* gene. A significant interaction between vitamin D and the *AR* gene and the risk of CRC was detected only in men. Men with lower vitamin D intake and lower sun exposure are more likely to develop CRC[116]. In turn, a study on polymorphisms of the

VDR gene and CRC confirmed that the *BsmI* variant is significantly associated with CRC in women[117]. Additionally, studies on colorectal adenomas suggest sex-dependent effects of vitamin D. In general, according to the outcome of this study, colorectal adenomas are much more common in men than in women ($P < 0{\cdot}001$); however, in men, the level of vitamin D was not associated with the place or amount of lesions in the large intestine. In women, on the other hand, the total number of neoplastic lesions was inversely correlated with the serum level of vitamin D[118]. Similar conclusions were reached by researchers from Arizona. Admittedly, they did not find a significant statistical relationship between 1,25(OH)$_2$D and the chance of metachronic adenoma. Yet according to their results, women – but not men – with the highest concentration of 1,25(OH)$_2$D have a significantly reduced chance of developing adenomas in the distal part of the large intestine. In addition, these researchers suggest that the supply and activity of vitamin D metabolites in different parts of the large intestine may vary according to sex[119]. Further, research on the carcinogenesis of esophageal adenocarcinoma implies a different effect of vitamin D on females and males. Zhou *et al.* verified a significantly higher level of VDR expression in precancerous lesions and esophageal adenocarcinoma in males than in females[120]. In another study, a high expression of TGR5, a cell membrane surface receptor-G protein coupled receptor associated with the development of gastrointestinal cancer, was significantly increased in males compared with females. Moreover, VDR was significantly correlated with TGR5 expression. According to the authors of this study, sex differences in TGR5 and VDR expression may explain why the prevalence of esophageal adenocarcinoma is higher in men than in women[121].

On the other hand, the results of a large population study in China revealed an unexpected direct link between higher serum concentrations of 25(OH)D and an increased risk of esophageal squamous cell carcinoma in men, but not in women[122]. The detrimental effect of higher vitamin D levels on non-Hodgkin's lymphoma was observed in the San Francisco Bay Area population[123]. The authors indicated that high levels of vitamin D are associated with an increased risk of non-Hodgkin's lymphoma, which is surprisingly more significant in men than in women. However, as the authors themselves pointed out, this study is ambiguous and needs to be continued[123].

Suggestions about the sex-dependent effect of vitamins can be found in a study on lung cancer conducted in Finland. This study did not demonstrate a generally significant link between vitamin D and the risk of lung cancer. However, serum concentrations of 25(OH)D were inversely related to lung cancer incidence in women ($P < 0{\cdot}001$), but not in men ($P = 0{\cdot}81$)[124].

When considering the effects of vitamin D supplements in women and men, we must take into account its impact on various types of cancer that occur only, or predominantly, in one sex, that is, ovarian, uterine and BC in women, and prostate cancer in men. There is a strong theoretical support for the beneficial effects of vitamin D in preventing BC. First of all, vitamin D has the potential to reduce progesterone and estradiol, which is connected with decreasing BC risk in young women. Moreover, it was shown that ER-positive BC cells treated with 1,25(OH)$_2$D induce a cell cycle shutdown.

Available data strongly suggest an association of 25(OH)D with BC risk in women[125–127]. The analysis by Song *et al.* showed that a 5 nmol/l increase in blood vitamin D levels was associated with a 6 % decrease in BC risk[126]. Furthermore, epigenetic analysis has shown that the interaction of 25(OH)D levels with methylation of CpG in vitamin D-related genes (specifically cg21201924 in *RXRA*) may influence BC risk[128]. Moreover, in the case of the most lethal gynaecological cancer among women, that is, ovarian cancer, the beneficial effect of 1,25(OH)$_2$D was observed[129–131]. For example, the results of the Prejovic *et al.* study suggest a chemo-preventive role of vitamin D in the development of epithelial ovarian cancer in women. This study investigated the association between vitamin D and ovarian cancer development in breast cancer susceptibility gene (*BRCA*) mutation carriers. *BRCA* gene mutation is strongly associated with the occurrence of epithelial ovarian cancer. The results showed that VDR protein levels in *BRCA1mut* ovarian surface epithelial cell cultures treated with calcitriol were increased by about 95 % compared with untreated cells. Furthermore, the same cancer cells treated with calcitriol showed a lower proliferation rate in a dose-dependent manner[132].

Another possible target for vitamin D is the prostate gland. In men with prostate cancer, higher 25(OH)D plasma concentrations were associated with longer survival[133]. A controlled clinical trial by Deschasaux *et al.* found that high 25(OH)D concentrations were associated with a reduced rate of prostate cancer[134]. Furthermore, a randomised controlled trial by Li *et al.* showed that both 25(OH)D and 1,25(OH)$_2$D may play an important role in preventing prostate cancer progression[135]. In addition, these researchers showed that the *VDR FokI* polymorphism and plasma 25(OH)D concentration interact and alter prostate cancer risk. According to this study, men with low 25(OH)D concentrations and the *FokI ff* genotype are at the highest risk of developing prostate cancer. Similarly, high plasma 25(OH)D concentrations correlated with an approximately 70 % reduction in the risk of aggressive prostate cancer in men with the *ff* genotype[135].

Vitamin D exhibits a number of actions, including anti-inflammatory activity, indicating that it may influence the process of cancer formation[136]. However, not all studies confirm the beneficial effect of vitamin D on preventing cancerogenesis. Remarkably, the study of the population of 217 244 individuals in Denmark revealed that vitamin D levels are not associated with the incidence of several major cancer types including breast, ovary or corpus uteri cancer, but higher vitamin D concentrations are associated with a significantly higher incidence of skin, prostate and haematological cancer, as well as a lower incidence of lung cancer[137]. Although these are correlations, and do not indicate a direct negative impact of vitamin D supplementation on the development of prostate cancer, they show how important it can be to understand sex-specific vitamin D actions.

Overall, the current knowledge does not allow us to regard vitamin D as a strong anticancer agent yet, and it only indicates the need to maintain its blood level within the laboratory norms. Furthermore, there are many scientific indications of potentially different effects of vitamin D on tumour formation in females and males.

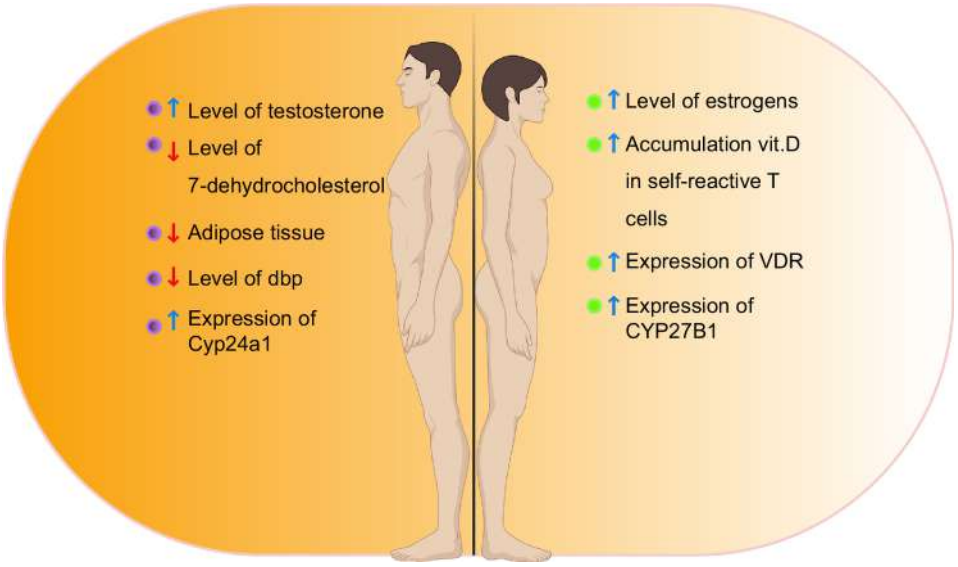

Fig. 2. The difference in the production of sex hormones is the most significant relationship leading to the conclusion that vitamin D metabolism is sex-dependent. Sex hormones characteristic of females stimulate vitamin D metabolism, whereas testosterone characteristic of males inhibits vitamin D metabolism. Studies show that males are characterised by lower levels of vitamin D precursors (7-dehydrocholesterol), lower levels of vitamin D transporting protein (DBP), lower levels of body fat and a higher expression of the vitamin D catabolising enzyme (CYP24A1). On the other hand, females are characterised by higher levels of self-reactive T cells and higher levels of vitamin D-activating enzyme (CYP27B1) and vitamin D receptor (VDR). (Figure made with BioRender).

Immunity/autoimmunity

The link between vitamin D action and immune system function has been known for a long time. The beneficial effects of vitamin D on tuberculosis were observed in 1943[138]. However, only a few years ago, it was found that calcitriol induces the expression of antimicrobial peptides, such as CAMP and DEFB4[139]. Additionally, several meta-analyses[140] have proven the beneficial effect of vitamin D supplementation on the incidence of upper respiratory tract infections. However, it is not known whether vitamin D improves the immunity of males and females to the same extent, or whether it is responsible to some degree for the differences between female and male immunity. Currently, the improvement of immunity is of great importance due to the widespread COVID-19 infection. Recently, it has been hypothesised that the interaction between vitamin D and oestrogen could affect the sex imbalance in the outcomes of patients with COVID-19[141]. However, further research to elucidate these relationships is still required. A good example of the investigation of sex hormones and vitamin D relationships in the context of immunity is a study by Olmos-Ortiz *et al.*, which points out that male newborns are less resistant to postpartum infections[44]. At the same time, cotyledons of male fetuses expressed lower *CYP27B1* gene expression than those of female fetuses. This led to lower local production of active vitamin D, and consequently, to lower expression of the antimicrobial peptide cathelicidin[44]. Sex differences were also observed in the immune system response to vitamin D through the secretion of inflammatory cytokines (TNF-α and IL-6) in human adipose tissue. According to the results, 25(OH)D and 1,25(OH)$_2$D reduced the lipopolysaccharide-induced increase in cytokine concentration in adipose tissue in women, but not in men[142]. Further, a randomised control trial that investigated the effect of long-term vitamin D supplementation on gene expression in blood identified a much stronger effect in women than in men. Gene set enrichment analysis highlighted the engagement of identified genes in the immune system, among others[11].

Strong evidence of the importance of sex in the action of vitamin D in shaping immunity is provided by studies on autoimmune diseases. The occurrence of autoimmune diseases such as MS, systemic lupus erythematosus, rheumatoid arthritis, irritative bowel syndrome or Hashimoto's disease is much more common in women than in men[143–144]. However, male sex is associated with more severe clinical manifestations of these ailments[14]. This generally indicates greater reactivity of the female immune system. The link between vitamin D deficiency and the change in the incidence of MS in women and men over the years has already been discussed in the section on the nervous system. For other autoimmune diseases, an association between oestrogen level, the course of the disease and the vitamin D content of the serum was also observed, although the role that vitamin D plays in the course of these diseases is less well established[145]. Undoubtedly, vitamin D deficiency is a risk factor in autoimmune diseases, but further studies are needed to assess the beneficial role of vitamin D supplementation in therapy for males and females with particular disorders.

Conclusion

Vitamin D has been the subject of several research studies over the years. Nonetheless, due to the large number of factors modulating its levels in organisms (Fig. 2), these studies seem to be insufficient. Our article is a narrative, not a systematic review, which may be a limitation. However, it indicates the need for comprehensive intervention studies to establish the real impact of vitamin D on individual diseases. Future research should take

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into account both sexes and possible differences in the responses of females and males, as well as inter-individual differences in vitamin D metabolism. Particularly at this time of dynamic progress in personalised medicine, differences in physiological processes between the patients require special attention. A better understanding of differences in the levels and effects of vitamin D in females and males may lead to sex-specific dietary recommendations.

The 2020–2021 quarantine, covering a large part of the world's population, may undoubtedly contribute to an increase in the number of people suffering from vitamin D deficiency[146]. Future epidemiological studies are likely to present even more worrying results as an effect of the SARS-CoV-2 pandemic. Further, in light of the current global situation, knowledge already acquired about the effects of vitamin D should be disseminated to improve the general health of the population.

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Effect of different doses of cholecalciferol and calcidiol on meat quality parameters and skeletal muscle transcriptome profiles in swine

Alicja Wierzbicka [a,*], Małgorzata Świątkiewicz [b], Mirosław Tyra [c], Tomasz Szmatoła [a,d], Maria Oczkowicz [a]

[a] Department of Animal Molecular Biology, National Research Institute of Animal Production, Ul. Krakowska 1, 32-083 Balice, Poland
[b] Department of Animal Nutrition and Feed Science, National Research Institute of Animal Production, Ul. Krakowska 1, 32-083 Balice, Poland
[c] Department of Pig Breeding, National Research Institute of Animal Production, Ul. Krakowska 1, 32-083 Balice, Poland
[d] Center for Experimental and Innovative Medicine, The University of Agriculture in Kraków, Rędzina 1c, 30 248 Kraków, Poland

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ABSTRACT

Skeletal muscle tissue is one of the potential targets for vitamin D actions. There are indications that vitamin D supplementation to swine has a positive effect on meat quality. However, these issues need further study, especially in terms of response to the use of different forms of vitamin D. We carried out a multi-purpose study to compare the effects of cholecalciferol and calcidiol on meat quality and muscle tissue transcriptome. Meat quality assessment and gene expression analysis were performed on *longissimus dorsi* samples collected from swine fed grower/finisher diets containing 2000 IU cholecalciferol/1500 IU cholecalciferol per kg ($n = 8$), 3000 IU cholecalciferol/2500 IU cholecalciferol per kg ($n = 10$), 2000 IU cholecalciferol +1000 IU calcidiol/1500 IU cholecaliferol +1000 IU calcidiol per kg (n = 8), and 2000 IU calcidiol/1500 IU calcidiol per kg (n = 8). The results suggest that increasing the dose of cholecalciferol and using calcidiol in the diet of finishers may improve meat texture parameters (shear force $P = 0{,}014$, toughness P = 0,048, cohesiveness P = 0,017, resilience P = 0,002). Shear force (68.46 N–51.42 N) and toughness (145.85 N–114.52 N) decreased the most under the effect of increasing cholecalciferol dosage. In turn, cohesiveness (0.60 N–0.65 N) and resilience (0.23 N–0.28 N) increased most strongly under the use of cholecalciferol+calcidiol. Moreover, the results indicate no significant effect of increasing cholecalciferol dose and use calcidiol in the swine diet on muscle tissue transcriptome.

1. Introduction

The appropriate serum concentration of vitamin D maintains calcium-phosphate homeostasis. This main non-genomic effect of vitamin D is necessary for the development and functioning of the skeletal system. However, vitamin D displays a much wider spectrum of actions in mammalian bodies via vitamin D receptors (VDR). One study showed that the VDR overexpression stimulates muscle hypertrophy through increased protein synthesis, translation efficiency and ribosomal expansion (Bass et al., 2020).

Vitamin D receptor is the DNA-binding factor regulating a lot of genes in mammalian organisms. VDR linked with $1{,}25(OH)_2D$, the active form of vitamin D, and with retinoid X receptor (RXR) create an active signal transduction complex. VDR-RXR complex recognises vitamin D responsive elements (VDREs) in the genes regulated by vitamin D. Thus, the availability of the active form of vitamin D regulates the expression of many genes connected with bones (*RANKL, SPP1*), mineral homeostasis (*PTH*), detoxification (*CYP3A4*), cell cycle control (p21), immunity (*CAMP*) and metabolism (*CBS*) (Haussler, Jurutka, Mizwicki, & Norman, 2011). Vitamin D receptor is also expressed in the muscle (Garcia, Seelaender, Sotiropoulos, Coletti, & Lancha Jr, 2019), therefore the assessment of the effect of vitamin D supplementation on the level of gene expression in the muscle may provide interesting results with clinical implications. The transcriptome analysis conducted on human skeletal muscle "biopsies" from older people did not show significant changes in gene expression after calcidiol (25(OH)D) supplementation (Hangelbroek et al., 2019). In contrast, the RT2 Profiler PCR Arrays in rats indicated that vitamin D deficiency altered the functions of skeletal muscles by changing several genes (Gogulothu et al., 2020). Furthermore, an RNA-seq study of human

* Corresponding author.
E-mail addresses: alicja_wierzbicka@iz.edu.pl (A. Wierzbicka), malgorzata.swiatkiewicz@iz.edu.pl (M. Świątkiewicz), miroslaw.tyra@iz.edu.pl (M. Tyra), tomasz.szmatola@iz.edu.pl (T. Szmatoła), maria.oczkowicz@iz.edu.pl (M. Oczkowicz).

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biopsies showed that combining vitamin D3 supplementation with resistance training had an impact on the expression of gene sets involved in vascular functions in muscle tissues and on strength gains in participants with high-fat mass (Mølmen et al., 2021).

In addition to improving health, vitamin D supplementation in livestock may also affect meat quality, but this has not yet been comprehensively evaluated. Recent study in this field indicates that the use of vitamin D_3 (cholecalciferol) in finishers nutrition by the short-term supplementation in the water can improve pork meat quality such as improving drip loss and tenderness of muscle (Rey et al., 2020). Vitamin D_2 (ergocalciferol) also has a positive effect on swine meat quality. According to recent findings, the use of ergocalciferol in the diet of swine increases the antioxidant activity and improves the overall colour stability of fresh pork meat (Conway et al., 2022). Nevertheless, it is worth noting that the use of vitamin D_2 in swine feeding is not allowed in the European Union (Commission Implementing Regulation (EU) 2019/849).

Due to its pleiotropic effect, vitamin D is widely recommended in both animals and humans. However, the current EU guidelines on vitamin D supplementation in swine (Commission Implementing Regulation (EU) 2019/849) do not indicate a minimum dose, while the maximum daily dose is 50 μg/kg of feed (2000 IU) regardless of whether it is supplemented in the form of cholecalciferol or calcidiol. Interestingly, the studies on pork biofortification with vitamin D indicate the effectiveness of this process may vary depending on its form - either cholecalciferol or calcidiol (Neill, Gill, McDonald, McRoberts, & Pourshahidi, 2021). In fact, according to other research, calcidiol supplementation has a much more effective impact on its blood concentration than cholecalciferol (Duffy et al., 2018; Upadhaya, Chung, Jung, & Kim, 2022).

We decided to conduct this study due to the above-mentioned inconsistencies, particularly considering the current scale of pork production in Europe. The experiment we designed allowed us to compare the effects of supplementation with standard doses of cholecalciferol and calcidiol, as well as increased doses of cholecalciferol and a combination of cholecalciferol and calcidiol, on both transcriptome of muscle tissue and pork meat quality. The problems discussed above indicate the necessity of further research on vitamin D in the context of public health, animal welfare and economic issues (Organisation for Economic Co-operation and Development (OECD) and Food and Agriculture Organization of the United Nations (FAO), 2017, Yang & Ma, 2021). We hope that the new data will help to establish recommendations for vitamin D supplementation in swine.

2. Materials and methods

2.1. Animals and diets

Animals were kept at the Research Station of the National Research Institute of Animal Production in Grodziec Śląski. All procedures included in this study relating to the use of live animals agreed with the local Ethics Committee for Experiments with Animals in Cracow (Resolution No. 427/2020 of 22.07.2020). In this study, we used 34 samples of muscle collected from crossbred swine. In the nutritional experiment, animals were randomly divided into four dietary groups in which the diets differed from each other only in dose and form of vitamin D. There was the same number of female and male swine in each group. It should be noted that the males used in the experiment were previously castrated (barrows).

The vitamin D dosage of the groups was as follows (Fig. 1.):

- **1 group** (control group) 2000 IU cholecalciferol (grower) and 1500 IU cholecalciferol/ kg feed (finisher)- 8 individuals.
- **2 group** 3000 IU cholecalciferol (grower) and 2500 IU cholecalciferol/ kg feed (finisher)- 10 individuals.
- **3 group** 2000 IU cholecalciferol +1000 IU calcidiol (grower) and 1500 IU cholecalciferol +1000 IU calcidiol/kg feed (finisher)- 8 individuals.
- **4 group** 2000 IU calcidiol (grower) and 1500 IU calcidiol/kg feed (finisher)- 8 individuals.

All animals were kept in individual straw-bedded pens where environmental conditions were uniform. The body weight of animals was measured at the beginning, every two weeks and at the end of experiment. The diets of all the groups were isonitrogenous, isoenergetic. The diets were formulated to cover all nutritional requirements of the swine (GROWER MIX: metabolizable energy - 13.3 MJ, total protein - 172 g/kg; FINISHER MIX: metabolizable energy - 13.3 MJ, total protein - 156 g/kg). The animals were fed with dosed amounts of feed, depending on body weight (first fattening period - grower - 30-60 kg, second fattening period - 60-110 kg - finisher). The experimental fattening lasted approximately from 30 to 110 kg of live weight of the animals. At the end of the experiment, all the finishers were slaughtered with used stunning high-voltage electric tongs (voltage 240–400 V).

Immediately after slaughter, the blood and muscle tissue samples (*longissimus*) were taken. Muscle tissue samples for the genomic analysis from the area between the last thoracic and the first lumbar vertebrae

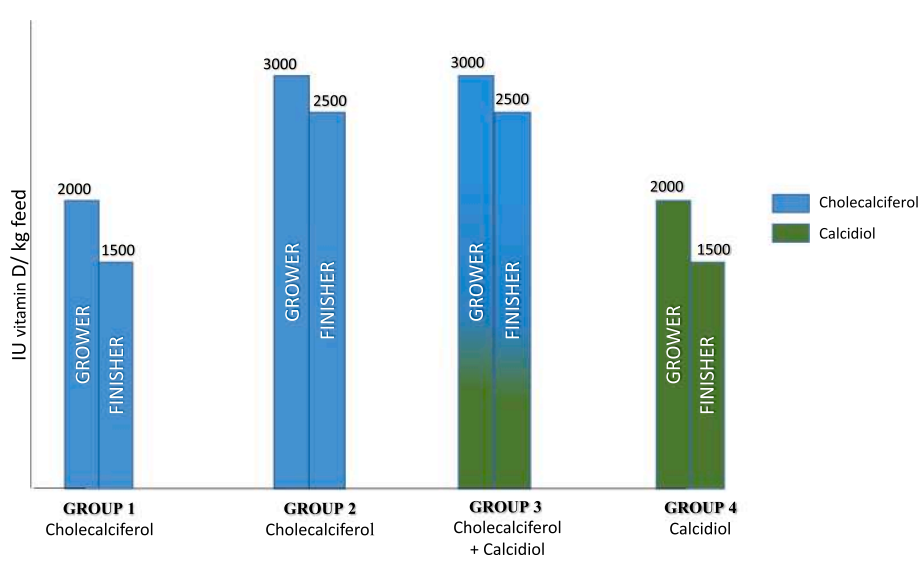

Fig. 1. Doses and forms of vitamin D used in groups of swine.

were stored in −85 °C until the analysis. Samples for meat the quality analysis were collected from the same part of meat cutting after 24 h cooling the carcass at +4 °C. Then the samples were used for the investigation of chemical components, acidity, colour, and texture parameters and the Warner-Bratzler shear force analysis. The samples for thiobarbituric acid reactive substances (TBARS) and colour analysis were stored frozen in −20 °C until the analysis.

Blood for measurement of 25(OH)D concentration was collected into tubes with ACD anticoagulant (acid dextrose citrate/glucose). The samples were stored at +6 °C until plasma was obtained. The blood samples were then transported to the laboratory where blood plasma was obtained in a gradient centrifuge (3000 rpm). Subsequently, the obtained plasma was stored at −20.

2.2. Measurement of 25(OH)D concentration

Determination of total plasma vitamin D concentration was performed by an external company (ANCHEM Laboratorium, 20 Fredry Street, Katowice, Poland). Frozen blood plasma samples were sent to the laboratory with care to maintain an even temperature in transport. The determination was performed by RIA method, using DIAsource 25OH Vitamin D total -RIA-CT Kit (Rue de Bosquet 2, 1348 Louvain-La-Neuve, Belgium) and Multigamma 1260 multidetector instrument (LKB WALLAC, Finland). The result 25OH Vitamin D total was obtained in ng/ml. Possible measurement error according to the protocol was > ± 1.5 ng/ml.

2.3. Analysis of chemical components, acidity, colour, and texture parameters of meat

Acidity of *longissimus.* Was checked 45 min. After the slaughter and after 24 h of cooling at +4 °C. It was measured using pH-meter HI99163 (Grupa Hanna Instruments, Olsztyn, Polska), equipped with a combined electrode pH/T°FC 232 (blade FC 099, length 35 mm). Calibration of the apparatus is performed each time when a new batch of samples is measured. Calibration is performed using two buffers with pH 4.01 and 7.0. The compensation of temperature in the range of −5 to 105 °C in the measuring device is automatic, the temperature resolution is 0.1 °C, and the accuracy of temperature is ±0.5 to 60 °C, ±1 °C to 105 °C.

Basic chemical analyses (dry matter, crude protein, crude fat) of *longissimus* samples were performed according to standard methods AOAC (2005).

TBARS were analysed in the meat samples after 3 months of storage at −20 °C using a modified method proposed by Pikul, Leszczynski, and Kummerow (1989). Briefly, 10 g of comminute sample was homogenised with 50 mL of 4% perchloric acid with butylated hydroxytoluene (BTH) additive. Next, after filtering, 5 ml of the filtrate was mixed with 5 ml of 2-thiobarbituric acid (0.02 M). The solution was heated in a boiling water bath for 1 h, then cooled under running cold water for 10 min. The measurement was carried out at 532 nm against a calibration curve including a blank sample. The colour of the meat was assessed with the CIE L^* a^* b^* system using a Minolta CR-310 colourimeter. The operating parameters of the device were as follows: degree of observer - 2°, aperture - 50 mm, illuminant - C. Measurements were carried out after 2 h of sample stabilisation. The first measurement was performed 24 h after slaughter. Next, brightness (L^*), colour saturation towards red (a^*), and saturation towards yellow (b^*) were measured in fresh samples. Then, the meat samples were frozen to the temperature of −20 °C and the meat colour was examined again after storage for 3 months. The psychometric colour saturation (C) and colour change during storage (ΔE) were calculated (McDougall, 2002):

$$C = \left(a^2 + b^2\right)^{0.5}$$

$$\Delta E = \left[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\right]^{0.5}$$

200 g of meat was cooked to the internal temperature of +80 °C. After cooling for 45 min at room temperature, samples were cut parallel to the muscle fibres in the form of cylinders 15 mm in diameter and 15 mm high. Cooking of the samples was carried out in one batch in a large water bath. The samples were placed in the central part of the bath for uniform heat treatment. Each sample was fitted with a temperature probe inserted into its centre. When the temperature reached 80 °C, the sample was removed from the bath. Therefore, the linear model of calculation did not need to include the effect of batches. Measurements were made with five technical repetitions. The pork shear force was established using a TA. XT Plus texturometer from Stable Micro Systems (Vienna Cort, Lampas Road, Godalming, Surrey GU7 1JG, England) with the Warner-Bratzler attachment fitted with a triangular cut-out knife. During the test, the knife speed was 4.5 mm/s. The shear force is expressed as Fmax at the highest point of the cut curve (N). The cutting energy is presented as the value of the force acting on the cross-sectional area ($N/cm^2/s$). Texture Profile Analysis (TPA) was conducted using the same texturometer with an adapter, which was a cylinder with a diameter of 50 mm. A test of double compression of the samples to 70% deformation of their height was performed. The rapidity of the roller was 2 mm/s, the pressure interval was 3 s, and the test detection threshold was 5 g. The analysis of texture included measuring the parameters such as hardness, springiness, cohesiveness, chewiness, and resilience.

The water holding capacity of the meat was measured according to Grau and Hamm (Grau & Hamm, 1953). To assess the weight loss during cooking, a thermal drip loss analysis was performed (Bertram, Karlsson, & Andersen, 2003). The meat samples were boiled in bags until the internal temperature reached +75 °C and then cooled. Pork weight loss during cooking was intended according to the following formula:

$$\text{Thermal drip loss } (\%) = ((\text{sample mass before cooking} - \text{sample mass after cooking}) \times 100)/\text{sample mass before cooking}$$

2.4. RNA isolation, 3′quant mRNA library construction and sequencing

RNA isolation from 34 samples was performed using the TRizol reagent (Invitrogen 15,596,026) according to the recommendations. Next, the isolated genetic material was cleaned up using RNAClean XP (Beckman Coulter, Brea, California, USA). The quality of RNA was assessed using a Tapestation2200 (Agilent, Santa Clara, California, USA), while quantity was evaluated by NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific™, Foster City, California, USA). RIN (integrity number equivalent) scores in all RNA samples were higher than 7. Then, genetic material was used for library preparation using the QuantSeq 3'mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). Assessment of library (quantity and quality) was performed using a Qubit (Thermo Scientific™, Foster City, California, USA) and Tapestation 2200 (Agilent, Santa Clara, California, USA) devices. The sequencing of the pooled libraries (50 bp single read) was performed on Nextseq 5500 device (Illumina, San Diego, California, USA) at the National Research Institute of Animal Production in Balice.

2.5. Statistical analysis

2.5.1. 25(OH)D concentration and meat quality assessments

Statistical analyses of 25(OH)D concentration and meat quality were conducted by one-way analysis of variance ANOVA, where each swine is a data point (n). The comparison of means was performed using Duncan's multiple range test at the $P \leq 0.05$ level of significance. All the analyses of variance were conducted using the Statistica 12 package (Copyright©StatSoft, Inc. 1984–2014).

2.5.2. RNA-seq

After downloading demultiplexed fastq files from the sequencing provider server, the quality check, trimming of reads, and mapping of reads were conducted with FastQC 11.8, FLEXBAR 3.5.0, and TopHat 2.1.1 software, respectively. For evaluation of the mapping statistics and read counts, Samtools 1.9, RSeQC, HTSeq-count 0.11.1 software, and Gtf-Ensembl annotation 96 were used. The DEseq 2 software was used to perform the differential expression analysis. Genes with $Padj < 0.05$ (FDR-False Discovery Rate) Benjamini–Hochberg (BH) adjustment and no fold-change threshold were regarded as differentially expressed. Functional analysis of RNA-seq results was conducted with BioMart and STRING software using the *Sus scrofa* 11.1 database.

2.6. qPCR validation

RNA from 18 samples was reverse transcribed using a high-capacity cDNA archive kit (Thermo Scientific™, Foster City, California, USA). As there were no significant differences in group 3, individuals from groups 1,2 and 4 were used for validation. We performed qPCR on the *VDR, MYH2, NDUFC2* and *SLC30A9* genes using gene-specific primers. The *VDR* and *MYH2* genes used for validation were selected in relation to the study subjects, while the *NDUFC2* and *SLC30A9* genes were selected using the obtained NGS data. 100 ng RNA was reverse transcribed to cDNA using a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Waltham, Massachusetts, United States). Real-time PCR was performed using RT PCR Mix SYBR® (A&A Biotechnology, Gdansk, Poland) on a QuantStudio ™ 7 Flex Real-Time PCR System (Applied Biosystems™, Waltham, Massachusetts, United States). Relative quantity data were analysed on the Thermo Fisher Cloud (Thermo Scientific). Statistical significance was assessed using the Relative Quantification application on ThermoFisher Connect.

3. Results

The results of the vitamin D concentration test in the blood serum showed that the applied supplementation was effective. There was a significantly ($P \leq 0.01$) higher concentration of 25(OH)D in finishers from all experimental groups compared to the control group (Fig. 2.). The highest blood serum concentration of calcidiol was found in animals receiving calcidiol in feed (group 3 and 4).

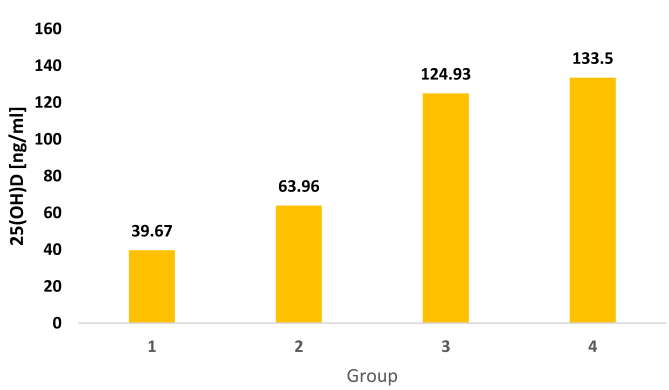

Fig. 2. Effect of vitamin D supplementation on blood 25(OH)D concentration of swine.

Table 1
Effects of vitamin D levels and forms on meat quality.

Parameters	Group*				SEM	
	1 (control)	2	3	4		*P value*
Meat acidity:						
pH 45 min. After slaughter	6.14	6.09	6.12	6.09	0.033	0.959
pH 24 h of cooling (4 °C)	5.73	5.66	5.69	5.66	0.019	0.592
Thermal drip loss after 48 h cooling in 4 °C (%)	27.94	27.03	26.89	27.08	1.030	0.986
Water holding capacity (cm^2/g):						
24 h after slaughter	18.68 b	17.28ab	16.85 a	16.60 a	0.304	0.064
after 3 months of frozen (−20 °C)	21.06	21.17	21.91	21.51	0.496	0.938
Meat colour 24 h after slaughter:						
brightness, *L	51.80	51.40	51.46	52.53	0.250	0.367
saturation in red, *a	15.83	16.34	16.06	15.48	0.152	0.245
saturation in yellow, *b	2.85	3.13	3.30	3.33	0.094	0.248
colour saturation, C	16.09	16.64	16.40	15.85	0.153	0.285
Meat colour after 3 months of frozen (−20 °C):						
brightness, *L	51.65	50.73	51.46	52.29	0.368	0.538
saturation in red, *a	16.20	17.09	17.02	17.53	0.285	0.432
saturation in yellow, *b	4.28	4.79	4.57	4.46	0.251	0.920
colour saturation, C	16.81	17.81	17.68	18.12	0.291	0.446
Colour change after 3 months of frozen (−20 °C), ΔE	3.21	3.23	2.87	3.07	0.209	0.933

a, b – mean values in the same row marked with different letters are significantly different at $P \leq 0.05$.
* 1- standard dose of cholecalciferol ($n = 8$), 2- increased dose of cholecalciferol (n = 8), 3- increased dose of cholecalciferol+calcidiol (n = 8), 4- standard dose of calcidiol (n = 8).

3.1. Effect of different doses and forms of vitamin D on meat quality parameters

The analysis of *longissimus* quality showed that the meat acidity was similar in all groups of the animals (Table 1.), irrespective of the dose and form of vitamin D used. The pH of the meat measured after both 45 min and 24 h of chilling at 4 °C did not differ significantly between the groups. Similarly, the analysis of free water loss from the meat, measured after 48 h of cooling at 4 °C did not show any significant differences between the groups. However, the meat from the animals receiving calcidiol (groups 3 and 4) had approximately 10% lower water

Table 2
Effects of vitamin D levels and forms on meat chemical components.

Components	Group*				SEM	
	1 (control)	2	3	4		*P value*
Dry matter (%)	24.10	24.44	24.43	24.54	0.176	0.848
Protein (%)	23.34	23.25	22.97	23.21	0.116	0.728
Fat (%)	1.52	1.51	1.57	1.58	0.080	0.991
TBARS (mg/kg)	0.518	0.548	0.517	0.538	0.018	0.925

* 1- standard dose of cholecalciferol ($n = 6$), 2- increased dose of cholecalciferol (n = 6), 3- increased dose of cholecalciferol+calcidiol (n = 6), 4- standard dose of calcidiol (n = 6).

holding capacity than that of the control group ($P \leq 0.05$). On the other hand, meat water absorption tested after 3 months of freezing at −20 °C did not show any differences between the groups. Likewise, the analysis of the meat colour and meat water absorption did not show any statistically significant differences.

The results of the chemical analyses of the meat also showed no statistically significant effect of supplementation with different forms and doses of vitamin D (Table 2.). The dry matter, protein and fat content of the meat were very similar in all groups. Also, the oxidative stability of the tested meat showed no significant differences between the groups after 3 months of frozen storage.

The results of the Warner-Bratzler test showed that the meat of animals from groups 2 and 3 was characterised by a significantly lower ($P \leq 0.05$) shear force compared with the control group (Table 3.). The meat texture analysis showed significant differences in traits such as toughness, cohesiveness, and resilience. The meat of animals in the experimental groups (groups 2–4) was characterised by lower toughness compared to the control group. Furthermore, the meat of animals receiving the combination of cholecalciferol and calcidiol was characterised by higher cohesiveness compared to the control group. Furthermore, the results of the Warner-Bratzler test showed that the meat from animals in the experimental groups was characterised by a highly significantly higher resilience compared to the meat from animals in the control group. It is worth mentioning that also in the case of this trait, supplementation of the combination of cholecalciferol and calcidiol at an increased dose showed the greatest effect, however, not statistically different from the other experimental groups (groups 2 and 4).

3.2. Effect of different doses and forms of vitamin D on transcriptome profiles of skeletal muscle

3.2.1. RNA-seq parameters of the results

Sequencing proceeded correctly and it was possible to use the results for all samples for further analysis (Table 4.). The sequencing results have been published on the GEO server and will be available from 1.01.2023.

3.2.2. RNA-seq statistics

The comparison of the transcriptome profiles of finishers supplemented with normal and increased doses of cholecalciferol (group 1 and 2) demonstrated only three changed genes. Table 5. shows the results of all the analyses that identified statistically significant changes according to the adjusted *P-value* (*Padj* < 0.05). Increase of cholecalciferol doses resulted in downregulation of *ENSSSCG00000044439* (log2FC = −0.543), *ENSSSCG00000025403* (log2FC = −0.564) and *SLC30A9* (log2FC = −0.568) genes. 13 genes, including *RBFOX2* (log2FC = −0.444) and *APOA5* (log2FC = −0.547), were on the border of statistical significance (*Padj* = 0.052). However, due to the functions of these genes, they should be considered. Interestingly, a comparison of the transcriptome profiles of swine supplemented with increased doses of cholecalciferol and increased doses of combination- cholecalciferol and calcidiol (group 2 and 3) – didn't show any altered genes. As well as comparing gene expression of the animals receiving a normal dose of cholecalciferol with gene expression of the animals receiving an increased dose of a combination of cholecalciferol and calcidiol (group 1 and 3). Also, according to *Padj*, the comparison of mRNA of finishers supplemented with normal doses of cholecalciferol and the finishers in which cholecalciferol was replaced with calcidiol showed no altered genes (group 1 and 4). However, the altered genes were found when compared to the mRNA of swine supplemented with increased doses of cholecalciferol and normal doses of calcidiol (group 2 and 4). There was found that two genes were downregulated- *TMEM127* (log2FC = −0.969) and *NDUFC2* (log2FC = −2.269).

The PCA graphs presented in Supplementary Material 2. show no significant effect of the supplementation used. Interestingly, the analysis

Table 4
RNA-seq parameters of muscle samples from swine received different levels and forms of vitamin D.

Sample	Number of input reads	Uniquely mapped reads number	Uniquely mapped reads %
1	15,379,262	9,690,960	63.01%
2	14,673,923	12,086,529	82.37%
3	14,150,442	10,373,066	73.31%
4	12,729,264	9,508,130	74.70%
5	15,375,411	11,286,264	73.40%
6	12,296,131	9,316,169	75.77%
7	9,428,628	7,019,892	74.45%
8	11,590,123	8,806,574	75.98%
9	14,860,692	10,651,971	71.68%
10	13,636,401	9,837,692	72.14%
11	12,800,784	9,445,004	73.78%
12	13,748,146	10,019,727	72.88%
13	25,267,856	18,738,362	74.16%
14	16,143,912	11,498,948	71.23%
15	16,372,471	11,608,864	70.90%
16	13,255,376	9,057,274	68.33%
17	10,011,650	7,120,922	71.13%
18	8,515,777	6,413,113	75.31%
19	14,477,786	11,023,250	76.14%
20	15,025,701	11,338,759	75.46%
21	13,277,640	9,923,000	74.73%
22	7,908,576	5,757,974	72.81%
23	9,096,168	6,284,202	69.09%
24	9,416,199	6,176,293	65.59%
25	59,017,472	42,433,005	71.90%
26	38,354,937	27,855,454	72.63%
27	35,318,849	25,854,335	73.20%
28	32,906,371	23,706,632	72.04%
29	46,779,763	31,701,386	67.77%
30	3,303,569	2,065,908	62.54%
31	4,849,117	3,213,388	66.27%
32	4,993,503	3,077,584	61.63%
33	6,498,348	3,944,580	60.70%
34	4,185,836	2,543,198	60.76%

Table 3
Effects of vitamin D levels and forms on shear force and meat texture parameters.

Parameters	Group*				SEM	*P value*
	1(control)	2	3	4		
Shear force, N	68.46 b	51.42 a	53.07 a	56.22 a	2.156	**0.014**
Texture profile analysis:						
Toughness	145.85 b	114.52 a	116.51 a	120.45 a	4.601	**0.048**
Hardness	8.99	7.96	10.44	8.79	0.796	0.757
Springiness	0.66	0.66	0.71	0.67	0.009	0.227
Cohesiveness	0.60 a	0.63 ab	0.65 b	0.63 ab	0.007	**0.017**
Chewiness	3.90	3.99	4.81	4.06	0.318	0.748
Resilience	0.23 a	0.26 b	0.28 b	0.26 b	0.005	**0.002**

a, b – mean values in the same row marked with different letters are significantly different at $P \leq 0.05$.
* 1- standard dose of cholecalciferol (n = 8), 2- increased dose of cholecalciferol (n = 8), 3- increased dose of cholecalciferol+calcidiol (n = 8), 4- standard dose of calcidiol (n = 8).

Table 5
Results of RNA-seq analysis of muscle samples from swine received different levels and forms of vitamin D.

Ensembl ID	baseMean	log2FoldChange	*Padj*[*]	Gene name
1 vs 2[**]				
ENSSSCG00000044439	2143.117	−0.543	0.008	–
ENSSSCG00000025403	1191.058	−0.564	0.008	–
ENSSSCG00000008801	1313.035	−0.568	0.040	**SLC30A9**
ENSSSCG00000028850	3785.044	−0.566	0.052	**RPL26**
ENSSSCG00000001705	2567.781	−0.562	0.052	**TCTE1**
ENSSSCG00000037310	2333.483	−0.435	0.052	**ATP6AP1**
ENSSSCG00000000152	1306.383	−0.444	0.052	**RBFOX2**
ENSSSCG00000036106	1213.265	−0.511	0.052	**TUBG1**
ENSSSCG00000030241	1090.864	0.509	0.052	**TSC22D3**
ENSSSCG00000034153	782.850	−0.431	0.052	**TAF6**
ENSSSCG00000008250	745.055	−0.365	0.052	**–**
ENSSSCG00000015486	652.409	−0.411	0.052	**PIGC**
ENSSSCG00000017476	607.512	−0.418	0.052	**MSL1**
ENSSSCG00000033982	515.959	0.462	0.052	**ZNHIT1**
ENSSSCG00000017104	506.971	−0.465	0.052	**NSUN2**
ENSSSCG00000015067	492.612	−0.546	0.052	**APOA5**
2 vs 4[**]				
ENSSSCG00000034105	136.251	−0.969	0.034	**TMEM127**
ENSSSCG00000039494	33.798	−2.269	0.034	**NDUFC2**

[*] all *P* values <0.001.
[**] 1- standard dose of cholecalciferol (n = 8), 2- increased dose of cholecalciferol (n = 8), 3- increased dose of cholecalciferol+calcidiol (n = 8), 4- standard dose of calcidiol (n = 8).

of 1 vs 2 PCA graph (Supplementary Material 1.) shows that the samples are divided into two groups, but the dividing factor is not supplementation, gender, or relatedness.

3.3. qPCR validation

Quantitative qPCR performed for 18 samples (6 samples each from groups 1, 2 and 4) partially confirmed the NGS results (Supplementary Material 2.). The Pearson correlation (r2) coefficient for the *VDR* and *MYH2* genes were > 0.75 but for the *NDUFC2* and *SLC30A9* genes were < 0.1.

4. Discussion

We analysed the effect of supplementation with standard and increased doses of cholecalciferol alone or with calcidiol on swine muscle tissue. Firstly, we determined the effect of different forms and doses of vitamin D supplementation on the quality parameters of swine meat. Subsequently, we analysed the data to find out if vitamin D supplementation in the form of cholecalciferol and/or calcidiol affects the transcriptome of swine muscle tissue.

Cholecalciferol is commonly used to supplement the diet with vitamin D in humans and animals. However, the results of the comparison of bioavailability and bioactivity of cholecalciferol and calcidiol$_3$ are not conclusive (Duffy et al., 2018; Neill et al., 2021). It seems that the use of the 25(OH) form of vitamin D may be more effective, inter alia due to the different ability of organisms to vitamin D hydroxylation into active form.

The analysis of the concentration of calcidiol in blood serum and fattening parameters of swine used in the experiment was presented in breeders' newsletter published by the National Research Institute of Animal Production in Balice (Fig. 1. and Supplementary Material 3.) (Świątkiewicz & Nabożny, 2015). Consistently, it is worth mentioning that according to our results, the finishers supplemented with calcidiol for about 13 weeks had 3.4 higher plasma concentration of this metabolite compared to the finishers receiving the same dose of cholecalciferol (133,6 ng/ml vs 39,67 ng/ml), which indicates that calcidiol supplementation is much more effective (Świątkiewicz & Nabożny, 2015). The same was observed by Duffy et al., confirming that the use of calcidiol results in significantly higher plasma calcidiol concentration in finishers compared to the use of the same dose of cholecalciferol (27,6 ng/ml vs 59,2 ng/ml) (Duffy et al., 2018). Additionally, based on our previous analysis, the application of a higher dose of vitamin D in the diet of finishers resulted in a slightly higher daily body weight gain. Moreover, the application of a standard dose of calcidiol in the diet of swine increased by 4.6% of the average daily body weight gain (ADG) compared to the group receiving the same dose of cholecalciferol. However, this increase was not statistically significant, nor was the increase in feed conversion ratio (FCR) and the final weight of the animals (Świątkiewicz & Nabożny, 2015). In this respect, our results are in concordance with the analysis carried out by Duffy et al., in which 2000 IU/kg cholecalciferol or calcidiol in swine feeding did not show significant differences in ADG, FCR and the final weight of animals (Duffy et al., 2018).

The first stage of this study was the analysis of parameters determining the quality of meat. The pH analysis did not show any changes in the *longissimus* of finishers supplemented with different doses and/or forms of vitamin D. The lack of significant effect of vitamin D in doses near recommended ones on meat pH is also confirmed by other researchers (Rey et al., 2020). Interestingly, the pH analysis performed by Wilborn et al. showed that only the application of 80,000 IU cholecalciferol per kg of feed caused a statistically significant increase of pH in the *longissimus* of swine (Wilborn, Kerth, Owsley, Jones, & Frobish, 2004).

Furthermore, our analysis of meat quality parameters performed on *longissimus* showed that the meat from animals receiving feed with calcidiol was characterised by slightly lower free water loss and a significantly more favourable water holding capacity index compared to meat from animals receiving the recommended dose in the form of cholecalciferol. Recent studies on the effect of calcidiol on pork quality confirm the positive influence of this form of vitamin D on water holding capacity. Moreover, this study indicates reduced drip loss during day 7 of meat sample storage after calcidiol supplementation (Upadhaya et al., 2022). On the other hand, according to the results of Duffy et al. meat parameters such as cook loss %, water holding capacity and the Warner-Bratzler shear force did not differ significantly between the groups that received calcidiol or cholecalciferol. However, in this study, a higher percentage of water holding capacity was observed in the meat from animals receiving calcidiol (10.2% vs 11.7%) (Duffy et al., 2018). Increased water holding capacity in meat is extremely important in the context of its technological processing and culinary qualities. Based on the results presented above, it seems that the use of vitamin D in the form of calcidiol in the diet of swine may be more beneficial than the use of cholecalciferol.

Nevertheless, based on our Warner-Bratzler test data, it appears that both the use of an increased dose of cholecalciferol and the calcidiol form in the feeding of swine may prove to be beneficial from the consumer's point of view. The results of our test showed that the use of increased doses of cholecalciferol improved meat toughness by about 22% and meat resilience by 13%. Furthermore, the application of the combination of cholecalciferol and calcidiol at an increased dose not only improved meat toughness by 20%, but also had a significant effect on its cohesiveness. Additionally, this combination had the greatest effect on meat resilience, increasing this parameter by 22% comparing to the control group. The total replacement of cholecalciferol with calcidiol improved meat toughness by 11% and, similarly to the higher dose of cholecalciferol, increased pork resilience by 13% compared to the control group. Interestingly, a recent study on the effects of maternal vitamin D$_3$ supplementation on meat quality in the swine offspring confirms the positive effect of an increased dose of cholecalciferol (Guo, Miao, Ma, & Melnychuk, 2021). In this research, the offspring born during the experiment were receiving cholecalciferol until slaughter in the same dose as their mothers during pregnancy. The results indicate that the meat from offspring receiving a high dose of cholecalciferol (3200 IU/kg feed) had the best parameters compared to the meat from

the groups receiving medium (800 IU/kg feed) and low (200 IU/kg feed) doses. Drip loss of meat from animals in this group was significantly lower ($P < 0.05$). Furthermore, the pork from animals receiving the medium cholecalciferol dose had significantly ($P < 0.05$) lower shear force compared to the meat from swine receiving the low cholecalciferol dose (shear force 35.28 N vs 31.36 N). Moreover, these researchers observed the greatest results (shear force = 27.44 N) in the pork from animals receiving a high dose of cholecalciferol compared to the other groups (Guo et al., 2021). On the other hand, one study on texture parameters of the same cut from swine receiving extremely high doses of vitamin D indicates that such doses have no effect on the shear force of pork (Wilborn et al., 2004).

In our experiment, we also carried out a meat colour test. Our analysis shows no significant effect of calcidiol supplementation on pork colour. However, we observed that the meat from animals receiving calcidiol changed colour to a lesser extent after a 3-month freezing period compared with the meat from animals receiving cholecalciferol. The pork from animals receiving the combination of cholecalciferol and calcidiol showed a 10% lower colour change after freezing compared to the group with the standard dose of cholecalciferol. In contrast, according to the results obtained by Duffy et al. meat from animals receiving calcidiol or cholecalciferol differed significantly ($P < 0.01$) in colour. These researchers conducted an analysis which showed that calcidiol supplementation increased the redness and yellowness of pork (Duffy et al., 2018). Moreover, the results of Guo et al. confirm the effect of cholecalciferol on meat colour. Their results indicate a significant improvement in subjectively assessed pork colour and marbling under the influence of 3200 IU/kg feed supplementation of cholecalciferol (Guo et al., 2021).

The final element of our experiment was the RNA-seq of porcine skeletal muscle. This study showed that the differences in gene expression in the muscles of swine supplemented with the recommended and increased doses of cholecalciferol and of those receiving calcidiol are not large. Similar conclusions were reached by researchers from the Netherlands, who found that calcidiol supplementation did not significantly affect the muscle tissue gene expression (Hangelbroek et al., 2019). Thus, we confirm the suggestions of Hangelbroek et al. that muscles may not be a direct objective for vitamin D (Hangelbroek et al., 2019).

Our comparison of transcriptomes of the animals from the groups receiving the standard dose of cholecalciferol and the animals receiving the increased dose of cholecalciferol showed that the expression of genes has changed marginally (log2FoldChange > −0,568 and < 0,509). The genes *ENSSSCG00000044439, ENSSSCG00000025403* and *SLC30A9* have changed the most. These genes were downregulated. The *SLC30A9* gene functions as a zinc transporter involved in intracellular zinc homeostasis. The *SLC30A9* gene is associated with the Birk-Landau-Perez Syndrome causing psychomotor regression and movement disorder. Importantly, the study by Perez et al. confirms that the *SLC30A9* is highly expressed in muscles (Perez et al., 2017). It demonstrates the rank of this gene for muscle tissue. Instabilities of zinc homeostasis in the body can lead to many health problems such as developmental delay or immunodeficiency. Moreover, studies show that mutations in the *SLC30A9* gene reduce its activity cause weakness of muscles (Perez et al., 2017). Our RNA-seq results suggest that increasing the dose of cholecalciferol may affect intracellular Zn homeostasis by downregulating the expression of the *SLC30A9*, however, we did not manage to confirm this by qPCR (r2 = 0.041). Noteworthy are also the genes that in this comparison, according to the adjusted *P-value*, were on the borderline of statistical significance (*Padj* = 0.052). These genes include *RBFOX2* and *APOA5*. The first of these genes is associated with cardiovascular diseases, heart defects, and development of skeletal muscle (Cao et al., 2021). In our experiment, *RBFOX2* was downregulated due to the increased dose of cholecalciferol. One recent paper on the role of the *RBFOX2* gene reports that cells of *RBFOX2* knockout rats shows significant disruption of alternative poly(A) (APA) in the *Slc25a4* gene. Interestingly, *Slc25a4* gene is critical for energy production in mitochondria. Furthermore, study on humans indicates that mutations in the *SLC25A4* gene are known to be associated with hypertrophic cardiomyopathy and skeletal muscle myopathy (Cao et al., 2021). Therefore, our results may suggest a potentially adverse effect of increasing the cholecalciferol dose.

Interestingly, the activity of *APOA5*, another gene on the borderline of statistical significance, is mainly associated with biosynthesis and absorption of cholesterol, biosynthesis of fatty acid and catabolic processes of triglycerides. It has been known that *APOA5* is a crucial fat transfer factor in the *PPAR* signalling pathway. Furthermore, *APOA5* is important for the regulation of triglycerides concentration and fat deposition (Hui et al., 2013). According to our results, increasing the dose of cholecalciferol may cause downregulation of the *APOA5* gene expression. It means that increasing the dose of cholecalciferol supplementation may enhance the number of triglycerides, thus enhancing intramuscular fat (IMF) in pork (Perez et al., 2017). IMF is dependent on triglycerides concentration and is one of the determinants of meat quality. Regrettably, this parameter of pork quality was significantly reduced during the selection for high growth rate in swine (Fernandez, Monin, Talmant, Mourot, & Lebret, 1999; Zuo et al., 2003). Nevertheless, our chemical analysis of pork does not support this assumption and shows that there is no difference in the percentage fat content of meat from animals receiving the standard and increased dose of cholecalciferol (1.52% vs 1.51%).

Additionally, we performed the expression analysis of the genes most known for their function in myogenesis (Upadhaya et al., 2022). One of the genes we analysed was *MYH2*, however, our results (RNA-seq and qPCR r2 = 0.772) do not confirm the effect on neither this gene, nor *MSTN, MYOD* and *MYF5* genes (RNA-seq) under the influence of calcidiol application compared to cholecalciferol-only supplementation. Changes in the expression of these genes after 25(OH)D supplementation were detected using qPCR and recently published by Upadhaya et al. (Upadhaya et al., 2022). The lack of identification of these changes may be due to sensitivity of the methods used (NGS vs qPCR).

Admittedly, our analysis (the increased dose of cholecalciferol vs standard dose of calcidiol) allowed us to detect (RNA-seq) significant differences in only two genes, but the functions of these genes are crucial for myogenesis. First, the *TMEM127* gene controls cell proliferation by acting as a suppressor of the *TOR* signalling pathway. The *TOR* signalling pathway regulates protein syntheses and degrades proteins via the ubiquitin-proteasome signalling pathway. This is supported by a study showing that the loss of muscle mass in rats with vitamin D deficiency was caused by a robust increase in protein degradation (Bhat, Kalam, Qadri, Madabushi, & Ismail, 2013). According to the results obtained in our study, calcidiol supplementation caused a significant downregulation of *TMEM127* compared to cholecalciferol supplementation. If *TMEM127* causes inactivation of *TOR* signalling and calcidiol supplementation significantly downregulates *TMEM127* expression, then calcidiol seems to be more beneficial for myogenesis, energy production and muscle fibre formation than cholecalciferol (Gogulothu et al., 2020). The recent study by Gogulothu et al. indicates that the inactivation of *mTOR* signalling observed in vitamin D deficient rats is detrimental to the muscle tissue of these animals (Gogulothu et al., 2020). Furthermore, also the study by Akagawa et al. using type 2 diabetes mellitus model rats, showed that vitamin D after hydroxylation shows beneficial effects on muscle tissue, probably by affecting the *TOR* pathway (Akagawa et al., 2018).

Furthermore, a comparison of the transcriptomes of swine that received an increased dose of cholecalciferol and a standard dose of calcidiol showed that the *NDUFC2* gene was strongly downregulated. *NDUFC2* is an accessory subunit of the mitochondrial membrane respiratory chain *NADH* dehydrogenase, and it is implicated in respiratory electron transport, ATP synthesis by chemiosmotic coupling and heat production by uncoupling proteins. According to the results of Nitert et al., physical exercise reduces the methylation of the *NDUFC2* in

skeletal muscle, i.e., activates the *NDUFC2* gene (Nitert et al., 2012).

Many advantages of determining muscle respiratory capacity have already been recognised (Laveneziana et al., 2019). Research on this issue is still being developed because muscle respiratory capacity measurements are a useful tool in diagnosis, phenotyping, and the assessment of treatment efficacy in patients with respiratory symptoms and neuromuscular diseases (Laveneziana et al., 2019). Negative effects of decreased the *NDUFC2* mRNA expression have also been shown in the acute coronary syndrome (ACS) studies (Raffa et al., 2019). According to Raffa et al., the *NDUFC2* silencing in human endothelial and vascular smooth muscle cells caused significant increases in ROS concentrations, reduced ATP levels, a higher degree of mitochondrial structural damage and dysfunction (Raffa et al., 2019). Our results from NGS indicate that the use of calcidiol may reduce muscle respiratory capacity as it strongly decreases the *NDUFC2* expression in the muscle. From this point of view, the use of cholecalciferol is preferable.

Despite these interesting clues, the *TMEM127* and *NDUFC2* data from NGS were not confirmed by the qPCR study (*NDUFC2* $r2 = -0.016$).

The presented results provide a basis for further research into increasing the dose of vitamin D and replacing cholecalciferol with calcidiol in swine nutrition. This is the first study to simultaneously investigate the effects of vitamin D on pork quality and muscle tissue transcriptome profiles. Our results suggest that increasing the dose of cholecalciferol and using calcidiol is safe and does not significantly affect skeletal muscle function. On the other hand, the use of calcidiol in the diet of swine results in an increase in meat quality, with no change in the chemical composition of the product. It is particularly significant in the light of ongoing attempts to biofortify pork with vitamin D. Remarkably, current research shows that vitamin D biofortified foods of animal source are the most efficient in raising blood 25(OH)D concentrations in humans compared to other products biofortified with this vitamin (Neill et al., 2021). Therefore, a limitation of our experiment is the lack of analysis of the total vitamin D content of the meat tested. In our opinion, such a study would complement the information on the effect of calcidiol in the diet of swine.

5. Conclusion

In conclusion, the meat from swine supplemented with calcidiol was characterised by a significantly more favourable water holding capacity index compared to the meat from animals receiving the recommended dose in the form of cholecalciferol. Furthermore, the meat from swine supplemented with calcidiol showed an improvement in such parameters as toughness, cohesiveness, and resilience. At the same time, we did not observe any strong effect neither of the increased dose or the calcidiol form of vitamin D supplementation on muscle transcriptome. Nevertheless, there are some indications that the application of vitamin D in the form of calcidiol in swine diet can be beneficial for myogenesis, energy production and muscle fibre formation by influencing the TOR signalling pathway. However, the use of vitamin D in the form of calcidiol may reduce muscle respiratory capacity.

Our results indicate that using calcidiol form instead of the cholecalciferol form in the diet of swine may have slight beneficial effects and that the risk of adverse effects of this form of vitamin D is low.

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Authorship statement

Conceptualization: Małgorzata Świątkiewicz, Maria Oczkowicz.

Data curation: Małgorzta Świątkiewicz, Alicja Wierzbicka

Formal analysis: Małgorzta Świątkiewicz, Mirosław Tyra, Maria Oczkowicz

Funding acquisition: Małgorzata Świątkiewicz

Investigation: Alicja Wierzbicka, Małgorzta Świątkiewicz, Mirosław Tyra

Methodology: Małgorzta Świątkiewicz, Mirosław Tyra, Alicja Wierzbicka

Project administration: Małgorzata Świątkiewicz

Resources: Małgorzta Świątkiewicz, Mirosław Tyra

Software:Tomasz Szmatoła, Alicja Wierzbicka, Małgorzata Świątkiewicz

Supervision: Małgorzta Świątkiewicz, Maria Oczkowicz, Mirosław Tyra

Validation: Alicja Wierzbicka

Visualization: Alicja Wierzbicka, Tomasz Szmatoła

Writing- original draft: Alicja Wierzbicka

Writing- review & editing: Maria Oczkowicz, Alicja Wierzbicka, Małgorzata Świątkiewicz, Mirosław Tyra, Tomasz Szmatoła

Declaration of Competing Interest

None.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2022.109071.

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ORIGINAL ARTICLE

Changes in miRNA expression in the lungs of pigs supplemented with different levels and forms of vitamin D

Alicja Wierzbicka[1] · Klaudia Pawlina-Tyszko[1] · Małgorzata Świątkiewicz[2] · Tomasz Szmatoła[1,3] · Maria Oczkowicz[1]

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Abstract

Background Vitamin D is an immunomodulator, and its effects have been linked to many diseases, including the pathogenesis of cancer. However, the effect of vitamin D supplementation on the regulation of gene expression of the lungs is not fully understood. This study aims to determine the effect of the increased dose of cholecalciferol and a combination of cholecalciferol + calcidiol, as well as the replacement of cholecalciferol with calcidiol, on the miRNA profile of healthy swine lungs.

Methods and results The swine were long-term (88 days) supplemented with a standard dose (2000IU/kg) of cholecalciferol and calcidiol, the increased dose (3000 IU/kg) of cholecalciferol, and the cholecalciferol + calcidiol combination: grower: 3000 IU/Kg of vitamin D (67% of cholecalciferol and 33% of calcidiol), finisher 2500 IU/Kg of vitamin D (60% of cholecalciferol and 40% of calcidiol). Swine lung tissue was used for Next Generation Sequencing (NGS) of miRNA. Long-term supplementation with the cholecalciferol + calcidiol combination caused significant changes in the miRNA profile. They embraced altered levels of the expression of miR-150, miR-193, miR-145, miR-574, miR-340, miR-381, miR-148 and miR-96 (q-*value* < 0.05). In contrast, raising the dose of cholecalciferol only changed the expression of miR-215, and the total replacement of cholecalciferol with calcidiol did not significantly affect the miRNAome profile.

Conclusions The functional analysis of differentially expressed miRNAs suggests that the use of the increased dose of the cholecalciferol + calcidiol combination may affect tumorigenesis processes through, inter alia, modulation of gene regulation of the TGF- β pathway and pathways related to metabolism and synthesis of glycan.

Keywords Cholecalciferol · Calcidiol · Lungs · miRNA-seq · Swine

Introduction

Vitamin D_3 (cholecalciferol), also known as the sun vitamin, is currently one of the most recommended dietary supplements for improving general health in humans and animals.

It is assessed that the vitamin D receptor (VDR) regulates up to 3% of genes in both the human and the mouse genomes [1]. Vitamin D affects *VDR* expression and can influence epigenetic processes by regulating miRNA expression [2]. Interestingly, although the expression of the enzyme CYP27B1 responsible for converting vitamin D into its active form is most pronounced in the kidney, its production is observed in many other tissues including the lungs, suggesting the possibility of local activation of this vitamin [3].

In recent years, interest in vitamin D has increased because there have been many reports of its extra-skeletal effects, e.g. on the immune system. Strengthening the immunity of animals is currently one of the priorities, therefore assessing the effects of vitamin D supplementation on changes in the lungs, which is the repertoire of tissue-resident immune immune cells, seems to be crucial. The domestic pig is an animal of great economic importance around the world. At the same time, it is considered a very good model animal due to its similar physiology and size of main organs to humans. Therefore, our experiment, on the

✉ Maria Oczkowicz
maria.oczkowicz@iz.edu.pl

1 Department of Animal Molecular Biology, National Research Institute of Animal Production, Ul. Krakowska 1, Balice 32-083, Poland

2 Department of Animal Nutrition and Feed Science, National Research Institute of Animal Production, Ul. Krakowska 1, Balice 32-083, Poland

3 Center for Experimental and Innovative Medicine, University of Agriculture in Kraków, Rędzina 1c, Kraków 30 248, Poland

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one hand, assessed the validity of using an increased dose and various forms of vitamin D to improve the functioning of the respiratory system of animals and, on the other hand, allowed us to trace the impact of these modifications on the miRNAome of lungs in the context of lung diseases in humans.

Vitamin D exerts antibacterial and anti-inflammatory effects, as well as plays an antioxidant function [4]. Nevertheless, knowledge about the effect of vitamin D supplementation on the health of the respiratory system in farm animals is scarce, despite that they are often exposed to dust, what makes them prone to respiratory diseases. Contrary, in humans, vitamin D deficiencies are linked to several lung diseases such as acute lung injury, asthma, pneumonia, cystic fibrosis, pulmonary fibrosis, tuberculosis, and COPD (chronic obstructive pulmonary disease) [3]. Due to its significance in inflammatory mechanisms, vitamin D has emerged as a potential therapeutic agent in patients with chronic and acute respiratory diseases [4]. Interestingly, both association and clinical trial studies demonstrated that vitamin D concentration in blood serum is closely related to the course of COVID-19 disease [5]. Moreover, the results of one randomized controlled trial in the field of oncology indicated that vitamin D_3 supplementation can improve the survival of patients with early-stage lung cancer [6]. It has also been proven that the chemopreventive effects of vitamin D are associated with the regulation of tumour suppressor miRNAs (miR-100 and miR-125b) [2].

Recently, there has been a lot of discussion about the need to update the recommendations concerning the dosage and supplementation of calcidiol (calcifediol) in humans [7, 8].

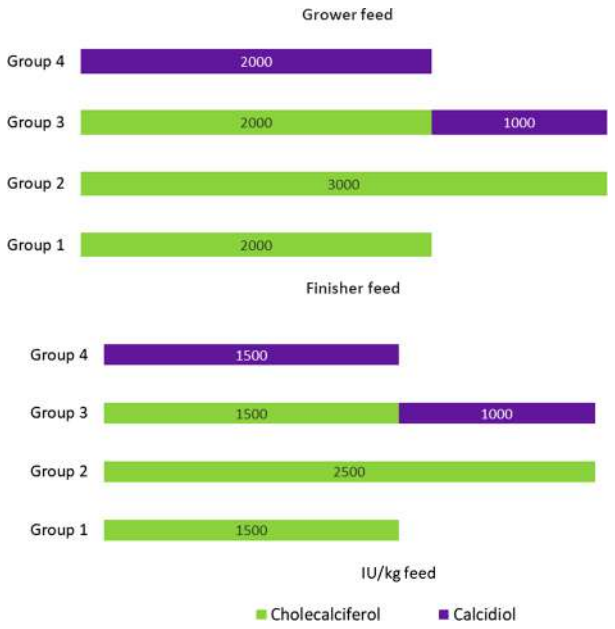

Fig. 1 The content of cholecalciferol and calcidiol in the grower and finisher feeds

According to some researchers, current recommendations for vitamin D intake are adequate for skeletal disorders, while for extra-skeletal disorders treatment much higher vitamin D doses are needed. It is also known that high-dose vitamin D supplementation is effective and well tolerated by the body, which is supported by randomized control trials of high-risk COVID-19 patients [9, 10]. In pigs, the maximum dose of vitamin D in the feedstuff is 2000IU/Kg, according to the European Union regulations ref. However, in the light of current knowledge, the question arises whether these recommendations should not be changed. Moreover, attempts to replace traditional cholecalciferol supplementation with calcidiol seem to be important for this issue. This is evidenced by our previous study, but also the findings of other researchers, indicate that calcidiol supplementation is more effective and faster in increasing 25(OH)D serum levels than cholecalciferol supplementation in pigs and in humans [11–13].

Although the effects of vitamin D have been researched for many years, the effect of cholecalciferol and calcidiol supplementation on the whole miRNAome has not been studied yet. It is well known that miRNAs, which belong to the class of short non-coding-RNAs, act as post-transcriptional regulators. They bind complementary mRNAs and prevent further protein synthesis. It is recognised that miRNA sequences are involved in the regulation of more than 60% of protein-coding genes [14]. Thus, the analysis of changes in the global miRNA profile can provide data which either confirms previous findings or points to new directions in the study of the effects of vitamin D.

Materials and methods

Animals and diets

All procedures on live animals included in this study received consent from the local Ethics Committee for Experiments with Animals in Cracow, Poland (Resolution No. 427/2020 of 22.07.2020). The animals were kept in individual straw-bedded pens at the Research Station of the National Research Institute of Animal Production in Grodziec Śląski. Swine were randomly divided into four dietary groups but all the animals were kept in the same environmental conditions. Group 1, $n = 12$ individuals, group 2, $n = 12$, group 3, $n = 12$, and group 4, $n = 12$. Each group included an equal number of males and females, except group 2 (3 females and 4 males). The male swine had previously been castrated.

The diets of animals differed from each other only in the dose and form of vitamin D (Fig. 1.). In this experiment, animals receiving a standard dose (grower: 2000 IU/Kg, finisher: 1500 IU/Kg) of cholecalciferol in feed constituted the

control group. The diets used were isonitrogenous and isoenergetic, and they covered all nutritional requirements of the swine (grower: metabolizable energy – 13.3 MJ, total protein – 172 g/kg; finisher: metabolizable energy – 13.3 MJ, total protein – 156 g/kg) [15]. The diet was determined by the needs of the animals and accounted for their current age and weight (grower – 30-60 kg, finisher – 60-110 kg).

After 88 days, when the animals reached a weight of ~ 110 kg, the feeding experiment ended, and 48 lung samples were collected from the animals. All the samples came from the middle part of the upper lobe of the left lung. Each swine was slaughtered using stunning high-voltage electric tongs (voltage 240–400 V). Immediately after, the lung samples were placed in a ULT freezer (– 85 °C) and stored until the miRNA analysis.

During slaughter, animals' blood was collected into tubes with anticoagulant (acid dextrose citrate/glucose from the animals. The blood was used to assess serum vitamin D concentrations. The blood samples were then transported at + 6 °C to the laboratory, where blood plasma was obtained by centrifugation at 3000 RPM. The plasma samples were stored at -20.

Measurement of 25(OH)D concentration

The measurement was conducted for 8 animals from each group. Assessment of total plasma vitamin D concentration was performed by ANCHEM Laboratorium, 20 Fredry Street, Katowice, Poland. The blood plasma samples were transported to the laboratory in a frozen state. The assessment was performed by RIA method. The DIAsource 25OH Vitamin D total-RIA-CT Kit (Rue de Bosquet 2, 1348 Louvain-La-Neuve, Belgium) and Multigamma 1260 multidetector instrument (LKB WALLAC, Finland) were used. The result of vitamin D concentration was obtained in ng/ml, and the possible measurement error was estimated at > ± 1.5ng/ml. Statistical analysis of measurement results was performed using SAS software (mean, *p-value*, standard deviation).

RNA isolation, miRNA library construction and NGS sequencing

Total RNA isolation from 48 lung samples was performed using the Direct-zol RNA Miniprep Kits (ZYMO Research, Orange, California) in line with the recommendations of the manufacturer. Then, the isolated genetic material was cleaned up using Monarch® RNA Cleanup Kit (New England Lab, Woburn, USA). The quality and quantity of RNA were assessed using the Tapestation 2200 (Agilent, Santa Clara, California, USA). The integrity number equivalent (RIN) scores in all RNA samples were higher than 7. The quantity of RNA was additionally evaluated by NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific™, Foster City, California, USA). Next, the isolates were used for library preparation with the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Lab, Woburn, USA). The quantity assessment of pooled libraries was performed using the Qubit (Ther-mo Scientific™, Foster City, California, USA), while the quality was assessed using the Tapestation 2200 (Agilent, Santa Clara, California, USA) devices. Sequencing of pooled libraries (75 bp single read) was performed on the NextSeq 550 device (Illumina, San Diego, California, USA) at the National Research Institute of Animal Production in Balice. The libraries for sequencing were prepared according to the Standard Normalization Method from NextSeq 500 and NextSeq 550 Sequencing Systems-Denature and Dilute Libraries Guide protocol. We used a 2nM starting library concentration and 10% PhiX addition.

Statistics of the miRNA-seq results

The obtained reads were demultiplexed with bcl2fastq software (Illumina) and quality controlled with FastQC software. The reads were also subjected to adapter and length trimming (18–25 nt) with the TrimGalore package. miRDeep2 was applied to identify known and potentially novel miRNAs using default settings, Sus scrofa 11.1 reference genome, miRBase 22.1. R package and DESeq2 software were used to perform the differential expression analysis. miRNAs with *p-adjusted* < 0.05 Benjamini–Hochberg (BH) adjustment and no fold-change threshold were regarded as differentially expressed (DE). Only those miRNAs for which the number of reads per sample was greater than zero in a minimum of four samples in each group were used for further analysis. Functional analysis of differentially expressed (DE) miRNAs was conducted with DIANA-miRPath v3.0 online tool for human miRNA homologs.

qPCR analysis

The RNA from 28 samples (7 samples/group) was reverse transcribed using a miRCURY LNA RT Kit (QIAGEN, Hilden, Germany), according to the manufacturer protocol. The following miRNAs were selected to confirm the results obtained from miRNA-seq: miR-215-5p, miR-96-5p and miR-381-3p. The selection was guided by the average number of reads and the log2FoldChange value. Real-time PCR was performed using miRCURY LNA SYBR Green PCR Kit (QIAGEN, Hilden, Germany) and miRCURY LNA miRNA PCR Assays (QIAGEN, Hilden, Germany) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™, Waltham, Massachusetts, United States).

Relative quantity data were analysed on the Thermo Fisher Cloud (Thermo Scientific). The results obtained from NGS (Next Generation Sequencing) and those obtained from qPCR analysis were compared by checking the level of Pearson correlation (r2).

The RNA isolated from the 28 samples was additionally reverse transcribed using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Waltham, Massachusetts, United States). The resulting cDNA was used to analyse changes in the expression of the *NEU1* (Neuraminidase 1) and *FUT1* (Fucosyltransferase 1) genes using *RPS29* endogenous control. These genes are targets of miRNAs altered by the vitamin D supplementation and were selected based on the results of functional analysis of miRNA-seq results. Analysis of the obtained results was conducted using the Thermo Fisher Cloud (Thermo Scientific). The level of significance of differences between groups was checked using the Mann-Whitney U and t-tests.

Results

The 25(OH)D blood test confirmed that the applied supplementation was effective. The plasma concentration of 25(OH)D in animals from all experimental groups differed significantly (*p-value* < 0.05) from that of the control group.

Pigs supplemented with a standard dose of cholecalciferol had the lowest plasma 25(OH)D concentration (39.67 ng/ml, +/- 10.25). The mean 25(OH)D concentration in animals receiving the increased cholecalciferol dose was 24.29 ng/ml higher (63.96 ng/ml, +/- 22.68). Animals treated with calcidiol (groups 3 and 4) had significantly (*p-value* < 0.05) higher vitamin D concentrations compared to the others. Animals receiving cholecalciferol + calcidiol combinations had an average of 124.93 ng/ml (+/- 23.20), while those receiving calcidiol alone had non-significantly higher plasma 25(OH)D concentrations-133.5 ng/ml (+/- 20.5).

RNA assessment and miRNA-seq statistics

RNA evaluation indicated that 39 samples met the quality criteria (RIN scores > 7). The remaining 8 samples were excluded from further analysis.

The NGS of lung tissue samples proceeded correctly for 38 of 39 samples. The average number of mapped reads was 345 116 per sample. One sample was discarded due to a low number of mapped reads (8569 reads). All 38 samples were used for further analysis, including miRNA identification and differential expression analysis. The sequencing results have been deposited in the NCBI GEO database (accession number: GSE217599).

Differential expression analysis results

Table 1. shows the miRNAs which, according to adjusted *p-value* (*q-value* < 0.05), were most significantly altered. The properties of individual miRNAs recognized so far are compiled in the table in Table S1. Nine significantly altered miRNAs were excluded from further analysis because the number of reads for these miRNAs was zero for most samples in each group.

The obtained results (Table 1.) indicate that the use of the increased dose of cholecalciferol compared to the standard dose (1 vs. 2) caused a significant change (*q-value* = 0,004) in the miR-215-5p expression only (log2FoldChange = 2,652). In turn, supplementation with the increased dose of the cholecalciferol + calcidiol combination (1 vs. 3) resulted in the altered expression of 13 miRNAs, including 7 upregulated and 6 downregulated. In contrast, supplementation with the standard dose of calcidiol instead of cholecalciferol (1 vs. 4) showed no effect on the miRNA profile.

Subsequent analysis showed that the increased dose of cholecalciferol + calcidiol compared to the increased dose of cholecalciferol (2 vs. 3) showed a change in the expression of 12 miRNAs (Table 1.). Among them, 9 miRNAs were upregulated and 3 were downregulated. The comparison of the standard dose of calcidiol with the increased dose of cholecalciferol + calcidiol (4 vs. 3) showed 17 altered miRNAs. However, 11 of these miRNAs are repeated in the results of the other comparisons, and the direction of their changes is the same. Only 6 of the altered miRNAs were specific for the 4 vs. 3 comparison, of which 5 miRNAs were downregulated and 1 was upregulated.

qPCR analysis

One of the reverse-transcribed miRNA samples was excluded at a further stage of analysis due to a significant concentration deviation from the other cDNA samples. The qPCR validation performed for 27 samples partially confirmed the NGS results (Table S2.). The Pearson correlation (r2) coefficient for the miR-215-5p was > 0.63 (*p-value* = 0.0004), for miR-381-3p was > 0.60 (*p-value* = 0.0008), and for the miR-96-5p was > 0.58 (*p-value* = 0.0015).

In turn, analysis of *NEU1* and *FUT1* gene expression carried out on 28 samples showed no significant differences between groups (Figure S1.). The *FUT1* expression results were not characterized by a normal distribution, because of this we performed a non-parametric analysis for this data.

Functional analysis

For the functional analysis we used the differentially expressed miRNAs from 1 vs. 3, 2 vs. 3 and 4 vs. 3. The

Table 1 The miRNAs altered by different doses and forms of vitamin D supplementation (*q-value* < 0.05). The level of change is presented in log2FoldChange (log2FC).

2 vs. 1		3 vs. 1		3 vs. 2		3 vs. 4	
miR name	log2FC	miR name	log2FC	miR name	log2FC	miR name	log2FC
miR-215-5p	2,652	miR-574-3p	1,663	miR-4334-3p	2,727	miR-885-5p	1,944
		miR-193a-5p	1,249	miR-205-5p	2,69	miR-574-3p	1,503
		miR-145-5p	1,101	miR-885-5p	2,337	miR-193a-5p	1,329
		miR-150-5p	1,015	miR-125b-5p	2,157	miR-150-5p	0,938
		miR-181a-5p	0,756	miR-574-3p	2,047	miR-181a-5p	0,72
		miR-191-5p	0,674	miR-133a-3p	1,691	miR-676-3p	0,604
		miR-181b-5p	0,519	miR-145-5p	1,311	miR-181b-5p	0,505
		miR-340-5p	-0,86	miR-193a-5p	1,145	miR-21-5p	-0,869
		miR-450a-5p	-0,885	miR-150-5p	0,887	miR-340-5p	-0,917
		miR-101-3p	-1,228	miR-148b-3p	-0,984	miR-218-5p	-0,923
		miR-96-5p	-1,653	miR-151a-3p	-1,695	miR-450a-5p	-0,963
		miR-182-5p	-2,117	miR-215-5p	-2,313	miR-148b-3p	-0,972
		miR-381-3p	-2,599			miR-299-3p	-1,176
						miR-183-5p	-1,203
						miR-96-5p	-1,36
						miR-101-3p	-1,504
						miR-381-3p	-2,504

Table 2 KEGG pathways that were stimulated by the combined supplementation of cholecalciferol + calcidiol in comparison to cholecalciferol at standard doses in the lungs of finishing pigs

KEGG pathway	*p-value*	KEGG class
Estrogen signalling pathway	8.7e-09	Organismal Systems; Endocrine system
TGF-β signalling pathway	2.7e-08	Environmental Information Processing; Signal Transduction
Signalling pathways regulating pluripotency of stem cells	1.8e-05	Cellular Processes; Cellular community - eukaryotes
Phosphatidylinositol signalling system	2.5e-05	Environmental Information Processing; Signal Transduction
Proteoglycans in cancer	6.4e-05	Human Diseases; Cancer: Overview
Amphetamine addiction	0.0001	Human Diseases; Substance dependence
Metabolism of xenobiotics by cytochrome P450	0.004	Metabolism; Xenobiotics biodegradation and metabolism
Transcriptional Misregulation in Cancer	0.005	Human Diseases; Cancer: Overview
Adherens junction	0.012	Cellular Processes; Cellular community - eukaryotes
Axon guidance	0.028	Organismal Systems; Development and regeneration
Vasopressin-regulated water reabsorption	0.033	Organismal Systems; Excretory system
Glioma	0.037	Human Diseases; Cancer: specific types
GABAergic synapse	0.039	Organismal Systems; Nervous system
Oxytocin signalling pathway	0.040	Organismal Systems; Endocrine system
Pathways in cancer	0.044	Human Diseases; Cancer: Overview

analysis was conducted using DIANA software ('pathways union' option) based on a human database. The results indicated that the increased dose of cholecalciferol + calcidiol significantly (*p-value* < 0.05) affected 15 KEGG pathways compared to the standard cholecalciferol supplementation (Table 2.). Among them, there were 5 disease-related pathways, and 4 were connected to cancers (cancer overview, glioma). On the other hand, the pathways from the organismal systems class are related to the endocrine system (2 pathways), nervous system (1 pathway), excretory system (1 pathway) as well as development and regeneration (1 pathway). The remaining altered pathways were associated with signal transduction, cellular community and xenobiotics biodegradation and metabolism.

Supplementation of the increased dose of cholecalciferol + calcidiol had the most significant effect on the estrogen signalling pathway (*p-value* = 8.74E-09), TGF-β signalling pathway (*p-value* = 2.69E-08), signalling pathways regulating pluripotency of stem cells (*p-value* = 1.80E-05), phosphatidylinositol signalling system (*p-value* = 2.45E-05) and proteoglycans in cancer (*p-value* = 6.42E-05). Figure 2 presents miRNAs and pathways clusters, as well as the number and significance of each miRNA's contribution to the regulation of the altered pathway. The heatmap shows

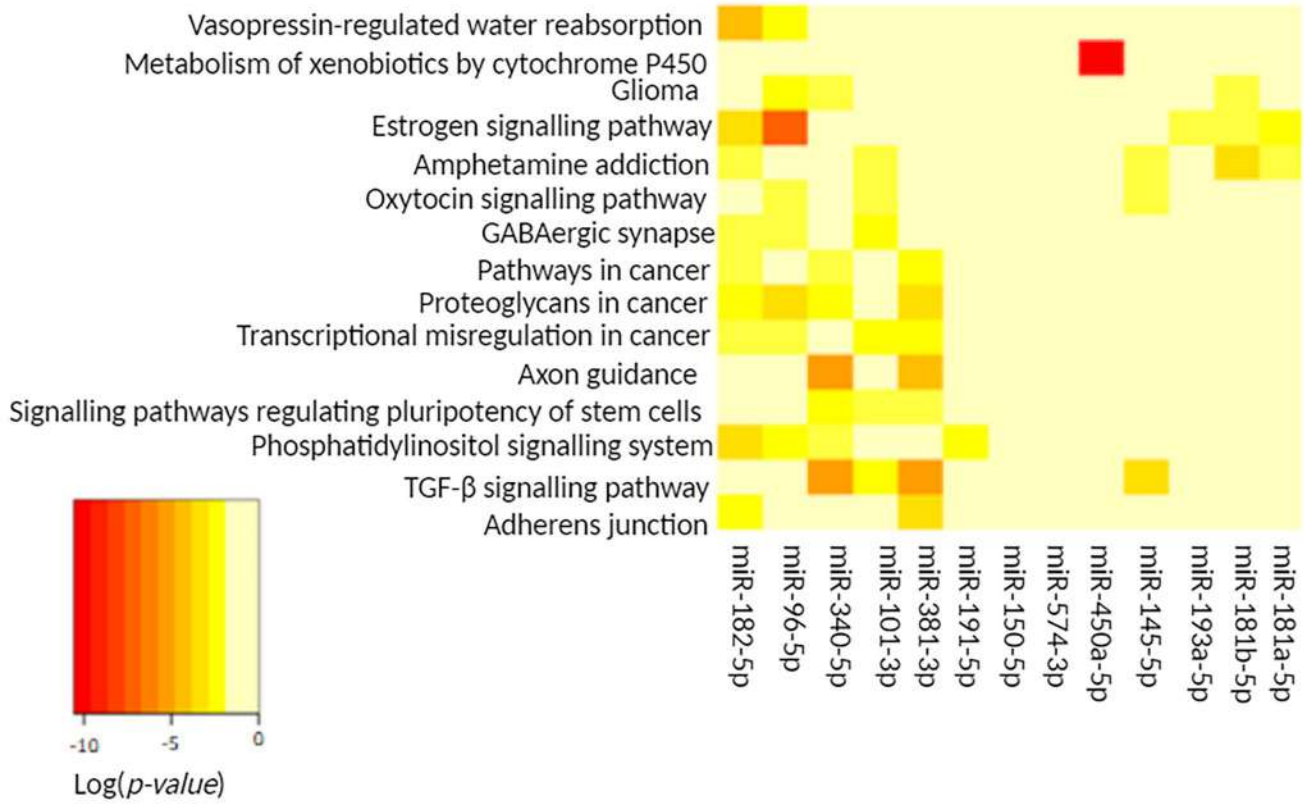

Fig. 2 The heat map shows the miRNAs altered by the increased dose of cholecalciferol + calcidiol (1 vs. 3), the pathways affected, and the clusters formed by each of the altered miRNAs and pathways

Table 3 KEGG pathways that were stimulated by the combined supplementation of cholecalciferol + calcidiol in comparison to cholecalciferol at the increased dose (2 vs. 3) of finishing pigs

KEGG pathway	*p-value*	KEGG class
Prion diseases	< 1e-325	Human Diseases; Neurodegenerative disease
ECM-receptor interaction	0.0002	Environmental Information Processing; Signalling molecules and interaction
Hippo signalling pathway	0.020	Environmental Information Processing; Signal Transduction
Glycosphingolipid biosynthesis-lacto and neolacto series	0.021	Metabolism; Glycan biosynthesis and metabolism
Other glycan degradation	0.048	Metabolism; Glycan biosynthesis and metabolism

that 5 of the 13 differentially expressed miRNAs are associated with the estrogen signalling pathway. Similarly, in the case of amphetamine addiction, 5 of the changed miRNAs are related to this substance-dependence disease. In contrast, only 1 of the altered miRNAs (miR-450a-5p) is associated with the pathway of metabolism of xenobiotics by cytochrome P450, but the significance of miR-450a-5p for this pathway is particularly high.

Among the altered miRNAs engaged in the highest number of pathways are miR-182-5p (9 pathways), miR-96-5p (8 pathways), miR-340-5p (7 pathways), miR-381-3p (7 pathways) and miR-101-3p (6 pathways).

The partial replacement of cholecalciferol with calcidiol at the increased dose (2 vs. 3) had a significant effect on 5 pathways (*p-value* < *0.05*) (Table 3.). The strongest effect was observed on the prion disease pathway (*p-value* = < *1e-325*). This effect was caused by a change in only one miRNA expression level - miR-148b-3p (Fig. 3.). Interestingly, the results also indicate activation of two pathways related to glycan biosynthesis and metabolism; glycosphingolipid biosynthesis - lacto and neolacto series (*p-value* = 0.022) and other glycan degradation (*p-value* = 0.049). Simultaneously, the ECM-receptor interaction pathway, which can also bind to glycans, was also significantly activated (*p-value* = 0.0003).

Only 5 of the 11 altered miRNAs had a significant impact on the listed pathways (Fig. 3.). Admittedly, the arrangement of clusters presents an intermediate connection between all

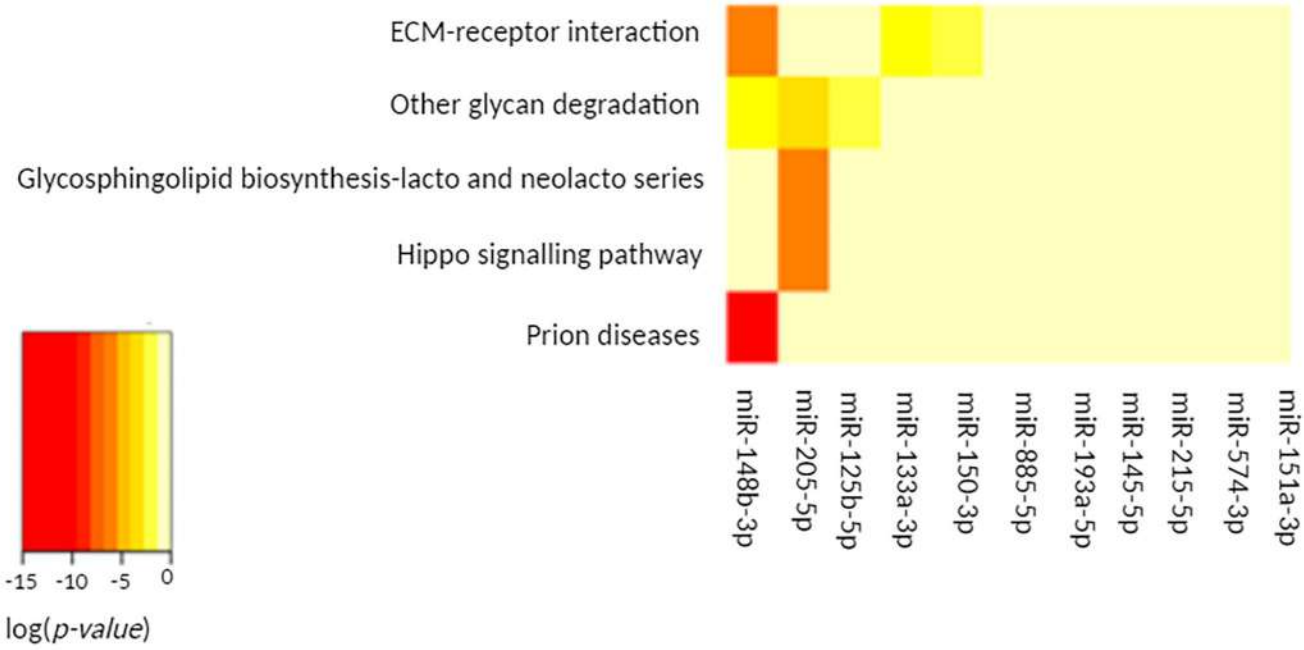

Fig. 3 The heat map shows the miRNAs altered by replacing a part of cholecalciferol with calcidiol at the increased dose (2 vs. 3), the pathways affected, and the clusters formed by each altered miRNA and pathway

Table 4 KEGG pathways that were stimulated by the combined supplementation of cholecalciferol + calcidiol in comparison to calcidiol (4 vs. 3) of finishing pigs

KEGG pathway	*p-value*	KEGG class
Prion diseases	< 1e-325	Human Diseases; Neurodegenerative disease
Signalling pathways regulating pluripotency of stem cells	1.1e-07	Cellular Processes; Cellular community - eukaryotes
Estrogen signalling pathway	9.9e-06	Organismal Systems; Endocrine system
TGF-β signalling pathway	1.7e-05	Environmental Information Processing; Signal Transduction
Proteoglycans in cancer	9.3e-05	Human Diseases; Cancer: Overview
Amphetamine addiction	0.0003	Human Diseases; Substance dependence
Phosphatidylinositol signalling system	0.002	Environmental Information Processing; Signal Transduction
GABAergic synapse	0.007	Organismal Systems; Nervous system
Morphine addiction	0.026	Human Diseases; Substance dependence
Glioma	0.029	Human Diseases; Cancer: specific types
Hippo signalling pathway	0.030	Environmental Information Processing; Signal Transduction
Pathways in cancer	0.048	Human Diseases; Cancer: Overview
Metabolism of xenobiotics by cytochrome P450	0.049	Metabolism; Xenobiotics biodegradation and metabolism

the miRNAs; however, it is miR-148b-3p and miR-205-5p that are most relevant to the biological pathways presented in Table 3.

The functional analysis led to the finding that the use of the cholecalciferol + calcidiol combination compared to the standard calcidiol dose (4 vs. 3) significantly affects 13 biological pathways (Table 4.). Most of these (10 pathways) are identical in the comparison of the standard cholecalciferol dose with the combination (1 vs. 3) (Figure S2.). Moreover, two of the remaining pathways are specific for the comparison of the increased cholecalciferol dose with the combination (2 vs. 3). The most significant difference between the results of these comparisons is the change in miR-148 expression. This miRNA is highly significant for the prion diseases pathway, which is also the most significantly altered pathway in the results of the 2 vs. 3 and 4 vs. 3 comparisons.

The heatmap (Fig. 4.) illustrating the effect of supplementation with the standard dose of calcidiol compared to the increased dose of cholecalciferol + calcidiol (3 vs. 4) shows similarities to the heatmap 1 vs. 3 comparison (Fig. 2.). This underlies the similarity of miRNA profiles of samples with the standard doses of cholecalciferol or calcidiol.

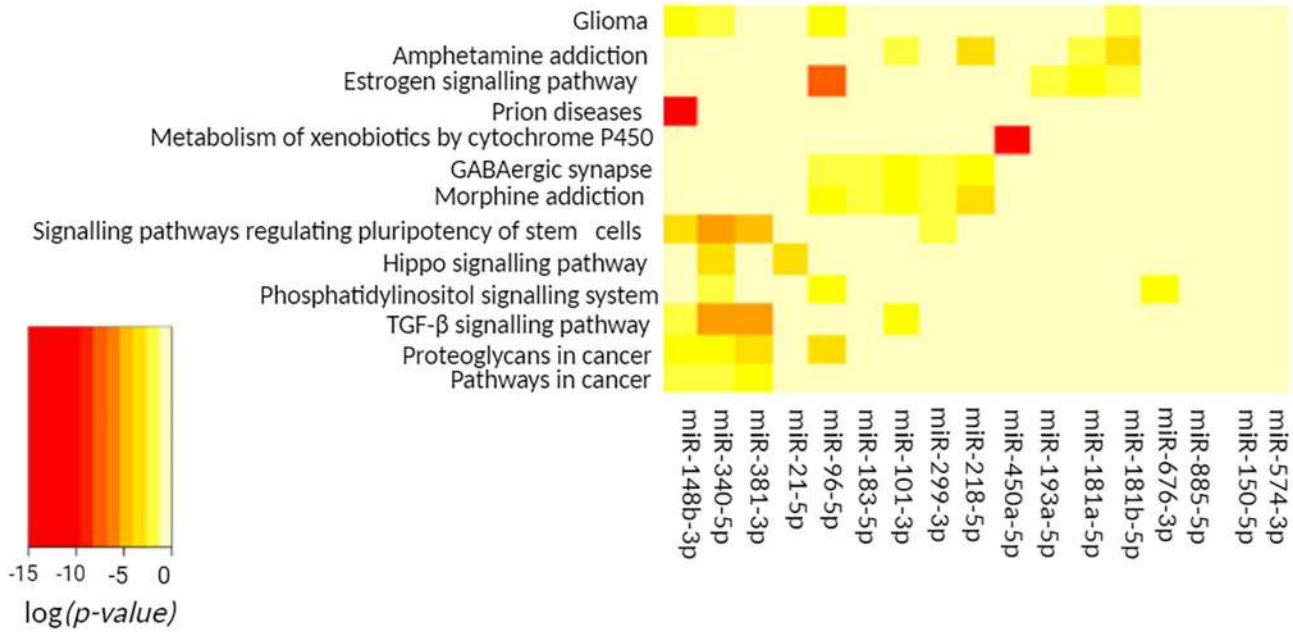

Fig. 4 The heatmap shows the miRNAs altered by the increased dose of cholecalciferol + calcidiol compared to the standard dose of calcidiol (4 vs. 3), the pathways affected, and the clusters formed by each altered miRNA and pathway

Discussion

In this study, we performed pairwise comparisons of different doses and forms of vitamin D to elucidate its effect on miRNA expression levels in healthy swine lungs. Previous studies indicate a significant association of vitamin D with the development of lung diseases, and the use of alternative forms of vitamin D such as calcidiol to treat lung diseases is of growing interest [16]. However, this is the first study describing the effects of calcidiol and choleclaciferol + calcidiol supplementation on the lung tissue miRNAome of any species.

We found that supplementation with the increased dose of the combination of cholecalciferol and calcidiol (group 3) in comparison to the other treatments induced the greatest changes in the miRNA profile of the lungs. Importantly, many of the miRNAs presented in our results appear to be typical for the lung (miR-148b, miR-101, miR-21, miR-145, miR-181, miR-191, miR-215), and they also have been mentioned in the results of other studies on this tissue [17, 18].

Many studies on the effects of vitamin D supplementation conducted to date indicate that increasing plasma 25(OH)D concentration has an immunomodulatory effect on lung tissue [19, 20]. However, the results presented here shed new light on these findings. We observed that even a significant increase in 25(OH)D concentration induced by calcidiol supplementation (group 4) did not remarkably affect the lung miRNA profile. Moreover, an almost equally high increase in plasma 25(OH)D concentration induced by supplementation with the cholecalciferol + calcidiol combination caused significant changes in the lung tissue transcriptome. This may suggest that simple increasing plasma 25(OH)D concentration may be insufficient to cause a change at the level of gene regulation and metabolic transformation of inactive vitamin D in the liver are necessary to achieve this.

The effect of increased dose of cholecalciferol

The first stage of our analysis was to determine how increasing the dose of commonly used cholecalciferol affects the miRNA profile of healthy lungs. We found that increasing the dose of cholecalciferol from 2000 IU to 3000 IU had little effect regarding the number of altered miRNAs. MiR-215, the only differentially expressed miRNA, is involved in several basic processes such as cell and tissue development, cell survival and migration, cell cycle and proliferation, as well as cellular metabolism [21]. Due to its crucial functions, the dysregulation of miR-215 expression has been implicated in the pathogenesis of many diseases. Changes in miR-215 expression in different types of cancer have been researched extensively. For some types of tumours, this miRNA is a suppressor, for others - an oncogene [21]. Moreover, one recent finding demonstrated a strong link between miR-215 and the development of pulmonary fibrosis [22]. The study also indicated that miR-215 was upregulated in herbicide-induced pulmonary fibrosis (in vitro and in vivo) causing the activation of the TGF-β pathway by inhibiting the expression of the *BMPR2* target gene.

The research concluded that reducing miR-215 expression decreased the progression of irreversible pulmonary fibrosis [22]. In our experiment, the increased dose of cholecalciferol (1 vs. 2) showed a significant upregulation of the expression of miR-215 (log2FoldChange = 2, 65). In contrast, subsequent analyses indicated that the use of the same dose of the cholecalciferol + calcidiol combination (1 vs. 3) did not affect this miR-215 Therefore, further research is necessary to clearly determine how vitamin D supplementation is related to the dysregulation of this miRNA.

The effect of replacing cholecalciferol with calcidiol

The second step of our analysis was to test the effect of calcidiol vs. cholecalciferol on the miRNA profile. Calcidiol is significantly more effective in raising the concentration of 25(OH)D in plasma compared to cholecalciferol. Therefore, we wanted to investigate if this higher efficacy may also be followed by the changes related to gene expression regulation. Surprisingly, we found that the miRNA lung profile of swine supplemented with the same dose (2000 IU/kg) of calcidiol and cholecalciferol did not differ significantly. In vitro studies demonstrated that 25(OH) vitamin D and 1,25$(OH)_2$ vitamin D have overlapping effects on gene expression but each of this metabolite also display partially independent gene transcriptional effects [23]. Nevertheless, the concentrations of vitamin D metabolites used in in vitro studies are much higher than that observed in living organisms, thus 1,25(OH)2 is the sole active metabolite under physiologic conditions. The results of our studies suggest that increasing the level of 25(OH) vitamin D by providing calcidiol, bypassing enzymatic transformations in the liver, has a negligible effect on the miRNA expression in the lungs. Perhaps greater differences in the action of two forms of vitamin D could be observed in the liver or kidneys - places where the main steps of vitamin D metabolism occur.

The effect of replacing cholecalciferol with the cholecalciferol and calcidiol combination

In contrast, the use of the increased dose of cholecalciferol + calcidiol showed several changes in miRNA expression compared to supplementation with cholecalciferol (1 vs. 3, 2 vs. 3) or calcidiol alone (4 vs. 3). This result was also surprising as one could rather expect a linear increase in the number of miRNAs changed with increasing 25(OH) vitamin D plasma concentration. Although there are several experiments describing the effects of replacing calciferol with calcidiol in pigs [24] we could not find any reports on the effect of the combination of these two substances. Molecular mechanisms that regulate vitamin D metabolism and activation are very complicated and include among others the availability of Ca ions. There is also a negative feedback loop by which 1,25$(OH)_2$ vitamin D inhibits the expression of 1α-hydroxylase (CYP27B1), preventing excessive 1,25 $(OH)_2$ vitamin D concentration. Thus the possible explanation of the highest effect of calciferol/calcidiol combination on miRNAs expression in our study could be that there is some optimal concentration of different vitamin D metabolites that triggers the changes at the miRNA level.

The effect of the cholecalciferol + calcidiol combination compared to cholecalciferol alone (3 vs. 1 and 2 vs. 3) provides interesting implications based on the changes in the expression of 4 common findings: miR-150, miR-193, miR-145, miR-574.

First- miR-150 - regulates the expression level of TLR2, a receptor involved in the primary mechanism of the immune response that protects against bacterial and viral infections [25]. Presumably, miR-150 may also play an important role in virulence through the regulation of transcription factor- c-Myb [26]. Moreover, Zheng et al. found, that miR-150 is downregulated in macrophages in tuberculosis (TB) patients [26]. On the other hand, miR-193, identified by us as upregulated by cholecalciferol + calcidiol, is overexpressed in macrophages in TB patients [26].

Nevertheless, due to the biological function of miR-193a (modulation of cell proliferation), it is worth taking a closer look at the changes in the expression level of this miRNA. According to Khordadmehr et al., miR-193a can be a valuable tool for lung cancer prognosis and diagnosis [27]. Furthermore, Yu et al. indicated that miR-193a overexpression inhibits non-small cell lung cancer (NSCLC) cell migration, invasion and epithelial-mesenchymal transition, and lung metastasis formation in vivo [28]. Thus, it may suggest that miR-193a acts as a tumour suppressor and cholecalciferol + calcidiol supplementation has a valuable effect on lung tissue in this regard. This is also confirmed by the change in expression of another tumour suppressor - miR-145 - which was also significantly upregulated under the cholecalciferol + calcidiol combination [29]. A study by Li et al. proves that miR-145 shows high expression in healthy lung tissue, while in NSCLC cells its expression is strongly reduced [30]. Moreover, it was observed that miR-145 may regulate pro-inflammatory and anti-inflammatory effects after intracellular bacterial infection in epithelial cells [31]. It has been found that downregulation of miR-145 expression inhibits eosinophilic inflammation, excessive mucus secretion, T(H)2 cytokine production and airway hyperresponsiveness [32]. Additionally, studies on the effects of exposure to tobacco smoke, conducted on rats, have shown that air pollution causes significant downregulation of miR-145 [33]. This finding is also supported by a study which used human tissues [34]. This study suggested that increased miR-145 expression alleviated apoptosis and inflammatory response by regulating apoptotic signalling mediated (*p53*) and pre-inflammatory factors (*TNF-α, IL-6, IL-8*) in bronchial epithelial cells [34]. Considering these results, the increase in miR-145 expression

under the influence of cholecalciferol + calcidiol supplementation appears to be beneficial for mammals including those constantly exposed to air pollution.

On the other hand, we can reach different conclusions by analyzing the change in miR-574 expression (upregulation). The results of research conducted on a group of healthy individuals exposed to feed dust suggest that increased expression of miR-574 is a good predictor of such exposure [35]. Moreover, oncology studies also point to negative effects associated with increased miR-574 expression. It seems to be well-confirmed that *TLR9* signalling increases the expression of miR-574 in human lung cancer cells. A meticulous in vitro and in vivo study indicated that Ches1-mediated upregulation of miR-574 enhanced tumour progression [36]. However, the in-silico study we performed showed that miR-574 is not significantly associated with any of the four cancer-related pathways. Therefore, the results of a recent study by Wei et al. seem to be more interesting [37]. These researchers discovered a mechanism of circ_0001498 action that stimulates sepsis-induced acute lung failure syndrome. Circular RNAs (circRNAs) are stable and conserved RNAs that serve as miRNA sponges affecting gene regulation. The circ_0001498 is a miR-574 sponge, and *SOX6* is a target gene of miR-574. Wei et al. found that overexpression of circ_0001498 mediated by miR-574 upregulates *SOX6*. In contrast, overexpression of miR-574 had the opposite effect (downregulation of *SOX6*), resulting in attenuation of cell damage [37]. The effect of the increased miR-574 expression on attenuating sepsis-induced lung injury was also confirmed in another study [38]. However, their effect was presumably associated with a reduction in *C3* (inflammatory transmitter regulated by miR-574) levels and a decrease in sepsis-induced endoplasmic reticulum stroma [38]. With the results cited, it can be concluded that upregulation of miR-574 induced by cholecalciferol + calcidiol supplementation may show a protective effect on lung tissue cells, both by downregulation of *SOX6* and *CD3* levels.

Among the miRNAs altered by the cholecalciferol + calcidiol combination, it is also worth mentioning about downregulation of miR-148b. Results of an experiment performed by Pacholewska et al. on healthy and asthmatic horses showed significantly higher expression (*p-value* = 0,043) of this miRNA in the blood of asthmatic horses [39]. The association of miR-148 with asthma was also pointed out by researchers experimenting on human cell lines. They suggested that miR-148b may contribute to the risk of asthma by regulating the HLA-G-a molecule with immunomodulatory properties [40]. Moreover, asthmatic horses have also been characterized by significantly (*p-value* = 0,05) higher expression of miR-215 [39]. Interestingly, our study showed a significant decrease in miR-215 expression levels under cholecalciferol + calcidiol supplementation (2 vs. 3) and a significant rise in the expression of this miRNA caused by the increased dose of cholecalciferol alone (1 vs. 2).

The opposing functions of individual miRNAs presented above illustrate the complexity of the mechanisms in which they participate. Moreover, the vast majority of miRNA profile analyzes concern patients. For these reasons, a clear assessment of the effect of changes in the expression of individual miRNAs requires additional research, including mRNA analysis.

Nevertheless, our results indicate that the cholecalciferol + calcidiol combination can regulate the cancer formation processes and functioning of the immune system by influencing the mentioned miR-150, miR-193, miR-145 and miR-574.

Functional analysis of differentially expressed miRNAs after cholecalciferol + calcidiol supplementation

In the functional analysis, we focused on miRNAs that were differentially expressed after the increased dose of cholecalciferol + calcidiol compared to a standard dose of cholecalciferol or calcidiol (1 vs. 3 and 4 vs. 3). There were 10 pathways common for these comparisons. Among them, the most significantly altered were signalling pathways regulating pluripotency of stem cells, estrogen signalling pathway, TGF-β signalling pathway and proteoglycans in cancer (Table 2. and Table 4.). Most of the identified pathways are associated with tumorigenesis, and only a few are linked to other functions attributed to vitamin D (e.g. Metabolism of xenobiotics by cytochrome P450). The reason for this may be that oncology research accounts for the vast majority of all miRNA research.

The signalling pathway regulating stem cell pluripotency is, among others, influenced by miR-340, miR-381 and miR-148. The results of our study indicate that replacing standard cholecalciferol supplementation with the increased dose of the combination of different forms of vitamin D has a significant effect on the expression of miR-340 and miR-381. Additionally, the combination use compared to the standard dose of calcidiol also alters the expression of miR-148. One of the targets of miR-340 is *Sox2*, and for miR-148 it is the *Klf4* gene. Both genes belong to a subsatellite transcriptional network that activates the reprogramming of somatic cells back to a pluripotent state. Moreover, both genes are thought to be crucial to the mechanisms of cancer cell proliferation [41]. Another pathway of interest in this regard and highly activated under increased cholecalciferol + calcidiol supplementation was the TGF- β signalling pathway. Our functional analysis showed that this pathway was regulated by changes in the expression of miR-381, miR-101, miR-148 and miR-340. *SMAD* genes (*SMAD2, SMAD9, SMAD5, SMAD4*) targeted by these miRNAs

modulate both proliferation and apoptosis as well as cell differentiation and migration [42]. One research showed that vitamin D, through its effect on the TGF- β pathway, prevents cancer cell-induced apoptosis of inflammatory cells [43]. Moreover, Moz et al., demonstrated that vitamin D can reduce the depletion of peripheral blood mononuclear cells (PBMCs) induced by cancer cells. Furthermore, this research also indicated that vitamin D inhibits tumour cell-induced release of tumour necrosis factor-alpha (TNF-α) and reduces intracellular transforming growth factor beta (TGF-β) levels [43].

Another activated path - estrogen signalling - is closely linked to the regulation of gene expression via, e.g., miRNAs [44]. We observed that the significance of the estrogen signalling pathway was determined particularly by the change in expression of miR-96, followed by miR-181a, miR-181b and miR-193a (Figs. 2 and 3.). Action of calcidiol related to the regulation of estrogen synthesis and signalling may determine the anticancer effect of this vitamin D metabolite [45]. Furthermore, calcidiol has been found to inhibit *COX-2* expression and increase *15-PGDH* expression, thereby reducing inflammatory mediator expression (prostaglandins). Thus, the putative inhibition of estrogen synthesis and signalling by calcidiol, as well as its anti-inflammatory properties, may play an important role in the prevention and treatment of cancer [45].

Also, the last of the above-mentioned pathways - proteoglycans in cancer - shows a very wide range of activities with tumour cells. Proteoglycans contribute to proliferation, adhesion, angiogenesis and metastasis, thus influencing the biology of various types of cancer significantly [46–48]. One well-known proteoglycan is hyaluronan (HA), which acts with *CD44* to enhance the growth and migration of tumour cells. The significance of the effect on this pathway was determined by the cholecalciferol + calcidiol-induced changes in miR-381, miR-96, miR-148 as well as miR-340 expression levels. Remarkably, the use of the increased dose of the cholecalciferol + calcidiol combination compared to the same dose of cholecalciferol alone could also activate glycosphingolipid biosynthesis - lacto and neolacto series - and other glycan degradation pathways belonging to the class of glycan biosynthesis and metabolism (Table 3.). A significant effect on the former pathway is related to the change in the expression of miR-205. The latter-another glycan degradation pathway is additionally regulated by changes in the expression of miR-125 and miR-148 (Fig. 3.). Interestingly, the potential link between vitamin D and proteoglycans was also highlighted in another experiment. A study by D'arrigo et al. showed that activation of the vitamin D receptor (VDR) through applications of paricalcitol raises the level of one of the proteoglycans – thrombomodulin (TM), which in turn, improves endothelial function [49]. All this information taken together, suggests that increased cholecalciferol + calcidiol supplementation may modulate the cell microenvironment by affecting glycan synthesis and metabolism.

Based on these findings, we decided to test the potential of vitamin D in regulating glycoprotein metabolism. For this purpose, we selected two genes (*NEU1* and *FUT1*) that are involved in glycoprotein metabolism and which are the targets of miRNAs significantly altered under cholecalciferol + calcidiol supplementation (miR-125b, miR-205). The first of the selected genes- *NEU1* encodes neuraminidase1. This enzyme plays an important role in various biological processes such as cell recognition, adhesion, cell signalling as well as degradation of glycoprotein molecules. The second gene - *FUT1* encodes an enzyme called fucosyltransferase 1, which is involved in the process of adding fucosyl sugars to glycans on the surface of cells. However, the analysis we performed did not show statistically significant differences in the expression of these genes, suggesting different ways of action of differentially expressed miRNAs.

Conclusions

Our results indicate that the increased dose of cholecalciferol + calcidiol causes many significant changes in the miRNA profile compared to cholecalciferol-only supplementation in the lungs of finishing pigs. Among the altered miRNAs, we can distinguish those that appeared in the results of several comparisons - miR-150, miR-193, miR-145, miR-574, and those that turned out to be the most significant from the point of view of functional analysis- miR-340, miR-381, miR-148 and miR-96. Simultaneously, we showed that the total exchange of standard cholecalciferol for calcidiol does not cause significant changes in the miRNA profile. The results of the functional analysis suggest that the cholecalciferol + calcidiol combination may affect tumorigenesis processes through, inter alia, modulation of the TGF- β pathway and pathways related to metabolism and glycan synthesis.

The miRNAs we have identified have the potential to affect dozens of genes. Therefore, the results presented above provide valuable information and a stimulus for further research including mRNA profiling results, DNA methylation analysis and clinical trials to fully report on the global molecular effects of dietary use of different doses and forms of vitamin D.

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Author contribution Conceptualization Maria Oczkowicz, Małgorzata Świątkiewicz; Methodology Maria Oczkowicz, Alicja Wierzbicka, Klaudia Pawlina-Tyszko; Software Tomasz Szmatoła, Klaudia Pawlina-Tyszko; Validation Alicja Wierzbicka; Formal analysis Klaudia Pawlina-Tyszko, Alicja Wierzbicka; Investigation Alicja Wierzbicka; Resources Małgorzata Świątkiewicz, Maria Oczkowicz; Data curation Alicja Wierzbicka; Writing Alicja Wierzbicka; Writing - review & editing Maria

Oczkowicz, Alicja Wierzbicka; Visualization Alicja Wierzbicka; Supervision Maria Oczkowicz, Klaudia Pawlina-Tyszko; Project administration Alicja Wierzbicka; Funding acquisition Maria Oczkowicz, Małgorzata Świątkiewicz.

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Data Availability The sequencing results have been deposited in the NCBI GEO database (accession number: GSE217599).

Declarations

Ethics approval The procedures included in this study related to the use of live animals were in accordance with Directive 2010/63/EU and were approved by the local Ethical Committee for Animal Experiments in Cracow, Poland (Resolution No. 427/2020, dated 22/07/2020).

Competing interests The authors declare no competing interests.

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Changes in DNA Methylation and mRNA Expression in Lung Tissue after Long-Term Supplementation with an Increased Dose of Cholecalciferol

Alicja Wierzbicka [1], Ewelina Semik-Gurgul [1], Małgorzata Świątkiewicz [2], Tomasz Szmatoła [1,3], Anna Steg [1] and Maria Oczkowicz [1,*]

[1] Department of Animal Molecular Biology, National Research Institute of Animal Production, ul. Krakowska 1, 32-083 Balice, Poland; alicja.wierzbicka@iz.edu.pl (A.W.); ewelina.semik@iz.edu.pl (E.S.-G.); tomasz.szmatola@iz.edu.pl (T.S.); anna.steg@iz.edu.pl (A.S.)
[2] Department of Animal Nutrition and Feed Science, National Research Institute of Animal Production, ul. Krakowska 1, 32-083 Balice, Poland; malgorzata.swiatkiewicz@iz.edu.pl
[3] Center for Experimental and Innovative Medicine, The University of Agriculture in Kraków, Rędzina 1c, 30-248 Kraków, Poland
* Correspondence: maria.oczkowicz@iz.edu.pl

Abstract: Maintaining an appropriate concentration of vitamin D is essential for the proper functioning of the body, regardless of age. Nowadays, there are more and more indications that vitamin D supplementation at higher than standard doses may show protective and therapeutic effects. Our study identified differences in the body's response to long-term supplementation with cholecalciferol at an increased dose. Two groups of pigs were used in the experiment. The first group received a standard dose of cholecalciferol (grower, 2000 IU/kg feed, and finisher, 1500 IU/kg feed), and the second group received an increased dose (grower, 3000 IU/kg feed, and finisher, 2500 IU/kg feed). After slaughter, lung samples were collected and used for RRBS and mRNA sequencing. Analysis of the methylation results showed that 2349 CpG sites had significantly altered methylation patterns and 1116 (47.51%) identified DMSs (Differentially Methylated Sites) were related to genes and their regulatory sites. The mRNA sequencing results showed a significant change in the expression of 195 genes. The integrated analysis identified eleven genes with DNA methylation and mRNA expression differences between the analyzed groups. The results of this study suggested that an increased vitamin D intake may be helpful for the prevention of lung cancer and pulmonary fibrosis. These actions may stem from the influence of vitamin D on the expression of genes associated with collagen production, such as *SHMT1*, *UGT1A6*, and *ITIH2*.The anti-cancer properties of vitamin D are also supported by changes in *KLHL3* and *TTPA* gene expression.

Keywords: cholecalciferol; lungs; swine; mRNA; DNA methylation

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1. Introduction

Vitamin and mineral supplementation is becoming an increasingly integral part of both human and animal diets. Among the range of available agents, vitamin D is one of the most commonly recommended dietary supplements.

Vitamin D is unique because it can be synthesized by the body. However, skin contact with sun-emitted UV-B radiation (290–315 nm) is necessary for its synthesis. Moreover, several factors, including sex, skin color, and obesity, may influence the availability of this vitamin [1]. In humans, inadequate environmental conditions e.g., staying indoors, covering the skin, and using cosmetics with UV filters, are the main cause of vitamin D deficiencies. Similarly, in the case of animal husbandry, constant or seasonal habitation with no or only poor access to sunlight determines the need for vitamin D supplementation. Nowadays, vitamin D deficiencies are frequently diagnosed and cause multifaceted irregularities in the body. In humans, adequate vitamin D status in young individuals is one of the main

elements determining their proper development and growth. Later in life, vitamin D continues to regulate the body's calcium–phosphate balance; however, the significance of its action manifests itself evenly in other areas as well.

To date, many studies have investigated the effects of vitamin D on lung diseases of different etiologies such as pneumonia, chronic obstructive pulmonary disease, asthma, or COVID-19. However, reviews compiling this research have indicated ambiguous results [2–4]. A possible reason for these inconsistencies is the variety of doses and methods of vitamin D supplementation used in the experiments.

In farm animals, especially in pigs, lung diseases caused mainly by mycoplasma pneumonia infections and high ammonium concentrations in the air significantly impact the animals' welfare and reduce breeders' incomes. However, the maximal dose of vitamin D in pig nutrition is 2000 IU/kg, as recommended by the European Union (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R0849&rid=19, accessed on 1 May 2019). Therefore, similarly, in human nutrition, the question arises as to whether a higher dose should be used.

While the beneficial effects of vitamin D on the skeletal system are widely recognized, its effects on non-skeletal diseases are the subject to debate in the scientific community. Therefore, studies on changes caused by vitamin D supplementation at the molecular level, such as gene expression, proteins, and DNA methylation, can be important voices in this debate. Nevertheless, so far, there have been few studies describing the effect of vitamin D supplementation on gene expression in lungs on the scale of the whole transcriptome in vivo. It has been shown previously that maternal vitamin D deficiencies induce changes in 2233 transcripts in newborn rats' lungs [5]. However, to our knowledge, the impact of the use of increased doses of vitamin D on the lung transcriptome or methylome in any model animal has not yet been investigated.

In recent years, the domestic pig has been increasingly used as a model animal in transcriptomic and epigenomic studies. Using the pig as a model may allow us to understand the changes at the molecular level that occur under the influence of vitamin D and assess its potential benefits on lung health. At the same time, this approach may contribute to the development of new recommendations in animal nutrition, contributing to improved health and well-being and reducing the amount of antibiotics used in animals on commercial farms.

The methodology used in this study enabled a bilateral analysis of supplementation-induced changes at both the epigenome and transcriptome levels. Our study—by combining changes in DNA methylation and gene expression in lung tissues—provides comprehensive knowledge on the effect of the increased long-term daily intake of chole-calciferol in healthy animals.

2. Results

2.1. Plasma Vitamin D Concentration

The average plasma 25(OH)D concentration in group 1 amounted to 39.67, and it was 63.96 ng/mL in group 2.

2.2. Methyl-Seq

The sequencing of RRBS libraries proceeded correctly for all 16 samples. The sequencing results are available in the Gene Expression Omnibus (GEO) database under access number GSE248607. The average number of raw reads per sample was 30.2 mln, and the average number of reads after filtering was 29.7 mln. The average number of uniquely mapped reads was 19.02 mln, which was an average of 64.16% for all the mapped reads. Detailed information on the individual samples is provided in Supplementary Table S1.

A total of 2349 CpG sides with statistically significant differences in methylation levels were identified. Of the DMSs (differentially methylated sites), 955 were hypomethylated (40.66%) and the remaining 1394 (59.34%) were hypermethylated. Most DMSs were identified on chromosomes 6 (n = 248) and 3 (n = 209). The smallest number of alterations

were on the sex chromosomes (X, 45 and Y, 12) and chromosome 16 (n = 47). On all other chromosomes, the number of DMSs ranged from 70 to 158. A total of 1116 (47.51%) of the identified DMSs were related to genes and their regulatory sites (Supplementary Table S2), and 628 of these DMS were hypermethylated (56.27%) while 487 were hypomethylated (43.73%). Annotation of the DMSs according to the gene features revealed that most of the DMSs were in introns (47.17%) (n = 1108) and intergenic regions (34.40%) (n = 808). In contrast, the DMSs in the coding parts of the genes accounted for 5.24% (n = 123).

2.3. mRNA-Seq

Sequencing proceeded correctly for 9 of the 10 samples. One sample was rejected due to a significantly lower number of reads. The sequencing results are available in the GEO database under access number GSE242293. The average number of raw reads for the nine mRNA libraries was 11.3 million, with an average of 10 million uniquely mapped reads, constituting an average mapping rate of 88.3% for all the reads. Further details on the individual samples can be found in Supplementary Table S3.

A comparison of the transcriptomic profiles of the animals receiving the increased dose and those receiving the standard dose of cholecalciferol showed that the expression of 195 genes was significantly altered (q-value of <0.05) (Supplementary Table S4). Among the altered genes, 168 were downregulated (86.15%) while the remaining 27 were upregulated (13.85%) in the animals receiving increased doses of cholecalciferol (Supplementary Figure S1). The most highly altered genes (log2FoldChange > 3 or <−3) included *EXTL1* (log2FoldChange = −4.04), *ENSSSCG00000057577* (log2FoldChange = −3.619), *ENSSSCG00000018197* (log2FoldChange = −3423), *GLP2R* (log2FoldChange = −3.36), *NPC1L1* (log2FoldChange = −3.279), *ENSSSCG00000042623* (log2FoldChange = −3.051), *SCEL* (log2FoldChange = 3.448), and *SCPEP1* (log2FoldChange = −3.091). The remaining genes all showed log2Foldchange > 0.928 or <−1.02.

2.4. Integration of the Methyl-Seq and mRNA-Seq Results

A comparison of the sets of genes whose expressions and methylation profiles were significantly altered under the influence of the increasing cholecalciferol dosages identified 11 genes (Table 1). However, only the changes in the methylation of the *ITIH2* gene (log2FoldChange = −2.164) were localized in the gene promoter.

Table 1. Similarities in the results of the mRNA-seq and methyl-seq data analyses.

Gene	mRNA-Seq		Methyl-Seq	
	Log2FoldChange	Base Mean	Meth. Diff.	Consequence
ITIH2	−2.164	1543.602	30.606	upstream gene
HSD17B6	−1.796	318.769	−32.363	intron
CYP3A22	−1.884	1599.78	−30.897	intron
TTPA	−2.297	201.53	−29.991	intron
SHMT1	−1.72	1007.481	−28.281	intron
MIPEP	−1.021	196.128	−27.29	intron
PSMA1	1.658	41.835	25.747	intron
HDLBP	−2.089	291.196	30.216	intron
KLHL3	−2.213	227.073	30.429	intron
BHMT	−2.227	1523.91	38.036	intron
UGT1A6	−1.603	260.41	50.914	intron

2.5. qPCR Validation

The *ITIH2* gene, which exhibited a significant downregulation in expression and demonstrated hypermethylation in its promoter region, underwent qPCR analysis. This analysis confirmed the significant downregulation of the *ITIH2* gene detected in the mRNA and RRBS sequencing results. Additionally, we selected three other genes (*TTPA*, *UGT1A6*, and *KLKL3*) whose expression as well as methylation levels changed under the influence of

the increasing cholecalciferol dosages (Figure 1). The qPCR results confirmed significant reductions in the expression of *TTPA* (p-value = 0.001) and *UGT1A6* (p-value = 0.038) genes. However, the decrease in the *KLHL3* gene expression according to the qPCR results was not statistically significant (p-value = 0.076).

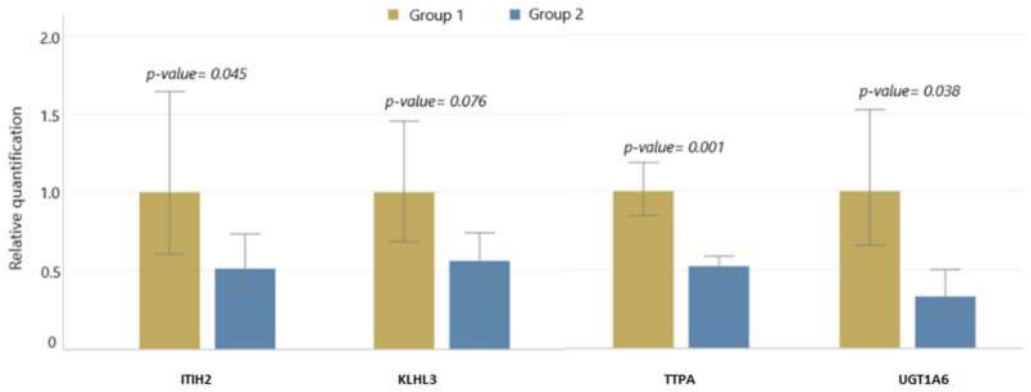

Figure 1. The qPCR results (RQ, relative quantification) for the groups of animals supplemented with different doses of vitamin D. The error bars represent the RQ min and RQ max values within the groups.

2.6. Functional Analysis

2.6.1. Methyl-Seq

Table 2 shows the top 10 (FDR < 0.0017 and strength > 0.3) functional effects identified by the RRBS sequencing. The analysis of a set of genes with altered methylation levels showed changes in 82 biological processes. According to the FDR values (<0.00075) and strength level (0.35), the actin filament-based process and actin cytoskeleton organization were most altered in this group. Potential effects on 39 biological functions were also observed. Within this group, according to the FDR values (<0.00031) and strength level (>0.35), the greatest effects of the increasing vitamin D intake were observed within the guanyl-nucleotide exchange factor activity, GTPase regulator activity, and actin binding. On the other hand, within the biological components, the results of the functional analysis showed effects on 26 areas. According to the FDR value (<0.00072) and strength level (>0.35), the greatest changes occurred in the actin cytoskeletons. All the results of the gene-set analysis are presented in Supplementary Table S5.

Table 2. Most significant results from the functional analysis of the RRBS sequencing data from the lung tissues from pigs supplemented with increased doses of cholecalciferol.

Base	Term Description	No. of Genes	Strength	FDR
GO molecular function	GTPase regulator activity	55	0.45	3.3×10^{-7}
GO molecular function	Cytoskeletal protein binding	84	0.31	1.34×10^{-6}
GO molecular function	Enzyme regulator activity	92	0.28	4.21×10^{-6}
GO molecular function	Kinase binding	60	0.35	7.62×10^{-6}
GO molecular function	Guanyl-nucleotide exchange factor activity	28	0.47	0.00025
GO molecular function	Actin binding	43	0.36	0.00031
GO biological processes	Actin filament-based process	51	0.35	0.00035
GO cellular component	Actin cytoskeleton	41	0.35	0.00072
GO biological processes	Actin cytoskeleton organization	47	0.35	0.00075

The separate analyses of the hypomethylated and hypermethylated DMSs were additionally carried out. The results of the analyses showed that hypomethylated DMSs enriched 16 biological process, 7 molecular functions, and 8 cellular components. According to the FDR and strength levels, the increasing vitamin D doses enriched the actin-binding (FDR = 0.016 and strength = 0.45) and cytoskeletal protein-binding (FDR < 0.001

and strength = 0.41) functions most potently. An analysis of the hypermethylated DMSs showed that 24 biological processes, 23 molecular functions, and 16 cellular components were enriched. According to the FDR and strength levels, the GTPase regulator activity was the most enriched (FDR < 0.001 and strength = 0.46). Interestingly, the analysis of the hypomethylated DMSs also showed a strong strength level of this molecular function (0.43; FDR = 0.02). On the other hand, according to the KEGG database, based on a set of hypermethylated DMSs, the phospholipase D signalling pathway was altered (FDR = 0.0153 and strength = 0.61).

The results of the hypomethylated/hypermethylated DMSs analysis are presented in Supplementary Table S7.

2.6.2. mRNA-Seq

Table 3 shows the top 10 strongest functional changes (5 from the GO database and 5 from KEEG) caused by the increasing cholecalciferol doses based on the mRNA sequencing results. The analysis showed that the applied changes in the diets of the pigs could significantly affect 103 biological processes. Based on the FDR (<0.0001) and strength (>1.2) values, we highlighted such processes as the negative regulation of blood coagulation, regulation of blood coagulation, retinoid metabolic process, and regulation of wound-healing. In turn, among the 19 significantly altered biological functions, we identified (FDR < 0.0001 and strength > 0.2) oxidoreductase activity and catalytic activity. Moreover, eight significant differences in the biological components were identified. In this area, the largest changes (FDR < 0.001 and strength > 0.49) occurred in the extracellular region and extracellular space. The results from the KEGG database indicated the effects of the increased vitamin D intake on 24 pathways, of which as many as 12 pathways with strength values of >1 and FDR values of <0.0001 could be distinguished. The results of the gene set analysis of the mRNA-seq results are presented in Supplementary Table S6.

Table 3. Most significant results from the functional analysis of the mRNA sequencing data for the lung tissues from pigs supplemented with increased doses of cholecalciferol.

Base	Term Description	No. of Genes	Strength	FDR
KEGG	Chemical carcinogenesis	12	1.55	1.66×10^{-12}
KEGG	Metabolism of xenobiotics by cytochrome P450	11	1.57	9.57×10^{-12}
KEGG	Retinol metabolism	10	1.52	2.77×10^{-10}
KEGG	Drug metabolism–cytochrome P450	9	1.5	3.59×10^{-9}
GO biological process	Negative regulation of blood coagulation	8	1.53	2.94×10^{-7}
GO biological process	Regulation of blood coagulation	9	1.4	2.94×10^{-7}
GO biological process	Regulation of wound-healing	10	1.21	8.14×10^{-7}
GO biological process	Blood coagulation	10	1.17	0.0000017
GO biological process	Retinoid metabolic process	8	1.29	7.68×10^{-6}
KEGG	Tyrosine metabolism	5	1.34	0.00022

The results of the analysis of the set of downregulated genes showed the enrichment of 106 biological processes, 28 molecular functions, and 8 cellular components. According to the FDR and strength levels, the most significantly enriched appeared to be the propionate metabolic process (FDR = 0.0007 and strength = 2.22). According to the KEGG database, the set of downregulated genes was most strongly involved (FDR < 0.001 and strength > 1.6) in the metabolism of the xenobiotics by the cytochrome P450 and chemical carcinogenesis pathways. The set of upregulated genes was not significantly involved in any pathway, nor did they significantly enrich any of the processes. The entire results of the analysis are presented in Supplementary Table S7.

2.6.3. Integration of the Methyl-Seq and mRNA-Seq Results

The analysis of a set of 11 genes, presented in Table 1, showed the significant affectation of 3 pathways (Table 4). The effects on the steroid hormone biosynthesis and

retinol metabolism pathways were conditioned by the altered expression of *CYP3A22* and *HSD17B6*. The glycine, serine, and threonine metabolism pathways, on the other hand, were found to be significantly altered due to the effects on the *SHMT1* and *BHMT* genes.

Table 4. Results of the functional analysis of the set of 11 genes identified in the RRBS and mRNA sequencing data from the lung tissues from pigs supplemented with increased doses of cholecalciferol.

Base	Term Description	No. of Genes	Strength	FDR
KEGG	Steroid hormone biosynthesis	2	2.03	0.0387
KEGG	Glycine, serine, and threonine metabolism	2	2.11	0.0387
KEGG	Retinol metabolism	2	2	0.0387

3. Discussion

The present study showed that increasing doses of cholecalciferol caused significant changes in the transcriptomes and methylomes of swine lung tissues. We found that increased vitamin D intake significantly altered the expression of nearly 200 genes. Moreover, we identified 1116 differentially methylated sites related to genes and their regulatory sites. Among all the DMSs, only 11 were associated with genes with significant changes in expression levels (Table 1). A presumed reason for the poor correlation of the results (RRBS and mRNA) was the different number of samples used in each NGS study. However, based on the results of other researchers, it should be noted that relatively low correlations are common, especially in experiments where the effect of the studied factor is not exceptionally strong [6].

It has been assumed that, via the VDR, vitamin D regulates key mechanisms such as metabolism and cell proliferation [7,8]. Increased vitamin D intake causes activation of the VDR, which, in turn, intensifies gene transcription. However, our results indicated that more than 86% of the genes altered by the increased vitamin D intake were downregulated. Also, among the genes identified as those with altered methylation, there was little bias toward hypermethylation (56%).

A functional analysis of the methylome sequencing results showed that the increasing vitamin D intake affected the GTPase-, cytoskeleton-, and actin-related processes most significantly (Table 2). Interestingly, a recent finding showed that reduced SARS-CoV-2 lung disease severity was associated with methylation changes within processes related to GTPase and actin [9]. Moreover, it appeared that increasing the dose of cholecalciferol induced changes in the methylome associated with pulmonary fibrosis. Our results indicated methylation changes in the *MeCP2* (methyl-CpG binding protein 2) gene, which is considered a key regulator of fibrosis (Supplementary Table S2) [10]. It has been shown, for example, that *MeCP2* KO mice are resistant to pulmonary fibrosis [11]. Another study found that vitamin D could inhibit the TGFβ1 stimulation of α-smooth-muscle actin expression and polymerization and prevent the upregulation of fibronectin and collagen in fibroblasts in vitro. These results indicated that vitamin D may inhibit the pro-fibrotic phenotype of lung fibroblasts and epithelial cells [12]. Additionally, we identified four genes encoding collagen (*COL4A1*, *COL5A1*, *COL6A3*, and *COL24A1*) whose methylation levels were also changed (Supplementary Table S2). One of these, *COL4A1* is considered a target of miR-29 in pulmonary fibrosis [10].

The functional analysis of the mRNA-seq results (Supplementary Table S6) identified genes related to complement and coagulation cascades (FDR < 0.0001) and chemical carcinogenesis (FDR < 0.0001). We detected the significant downregulation of the expression of genes encoding acute phase proteins (e.g., *FBG*, *FGA*, and *FGG*) and tumor-associated genes such as *SERPINC1* and *F2* (Supplementary Table S4). *SERPINC1* is recognized as a key gene in the processes of cancer cell proliferation and migration. It has been shown that reducing *SERPINC1* expression can be an effective treatment for lung cancer [13,14]. Furthermore, the *F2*-thrombin factor 2 gene may not only affect interactions between viral proteins and cytokine receptors [15] but also play an essential role in blood coagulation, angiogenesis, tissue repair, and vascular integrity.

Our study also showed that increasing vitamin D intake results in changes in the methylation and expression levels of genes linked to retinol and retinoid metabolism (Tables 3 and 4). This was in line with the fact that the active form of vitamin D, combined with vitamin D receptor (VDR), forms a heterodimer with the retinoid X receptor (RXR). The heterodimer formed in this way can interact with the vitamin D gene's response elements (VDRE) [16]. Interestingly, other researchers have shown that vitamin D supplementation can affect methylation within the RXR promoter [17].

The functional analysis of the results shared by the methylome and mRNA sequencing indicated the anti-tumor and anti-fibrotic effects of increasing the cholecalciferol dosage. We identified changes in the expression of the *PSAT1* and *SHMT1* genes, as well as methylation changes in the *SHMT1* gene. The in vitro and in vivo studies on mice by Zhu et al. showed that *PSAT1* is a strong promoter of pulmonary fibrosis and that VDR regulates the expression of this gene [18]. Moreover, an in vitro study on human cell lines showed that silencing the *PSAT1* gene resulted in the inhibition of tumor proliferation and growth in non-small cell lung cancer [19]. The *PSAT1* plays an important role in connecting pathways involving glycolysis and the biosynthesis of amino acids. Its decreased expression inhibits the synthesis of serine and, consequently, glycine. Glycine is a major component of collagen, which is the building material of connective tissue. Alterations in collagen production cause lung fibrosis and may regulate cell proliferation in lung tumors. Increasing the dose of cholecalciferol resulted in a significant decrease in *PSAT1* expression and an effect on both the glycolysis and amino acid synthesis pathways, including glycine and serine. Therefore, our findings supported the suggestions that vitamin D may be a therapeutic agent in patients with pulmonary fibrosis and lung cancer, though not only through the regulation of *PSAT1* but also the *SHMT1* gene. *SHMT1* encodes serine hydroxymethyltransferase 1, an enzyme essential for converting serine to glycine [18]. We identified the downregulation and hypermethylation of the *SHMT1* gene under the influence of increasing cholecalciferol doses. Based on these results, it could be assumed that the effect of vitamin D in inhibiting collagen production in the lungs was bidirectional: first, through the inhibition of the synthesis of serine, the material necessary for the formation of collagen, and second, through the downregulation of the enzyme directly involved in collagen synthesis. Interestingly, the *UGT1A6* gene, whose variations in expression and methylation were observed, is also significantly associated with the development of pulmonary fibrosis. There have been some indications that this gene is significantly upregulated in idiopathic pulmonary fibrosis patients compared to healthy controls [20]. Moreover, the increased expression of *UGT1A6* is also characteristic of patients with cancers, including lung cancers [21,22]. UDP-glucuronosyltransferases are a group of enzymes associated with the catabolism of drugs and xenobiotics. Therefore, it is believed that altering the expression of *UGT1A* genes, including *UGT1A6*, can significantly modulate the response to the treatment, development, and progression of cancer [22]. A decrease in *UGT1A6* gene expression under vitamin D (calcitriol) supplementation has already been observed by other researchers, though in rat liver tissues [23].

The effects of vitamin D on processes associated with pulmonary fibrosis and cancer were also suggested by changes within the *ITIH2* (*SHAP*) gene. The *ITIH2* gene in our study was the only one to have significantly altered mRNA expression via a change in methylation within the promoter. The *ITIH2* gene, through the inter-alpha-trypsin inhibitor protein it encodes, is related to the serine inhibitor group. This gene may be involved in the control of inflammation and immune processes [24]. There have been indications that the ability to regulate angiogenesis may link *ITIH2* to the process of pulmonary fibrosis [25]. A study by Garantziotis et al. showed that induced lung injury resulted in a greater than sixfold increase in *ITIH2* expression in the liver and a decrease in its expression in the lungs [25]. Similarly, in lung cancers, *ITIH2* shows significant downregulation in the altered tissues [24]. ITIH2 is a part of IaI protein, which is synthesized in the liver. IaI consists of a light chain and two heavy chains (ITIH1 and ITIH2). It is generally considered that IaI is a systemic factor that enhances angiogenesis. Histological studies have shown the

strong colocalization of inter-α-trypsin inhibitor protein (IaI) in disease focus in patients with pulmonary fibrosis. In contrast, the lungs of healthy subjects were weakly stained for IaI [25]. It is noteworthy that angiogenesis in patients with pulmonary fibrosis and cancer may have a dual effect. In the early stages of the disease, it may promote tissue regeneration; however, in the exacerbated stages, it may accelerate the development of the disease. The results cited above have indicated that IaI shows higher levels in patients with fibrosis; however, the expression of the lung *ITIH2* gene decreases. This result may suggest another role, unrelated to the IaI, for *ITIH2* in lung tissues.

KLHL3 (kelch-like family member 3) is another gene that was downregulated by the increasing cholecalciferol intake in our study. There are many indications that the *KLHL3* gene is linked to cancer development. This gene appears to be downregulated in the plasma samples of patients with lung cancers [26]. However, findings regarding the expression and regulation of this gene in lung tissues are lacking. Studies presenting the expression of this gene in tumor tissues have indicated that it can be both overexpressed and downregulated under the influence of disease [27]. Nevertheless, the role of KLHL3 in the pathogenesis of cancers formed through viral interaction appears to be well-proven [28]. Indeed, it appears that KLHL3 protein expression is significantly higher in cells expressing vIRF1 (viral interferon regulatory factor 1), as well as in cells infected with KSHV (Kaposi's sarcoma-associated herpesvirus). Researchers have found that KLHL3 mediates the infection and replication of KSHV-induced tumorigenesis [28].

Another interesting result was the significant downregulation of the *TTPA* (alpha tocopherol transfer protein) gene, which we confirmed by the mRNA-seq (log2FoldChange = −2.297) and qPCR results. In addition, we observed significant methylation changes in the introns of this gene. TTPA binds the biologically active form of vitamin E (α-tocopherol) and plays an important role in regulating the levels of this vitamin in the body. Due to its strong antioxidant properties, numerous studies have been conducted to determine whether vitamin E supplementation can protect against cancer. The first clinical trial conducted on a large group of male tobacco smokers surprisingly showed that mortality was 2% higher in the group of those taking a daily dose of alpha-tocopherol compared to the group of those not supplemented with alpha-tocopherol [29]. The lack of protective effects of vitamin E supplementation in cancer and cardiovascular disease was also confirmed in another clinical trial conducted on a group of healthy women [30]. The change in the expression level of the *TTPA* gene we observed under the influence of vitamin D indicated a possible interaction between simultaneous dietary supplementation with vitamin D and E, and we recommend further studies in this area.

Altogether, our findings, along with those of other researchers, have suggested that increasing the intake of cholecalciferol in the daily diet may exhibit anti-cancer and anti-fibrotic effects (Figure 2). It is assumed that respiratory infections can cause chronic fibrotic reactions even up to several months after infection [31]. Therefore, our results appear to be important both for breeders of animals at risk of lung disease and from a human health point of view. It is important to highlight that our mRNA sequencing analysis was conducted on a limited number of samples exclusively from males. Nevertheless, the robustness of these findings was validated across a more extensive cohort encompassing both males and females through RRBS and qPCR testing. Nonetheless, further investigations are warranted to comprehensively explore the correlation between vitamin D and the development of pulmonary fibrosis and cancer formation.

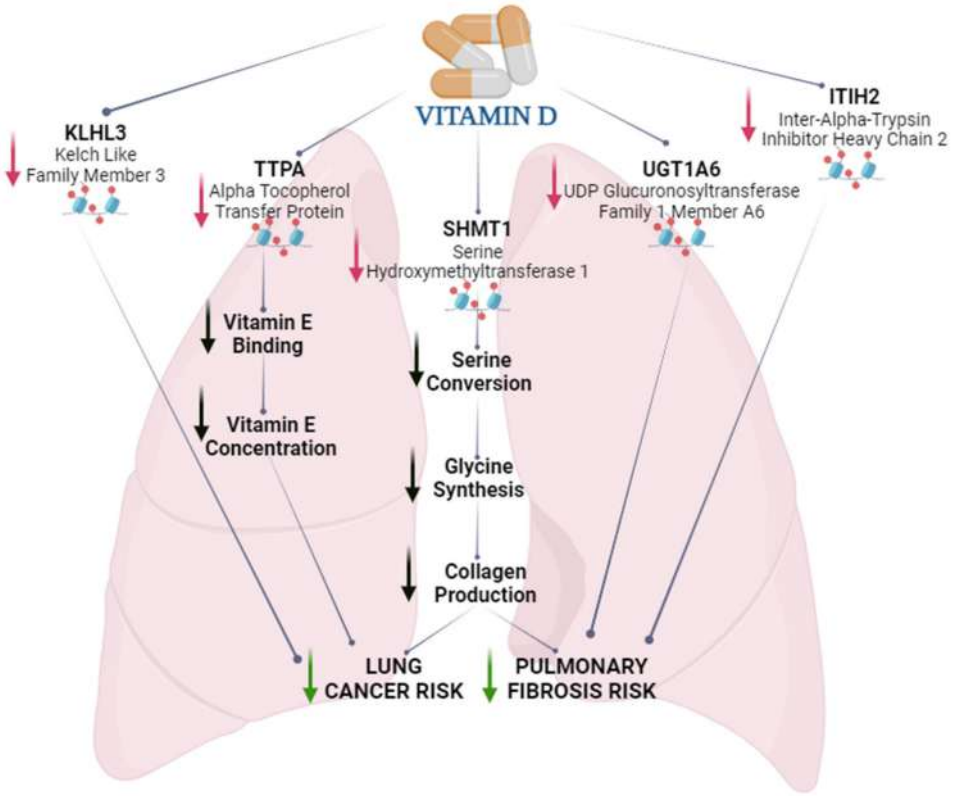

Figure 2. Putative molecular mechanism of the effect of increasing vitamin D intake on lung cancer and pulmonary fibrosis risks. The red and blue dots illustrate the change in gene methylation, the red arrows show decreases in gene expression, the black arrows illustrate the potential inhibition of processes, and the green arrows show the likely final effects of the changes that occurred.

4. Materials and Methods

4.1. Animal, Diets, and 25(OH)D Blood Serum Concentration Measurements

All the procedures conducted on live animals had the approval of the local Ethical Committee for Experiments with Animals in Cracow (Resolution No. 427/2020 dated 22 July 2020). The animals were kept under the same conditions, in individual pens, at the Research Station of the State Research Institute of Animal Production in Grodziec Śląski. In the experiment, animals from two groups were utilized. Animals in the first group (group 1) received a standard dose, while those in the second group (group 2) received increased doses of cholecalciferol (Figure 3). Both groups contained 10 individuals, with 5 males and 5 females in each group. The animals received the same feed, though it differed in the levels of cholecalciferol. The feed covered all their current requirements (grower, 30–60 kg: metabolizable energy, 13.3 MJ and total protein, 172 g/kg; finisher, 60–110 kg: metabolizable energy, 13.3 MJ and total protein, 156 g/kg).

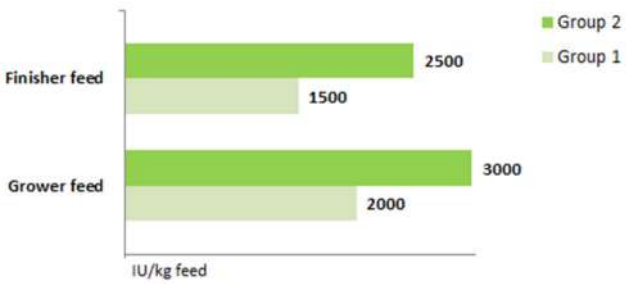

Figure 3. The content of cholecalciferol in the grower and finisher feeds used in the groups of animals.

The feeding experiment ended when the animals reached individual weights of 110 kg (88 days). Immediately after the animals were slaughtered, samples from the middle parts of the upper lobes of the left lungs were collected. The samples were stored in a freezer (−85 °C) until further analysis.

The assays of total 25(OH)D concentrations in the animal plasma samples were carried out by the ANCHEM Laboratorium from Katowice in Poland, in line with the RIA method, using a DIAsource 25OH Vitamin D total-RIA-CT Kit (Rue de Bosquet 2, 1348 Louvain-La-Neuve, Belgium) and Multigamma 1260 multidetector instrument (LKB Wallac, Turku, Finland). The plasma vitamin D levels were measured using blood samples collected at slaughter.

4.2. DNA and RNA Isolations, Library Construction, and Sequencing

The total DNA from 16 lung samples (8 females and 8 males) was isolated using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The concentrations of the genetic materials (DNA) were confirmed using NanoDrop™ 2000/2000c spectrophotometers (Thermo Scientific™, Waltham, MA, USA). Then, high-quality DNA samples were used to prepare the libraries. The libraries for the methyl-seq were prepared using an Ovation® RRBS Methyl-Seq System 1–16 kit (Tecan, San Jose, CA, USA). The RRBS libraries were sequenced in the United States by Medical Research Foundation NGS Core using Illumina NovaSeq 6000 device (Illumina, San Diego, CA, USA) as 150 bp-paired end reads and using PhiX control.

RNA was isolated from 10 lung samples (5 samples from group 1 and 5 samples from group 2, only females) using a PureLink™ RNA Mini Kit (Invitrogen, Waltham, MA, USA). The isolated genetic materials were quantitatively and qualitatively assessed using Tapestation 2200 (Agilent, Santa Clara, CA, USA). The high-quality RNA samples were used for the library preparation using a QuantSeq 3′mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). The procedures were carried out in accordance with the manufacturers' recommendations. Quantitative evaluation of the prepared libraries was performed using Qubit (Thermo Scientific™, Waltham, MA, USA), while a qualitative evaluation was assessed using Tapestation 2200 device (Agilent, Santa Clara, CA, USA). Sequencing of the mRNA pooled libraries (75 bp single read) was performed using a Nextseq 5500 device (Illumina, San Diego, CA, USA). The libraries were prepared for sequencing according to the standard normalization method from the NextSeq 500 and NextSeq 550 Sequencing Systems-Denature and Dilute Libraries Guide protocol. We used a 2 nM starting library concentration and a 10% PhiX addition.

4.3. qPCR Validation

Twenty samples were used for the qPCR analysis. The experiment began with RNA isolation from the 10 remaining samples. RNA from 20 lung samples was reverse transcribed. We performed qPCR on the *KLHL3*, *TTPA*, *UGT1A6*, and *ITIH2* genes using *RPS29* as an endogenous control. The genes were selected based on the results of the RRBS and mRNA sequencing, and 500 ng of RNA was reverse-transcribed to cDNA using a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, Waltham, MA, USA). The real-time PCR was performed using TaqMan™ Fast Advanced Master Mix for qPCR (Applied Biosystems™, Waltham, MA, USA) and TaqMan Real-Time PCR assays on a QuantStudio ™ 7 Flex Real-Time PCR System (Applied Biosystems™, Waltham, MA, USA). The relative quantity data were analyzed on a Thermo Fisher Cloud (Thermo Scientific™, Waltham, MA, USA). Analysis of the results was carried out using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

4.4. Statistical Analysis

4.4.1. Methyl-Seq

The first step of the data analysis was the quality control of the raw sequencing reads using FastQC v. 0.12.1 software. Low-quality reads (quality level of <20 and read length

of <36) and fragments containing adapter sequences were filtered using FlexBar v. 3.5.0 software. Matching to the swine reference genome (Sscrofa11.1) was performed using bisulfite mapping software-BSMAP v. 2.9.0, with the default options recommended for RRBS data specifying the enzyme cleavage site (MspI) and mapping to the two forward strands. The Methylation Caller software provided in the BSMAP v. 2.9.0 package was used to determine the percentage of methylation in the individual CpG sites with the coverage of more than 5 reads. The CpG methylation analysis included the distribution in the swine chromosomes and the distribution in the upstream regions, 5′-UTRs, 3′-UTRs, exons, introns, and intergenic regions. Next, the files were processed using R package (version 4.3) to obtain the input data for the Methylkit software. Methylkit v. 1.26.0 software was used to identify the differentially methylated sites (DMS) with cutoff values of at least 25% methylation differences between the two groups and q-values of <0.05. Gene annotations were obtained from the Sscrofa11.1 Ensembl GTF annotation.

4.4.2. mRNA-Seq

The demultiplexed fastq files downloaded from the sequencing server were quality-checked, trimmed from reads, and mapped from reads using FastQC 11.8, FLEXBAR 3.5.0, and TopHat 2.1.1, respectively. Samtools 1.9, RSeQC, HTSeq-count 0.11.1 software, and Gtf-Ensembl annotation 96 were used to assess the mapping statistics and read counts. Then, to perform the differential expression analysis, the R program and DESeq 2 software suites were used. Differentially expressed genes were regarded as genes with q-values < 0.05 (FDR, false discovery rate), Benjamini–Hochberg (BH) adjustments, and no fold-change thresholds. Only the genes that showed base means > 20 were used for further analyses. The gene annotations were obtained from the Sscrofa11.1 Ensembl GTF annotation.

4.4.3. Integration of the Methyl-Seq and mRNA-Seq Results

We used the RRBS and RNA-seq datasets to relate the changes in the CpG site methylation to the changes in the expression levels of the associated genes. The Venny 2.1 program was used for this comparison.

4.4.4. Functional Analysis

An analysis of the methyl-seq results, mRNA-seq results, and the combined data was performed. The upregulated/hypermethylated and downregulated/hypomethylated genes were used for joint (gene set analysis (GSA)) and separate analyses.

The functional analyses were carried out using STRING software (version 12.0), with which Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed. In addition, the BioMart (release 110) and Venny 2.1 programs were used during these analyses. The top 10 results from the functional enrichment analysis were selected based on the FDR (false discovery rate) and strength level values.

5. Conclusions

Our results showed the putative mechanism by which increasing vitamin D intake may reduce the risk of lung cancer and pulmonary fibrosis in healthy individuals. These actions may have been due to the effects of vitamin D on collagen production-related genes, such as *SHMT1*, *UGT1A6*, and *ITIH2*. The anticancer properties of vitamin D are further supported by changes in the expression levels of the *KLHL3* and *TTPA* genes. Changes in *TTPA* gene expression also indicated that vitamin D can affect vitamin E action. The likely epigenetic mechanism regulating these processes is DNA methylation; however, further experimental studies are needed to confirm our hypothesis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25010464/s1.

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Conflicts of Interest: The authors declare no conflicts of interest.

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