Maternal loss of *Cyp24a1* causes increased intestinal calcium absorption and hypercalcemia during pregnancy but reduced skeletal resorption during lactation in mice

By

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I. ABSTRACT

Inactivating mutations of 24-hydroxylase (*CYP24A1*) cause mild hypercalcemia in humans that can become severe during pregnancy. We studied *Cyp24a1* null mice (NULL) during reproductive cycles, hypothesizing that they have a greater increase in calcitriol during pregnancy, leading to a greater increase in intestinal calcium absorption that causes hypercalcemia. We also hypothesized that bone loss would be reduced during lactation due to a persistent increase in intestinal calcium absorption.

Wild-type (WT) and NULL females were mated with heterozygous (HET) males. We examined them at baseline (BL), late pregnancy (LP), mid-lactation (ML), late lactation (LL), and during four weeks of post-weaning recovery (R1-4). Tests included intestinal calcium absorption, bone mineral content (BMC), μCT of femurs, 3-point bending tests of tibias, serum hormones, serum and urine minerals, hematocrit, milk analysis, and intestinal gene expression.

At LP, both NULL and WT mice saw a ~12% increase in BMC. In NULLs, calcitriol was 2.5-fold higher, with a 3-fold increase in intestinal calcium absorption, and marked hypercalcemia. By LL, NULLs remained hypercalcemic compared to WT and had reduced lactational BMC loss in the lumbar spine (11% vs. 21%, $p<0.02$).

In summary, *Cyp24a1* ablation raises intestinal calcium absorption and causes hypercalcemia during pregnancy and lactation, with reduced lactational BMC loss. Treatment for women with gestational hypercalcemia due to *Cyp24a1* mutations should target lowering calcitriol or intestinal calcium absorption, as increased bone resorption is not the underlying issue.

II. GENERAL SUMMARY

The primary function of calcitriol, the active form of vitamin D, is to increase calcium levels in the blood. The maternal body increases calcitriol levels during pregnancy to provide sufficient calcium for the developing fetus. Without adequate catabolism of calcitriol, an excessive increase in calcium can occur, leading to potential complications during pregnancy. CYP24A1 is the primary enzyme responsible for degrading calcitriol, thus keeping this hormone in adequate levels.

In this project, we studied mice lacking a functional *CYP24A1* gene before and during pregnancy, lactation, and post-weaning. We hypothesized that these mice would experience a more significant increase in calcitriol during pregnancy and lactation, leading to increased intestinal calcium absorption and reduced bone loss.

In summary, the *CYP24A1* ablation increased intestinal calcium absorption, caused hypercalcemia during pregnancy and lactation, and reduced lactational BMC loss. Treatment for women with gestational hypercalcemia from CYP24A1 mutations should focus on lowering calcitriol or intestinal calcium absorption.

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Most of this thesis is based on data and findings that have been previously published in the Journal of Bone and Mineral Metabolism (JBMR),[1] for which I am the first author. I would like to acknowledge the contributions of my co-authors and collaborators to the publication, as their support and insights were integral to the success of this work. I am grateful for the opportunity to incorporate this material into my thesis with the permission of Oxford University Press (Appendix 1).

IV. TABLE OF CONTENTS

V. LIST OF FIGURES

VI. LIST OF TABLES

VII. LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bovine Serum Albumin
BGLAP	Bone gamma-carboxyglutamic acid-containing protein
BL	Baseline
BMC	Bone mineral content
BMD	Bone mineral density
BMPs	Bone morphogenic proteins
BMU	Basic multicellular unit
CaM	Calcium-binding protein, calmodulin
CaSR	Ca^{+2}-sensing receptor
cDNA	Complementary DNA
CIHR	Canadian Institutes of Health Research
CT	Computed tomography
CTX	C-terminal telopeptide
CTX-1	C-terminal telopeptide of type 1 collagen
CYP24A1	Cytochrome P450 family 24 subfamily A member 1
CYP27A1	Cytochrome P450 family 27 subfamily A member 1

CYP27B1	Cytochrome P450 family 27 subfamily B member 1
CYP2R1	Cytochrome P450 family 2 subfamily R member 1
CYP3A4	Cytochrome P450 family 3 subfamily A member 4
DBP	Vitamin D binding protein
DXA or DEXA	Dual-energy x-ray absorptiometry
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FGF23	Fibroblast growth factor 23
FGFR1	FGF binds to FGF tyrosine kinase receptor 1
FGFs	Fibroblast growth factors
HET	*Cyp24a1*$^{+/-}$ mice
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IGF-1	Insulin-like growth factor-1
IIH	Idiopathic infantile hypercalcemia
IOF	International Osteoporosis Foundation
JAMs	Junctional Adhesion Molecules

KCNma1	Calcium-activated potassium channel subunit alpha-1
LBD	Ligand-binding domain
LL	Late lactation
LP	Late pregnancy
M-CSF	Macrophage colony stimulating factor
MAPK	Mitogen-activated protein kinase
Micro-CT	Micro Computed Tomography
ML	Mid-lactation
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaPi2a	Sodium-dependent phosphate transport protein 2A
NaPi2b	Sodium-dependent phosphate transport protein 2B
NBF	Neutral buffered formalin
NCBI	National Center for Biotechnology Information
NCoA62	Nuclear coactivator-62 kDa
NCX1	Solute Carrier Family 8 Member 1
NF-kappa	Factor nuclear kappa B
NHANES	National Health and Nutrition Examination Survey

NIHR	National Institute for Health and Care Research
NO	Nitric oxide
NPT2a	Sodium-Dependent Phosphate Transport Protein 2A
NPT2c	Sodium-Dependent Phosphate Transport Protein 2C
NULL	*Cyp24a1*$^{-/-}$ mice
OPG	Osteoprotegerin
OPN	Osteopontin
P1NP	Procollagen type I N-terminal propeptide
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PGK-Neo	Phosphoglycerine Kinase - Neomycin
PHPT	Primary hyperparathyroidism
PMCA1b	ATPase Plasma Membrane Ca2+ Transporting 1
PTH	Parathyroid hormone
PTH1R	Parathyroid Hormone Receptor Type 1
PTH2R	Parathyroid Hormone Receptor Type 2
PTHrP	Parathyroid hormone related peptide
qPCR	Quantitative polymerase chain reaction
RANKL	Receptor activator of NF-kappa B ligand

RDA	Recommended Dietary Allowance
RER	Rough endoplasmic reticulum
RCF	Relative centrifugal field
RNA	Ribonucleic acid
RNase	Ribonucleases
ROI	Region of interest
RT-qPCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SLC34A1	Sodium-dependent phosphate transport protein 2A
SOST	Sclerostin
SPX	SYG1/Pho81/XPR1 domain
SRC1	Nuclear receptor coactivator 1
SYBR	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TF	Transcription factors
TNF	Tumor necrosis factor
TRPV5	Transient receptor potential vanilloid 5
TRPV6	Transient receptor potential vanilloid 6

UV	Ultraviolet light
VDR	Vitamin D receptor
VDREs	Vitamin D response elements
WT	*Cyp24a1*$^{+/+}$ mice

VIII. LIST OF APPENDENCIES

1. INTRODUCTION

1.1. Preamble

This thesis builds upon and expands the content of the homonymous paper published in JBMR,[1] where I am the first author. The use of this content has been authorized by Oxford University Press (Appendix 1).

The project involves the study of the global knockout mice not expressing 24-Hydroxylase Cytochrome P450 family 24 subfamily A member 1 (Cyp24a1). The objective was to understand the mechanisms by which the deletion of *Cyp24a1* leads to gestational hypercalcemia and the impact of this on bone resorption during lactation.

I will begin by explaining bone and mineral homeostasis in adults, followed by the mother's adaptations during pregnancy and lactation and their relationships with calcitriol and Cyp24a1. Then, I'll review previous relevant studies for my project and provide the rationale, hypothesis, purpose, and objectives.

Proteins and genes follow the international standards of nomenclature in humans and mice.[2] For mice, gene names start with a capital letter followed by lowercase letters and are italicized (e.g., *Cyp24a1*), while protein names are not italicized (e.g., Cyp24a1). For humans, gene names are entirely in uppercase and italicized (e.g., *CYP24A1*), while protein names are not italicized (e.g., CYP24A1). When both models are referenced, we used human nomenclature. The protein form is used when both the gene and the protein are implied.

Serum phosphorus primarily exists as inorganic phosphates (dihydrogen and monohydrogen phosphate). In bone, it is mostly found in the form of hydroxyapatite, while in soft tissues and extracellular fluid (ECF), it predominantly exists as an organic phosphates complex with proteins, carbohydrates, and lipids. For simplicity and consistency, and since phosphorus is reported in serum and urine assays, the term "phosphorus" will be used to refer to all its forms throughout this thesis.

1.2. Bone and mineral homeostasis

1.2.1. Bone Tissue

Bone is a mineralized connective tissue with four types of cells: osteoblasts, bone lining cells, osteocytes, and osteoclasts.[3] It has several vital functions in the body, including locomotion, support and protection of soft tissue and bone marrow, and calcium and phosphate storage. Despite its apparent inertia, it is constantly being remodelled by tightly regulated interactions.[3]

1.2.2. Bone structure

Bone can be morphologically divided into cortical and trabecular (spongy or cancellous) bone.[3,4] Cortical bone is a dense tissue containing less than 10% soft tissue. It forms the external layer of all bones, particularly in the mid-portion of long bones (diaphysis). Cortical bone represents 80% of the weight of the human skeleton and, therefore, supports most of the mechanical functions. Trabecular bone comprises trabeculae shaped like plates or rods, interspersed with bone marrow. It is found mainly in the axial skeleton, such as the scapulae, pelvis, and vertebrae. Although trabecular bone forms only 20% of the skeleton, it is metabolically four times more active per unit volume than cortical bone.[5]

1.2.3. Bone Matrix

Collagen is a primary component of the bone matrix, with type I collagen fibers composed of two α1 chains and one α2 chain.[6] Along with noncollagenous proteins, collagen fibers make up about 90% of the organic composition of bone tissue. These fibres create arches in lamellar bone formation, allowing for the greatest collagen density per unit of tissue volume. In the trabecular bone and the periosteum, the lamellae may be parallel to each other. In contrast, in the cortical bone, they are concentrically positioned around a central blood vessel in the Haversian system.[6] Hydroxyapatite

crystals ($Ca_{10}(PO_4)_6(OH)_2$) are present on and within the collagen fibers and throughout the matrix. Among the noncollagenous proteins, the most important is osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), which plays a crucial role in regulating bone formation.[6,7]

1.2.4. Osteoblasts

Osteoblasts compose approximately 4-6% of the human skeleton[3]. They are mononuclear cells that form tight junctions with neighbouring osteoblasts and use their membranes for communication.[3] Their primary role is to produce the organic collagenous matrix and coordinate its mineralization.

Many factors affecting osteoblast differentiation will impact bone formation. One key influencer is the transcription factor RUNX2, which triggers downstream cell signal cascades to express osteoblast-specific genes such as type I collagen (COL1), alkaline phosphatase (ALP), osteopontin (OPN), osteonectin (ON), and osteocalcin.[8] RUNX2 also regulates Osterix (OSX) downstream, which interacts with the Nuclear factor of activated T cells 2 (NFATC2) to control the transcription of target osteoblastic genes.[9] Other transcription factors influencing bone formation include parathyroid hormone (PTH), fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), transforming growth factor β (TGF-β), WNTs, hedgehogs, insulin-like growth factor-1 (IGF-1), and Notch.[10] The hormones PTH and FGF23 will be explained in more detail in the session 1.4.3.

1.2.5. Bone Lining Cells

Bone lining cells are inactive, flat-shaped osteoblasts that cover bone surfaces where neither bone resorption nor formation occurs.[3] These cells have a thin, flat nuclear profile and a cytoplasm with few organelles, extending along the bone surface.[3] They are know to prevent osteoclasts from

interacting with the bone matrix when resorption is unnecessary and to participate in osteoclast differentiation by producing OPG and RANKL, thus regulating calcium balance.[3]

1.2.6. Osteocytes

Approximately 90-95% of all bone cells are osteocytes.[7] Osteocytes are the most mature differentiation state of osteoblast lineage that become embedded in the bone matrix.[3] The mineralized matrix around them forms an extensive lacunocanalicular network, where the cell body is encaged in a lacuna, and the dendrites permeate through the bone.[3]

Osteocytes are responsible for sensing various stimuli and regulating bone remodelling accordingly. Their unique arrangement allows them to translate mechanical stimuli into biochemical signals, a process known as the piezoelectric effect.[11] When mechanically stimulated, osteocytes produce several secondary messengers, such as ATP, nitric oxide (NO), Ca2+, and prostaglandins (PGE2 and PGI2), which influence bone physiology.[3]

Additionally, osteocytes regulate phosphate homeostasis and act as an endocrine gland.[12] The enzymes PHEX and DMP1 are highly expressed in osteocytes and are responsible for downregulating the expression of FGF23, which in turn increases phosphate reabsorption by the kidneys, helping to maintain homeostasis.[12]

1.2.7. Osteoclasts

Osteoclasts are large, multinucleated, highly motile cells that comprise about 1-2% of the cells in the human skeleton.[8] They are shaped with finger-like cytoplasmic projections and originate from hematopoietic stem cells.[13] Their development occurs in the presence of the cytokines macrophage colony-stimulating factor (M-CSF) and RANKL.[13]

The primary function of osteoclasts is to promote bone resorption. This process begins when there is physical contact between osteoclasts and bone matrices, mediated by integrin αvβ3, forming a sealed zone under the osteoclast with a pH of 4.5.[14,15] The acidic environment releases the mineral content from the bone and exposes the organic matrix. The main organic compound, type I collagen, is further degraded by the lysosomal enzyme cathepsin K.[16] Factors such as parathyroid hormone (PTH) and prostaglandin E2 increase the secretion of acid, promoting bone resorption, whereas calcitonin inhibits this process.[17]

1.2.8. Bone remodeling

Bone remodelling is a highly complex process by which old bone is replaced by new bone in a cycle comprised of three phases: (1) initiation of bone resorption by osteoclasts (the cutting cone), (2) the transition (or reversal zone) from resorption to new bone formation, and (3) bone formation by osteoblasts (closing zone).[3,18]

Osteoclasts, osteoblasts, osteocytes, and bone lining cells act in coordination to form a temporally anatomical structure named the basic multicellular unit (BMU).[6] The idealized creation of a Haversian system with a central blood vessel, represented in Figure 1, illustrates the BMU in cortical bone. In contrast, the BMU in trabecular bone forms scallop-like depressions, as shown in Figure 2. Despite these morphological differences, the biological processes of BMUs in trabecular and cortical bones are fundamentally similar.

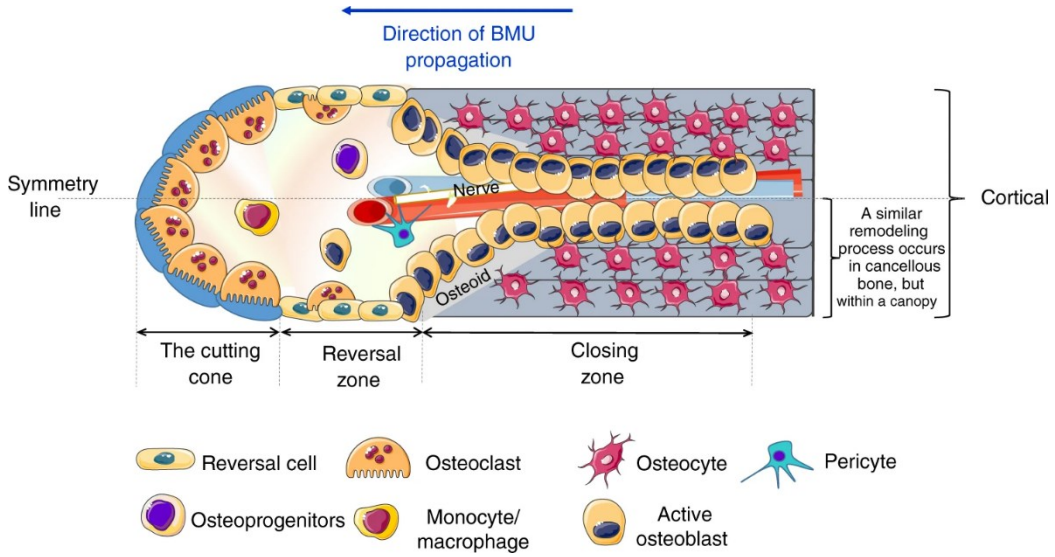

Figure 1. Schematic bone remodeling in cortical bone

The cutting cone comprises clusters of osteoclasts that erode the bone using acid and enzymes. The reversal zone is formed by osteoblasts, reversal cells, and secondary osteoclasts, which release signals to initiate bone formation. Osteoblasts compose the closing zone and promote bone formation by depositing an unmineralized protein matrix material (osteoid) and its subsequent mineralization. A line of symmetry divides the representation of a complete BMU in the cortex, moving toward the longitudinal axis of the long bone. Used with permission from Nature (licensed under a Creative Commons Attribution 4.0 International License).[18]

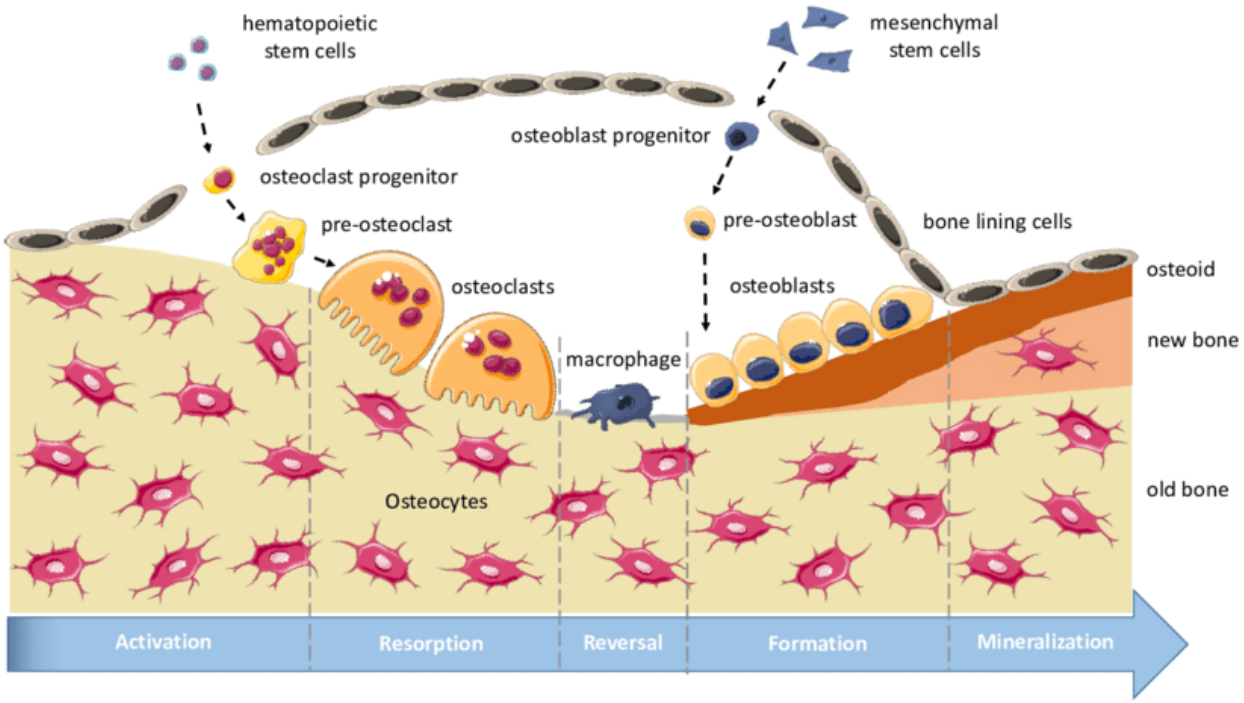

Figure 2. Schematic bone remodeling in trabecular bone

The diagram illustrates the distinct phases of BMU of bone remodeling in trabecular bone. The process begins with **activation**, where hematopoietic stem cells differentiate into osteoclast progenitors, and pre-osteoclasts migrate to the bone surface. During **resorption**, large, multinucleated osteoclasts resorb bone, creating cavities, while osteocytes embedded in the bone matrix play a regulatory role. In the **reversal** phase, macrophages clean and prepare the bone surface, and osteoblast progenitors derived from mesenchymal stem cells migrate to the site. The **formation** phase involves pre-osteoblasts differentiating into mature osteoblasts, which synthesize new bone matrix (osteoid). Finally, in the **mineralization** phase, bone lining cells contribute to the mineralization of the osteoid, forming new bone that replaces the resorbed bone. The structure formed by bone lining cells and other supporting cells that covers the remodeling site is called a canopy. Used with permission from AIMS Press (licensed under a Creative Commons Attribution 4.0 International License).[19]

The bone remodeling cycle begins with the activation of osteoclasts, primarily by osteocytes, but it may also involve contributions from lining cells and pre-osteoblasts in the bone marrow.[6] These cells change in shape and start to secrete digestive proteins on the bone surface and start to express receptor activator of NF-kappa B ligand (RANKL), a member of the superfamily tumor necrosis factor (TNF).[6] The interaction of RANKL with its receptor RANK initiates the differentiation of and fusion of hematopoietic cells of the osteoclast lineage, and it also suppresses osteoclast apoptosis, leading

to prolonged survivability.[6] Both bone resorption and bone formation are coupled by RANKL. When osteoblast or bone marrow produces osteoprotegerin (OPG), a secretory dimeric glycoprotein that also belongs to TNF family, the effects of RANKL are blocked. OPG achieve this by serving as a decoy for RANKL, limiting the number of RANKL molecules available to bind to RANK (Figure 3).[6]

The reversal phase starts when osteoclasts have moved further away and is characterized by the appearance of a mononuclear cell layer on the bone surface. These cells are responsible for signaling osteoblasts to migrate and differentiate. The final process involves osteoblasts laying down new bone tissue until the old bone is replaced. In the end, the final surface is covered with flattened lining cells that remain there until a new remodeling cycle is initiated.[6]

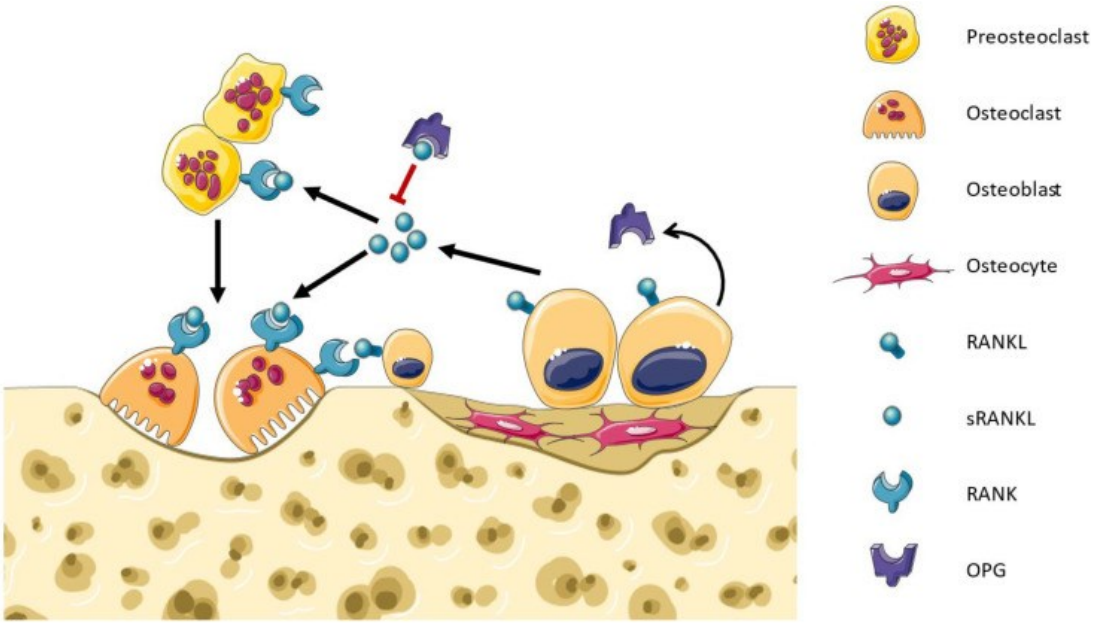

Figure 3. A simplified representation of the RANK-RANKL-OPG signaling pathway
Osteoblasts produce RANKL, which binds to RANK receptors on the precursor cells of osteoclasts, promoting their differentiation. Additionally, osteoblasts and osteocytes secrete OPG, which competitively inhibits RANKL, thereby regulating the development of osteoclasts and preventing excessive bone resorption. Used with permission from MDPI (licensed under a Creative Commons Attribution 4.0 International License).[20]

1.2.9. Bone remodelling biomarkers

There are several biomarkers used to measure bone formation and bone resorption,[21,22] but the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have recommended using serum procollagen type I N-terminal propeptide (P1NP) and serum C-telopeptide of type I collagen (CTX-1 or CTX) and as reference markers for bone formation and resorption, respectively.[21] These markers are intended for assessing fracture risk, monitoring therapy in clinical settings, and being used in research.[21]

Bone formation markers are molecules produced by osteoblasts at various stages of development that indicate bone formation activity and its different aspects.[21] Type I collagen constitutes 90% of bone proteins and is synthesized as type I procollagen.[23] P1NP is produced by the post-translational cleavage of type I procollagen molecules by proteases at the N-terminus and circulates in serum in either trimeric or monomeric forms.[21,23] The primary advantage of P1NP over other markers is that it remains unaffected by other metabolic pathways that may challenge the result interpretation.[21]

Bone resorption markers are molecules made during the resorption phase of bone remodelling, including byproducts of osteoclast activity or osteocytic osteolysis released during bone resorption.[21,24] CTX is formed by the degradation products of Type 1 collagen in bone generated by the enzyme cathepsin K and exists in two native forms: α and β isomers. Despite being the gold standard for measuring bone resorption, CTX levels exhibit circadian variation, necessitating samples being collected during the same day period for accurate comparison.[21,24]

1.3. Serum Minerals

1.3.1. Calcium

Calcium is the most abundant mineral in the body.[25] It is often associated with healthy bones and teeth, although it also plays an essential role in blood clotting, muscle contraction, and maintaining normal heart rhythms and nerve functions.[26] An adult has roughly 1200 g of this element.[27] Most of it (99%) exists in the form of hydroxyapatite in the skeleton, and the remaining is found in the blood, extracellular fluid, and soft tissues.[27] Around 50% of the calcium in blood is free or ionized, 40% is bound to albumin, and 10% is bound to citrate or phosphate ions.[5] The ionized portion is biologically functional, and it's often used as a reference for clinical calcium status. The normal concentration of calcium in serum is 2.2-2.6 mmol/L, whereas for ionized free calcium is 1.1-1.4 mmol/L.[28] These levels are tightly regulated and rarely oscillate more than 5%. The main receptor responsible for calcium homeostasis is the Ca^{+2}-sensing receptor (CaSR), which is primarily expressed in parathyroid, can also be found in kidneys, intestine, C-cells of the thyroid gland, and osteoblasts, osteocytes, and osteoclasts in bone.[8]

The Recommended Dietary Allowance (RDA) for calcium is 1200 mg/day for healthy adults.[27] according to the Institute of Medicine (IOM),[29] In the USA and Canada, the 50th percentile of calcium intake for women between 18 and 50 is 800-1000 mg/day, of which 200-250 mg is absorbed.[29] Therefore, most of them barely or don't achieve the minimum calcium requirement.

Pregnant and breastfeeding women require enough calcium to support the healthy development of their babies.[30] The increase in calcium intake during these periods is not necessary if the mother is already consuming 1200 mg of calcium per day (Section 1.6).[29,30]

1.3.2. Phosphorus

Phosphorus is the second most abundant mineral in the body, composing 0.65-1.1% of adults bodyweight.[29,31,32] It acts as a structural component of bones, teeth, and nucleic acids and enables the bipolarity of lipid membranes and circulating lipoproteins.[31] Of the total body phosphorus content in adults (500-800 g), roughly 80% are stored in the bones as hydroxyapatite, 14% are distributed in soft tissues, and trace amounts are found in extracellular fluids.[27,33] There are two forms of

phosphorus in extracellular fluids: the inorganic phosphate (Pi) and the organic phospholipids.[31,34] The average amount of phosphorus commonly found in the blood is 13 mmol/L (40 mg/dL).[34] Most of this phosphorus is located within the phospholipids of red blood cells and lipoproteins in the plasma, while about 1 mmol/L (3.1 mg/dL) exists in the form of inorganic phosphate. Phosphorus homeostasis is not fully understood, but Wild et al[35] proposed that inositol polyphosphates communicate information about the levels of phosphate within cells to SYG1/Pho81/XPR1 (SPX) domains, which then allows them to interact with various proteins to control the uptake, transport, and storage of phosphate in organisms such as fungi, plants, and animals.

The Recommended Dietary Allowance (RDA) for phosphorus is 700 mg/day for healthy adults.[34] According to the National Health and Nutrition Examination Survey (NHANES) of 2013–2016,[36] in North America, adults aged 20 and older, have an average daily phosphorus intake from foods of 1,189 mg for women and 1,596 mg for men. Therefore, most of them must excrete the excess.

1.3.3. Magnesium

The adult body contains nearly 25g of magnesium, of which 50 – 60% is stored in bones.[37] Magnesium influences many functions, including protein, DNA, and RNA synthesis, nerve impulse conduction, structural development of bone, and many others.[38] Less than 1% is found in blood serum, and these levels are tightly regulated. The normal serum concentration is generally between 0.75 and 0.95 mmol/L.[34]

The RDA for adult men is 400 mg/day, while for women it is 310 mg/day when non-pregnant, 350 mg/day when pregnant, and 310 mg/day when lactating.[37] According to the NHANES of 2013–2016,[37] 48% of Americans ingest less magnesium than recommended.

1.4. Hormonal Regulation of Calcium and Phosphorus

Several hormones and organs work in coordination to tightly regulate calcium and phosphorus. These hormones include parathyroid hormone (PTH), calcitriol ($1,25(OH)_2D$), and fibroblast growth factor 23 (FGF23).[39,40] Other hormones such as Parathyroid hormone-related peptide (PTHrP), estradiol (E2), and testosterone also influence the regulation.[39,40]

1.4.1. PTH

PTH is responsible for raising the amount of calcium in the bloodstream back to the desired range of 1.1-1.3 mmol/L, decreasing the level of phosphorus, promoting the production of calcitriol and inhibiting its catabolism, and regulating the process of bone formation and resorption.[5] The hormone is a polypeptide produced and divided into an active form inside the rough endoplasmic reticulum (RER) of the parathyroid glands.[5]

PTH production is regulated by the levels of ionized calcium and calcitriol in the blood.[41,42] The calcium-sensing receptor (CaSR), located on the plasma membrane of chief cells in the parathyroid glands, detects the amount of free calcium in the blood and regulates PTH secretion accordingly.[41] Once free calcium binds to the receptor, it triggers a signal transduction process that temporarily suppresses PTH secretion and reduces PTH mRNA transcription in the long term.[43] On the other hand, when extracellular calcium levels decrease, PTH transcription and secretion are increased. High levels of phosphorus also increase the release of PTH, although to a lesser extent than calcium concentrations affect PTH release.[44,45] This is because PTH is suppressed by CaSR when calcium levels are high, which prevents PTH from fully correcting hyperphosphatemia.

Calcitriol can also inhibit PTH directly and indirectly.[42,46] The direct pathway occurs when calcitriol binds to the VDR inside the cell, triggering its translocation to the nucleus. Once there, the calcitriol–VDR complex can assemble at various promoter regions, ultimately suppressing PTH gene

transcription and thus reducing PTH production and secretion.Calcitriol also indirectly inhibits PTH by increasing intestinal calcium absorption, thereby elevating serum calcium levels, which are then sensed by the CaSR.[42,46] Lastly, FGF23 also has a role in blocking the release of PTH, by phosphorylating ERK1/2 and activating the MAPK pathway.[47]

Its response is rapid, with PTH being secreted within seconds via exocytosis.[48] However, the active form of PTH has a short serum half-life of just a few minutes and is quickly eliminated from the bloodstream by the kidneys and liver.

PTH, along with PTHrP, binds and activates the Parathyroid Hormone Receptor Type 1 (PTH1R) expressed mainly in the bones, kidneys, and intestines, but also the heart, brain, liver, testis, pancreas, uterus, placenta, and blood cells.[49] The hormone also activates Parathyroid Hormone Receptor Type 2 (PTH2R), but it seems to have less effect on Ca and P homeostasis.[49]

1.4.1.1. Effects of PTH on Bone

The main target of PTH is the osteoblast since its receptor is located within these cells. Intermittent elevation of PTH can increase the number and life span of osteoblasts by suppressing the expression of the bone formation inhibitor SPX and also raising the expression of RANKL, a signaling molecule that helps promote the differentiation of osteoblasts into osteocytes.[48,50] PTH also promotes the differentiation of osteoclast precursors into osteoclasts by reducing the amount of OPG produced by osteoblasts.[51] OPG competes with RANKL for binding to receptors so when its level decreases, RANKL is more successful in promoting differentiation into osteoclasts. Therefore, if PTH stimulation is prolonged or at a higher level, it can cause excessive bone turnover, resulting in an overall decrease in bone.[52]

In addition, PTH has been shown to suppress sclerostin (SOST) expression in osteoblasts through the cAMP signaling pathway downstream of the PTH/PTH-related peptide (PTHrP) receptor (PPR).

Since sclerostin inhibits bone formation by osteoblasts, PTH can decrease bone formation via this pathway.[53]

Overall, PTH operates through multiple, sometimes opposing pathways in bone turnover. The overall effect depends on factors like timing and dosage, although many details of this regulatory process remain unknown.[53]

1.4.1.2. Effects of PTH on the Kidneys

PTH plays a role in regulating calcium and phosphate levels in the kidney.[51,54] The hormone stimulates calcium resorption in the distal convoluted tubule through the use of specific ion channels, such as TRPV5.[54,55] It also promotes phosphorus excretion by decreasing phosphate reabsorption in the proximal convoluted tubule. This mechanism involves removing the sodium-phosphate cotransporters NaPi2a and NaPi2c from the brush border membrane (BBM) via protein kinase A- and C-dependent pathways.[54,55] Moreover, the hormone indirectly increases the absorption of calcium and phosphate in the intestine by stimulating the production of calcitriol in the kidney.[51,54]

1.4.2. Calcitonin

Calcitonin (CT) is a hormone whose main effect is to inhibit osteoclast-mediated bone resorption.

CT is secreted by the C-cells of the thyroid gland all the time, and breast and placenta during pregnancy.[56] It primarily lowers calcium levels in the blood but also reduces serum phosphorus, thereby serving as an opposing mechanism to PTH.[56] When calcium levels are high, CaSR signals this hormone's release. CT acts by inhibiting the osteoclast's breakdown in the bone and promoting urine calcium and phosphorus excretion. Pharmacologic doses of CT have been employed to reduce bone resorption in conditions such as osteoporosis, Paget's disease of bone, and hypercalcemia

associated with malignancy. It primarily lowers calcium levels in the blood but also reduces serum phosphorus, thereby serving as an opposing mechanism to PTH.[56] However, the physiological influence of calcitonin in calcium and phosphorus homeostasis seems to be vestigial compared to other hormones, such as PTH, calcitriol, and FGF23. This was demonstrated by normocalcemia in both thyroidectomized patients and patients with medullary thyroid cancer who have very high calcitonin levels.[56,57]

1.4.3. FGF23

The fibroblast growth factor-23 (FGF23) is a pivotal hormone regulating mineral homeostasis and many other processes in the body.[55,58] One of its main purposes is to downregulate serum phosphorus by primarily reducing renal phosphate reabsorption, as well as decreasing intestinal phosphate absorption and bone phosphate resorption phosphate.[55,58]

The hormone is mostly expressed by osteocytes and osteoblasts, but can also be found in smaller amounts in salivary glands, stomach, etc.[52,55] FGF23 regulates itself by negative feedback, along with other regulators, such as bone mineralization and remodeling, phosphate and calcium.[55]

The FGF-receptor-α-Klotho complexbinds to FGF23 with higher affinity than FGFR or Klotho alone[55,59]. More specifically, FGF binds to FGF tyrosine kinase receptor 1 (FGFR1) and the FGF23-specific co-receptor Klotho to inhibit the translation of the sodium phosphate cotransporters NaPi2a and NaPi2c.[58]

FGF-receptor-α-Klotho complexes are primarily located in the renal distal tube. However, FGF23 mainly inhibits phosphate transport in renal proximal tubular cells through weaker interactions with FGFR and co-factors other than Klotho.[59]

In addition, the hormone inhibits Cyp27b1, which is responsible for calcitriol synthesis, and enhances Cy24a1 expression and activity, which is responsible for calcitriol degradation, thereby lowering calcitriol to influence phosphate and calcium homeostasis.[55,58]

1.4.4. Calcitriol synthesis and catabolism pathway

Vitamin D is a group of fat-soluble vitamins that includes different vitamers, such as Vitamin D_3 (Cholecalciferol), Vitamin D_2 (Ergocalciferol), among others.[37,60] It can be either ingested in the form of Vitamin D_3 or Vitamin D_2 or converted from 7-dehydrocholesterol (7-DHC) in the skin (Figure 4) [61]. Several 25-hydroxylase enzymes, such as CYP27A1 and CYP2R1, convert both molecules into Calcifediol (25-hydroxyvitamin D; 25(OH)D), which is the main form of vitamin D found in the blood.[61] This conversion occurs mainly in the liver but can also happen in other tissues.

However, vitamin D has no biological effects. To become biologically effective, it must be further hydroxylated in the 1α position by the enzyme CYP27B1.[61] This leads to the formation of the most potent form of vitamin D, calcitriol (1,25-dihydroxyvitamin D; $1,25(OH)_2D$). This 1α-hydroxylation primarily occurs in the kidney but can also happen in other tissues, such as placenta, monocytes, and macrophages.[61] The calcitriol and its precursor calcifediol are mostly transported in blood bound to vitamin D binding protein (DBP) and albumin, with a minor portion circulating as the free form.[61]

Both calcitriol and its precursor, calcifediol, are converted to inactive forms by CYP24A1.[62] This enzyme is responsible for the five-step, 24-oxidation pathway that converts calcitriol into calcitroic acid, a known biliary catabolite.[62] Additionally, it catalyzes a similar pathway starting with 23-hydroxylation, resulting in the formation of $1,25\text{-}(OH)_2D_3$-26,23-lactone. Furthermore, CYP24A1 efficiently hydroxylates the vitamin D_2 side chain of 25-OH-D_2 and $1,25\text{-}(OH)_2D_2$, producing a series of polyhydroxylated products. Other enzymes, such as CYP3A4, can represent an alternative pathway to calcitriol breakdown.[63]

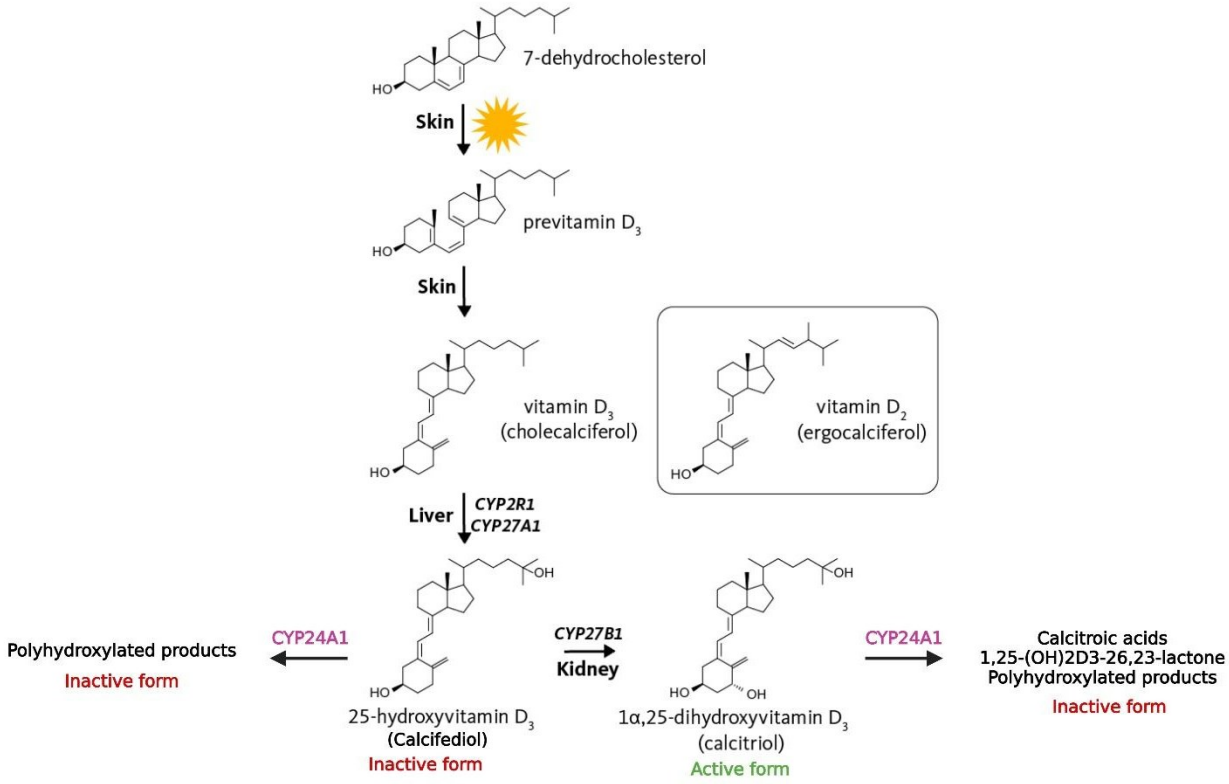

Figure 4. Calcitriol synthesis and catabolism pathway

The initial form of vitamin D, known as previtamin D, is produced in the top layers of the skin when 7-dehydrocholesterol is exposed to ultraviolet light B (UVB). As the previtamin D_3 moves to the lower layers of the skin, it is converted into vitamin D_3 (cholecalciferol) through a nonenzymatic process. Vitamin D_3 is then rapidly transported by vitamin D binding protein (DBP) to either be stored in adipose tissue or to be activated in the liver. Within the liver, various cytochrome P450 enzymes can catalyze the 25-hydroxylation of vitamin D3 (or plant-based vitamin D2 or ergocalciferol). This step produces 25-hydroxyvitamin D2, which is then converted into the active form of vitamin D3, 1a,25-dihydroxyvitamin D, by the enzyme CYP27B1. This 1α-hydroxylation primarily takes place in the kidney. Both calcitriol and calcifediol are converted to several inactive forms by CYP24A1. Used with permission from the American Physiological Society (Appendix 4).[60] Adapted using BioRender.com (Appendix 5).

1.4.5. Alternative pathway for calcitriol and calcifediol catabolism

CYP24A1 is the primary and most well-established pathway for calcitriol and calcifediol catabolism. However, recent studies suggest that CYP3A4 may serve as an alternative pathway to degrade both molecules (Figure 5).[64]

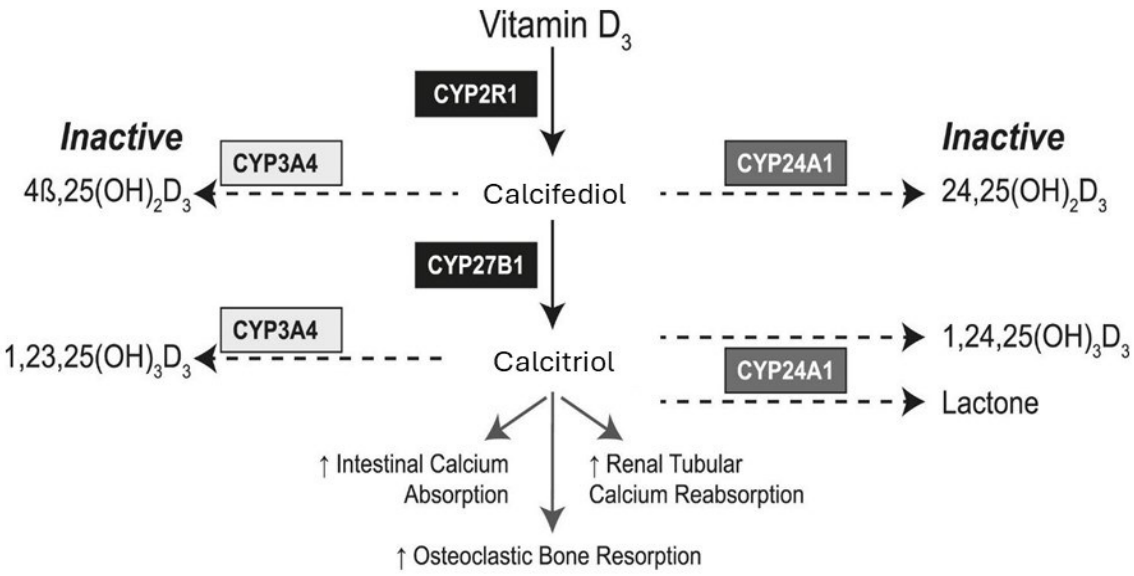

Figure 5. Alternative pathways for calcitriol and calcifediol catabolism by CPY3A4
CYP24A1 is the canonical pathway for the inactivation of calcifediol and calcitriol, while CYP3A4 represents an alternative pathway. Used with permission from The Endocrinology Society (Appendix 6).[64]

CYP3A4 is a member of the cytochrome P450 superfamily of enzymes. This enzyme is expressed in the liver, small intestine, and other tissues, metabolizing many xenobiotics, steroids, and drugs. Under physiological conditions, this enzyme does not have a significant impact on the vitamin D catabolism. However, Hawkes et al. (2017)[64] used rifampin to enhance CYP3A4 activity as a therapeutic intervention when CYP24A1 function was compromised. They treated two patients with IIH caused by mutations in CYP24A1 with daily doses of 600 mg of rifampin for one month. The treatment resulted in reductions in serum levels of calcitriol and calcium, with PTH levels returning to the

normal range. Additionally, urinary calcium excretion decreased, and serum creatinine levels improved to 1.1 mg/dL. Although promising, further studies should test the toxicology and increase the sample size to confirm the benefits of rifampin in treating *CYP24A1* loss of function.

1.4.6. VDR

Most of calcitriol's biological activity occurs when it binds to the vitamin D receptor (VDR).[65] VDR belongs to a superfamily of nuclear receptors for steroid hormones that regulate gene expression through transcriptional regulators.[65] Several steps are required to control the gene expression, starting with (1) ligand binding, followed by (2) heterodimerization with retinoid X receptor (RXR), binding of the heterodimer to vitamin D response elements (VDREs) in the promoter of calcitriol responsive genes, and (4) recruitment of VDR-interacting nuclear proteins (coregulators) into the transcriptional pre-initiation complex that will enhance or suppress the gene transcription by VDR.[65]

Calcitriol binds to the ligand-binding domain (LBD), located in the carboxyterminal portion of the VDR molecule, with an affinity 100 times higher than calcifediol and $24,25(OH)_2D_3$.[65] Upon binding, VDR dimerizes with RXR to form an essential conformation for transactivation. The DNA-binding domain of VDR is a two-zinc nucleated module highly conserved among nuclear steroids.[65–67] This domain interacts with promoters of calcitriol target genes and modulates their expression. Coregulators then play a role in up or downregulating the expression of VDREs. Co-activators such as SRC1, -2, and -3, CBP/p300 unfold and expose the DNA. Then, the DRIP/TRAP complex and VDR interact with the exposed DNA to enhance gene expression.[65–67] On the contrary, PTH is suppressed by the recruitment of co-repressors of the family of histone deacetylases, which prevent the DNA exposures and access of transcription factors (TF) essential to initiating transcription.[65] Other molecules are co-modulators that can enhance or repress the expression depending on the cell and cellular environment conditions, such as NCoA62/Skip and ATP-dependent chromatin

remodelling complex.[65] The most upregulated gene by VDREs is *CYP24A1*, the primary enzyme responsible for degrading vitamin D.[62]

VDR is vastly expressed throughout the body, including osteoblasts, enterocytes, distal renal tubule cells, parathyroid gland cells, skin keratinocytes, promyelocytes, lymphocytes, colon cells, pituitary gland cells, and ovarian cells.[68] Its vast expression in the body led to the hypothesis that calcitriol has many extra skeletal roles that still need to be proven.[68]

1.4.7. Summary of calcium and phosphorus regulation

As seen in the previous chapters, the regulation of Ca and P involves a multitude of biological systems and regulators. The pleiotropism of the primary regulators, PTH, FGF23, and calcitriol, are illustrated in Figure 6. However, their interplay may shift depending on specific conditions. PTH, for example, can promote bone formation or resorption depending on exposure time (Section 1.4.1.1), and many other molecules, such as sex steroids and PTHrP, can also influence the dynamic.[30,69,70]

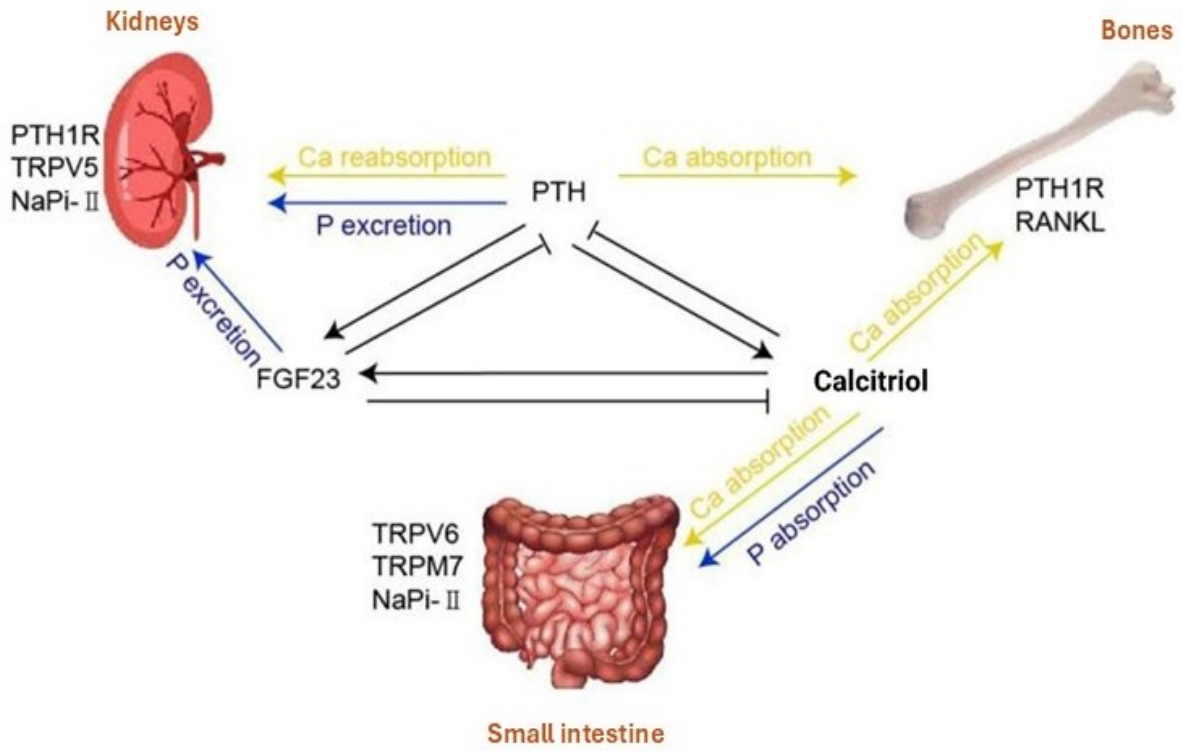

Figure 6. Calcium and phosphorus homeostasis

The small intestine, kidneys, and bones are the primary organs influencing Ca and P homeostasis. They are tightly regulated by PTH, calcitriol, FGF23, and other factors not shown. PTH influences effectors like TRPVs, NaPi-II counterorders, PTH1R, etc., to increase bone resorptions and kidney reabsorption of Ca and P. Calcitriol mainly acts by increasing the intestinal Ca and P absorption but can also act in bone resorption e kidney reabsorption. FGF23 promotes P excretion by primarily decreasing NaPi-II cotransporters in proximal tubules. The yellow and blue arrows show how hormones influence Ca and P metabolism in each organ, while the black arrows indicate whether these hormones enhance or inhibit one another. Used with permission from the Frontiers (licensed under a Creative Commons Attribution 4.0 International License).[71]

1.5. Intestinal calcium absorption

Calcium absorption in the intestine occurs through two primary mechanisms: a transcellular active transport process that mainly occurs in the duodenum and upper jejunum, and a paracellular passive process throughout the intestine (Figure 7).[72,73] The proportion of each calcium absorption mechanism depends on the luminal Ca^{2+} concentration. When calcium concentrations are high due to a calcium-

rich diet, paracellular transport predominates. Conversely, when calcium intake is low, transcellular transport is upregulated.

Both pathways are regulated by calcitriol, but the active transcellular calcium transportation is significantly more sensitive to calcitriol concentration.[72,73] During pregnancy, the maternal body increases the conversion of calcitriol to meet the fetal needs for calcium (Section 1.6).[30]

1.5.1. Paracellular transportation

Paracellular transportation (passive diffusion) process is generally less regulated than transcellular transport but involves complex mechanisms related to the structure and function of tight junctions between epithelial cells.[72,73]

Paracellular transport occurs between the epithelial cells through tight junctions, which are complex protein structures that seal the space between adjacent cells.[72,73] Most of the calcium moves passively down its concentration gradient. No metabolic energy is required for this process, as it relies on the differences in concentration and electrical gradients across the epithelial layer. Moreover, the water movement through the paracellular pathway can drag some of the dissolved calcium ions along with it, a process known as solvent drag.

Once calcium passes through the tight junctions and enters the intercellular space, it can diffuse into the capillaries in the lamina propria (a layer of connective tissue underlying the epithelium) and subsequently enter the bloodstream.[72,73]

Claudins are critical components of tight junctions that regulate paracellular permeability. Claudin-2 and claudin-12 have been shown to increase calcium permeability in the intestine.[72,73] Claudin-16 (also known as paracellin-1) is another claudin involved in calcium transport, although it is primarily expressed in the kidney. Occludin and Junctional Adhesion Molecules (JAMs) are also proteins that contribute to the structural integrity and selective permeability of tight junctions.

Calcitriol enhances the expression of various proteins involved in tight junction integrity and function, ultimately leading to increased permeability of tight junctions to calcium.[72,73]

1.5.2. Transcellular Calcium Transport

Transcellular calcium transport requires the energy stored in adenosine triphosphate (ATP) to pump calcium against its concentration gradient, making it an active transport process.[72,73] This is a highly regulated process that allows for efficient calcium absorption in the intestine, particularly when dietary calcium intake is low. There are three main steps involved: apical entry, intracellular transport, and basolateral exit.

Apical entry: The primary channels involved in the apical entry of calcium are the Transient Receptor Potential Vanilloid 6 (TRPV6) and, to a lesser extent, Transient Receptor Potential Vanilloid 5 (TRPV5). These channels are located on the apical membrane of enterocytes (intestinal epithelial cells). The voltage-dependent L-type calcium channel, alpha 1D subunit (Cav1.3) also plays an important role in calcium transportation, particularly during periods of dietary calcium insufficiency.[72,73] Calcium enters the enterocytes through these channels, driven by a steep electrochemical gradient. The luminal concentration of calcium is typically much higher than the intracellular concentration, facilitating passive entry of calcium into the cells. When bound to calcitriol, the VDR acts as a transcription factor that upregulates the expression of these and many other calciotropic genes.

Intracellular transport: Once inside the enterocytes, calcium ions bind to calcium-binding proteins, such as calbindin-D9k.[72,73] Calbindin-D9k transports the bind calcium from the apical side of the cell to the basolateral side. This binding prevents free calcium from disrupting cellular functions and ensures efficient transport across the cell. Another calcium-binding protein, calmodulin (CaM), may interact with various intracellular signaling pathways to regulate calcium homeostasis. Although calmodulin's role in direct calcium transport is less clear, it is involved in modulating the activity of

calcium channels and transporters. Vesicles formed by TRPV-mediated Ca^{2+} uptake can be transported through the cells via microtubules. These vesicles can pass through the endoplasmic reticulum (ER) and be released at the basolateral membrane. Alternatively, they can merge with lysosomes and be released by exocytosis.

Basolateral Exit: The primary mechanism for extruding calcium from enterocytes into the bloodstream is the plasma Membrane Calcium ATPase 1b (PMCA1b) pump.[72,73] This ATP-dependent pump actively transports calcium ions out of the cell against their concentration gradient. Although less significant in the intestine compared to PMCA1b, the Sodium-Calcium Exchanger 1(NCX1) also contributes to calcium extrusion. This exchanger typically operates in a mode that swaps three sodium ions for one calcium ion, driven by the sodium gradient maintained by the Na+/K+ ATPase.

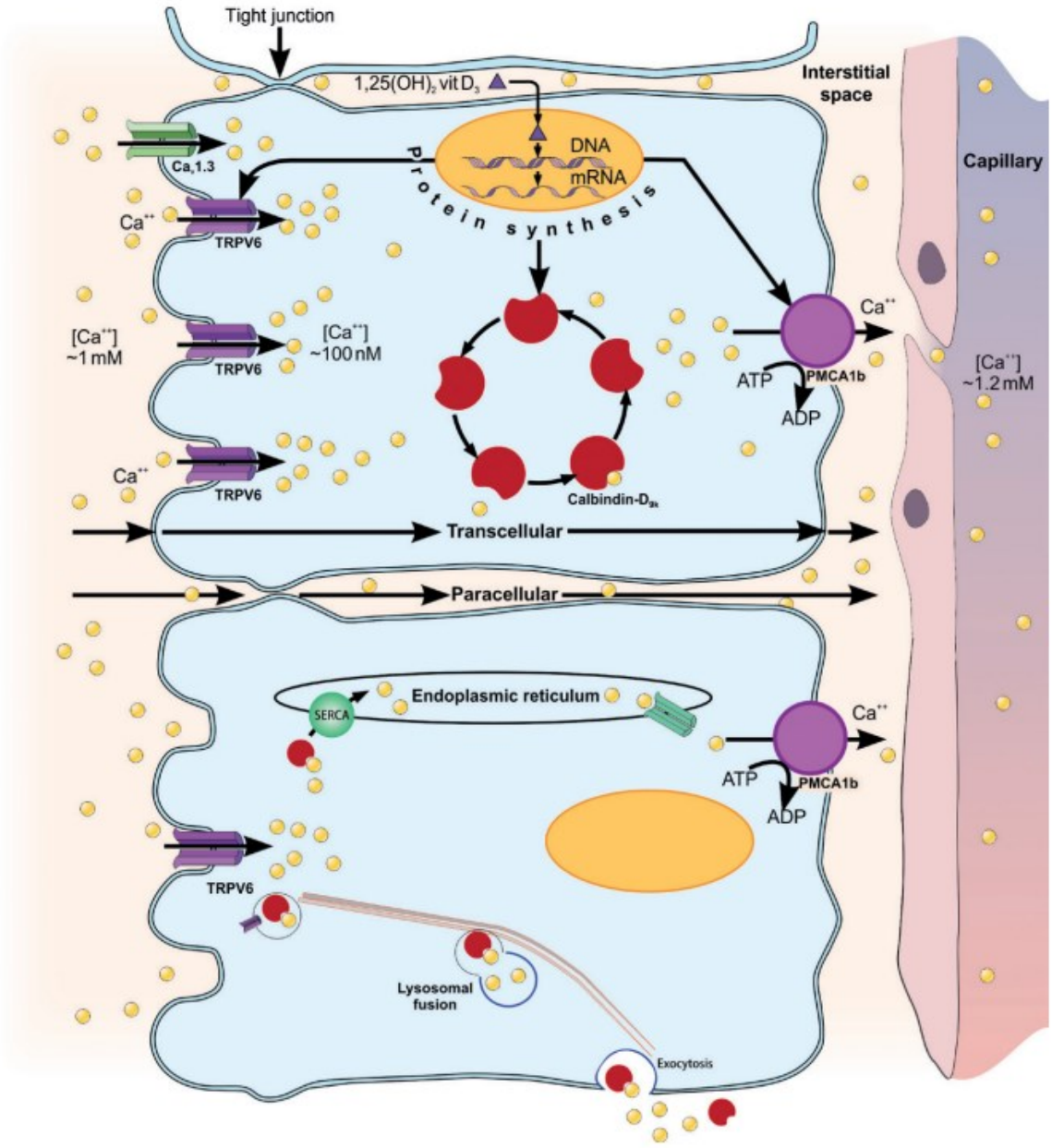

Figure 7. Model of transepithelial calcium transport in the duodenal epithelium

Three adjacent enterocytes are illustrated along with the key proteins involved in transepithelial intestinal calcium transport. Both transcellular and paracellular pathways are shown. Transcellular transport primarily starts with TRPV6 facilitating calcium influx at the brush border, where it subsequently binds to the intracellular buffering protein calbindin-D9k. Calcium is then released from calbindin-D9k and exits the enterocytes through the basolateral membrane via PMCA1b. The expression of these proteins is regulated by calcitriol in response to the body's calcium requirements. Paracellular ion movement occurs through tight junctions between adjacent enterocytes, and it's more prominent with high calcium intake. Additionally, the Cav1.3 calcium channel on the apical membrane may be involved in transcellular calcium transport during periods of dietary sufficiency.

The lower enterocyte shows a schematic representation of vesicular Ca^{2+} transport and Ca^{2+} tunneling through the endoplasmic reticulum (ER). Used with permission from Elsevier (Appendix 7).[72]

1.6. Maternal body adaptations during pregnancy, lactation and post-weaning to maintain calcium homeostasis.

In the USA and Canada, the 50th percentile of calcium intake for women between 18 and 50 is 800-1000 mg/day, while the recommended intake is 1200 mg/day.[27,30] The situation can be even more severe in developing regions such as Asia, Africa, and South America, where daily calcium intake ranges from 400 to 700 mg/day.[74] If the normal efficiency of 25% of intestinal Ca absorption remained unchanged, women would have to consume an extra 1,200 mg/day during the third trimester of pregnancy, whereas lactating women would have to consume an extra 800 mg daily during the first semester and 480 mg daily during the second semester.[30] However, the increase in calcium intake is not required for women who already consume adequate levels of calcium and don't have preexisting disorders that cause skeletal fragility because of several adaptations during pregnancy and lactation (Figure 8).[30,75] The maternal adaptations may include the increased intestinal absorption of calcium, renal conservation of calcium, and increased skeletal resorption of calcium.[30] During pregnancy, the main adaptation is the doubling of intestinal calcium absorption (Section 1.6.1), whereas during lactation, the main adaptation is the increased skeletal resorption (Section 1.6.2). It is unclear why different adaptations arose in these two distinct periods, but it is known that these hormone-induced adaptations are generally sufficient to meet daily mineral requirements for the fetus and infant without long-term negative effects.[30]

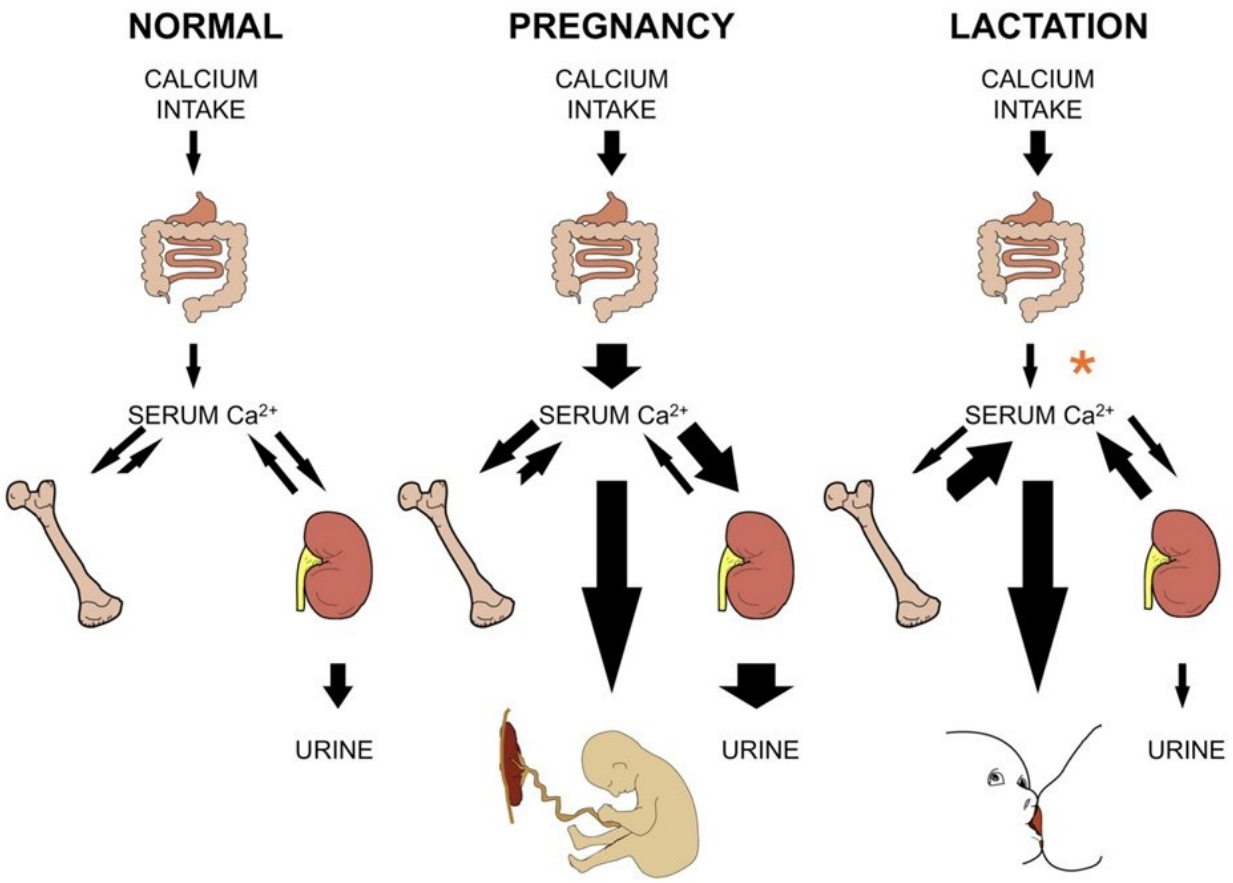

Figure 8. Adaptive processes of calcium homeostasis in humans during pregnancy and lactation.

The thickness of the arrows indicates a relative increase or decrease compared to normal. The main adaptation during pregnancy is increased intestinal calcium absorption, whereas the main adaptation during lactation is increased bone resorption. *In rodents, the intestinal calcium absorption also remains upregulated during lactation. Used with permission from the Europe PMC (Licensed under a Creative Commons (CC BY-NC-ND) license).[76]

1.6.1. Pregnancy

During pregnancy, the maternal body adapts to provide enough minerals for the fetus. Around 80% of the fetus's calcium content is accreted during the third trimester.[30] During this period, the placenta transports between 5 to 10% of the calcium available in the mother's serum to the fetus hourly.[30]

The main adaptation during pregnancy is the doubling of intestinal calcium absorption.[30] The primary mechanism behind the upregulation is likely paracellular passive calcium transport, which operates independently of calcitriol or VDR. However, calcitriol levels increase significantly during this period—ranging from a 2- to 5-fold rise in humans and a 3- to 5-fold rise in rodents. Calcitriol enhances calcium permeability during paracellular transport and upregulates the expression of calcium transporters involved in transcellular active transport.[72,73,77] Therefore, calcitriol is partially attributable to the increased doubling of intestinal calcium absorption.

Animal research indicates that calcitriol levels rise in maternal blood due to the increased activity of renal CYP27B1, where placentas and fetuses contribute very little calcitriol[30]. This increase in maternal calcitriol is not triggered by PTH, which typically drops to low levels early in human and rodent pregnancies. Instead, both animal and some human studies suggest that the boost in calcitriol production might be influenced by factors like estradiol, prolactin, placental lactogen, oxytocin, or PTH-related protein (PTHrP).[30]

Early research in humans indicated that serum calcium levels were lower during pregnancy, suggesting hypocalcemia.[78,79] However, later studies demonstrated that the observed decrease in serum calcium was actually due to reduced albumin-bounded calcium serum, which resulted from the expansion of intravascular volume typical of normal pregnancy.[80] Since albumin-bound calcium is not physiologically significant, subsequent studies that measured ionized (physiologically active) calcium found no significant changes in its levels, indicating no physiological consequences from the drop in total serum calcium.[80,81]

Studies in mice have shown no changes in either ionized calcium or albumin-bound calcium during pregnancy.[57,77,82–85] The exception occurs when the mother has a deleterious condition such as vitamin D deficiency[68] or a lack of *Vdr*[86] or *Cyp27b1*.[87]

1.6.2. Lactation

During lactation, the neonate demands that nearly 200 mg of calcium be provided daily through milk to a singleton during the first 6 months and approximately 120 mg during the next semester.[30,88] However, the demand can vary greatly depending on individual aspects of both mothers and babies, but it is shown to be positively correlated with the litter size and weight.[30]

Humans mostly rely on bone resorption to keep up with the babies' demands for calcium, while intestinal calcium absorption returns to pre-pregnancy levels.[30] During this period, the maternal body loses between 4-7% in the lumbar spine and femoral neck.[89] Other adaptations do not, or only marginally, compensate for the expected bone resorption.[30,75] On the contrary, rodents keep both bone resorption and intestinal calcium absorption upregulated.[30] The amount of bone resorption in rodents depends on how much calcium is being absorbed by the intestines.Therefore, elevated intestinal calcium absorption due to high calcitriol levels or a calcium-rich diet reduces bone resorption, while reduced intestinal calcium absorption, such as from a low-calcium diet, increases bone resorption to compensate for the lower calcium availability.. [30] Likewise, The reason why rodents differ from humans regarding the adaptations during lactation is unclear, but it might be an evolutionary advantage to have both mechanisms when nursing many pups.

The mechanisms involved in bone resorption during lactation are osteoclast-mediated bone resorption and osteocytic osteolysis.[30] PTH and calcitriol do not seem to be the primary regulators of these processes. Rather, bone resorption is mainly driven by PTHrP secreted from breast tissue and low systemic estradiol levels.[30] Adaptations in humans have consistently been shown not to affect either free or albumin-bound calcium levels in the serum of breastfeeding women.[30,90–92] Similar findings were observed in studies using WT Black Swiss or C57BL/6 mice on a standard 1% calcium diet, where both ionized and total serum calcium levels remained consistent during lactation, regardless of whether the samples were collected from fasting or non-fasting mice.[57,77,82,83,87]

1.6.3. Post-weaning

After weaning, the maternal skeleton recovers its mineral and biomechanical properties. This recovery takes between 9 to 12 months in humans and up to four weeks in mice.[30]

Studies in humans have been focusing on longitudinal changes in bone mass and mineralization.[30] The available DXA scan data revealed that, although women generally recover to their pre-pregnancy BMD levels on average, individual responses can vary significantly, with some surpassing and others falling below their baseline levels.[30,91,93,94]

Rodent studies have encompassed a broader range of measurements compared to human studies. Molecular pathways indicate that recovery begins as early as 24 to 48 hours after artificially weaning the pups. Changes include the osteoclast number and activity, accompanied by a significant decrease in RANKL and RANK expression, and a notable increase in osteoblast precursors, osteoblast number, osteoid surface, and bone formation rate.[95–97] During this period, PTH and calcitriol levels decrease while calcitonin levels surge. Bone resorption markers decline rapidly, while bone formation markers rise.[82,83,95] Osteolysis also ceases, with osteocytes beginning to express osteoblast-specific genes, leading to the restoration of the matrix to its previous mineral content, as evidenced by tetracycline-labeled bands in their lacunae.[98] This anabolic activity of osteoblasts and osteocytes continues for several weeks, ultimately improving bone strength, mineralization, and microarchitecture, potentially reaching or exceeding pre-pregnancy levels.[30]

1.7. Physiological consequences of *CYP24A1* mutations during infancy and adulthood

The mitochondrial enzyme CYP24A1 was first described in the early 1970s and initially believed to be involved solely in the degradation of calcifediol, the most abundant circulating form of vitamin D and precursor of calcitriol[62]. It is now known that CYP24A1 is the main enzyme responsible for catabolizing both calcifediol and calcitriol into several inactive metabolites (Section 1.4.4). Without

proper catabolism due to homozygous or compound heterozygous inactivating mutations of *CYP24A1*, calcitriol can rise above normal. Many individuals appear asymptomatic whereas others develop physiological consequences of the hypercalcemia, including hypercalciuria, low parathyroid hormone (PTH), vomiting, dehydration, fever spikes, nephrocalcinosis, and likely increased intestinal calcium absorption.[62,99–101]

When symptoms manifest early in life, it leads to Infantile Idiopathic Hypercalcemia Type I, which can cause developmental problems affecting both bone and neurological function, in addition to the previously described symptoms.[102–104] The condition may be transient or prolonged. David et al.[105] have shown that hypercalcemia can begin as early as in utero in mice.

Multiple allelic variations in the *CYP24A1* gene have been reported to cause hypercalcemia.[106] Most of the variations exhibit recessive traits, affecting individuals with varying degrees of penetrance.[100,104,106] Therefore, individuals who are heterozygous for inactivating mutations generally exhibit no symptoms, whereas individuals who are homozygous or compound heterozygous for mutations in *CYP24A1* are more likely to be symptomatic.[62,99–101,106]

Still, many affected infants and adults show no symptoms, and the loss of CYP24A1 function may not be clinically evident even in the homozygous state.[75,77,88,89] However, factors such as intense sunlight exposure, vitamin D supplementation, or pregnancy can lead to more severe hypercalcemia in those adults.[30,75] These factors increase calcitriol synthesis, potentially overwhelming a deficient catabolic pathway and leading to hypercalcemia. Conversely, patients who are vitamin D deficient or insufficient are unlikely to develop symptomatic hypercalcemia.

The condition is considered rare, with homozygous and compound heterozygous inactivating mutations of CYP24A1 having a biallelic frequency of 4-20% to cause infantile hypercalcemia type 1 (OMIM#143880).[104,106–108] It is likely underdiagnosed due to the hypercalcemia remaining undetected (serum calcium is not routinely measured) and to the potential for physicians to misattribute the symptoms from hypercalcemia to other causes. This is particularly true during

pregnancy, when symptoms caused by hypercalcemia, such as nausea and lethargy, can easily be mistaken for typical pregnancy-related conditions. Additionally, the definitive diagnosis can only be obtained through DNA sequencing, which is not routinely requested by the physicians.

1.7.1. Gestational hypercalcemia

Hypercalcemia during pregnancy (gestational hypercalcemia) is a rare condition, affecting roughly 0.03% of women of reproductive age.[109] The symptoms may include nausea, dehydration, preeclampsia, pancreatitis, and nephrolithiasis.[30,99,109] In more severe cases, it can cause maternal or fetal death.[30,99,109] Especially when the symptoms are mild, the diagnosis of hypercalcemia in pregnancy can be challenging because its symptoms often resemble those of a typical pregnancy.[30,99,109,110]

The condition may have different etiology, such as primary hyperparathyroidism (PHPT), Familial Hypocalciuric Hypercalcemia (FHH), Pseudohyperparathyroidism caused by breast and placenta PTHrP production, and the lack of a functional CYP24A1 enzyme.[71,76]

Calcitriol has a crucial role during pregnancy, increasing from 2 to 5-fold, which partially explains the doubling of intestinal calcium absorption to meet the fetal demand for minerals (Section 1.6).[30,111] Consequently, it reaches levels that would be toxic in non-pregnant individuals but are essential during gestation[30].

When unopposed by catabolism from CYP24A1, the upregulation of calcitriol during pregnancy may exceed the expected values, possibly contributing to excessive intestinal calcium absorption and marked hypercalcemia (**Figure 9**).[102,110,112–114]

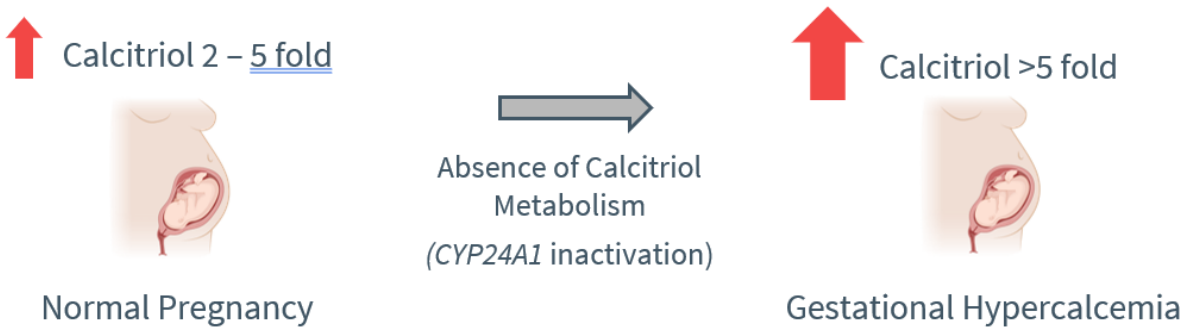

Figure 9. Illustration of how *CYP24A1* inactivation causes gestational hypercalcemia.

Calcitriol normally increases between 2-5 folds during pregnancy, but the lack of calcitriol catabolism by C*YP24A1* exacerbates the increase, leading to hypercalcemia during pregnancy. Created with BioRender.com (Appendix 8).

1.7.2. Consequences during lactation and pos-weaning

Few cases have been reported whether the loss of CYP24A1 has any impact during lactation, as most have focused on hypercalcemic crises during pregnancy. In a case involving a breastfeeding woman with CYP24A1 deficiency, her hypercalcemia was less severe, and her serum calcitriol levels were normal.[113] Normally, during lactation, the maternal skeleton undergoes resorption to supply minerals to the milk, primarily due to low estradiol levels and high levels of PTHrP from the lactating breast tissue.[30] PTH remains low, while calcitriol drops to non-pregnant levels.[30] Intestinal calcium absorption in lactating women returns to normal,[30] whereas in rodents, it remains high, similar to during pregnancy.[30,115] Women only rely on skeletal resorption to provide the necessary minerals to the milk, while rodents require both a high rate of intestinal calcium absorption and skeletal resorption to meet the higher calcium demand of a litter of pups.[30] If elevated calcitriol and increased intestinal calcium absorption continue during lactation, this might lead to a compensatory reduction in skeletal resorption in mice.[30]

1.8. Rationale, purpose, and hypothesis

There are several case reports linking gestational hypercalcemia to *CYP24A1* mutations,[102,110,112–114] but they do not clarify whether this condition is driven by increased intestinal absorption, bone resorption, renal calcium conservation, a combination of these factors, or another mechanism entirely. Other studies have explored the mechanisms and consequences during fetal development and idiopathic infantile hypercalcemia type 1,[38,105] but did not extend their research into pregnancy. Our lab also studied the lack of calcitriol anabolism during pregnancy, lactation, and post-weaning,[87] but the effect of the lack of calcitriol catabolism remained unclear.

Thus, our objective was to determine the mechanism by which the loss of *Cyp24a1* leads to gestational hypercalcemia, and to what extent it influences bone resorption and bone strength during lactation.

We hypothesized that since calcitriol levels typically increase 2-5-fold during pregnancy, its rise may be even more pronounced with Cyp24a1 inactivation due to the lack of catabolic regulation. This unopposed increase could lead to significant effects during pregnancy. Some consequences may include increased calcium absorption, hypercalcemia, and potentially decreased bone resorption during pregnancy. We also hypothesized that calcitriol would not be rapidly cleared after pregnancy, resulting in the persistence of its physiological effects during lactation. In mice, it is known that both increased intestinal calcium absorption and bone resorption play crucial roles in supplying sufficient calcium for the litter and that one mechanism will compensate for the other. Therefore, the hypothesized sustained increase in intestinal calcium absorption would likely reduce the typical bone resorption and loss of bone strength.

2. MATERIALS AND METHODS

2.1. Animals

2.1.1. Animal care approval

All studies involving live mice were performed with the prior approval of the Institutional Animal Care Committee of Memorial University under protocol 21-02-CK (Appendix 2).

2.1.2. Mouse Model

The *Cyp24a1 null* mice were provided by our collaborator from McGill University in Montreal, Dr. René St-Arnaud. To produce the genetically engineered mice, Dr. St-Arnaud and colleagues constructed a vector containing a PGK-Neo cassette in the opposite orientation, flanking the exons 9 and 10 that encode the Heme-biding domain (Figure 10).[38] The J1 ES cell line was cultured with the DNA and electroporated as described.[116] The DNA from ES cells or tail was extracted, and the transformation was confirmed by Southern Blot.[38] The ES cells carrying the disrupted *Cyp24a1* gene were injected into C57BL/6 embryos at the blastocyst stage.[116]

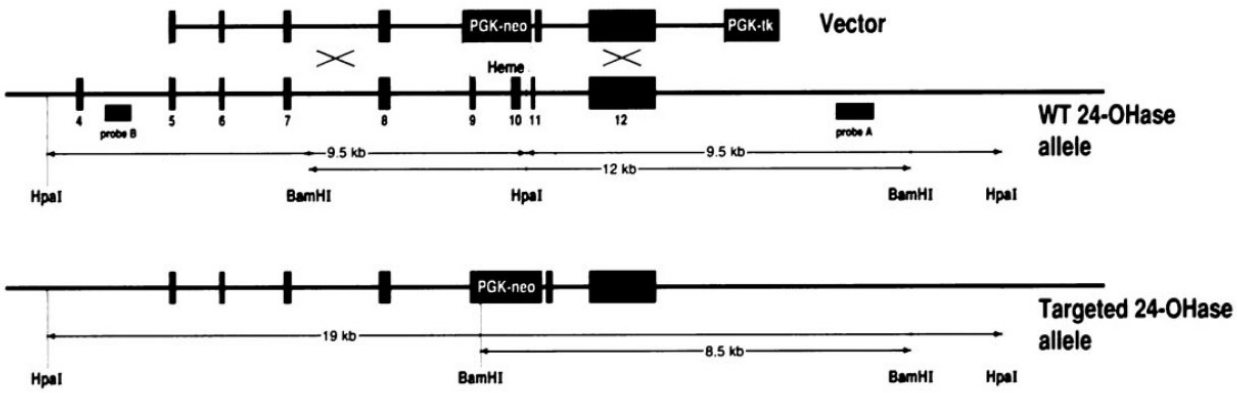

Figure 10. Schematic representation of *Cyp24a1* NULL mice allele.
In summary, a PGK-Neo cassette substituted the Heme-binding domain flanking exons 9 and 10, disrupting the normal gene function. The figure is not in scale. Used with permission from the Oxford Press (Appendix 9).[38]

We generated three genotypes by crossing heterozygous male and female mice: wild type (*Cyp27b1*$^{+/+}$ or WT), with two normal *Cyp24a1* alleles, heterozygous (*Cyp27b1*$^{+/-}$ or HET) with one normal and one ablated allele and null (*Cyp27b*$^{-/-}$ or NULL) with both ablated alleles.

2.1.3. Housing

The animals were housed in individually ventilated rodent cages (GM500, Techniplast, Canada) and Bed-O-Cobs corn cob absorbent bedding (The Andersons, Maumee, OH, USA). The humidity in the animal room was kept between 40% and 60%, and the room temperature was between 22-25 ºC. All mice were kept on a light and dark cycle of 12 h each.

2.1.4. Diet and water

All mice were fed Teklad Global 18% Protein Rodent Diet (Envigo, Medison, USA), *ad libitum.* A complete list of ingredients can be found in Appendix 3. Filtered water was provided *ad libitum*. The filtration system removes small particles, but not minerals, such as calcium.

2.2. Genotyping

2.2.1. Animal Identification

The mice were weaned at 21 days of age and separated by sex, with a maximum of four mice per cage. After brief anesthetization using isoflurane, each mouse had its right ear tagged with a letter and number for identification.

2.2.2. Tissue Collection and DNA extraction

Immediately after tagging, the ear was notched with a sanitized ear puncher and placed in a 1.5 mL microtube for genotyping. The tissue was then digested in 250 µL of 50 nM NaOH for 30 minutes at 98°C. After digestion, the sample was vigorously homogenized and centrifuged for 5 minutes at 15,000 RCF. Then, 20 µL of the supernatant was placed in a new tube containing 80 µL of TE buffer (10 mM Tris-HCl with 1 mM EDTA, pH 7.4) and stored at -20°C until further processing.

2.2.3. Polymerase Chain Reaction (PCR)

PCR was performed using a four-primer system. Primers WT_F (CCA AGT GTG CCA TTC ACA AC) and WT_R (TCT CTC GCT GAA CCT GGA TT) identify the WT mice and generate a band at 557 bp, whereas primers NEO_F (GAT CGG CCA TTG AAC AAG AT) and NEO_R (TCG TCC TGC AGT TCA TTC AG) amplify the Neomycin/Kanamycin resistance gene present in the

CYP24A1 null mice, generating a band at 193 bp (Figure 11 and Figure 12). Heterozygous mice (HETs) were expected to generate both fragment bands (557 bp and 193 bp).

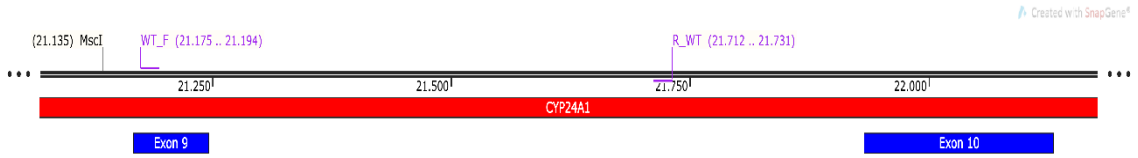

Figure 11. Map of the region near the binding sites of WT primers.
Primer WT_F binds to exon 9, while WT_R binds to the intron between exons 9 and 10, a region that is only present in WT mice. Created with SnapGene.[117]

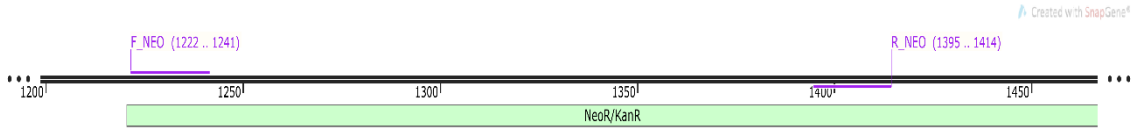

Figure 12. Map of the region near the binding sites of NULL primers.
Primers F_NEO and R_NEO bind to the Neomycin/Kanamycin resistance gene present in NULL mice. Created with SnapGene.[117]

The PCR was done using the Invitrogen Platinum II Taq Hot-Start DNA Polymerase kit (Invitrogen, Carlsbad, USA). Each reaction consisted of a mastermix with 1X Platinum II PCR Buffer (without Mg), 0.2 mM dNTP mix, 0.2 µM of primers Forward and Reverse, 2 µL of DNA (varied concentration) and 0.04 U/µL of Platinum II Taq Hot-Start DNA Polymerase.

The PCR program was as follows:

Step 1: 94°C for 5 minutes to degrade the antibody attached to the Taq polymerase, exposing its active site (activation).

Step 2: 94°C for 30 seconds to denature the double-helix

Step 3: 58°C for 30 seconds to anneal the primers

Step 4: 72°C for 1 minute to synthesize the new strand.

Step 5: Repeat Steps 2-4 for 30 cycles

Step 6: 72°C for 5 minutes to elongate the remaining strands.

Step 7: Hold at 4°C until the samples were removed from the thermocycler.

2.2.4. Gel Electrophoresis

The PCR products were separated by size using agarose gel electrophoresis. 10X Tris-actetate-EDTA (TAE) buffer (0.12 M EDTA, 0.40 M Tris, 11.5% glacial acetic acid, pH 8.0), was diluted to 1X and a 1.2% gel was prepared by adding 100ml 1X TAE, 1.2 g agarose (Invitrogen, Carlsbad, USA) and 10 µL of SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) The solution was heated until dissolved and poured into a casting tray and left for 30 minutes to polymerize. PCR samples were prepared for electrophoresis by adding 4 µL of 6X loading dye (10 mM Tris, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA, and 1.5 M dH_2O) to each reaction. The gel was covered with 1X TAE running buffer and 10 µL of each PCR reaction was pipetted into the wells. The gel ran for 20 minutes at 200 V. The bands were visualized with UV light and analyzed using the Bio-Rad Gel Doc XR+ imaging system, software version 5.1. (Bio-Rad, Hercules, USA). Figure 13 is a representative gel image. The presence of a single band at 557 bp indicated the mouse was WT, a single band at 193 bp indicated the mouse was NULL, and two bands indicated the mouse was HET.

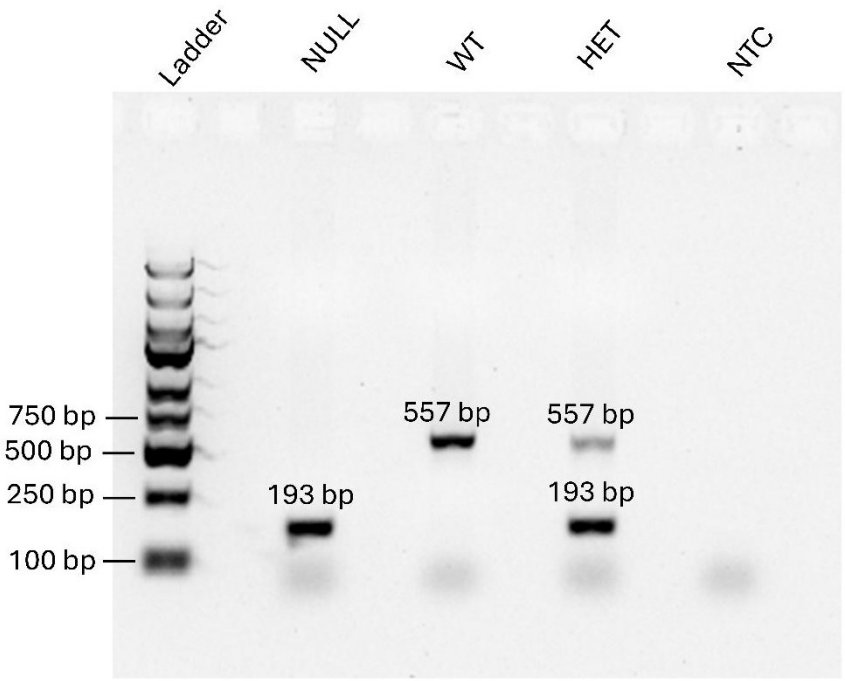

Figure 13. Representative gel image.
Mice with a single band at 193 bp were identified as NULLs, while mice with a single band at 557 bp were identified as WT. Mice showing both bands were identified as HETs.

2.3. Mating scheme

The colony was maintained using HET females and males, which were crossed to each other to generate all genotypes following Mendelian ratios of 1 WT: 2 HETs: 1 NULL. In each generation, some HETs were separated to replace older HETs and maintain a constant colony size. HETs not used for colony maintenance were utilized for fetal studies being completed by another student, while WTs and NULLs became the experimental mice used for this project.

Mice used for colony maintenance were bred starting at 8 weeks of age, and each female was allowed to have up to three litters. Some retired from the breeding colony and became the "experienced mothers" who were placed with experimental mice to reduce stress, thereby decreasing the likelihood of litter destruction by the new mom.

Experimental mice of all genotypes were mated starting at 10 weeks of age, at which time bone mass reached a relatively stable plateau. We maintained this starting point even for mice that did not undergo bone measurements.

Once a week, the experimental female mice were placed in the same cage as the male HETs around 4:00 PM and removed at approximately 10:00 AM the next day. This process was repeated until the mice were visibly pregnant or had undergone 8 unsuccessful attempts, at which point they were considered too old for further breeding attempts. The detection of vaginal mucus plugs the following day confirmed successful mating, designating that day as embryonic day 0.5. Once pregnancy was further verified by a noticeable size increase, the pregnant mice were individually housed alongside an "experienced mother" mouse, as previously described. The average gestation period lasted approximately 19.5 days.

2.4. Litter Size

To accurately assess changes in BMC and other parameters throughout the reproductive cycle in mice, it is essential that both WT and NULL mothers have similar litter sizes, especially during lactation. The number of pups nursed during lactation directly affects calcium requirements, influencing the extent of bone resorption from the maternal skeleton and calcium absorption from the intestines. In this study, breeding pairs were well-matched in terms of litter sizes, with averages close to 6 for both groups. Larger litters were culled to 6 by 48 hours after delivery, and litters smaller than 4 were not used for analysis.

2.5. Timeline, data collection points, and sample size

The timeline, along with all the collection points, can be tracked in Figure 14. In the first cohort, blood, urine, bone, and DXA scans were collected at BL, LP, LL, R1, R2, R3, and R4. Subsequently,

an additional collection point (ML) was introduced in a second cohort to study hormonal and mineral changes during lactation; however, urine samples were not collected at that time. As the reviewers of JBMR requested additional data to publish our project, we introduced a third cohort to collect more blood and urine samples for measuring albumin-adjusted serum calcium and creatinine, fractional excretion of calcium, phosphorus, and magnesium, and the renal expression of *Cyp24a1* and *Cyp27b1*.

Instead of performing power calculations for each assay, we targeted an empirical sample size of 10 for each genotype. This empirical approach stems from decades of experience in our laboratory, which has shown that a sample size of 10 generally provides sufficient power in most in vivo assays. The hormonal and gene expression assays were capped at 7 samples in order to fit everything on a single plate, while 5 samples per genotype is a widely accepted standard for qPCR and µCT analyses. The sample size for the fractional excretion of phosphorus and magnesium was further constrained due to a shortage of remaining samples.

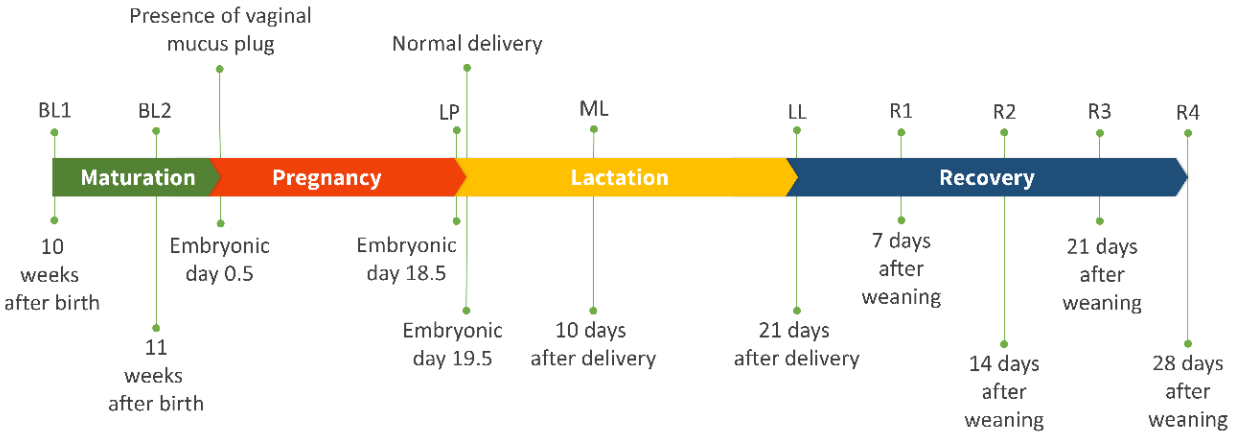

Figure 14. Schematic showing sample collection time points during the reproductive cycle. The time from birth to R4 lasts around 21 weeks (5 months). **BL1:** Baseline one, data collected from virgin mice. **BL2:** Baseline two, data collected one week after BL1. **LP:** Late pregnancy, 18.5 days of pregnancy. **ML:** Mid lactation, 10 days of lactation. Milk production and bone resorption are at their peak. **LL:** Late lactation, 21 days of lactation. The pups are weaned on this day. **R1:** Recovery 1: 7 days after weaning. The skeleton is regaining the minerals lost during lactation. **R2:** Recovery 2,

14 days after weaning. Recovery of the skeleton should be complete for most mice at this point. **R3:** Recovery 3, 21 days after weaning. Some mice may require more time to recover fully. **R4:** Recovery 4, 28 days after weaning. All the mice be recovered completely by this point.

2.6. Bone analysis

2.6.1. Bone Mineral Content (BMC)

Dual-energy X-ray absorptiometry (DXA or DEXA) is a non-invasive diagnostic imaging technique used to assess body composition.[118,119] The DXA machine emits two low-current X-ray wavelengths that penetrate the subject's body and are subsequently detected by a sensor[118]. The differential absorption of these X-rays by various tissues allows body composition measurements, such as bone mineral content (BMC), bone mineral density (BMD), and body fat percentage.[118]

BMC of mice in this study was measured using the Lunar PIXImus 2 DXA (General Electric, Boston, USA), and quality control measurements were performed before each scan by calibrating with a "phantom" mouse (BMD=0.0630 g/cm, % fat =11.9%).

For immobilization during the scan, mice were given an intraperitoneal injection of a mixture of ketamine (50 mg/ml) and xylazine (20 mg/ml). The amount of anesthetic was adjusted accordingly to the average mouse size at each time point, varying from 25 µL at BL, 40 µL at LP, 30 µL at LL, and 25 µL at R1-4. The amount of anesthetic was optimized to use the minimum amount to keep the mice immobilized during the scan while ensuring a fast recovery. Once anesthetized, mice were placed in the prone position (Figure 15) by the same person throughout all measurements. Each measurement took approximately 3 minutes to complete.

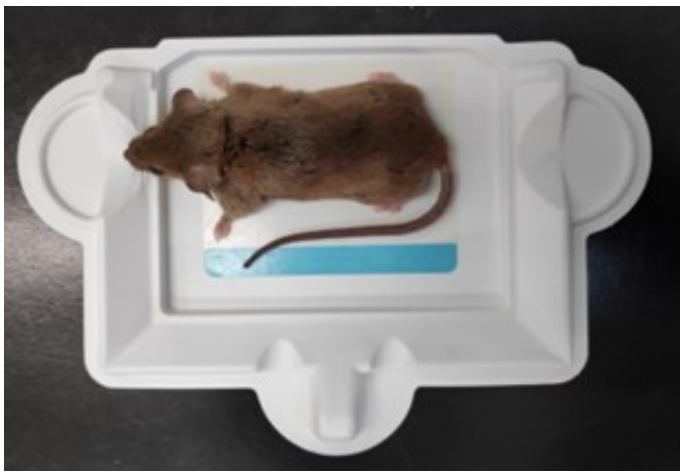

Figure 15. Representative photo of a mouse in the prone position for DXA scan.
The image was analyzed using Lunar PIXImus software (v1.45). To reduce variability, the head and neck were excluded from all scans, and the ear tag was kept out of the region of interest (ROI).

All ROIs were manually adjusted to measure the whole body (except the head and neck), lumbar spine, and hind limb. Three representative images of the scans are illustrated in Figure 16.

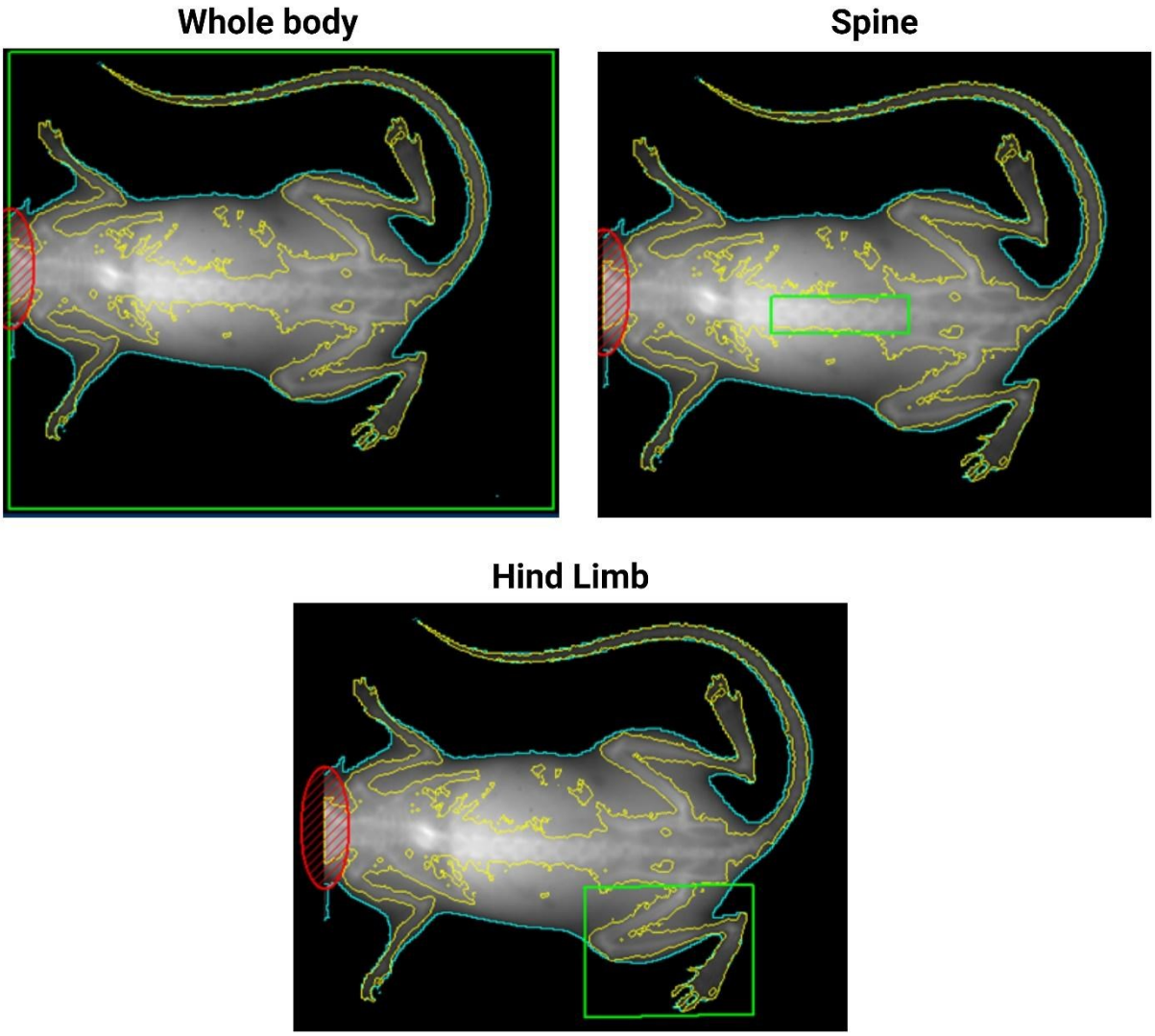

Figure 16. Representative BMC scans of the whole body, lumbar spine, and hind limbs. The green box represents the ROI, while the red ellipse indicates the excluded region.

2.6.2. Micro-computed tomography

Computed tomography (CT) is a non-invasive x-ray imaging technique that permits the reconstruction of a three-dimensional image of the sample.[120] Continuous improvements of tomography technology led to the development of micro-computed tomography (μCT) scanners with resolutions at the sub-micron level.[120]

The principle of μCT involves generating a series of radiographs (projection images) around a fixed axis of a sample placed between the X-ray source and detector.[120,121] The sample can either be fixed at the center while the X-ray source and detector rotate around it, or the sample can rotate around its axis while the X-ray source and detector remain fixed.[122] Both methods reconstruct a three-dimensional image using reconstruction algorithms on two-dimensional images (Figure 17).[120–122] More recent versions of this technique use helical scans, but the principle remains the same[123]. Both soft and hard tissues can be analyzed using μCT scans; however, hard tissues like bone interact strongly with X-rays, eliminating the need for contrast agents.[120–122]

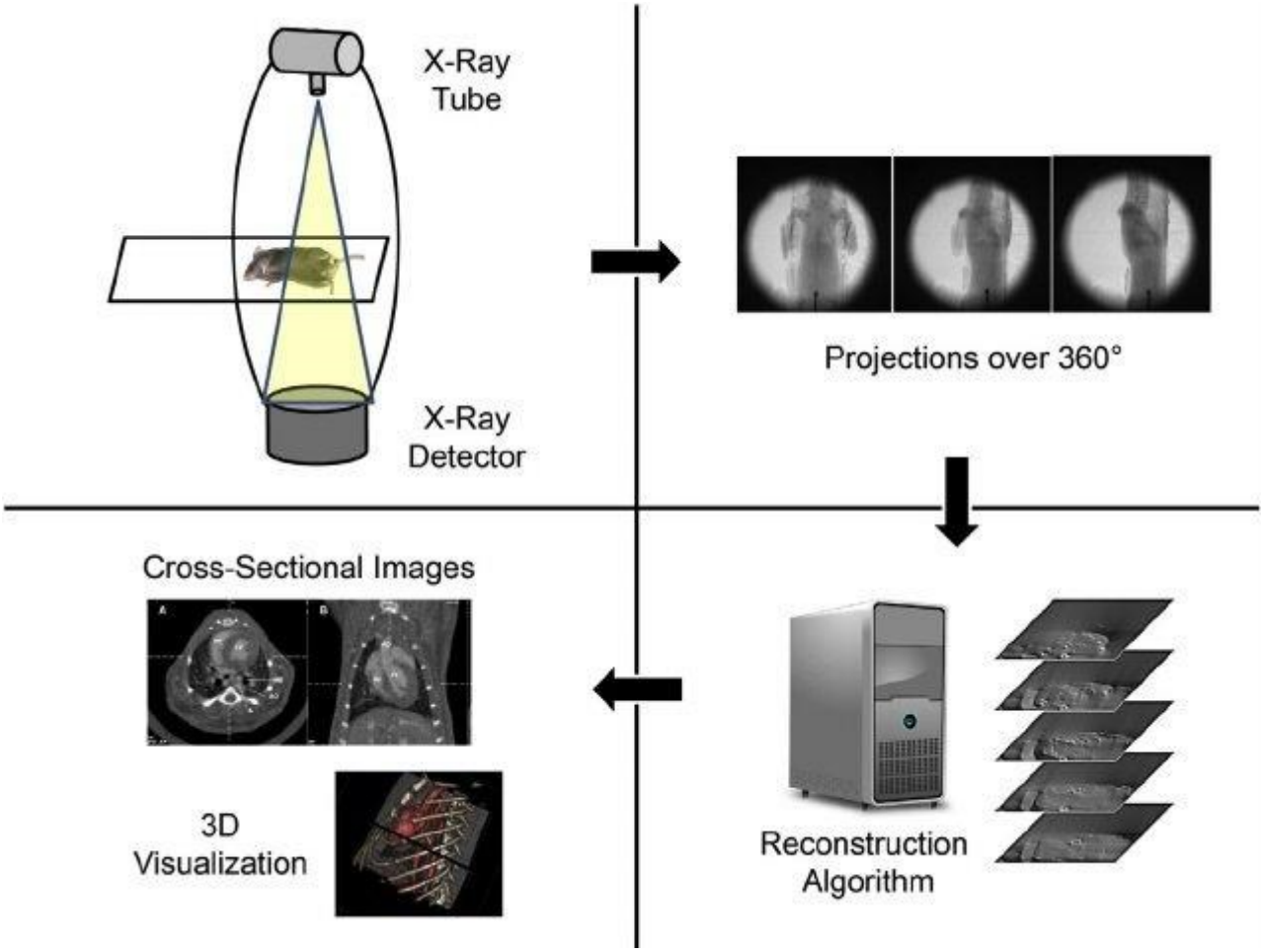

Figure 17. Schematic of the μCT imaging process, including image acquisition of cone beam projections, reconstruction, and visualization of tomographic data.

The mouse sample is placed between an X-ray tube and an X-ray detector, which rotates around it. Projections are typically taken over 360º at intervals of 0.1 to 1º. The images are then reconstructed to form three-dimensional images. Used with permission of Elsevier (Appendix 10).[124]

WT and NULL mice were anesthetized with isoflurane and then euthanized via cervical dislocation. Lumbar vertebrae and the right hind limb were collected and placed in 10% neutral buffered formalin (NBF) at 4º C overnight. The next day, the samples were washed with 1X PBS for 10 min and stored in 70% ethanol in the dark until they were analyzed.

µCT was performed Dr. Natalie Sims' group at St Vincent's Institute of Medical Research in Melbourne, Australia. A high-resolution µCT scanner (Skyscan 1076, Kontich, Belgium) was used for imaging. The settings included a 9 mm voxel resolution, 0.5 mm aluminum filter, 50 kV voltage, and 100 mA current, with an exposure time of 2600 ms, a rotation of 0.5 degrees, and frame averaging set to 1. Image reconstruction and analysis were carried out using Skyscan's NRecon (version 1.6.3.3), DataViewer (version 1.4.4), and CT Analyzer (version 1.12.0.0) software. Femoral lengths were measured, and the diaphyseal (cortical) region analyzed commenced at a distance of 30% of the length of the bone proximal to the distal end of the femoral growth plate, while the metaphyseal (trabecular) region commenced at 10% of bone length proximal to the distal end of the femoral growth plate; both had regions of interest with a length of 15% bone length. The lower thresholds for detecting bone were equivalent to 0.3579 g/cm3 CaHA for trabecular and 0.5229 g/cm3 CaHA for cortical bone.

2.6.3. Biomechanical test (Three-point bend test)

Hind limbs were collected, and the tibias were isolated. The surrounding tissues were removed, the fibula was detached from each specimen, and all bones were stored at -20ºC until analysis. The three-point bend test was performed on the left tibia from both WT and NULL mice. On the day the test was performed, the bones were thawed at room temperature in 1X PBS for 2 h to rehydrate and stabilize the samples. The assessment of cortical bone strength was conducted using an Instron Series 3340 single-column electromechanical testing device (Instron, Norwood, MA) in conjunction with the Instron Series IX software (v8.30.00). Each tibia was consistently positioned on the platform, and the ends were securely fixed (Figure 18). The crosshead of the machine was positioned roughly 2-3

cm above the tibia's midshaft and calibrated before each test. The crosshead descended at a rate of 10 mm/min until the tibia fractured. Each test generated a range of biomechanical data, including maximum load (gf), displacement (μm), maximum strain (gf/mm2), strain percentage, and slope (gf/mm). All collected bones were analyzed on the same day.

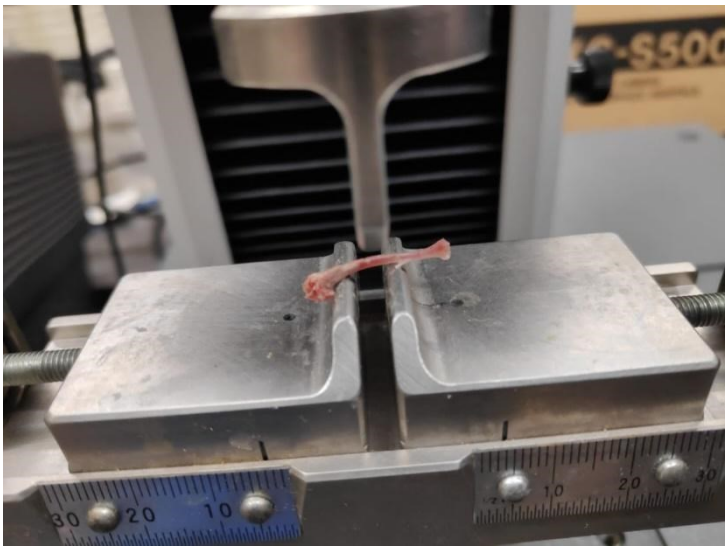

Figure 18. Representative image of a tibia positioned on the Instron Series 3340 single-column electromechanical testing device platform.
Tibias were placed at fixed positions on the platform, and a flexural device applied pressure to the mid-shaft of each tibia until it fractured.

2.7. Blood and urine collection

2.7.1. Blood

Blood samples were collected from the tail vein for longitudinal studies and heart puncture for terminal procedures.

Tail bleeding was performed by cutting a thin section at the end of the tail with a sharp razor. The blood was collected into a 0.6 mL microtube.

A cardiac puncture was performed to collect larger amounts of blood for terminal procedures. Immediately before the procedure, all mice were deeply anesthetized with isoflurane. A sterile 1 mL

syringe with a 20-gauge needle was then used to pierce the skin below the ribcage to access the heart. After collecting the blood, the mice were immediately euthanized by cervical dislocation.

Blood from tail bleeding and heart puncture were centrifuged at 1500 x g for 15 minutes to separate the serum from white blood cells (leukocytes), red blood cells (erythrocytes), platelets, or clotting factors. The supernatant (serum) was pipetted into a new microtube and stored at -20 °C until further analysis. The samples were aliquoted to avoid freezing and thawing.

2.7.2. Hematocrit test

A hematocrit test, also referred to as packed cell volume (PCV), volume of packed red cells (VPRC), or erythrocyte volume fraction (EVF), measures the percentage of red blood cells in the blood.[125,126] Abnormally high or low hematocrit levels can indicate various conditions, including dehydration. Dehydration reduces the overall water content in the body, including the plasma in the blood. Therefore, reduced plasma levels increase the proportion of hematocrit to total blood volume, indicating dehydration, with the opposite also being true.[125,126]

Hematocrit is expressed as a unitless value between 0.00 and 1.00. In mice, hematocrit values typically range between 0.40 and 0.50, with levels during pregnancy being comparatively lower than pre-pregnancy values due to plasma volume expansion during this period.[127]

Blood for the test was collected from the tail vein using capillary tubes coated with ammonium heparin. The microtubes were sealed with wax and centrifuged for 10 minutes at 15,000 x g. The microtubes were positioned with a ruler placed underneath them to measure the length of the red cell fraction. The length of the red cell fraction was recorded in centimeters and then divided by the length of the total blood volume fraction, also recorded in centimeters. The measurement was performed by the same person for consistency.

2.7.3. Urine

Mice were placed in clean, empty cages to urinate in the morning. Urine was collected with a pipette, placed in 0.6 mL microcentrifuge tubes, and stored at -20°C until analysis.

2.8. Serum and urine mineral analysis

All mineral levels in serum and urine were measured using colorimetric assays with absorbance determined using the Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Piscataway, NJ).

2.8.1. Calcium content

The calcium colorimetric assay by Sekisui Diagnostics[128] (Sekisui Diagnostics, Charlottetown, PEI) was used to measure serum and urine calcium concentrations. Arsenazo III [2,2'-(1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo) bisbenzenearsonic acid] reacts with calcium in an acid solution to form a blue-purple complex. The color developed has a maximum absorbance of 650 nm and is proportional to the calcium concentration in the sample. The linear range of the kit was 0.01 mmol/L to 3.75 mmol/L. The stoichiometric reaction is shown below:

$$2\ \text{Arsenazo III} + Ca^{++} \rightarrow \text{Ca–Arsenazo Complex}^{++}\ \text{(blue-purple)}$$

To perform the test, 10 µL of the sample (serum or urine) or calcium standard of known concentration were pipetted into 1000 µL of Calcium Reagent in 1.5 mL plastic cuvettes. The absorption at 650 nm was measured using a spectrophotometer, and the concentration of calcium was calculated using the absorbance of the calcium standard. Some samples were diluted with saline (0.9% NaCl) when concentrations were beyond the linear range of detection.

2.8.2. Phosphorous content

The phosphorus-SL colorimetric assay by Sekisui Diagnostics (Sekisui Diagnostics, Charlottetown, PEI) was used to measure serum and urine phosphorus concentrations.[129] A reaction between inorganic phosphorus and ammonium molybdate in the presence of sulfuric acid results in the formation of an unreduced phosphomolybdate complex. The absorbance intensity at 340 nm is directly proportional to the amount of inorganic phosphorus in the sample. The reportable range of the kit was 0.03 mmol/L to 6.46 mmol/L. The stoichiometric reaction is written below:

$$\text{Inorganic Phosphorus} + \text{Ammonium Molybdate} \xrightarrow{H_2SO_4} \text{unreduced Phosphomolybdate complex}$$

To perform the test, 10 µL of the sample (serum or urine) or phosphorous standard of known concentration were pipetted into 1000 µL of Reagent 1 and 200 µL of Reagent 2 in 1.5 mL plastic cuvettes. Samples were incubated for 10 min at room temperature. The absorption at 340 nm was measured using a spectrophotometer, and the concentration of phosphorous was calculated using the absorbance of the phosphorous standard. Some samples were diluted with saline (0.9% NaCl) when concentrations were beyond the linear range of detection.

2.8.3. Magnesium content

The magnesium colorimetric assay by Sekisui Diagnostics (Sekisui Diagnostics, Charlottetown, PEI).[130] When magnesium from a sample is mixed with the Magnesium reagent under alkaline conditions, it forms a red complex with xylidyl blue diazonium salt. The concentration of magnesium in the sample can be determined by measuring the decrease in absorbance at 660 nm using spectrophotometry. The reportable range of the kit is 0.12 mmol/L to 3.29 mmol/L. The stoichiometric reaction is as follows:

$$\text{Xylidyl blue-1} + Mg^{++} \rightarrow \text{Mg-xylidyl blue complex (red)}$$

The first step was adding 10 µL of the sample (serum or urine) or magnesium standard of known concentration into 1000 µL of Magnesium Reagent in 1.5 mL plastic cuvettes. The mix was incubated for 5 min at room temperature, the absorption at 520 nm was measured, and the concentration of magnesium was calculated using the absorbance of the magnesium standard. Some samples were diluted with saline (0.9% NaCl) when concentrations were beyond the linear range of detection.

2.8.4. Creatinine content

Creatinine was measured using a colorimetric assay by Sekisui Diagnostics (Sekisui Diagnostics, Charlottetown, PEI).[131] The kit is based on the Jaffe chemical reaction:

$$\text{Creatinine} + \text{alkaline picrate} \longrightarrow \text{creatinine-picrate complex (Janvovsky complex)}$$

At alkaline pH, creatinine reacts with picrate to form Janvovsky complex. The rate of increase in absorbance (measured at 20 sec and 80 sec) is due to the formation of the creatinine-picrate complex is directly proportional to the concentration of creatine in the sample. The limit of detection was 4 µmol/L to 1945 µmol/L.

First, one part of the Creatinine Picrate reagent and four parts of the Creatinine base reagent were mixed to form the working reagent. Next, 50 µL of the sample (serum or urine) was pipetted into 1000 µL of working reagent in 1 mL plastic cuvettes. The absorbance at 510 nm was recorded after 20 and 80 s the addition of the working reagent, and the concentration of creatinine was calculated using a standard. Some samples were diluted using a saline solution (0.9% NaCl) to fit within the linear range of detection.

2.9. Albumin-adjusted calcium

Ionized calcium, the biologically active form of calcium, is regarded as the gold standard for calcium assessment, but obtaining precise measurements can be challenging due to the time-sensitive nature of sample processing.[132] Consequently, calcium levels are typically reported as total calcium, which includes both free (ionized) and albumin-bound calcium[133]. Fluctuations in bound calcium can sometimes lead to misdiagnoses of hypo- or hypercalcemia when the variations involve non-biologically active calcium.[133] To determine if hypercalcemia in the NULL mice was related to changes in albumin, we calculated albumin-adjusted calcium.

Both albumin-adjusted calcium and fractional mineral excretion were performed in the same group of mice. The group comprised a mix of cross-sectional and serial samples due to the large sample volume required.

Albumin was measured by the Pierce™ BCA Protein Assay Kit[134] (Thermo Fisher Scientific, Waltham, USA), and the calcium measuements calculated for fractional excretion were adjusted using the formula[135]:

$$Albumin\ Ca\ (mmol/L) = Total\ Ca(mmol/L) + 0.02\ (40 - albumin(g/L)$$

2.10. Fractional mineral excretion

Fractional excretion refers to the percentage of plasma component filtered by the glomerulus that is excreted into the urine.[136] This method, used to assess kidney function, involves measuring the concentration of the mineral in both urine and serum and adjusting for creatinine, which is excreted at a relatively constant rate.[136] The technique can also be utilized to determine whether renal calcium retention is contributing to hypercalcemia.

Calcium, phosphorus, magnesium, and creatinine levels were measured in both serum and urine samples from the same mice, following the previously described protocol (Section 2.8). To eliminate inter-assay variability, all samples for any assay were run at the same time. After measuring the amount of mineral in all samples, the fractional excretion for each mineral was calculated using the formula[137]:

$$Fractional\ excretion\ of\ X = \left(\frac{Urine\ X/Serum\ X}{Urine\ Creatinine/Serum\ Creatinine}\right)$$

Calcium analysis was prioritized; however, due to the limited remaining sample volume, only a few samples from *Cyp24a1* null mice at late pregnancy could be measured for phosphorus and magnesium.

2.11. Hormonal analysis

The hormones were measured using separate aliquots of serum, thus thawing only once for each sample. All hormones were measured using ELISA kits, and the results were measured at their specified wavelengths using a Biotek Epoch microplate reader (Agilent Technologies, Santa Clara, CA) and analyzed with Gen5 software v 3.15.15. Values below the detection limit were set to the detection limit, while those exceeding the maximum were set to the maximum.

2.11.1. Calcitriol

Calcitriol levels in mouse serum were determined using a 1,25-Dihydroxy Vitamin D Enzyme Immunoassay (EIA) (Immunodiagnostic Systems, Inc., Gaithersburg, MD).[138] This 2-day procedure involved immune extraction for the purification of calcitriol from serum, followed by its quantification through EIA. Each sample consisted of 50 µL of undiluted serum. The technique is based on the competitive binding of free calcitriol or biotin-conjugated calcitriol to a limited number

of specific sheep anti-calcitriol receptors. The degree of biotin linked to the anti-sheep antibody is inversely related to the calcitriol levels present. The procedure followed an alternative approach for sample preparation, with immune extraction and assay processes as outlined in the kit instructions. Sample absorbances were measured at a wavelength of 450 nm using a spectrophotometer. The detection range for the kit was from 6 pmol/L to 333 pmol/L.

2.11.2. Calcifediol

Calcifediol levels in mouse serum were determined using a 25-Hydroxy Vitamin D Enzyme Immunoassay (EIA) (Immunodiagnostic Systems, Inc., Gaithersburg, MD).[139] A 12.5 µL serum sample was diluted with 25 µL of saline. Initially, calcifediol molecules in the sample are captured by a dedicated calcifediol antibody fixed to the surface of the assay plate well. Subsequently, a horseradish peroxidase (HRP)-tagged calcifediol antibody is introduced, binding to the anchored calcifediol to form a highly selective sandwich complex. The strength of the enzymatic activity demonstrated by this bound antibody complex is directly related to the amount of calcifediol in the serum sample. This relationship enables the determination of calcifediol concentrations by measuring absorbance at 450 nm. . The detection range for the kit was from 0 nmol/L to 260 nmol/L.

2.11.3. Serum parathyroid hormone (PTH)

PTH levels in mouse serum were determined using a MicroVue™ Mouse PTH ELISA (1-84) (QuidelOrtho, San Diego, USA).[140] This assay employs a two-site ELISA approach to measure serum PTH concentration. Intact PTH, consisting of 84 amino acids, is detected in two parts: the C-terminal portion is identified by a biotinylated anti-mouse PTH antibody fixed to the well surface, while the N-terminal portion is recognized by a horseradish peroxidase (HRP)-labeled anti-mouse antibody. These two antibodies form a 'sandwich' complex, with the enzymatic activity of this complex being

directly proportional to the PTH quantity in the sample. The process was conducted according to the kit's guidelines, using 25 uL of sample, and the absorbances were read at 450 nm. The kit's detection range was from 4 pmol/mL to 1,045 pg/mL

2.11.4. Serum fibroblast growth factor 23 (FGF23)

Mouse serum FGF23 was measured using an FGF23 ELISA Kit (Kainos Laboratories, Tokyo, Japan).[141] A 20 µL serum sample was analyzed undiluted. The FGF23 molecules in the sample are initially bound by a specific FGF23 antibody that is immobilized on the surface of the assay plate well. This is followed by the addition of a horseradish peroxidase (HRP)-labeled FGF23 antibody, which attaches to the immobilized FGF23, creating a highly specific sandwich complex. The intensity of the enzymatic reaction exhibited by this antibody complex, firmly anchored to the well, is in direct correlation with the FGF23 concentration present in the serum sample, allowing the measurement of FGF23 levels by absorbance at 540 nm. The kit's detection range was from 6 pg/mL to 800 pg/mL.

2.12. Intestinal calcium absorption

The intestinal ^{45}Ca absorption protocol is based on a method by Delucca et al. (2008)[142] with several modifications. Mice were fasted overnight and given a 0.3 mL solution containing 0.5 µCi ^{45}Ca in a buffer (0.5 mM CaCl2, 10 mM Tris-acetate, pH 7.5) by oral gavage. After 10 minutes, blood was collected by cardiac puncture, and serum was separated by centrifugation at 1500 x g for 15 min. 50 µL of serum was used for counting ^{45}Ca, and the rest was stored at -20°C for further analysis.

For ^{45}Ca counts, 50 µL of serum was bleached with 30% H_2O_2 and 3N KOH for 30 minutes, then neutralized with 5N HCl. 5 mL of ScintiSafe™ 30% Cocktail (Fisher Chemical, Canada) was added,

and the radioactivity in the sera was measured using a liquid scintillation counter (Beckman Coulter LS6500, Brea, California, USA). Counts were adjusted for total blood volume and expressed as a percentage of the administered dose.

2.13. Gene expression analysis by qPCR

Duodena and kidneys were collected to analyze gene expression using qPCR. The duodenum is the main portion of the intestine where intestinal calcium absorption takes place. The kidneys are where most of the calcifediol is converted into calcitriol.

The genes analyzed in the duodenum are involved in calcium and phosphate transport. This included calbindin-D9k (*S100g*), Ca2+-ATPase (*Atp2b1*), potassium calcium-activated channel subfamily M alpha 1 (*KCNma1*), sodium-calcium exchanger type 1 (*Ncx1*), transient receptor potential vanilloid 6 (*Trpv6*), sodium-phosphate transporter *NaPi2b* and phosphate transporters *Pit1* and *Pit2*, *Cyp27b1*, *Cyp24a1*, *Vdr*, and protein disulfide isomerase family A, member 3 (*Pdia3*).

Genes analyzed in the kidneys were *Cyp27b1*, responsible for calcitriol synthesis, and *Cyp24a1*, involved in its degradation.

Tissue was collected at BL and LP, as these were the most relevant time points for observing gene expression changes. 5 samples were collected at each time point for each tissue. Values below the detection limit were set to the detection limit, while those exceeding the maximum were set to the maximum.

2.13.1. Tissue collection

The mice were anesthetized with isoflurane and sacrificed by cervical dislocation. The duodenum and kidneys were quickly removed and snap-frozen in liquid nitrogen and then stored at -80°C for

gene expression analysis. All procedures were performed as quickly as possible to minimize RNA degradation.

2.13.2. RNA extraction

Total RNA was purified using the RNeasy Midi Lipid kit (Qiagen, Toronto, ON). The frozen tissues were transferred immediately to a tube containing 1 ml of cold QIAzol Lysis Reagent (Qiagen, Toronto, ON) containing 2.8 mm zirconium oxide beads (Bertin Technologies, Paris, FR). The samples were homogenized using the Percellys® Tissue Homogenizer (Bertin Technologies, Paris, FR) 2 times for 30 seconds at speed 5, with the samples incubating on ice between the two steps. The homogenized tissues were placed at room temperature for 5 min, 1 mL of chloroform was added, and vigorous shaking was performed for 15 s. Tubes were let to rest for 3 min and centrifuged at 5,000 g for 15 min at 4 ºC. The upper phase was transferred to a new tube, 3 mL of 70% ethanol was added, and the mixture was mixed thoroughly by vortexing. 4 mL of sample was transferred to a Qiagen RNeasy Midi spin column placed in a 15 mL collection tube. The lid was closed, and the tube was centrifuged at 5,000 g for 5 min at 25 ºC. The overflow was discarded, and 4 mL of Buffer RW1 was added, followed by centrifugation at 5000 g for 5 min. After centrifugation, the collection tube was replaced, 2.5 mL of Buffer RPE was added to the column, and the tube was centrifuged at 5,000 g for 5 min. The previous step was repeated one more time. Another centrifugation was done without putting RPE Buffer in order to clean the residues. The column was then transferred to a new 15 mL collection tube, and 200 uL of RNase-free water eluted the RNA column for 2 min. Lastly, the tube was centrifuged at 5,000 g for 3 min to collect the eluted RNA. All the procedure was carefully performed in an RNAse-free environment.

2.13.3. RNA quality control

The RNA was quantified using the Take 3 plate accessory for the Biotek microplate reader (Agilent Technologies, Santa Clara, CA). 2µL of RNA was loaded onto the plate and concentration and quality were determined with the method "Quantification of single strand RNA" using the Gen5 software (v 3.15.15) (Agilent Technologies, Santa Clara, CA). Samples with high concentration (>250 ng/µL) and ratios of 260/230 and 260/280 greater than 1.8 were selected for qPCR reactions.

2.13.4. cDNA synthesis

RNA samples were diluted to 200 ng/µL. cDNA was synthesized using the Taqman® High Capacity cDNA Reverse Transcription Kit (ThermoFisher, California, US). The RT master mix was prepared on ice. The mastermix consisted of: 2.0 µL of 10X RT Buffer, 0.8 µL of 25X dNTP Mix (100 mM), 2.0 µL of 10X RT Random Primers, 0.1 µL of MultiScribe™ Reverse Transcriptase, 1.0 µL of RNase Inhibitor, and 3.2 µL of nuclease-free H_2O. 10 uL of diluted RNA was added to 10 uL of mastermix and the tubes were placed in a thermocycler with the following program: 10 min at 25 ºC, 120 min at 37ºC, 5 min for 85 ºC and held at 4º C.

2.13.5. Quantitative real-time polymerase chain reaction (qPCR)

Pre-designed TaqMan® Gene Expression Assays with primers and probes for optimal amplification and TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, USA) were used to measure gene expression with the manufacturer's (Table 1).

Table 1. List of primers and probes used in qPCR

Gene Abbreviation	Gene Name	Catalogue number
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1

Atp2b1, Pmca1	ATPase, Ca++ transporting, plasma membrane 1	Mm01245805_m1
Slc20a2, Pit2	solute carrier family 20, member 2	Mm00660203_m1
Slc20a1, Pit1	solute carrier family 20, member 1	Mm00489378_m1
Trpv6	transient receptor potential cation channel, subfamily V, member 6	Mm00499069_m1
Vdr	vitamin D receptor	Mm00437297_m1
Cyp27b1	cytochrome P450, family 27, subfamily b, polypeptide 1	Mm01165918_g1
Slc34a2, NaPi-2b	solute carrier family 34 (sodium phosphate), member 2	Mm01215846_m1
S100g, Calb3	S100 calcium binding protein G	Mm00486654_m1
Kcnma1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	Mm01268569_m1
Slc8a1, Ncx1	solute carrier family 8 (sodium/calcium exchanger), member 1	Mm01232254_m1
Cyp24a1	cytochrome P450, family 24, subfamily a, polypeptide 1	Mm00487244_m1
Pdia3	protein disulfide isomerase associated 3	Mm00433130_m1

qPCR was performed in a 96-well plate, in triplicate, by mixing 10 µL (2µg) of cDNA with 10 µL of TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, USA). The reaction was run in the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA) with the following parameters: 2 minutes at 50°C, 20 seconds at 95°C, 40 cycles of 1 second at 95°C and 20 seconds at 60°C. Relative expression was determined using the threshold cycle (Ct) value of the gene of interest normalized to the Ct of the reference gene (*Gapdh*).

2.14. Milk analysis

Milk was collected at ML to measure calcium and total protein levels. Lactating dams were separated from their pups for 2 hours, anesthetized with 30 µL of ketamine (50 mg/mL) and xylazine (20 mg/mL) mixture, and given 1 IU of oxytocin intraperitoneally while in a supine position. After 5 minutes, all mammary glands were manually massaged from the base to the apex, and milk was

collected using a 200 µL pipette. Fresh milk was used to measure creamatocrit, whereas the samples for calcium and protein were stored at -20°C until analysis.

2.14.1. Calcium content

Calcium content was analyzed using the method described in Section 2.8.1. Frozen samples were thawed, homogenized by vortexing for 1 minute, and diluted 400 times with distilled H_2O to fit within the linear range of detection.

2.14.2. Protein content

In milk, the primary calcium-binding and chelating proteins are caseins and whey proteins,[143] which together account for nearly all of the milk's proteins.[144] Because of this intrinsic relationship, expressing calcium per unit of protein is a globally accepted standard. Otherwise, a small difference in protein concentration will lead to a large artifactual difference in calcium concentration. Protein content was measured using the Pierce™ BCA Protein Assay Kit[134] (Thermo Fisher Scientific, Waltham, USA). The kit uses bicinchoninic acid (BCA) for colorimetric detection and quantification of total protein. The purple-colored product of this assay results in a complex that has a strong absorbance at 562 nm, which is almost linear with increasing protein concentrations across a wide range (20–2000 µg/mL). The BCA method is not a true end-point method, meaning the final color continues to develop even after incubation. However, the rate of continued color development is sufficiently slow, allowing for the simultaneous assay of large numbers of samples.

The protocol can be subdivided into two parts:

Preparation of the BCA working reagent (WR) and albumin (BSA) standards:

The following formula was used to determine the total volume of WR required: (# standards + # unknowns) × (# replicates) × (volume of WR per sample). 200 µl of WR reagent was needed per sample. The required amount of WR was prepared by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B. Standards were made by serial dilution of 2 mg of Bovine Serum Albumin (BSA) (Sigma-Aldrich, Saint-Louis, USA) in 1 mL of saline.

Microplate procedure:

For the assay, 25 µL of each standard or unknown sample was pipetted in duplicate into a microplate well. 200 µL of WR was added to each well, and the plate was thoroughly mixed on a plate shaker for 30 seconds to ensure uniform distribution of the reagent. The plate was covered and incubated at 37°C for 30 minutes. The absorbance of each well was measured at 562 nm using a plate. A standard curve was generated by plotting the concentration of the standards against their corresponding absorbances. This curve was then used to determine the protein concentration in each sample.

2.14.3. Creamatocrit

The creamatocrit test is a simple test to estimate milk's fat content[145], and it was performed as follows: Fresh milk was collected as described in Section 2.13. The milk was then transferred into a capillary tube, filling approximately 4/5 of its capacity. The capillary tube was then sealed with wax and centrifuged for 40 minutes at 15.000 RFC. Finally, the length of the cream portion was divided by the total milk volume to calculate the creamatocrit. The percentage of cream relative to the total milk volume was calculated to estimate the lipid content.

2.15. Statistical Analysis

Instead of performing power calculations for each assay, we targeted an empirical sample size of 10 for each genotype. This empirical approach stems from decades of experience in our laboratory, which has shown that a sample size of 10 generally provides sufficient power in most in vivo assays. The hormonal and gene expression assays were capped at 7 samples in order to fit everything on a single plate, while 5 samples per genotype is a widely accepted standard for qPCR and μCT analyses. The sample size for the fractional excretion of phosphorus and magnesium was further constrained due to a shortage of remaining samples.

Data were analyzed using GraphPad Prism 10 for MacOS, Version 10.0.3 (GraphPad Software, Boston, MA). qPCR results were evaluated through the Comparative CT Method (2ΔCT).[146] The primary prespecified outcomes were assessed via paired t-tests, comparing WT and NULL mice for serum calcium levels and intestinal calcium absorption at the end of pregnancy, as well as BMC loss measured by DXA at the end of lactation. All other analyses involved two-way ANOVA across the full dataset (covering both genotypes and all time points), followed by Tukey's post-hoc test to adjust for multiple comparisons. All data are presented as mean ± SD with two-tailed p-values.

3. RESULTS

3.1. Litter size and genotype ratio

The ratio of WT:HET:NULL born from colony breeders was 1:2:1, as verified by David Bennin's project.[105] However, consistent with the previous findings reported by the laboratory that developed the mice,[38] approximately half of the null mice survived to three weeks of age (expected time of weaning). This limited the availability of adult null mice for these experiments.

3.2. Longitudinal changes in calciotropic hormones

Serum calcitriol levels increased in both genotypes during pregnancy as expected, with a 2.5-fold higher increase in NULLs compared to WT (185.3±7.0 vs. 76.3±21.5 pmol/L, $p<0.001$). This increase was attenuated at mid-lactation but remained significantly higher in NULLs compared to WT (91.1±52.5 vs. 20.5±6.0 pmol/L, $p=0.0174$), and returned to normal levels by R1 (Figure 19A). The absence of Cyp24a1-mediated catabolism also contributed to apparently higher baseline levels of calcifediol in NULLs, though not statistically significant (56.4±8.6 vs. 40.5±3.8 mmol/L, $p=0.067$) (Figure 19B). During pregnancy, calcifediol levels significantly decreased in both genotypes, with a more pronounced drop in NULL mice (29.8±6.3 vs. 56.4±8.6 mmol/L, $p=0.0001$). Subsequently, calcifediol levels returned toward baseline values during post-weaning recovery but did not reach levels significantly higher than controls at these time points (Figure 19B).

Mean serum PTH was not significantly different in NULLs at baseline compared to WTs. During pregnancy and mid-lactation, PTH was suppressed to at or near the lower limit in both genotypes. Afterward, PTH levels in both genotypes rose toward baseline values (Figure 19C). Serum FGF23 rose 6.5-fold higher in NULL compared to WT mice during pregnancy (1,716.3±867.4 vs 269.0±162.8, $p>0.001$) (Figure 19D).

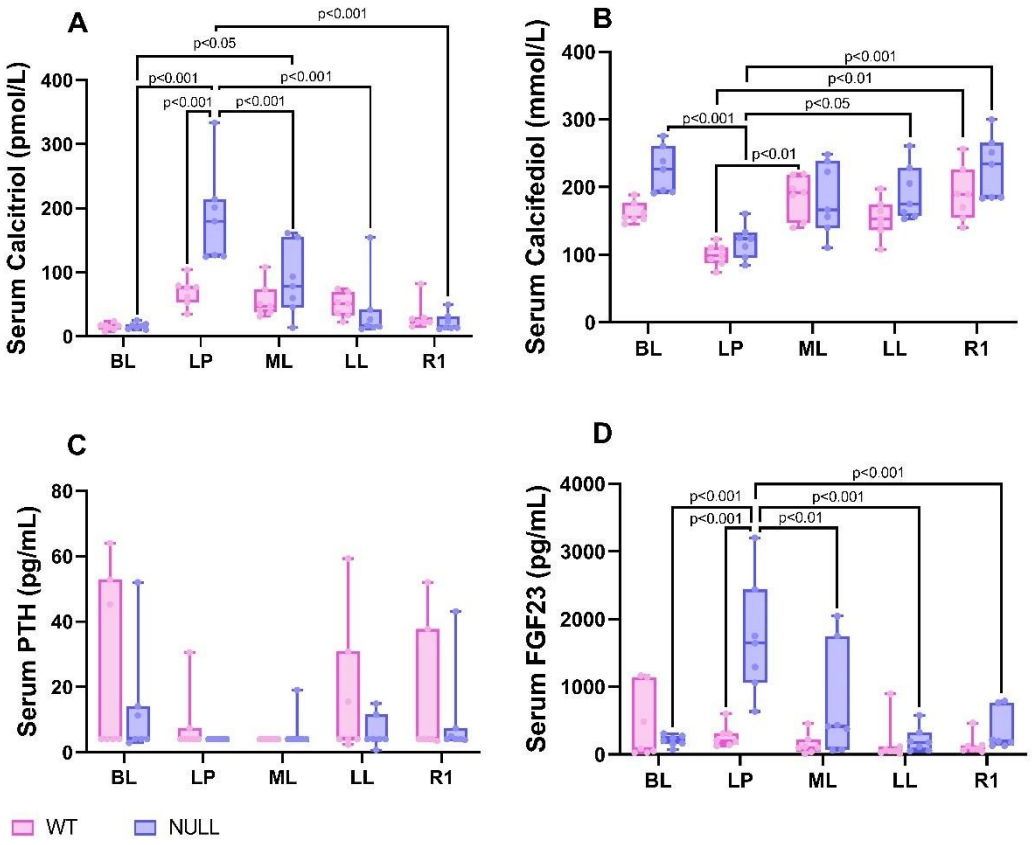

Figure 19. Longitudinal changes in calciotropic hormones.

Serum calcitriol levels increased during pregnancy as expected, with a 2.5-fold greater increase in NULLs, although this increase was less pronounced at mid-lactation (**A**). Calcifediol levels were higher in NULLs at baseline but decreased more than in WTs during pregnancy (**B**). PTH levels were slightly lower in NULL mice compared to WT mice during baseline. Still, the difference was not significant, and PTH became suppressed during pregnancy and lactation in both genotypes (**C**). Serum FGF23 levels rose 6.5-fold in NULLs compared to WTs during pregnancy (**D**).

3.3. Longitudinal changes in serum and urine mineral

The serum calcium levels, unadjusted for serum albumin, were not significantly different between WT and NULL dams at baseline (Figure 20A). However, serum calcium of NULLs rose 17% higher than WTs during pregnancy (3.1±0.5 vs 2.7±0.4, p=0.041) before returning to values no different from WT at subsequent time points (Figure 20A). About 20% of our NULL mice used for longitudinal studies had some problems during the perinatal period and had to be euthanized. Issues included

failure to deliver, offspring becoming stuck in the vagina (dystocia) or extreme distress symptoms post-partum. The serum calcium of these mice, measured the day before delivery, was not significantly higher than that of NULLs that successfully delivered (3.3±0.3 vs 3.1±0.5 mmol/L, p=ns) (Figure 20A, compare magenta to blue bars at late pregnancy). Urine calcium excretion did not differ between genotypes at baseline but rose significantly in NULLs at late pregnancy (9.4±8.2 vs 0.6±0.4 Ca/Cr, $p<0.001$) (Figure 20B).

There were no differences in serum phosphorus levels between WT and NULL mice at any time point (Figure 20C). However, both groups exhibited elevated phosphorus levels at mid-lactation, aligning with bone resorption and the release of skeletal phosphate (as hydroxyapatite) during lactation (Figure 20C). Urine phosphorus showed no statistically significant changes (Figure 20D).

Unlike phosphorus levels, NULLs had significantly higher serum magnesium levels from the start (1.1±0.049 vs 1.0±0.1 mmol/L, p=0.029), which continued to rise, reaching their peak during mid (1.6±0.1 vs 1.1±0.1 mmol/L, $p<0.001$) and late lactation (1.5±0.1 vs 1.1±0.1, $p<0.001$) (Figure 20E). Urine magnesium levels significantly increased in both genotypes from baseline to late pregnancy (NULLs: 3.6±3.0 vs 3.0±2.5 Mg/Cr, p=0.015; WTs: 0.7±0.4 vs 3.0±2.5, p=0.04) before returning to baseline values in late lactation (Figure 20E).

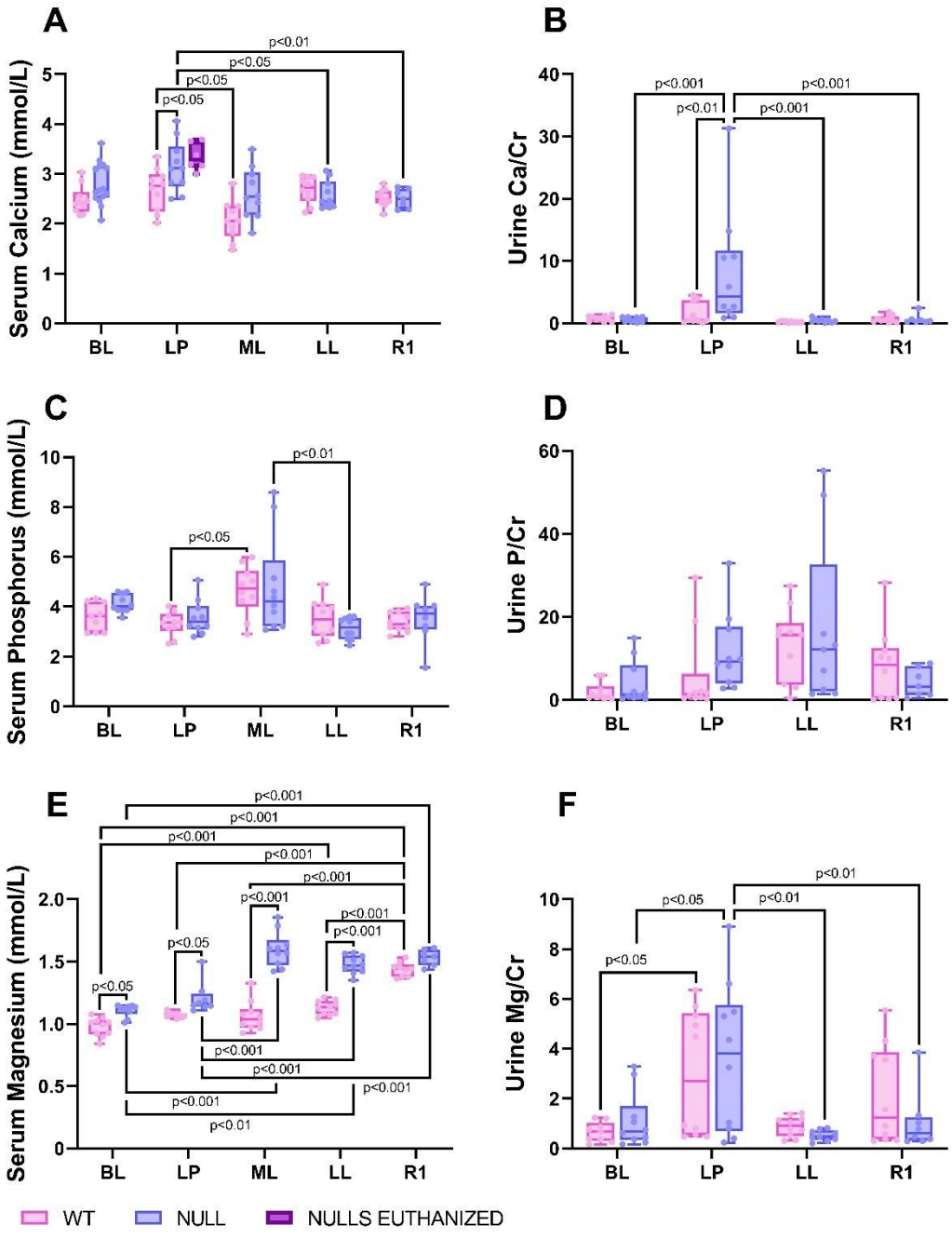

Figure 20. Longitudinal changes in serum and urine minerals.

NULL mice became hypercalcemic during pregnancy, but their calcium levels returned to values that were not significantly different from WT mice at subsequent time points (**A**). The magenta bars represent the serum calcium levels the day before delivery in the 20% of NULL mice that had to be euthanized during parturition (**A**). Urine calcium excretion rose significantly in NULL at late pregnancy (**B**). There were no differences in serum phosphorus levels between WT and NULL mice at any time point (**C**), while urine phosphorus increased numerically but not significantly in both genotypes from baseline to late lactation (**D**). NULLs exhibited elevated serum magnesium levels

during pregnancy and lactation (**E**), whereas urine magnesium rose significantly in both genotypes during pregnancy before returning to the baseline in late lactation (**F**).

3.4. Baseline and pregnancy values of albumin-adjusted serum calcium, fractional excretion of minerals, and creatinine.

In separate experiments, blood and urine samples were collected to measure the albumin-adjusted serum calcium and the fractional excretion of minerals.

NULL mice had a 19% albumin-adjusted serum calcium than WT by late pregnancy (2.8±0.4 vs 2.2±0.2, $p<0.001$) (Figure 21A), which shows that the hypercalcemia was not due to fluctuations of albumin content.

Fractional excretion of calcium (Figure 21B) and phosphorus (Figure 21C) showed no differences between genotypes at baseline. However, during pregnancy, there was an increase of approximately 37 times in calcium fractional excretion (1.10±0.73 vs 0.03±0.04, $p<0.0001$) and almost 9 times in phosphorous fractional excretion (2.32±0.52 vs 0.24±0.26, $p<0.0001$) (Figure 21BC). Fractional excretion of magnesium (Figure 21D) remained unchanged between genotypes and time points. However, due to limited sample volumes, fractional excretion of phosphorus and magnesium during pregnancy could only be measured in a few NULL mice. Meanwhile, serum creatinine remained the same for both genotypes at all time points (Figure 21E).

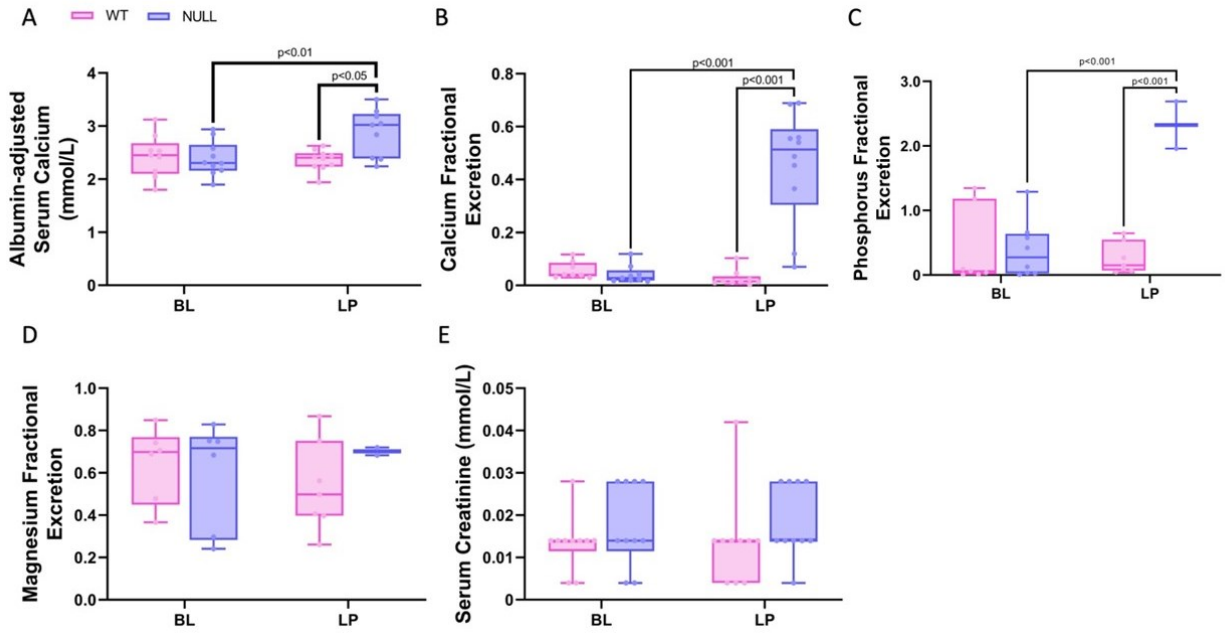

Figure 21. Comparison of non-pregnant and late-pregnancy values of albumin-adjusted serum calcium, fractional excretion of minerals, and serum creatinine

Simultaneous samples of serum and urine were collected, and it was determined that NULLs showed persistent hypercalcemia during pregnancy after adjusting the serum calcium for albumin (**A**), accompanied by increased renal fractional excretion of calcium (**B**). Renal fractional phosphorus excretion was also increased (**C**), whereas magnesium remained stable (**D**), but the pregnancy samples from the null were limited for phosphorus and magnesium. Serum creatinine did not differ between genotypes at any time point (**E**).

3.5. Hematocrit test

The hematocrit was measured to assess whether the hypercalcemia in NULL mice resulted in volume depletion.

The hematocrit in both genotypes decreased by approximately 11% during pregnancy (0.48±0.03 vs 0.43±0.02, p=0.01 for NULLs; 0.44±0.02 vs 0.48±0.02, p=0.02 for WTs) and returned to baseline values afterward (Figure 22). This indicates that hypercalcemia in NULLs during pregnancy was not associated with significant volume depletion or renal dysfunction but does not exclude the possibility of volume depletion in NULLs that died during delivery.

WT and NULL mice experienced a small decline in hematocrit during pregnancy with no differences between genotypes (Figure 22).

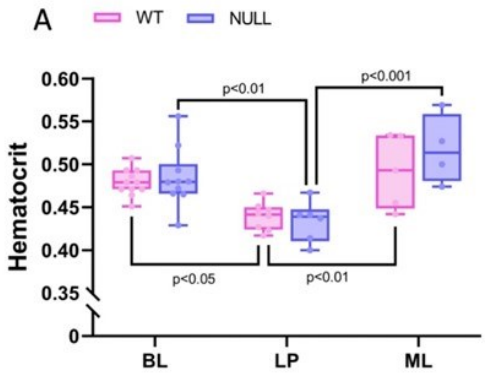

Figure 22. Volume depletion indicator

WT and NULL mice experienced a slight decline in hematocrit during pregnancy, with no differences between genotypes, and returned to baseline levels by mid-lactation.

3.6. Intestinal calcium absorption and the expression of calciotropic genes

During pregnancy, intestinal calcium absorption increased in both genotypes but achieved a significantly greater increase in NULLs, with levels becoming three times that of WT (3.6±1.6 vs 1.0±0.9, $p<0.001$) (Figure 23A). The increased intestinal absorption in NULL mice was attenuated during mid-lactation but remained almost significantly higher than pre-pregnancy levels (2.5±1.9 vs 1.0±0.9, $p=0.062$) (Figure 23A). This increase in NULLs was accompanied by higher duodenal mRNA levels of *Trpv6*, *Atp2b1*, and *S100g* ((Figure 23BCD). In both genotypes, mRNA levels of *Ncx1* were significantly lower (Figure 23E), while *Kcnma1* remained unchanged (Figure 23F). *Vdr* mRNA levels were reduced during pregnancy in both genotypes, while *Cyp27b1* remained unchanged (Figure 23GH). *Cyp24a1* expression was low in the duodenum, increasing significantly in WT but remaining low to undetectable in NULLs (Figure 23I). Regarding phosphate metabolism, duodenal

Napi2b expression increased significantly in *Cyp24a1* nulls, while *Pit1* and *Pit2* expression remained unchanged (Figure 23KJ).

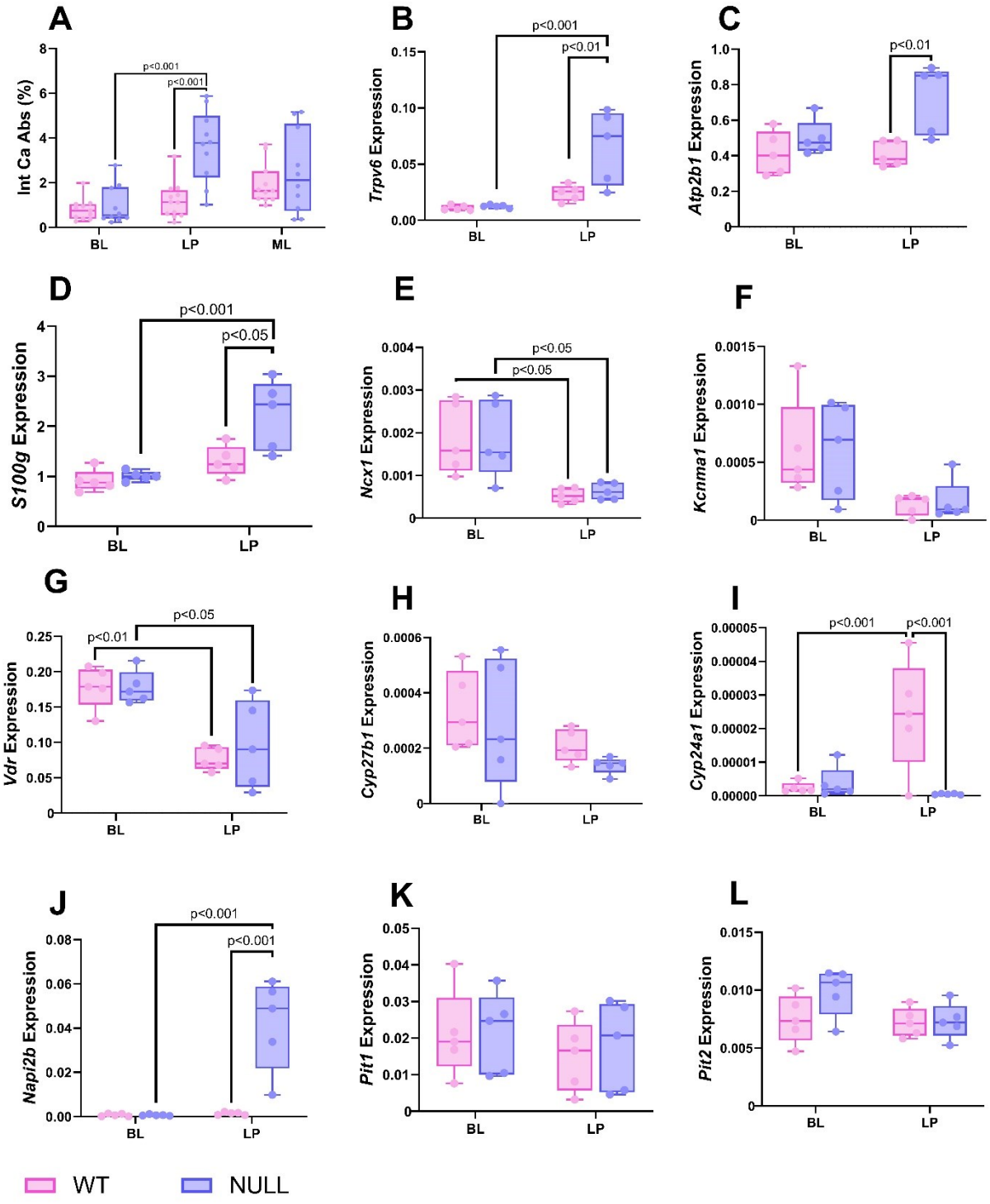

Figure 23. Intestinal calcium absorption and duodenal gene expression.

BL WT was chosen as the control, and its mean value was set to 1. All other gene expression levels were expressed relative to the control. Intestinal calcium absorption increased during pregnancy and

lactation in both genotypes, with NULL mice showing a 3-fold higher value than WT mice in late pregnancy (**A**). NULL mice also exhibited increased expression of calcium-relevant channels and transporters, including *Trpv6* (**B**), *Atp2b1* (**C**), and *S100g* (**D**). In both genotypes, *Ncx1* expression was significantly reduced (**E**), and *Kcnma1* expression was non-significantly reduced (**F**). *Vdr* expression was also reduced during pregnancy in both genotypes (**G**), while *Cyp27b1* expression remained unchanged (**H**). *Cyp24a1* expression in the duodenum was low but rose significantly in WT mice, while it remained low to undetectable in NULLs (**I**). Expression of the phosphate-relevant transporter *Napi2b* increased significantly in NULLs (**J**), while *Pit1* (**K**) and *Pit2* (**L**) expression remained unchanged.

3.7. Renal expression of calcitriol synthesis and catabolic pathway genes

As expected with the global gene ablation, the renal expression of the gene responsible for calcitriol synthesis, *Cyp24a1*, was absent in NULL mice both at baseline and during pregnancy, while it increased 3.9-fold in WT mice during pregnancy (Figure 24A). In contrast, renal *Cyp27b1* expression was reduced by more than 95% in Cyp24a1 nulls compared to WT at baseline, and this reduction persisted during pregnancy (Figure 24B).

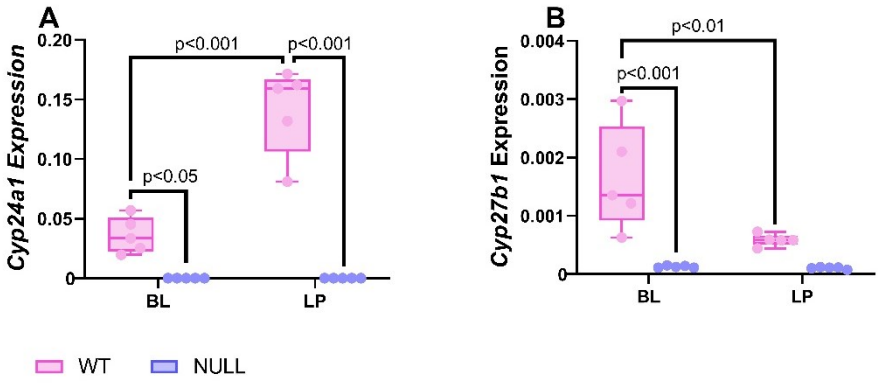

Figure 24. Renal gene expression of calcitriol synthesis and catabolic pathway genes.
In keeping with the global gene deletion, renal expression of *Cyp24a1* was absent in the *Cyp24a1* null at baseline and pregnancy, whereas it increased 3.9-fold during pregnancy in WT (Figure 24A). Conversely, renal expression of *Cyp27b1* was downregulated more than 95% compared to WT at baseline and remained so during pregnancy (Figure 24B).

3.8. Milk calcium, protein and creamatocrit

Milk from NULLs contained higher concentrations of calcium (67.1±11.0 vs. 52.0±9.8, p=0.006) and protein (2.8±0.4 vs. 2.2±0.4, p<0.001) compared to WT at mid-lactation (Figure 25AB). However, when adjusted for protein content, the calcium levels were similar to those in WT milk (Figure 25C). Additionally, the creamatocrit levels were the same for both types of mice, indicating similar water content (Figure 25D).

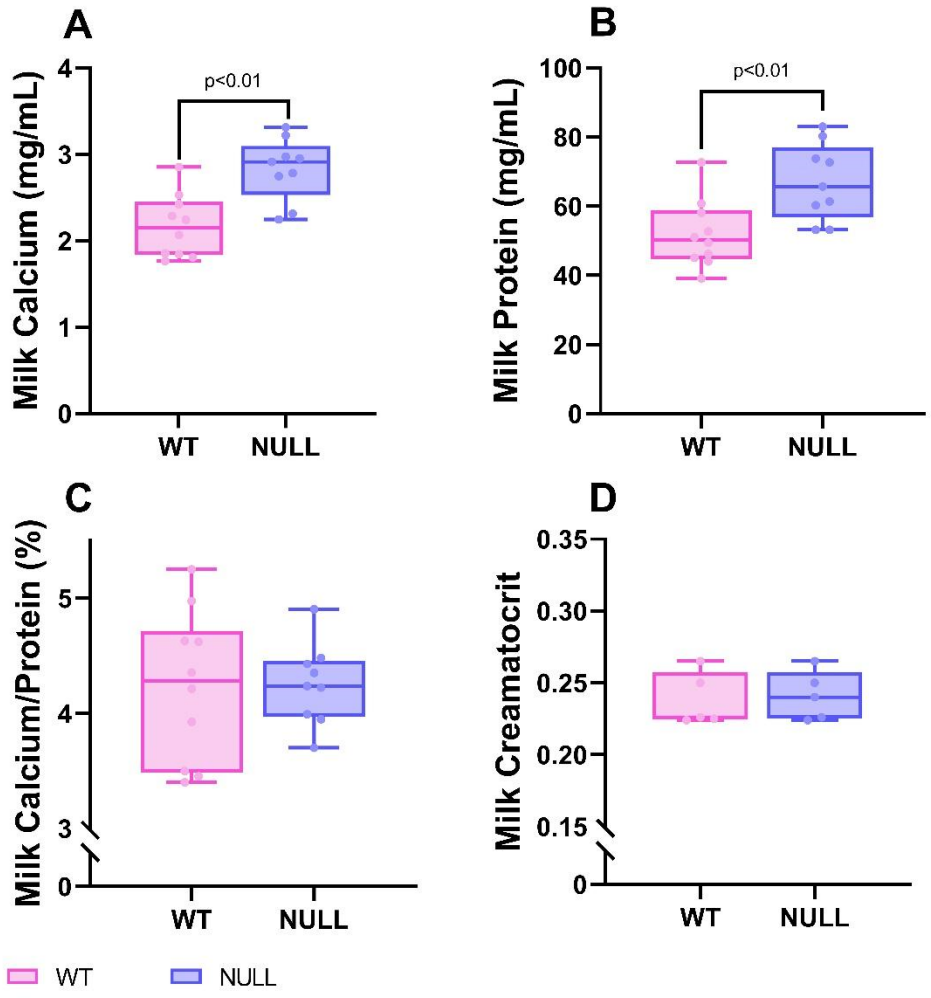

Figure 25. Milk calcium and protein content.
The milk from *CYP24A1* null mice had higher concentrations of calcium (**A**) and protein (**B**) at mid-lactation, but when the calcium content was adjusted for protein, it was not different from that of WT mice (**C**). Milk cream content remained the same for both genotypes (**D**).

3.9. Longitudinal Assessment of Skeletal Mineral Content by DXA

Whole body, lumbar spine, and hind limb BMC were similar between NULLs and WT at the start of the study (Figure 26).

BMC increased similarly during pregnancy in the whole body and hindlimb for each genotype, becoming statistically significant in WT (0.576±0.05 vs 0.638±0.078, p=0.021). (Figure 26). Meanwhile, both genotypes experienced BMC loss in the lumbar spine, with statistically significant change for WT (0.059±0.005 vs 0.05±0.01, p=0.004) (Figure 26).

By late lactation, BMC had decreased in both genotypes (Figure 26). NULLs lost ~12% less BMC between BL and LL (0.581±0.052 vs 0.572±0.056 g, p=0.973) compared to WT (0.576±0.045 vs 0.519±0.035 g, p=0.038), ultimately leading to a significant difference in BMC in the whole body (0.572±0.056 vs 0.519±0.035 g, p=0.014) (Figure 26A). A significant difference was also observed in the spine (0.054±0.006 g vs 0.049±0.004 g, p<0.001), while a non-significant decline was observed in the hind limb (0.08±0.006 g vs 0.075±0.004 g, p=0.08) (Figure 26BC).

During the recovery phase, BMC increased at all three sites in both genotypes to levels that were equal to or higher than baseline (Figure 26). By R4, NULL mice had higher BMC than BL in the whole body (0.674±0.05 vs 0.581±0.052 g, p=0.009) and hind limb (0.092±0.006 vs 0.08±0.09 g, p=0.044), but the increase was not significantly high in the spine (0.067±0.007 vs 0.059±0.006, p=ns) (Figure 26). Meanwhile, the increase in BMC during the same period for WT mice was significant only in the hind limb (0.087±0.004 g vs 0.078±0.008 g, p=0.017) (Figure 26).

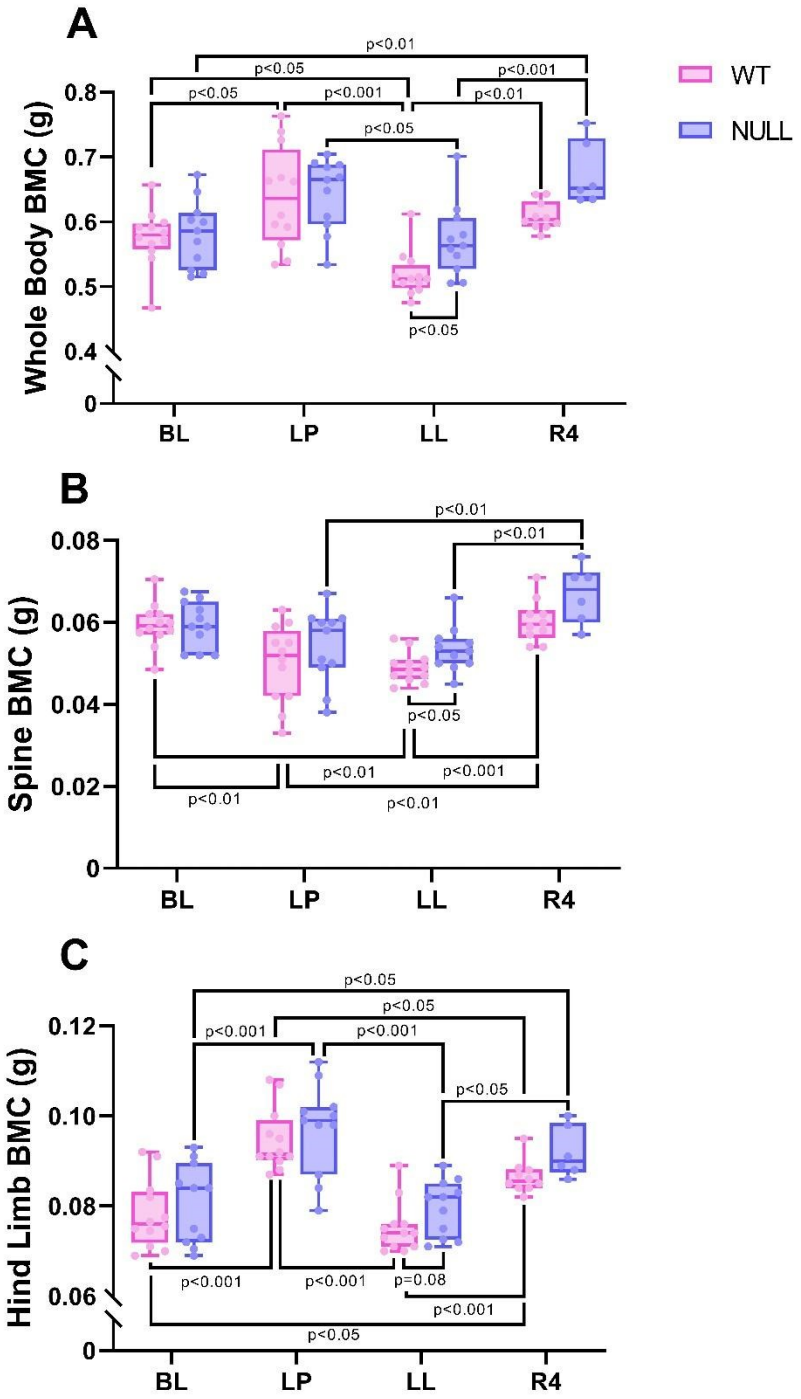

Figure 26. Longitudinal Assessment of Skeletal Mineral Content by DXA.

There were no differences in whole body, lumbar spine, and hind limb BMC between WT and NULL mice at BL (**ABC**). Both genotypes experienced increases in whole body and hind limb BMC during pregnancy, with minimal changes in the lumbar spine, and no significant differences between genotypes (**ABC**). NULL mice exhibited significantly less BMC loss than WT mice in both the whole body and spine by late lactation (**ABC**). Subsequently, BMC in both genotypes increased to levels

that were significantly higher than BL in the hind limb, but not in the whole body or lumbar spine (ABC).

3.10. Bone resorption marker (CTX)

CTX levels were similar in both genotypes before pregnancy (Figure 27). By late pregnancy, CTX increased by approximately 80% in WT mice (28.2±9.9 vs 50.7±0.7, p<0.001), whereas it rose by about 31% in NULL mice (23.2±9.3 vs 30.3±12.3, p=ns) (Figure 27). The less marked increase in NULL mice led to a significant difference between genotypes at this time point (30.3±12.3 vs 50.7±10.7 ng/mL, p<0.001). During lactation, CTX levels declined at different rates for each genotype. NULLs declined about 20% by mid-lactation (24.1±9.9 vs 30.3±12.3, p=ns), while WTs dropped close to 32.6±6.6 vs 50.7±10.7, p<0.001), which diminished the difference between them to non-significant levels(24.1±9.9 vs 32.6±6.6 ng/mL, p=ns) (Figure 27). CTX continued to decline, reaching the same low plateau for both genotypes in late lactation and remaining stable through R1 (Figure 27).

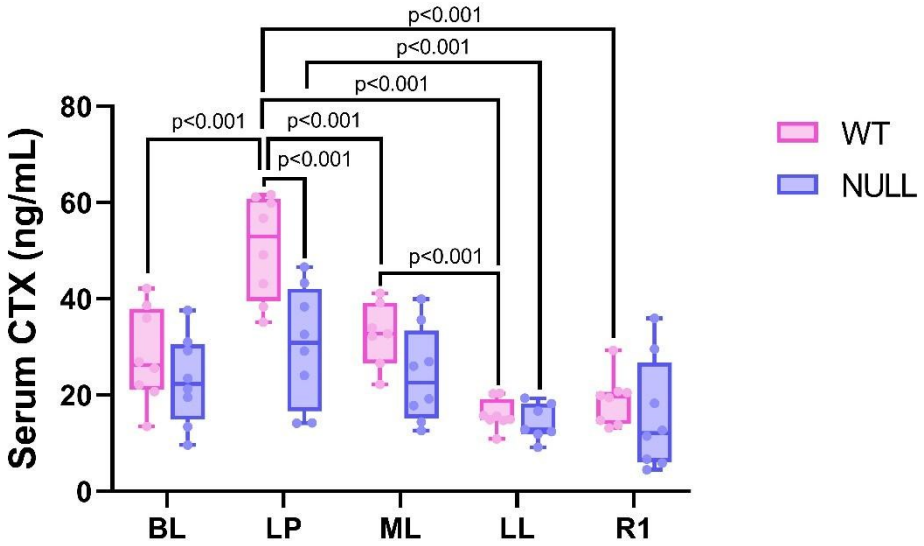

Figure 27. CTX serum levels.
WT mice exhibited significantly increased bone resorption at LP compared to NULL mice. Although this heightened bone resorption persisted during ML, the difference was not statistically significant.

3.11. µCT assessment of femur macro and microstructure

Both WT and NULL mice experienced similar growth during their reproductive cycles, reaching their peak towards the end of the experiments, as indicated by the increased lengths of their femurs (Figure 28A). The endocortical and periosteal perimeters remained constant during pregnancy but increased similarly by late lactation in both genotypes and remained high afterwards (Figure 28BC). Their polar moment of inertia progressively increased but was only significant for NULLs at R4 (0.452±0.084 mm^4, p=0.003) (Figure 28D). Cortical thickness and cortical bone area were similar between genotypes at the start and did not change during pregnancy (Figure 28EF). Still, they decreased significantly and equally during lactation before returning to baseline levels by the end of the 4-week recovery period in both genotypes (Figure 28F). Trabecular bone volume and number were initially the same between NULL and WT mice but the gap increased significantly during pregnancy (1.644±0.119 vs 0.756±0.196 %, p=0.001; 0.334±0.024 vs 0.162±0.013 /mm, p<0.001) before decreasing during lactation to baseline levels (Figure 28GH). Trabecular separation (Figure 28I) increased significantly by late lactation in both genotypes before returning to baseline levels, while trabecular thickness (Figure 28J) increased in WT during lactation and showed a similar but nonsignificant increase in NULLs (Figure 28IJ).

Figure 29 illustrates representative images from distal femurs that were used to collect data used in Figure 28.

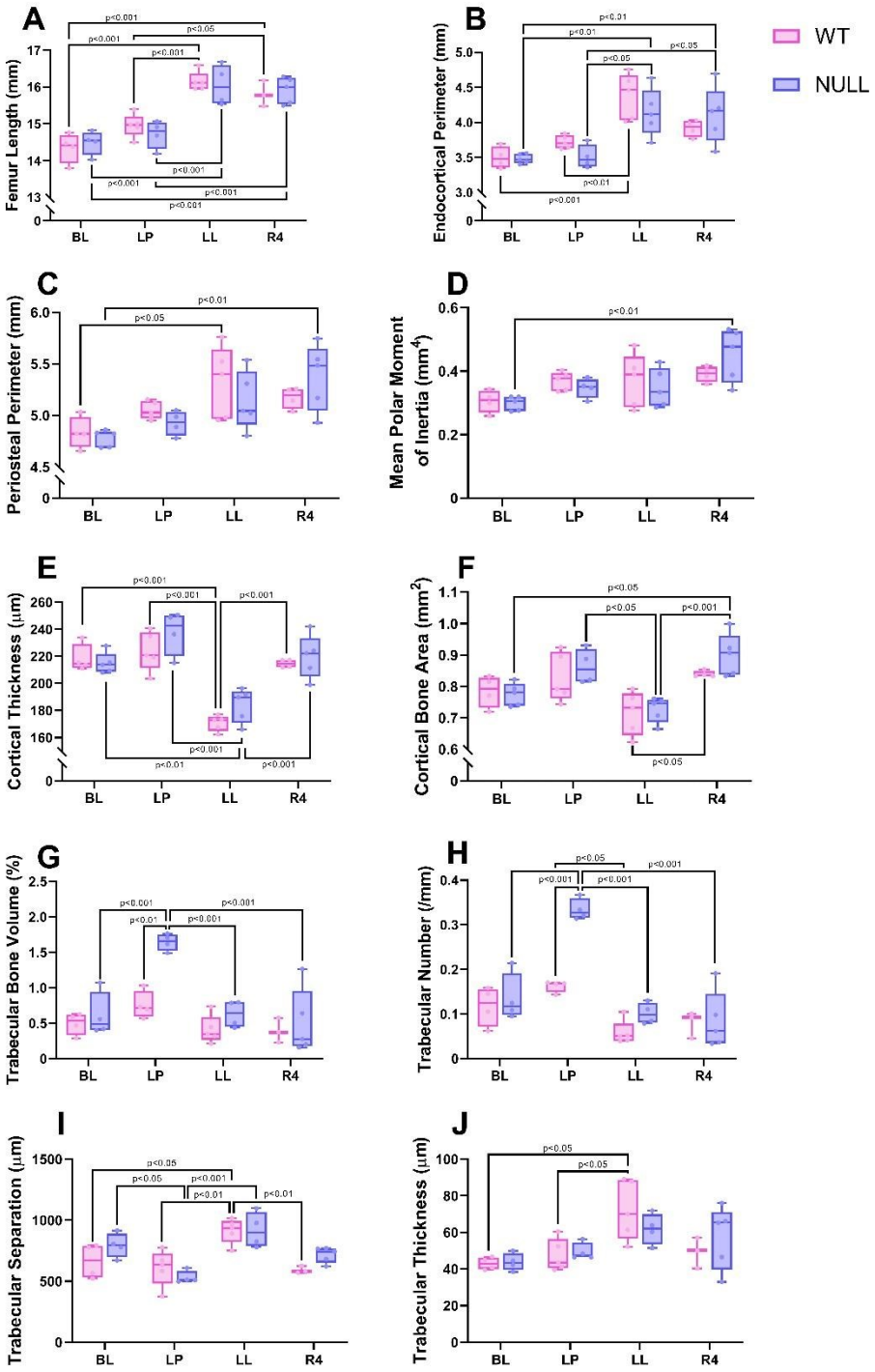

Figure 28. Cross-sectional µCT assessment of femur macro and microstructure.

WT and NULL mice exhibited comparable growth during their reproductive cycles, resulting in similar increases in femur lengths (**A**). Both endocortical (**B**) and periosteal perimeters (**C**) remained constant during pregnancy but expanded over three weeks of lactation in both genotypes, along with the polar moment of inertia (**D**). There were no differences in cortical thickness (**E**) and cortical area (**F**) between WTs and NULLs at the outset, both of which significantly decreased during lactation and then reverted to initial levels. Trabecular bone volume (**G**) and number (**H**) were identical

between WTs and NULLs initially but significantly rose during pregnancy in NULLs before falling back to starting values during lactation. Trabecular separation (**I**) significantly increased by the end of lactation in both genotypes before returning to initial levels, while trabecular thickness (**J**) increased in WT during lactation and exhibited a similar yet nonsignificant rise in NULLs.

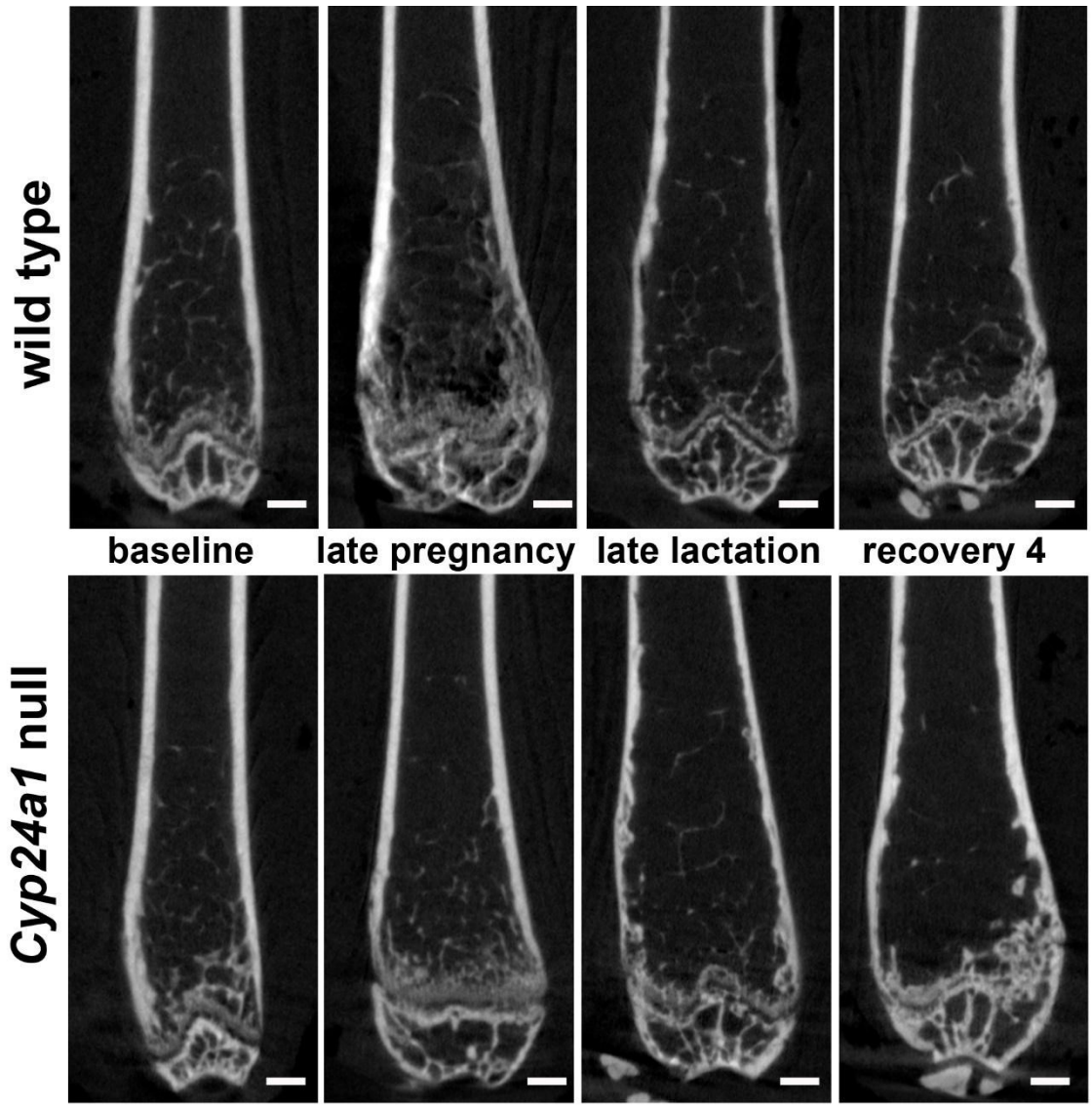

Figure 29. Representative µCT images of distal femurs.
Scale bars equal 600 microns.

3.12. Biomechanical properties

The three-point bend test revealed significantly lower tibial strength in NULL compared to WT at the start of the study, as shown by ultimate load (1.687±0.272 vs 2.171±0.333 gf, p=0.043), but the differences disappeared during pregnancy (7.176±1.34 vs 7.83±0.683, p=ns) (Figure 30A). During lactation, the ultimate load significantly decreased from baseline for NULL (1.687±0.272 vs 1.193±0.168 gf, p=0.037) and WT mice (2.171±0.333 vs 1.239±0.183 gf, p<0.001) (Figure 30A). Still, because WTs had more strength initially and experienced a more dramatic decline, there was no difference between genotypes by the end of lactation (Figure 28A). Following this period, bone strength returned to similar pre-pregnancy values (Figure 30A).

Maximum displacement did not change significantly in either genotype, while stiffness decreased only in NULL mice at late lactation (Figure 30BC).

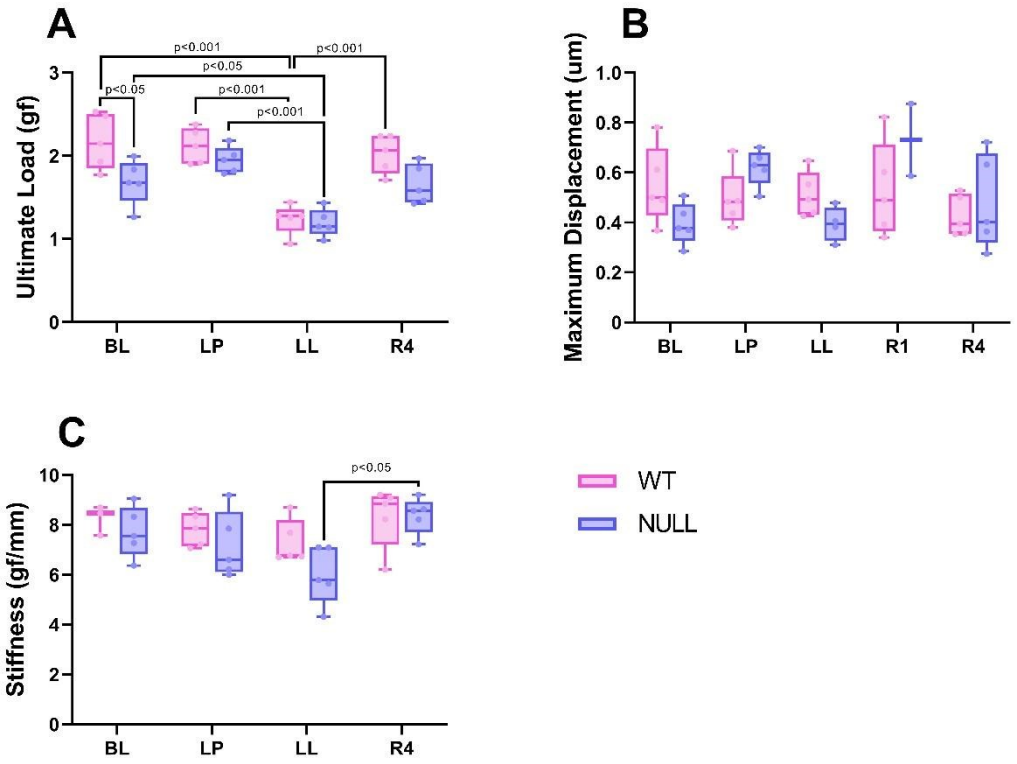

Figure 30. Cross-sectional assessment of tibial bone strength.

The three-point bend test showed that NULLs had significantly lower tibial strength at baseline, as indicated by the ultimate load graph (**A**). Ultimate load decreased during lactation and then increased

during post-weaning to reach baseline values in both genotypes (**A**). Bone strength in NULLs increased during pregnancy and late lactation to become similar to WTs but then trended downward in NULLs to approach the lower bone strength observed at baseline (**A**). Maximum displacement remained unchanged in both genotypes (**B**), while stiffness only decreased in NULLs at late lactation (**C**).

4. DISCUSSION

This study aimed to investigate the mechanism by which the loss of *Cyp24a1* leads to gestational hypercalcemia and to assess its impact on bone resorption and bone strength during lactation.

We hypothesized that calcitriol levels would rise above normal during pregnancy due to the disruption of the catabolic pathway caused by *Cyp24a1* inactivation. As a result, we would observe significant hypercalcemia, enhanced intestinal calcium absorption, and potentially increased skeletal resorption during pregnancy.

We also proposed that the elevated calcitriol levels would persist during lactation, sustaining increased intestinal calcium absorption, which would lead to reduced lactational bone resorption and less bone strength loss in mice.

Our main findings revealed that serum calcitriol was 2.5-fold higher in NULLs during pregnancy, accompanied by a 3-fold increase in intestinal calcium in NULL mice, and marked hypercalcemia. Both genotypes had equivalent bone resorption during pregnancy, whereas renal calcium excretion was 5-fold higher in NULL mice.

The increases in calcitriol and intestinal calcium absorption in NULLs were attenuated during lactation, but still sufficient to contribute to a reduction in bone resorption by about 12% in NULLS compared to WT. This implies that proportionately more of the milk calcium content was provided by intestinal calcium absorption in the NULLs. The loss in bone strength loss in NULLs was less

marked during lactation, but both genotypes reached equivalent strength by the end of lactation despite the reduction in BMC loss.

4.1. *Cyp24a1* inactivation causes mild effects on mineral metabolism in adult mice before pregnancy.

Deletion of *Cyp24a1* had mild effects on mineral metabolism. Serum levels of calcitriol, PTH, and FGF23 were similar between genotypes in the non-pregnant, baseline state. Consequently, the expression of calciotropic genes in the duodenum remained equivalent in both groups at baseline, with no differences in intestinal calcium absorption. The mean serum calcium levels in NULL mice were numerically higher, though not statistically significant, indicating that they were within the normal range or exhibited only mild hypercalcemia. This finding aligns with observations in both humans and mice, where *Cyp24a1* mutations can be asymptomatic and result in only mild increases in serum calcium.[38,62,99–101,106]

Without Cyp24a1 catabolism, NULL mice must rely on alternative pathways for calcitriol degradation. The alternative pathways for calcitriol catabolism are not well understood, but one known pathway involves the enzyme CYP3A4 (Section 1.4.5).[64] Additionally, calcitriol inhibits its own formation by suppressing the activity of Cyp27b1, thereby helping to maintain its levels within an adequate range.[30] This may, in part, explain why mRNA expression of *Cyp27b1* was significantly reduced in NULLs at baseline and late pregnancy.

The half-life of calcitriol in non-pregnant WT and HET mice is approximately 3-6[147,148] and 6-12 hours,[149] respectively. However, in non-pregnant NULL mice, clearance extends to 96 hours.[149] This suggests that, despite the extended clearance time, the combination of alternative pathways and self-regulation is sufficient to minimize the rise in calcitriol when non-pregnant and thereby minimize any disturbance in mineral metabolism. The half-life of calcitriol has not been measured in pregnant mice.

However, if synthesis of calcitriol surges in the absence of Cyp24a1, the alternative catabolic pathway becomes overwhelmed and is unable to effectively eliminate the excess calcitriol. As noted earlier, factors such as pregnancy (Section 1.6.1), vitamin D supplementation, and intense sunlight exposure can cause increased synthesis of calcitriol and decompensate mineral homeostasis in NULL mice.

Calcifediol levels were increased at baseline in NULLs compared to WT. This difference was evidently due to the combination of reduced catabolism of 25OHD and reduced conversion of 25OHD into calcitriol. Vitamin D is rapidly converted to calcifediol in the liver, but its catabolism should be slowed without Cyp24a1. Furthermore, we observed reduced mRNA expression of *Cyp27b1*, indicating reduced calcifediol conversion into calcitriol. Calcifediol normally has a half-life of 3-4 weeks,[150] as compared to the few hours of calcitriol,[149] but its half-life is likely increased in the absence of Cyp24a1. With the onset of pregnancy and upregulation of calcitriol synthesis, more substrate (25OHD) was consumed, thereby accounting for the marked fall in serum calcitriol.

BMC of the whole body, lumbar spine, or hind limb, as assessed by DXA, and cortical and trabecular microarchitecture of the femurs, as assessed by μCT, showed no significant differences. Still, tibial bone strength was lower in NULLs before pregnancy, as measured by the ultimate load. This may imply a difference in some aspect of bone composition that has yet to be detected by other methods.

Previous studies have shown that adult mice lacking *Cyp24a1* demonstrated negative effects on bone health, particularly during fracture repair.[151–153] These studies revealed that endochondral ossification was inadequate, leading to a smaller callus, reduced stiffness, decreased mineralization, and delayed healing. These adverse effects were reversed by treatment with 24,25-dihydroxyvitamin D (24,25(OH)2D), but not with calcitriol.[153] 24,25(OH)2D is a metabolite produced from the catabolism of calcifediol by CYP24A1, which is absent in NULLs. Thus, the lack of or reduced 24,25(OH)2D may at least partially explain the observed deficits in skeletal strength at baseline.

4.2. Gestational hypercalcemia in *Cyp24a1* mice is caused by increased intestinal calcium absorption, but not bone resorption or renal calcium conservation.

Calcitriol normally increases several-fold during pregnancy through PTH-independent mechanisms, contributing to the usual doubling of intestinal calcium absorption efficiency during this period.[30,83] In NULL mice, calcitriol levels increased 2.5 times more than in WT mice, likely due to the pregnancy-related increase in Cyp27b1 activity not being counteracted by Cyp24a1-mediated catabolism of calcitriol and calcifediol. The hypercalcemia was further confirmed on additional samples when the serum calcium was adjusted for the lower serum albumin during pregnancy.

The upregulation of calcitriol was accompanied by altered expression of calciotropic genes, including *Trpv6*, *Atp2b1*, and *S100g* in the duodenum, leading to a 3-fold increase in intestinal calcium absorption in the NULL mice, making them hypercalcemic compared to WT mice.

Skeletal resorption is also unlikely to have contributed to the hypercalcemia, as BMC increased equally in both genotypes in the whole body and hind limbs during pregnancy, with no changes observed in the lumbar spine. Additionally, NULL mice did not increase CTX levels during pregnancy, and were comparatively lower than those WT mice, indicating reduced bone resorption. Indeed, trabecular volume and thickness spiked in the NULL mice, and bone strength also showed a numerical increase, though it was not significantly different from the baseline.

Renal conservation does not seem to contribute to hypercalcemia in NULL mice, as they excreted over 5-fold the amount of calcium in their urine compared to WT mice. Further analysis of fractional calcium excretion revealed a 6-fold increase, suggesting that the NULL mice were attempting to maintain calcium homeostasis by excreting excess serum calcium.

Although there was a significant rise in calcitriol levels during pregnancy, we did not observe an increase in renal *Cyp27b1* expression in NULL mice during this period, which had been seen in previous studies.[149] This may be attributed to the prolonged 96-hour half-life of calcitriol clearance,

where even small changes can have a significant impact on the mice. Additionally, although fetal calcitriol contributes minimally to maternal serum,[30] the impaired ability to degrade calcitriol could have amplified this small contribution, making it more significant in the NULL mice.

Altogether, the data indicate that the hypercalcemia observed in *Cyp24a1* null mice was primarily due to an excessive increase in intestinal calcium absorption driven by elevated calcitriol levels, rather than increased bone resorption or enhanced renal calcium reabsorption.

4.3. Serum phosphorus levels remained constant throughout the reproductive cycle, while magnesium progressively increased in both genotypes but remained higher in Cyp24a1 NULL mice at all time points.

Calcitriol also promotes intestinal phosphate absorption and renal phosphate conservation; therefore, increased calcitriol concentrations would be expected to lead to increased serum phosphorus. Intestinal expression of *Napi2b* was significantly increased in NULLs during pregnancy compared to WT, which suggests that intestinal phosphate absorption was also higher in NULLs vs. WT, although this was not directly measured. However, phosphate regulation involves a complex interplay among calcitriol, PTH, and FGF23 across multiple organs.[32] FGF23 not only enhances renal phosphate excretion but also reduces intestinal phosphate absorption.[47,55,58] Therefore, the 6.5-fold increase in FGF23 in NULLs during pregnancy likely counteract calcitriol's effects on intestinal phosphate absorption. The net effect was normal serum phosphorus and a modest but non-significant increase in urine phosphate excretion during pregnancy and lactation. It is possible that with a larger sample size, differences in urine excretion might have reached statistical significance.

Unlike calcium and phosphorus, serum magnesium progressively increased in both genotypes during and after the reproductive cycle, remaining higher in NULLs at all time points. Meanwhile, urinary magnesium excretion peaked during pregnancy in both genotypes, with no difference observed at other time points. These findings suggest that the *Cyp24a1* knockout likely enhances intestinal magnesium absorption along with calcium. Notably, the peak in magnesium excretion during pregnancy corresponds to the peak in urinary calcium excretion in NULLs. Measuring intestinal magnesium absorption with ^{28}Mg in the same mice used for intestinal calcium absorption studies using ^{45}Ca would have confirmed this hypothesis. However, because both isotopes are beta-emitters, their signals would overlap, making the data unreliable. Consequently, a separate cohort of WT and NULL would be required at each time point to assess magnesium absorption, substantially increasing both the time commitment (by an additional 6–12 months) and costs. Furthermore, ^{28}Mg is rarely produced, extremely expensive, and has a short half-life of 21.3 hours,[154] necessitating on-site production timed precisely to mouse matings. Since no such facility is available here, using 28Mg is simply not feasible.

Research into magnesium metabolism regulation is less comprehensive compared to calcium or phosphate. Existing studies, although limited, offer some insights. Some rodent and human studies suggest that calcitriol may enhance intestinal magnesium absorption.[155–157] Conversely, other research indicates that calcitriol does not influence magnesium absorption.[158,158] Additionally, magnesium deficiency has been linked to reduced CYP27B1 activity and increased CYP24A1 mRNA levels, whereas other studies report that magnesium does not significantly impact the in vivo conversion of calcifediol to calcitriol or 24,25(OH)2D.[159]

4.4. Inactivation of *Cyp24a1* alters physiology during lactation, reducing bone resorption while maintaining calcium levels in milk.

During lactation, the maternal body must supply sufficient calcium for the offspring.[30] In humans, most of this calcium is sourced from the skeleton through osteoclast-mediated bone resorption and osteolysis. The mechanisms of skeletal resorption reduction may involve a compensatory reduction in PTHrP production by the mammary glands, although PTHrP was not measured in this study. A small portion of the calcium can come from renal calcium conservation, while intestinal calcium absorption returns to pre-pregnancy levels.[30]

Women with higher calcium intake during pregnancy experience little to no reduction in bone resorption compared to those who consume adequate levels of calcium, as they are likely to excrete the excess calcium through urine. In fact, hypercalciuria is a normal finding in human pregnancy. In rodents, however, both bone resorption and increased intestinal calcium absorption are upregulated to meet the maternal demands, likely due to their relatively large litters and short lactation periods.[30] Indeed, the upregulation of intestinal calcium absorption can reduce the need for bone resorption and vice versa.[30,83] Thus, a calcium-enriched diet, for example, reduces bone resorption during lactation, while a low-calcium diet is compensated for by increased bone resorption.

In this study, we observed that the elevated intestinal calcium absorption in NULL mice during pregnancy diminished by mid-lactation compared to late pregnancy but was numerically and almost statistically significantly ($p=0.057$) higher than baseline. Despite the diminished effects during lactation compared to pregnancy, BMC loss was still significantly less pronounced in NULL mice compared to WT mice at both the whole body and lumbar spine ($p<0.05$), with a borderline difference observed at the hind limb ($p=0.08$). The reduced bone loss in NULL mice suggests that increased intestinal calcium absorption persisted during lactation and was sufficient to minimize the need for skeletal resorption to support milk production.

Thus, we expected to observe a peak in bone resorption markers by mid-lactation as demonstrated by previous studies.[30,75] Instead, the serum CTX for both NULLs and WTs was not significantly higher than the baseline. Further investigation led us to conclude that the phenomenon was likely caused by an unanticipated technical issue during the blood collection at mid-lactation. CTX release is stimulated by the pups' suckling of the mammary gland and has a half-life of one hour.[160,161] However, the blood was collected two hours after the mother had been separated from the pups, as they needed to be separated for milk collection. Therefore, the samples did not reflect the CTX peak that occurs when pups are suckling. Previous studies have shown that CTX is 2-fold greater than baseline when the blood is drawn right after removing the dams from the pups.[75] The same was also observed with another bone resorption marker deoxypyridinoline, which is stably excreted into urine.[82,83]

Moreover, CTX is an indirect marker of bone resorption—resulting from osteoclast or osteocytic osteolysis at a given moment—and can fluctuate rapidly in response to factors such as circadian rhythms, fasting, therapy, or cessation of pup suckling.[21,24,162] Because of its short half-life, CTX levels decline quickly when osteoclast activity drops, even though osteoclasts already engaged in resorption may continue working for a time.[163] Thus, some bone resorption persists after CTX begins to decrease, albeit at a much slower pace. In contrast, the change in BMC during lactation directly reflects the cumulative bone resorption over a three-week period, and this decline was notably smaller in NULLs compared to WT.

The observed increased intestinal calcium absorption, coupled with reduced bone resorption observed in NULLs, maintained milk calcium levels (corrected for protein) equivalent to those in WTs. The same was observed for the estimated fat content measured by creamatocrit test.

4.5. *Cyp24a1* inactivation impacts macro- and microstructure and biomechanical properties throughout the reproductive cycle.

Macro- and microstructure and BMC were similar in both genotypes before pregnancy, but the 3-point bend test revealed that NULLs had significantly lower tibial strength than WT. The mechanism behind this difference is unclear, but it may be caused by an untested variation in bone composition.

Differences in bone structure started during the reproductive cycle. Cross-sectional studies of femoral bone structure using μCT revealed progressive increases in femur lengths during pregnancy and lactation, with similar patterns observed in both genotypes. This was anticipated, as the mice aged approximately 80 days from the start to the end of their reproductive cycles. Significant increases in endocortical and periosteal perimeters were also noted, occurring within the three weeks between late pregnancy and the end of lactation, rather than during the four weeks between baseline and late pregnancy. These changes suggest an expansion in femur diameter. Previous rodent studies have also reported increased cross-sectional diameters, cortical perimeters, and volumes of the tibiae or femora by post-weaning compared to pre-pregnancy or age-matched controls.[57,164,165] Limited human data similarly indicate that cortical width expansion may occur during lactation and post-weaning recovery.[30] Such increases in bone diameter enhance the cross-sectional and polar moments of inertia, thereby increasing breaking strength. This may help maintain bone strength during lactation, even as skeletal resorption progresses, and despite the potential incomplete recovery of cortical and trabecular microarchitecture afterward. However, continued growth could also contribute to these changes. Comparisons with age-matched non-pregnant controls could clarify this issue.

Despite having increased BMC, the NULL mice did not exceed the bone strength of their WT counterparts. Bone strength depends on the interplay of mineralization, spatial organization, and overall composition. Notably, the NULL mice had weaker bones even before pregnancy, despite having comparable BMC and similar micro- and macrostructure. A histomorphological analysis could have elucidated the underlying reasons for this discrepancy, and a larger sample size might

have more conclusively determined whether any true differences exist for both points.As cortical thickness increased in the femurs of both genotypes, it is likely that a similar change occurred in the tibia, even though it was not measured. The cortical bone is the primary structure responsible for providing strength when stress is applied to the mid-shaft of the tibia,[166,167] such as in the case of the 3-point bend test. Therefore, the increase in cortical bone thickness may partially explain the recovery of bone strength in both genotypes, which returned to levels comparable to those observed before pregnancy.

In outbred strains of mice, such as BlackSwiss, whole body, lumbar spine, and hind limb BMC all increase significantly during pregnancy.[30] However, in the inbred C57BL/6J strain (as used in this study), the pattern differs slightly: whole body and hind limb BMC increase during pregnancy, while the lumbar spine either remains unchanged or slightly decreases. The gains in hind limb BMC may be partly driven by the approximate doubling of body weight during pregnancy, which increases limb loading. These BMC increases likely prepare the maternal skeleton for the enhanced need to resorb minerals during lactation by creating more readily resorbed trabecular bone.[30]

The trabecular bone increases within the primary spongiosa of both WT and NULL mice between baseline and late pregnancy, but the same is not observed during lactation and is absent at later time points. This increase is likely more pronounced in NULLs for two reasons: first, the enhanced intestinal calcium absorption enables newly formed trabeculae to become more fully mineralized and thus detectable by μCT; second, the higher calcitriol levels in NULLs may stimulate osteoblast activity more than in WT.

4.6. **Study strengths and limitations**

4.6.1. Strengths

The study analyzed a wide range of parameters throughout the reproductive cycle, including serum levels of calcium, phosphorus, and magnesium; serum calcitriol, calcifediol, PTH, and FGF23; urine excretion of calcium, phosphorus, and magnesium; calciotropic and phosphotropic gene expression in the duodenum; intestinal calcium absorption; hematocrit; as well as milk calcium, protein, and creamatocrit. Additionally, bone mineral content, microstructure, and biomechanical properties were assessed. The extensive data collected allowed for a comprehensive understanding of the mechanisms underlying gestational hypercalcemia.

Most analyses of blood, urine, and milk samples, as well as all DXA scans, were longitudinal, meaning they were performed on the same set of mice over time. Longitudinal studies are advantageous for understanding changes across time because they are less affected by confounding factors.

Milk demand in mice varies depending on the number of pups being nursed, and to minimize differences that could affect bone resorption, the study maintained litter sizes close to six pups.

Both WT and NULL mice used in the experiments were closely related, being born from heterozygous parents who were either first-degree relatives or closely inbred relatives, which helped reduce confounding factors.

4.6.2. Limitations

The expected genotypic ratio from breeding two heterozygous mice carrying a mutation on an autosomal chromosome is 1 WT: 2 HETs: 1 NULL, meaning roughly 1/4 of the litter should be NULL. David Bennin, a member of our lab using the same colony, confirmed the expected proportion of genotypes in utero at embryonic day 18.5, one day before delivery.[105] However, as reported by Dr. René St-Arnaud,[38] who developed the mutated colony, roughly 50% of NULL mice die before 3 weeks of age. This high mortality rate is likely due to the murine equivalent of idiopathic infantile

hypercalcemia type 1 being more severe than the human condition, NULL mothers recognizing something wrong with their progeny and cannibalizing them, or a combination of both factors. Consequently, significantly fewer NULLs than WTs were available for experiments.

Additionally, about 20% of NULL mice used in longitudinal studies experienced issues during or immediately after delivery and had to be euthanized. Symptoms included dystocia (difficult or obstructed labor), severe lethargy, and hunched posture. Blood samples collected one day before parturition revealed no significant differences in hypercalcemia levels between these mice and NULLs that delivered normally, but it is conceivable that they experienced more severe hypercalcemia during delivery. Hematocrit measurements at all time points showed a similar decrease in both genotypes during pregnancy, returning to baseline afterward, suggesting dehydration from hypercalcemia was not a factor. Blood could not be collected during the delivery crisis, as affected mice were euthanized immediately to prevent further suffering, leaving the role of severe hypercalcemia uncertain.

Another potential contributing factor was anesthesia for BMC measurements performed one day before delivery. NULL mice might have been more sensitive to anesthesia than WTs, as subsequent studies in mice not subjected to DXA scans or anesthesia showed no signs of dystocia or perinatal distress. Both WT and NULL mice also displayed cannibalistic behavior, destroying their litters within a day or two of delivery. Social distress from solitude, compounded by their defective genotype, may have contributed to this behavior. To minimize litter destruction, experienced colony maintenance mothers were housed with inexperienced experimental mice, including both WTs and NULLs, which significantly reduced cannibalistic behavior.

Lastly, both μCT and the 3-point bend test provide cross-sectional data as they require sacrificing the mice before conducting bone analyses. While cross-sectional research is useful, it is less ideal for tracking changes over time due to its susceptibility to confounding effects. Our lab has been using DXA to analyze longitudinal changes in bone because it provides easy and non-invasive scans, but it

has its limitations. We have successfully tested in-vivo μCT (data not shown), and once Memorial University acquires its own μCT, we plan to transition to longitudinal in-vivo μCT instead of relying on ex-vivo μCT or DXA. Currently, there is no reliable method to measure biomechanical properties without using the 3-point bend test, which requires breaking the bones. Other techniques, including μCT, can only provide estimates of biomechanical properties.

4.7. **Implications for human health**

Our findings in the *Cyp24a1* null mouse model confirm that hypercalcemia primarily results from increased intestinal calcium absorption, with no apparent contributions from skeletal resorption or renal calcium conservation. We expect to observe a similar excessive increase in intestinal calcium absorption in affected women during pregnancy. Therefore, treatments for hypercalcemia that mainly reduce skeletal resorption, such as bisphosphonates and denosumab, are likely to be ineffective. Treatment options that should still be effective include increased hydration, dietary calcium restriction, phosphate supplementation to bind dietary calcium, oral glucocorticoids to suppress intestinal calcium absorption, and calcitonin or loop diuretics to promote urinary calcium excretion.

Primary hyperparathyroidism is the most common cause of hypercalcemia and, therefore, a clinician's first consideration when hypercalcemia occurs during pregnancy. However, hypercalcemia due to hyperparathyroidism is characterized by normal to elevated serum PTH. Instead, when PTH is suppressed in the presence of hypercalcemia (as it was in *Cyp24a1* NULL mice), it suggests other causes of hypercalcemia, such as excess PTHrP or calcitriol.. Therefore, the combination of hypercalcemia and low serum PTH during pregnancy should serve as key indicators to rule out PTHrP-mediated and calcitriol-mediated causes of hypercalcemia, with DNA sequencing of the *CYP24A1* gene to needed confirm or exclude the diagnosis of CYP24A1 deficiency.

4.8. **Future directions**

We plan to explore approaches that directly target the mechanism of gestational hypercalcemia. One potential strategy involves using azoles (ketoconazole, fluconazole, and itraconazole) to inhibit CYP27B1 activity and reduce calcitriol anabolism.[100] Additionally, we could employ rifampin to enhance calcitriol catabolism through an alternative pathway involving CYP3A4.[64,168] More generic treatments for hypercalcemia, such as dietary calcium restriction, phosphate supplementation, glucocorticoids calcitonin or loop diuretics could be used as controls.

There is currently no data on the safety of these specific approaches for treating hypercalcemia during pregnancy, and there are plenty of treatments that could be tested in this mouse model.

5. CONCLUSION

The hypercalcemia observed in *Cyp24a1* null mice during pregnancy is due to the normal stimulation of calcitriol production during pregnancy that is unopposed by catabolism. That increases intestinal calcium absorption by overexpressing calcium transporter genes while simultaneously suppressing bone resorption. During lactation, the increases in calcitriol and intestinal calcium absorption were reduced but still sufficient, resulting in NULL mice resorbing less BMC than normal to support milk production. While the skeleton experienced diminished losses during lactation with recovery during the post-weaning period, the long-term effects of the CYP24A1 deletion on bone health are still unknown. Evaluating women with inactivating mutations in CYP24A1 is necessary to determine whether there are lasting impacts on skeletal health following reproductive events.

The change in serum calcium didn't disrupt the normal calcium levels provided in the milk. Their skeleton exhibited reduced bone strength at baseline, a phenomenon that remains mostly unexplained. The bone macro- and microstructure followed a similar pattern across genotypes, with a notable

increase in trabecular bone in the NULLs during pregnancy. This spike may result from the elevated serum calcium levels observed during the same period, but further research would have to assess that.

Treatments for women carrying this mutation should prioritize reducing calcitriol levels or intestinal calcium absorption rather than targeting bone resorption. These approaches will be explored in future research projects.

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Maekawa, Alexandre Seiji <asmaekawa@mun.ca>

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Researcher Portal File #: 20220273
Event Form Name: AUP Amendment - Administrative
Event Reference No.: 20220273 - 639661

Project Title: (21-01-CK) Fetal, neonatal and lactational mineral homeostasis in normal and genetically engineered rodents
Date of Submission: 2024/02/20

Receipt of this automated response confirms that your "AUP Amendment - Administrative" event form was received.

The form will be reviewed at the next available meeting of the Institutional Animal Care Committee.

Sincerely,

ANULIKA MBAKWE | ACC COORDINATOR
Department of Animal Care Services
Memorial University of Newfoundland
Health Sciences Centre | Room H1848
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7.3. Appendix 3: Teklad Global 18% Protein Rodent Diet

2018

Teklad Global 18% Protein Rodent Diet

inotiv
analyze. answer. advance.

Product Description- 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients designed to support gestation, lactation, and growth of rodents. 2018 exludes alfalfa meal, which lowers phytoestrogen (coumestrol) content, and reduces chlorophyll, improving optical imaging clarity. A moderate inclusion of soybean meal results in an expected isoflavone range of 225-340 mg/kg diet (daidzein + genistein aglycone equivalents). Absence of fish meal minimizes the presence of nitrosamines. **Related codes 2018C (certified), 2918 (irradiated), 2918C (irradiated, certified), 2018X (extruded), 2918X (irradiated, extruded), 2018S/SC (sterilizable, certified), 2018SX (sterilizable, extruded).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: ***Pellet***

Macronutrients		
Crude Protein	%	18.4
Fat (ether extract) [a]	%	6.0
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.8
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.5
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	15.0
Vitamin D_3 [e, g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

Shelf life: With proper storage, diet is suitable for use out to 9 months.

www.inotivco.com/shelf-life-of-diets-used-in-research

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

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0223

7.4. Appendix 4: Permission Agreement (American Physiological Society)

CCC Marketplace

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Order Date	23-Aug-2024	**Type of Use**	Republish in a thesis/dissertation
Order License ID	1518872-2		
ISSN	1522-1210	**Publisher**	AMERICAN PHYSIOLOGICAL SOCIETY
		Portion	Image/photo/illustration

LICENSED CONTENT

Publication Title	Physiological reviews	**Publication Type**	e-Journal
Article Title	Current understanding of the molecular actions of vitamin D.	**Start Page**	1193
		End Page	1231
Author / Editor	American Physiological Society (1887-)	**Issue**	4
		Volume	78
Date	01/01/1921	**URL**	http://physrev.physiology.org
Language	English		
Country	United States of America		
Rightsholder	American Physiological Society		

REQUEST DETAILS

Portion Type	Image/photo/illustration	**Distribution**	Worldwide
Number of Images / Photos / Illustrations	1	**Translation**	Original language of publication
Format (select all that apply)	Print, Electronic	**Copies for the Disabled?**	No
		Minor Editing Privileges?	No
Who Will Republish the Content?	Academic institution	**Incidental Promotional Use?**	No
Duration of Use	Life of current and all future editions	**Currency**	CAD
Lifetime Unit Quantity	Up to 499		
Rights Requested	Main product		

NEW WORK DETAILS

Title	Master's student in Medicine	**Institution Name**	Memorial University of Newfoundland
Instructor Name	Dr. Christopher Kovacs	**Expected Presentation Date**	2024-12-01

ADDITIONAL DETAILS

Order Reference Number	N/A	**The Requesting Person / Organization to Appear on the License**	Memorial University of Newfoundland - Alexandre Maekawa

REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	Fig. 1. A: nutritional forms of vitamin D. B: steps involved in activation of vitamin D3 molecule. Note that names of cytochrome P-450 isoforms currently thought to be responsible for enzyme steps are also provided.	**Title of the Article / Chapter the Portion Is From**	Current understanding of the molecular actions of vitamin D.
		Author of Portion(s)	Jones, G; Strugnell, S A; DeLuca, H F
		Issue, if Republishing an Article From a Serial	4
Editor of Portion(s)	Jones, G; Strugnell, S A; DeLuca, H F	**Publication Date of Portion**	1998-10-01
Volume / Edition	78		
Page or Page Range of Portion	1193-1231		

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14) **Additional Terms for Specific Products and Services.** If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

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i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

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B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

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B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

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B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

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v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

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d) No amendment or waiver of any Terms is binding unless set forth in writing and signed by the appropriate parties, including, where applicable, the Rightsholder. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by or on behalf of the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the License described in the Order Confirmation, which terms are in any way inconsistent with any Terms set forth in the Order Confirmation, and/or in CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

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Last updated October 2022

7.5. Appendix 5: Permission Agreement (BioRender)

49 Spadina Ave. Suite 200
Toronto ON M5V 2J1 Canada
www.biorender.com

Confirmation of Publication and Licensing Rights - Open Access

October 19th, 2024

Subscription Type:	*Student Plan - Academic*
Agreement number:	*YP27G1N56Z*
Publisher Name:	*Memorial University of Newfoundland*
Figure Title:	*Figure 4. Calcitriol synthesis and catabolism pathway*
Citation to Use:	*Created in BioRender. Maekawa, A. (2024) BioRender.com/o41y644*

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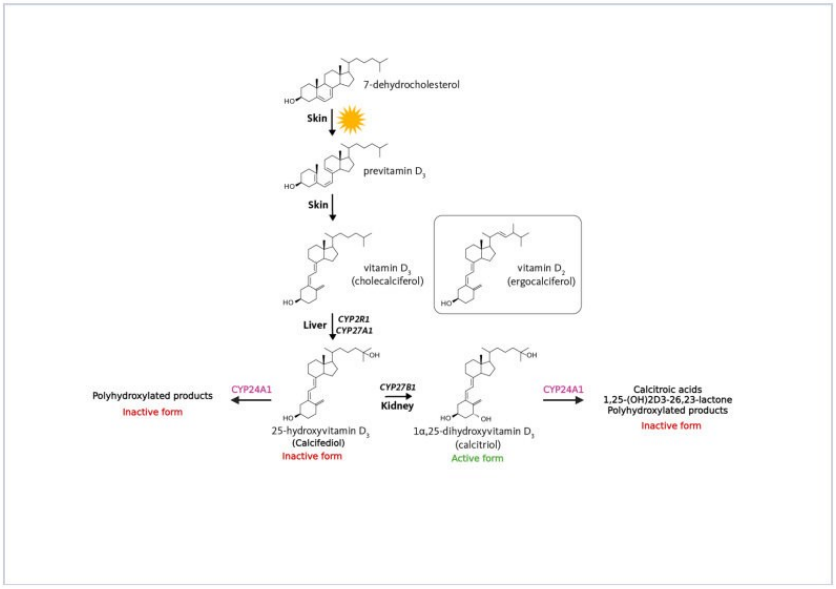

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7.6. Appendix 6: Permission Agreement (Endocrine Society)

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Order Date	23-Aug-2024	**Type of Use**	Republish in a thesis/dissertation
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ISSN	1945-7197	**Publisher**	ENDOCRINE SOCIETY
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LICENSED CONTENT

Publication Title	The Journal of Clinical Endocrinology & Metabolism	**Publication Type**	e-Journal
		Start Page	1440
Article Title	CYP3A4 Induction by Rifampin: An Alternative Pathway for Vitamin D Inactivation in Patients With CYP24A1 Mutations.	**End Page**	1446
		Issue	5
		Volume	102
		URL	http://press.endocrine.org/journal/jcem
Author / Editor	Endocrine Society.		
Date	01/01/1952		
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Title, Description or Numeric Reference of the Portion(s)	Figure 1.The activation and inactivation pathways of vitamin D, demonstrating the role of CYP24A1 in inactivating 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)2D] and the potential of CYP3A4	**Title of the Article / Chapter the Portion Is From**	CYP3A4 Induction by Rifampin: An Alternative Pathway for Vitamin D Inactivation in Patients With CYP24A1 Mutations.
		Author of Portion(s)	Hawkes, Colin Patrick; Li, Dong; Hakonarson, Hakon; Meyers, Kevin E; Thummel, Kenneth E; Levine, Michael A
Editor of Portion(s)	Hawkes, Colin Patrick; Li, Dong; Hakonarson, Hakon; Meyers, Kevin E; Thummel, Kenneth E; Levine, Michael A	**Issue, if Republishing an Article From a Serial**	5
		Publication Date of Portion	2017-05-01
Volume / Edition	102		
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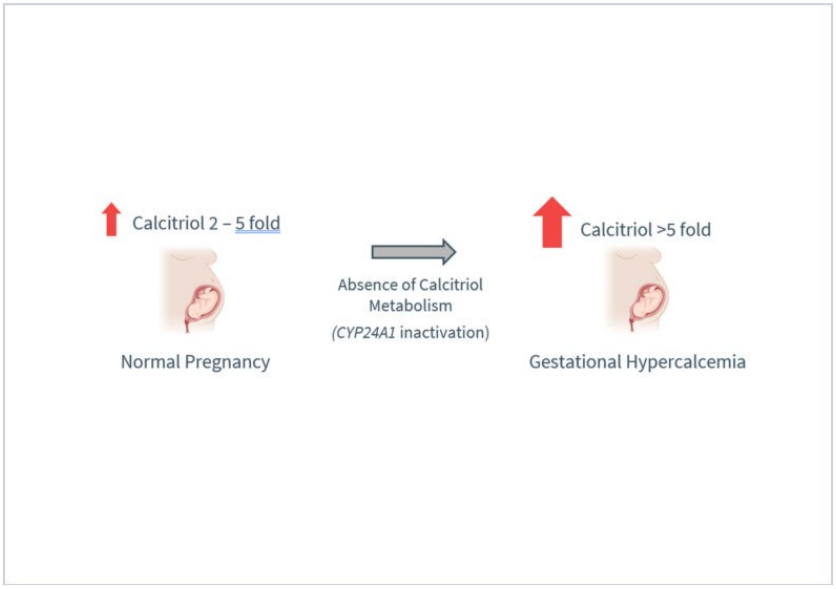

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