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**Pre-clinical development of siRNA Therapy for
Autosomal Dominant Osteopetrosis (ADO)**

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Abstract

Rare skeletal diseases are still in need of proper clinically available transfection agents as the major challenge for first-in-human translation relates to intrinsic difficulty intargeting bone without exacerbating any inherent toxicity due to used vector. SiSaf's sil-icone stabilized hybrid lipid nanoparticles (sshLNPs) constitute next-generation non-viral vectors able to retain the integrity and stability of constructs and to accommodate considerable pay-loads of biologicals, without requiring cold-chain storage. sshLNP was complexed with a small interfering RNA (siRNA) specifically designed against the human CLCN7 G215R mRNA. When tested via single intraperitoneal injection in pre-puberal autosomal dominant osteopetrosis type 2 (ADO2) mice, carrying a heterozygous mutation of the Clcn7 gene (Clcn7 G213R), sshLNP, this significantly downregulated the Clcn7 G213R related mRNA levels in femurs at 48 h. Confirmatory results were observed at 2 weeks and 4 weeks after treatments (3 intraperitoneal injections/week), with rescue of the bone phenotype and demon-strating safety. The pre-clinical results will enable advanced pre-clinical development of RNA-based therapy for orphan and genetic skeletal disorders by safely and effectively delivering biologicals of interest to cure human systemic conditions.



Chapter 1: “Introduction”

1 Introduction

1.1 The bone tissue

Bone tissue is a highly organized and specialized form of connective tissue that, in conjunction with cartilage, constitutes the skeletal system. The adult human skeleton comprises 206 individual bones, which collectively represent approximately 20% of total body mass. The skeletal system fulfills three fundamental physiological functions¹⁻³:

1. Mechanical function – Bones provide a rigid framework that supports the body's structure and posture. They also serve as anchor points for muscles, enabling voluntary movement through biomechanical leverage.
2. Protective function – The skeleton safeguards vital internal organs; for example, the skull encases the brain, the vertebral column protects the spinal cord, and the rib cage shields the heart and lungs.
3. Metabolic function – Bones act as a major reservoir for essential minerals, particularly calcium and phosphate, which are crucial for numerous cellular processes and are regulated in the bloodstream through bone activity.

Although bone may appear static and inert, it is in fact a highly dynamic tissue, constantly undergoing renewal through a tightly regulated process known as bone remodeling. This continuous cycle of bone resorption and formation is essential to preserve the tissue's mechanical integrity, repair micro-damage, and maintain systemic mineral homeostasis, especially concerning calcium and phosphate levels in the plasma.

Beyond its structural and metabolic roles, the skeleton is now recognized as a functional endocrine organ. It secretes signaling molecules that contribute to the regulation of various physiological systems, including renal function, energy metabolism, and even neurodevelopment, demonstrating its involvement in processes beyond traditional mechanical and protective roles^{4,5}.

As with all connective tissues, the extracellular matrix (ECM) in bone is abundant and complex. It consists predominantly of collagen fibers, primarily type I collagen, and a variety of non-collagenous proteins that regulate mineral deposition and cellular interactions. What sets bone apart from other connective tissues is its unique capacity for mineralization: the ECM becomes infiltrated with hydroxyapatite crystals, giving bone its exceptional hardness and load-bearing strength⁶.

In terms of cellular composition, bone contains three principal types of cells, each playing a distinct and essential role in bone physiology⁷:

- **Osteoblasts** – These are bone-forming cells responsible for synthesizing and secreting the organic components of the ECM, including collagen, and initiating the mineralization process.
- **Osteoclasts** – Multinucleated cells derived from the monocyte-macrophage lineage, osteoclasts are specialized for bone resorption. They degrade both the mineral and organic components of bone, releasing calcium and phosphate into the circulation.
- **Osteocytes** – These are mature bone cells derived from osteoblasts that have become embedded within the mineralized matrix. Osteocytes are critical regulators of bone homeostasis: they sense mechanical stress, coordinate remodeling processes, and modulate the activity of both osteoblasts and osteoclasts. They also play a key role in maintaining calcium and phosphate balance.

1.2 Anatomy and Macroscopic Organization

Bones can be macroscopically classified into three main types: long, short, and flat bones¹⁻³. They originate from two distinct developmental processes: intramembranous ossification and endochondral ossification.

Long bones, such as the femur and tibia, are characterized by a cylindrical central shaft known as the diaphysis, two expanded ends called epiphyses, and a transitional region between them referred to as the metaphysis (**Figure 1**).

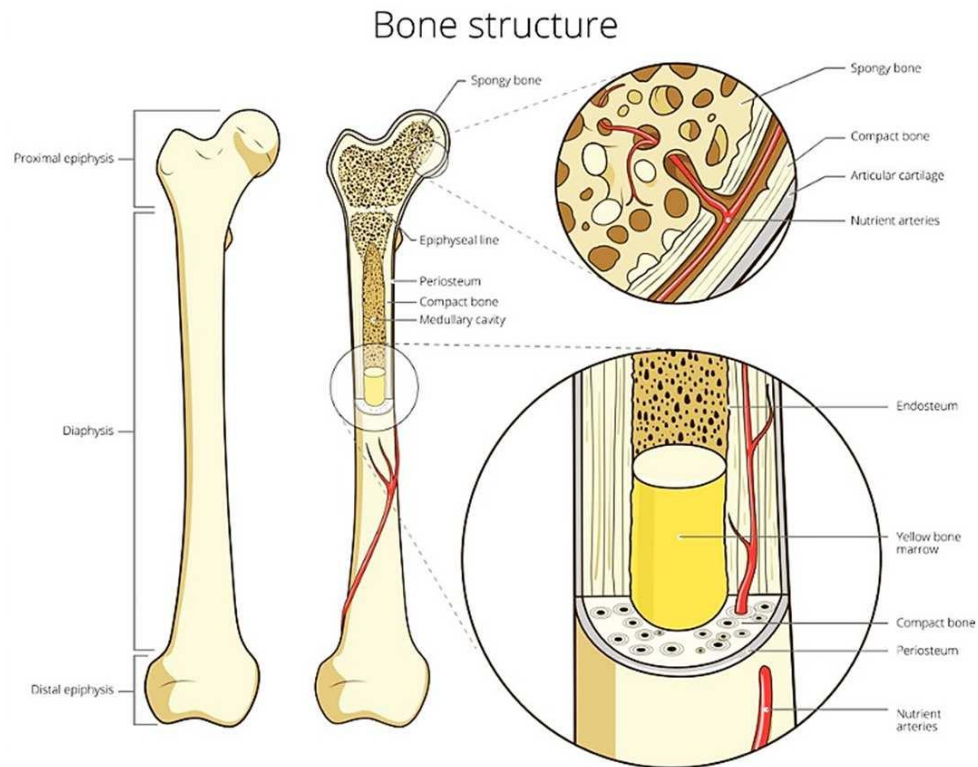


Figure 1 - Structure of the bone. Schematic representation of the macroscopic organization of the long bone.
(Source: adapted from <https://www.vectorstock.com>.)

The diaphysis contains a central medullary cavity, surrounded by a dense outer wall of compact bone (also known as the bone cortex). In the metaphysis and epiphysis, the compact bone thins out, and the internal structure is composed of a network of fine, calcified trabeculae, forming the cancellous or trabecular bone. The spaces between these trabeculae are continuous with the medullary cavity of the diaphysis and are filled with either hematopoietic (blood-forming) or adipose (fat-storing) bone marrow.

Flat bones are composed of two thin layers of compact bone enclosing a variable amount of cancellous (trabecular) bone. These bones serve primarily to provide broad protection for internal organs. A typical example is the skull, where the two compact layers—commonly referred to as the "tables" enclose a central layer of cancellous tissue known as the diploë¹⁻³.

Compact bone (also known as cortical bone) appears as a solid, dense mass, whereas cancellous bone (or trabecular bone) has a reticular, lattice-like structure formed by thin, interconnected trabeculae. Despite their different appearances, both cortical and trabecular bone are composed of the same cellular and extracellular matrix components. However, they differ significantly in both structure and function.

Cortical bone accounts for approximately 80–90% of the total calcified bone tissue, while trabecular bone comprises only 15–25%. The remaining intraosseous space is occupied by bone marrow, blood vessels, and stromal connective tissue.

This structural distinction underlies a key functional difference:

- **Cortical bone** is primarily responsible for mechanical strength and protection.
- **Trabecular bone**, in contrast, plays a major role in metabolic functions, such as calcium homeostasis and hematopoiesis. Nonetheless, it also contributes to the mechanical performance of bones.

Indeed, trabeculae within cancellous bone are oriented along the lines of greatest mechanical stress, which allows the bone to withstand forces efficiently while using minimal material. This adaptive architectural arrangement ensures maximum resistance to stress and strain with a minimal amount of bone mass¹⁻³.

Short bones, such as those of the carpus, tarsus, and vertebrae, are typically located in regions of the skeleton that require a combination of strength, compactness, and limited mobility. These bones are primarily composed of cancellous bone tissue, surrounded by a thin outer layer of compact bone.

1.3 Microscopic Organization

From a microscopical perspective, human bone can be classified into two types: woven bone and lamellar bone⁸.

Woven bone is found exclusively in embryonic and neonatal skeletons, as well as during the early stages of fracture healing. It is characterized by a disorganized arrangement of collagen fibers, which are laid down in randomly oriented bundles. This irregular structure results in bone that is mechanically weaker but more flexible than its mature counterpart (**Figure 2a**).

In contrast, lamellar bone represents the mature, fully developed form of bone found in the healthy adult skeleton. Its extracellular matrix is organized into microscopically thin, layered sheets called lamellae, within which collagen fibers are arranged in parallel bundles. Notably, the orientation of these bundles alternates between adjacent lamellae, creating a highly ordered and mechanically robust structure (**Figure 2b**)^{9,10}.

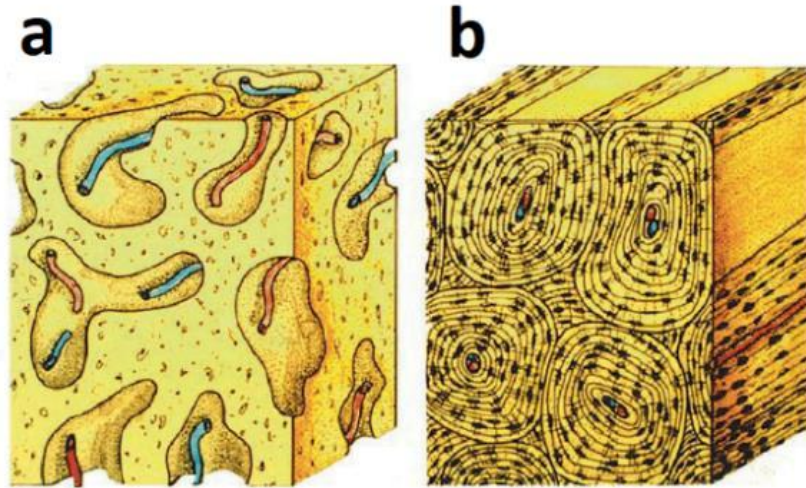


Figure 2: Woven and lamellar bone. Diagram illustrating the difference between (a) woven and (b) lamellar bone. Woven bone, due to the random organization of collagen fibres, displays a disorganised appearance, while in lamellar bone the matrix is organized in microscopically thin layered sheets known as lamellae in which collagen fibers are arranged in parallel oriented manner (Source: adapted from: Bone Structure, Development and Bone Biology: Bone Pathology -Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Diagram-of-immature-and-mature-bone-Immature-woven-bone-displays-a-disorganized_fig2_224929158).

In trabecular bone and periosteum, which is an envelope of connective tissue that lines external bone surfaces, the lamellae are arranged parallel to each other and lodged along a flat surface, while in cortical bone they are deposited around a central canal, known as Haversian canal. In fact, cortical bone is organized in the Haversian systems or osteons (**Figure 3**).

In the center of each osteon there is the Haversian canal, which is coated by endosteum, a layer of connective tissue that lines the inner surfaces of bones and contains blood vessels and nerves. In addition to Haversian canal, Volkmann's canals ensure communication between adjacent osteons by also connecting Haversian blood vessels. The appearance of an osteon is that of a cylinder running parallel to the long axis of the diaphysis. In the interstices between the osteons there are irregular areas of lamellar bone, which are residues of previous remodeled osteons.

In the outermost and innermost layers of cortical bone, called the outer and inner circumferential lamellae respectively, there are no Haversian canals and the lamellae run parallel with periosteal and endosteal surfaces^{9,10}.

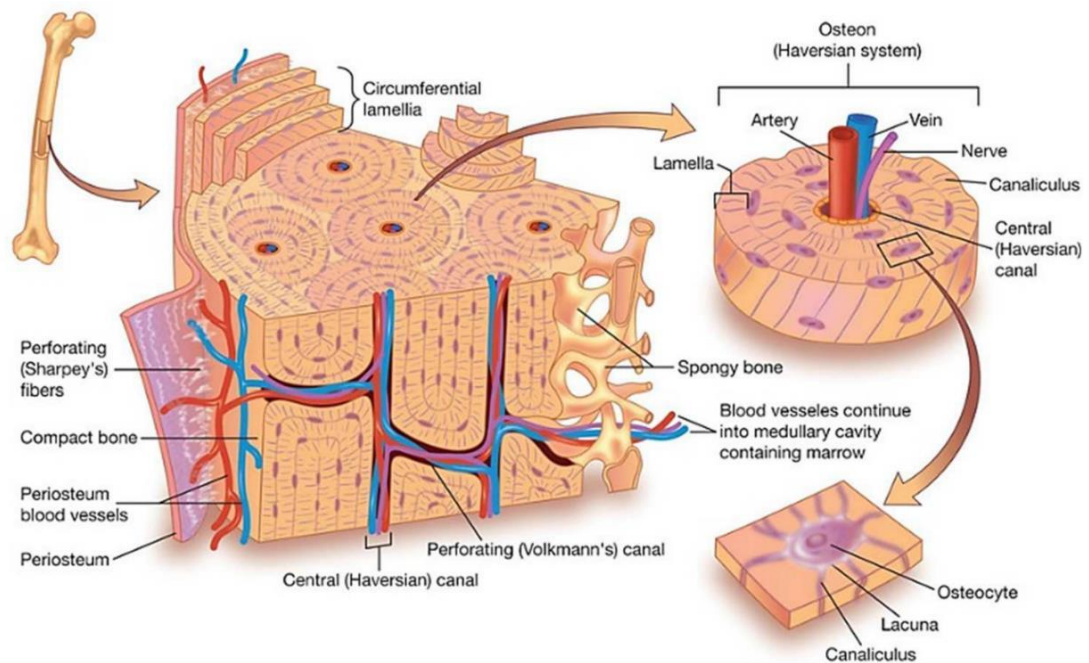


Figure 3: Microscopic structure of bone. Schematic representation of the Haversian system and of lamellar bone (Source: adapted from <https://www.vectorstock.com>.)

1.4 The Bone Matrix

The bone extracellular matrix is structurally and functionally unique due to its mineralized nature, comprising both inorganic and organic components¹¹. The inorganic component provides mechanical rigidity and the ability to withstand load-bearing stress, while the organic component contributes flexibility and elasticity to the tissue.

Approximately 90% of the inorganic matrix consists of hydroxyapatite crystals, which are responsible for the bone's hardness and structural strength. In contrast, the organic matrix—which makes up around 35% of the bone's dry weight—is primarily composed of type I collagen fibers. These fibers are aligned in parallel and organized into lamellae, the layered structural units that define the typical lamellar architecture of mature bone¹². This highly ordered collagen arrangement accounts for the high tensile strength and stiffness of bone tissue.

In addition to collagen, the organic matrix contains a variety of non-collagenous proteins, including osteonectin, osteocalcin, osteopontin, proteoglycans, as well as numerous cytokines and growth factors^{12,13}. These molecules play diverse roles—not only providing structural support, but also

contributing to the regulation of mineral deposition, bone remodeling, and the activity of bone cells, such as osteoblasts and osteoclasts.

1.5 Bone Cells

1.5.1 Osteoblasts

Osteoblasts are specialized bone-lining cells responsible for the production of new bone matrix on bone-forming surfaces^{1,14}. They are derived from mesenchymal stem cells (MSCs), multipotent progenitors capable of differentiating into chondrocytes, osteoblasts, myoblasts, or adipocytes, depending on the expression of specific transcription factors^{14,15}.

Microscopically, osteoblasts are characterized by a basally located round nucleus and a strongly basophilic cytoplasm. Under electron microscopy, they exhibit a well-developed rough endoplasmic reticulum and a prominent Golgi complex, positioned between the nucleus and the apical side of the cell that faces the bone matrix. These features reflect the intense biosynthetic and secretory activity typical of active osteoblasts (**Figure 4**).

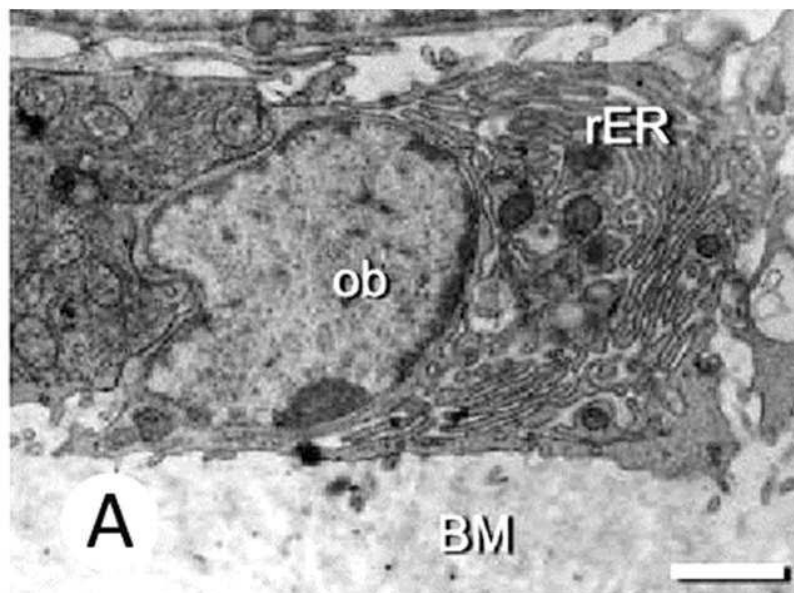


Figure 4 - Osteoblasts ultrastructure. Electron micrograph of an osteoblast lining the bone matrix. Ob: osteoblast, rER: rough endoplasmic reticulum; BM: bone matrix. Scale bar: 2 μ m. (Source: adapted from Toray et al., 2017¹⁶)

At the ultrastructural level, the secretory (apical) surface of osteoblasts shows cytoplasmic processes that extend deeply into the osteoid matrix, where they establish contact with the cell processes of

osteocytes within their canaliculi. Gap junctions and other junctional complexes are commonly observed between adjacent osteoblasts, facilitating intercellular communication and coordination of bone formation activity^{14,15,16}.

The surface membrane of osteoblasts is notably abundant in alkaline phosphatase (ALP), an enzyme widely recognized as a hallmark of cells belonging to the osteoblast lineage. The level of ALP detected in the bloodstream is commonly utilized as an indicator of bone formation activity. Furthermore, osteoblasts possess specific receptors that respond to various regulatory molecules, including parathyroid hormone (PTH), prostaglandins, estrogens, and vitamin D3.

Similar to other connective tissue cells such as adipocytes, chondrocytes, fibroblasts, and myoblasts, osteoblasts originate from mesenchymal stem cells (MSCs)¹⁷. The differentiation of MSCs toward the osteo/chondroprogenitor lineage is tightly controlled by signaling molecules, particularly those from the Wingless-related integration site (WNT) family and Bone Morphogenetic Proteins (BMPs)¹⁸ (see **Figure 5**).

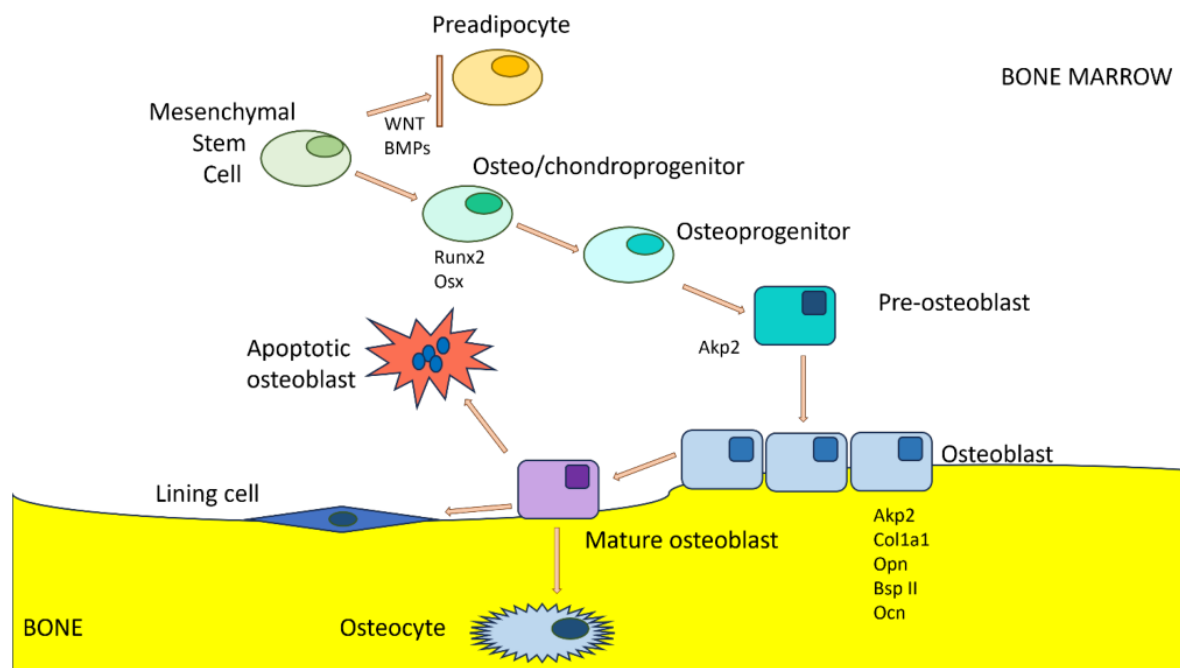


Figure 5: Schematic representation of the osteoblast differentiation process. Osteoblasts derive from mesenchymal stem cells and their differentiation is promoted by Wingless (WNT) and bone morphogenetic protein (BMP) pathways. The activation of these pathways promotes RUNX2 and OSX expression in osteo-chondroprogenitors, leading to their differentiation towards osteoprogenitors and then pre-osteoblasts, which express ALP, one of the earliest markers of osteoblast differentiation. Later on osteoblasts will express other

specific markers, such as COL1A1, OPN, BSP II and OCN. Mature osteoblasts ultimately will be embedded in the bone matrix as terminally differentiated osteocytes, or will become a bone lining cell, or will die by apoptosis.

Among these, WNT proteins play a crucial role through both canonical and non-canonical signaling pathways. The canonical WNT pathway begins when WNT ligands bind to the Frizzled (FZD) receptor and the Low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor, which are present on the plasma membrane of osteoprogenitor cells¹⁹. This ligand-receptor interaction triggers an intracellular cascade involving a multi-protein complex composed of Glycogen Synthase Kinase 3 β (GSK3 β), Axin, Adenomatous Polyposis Coli (APC), and Dishevelled (DSH). The assembly of this complex inhibits GSK3 β from phosphorylating β -catenin, preventing its ubiquitin-mediated degradation and leading to β -catenin stabilization. Stabilized β -catenin then translocates into the nucleus, where it binds to T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors, promoting the expression of key regulators of osteoblast differentiation such as Runt-related transcription factor 2 (RUNX2) and Osterix (OSX), along with other osteogenic genes²⁰.

The necessity of β -catenin for osteoblast development was confirmed genetically by *Hu and colleagues* in (2005)²¹, while *Tu X et al*²² demonstrated that the WNT-Gaq/11-PKC δ non-canonical signaling pathway also contributes to osteoblast differentiation in mammalian progenitors. Additionally, recent studies by *Chang J et al*²³ revealed a novel non-canonical pathway in which WNT-4 is essential for promoting osteogenic differentiation of MSCs.

As differentiation proceeds, pre-osteoblasts begin to express AKP2, the gene encoding alkaline phosphatase (ALP), a hallmark early marker of osteoblast commitment. Alongside ALP, genes such as Collagen 1A1 (COL1A1), which remains highly active throughout differentiation, and Osterix are expressed. Fully differentiated osteoblasts also express receptors for parathyroid hormone (PTH), estrogens (ERs), and vitamin D3 (VDR). These mature cells are responsible for producing bone matrix proteins including bone sialoproteins and osteocalcin.

Under transmission electron microscopy, mature osteoblasts display an extensive rough endoplasmic reticulum and a well-developed Golgi apparatus. Their cytoplasm is rich in ribosomes and secretory vesicles, while the nucleus appears euchromatic with prominent nucleoli. This cellular architecture reflects their primary function: the synthesis and secretion of the organic extracellular matrix (ECM), as well as the deposition of mineral components essential for bone formation¹⁷.

Osteoblasts also secrete various cytokines that regulate osteoclast activity, such as macrophage colony-stimulating factor (M-CSF), which supports the survival and proliferation of pre-osteoclasts; receptor activator of nuclear factor kappa-B ligand (RANKL), critical for osteoclast differentiation; and osteoprotegerin (OPG), a decoy receptor that binds RANKL, preventing its interaction with RANK and thereby inhibiting osteoclastogenesis²⁴⁻²⁷. Recent research has revealed unexpected osteogenic roles for RANKL, including the existence of an autocrine loop in MSCs. MSCs lacking RANKL show diminished osteogenic potential, which can be restored by reintroducing RANKL²⁸. Furthermore, osteoclast-derived vesicular RANK can stimulate bone formation by interacting with osteoblast RANKL, activating reverse signaling that promotes RUNX2 activity²⁹.

It is important to note that the osteoblast stage is transient in the osteoblast lineage. Once their bone matrix synthesis is complete, these cells have three possible fates: undergoing apoptosis, differentiating into osteocytes, or transforming into bone lining cells—flat, inactive cells that cover quiescent bone surfaces until they are reactivated by physiological stimuli^{17, 24-27}.

1.5.2 Osteocytes

The transition from osteoblasts to osteocytes involves a significant transformation in cell morphology. Mature osteoblasts typically exhibit a polygonal shape, but as they differentiate into osteocytes, they become noticeably more flattened and develop multiple elongated cytoplasmic extensions. These projections are polarized in nature—some reach toward the mineralization front, others extend toward blood vessels or neighboring osteocytes. This intricate network of cellular connections forms the lacunar-canalicular system (LCS)¹⁷, a critical structure for cell communication and nutrient transport within bone tissue.

Over the past few decades, several molecular markers have been identified that help distinguish different stages of osteocyte differentiation. For early osteocytes, markers include PHEX (phosphate-regulating neutral endopeptidase on the X chromosome), dentin matrix protein 1 (DMP1), and E11/gp38. In contrast, mature osteocytes predominantly express receptor activator of nuclear factor kappa-B ligand (RANKL), sclerostin (SOST), fibroblast growth factor 23 (FGF23), and matrix extracellular phosphoglycoprotein (MEPE)³⁰.

Although osteocytes make up approximately 90–95% of all bone cells, for a long time they were thought to be largely inactive—essentially osteoblasts that had become embedded in the mineralized

bone matrix. However, advancements in cell isolation methods and molecular biology techniques have led to a deeper understanding of their diverse and active roles in bone physiology⁹.

Osteocytes are remarkably long-lived, with a lifespan estimated to be as long as 25 years. Throughout this time, they function as key mechanosensors—detecting mechanical forces exerted on bone—and play a central role in regulating bone remodeling. They contribute to bone turnover locally and also secrete systemic factors, such as FGF23, which helps regulate phosphate reabsorption in the kidneys²⁵.

Another essential molecule secreted by osteocytes is sclerostin (SOST), a glycoprotein that negatively regulates bone formation. Sclerostin inhibits osteoblast proliferation and differentiation by binding to LRP5/6, thereby blocking the Wnt/ β -catenin signaling pathway³¹, which is crucial for bone growth. Due to its inhibitory effect, anti-sclerostin antibodies have been developed and are now successfully used in the treatment of osteoporosis³².

Being fully enclosed within the LCS allows osteocytes to detect shear stress resulting from mechanical stimulation. They are capable of converting mechanical signals into intracellular biochemical responses, which can subsequently influence a variety of biological processes. This mechanotransduction ability positions osteocytes as key regulators of bone remodeling³³.

Osteocytes modulate the activity of both osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells). They achieve this regulation not only through direct cell-to-cell communication but also by secreting soluble signaling molecules³⁴. Notably, osteocytes produce both RANKL and osteoprotegerin (OPG)—two critical regulators of osteoclast differentiation and activity. The balance between RANKL and OPG production is a mechanism by which osteocytes control osteoclast-mediated bone resorption³⁵.

Experimental evidence further underscores their importance. For instance, mice engineered to lack RANKL expression specifically in osteocytes develop an osteopetrotic phenotype, characterized by increased bone mass and resistance to bone loss—even under conditions of mechanical unloading (such as tail suspension).

Beyond RANKL and OPG, osteocytes also secrete a variety of molecules that influence bone metabolism. These include small signaling mediators such as prostaglandins, nitric oxide, and nucleotides, as well as numerous cytokines and growth factors. Examples include insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGF- β).

Furthermore, the hormone parathyroid hormone (PTH) stimulates RANKL production in osteocytes, highlighting their role as key mediators of PTH-induced bone resorption³⁵.

1.5.3 Osteoclasts

Osteoclasts are highly specialized cells responsible for the degradation and removal of aged bone matrix during the processes of bone modeling and remodeling. These activities are crucial for maintaining the biomechanical integrity of the skeleton as well as regulating the mineral balance throughout the whole organism^{36,37}.

The structural features of osteoclasts are intricately linked to their functional roles. These cells are large and multinucleated, containing between 4 to 20 nuclei, which result from the fusion of multiple mononuclear precursor cells originating from the monocyte/macrophage lineage^{36,37}. Osteoclasts are predominantly located in close contact with mineralized bone surfaces, where they create resorption cavities known as Howship's lacunae through their bone-resorptive activity.

At the microscopic level, the nuclei within a single osteoclast can exhibit heterogeneity: some appear round with a euchromatic (actively transcribing) appearance, while others are irregular and heterochromatic, likely reflecting the asynchronous fusion events of precursor cells. The cytoplasm is rich in vacuoles, indicative of intense secretory processes. On an ultrastructural scale, osteoclasts display multiple large Golgi apparatus clusters surrounding each nucleus, abundant mitochondria, and a variety of transport vesicles, lysosomes, and vacuoles. These features highlight the cell's high metabolic activity, energy demands, and the production of lysosomal enzymes necessary for bone degradation^{36,37}.

Osteoclasts exhibit a polarized morphology with distinct domains on their plasma membrane (see **Figure 6**). On the side facing the bone (apical membrane), they form a specialized "sealing zone" composed of two key regions: the "clear zone" and the "sealing membrane." The clear zone consists of cytoplasm with few organelles, creating a ring-like structure. The sealing membrane, which directly contacts the bone surface, is enriched with specialized adhesion complexes called podosomes^{38,39}. Podosomes are actin-rich, foot-like structures surrounded by signaling molecules, adhesion proteins, and integrin receptors (including $\alpha 2\beta 1$, $\alpha \nu \beta 3$, and $\alpha \nu \beta 5$), which collectively form a tight peripheral ring. This ring firmly anchors the osteoclast to the bone matrix during resorption, ensuring a sealed microenvironment necessary for effective bone degradation.

Just inside the sealing zone lies the ruffled border, characterized by intricate membrane folds that facilitate the fusion of lysosomal membranes with the plasma membrane. This allows the targeted secretion of lysosomal enzymes into the resorption lacuna⁴⁰. The ruffled border is the site where ion transporters are inserted, including the ATPase proton pump and the H^+/Cl^- antiporter (ClC7 protein). These transporters maintain an ion gradient and electrical balance across the membrane, which is essential for acidifying the resorption lacuna and dissolving the mineralized bone matrix⁴¹.

Recent studies have further divided the ruffled border into two functional subdomains⁴²: the fusion zone and the uptake zone. The fusion zone facilitates the fusion of endosomal and lysosomal vesicles with the plasma membrane, enabling enzyme secretion. The uptake zone is responsible for internalizing the degraded bone matrix components and recycling lysosomal enzymes^{42,43} for reuse within the cell.

On the side opposite to the bone (the basolateral membrane), osteoclasts feature a “functional secretory domain” specialized in expelling the digested matrix byproducts into the bloodstream⁴³. This membrane area is also enriched with various ion transporters, including sodium-potassium ATPases (Na^+/K^+ pumps), bicarbonate/chloride exchangers (HCO_3^-/Cl^-), sodium/hydrogen exchangers (Na^+/H^+), and several ion channels. Additionally, the basolateral membrane expresses key receptors involved in osteoclast biology, such as RANK (the receptor for RANKL), c-Fms (the receptor for macrophage colony-stimulating factor, M-CSF), and the calcitonin receptor³⁶.

Calcitonin, a hormone that rapidly inhibits osteoclast activity, acts by causing disassembly of actin filaments, loss of the clear zone, cell contraction, and subsequent detachment of the osteoclast from the bone surface, thus halting bone resorption⁴⁴.

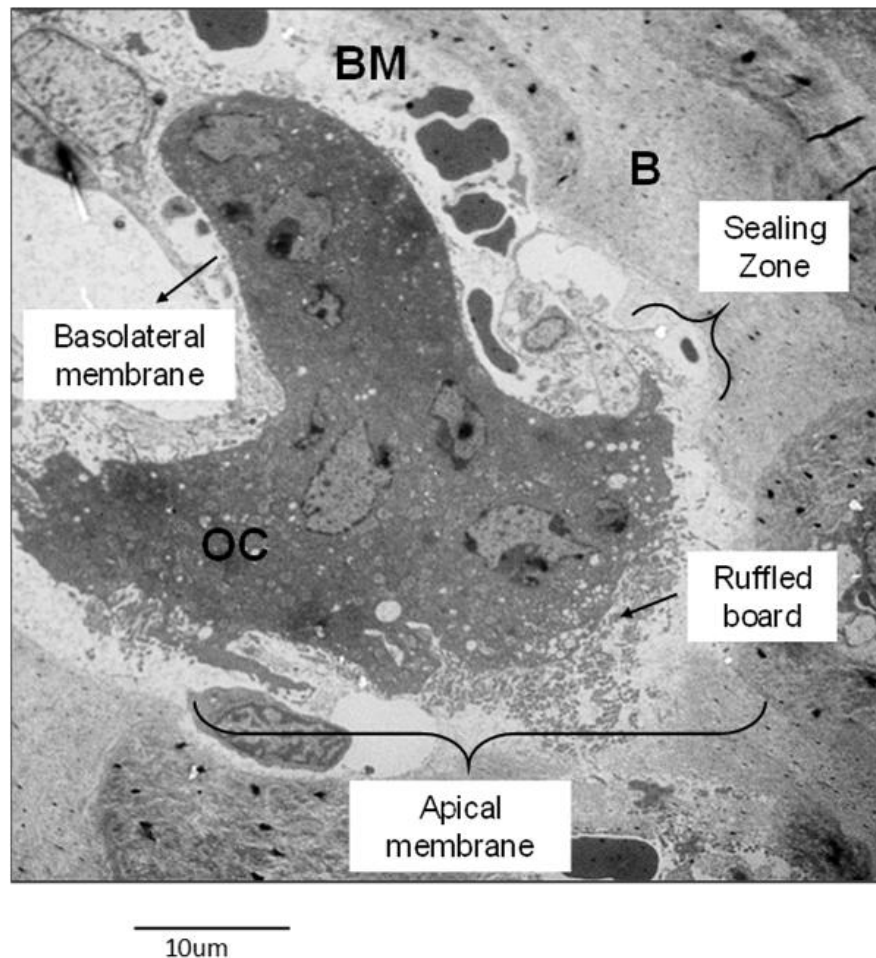


Figure 6. Osteoclast morphology. Transmission Electronic Microscopy (TEM) image of an osteoclast from an EPON-embedded tibia of C57/B6 mouse illustrating the osteoclast plasma membrane domains. Scale 10 μm . B: bone. OC: osteoclast. B: bone. (Source: Maurizi and Rucci, 2018⁴⁵)

Osteoclast formation and activation. Osteoclasts originate from cells belonging to the monocyte-macrophage lineage, and their development is tightly regulated by two essential cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). The process of osteoclast differentiation is complex and occurs in several distinct stages, which can be broadly categorized into three main phases (see **Figure 7**):

1. **Lineage Commitment:** Hematopoietic stem cells (HSCs), which reside in the bone marrow and give rise to all blood and immune cells, first commit to the monocyte/macrophage lineage under the influence of M-CSF. This step marks the beginning of osteoclastogenic potential.
2. **Acquisition of Osteoclast Identity:** These committed progenitor cells further differentiate into osteoclast precursors (OCPs). At this stage, they begin to express characteristic molecular

markers of the osteoclast lineage, including tartrate-resistant acid phosphatase (TRAcP) and the calcitonin receptor-both of which are indicative of cells primed for bone resorption.

3. **Cell Fusion and Maturation:** In the final step, under continued stimulation by RANKL, the mononuclear osteoclast precursors undergo cell-cell fusion, forming large, multinucleated cells that become fully functional mature osteoclasts capable of resorbing bone⁴⁵.

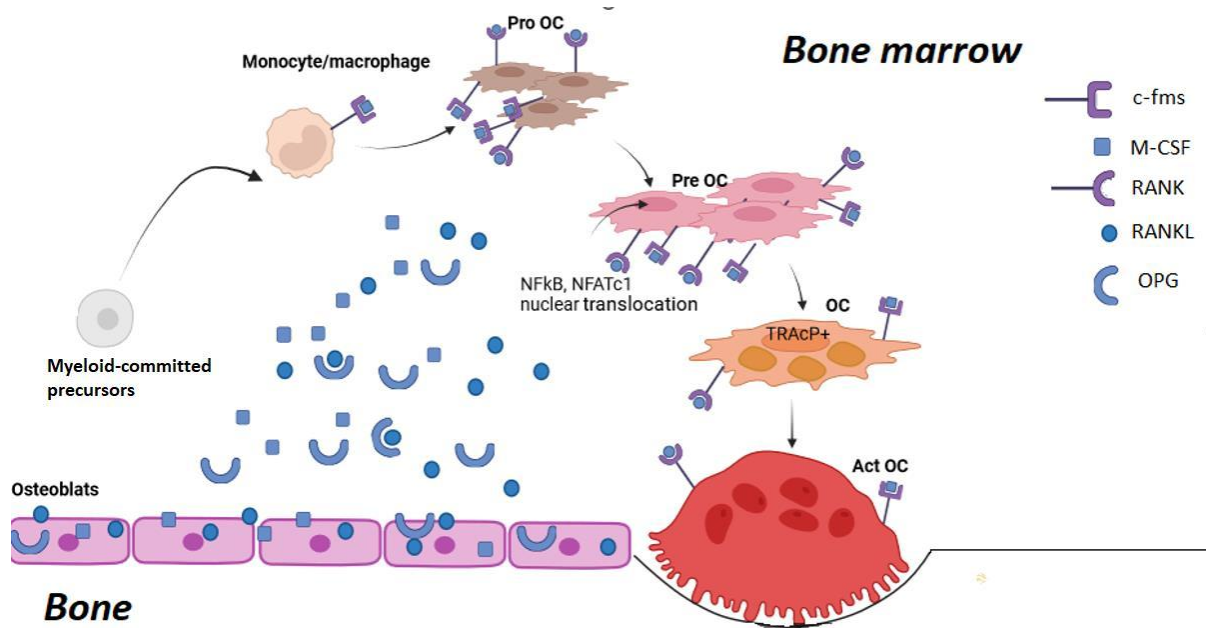


Figure 7: Osteoclast differentiation. Osteoclasts arise from myeloid-committed precursors that are able to differentiate into monocytes/macrophages or dendritic cells. Monocytes/macrophages then differentiate into osteoclast progenitors (ProOC), pre-osteoclasts (PreOC), osteoclasts (OC) and then active OC (Act OC). (Source: created in BioRender.com).

M-CSF signaling pathway

Macrophage Colony-Stimulating Factor (M-CSF) plays a pivotal role in the early stages of osteoclastogenesis by promoting the commitment of hematopoietic progenitors to the monocyte lineage, supporting their proliferation, and enabling the expression of RANK⁴⁶ on their surface, an essential step for their subsequent differentiation into osteoclasts.

The critical importance of M-CSF in osteoclast development has been clearly demonstrated in *op/op* mice, which are genetically deficient in M-CSF. These animals exhibit a pronounced osteopetrotic

phenotype⁴⁷, characterized by increased bone mass due to a severe deficiency in osteoclast formation and function.

M-CSF exerts its biological effects by binding to its specific receptor, c-Fms (also known as CSF1R), located on the surface of osteoclast precursors. This binding triggers receptor dimerization, which in turn activates the intrinsic tyrosine kinase activity of c-Fms. This activation leads to a cascade of autophosphorylation and transphosphorylation events on specific tyrosine residues within the cytoplasmic domain of the receptor⁴⁸.

Among these phosphorylated residues, tyrosine 559 (Y559) plays a particularly important role by recruiting and interacting with the non-receptor tyrosine kinase c-Src⁴⁹. This interaction initiates the activation of the PI3K/AKT signaling pathway⁵⁰, which promotes cell proliferation and survival by inhibiting the antiproliferative effects of molecules such as glycogen synthase kinase 3 (GSK3) and the transcription factor FOXO⁵¹.

Another major pathway activated downstream of M-CSF/c-Fms signaling is the ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) pathway. This pathway is triggered through the recruitment of the adaptor protein Grb2⁵², which binds to phosphorylated tyrosine residues Y697 and Y974 on c-Fms. Activation of this pathway further supports the proliferation and survival of osteoclast precursors.

In summary, the M-CSF/c-Fms signaling axis serves dual functions in osteoclastogenesis:

1. It drives the proliferation and maintenance of osteoclast precursor cells (OCPs).
2. It induces RANK expression, thereby rendering these precursors competent to respond to RANKL and progress further along the osteoclast differentiation pathway.

RANKL/RANK axis

The RANKL–RANK signaling pathway is a central mechanism regulating osteoclast differentiation and activation. This pathway is primarily mediated through the adaptor protein Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6), which binds to the intracellular domain of RANK upon RANKL engagement. Once recruited, TRAF6 initiates a cascade of downstream signaling events by assembling and activating a complex that includes TGFβ-activated kinase 1 (TAK1) and its binding partner TAB2 (TAK1-binding protein 2).

Activation of this signaling complex leads to the phosphorylation of multiple key kinases involved in osteoclastogenesis and osteoclast function⁵³. These include:

- Inhibitor of NF- κ B kinase (IKK)
- c-Jun N-terminal kinase (JNK)
- p38 MAP kinase
- Extracellular signal-regulated kinase (ERK)
- c-Src, a non-receptor tyrosine kinase

A closer look at this process reveals that TRAF6 activation stimulates IKK, which in turn phosphorylates I κ B, the inhibitor of NF- κ B. This phosphorylation marks I κ B for degradation by the proteasome, releasing NF- κ B from its cytoplasmic sequestration. Freed NF- κ B then translocates to the nucleus, where it binds to specific DNA elements and activates the transcription of genes essential for osteoclast differentiation, function, and survival⁴⁵.

Concurrently, TRAF6 also activates the JNK pathway, leading to the upregulation of c-Jun and c-Fos, two transcription factors that form the AP-1 complex when they heterodimerize. AP-1 plays a key role in osteoclast gene transcription and is also required for osteoclastogenesis.

Importantly, the activation of both NF- κ B and AP-1 (via c-Fos) is necessary to induce the expression of NFATc1 (Nuclear Factor of Activated T cells, cytoplasmic 1), a master transcription factor that governs the final stages of osteoclast differentiation⁵⁴. Evidence of NFATc1's critical role comes from studies showing that in cells lacking NF- κ B subunits (p50/p52) or c-Fos, RANKL-induced activation of NFATc1 is severely impaired⁵⁵.

NFATc1 directly regulates the transcription of numerous osteoclast-specific genes⁵⁶, including:

- Calcitonin receptor
- Tartrate-resistant acid phosphatase (TRAcP)
- Cathepsin K
- OSCAR (Osteoclast-associated receptor)
- β 3 integrin

Additionally, RANKL signaling elevates intracellular calcium (Ca^{2+}) levels, which activates calcineurin, a calcium/calmodulin-dependent phosphatase. Calcineurin dephosphorylates NFATc1,

enabling its translocation into the nucleus, where it can initiate the transcription of osteoclast-specific genes⁵⁷.

The essential role of NFATc1 in osteoclast biology has been further confirmed in genetic models: mice lacking NFATc1 develop osteopetrosis due to defective osteoclast formation. Conversely, forced overexpression of NFATc1 in NFATc1-deficient embryonic stem cells is sufficient to drive osteoclast differentiation, even in the absence of RANKL stimulation^{58,59}.

Finally, the RANKL–RANK–TRAF6 axis also activates additional signaling pathways involving JNK, c-Myc, and c-Src, all of which contribute to the regulation of osteoclast differentiation and activation⁵³. Notably, c-Src also plays a key role in linking RANK signaling to the PI3K/AKT pathway, which is involved in promoting cell survival and cytoskeletal organization during bone resorption⁶⁰.

The importance of c-Src in osteoclast function is supported by genetic studies. In particular, mice lacking c-Src (Src^{-/-} mice) exhibit an osteopetrotic phenotype due to a severe impairment in osteoclast activity, despite the presence of mature osteoclasts⁶¹.

Mechanisms of bone resorption

The unique characteristic of osteoclasts is their capacity to resorb the bone matrix. During the process of bone resorption, osteoclasts first dissolve the mineral portion of the matrix—primarily composed of crystalline hydroxyapatite—and subsequently degrade the organic component. This sequence of events takes place within a specialized compartment known as the Howship lacuna (or resorption lacuna), which is the area of bone located beneath the osteoclast's ruffled border, enclosed by a sealing membrane that isolates it from the surrounding environment.

Acidification of the resorption lacuna is a crucial step for dissolving hydroxyapatite. The molecular mechanisms responsible for acid secretion involve at least two distinct systems: the active transport of protons through an ATP-dependent proton pump known as the vacuolar H⁺-ATPase (V-H⁺-ATPase)^{62,63} (**Figure 8**), and the parallel movement of counter anions via a H⁺/Cl⁻ antiporter (specifically, the chloride channel ClC-7)^{64,65}. The ClC-7 channel, located in the ruffled border membrane, counterbalances the electrical potential created by the extensive outward flow of positively charged protons generated by the V-H⁺-ATPase.

Extensive experimental evidence indicates that the dissolution of the mineral phase represents the rate-limiting step of bone resorption, as the collagen-rich organic matrix can only be degraded after the mineral has been removed.

The acidification of the resorption lacuna is primarily mediated by the V-H⁺-ATPase situated in the ruffled border of osteoclasts. This proton pump is also found in various intracellular vesicles that acidify internal compartments⁶⁶. V-H⁺-ATPases are electrogenic enzyme complexes composed of multiple subunits, and several tissue-specific isoforms of these subunits have been identified (**Figure 8**). Notably, four isoforms of the a-subunit (a1, a2, a3, and a4) have been described, with the a3 isoform being characteristic of osteoclasts and the parietal cells of the gastric glands.

The absence of the a3 subunit in mice results in a severe osteopetrotic phenotype, demonstrating its essential role in bone resorption^{67,68}. Moreover, approximately 50% of patients with malignant osteopetrosis carry mutations in the gene encoding this subunit, highlighting its critical importance in osteoclast function and skeletal homeostasis⁶⁹.

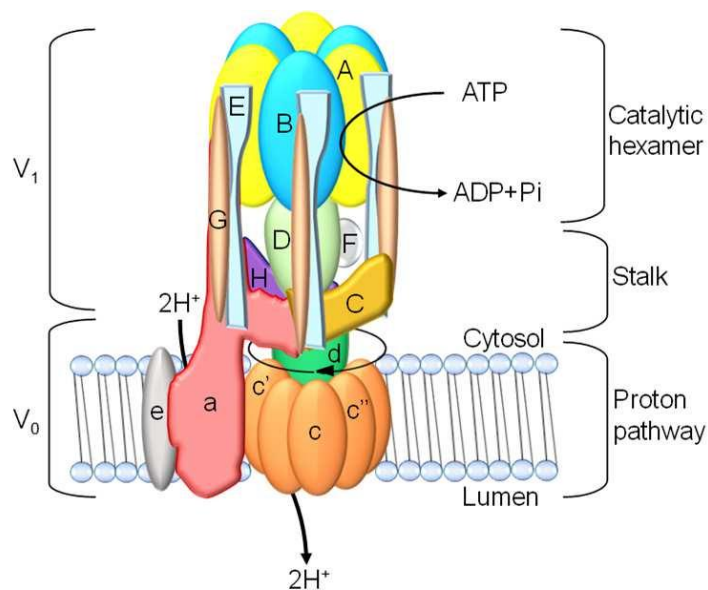


Figure 8. Molecular structure of the vacuolar H⁺-ATPase. Osteoclasts express the isoform 3 of the a subunit (a3) in the V0 domain (circle). (Source: Maurizi and Teti⁶³).

The initial evidence for the existence of chloride channels in osteoclasts originated from biochemical analyses of isolated membrane vesicles⁷¹. Subsequent studies demonstrated the crucial functional role of these channels at the ruffled border, revealing that loss of the chloride channel isoform ClC-7 results in osteopetrosis in both humans and mice⁶⁴. ClC-7 operates in close association with another membrane protein, Ostml, which is essential for the correct localization and proper function of ClC-

7. Interestingly, mutations in the *Ostm1* gene cause a comparable form of osteopetrosis observed in grey-lethal mice and in affected human patients⁷².

The acidic dissolution of apatite crystals during bone resorption releases substantial amounts of calcium, phosphate, and bicarbonate ions from the mineralized matrix. These ions must be efficiently removed from the resorption lacuna to allow the resorption process to continue effectively. In particular, bicarbonate represents a potential problem, as it can rapidly neutralize the acidic environment required for mineral solubilization (**Figure 9**). Studies by Riihonen⁷³ and Bouyer⁷⁴ demonstrated that the electroneutral sodium–bicarbonate cotransporter NBCn1 plays a key role in exporting HCO_3^- ions away from the lacuna, thereby maintaining the necessary low pH.

The generation of protons needed by the V-H^+ -ATPase in osteoclasts is driven by the enzyme cytosolic carbonic anhydrase II (CAII). This enzyme catalyzes the reversible hydration of CO_2 to form carbonic acid (H_2CO_3), which spontaneously dissociates into protons (H^+) and bicarbonate ions (HCO_3^-). The H^+ ions are then utilized by the V-H^+ -ATPase to acidify the resorption lacuna, while the HCO_3^- ions are expelled through an anion exchanger ($\text{HCO}_3^-/\text{Cl}^-$) located in the basolateral membrane of osteoclasts. This exchange process not only prevents excessive alkalinization of the cytosol but also provides chloride ions required for ClC-7 function at the ruffled border.

The energy (ATP) and carbon dioxide (CO_2) necessary for these metabolic and transport processes are supplied by the numerous mitochondria found within osteoclasts, reflecting the high energetic demands of bone resorption³⁶.

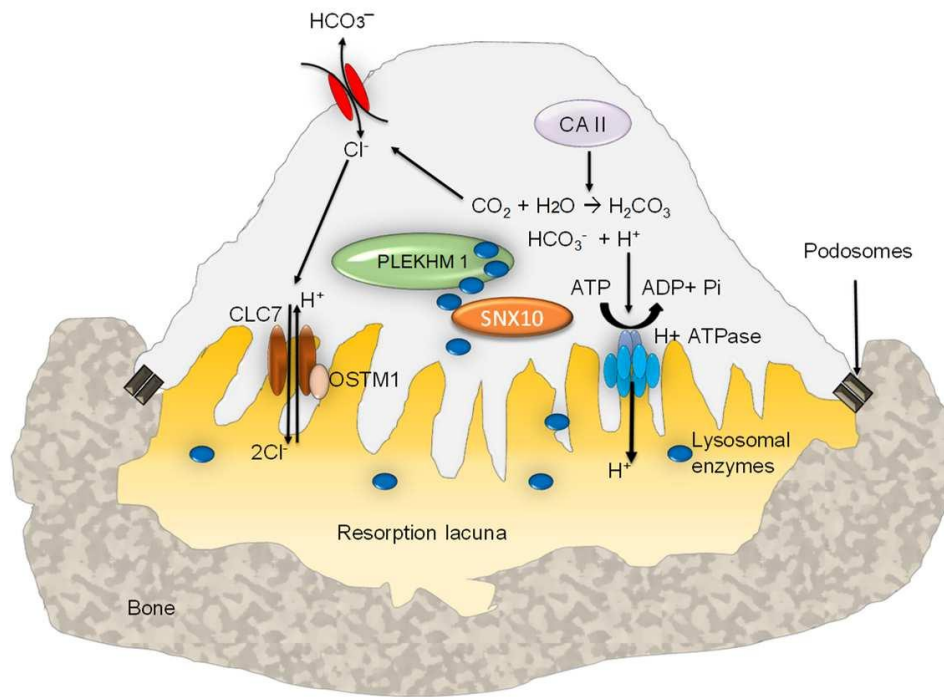


Figure 9. Osteoclast bone resorption. Cartoon illustrating the molecular mechanism involved in bone resorption. (Source: Maurizi and Teti⁶³).

The dissolution of mineral crystals during bone resorption exposes the collagen-rich organic matrix, making it accessible to proteolytic enzymes. Current evidence indicates that two major families of enzymes—the matrix metalloproteinases (MMPs) and the lysosomal cathepsins—play central roles in the extracellular degradation of the bone matrix. Among these, cathepsin K stands out as a crucial collagen-degrading enzyme. It is highly expressed in osteoclasts and secreted into the resorption lacuna, where it functions under acidic conditions to cleave insoluble type I collagen⁷⁵. Inhibition of cathepsin K activity, both *in vitro* and *in vivo*, effectively blocks matrix degradation⁷⁶, underscoring its pivotal role in this process. Moreover, genetic deletion of the cathepsin K gene in mice and humans leads to pycnodysostosis, a condition characterized by abnormally dense and brittle bones^{77,78}.

Osteoclasts also produce cystatin B, a natural inhibitor of cysteine proteases, and contain high levels of acid phosphatases, most notably tartrate-resistant acid phosphatase (TRAcP), which serves as a well-established biochemical marker of osteoclasts⁷⁹. TRAcP possesses two distinct enzymatic functions: besides acting as a phosphatase, it can also generate reactive oxygen species (ROS) through the Fenton reaction. The ROS produced by TRAcP contribute to collagen breakdown and may play a role in the intracellular degradation of resorption products after they have been internalized⁸⁰.

In contrast, the precise contribution of MMPs to osteoclast-mediated bone resorption remains a matter of debate. Although several MMPs are expressed in bone tissue, only a few most notably MMP-9 and MMP-14 are synthesized by osteoclasts themselves⁸¹.

Following extracellular digestion, the degradation residues are internalized by the osteoclast, transported across the cell through transcytosis, and subsequently released at the basolateral functional secretory domain. Alternatively, these products may be discharged during temporary disassembly of the sealing zone, completing the bone resorption cycle.

1.6 Bone Remodeling

To preserve its strength and structural integrity, bone continuously undergoes a remodeling process. Even after skeletal growth and shaping are complete, roughly 20% of the surface of cancellous (spongy) bone is actively remodeled at any moment, with approximately 8-10% of the entire bone tissue being renewed annually. This remodeling process is essential for replacing old or damaged bone with fresh, mechanically competent bone^{82,83}. Disruptions in bone remodeling are implicated in a wide range of common and rare human diseases, including osteoporosis, periodontitis, arthritis, tumor-related bone degradation, osteogenesis imperfecta, and osteopetrosis.

Bone remodeling is a highly complex and tightly regulated process influenced by numerous biochemical signals and mechanical forces. It involves two primary stages: bone resorption carried out by osteoclasts, followed by bone formation performed by osteoblasts. These specialized cells work together within structures known as basic multicellular units (BMUs)⁸⁴. The remodeling cycle proceeds through distinct sequential phases: (i) resorption, (ii) reversal, and (iii) formation. Although the exact signal that initiates remodeling remains unknown, research suggests that mechanical stresses sensed by osteocytes play a critical role in triggering changes in local bone structure⁸⁵.

i. Resorption phase: In this initial phase, stromal cells or bone lining cells secrete chemokines-such as monocyte chemoattractant protein-1 (MCP-1) -to recruit osteoclast precursors to the remodeling site. MCP-1 production is stimulated by parathyroid hormone (PTH) as well as inflammatory cytokines like tumor necrosis factor-alpha (TNF- α) and interleukins 1 β and 6. Osteoclasts then degrade the bone matrix using specialized enzymes, releasing breakdown products of type I collagen including N-terminal (NTx) and C-terminal (CTx) telopeptides, along with cross-linked molecules like deoxypyridinoline (DPD). Additionally, during resorption, growth factors stored in the bone matrix-such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), and

transforming growth factor-beta (TGF- β) -are released. These factors help signal the recruitment and differentiation of osteoblasts, shifting the process from bone breakdown to bone rebuilding^{85,86}.

ii. Reversal phase: Following resorption, the remodeling enters a reversal stage. The precise regulatory mechanisms that halt osteoclast activity are not fully understood but may involve apoptosis (programmed cell death) of osteoclasts triggered by extensive bone degradation and the release of calcium ions, TGF- β , and related peptides from the matrix. These molecules could deactivate osteoclasts while simultaneously attracting osteoblasts to the site^{84,85}. During this phase, osteoclasts disappear and macrophage-like cells emerge on the bone surface. These cells are believed to secrete factors that suppress osteoclast function and promote osteoblast activity, as well as help clear residual matrix debris due to their elevated collagenase content compared to osteoclasts.

iii. Formation phase: The final phase centers on the activation and differentiation of osteoblast precursors, potentially stimulated by molecules secreted by osteoclasts (often referred to as clastokines), including sphingosine-1-phosphate (S1P), myb-induced myeloid protein-1 (mim-1), platelet-derived growth factor homodimer (PDGF-BB), and hepatocyte growth factor (HGF)⁸⁷. Once activated, osteoblasts synthesize and deposit new bone matrix, fully replacing the resorbed bone. Upon completion of remodeling, some osteoblasts become embedded within the newly formed matrix and differentiate into osteocytes, while others remain on the bone surface as lining cells, maintaining bone homeostasis until the next remodeling cycle begins^{84,85}.

2.Osteopetrosis

2.1 Definition

Osteopetrosis refers to a group of rare genetic disorders characterized by increased bone density resulting from impaired osteoclast function⁸⁸. First identified in 1904 by Albers-Schönberg, the condition was originally described as “marble bone disease” due to the bones’ abnormally dense yet fragile appearance^{89,90}. The underlying defect in osteoclast activity leads to an accumulation of bone mass while simultaneously compromising its mechanical integrity, making bones more susceptible to fractures.

Osteoclast-mediated bone resorption plays a critical role during skeletal growth and development. It is essential not only for normal bone remodeling but also for replacing the mechanically weak primary woven bone with structurally superior secondary lamellar bone. This process ensures appropriate mechanical strength and structural functionality. Additionally, bone resorption is crucial for the expansion of medullary cavities and the proper formation of foramina through which nerves pass. Consequently, osteopetrosis is commonly associated with clinical features such as short stature, skeletal deformities, and frequent pathological fractures, as well as severe hematological and neurosensory complications^{70,88}.

Several forms of osteopetrosis have been identified, varying widely in severity—from life-threatening infantile forms to milder adult-onset variants and demonstrating different patterns of genetic inheritance. Collectively, osteopetrosis has an estimated incidence of more than 1 in 100,000 individuals in the general population, though certain ethnic groups may exhibit a higher prevalence⁹¹.

At present, no definitive cure exists for osteopetrosis. The only potentially curative treatment for the most severe forms is hematopoietic stem cell transplantation (HSCT). However, this therapeutic approach is not universally successful. Approximately 50% of patients undergoing HSCT experience graft failure or serious complications. Despite these challenges, HSCT remains the most effective intervention for the malignant forms of the disease and can significantly improve quality of life, particularly in patients who do not present with neurodegeneration or cognitive impairment⁹².

2.2 Clinical and Radiologic features

Clinically, osteopetrosis can be classified into three major forms based on severity: lethal, intermediate, and mild.

The lethal form is represented by Autosomal Recessive Osteopetrosis (ARO), also referred to as infantile or malignant osteopetrosis. This variant follows an autosomal recessive inheritance pattern and has an estimated incidence of approximately 1 in 200,000 to 1 in 300,000 live births⁸⁸. Radiologically, ARO is characterized by generalized skeletal sclerosis, affecting virtually all parts of the skeleton including the skull, spine, pelvis, and long bones with a hallmark "bone-in-bone" appearance⁹³. This radiologic sign reflects a dense bone structure enclosing a smaller, secondary bone formation within, a direct consequence of severely impaired bone resorption.

Due to defective osteoclast function, critical anatomical structures fail to develop normally or are subjected to pathological compression. This includes narrowing of bone marrow cavities, reduced skull space, and constricted cranial nerve foramina, all of which have severe clinical implications (**Figure 10 A**)⁹⁴. The most life-threatening complications stem from bone marrow failure, resulting in ineffective hematopoiesis. To compensate, extramedullary hematopoiesis may occur, often leading to hepatosplenomegaly.

Children affected by ARO typically exhibit a drastically reduced life expectancy, with most patients succumbing to complications such as severe anemia, recurrent infections, or sepsis within the first 3 to 4 years of life. Furthermore, visual and auditory impairments, including blindness and deafness, are common and usually result from compression of cranial nerves due to excessive bone growth^{70,88}.

In addition, a specific subtype of ARO presents with neurological involvement, including primary degeneration of the brain and retina, which is attributed to an underlying lysosomal storage disorder. This variant is associated with even more severe clinical outcomes due to the combined effects of skeletal, hematological, and neurological dysfunction^{64,95,96}.

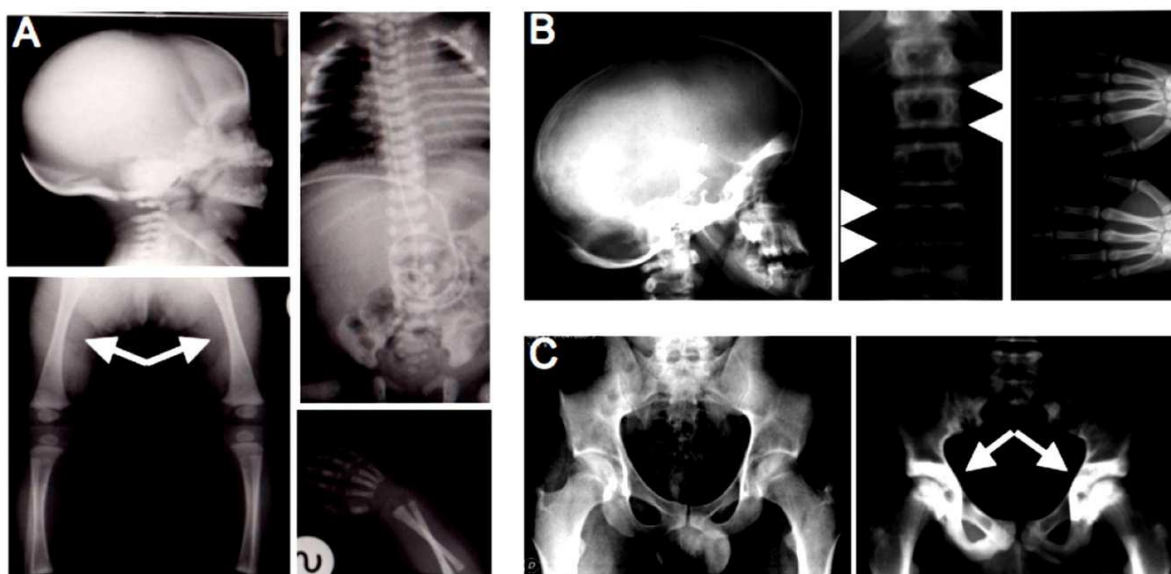


Figure 10. X-Ray analyses of osteopetrotic patients. (A) X-Ray images of ARO patients showing extensive sclerosis of the skull, spine, pelvis and appendicular bones with obliterated femoral cavities (arrows). (B) ADO2 patients presented with osteosclerosis, especially at the skull base (arrow), vertebral endplates (arrowheads) and appendicular bones. (C) Typical sclerosis at the pelvis (arrows) in an ADO2 patient (right panel) compared to a normal pelvis (left panel). (Source: Del Fattore et al., 2009⁹⁴).

The intermediate form of osteopetrosis, known as Intermediate Recessive Osteopetrosis (IRO), presents with milder clinical features compared to Autosomal Recessive Osteopetrosis (ARO) and is generally compatible with long-term survival. This form is extremely rare, with an undefined prevalence, and approximately 50 cases have been reported in the literature to date⁹⁷.

Typical clinical features of IRO include short stature, generalized bone sclerosis, dental abnormalities, and a predisposition to jaw osteomyelitis. However, the clinical presentation is highly heterogeneous, and some patients exhibit more severe manifestations. These may include renal tubular acidosis, cranial nerve compression syndromes, intracranial calcifications, developmental delays, moderate anemia, and intellectual disability^{97,98}.

The mildest form of osteopetrosis is represented by Autosomal Dominant Osteopetrosis (ADO). Although traditionally subdivided into Type 1 (ADO1) and Type 2 (ADO2)⁹⁹, it is now understood that ADO1 does not result from osteoclast dysfunction, but rather from altered osteoblast activity. As such, ADO1 is classified as a high bone mass syndrome rather than a true form of osteopetrosis.

In contrast, ADO2—the classical Albers-Schönberg disease—is caused by osteoclast dysfunction and is the only form of autosomal dominant osteopetrosis considered a genuine osteopetrotic disorder. ADO2 has an estimated prevalence of 1 in 20,000 live births and is characterized by incomplete

penetrance, affecting only about 66% of individuals carrying the mutation. Therefore, a significant portion of mutation carriers (over 30%) remain asymptomatic^{99,100}.

Despite being referred to as "benign osteopetrosis", ADO2 exhibits marked phenotypic variability, ranging from completely asymptomatic individuals to patients with severe complications—even among family members sharing the same genetic mutation¹⁰¹. Radiographically, ADO2 is characterized by localized (focal) sclerosis, particularly involving the skull base, pelvis, and vertebral endplates. The vertebral bodies often show dense sclerotic bands at the subendplate regions, leading to the classic "sandwich vertebrae" appearance. When multiple vertebrae are affected in this manner, the spine displays the so-called "rugger-jersey spine" pattern, with alternating zones of sclerosis and relative lucency^{99,102-104}.

Clinically, ADO2 patients frequently experience bone pain, low-energy fractures, and episodes of osteomyelitis (**Figure 10 B-C**). In more severe cases, cranial nerve compression may occur, leading to sensory deficits. Although early mortality is rare, the disease can still significantly impact quality of life, with some individuals becoming wheelchair-dependent due to skeletal complications⁸⁸.

Beyond these three classical forms of osteopetrosis, a novel clinical phenotype has recently been described, known as OL-EDA-ID syndrome, which combines osteopetrosis with ectodermal dysplasia, lymphedema, and immunodeficiency. Due to its X-linked inheritance, this extremely rare condition is also referred to as X-linked Osteopetrosis (XLO). To date, only five male patients have been documented with this syndrome. All presented with severe systemic manifestations and died at an early age due to infection-related complications, underscoring the gravity of the associated immunodeficiency¹⁰⁵.

Type of osteopetrosis	Acronym	Radiographic features	Clinical features
Autosomal recessive	ARO	• Dense bone with reduced or obliterated medullary cavity • Diffuse sclerosis • Bone-in-bone appearance ▪ Spine ▪ Pelvis ▪ Long bones • Misshape of the metaphysis • Incomplete dentation • Hypertelorism with 'hair-on-end' appearance of the calvarial bone	• Bone marrow failure • Anemia • Pancytopenia • Thrombocytopenia • Impaired blood clotting • Susceptibility to infections • Osteomyelitis • Hepatosplenomegaly • Hypocalcemia • Hyperparathyroidism • Stunted growth • Pain • Multiple low energy fractures • Impaired vision and hearing • Obstructive hydrocephalus • Nasal sinus abnormalities • Impairment of tooth eruption
Intermediate	IRO	• Similar to ARO but less severe	• All ARO symptoms but less severe • Renal tubular acidosis • Cerebral calcification • Mental retardation
Autosomal dominant	ADO2	• Focal sclerosis ▪ Skull basis ▪ Vertebral bodies ▪ Pelvis • Sandwich vertebrae • Rugger-jersey spine	• Multiple low energy fractures • Anemia • Mandibular osteomyelitis • Dental problems • Pain • Hypovision • Hypoacusis • Cognitive failures (rare cases)

Table1. Descriptive table with the radiological and clinical features of different type of osteopetrosis⁷⁰.

2.3 Histological features

As previously mentioned, osteoclast dysfunction is the hallmark of osteopetrosis. Due to their inability to properly resorb bone matrix, osteoclasts in osteopetrotic patients fail to maintain normal bone turnover, resulting in bones that, despite being abnormally dense, are paradoxically fragile. This fragility is largely attributed to the persistence of mineralized cartilage within the bone structure. Specifically, large regions of non-remodeled mineralized primary spongiosa remain embedded in the mature bone, often forming islands or trabecular bars of calcified cartilage, which severely compromise the bone's mechanical properties (**Figure 11**).

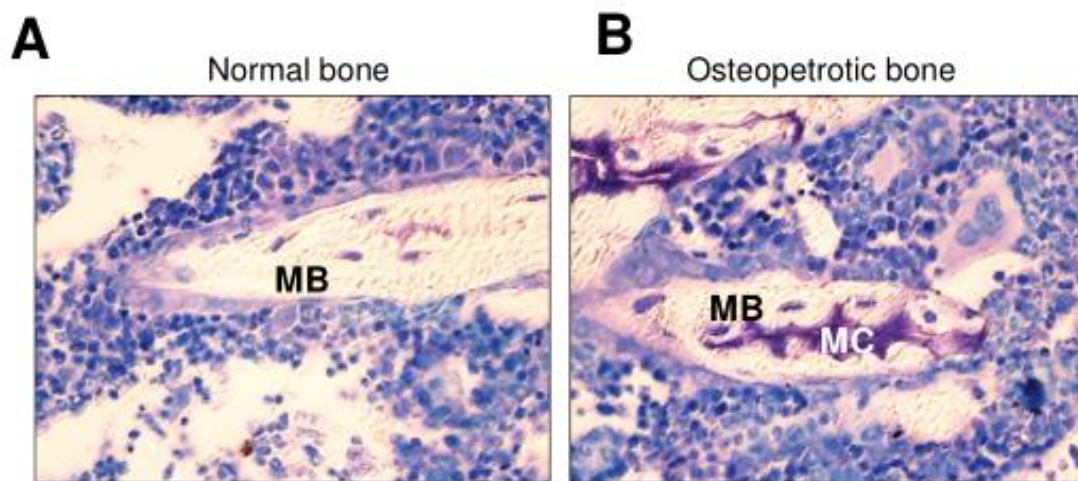


Figure 11. Non-remodelled mineralised cartilage in osteopetrotic bone. Toluidine blue stained histological section of tibia harvested from (A) wildtype and (B) ADO2 mice. MB: mineralized bone. MC: mineralized cartilage. Scale bar: 20μm

A notable histological feature in osteopetrosis is the variability in osteoclast number and morphology observed in bone biopsies. Based on these observations, osteopetrosis can be broadly categorized into two forms: osteoclast-rich and osteoclast-poor subtypes¹⁰⁶.

In osteoclast-rich osteopetrosis, patients typically exhibit a high number of osteoclasts, which are often enlarged. However, despite their apparent abundance, these osteoclasts may exhibit functional impairments-such as absence of ruffled borders or reduced bone-resorbing capacity. These dysfunctional cells can adhere to bone surfaces but are ineffective at matrix degradation^{88,107}.

The increased number of osteoclasts in many of these patients is often linked to elevated parathyroid hormone (PTH) levels, a compensatory response to hypocalcemia. PTH stimulates osteoblasts to produce RANKL, a key cytokine promoting osteoclast differentiation and activation. However, in Autosomal Dominant Osteopetrosis Type 2 (ADO2), PTH levels are typically within the normal range, despite the presence of numerous osteoclasts¹⁰⁸ (**Figure 12**). This paradox may be explained by an increased lifespan of osteoclasts, rather than enhanced osteoclastogenesis^{109,110}.

However, in a small number of cases, osteoclasts are absent in bone biopsies (osteoclast-poor)^{103,107,111}. This condition may result from a defect in RANKL production or a failure of osteoclast precursors to respond to RANKL signaling. In the former case, the dysfunction lies within the osteoblast lineage or other RANKL-producing cells, while in the latter, it is due to mutations in the RANK receptor on osteoclast precursors, preventing proper osteoclast differentiation. Both mechanisms ultimately lead to a failure in osteoclastogenesis.

Interestingly, bone marrow histology differs significantly between these two subtypes. In osteoclast-rich ARO, the bone marrow environment is typically fibrotic, rendering it inefficient for normal hematopoiesis (**Figure 12**). On the other hand, in osteoclast-poor osteopetrosis, the bone marrow is often less fibrotic and more functionally competent, though the reasons behind this distinction remain unclear.

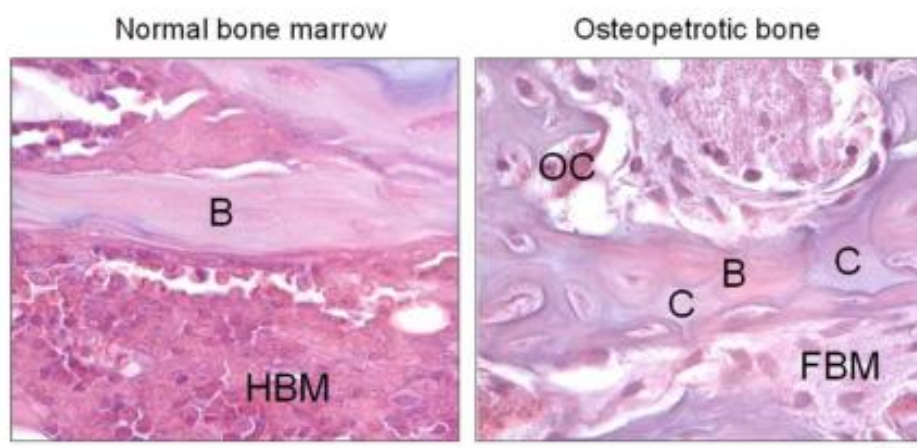


Figure 12. Bone marrow fibrosis. Histological section of wild type and osteoclast-rich ARO mice stained with haematoxylin and eosin showing the bone marrow fibrosis. B: bone. HBM: hematopoietic bone marrow. C: cartilage. FBM: fibrotic bonemarrow. OC: osteoclasts. Scal bar 20µm. (Source:adapted from Maurizi and Teti⁷⁰).

2.4 Genetics of Osteopetrosis

The significant phenotypic variability observed in osteopetrosis is largely due to its notable genetic heterogeneity. Several genes have been identified as contributing to the disease's development, although approximately 30% of patients still do not have a confirmed genetic diagnosis¹¹². In autosomal recessive osteopetrosis (ARO), around 50% of cases are caused by loss-of-function mutations in the *TCIRG1* gene. This gene encodes the osteoclast-specific $\alpha 3$ subunit of the vacuolar H^+ -ATPase, which is crucial for acidifying the resorption lacuna⁶⁹.

Other genes have also been linked to ARO. The second most common form is associated with biallelic loss-of-function mutations in the *CLCN7* gene, accounting for about 10–15% of cases^{64,113}. The *CLCN7* gene encodes the ClC-7 chloride/proton antiporter, which facilitates the exchange of chloride ions into the resorption lacuna and protons into the cytoplasm at a ratio of $2Cl^-: 1H^+$ ⁶⁵. This transporter is located in both the lysosomal membrane and the ruffled border of osteoclasts, where it helps maintain charge balance during vesicle acidification and bone resorption, functioning as an essential electrical shunt. Patients with *CLCN7* mutations exhibit impaired bone resorption due to disruption of this mechanism.

Mutations in the *OSTM1* (Osteopetrosis-associated TransMembrane protein 1) gene have been identified in about 5% of ARO cases^{114,115}. *OSTM1* encodes a single-pass transmembrane protein that serves as the β -subunit of ClC-7 and is vital for its proper localization and function. ARO caused by mutations in *OSTM1*- and to a lesser extent, *CLCN7*- is frequently associated with primary degeneration of the brain and retina, which resembles features of lysosomal storage disorders¹¹⁵.

Another gene implicated in ARO is *SNX10*¹¹⁶, which encodes sorting nexin 10, a protein involved in intracellular trafficking and the maintenance of endosomal homeostasis. Mutations in *SNX10* are found in about 4% of ARO patients¹¹⁶. The *SNX10* protein plays a role in the correct subcellular positioning of the V- H^+ -ATPase. Complete loss of SNX10 function results in a condition resembling both osteopetrosis and rickets (termed osteopetrorickets or osteopetromalacia), due to defective osteoclast function and impaired calcium absorption caused by a lack of gastric acid production¹¹⁷.

A milder variant of ARO has been linked to mutations in the *PLEKHM1* gene, which encodes the *Pleckstrin Homology Domain Containing Family M Member 1* protein. In one family, a severely truncated version of this gene was associated with disease¹¹⁸. Additionally, a heterozygous mutation (R714C) in *PLEKHM1* was found in a patient with generalized bone loss (osteopenia) but localized bone hardening (osteosclerosis), further indicating the gene's relevance in bone metabolism¹¹⁹.

Although its exact role in bone resorption is not yet fully understood, *PLEKHM1* appears to be involved in Rab7-mediated late endosomal trafficking, acidification of vesicles, and the release of tartrate-resistant acid phosphatase (TRAcP) by osteoclasts^{118,119}. Given the importance of vesicle trafficking in lysosome function and the formation of the osteoclast ruffled border, it is reasonable to conclude that loss-of-function mutations in *PLEKHM1* negatively affect bone resorption.

In addition to the more common forms of autosomal recessive osteopetrosis (ARO) characterized by abundant but dysfunctional osteoclasts, there also exists a particularly rare subtype known as osteoclast-poor ARO. In these cases, mutations have been identified in the *TNFSF11* and *TNFRSF11A*^{111,120} genes, which encode the osteoclast-stimulating molecule RANKL and its receptor RANK, respectively. The disruption of the RANKL-RANK signaling pathway in these patients directly accounts for the failure of osteoclast development.

Currently, mutations in *TNFSF11* account for about 5% of ARO cases. Since *TNFSF11* is expressed by various cells, including lymphocytes and osteoblasts—but not by osteoclasts or their precursors—this mutation holds significance not only for diagnosis but also for treatment strategies. Notably, patients with RANKL deficiency do not respond to hematopoietic stem cell transplantation (HSCT). Potential therapeutic alternatives include administration of soluble RANKL or transplantation of mesenchymal stem cells (MSCs) derived from bone marrow. However, these treatments are still experimental and not yet available for clinical use in humans.

RANK-deficient ARO, which presents with similar clinical features to RANKL-deficient ARO, was initially described in two siblings from a Turkish family. These patients exhibited a combination of osteopetrosis and hypogammaglobulinemia, resulting from defective development of immunoglobulin-producing B cells¹²⁰. Despite its phenotypic resemblance to RANKL-related osteopetrosis, this form is treatable with HSCT.

Another rare and severe form of osteopetrosis is X-linked osteopetrosis (XLO), caused by mutations in the *NEMO* gene (NF-κB Essential Modulator), which encodes a key regulatory subunit of the IκB kinase (IKK) complex. In affected individuals, a mutation replaces the normal stop codon with a codon for tryptophan, leading to the addition of 27 non-functional amino acids at the protein's C-terminal end. This alteration significantly destabilizes the NEMO protein, resulting in markedly reduced protein levels and contributing to the disease's complexity and severity¹⁰⁵.

The most common form of osteopetrosis overall is autosomal dominant osteopetrosis (ADO). Among its subtypes, ADO1 is no longer considered a true form of osteopetrosis but rather a high bone mass disorder. It results from mutations in the *LRP5* gene, which is expressed by osteoblasts.

In contrast, ADO2 is a genetically heterogeneous condition, with approximately 70% of cases attributed to mutations in the *CLCN7* gene. These mutations are typically heterozygous, dominant-negative, and often missense in nature. They are distributed throughout the gene, though many cluster in the C-terminal CBS (Cystathionine Beta Synthase) domains, which are considered a mutational hotspot. Despite this, about 30% of ADO2 patients still lack a clear genetic diagnosis^{101,103,113}.

Importantly, ADO2 shows incomplete penetrance (around 66%), and there is no straightforward correlation between genotype and clinical presentation. In fact, identical mutations in *CLCN7* can lead to widely variable clinical outcomes¹¹¹. This phenotypic variability may be influenced by single nucleotide polymorphisms (SNPs) in *CLCN7*, other genetic modifiers¹²¹, or instability of the mutant protein⁹⁴.

A similar degree of clinical variability is seen in intermediate recessive osteopetrosis (IRO), again without a clear genotype–phenotype relationship. In some IRO patients, mutations are found in the *CAII* gene, which encodes carbonic anhydrase II—an enzyme^{97,98} that accelerates the conversion of carbon dioxide and water into carbonic acid. Clinically, this form is distinguishable by symptoms such as renal tubular acidosis, brain calcifications, and intellectual disability. The intermediate severity of this form may be due to the fact that some spontaneous carbonic acid production still occurs in osteoclasts, allowing for residual bone resorption. Notably, *CAII* was the first gene ever linked to osteopetrosis, marking a milestone in the genetic understanding of the disease⁹⁸.

2.5 Current therapies for Osteopetrosis

Due to the inherent complexity and genetic diversity of osteopetrosis, there is currently no universal or definitive treatment for the disease.

At present, hematopoietic stem cell transplantation (HSCT) remains the only curative option for patients with autosomal recessive osteopetrosis (ARO)¹²². In contrast, no curative treatment is available for autosomal dominant osteopetrosis type 2 (ADO2)⁹². However, HSCT carries significant risks and is considered a high-risk intervention. Patients undergoing this procedure face the possibility of serious complications, including graft failure and early post-transplant adverse events, which can result in high mortality rates. Additionally, hypercalcemia is a common side effect post-transplantation and often requires management with anti-resorptive medications, such as bisphosphonates⁹⁷.

Importantly, HSCT is not suitable for patients with RANKL-dependent ARO, because the genetic defect lies in non-hematopoietic cells, specifically osteoblasts, which derive from the mesenchymal lineage. As such, HSCT cannot correct this form of the disease. A promising alternative for these patients might be mesenchymal stem cell (MSC) transplantation, but this therapy is not yet available in clinical practice.

On the other hand, ARO caused by mutations in *CLCN7* or *OSTM1* may, in principle, be treated with HSCT, as the defects affect the hematopoietic lineage. However, in *OSTM1*-related ARO, the profound neurological deterioration, particularly affecting the brain and retina, cannot be reversed by HSCT, limiting the treatment's overall effectiveness⁹².

Despite these challenges, recent advancements have improved HSCT outcomes, with success rates now reaching up to 60%, especially when HLA-matched donors are available.

For patients who are not eligible for transplantation, several alternative therapeutic approaches have been explored. These include attempts to stimulate osteoclast activity using calcium-restricted diets, calcitriol, steroids, parathyroid hormone (PTH), and interferon-gamma (IFN- γ). While some of these methods have shown potential in slowing disease progression or partially correcting the phenotype, their effectiveness has been limited, particularly in infants and young children¹²³.

Currently, patients with ADO2 are managed with supportive (palliative) care, as no effective treatment exists. This includes blood transfusions, orthopedic surgeries to address fragility fractures, pain management with analgesics, and antibiotic therapy to treat osteomyelitis, which commonly occurs after surgical procedures⁸⁸. Trials involving PTH, vitamin D3, and calcium supplementation have so far failed to demonstrate therapeutic benefit⁸⁸.

Given these limitations, there is a pressing need for the development of new and more effective treatment strategies for patients affected by osteopetrosis.

3. Aim of the work

As discussed in the introduction ADO2 is a heterogeneous disease ranging from asymptomatic carriers to severe clinical manifestations that may lead to death.

To date, there is not a resolutive therapy for the disease and the ADO2 patients experience a reduced quality of life.

Moreover, the gene implicated in the pathogenesis of the ADO2, the CLCN7 gene, is expressed in many tissues, suggesting that mutations on this gene could have a systemic impact.

An essential tool for studying the disease and developing a potential therapy, is represented by the ADO2 animal model developed in our laboratory in 2013. This mouse model, carrying the Clcn7 G213R mutation, homologue of the human CLCN7 G215R mutation, fully recapitulates the bone phenotype observed in the ADO2 patients.

Starting from the above-mentioned background and taking advantage of this animal model, my PhD project was based primarily on data obtained from previous work conducted by our research group, concerning the use of RNA interference therapy for Autosomal Dominant Osteopetrosis Type 2 and progress toward the preclinical development (**Chapter 2**).

Next, we will analyse the main study conducted during my PhD studies concerning Novel hybrid silicon-lipid nanoparticles deliver a siRNA to cure autosomal dominant osteopetrosis in mice. Implications for gene therapy in humans (**Chapter 3**).

Further details about our scientific rationale are explained in the next Chapters, along with the results obtained. In particular, in chapter 2 our results showed that the use of a mutation-specific siRNA is able to rescue the bone phenotype of the ADO2 mice in different experimental conditions. While, in chapter 3 has been demonstrated that SiSaf's silicon stabilised hybrid lipid nanoparticles (sshLNPTTM) constitutes a next-generation non-viral vectors able to retain both integrity and stability of constructs over time and offering considerable payloads of biologicals without requiring any cold-chain distribution. Finally, in **Chapter 4**, general discussion and conclusions are presented.

4.References

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Chapter 2: “RNA interference therapy for autosomal dominant osteopetrosis type2. Towards the preclinical development.”

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1. Abstract

Autosomal Dominant Osteopetrosis type 2 (ADO2) is a rare bone disease characterized by dense and brittle bones due to impairment of osteoclast bone resorption. Dominant negative mutations of the CLCN7 gene affect about 70% of ADO2 patients. ADO2 has no cure and our recent work established that it is suitable for gene silencing by a specific small interfering RNA that does not affect the normal mRNA, thus inducing a condition of pseudo-haplosufficiency and rescuing the bone phenotype. We performed a systematic study to test the likelihood that the therapy could progress towards clinical trials, treating Clcn7 G213R/WT ADO2 mice with Clcn7 G213R -specific siRNA and investigating the bone phenotype, by μ CT and histomorphometry, and safety, by histopathology and serology. We demonstrated that our Clcn7 G213R siRNA is not only effective in pre-pubertal ADO2 male mice as we showed in our previous study, but also in adult and ageing mice, in males and females, by intraperitoneal and subcutaneous administration. Furthermore, the study showed safety also after prolonged chronic administration and allowed to identify specific end-points to be potentially used in clinical trials. These results might plot the direction to progress towards regulatory toxicity studies, through which the therapy, that is patent-protected, can obtain approval from public health authorities for the transition to the Phase I/II clinical trials. The study also suggests that similar strategies could be applied to other autosomal dominant bone diseases, opening an avenue for a wider use of the RNA interference therapy in rare genetic disorders.

2. Introduction

The treatment of genetic diseases is extremely challenging and several of them have currently no cure. Precision medicine is likely to represent the way genetic diseases will be treated in the years to come ^{1,2}. However, it cannot be applied until new means to eliminate the deleterious effects of the mutant genes are identified.

Autosomal dominant inheritance is the most frequent means for the transmission of an abnormal genetic trait to the offspring ^{3,4}. Dominant mutant subunits are often altered in their conformation and make complexes with the normal subunits, impairing the function of multimeric proteins ⁵. In our previous work, we reasoned that interfering with the mutant mRNA would remove this altered RNA species, generating less abundant but fully functional proteins, plenty, in case of gene haplosufficiency, to rescue a nearly normal phenotype ⁶.

Paradigmatic of autosomal dominant diseases is ADO2 (Autosomal Dominant Osteopetrosis type 2) ⁷. In most cases, the gene implicated is *CLCN7*, encoding a homodimeric proton/chloride antiporter (*ClC7*) widely spread in many tissues ^{8,9}. However, the most prominent phenotype in this disease is with the bone and is caused by the reduced function of osteoclasts ^{10–13}. These cells remove old and damaged bone that is then replaced by new, fresh and functional bone, thus contributing to fulfil the remodeling of the tissue and keep the skeleton healthy ¹⁴. The osteoclast bone resorption mechanism requires the acidification of the extracellular space located between the cell and the bone matrix, called resorption lacuna. This acidification occurs through a V-type proton pump and requires to be charge balanced by the release of chloride ¹³. The *ClC7* accomplishes this function and its mutations lead to i) infantile severe osteopetrosis in homozygous status, ii) haplosufficiency and healthy carrier phenotype in the case of heterozygous loss-of-function mutations, and iii) ADO2 in the case of heterozygous dominant negative mutations ^{15–17}. This latter form of osteopetrosis is milder but still can be strongly debilitating, resulting in dense and fragile bones, with recurrent low energy fractures that can be very frequent (tens lifelong), difficult to heal and prone to develop osteomyelitis ¹⁸. Notably, this disease has currently no cure.

Because of the dominant negative nature of ADO2 mutations, our therapeutic strategy of RNA interference was very successful and the bone phenotype of our mouse model of *Clcn7* G213R/WT ADO2 was fully rescued by the treatment ⁶. *Clcn7* G213R is the mouse homologous of the most frequent ADO2 missense mutation in humans (*CLCN7* G215R). Our ADO2 mice were systemically treated with a highly effective and specific *Clcn7* G213R small interference (si)RNA that also

efficiently and selectively recognized the human mutant gene ⁶. This siRNA strongly downregulated the mutant transcript without altering the normal mRNA, thus mimicking the predicted condition of pseudo-haplosufficiency and rescuing the bone phenotype ⁶.

Our previous work focused on pre-pubertal male ADO2 mice, which achieved a bone mass similar to the wildtypes in 4 weeks of siRNA treatment. However, ADO2 is known to affect individuals at any age and it is not infrequent that the diagnosis is received during adolescence or adulthood ¹⁸. Furthermore, we believe that this therapy has the potential to move to pre-clinical and clinical developments. Therefore, we were prompted to design a comprehensive and systematic study to assess as many variables as possible and allow the therapy to progress towards regulatory toxicity studies that are indispensable to receive the Orphan Drug Designation and the approval from public health authorities for the transition to the Phase I/II clinical trials. Our results demonstrated that our Clcn7G213R siRNA is not only effective in pre-pubertal ADO2 male mice, but also in adult and ageing mice, in males and females, upon intraperitoneal (i.p.) and subcutaneous (s.c.) administration. Furthermore, the study allowed to identify end-point parameters to be potentially used in future clinical trials and showed safety also after prolonged chronic administration. Given the relevance of these results, we can predict that similar strategies can be adopted for other autosomal dominant bone diseases, thus opening up an avenue for a wider use of the RNA interference therapy in rare genetic disorders.

3. Material and Methods

3.1 Materials

Scrambled siRNA and siRNAs specific for the Clcn7 G213R mutant 6 (Patent application PCT/IB2015/053730, publication number WO2015177743 A1) were custom-made from GE Dharmacon (Lafayette, CO, USA). Plastic ware was from Falcon Becton-Dickinson (Cowley, Oxford, UK). Trizol® reagent and primers were from Invitrogen (cat# 15596026, Carlsbad, CA). RevertAid H Minus First Strand cDNA Synthesis Kit was from Thermo Scientific (cat# K1631, Waltham, Massachusetts, USA). The SensiMix™ SYBR® kit was from Bioline (cat# QT605, London, UK). The transfection reagent in vivo-jetPEI® was from Polyplus-Transfection (cat# 201-50G, Illkirch, France). The RatLaps EIA kit (cat# AC-02F1) for CTX detection was from Immunodiagnostic Systems (Gaithersburg, MD, USA). The Reflotron® kits were from Roche Diagnostics (Manheim, Germany). Biodent probes (type BP2) were provided by Biodent Hfc, Active Life Scientific (Santa Barbara, CA). Unless indicated otherwise, all the other reagents were of the purest grade from Sigma Aldrich Co. (St. Louis, MO, USA).

3.2 Animals

The Clcn7 G213R/WT (ADO2) mouse model (Mus Musculus, C57/BL6 background) has been described by Alam et al.¹². This ADO2 animal model carries the mouse homologue of the most frequent human ADO2 mutation, CLCN7 G215R, recapitulating the human ADO2 phenotype. The CLCN7 G215R mutant mRNA is recognized and downregulated by the Clcn7 G213R siRNA used in the study⁶. All the in vivo experiments were conducted in agreement with the national and international guidelines and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 4.03.2014, n.26, Gazzetta Ufficiale della Repubblica Italiana no. 61, March 4, 2014) and were approved by the Italian Ministry of Health (approval n. 564/2016-PR). The study was performed according to the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines, upon randomization of the mice, with usually >5 mice/group, unless otherwise stated. Blinding was not undertaken given that in vivo treatments could be performed only by personnel who was aware of the experimental settings. However, experiments were randomly blinded reevaluated by a person who was not aware of the experimental conditions, who confirmed the accuracy of the results. Mice were humanely sacrificed by CO₂ inhalation.

3.3 siRNAs preparation and in vivo treatments

For the in vivo treatment, we used a custom-made siRNA against the *Clcn7* G213R, whose sequence is published in Capulli et al., ⁶. The siRNA had modified 3'dAdT overhangs to enhance the conjugation with the in vivo-jetPEI® transfection reagent used as vehicle. The formulation was done in a 5% glucose solution to increase stability, as suggested by the manufacturer. Mice were injected with the solution within 15-20 minutes from its preparation. The injected volumes were 100 µl for young mice and 200 µl for adult and old mice. The treatment was performed i.p. or s.c., with 4mg/Kg of control scrambled siRNA (SCR) or *Clcn7* G213R -siRNA (siRNA), three times a week for 2 to 12 weeks. Treatments were performed in males and efficacy was confirmed in females.

3.4 Serological tests

Levels of Collagen Type 1 C-Telopeptide (CTX) were measured in the sera after 2 weeks of siRNA-treatment, using specific Enzyme-Linked Immunosorbent Assay (ELISA) kit, following the manufacturer's instructions. Biomarkers of liver, kidney, muscle and pancreatic diseases were measured using Reflotron® strips. Blood sampling was performed always in the morning in fasted mice, according to Christgau ¹⁹.

3.5 Real Time RT-PCR

Total RNA was extracted from tissues using Trizol® and following the manufacturer suggested protocol. RNA was quantified using Nanodrop, and quality checked by 1% agarose gel run. One µg of RNA was then reverse transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit. Real time PCR reaction was performed loading 0.1 µg of cDNA using the SensiMix™ SYBR® kit. Gene expression data were represented as fold change over the control and normalized by Gapdh. PCR conditions and primer sequences are listed in **Table1**.

Table 1- Primer sequences and PCR condition.

Primer pairs	Sequences	PCR conditions
<i>Clcn7^{G213R}</i>	Fw: CAAGTGCTTCCTCAATA Rv: GCCCTCTTCCAAGCTAAACA	95° 10' 35 cycles 95° 30", 60° 30", 72° 30"
<i>Il-6</i>	Fw: GTTCTCTGGGAAATCGTGGA Rv: GGAAATTGGGGTAGGAAGGA	
<i>Il-1β</i>	Fw: GCCCATCCTCTGTGACTCAT Rv: AGGCCACAGGTATTTTGTCTG	
<i>Tnf-α</i>	Fw: CTCCCTTTGCAGAACTCAGG Rv: AGCCCCCAGTCTGTATCCTT	
<i>Ifn-γ</i>	Fw: TTTGAGGTCAACAACCCACA Rv: CGCAATCACAGTCTTGGCTA	
<i>Gapdh</i>	Fw: TGGCAAAGTGGAGATTGTTGC Rv: AAGATGGTGTATGGGCTTCCCG	
<i>Clcn3</i>	Fw: GGTCAGGATGGCTTGTTGTT Rv: ACTCTGCCCATGTTTTCCAC	
<i>Clcn5</i>	Fw: GCCTTTGAAGCCACAGAAAG Rv: CTAGCCACCGACTTTTGCTC	

3.6 Micro Computed Tomography (mCT)

Tibias were fixed in 4% formaldehyde for 48 hours and then scanned by a μ CT SkyScan 1174. The scan was performed with a 6.4 μ m resolution using the x-ray voltage of 50 kV. The Skyscan NRecon software was used to reconstruct the images using a modified Feldkamp algorithm ²⁰.

Three-dimensional (3D) analysis was carried out employing a Marching Cubes type model with a rendered surface ²¹. The trabecular bone parameters were calculated on 550 consecutive slides starting from 100 μ m below the growth plate. Pratt's algorithm was adopted to take 2D measurements. Bone cortical analysis was performed on 350 consecutive slides, starting from 100 μ m below the growth plate. Threshold values were applied for segmenting trabecular bone. Bone trabecular and cortical variables were determined according to Bouxsein et al. ²².

3.7 Histology and Bone histomorphometry

Kidneys and livers were fixed in 4% paraformaldehyde, dehydrated in ascending ethanol series and processed for paraffin embedding. Histopathological analysis was carried out on 5 µm thick sections stained with hematoxylin and eosin.

Tibias were fixed in 4% paraformaldehyde, dehydrated in ethanol and processed for polymethylmethacrylate embedding without decalcification. Histomorphometric measurements were performed on 5 µm thick sections, 100 µm away from the growth plate (metaphyseal region), excluding the endocortical surfaces. The analysis was carried out using image analysis system IAS 2000 software (Delta Sistemi, Rome, Italy) ²³. Histochemical staining for Tartrate-Resistant Acid Phosphatase (TRAcP) activity was used to mark osteoclasts and measure Osteoclast Surface over Bone Surface (Oc.S/BS; %), Osteoclast Number over Bone Surface (Oc.N/BS; n/mm²) and the osteoclast Eroded Surface over Bone Surface (ES/BS; %). Osteoblast Surface over Bone Surface (Ob.S/BS; %) was evaluated after 0.1% toluidine blue staining. Images were captured with AxioCam MRC5 (Carl Zeiss, Inc.) using the AxioVision software (Carl Zeiss, Inc.).

3.8 Mechanical tests

Femurs were harvested from treated mice, cleaned thoroughly to remove any soft tissue and stored at -80°C. The mechanical test was performed on the distal portion of the femur using the Reference Point Indentation technique by Biodent®. Briefly, bones were kept in ice-cold PBS during the test to maintain tissue hydration. All the femur samples were tested using 5 to 10 indentation cycles at 2 Hertz (Hz) utilizing a force of 2 or 4 Newton (N) in accordance with the age of the mice. Indentation distance (ID) and Total indentation distance (TID) were calculated for each test.

3.9 Statistical analysis

Statistical analysis complies with the recommendations on experimental design and analysis in Pharmacology ²⁴. All results are presented as mean±SD of at least 5 mice/group, unless otherwise indicated in the figure or table legends. Only data obtained by real time RT-PCR were normalized and shown as fold changes to control for unwanted sources of variation. Statistical analyses were

carried out by the software Prism® by GraphPad v7.0, using the unpaired, two-tailed Student's t test reported in table and figure legends. P-values threshold was <0.05 .

4. Results

A summary of the results obtained in this study is provided in **Table 2**, which includes also our previous outcome⁶. The table shows the features of the mouse groups, the treatment regimens and their effects. It is clear from the summary that the therapy was effective at any age tested, on both genders and with i.p. and s.c. routes of administration. Outcomes included the reduced expression of the *Clcn7* G213R mutant mRNA in femurs, the increase of the serum bone resorption biomarker, CTX, the reduction of the trabecular bone volume and the improvement of the bone quality. In the following sections, we will describe each treatment regimen, specifically explaining the technical and scientific details.

Table 2 - Summary of the results.

Regimen ¹	Age	n	Gender	Duration (weeks)	Route	<i>Clcn7</i> ^{G213R} expression	CTX (2 weeks)	BV/TV%	Bone quality	Notes
1 ²	10 d	3-6	Male	4	i.p.	↓↓	↑	↓	↑	
2	3 m	5-6	Male	4	i.p.	↓↓	↑	↓	↑	
3	3 m	5-6	Male	12	i.p.	↓↓	↑	↓	↑	
4	6 m	3	Male	2	i.p.	↓	↑	=	n.a. ⁴	P.o.P.
1	10 d	6	Female	4	i.p.	↓↓	n.a. ³	↓	↑	
4	3 m	5	Female	2	i.p.	↓	↑	=	n.a. ⁴	P.o.P.
5	10 d	6	Male	4	s.c.	↓↓	n.a. ³	↓	↑	

d: days; m: months; ↑ and ↓: increase and decrease <50%, respectively; ↑↑ and ↓↓: increase and decrease >50%, respectively; =: no change; i.p.: intraperitoneal injection; s.c.: subcutaneous injection; n.a: not analyzed; P.o.P.: Proof of Principle; ¹dose of 4mg/Kg of scrambled (SCR)- or *Clcn7*^{G213R} (siRNA)-sticky siRNA/jetPEI® conjugate administered 3 times/week; ²Capulli et al., 2015⁶; ³due to young age preventing blood sampling; ⁴due to short-term treatment.

4.1 ADO2 siRNA efficacy in young, adult elderly ADO2 male mice

The first aim of the study was to address whether the treatment was effective at various ages. In fact, human ADO2 can be diagnosed at any life stages, from childhood to elderly, therefore it would be of great translational relevance to impact on the disease even in case of later diagnosis, when bone remodeling slows down and the effect of the drug could be mitigated. We had already demonstrated that treatment regimen 1 (4 mg/kg of *Clcn7* G213R -siRNA or SCR-siRNA 3 times a week for 4 weeks in young mice) (**Table 2**) was effective at rescuing the phenotype of ADO2 mice when it was started at 10 days of age ⁶. Here we demonstrated that treatment regimen 2 (4 mg/kg of *Clcn7* G213R -siRNA or SCR-siRNA 3 times a week for 4 weeks in adult mice) (**Figure 1A**), started at 3 months of age, significantly increased serum CTX levels (measured after 2 weeks from the first injection) (**Table 3**) and reduced the *Clcn7* G213R mutant mRNA expression in the femurs of siRNA- versus SCR-treated ADO2 mice (measured at end-point) (**Table 4**).

Table 3 - Serum CTX after 2 weeks of treatment.

Age	n	Gender	Route	CTX (ng/ml) mean±SD			p-value		
				WT SCR (1) ¹	ADO2 SCR (2) ¹	ADO2 siRNA (3) ¹	1 vs 2	1 vs 3	2 vs 3
10 d ²	3-6	Male	i.p.	n.a.	20.8±1.51	30.28±7.24	-	-	0.07
3 m	12	Male	i.p.	20.9±4.3	13.41±1.62	19.3±5.04	0.0001	0.46	0.004
3 m	5	Female	i.p.	n.a.	20.46±2.88	24.30±1.89	-	-	0.03

d: days; m: months; i.p.: intraperitoneal injection; n.a.: not analyzed because only ADO2 mice were treated in this experimental setting; SCR: scrambled siRNA; siRNA: *Clcn7*^{G213R}-siRNA. ¹Identify groups for statistics reported in the last three columns. ²Capulli et al., 2015⁶. Data are the mean±SD (unpaired two-tailed Student's *t* test).

Table 4. *Clcn7^{G213R}* gene expression in femurs at the indicated end-points.

Age	End-point (weeks)	n	Gender	Route	<i>Clcn7^{G213R}</i> relative expression ¹			p-value
					WT SCR	ADO2 SCR (1) ²	ADO2 siRNA (2) ²	
10 d ³	4	3-6	Male	i.p.	0	1±0.40	0.28±0.2	0.008
10 d	4	5	Female	i.p.	0	1±0.45	0.22±0.18	0.007
10 d	4	5	Male	s.c.	0	1±0.53	0.37±0.23	0.04
3 m	4	6	Male	i.p.	0	1±0.64	0.27±0.34	0.03
3 m	12	6	Male	i.p.	0	1±0.86	0.09±0.1	0.04
3 m	2	5 ⁴	Females	i.p	0	1±0.15	0.60±0.10	0.001

d: days; m: months; i.p.: intraperitoneal injection; s.c.: subcutaneous injection; SCR: scrambled siRNA; siRNA: *Clcn7^{G213R}* siRNA. ¹Primer pairs specific for *Clcn7^{G213R}* (Table 1) that do not detect WT *Clcn7*⁶. ²Identify groups for statistics reported in the last column. ³Capulli et al., 2015⁶. ⁴Proof of principle experiment. Data are the mean±SD (unpaired two-tailed Student's *t* test).

After 4 weeks of treatment, μ CT imaging showed a reduction of trabecular bone mass in siRNA-treated compared to SCR-treated ADO2 mice (**Figure 1B**), confirmed by the measurement of trabecular Bone Volume over total Tissue Volume (BV/TV) (**Figure 1C**) and Trabecular Number (Tb.N) (**Figure 1D**), while Trabecular Thickness (Tb.Th) was not altered in ADO2 mice and remained unchanged after the treatment (**Figure 1E**). Consistently, Trabecular Separation (Tb.Sp.) was increased in siRNA-treated ADO2 mice (**Figure 1F**). Histomorphometric analysis showed that Osteoclast Surface over Bone Surface (Oc.S/BS) (**Figure 1G**) and Osteoclast Number over Bone surface (Oc.N/BS) (**Figure 1H**) were high in ADO2 mice, consistent with the knowledge that this is an osteoclast-rich osteopetrosis ^{10,11}. These variables were reverted by the siRNA treatment (**Figure 1G,H**), alongside the eroded bone surface, which was incremented by the treatment (**Figure 1I**). Indentation analysis showed an improvement of the bone quality, which, however, was not statistically significant (**Figure 1J,K**).

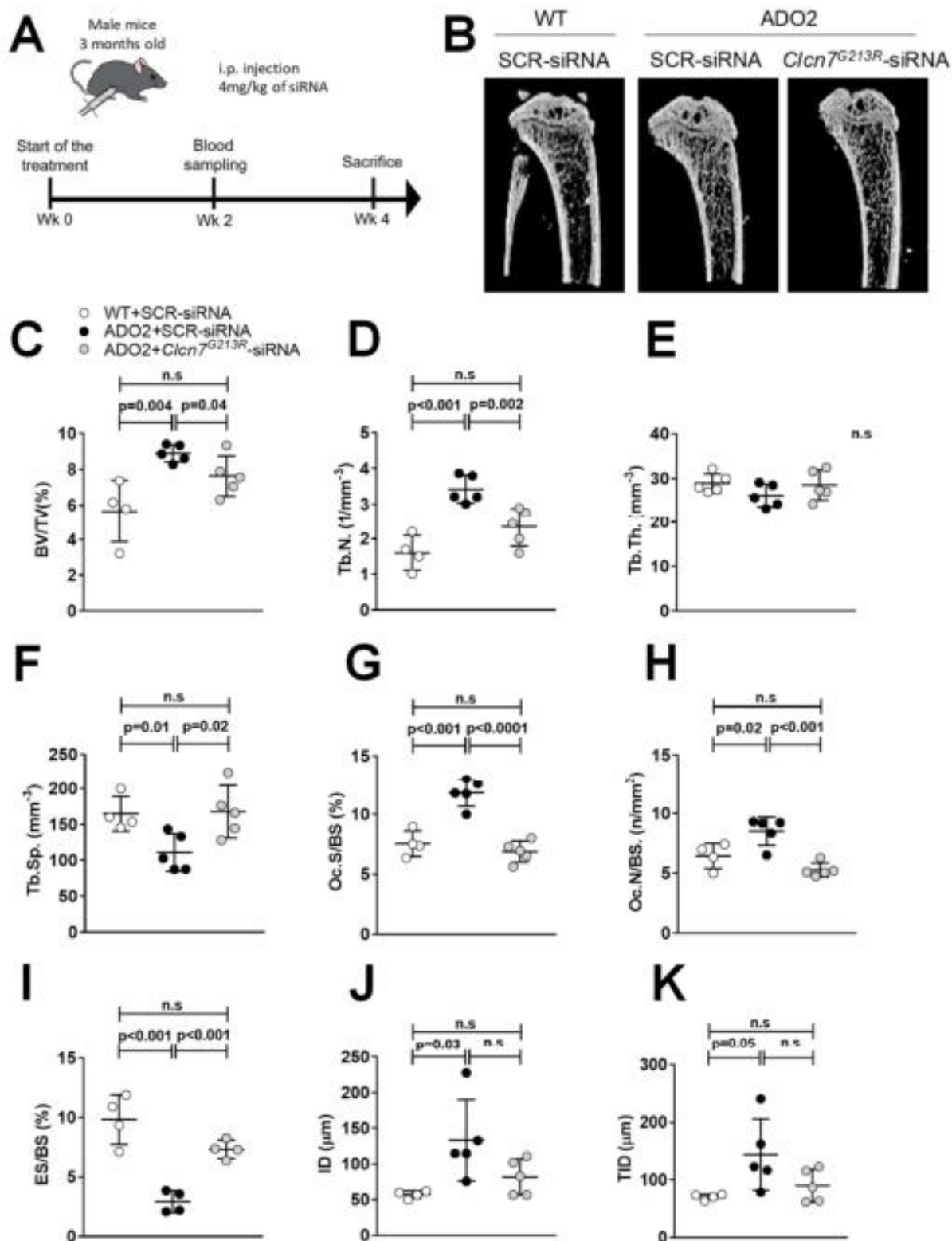


Figure 1-Bone phenotype of adult male mice treated intraperitoneally for 4 weeks. (A) Three-month-old wildtype (WT) and ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 4 weeks. After 2 weeks, blood was sampled from live mice. At the end of the experiments, mice were culled, the hind-limb bones isolated and analyzed. (B) Representative μ CT 3D reconstructions of proximal tibias. (C) Morphometric analysis of trabecular Bone

Volume over total Tissue Volume (BV/TV), **(D)** Trabecular Number (Tb.N), **(E)** Trabecular Thickness (Tb.Th) and **(F)** Trabecular Separation (Tb.Sp). **(G)** After μ CT scanning, the same bones were embedded in plastic, sectioned, stained for TRAcP enzymatic activity and evaluated histomorphometrically for Osteoclast Surface over Bone Surface (Oc.S/BS%), **(H)** Osteoclast Number over Bone Surface (Oc.N/BS) and **(I)** Eroded Surface over Bone Surface (ES/BS%). **(J)** Micro-indentation was performed on the contralateral femurs to assess bone quality by measuring the first cycle Indentation Distance (ID) and **(K)** the Total Indentation Distance (TID). Pictures are representative, and data are the mean $\pm$ SD of 5 mice per group (unpaired two-tailed Student's t test). n.s., not significant.

Like what we observed previously in young mice ⁶, osteoblast variables were not altered neither in adult ADO2 mice and remained unchanged upon treatment (**Figure 2A-B**).

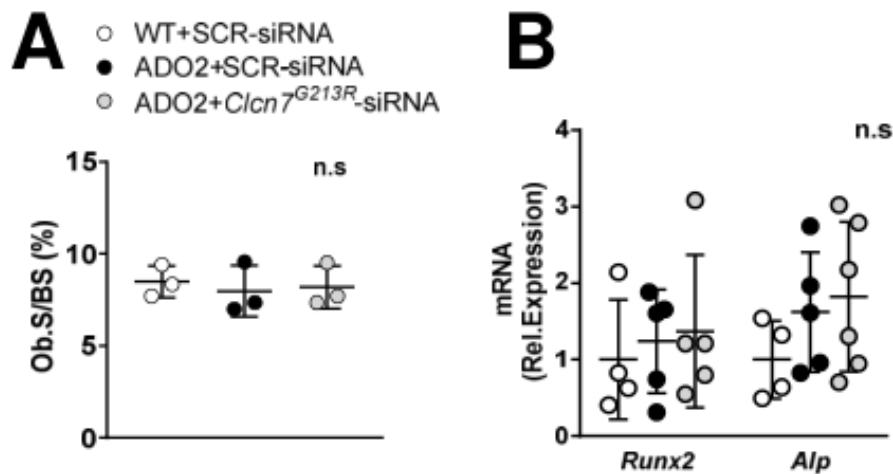


Figure 2 - Osteoblast phenotype of adult male mice treated intraperitoneally for 4 weeks. **(A)** Three months old wildtype (WT) and ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or Clcn7 G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 4 weeks. At the end of the experiment bones were embedded in polymethyl-methacrylate, sectioned, stained with 0.1% toluidine blue and evaluated histomorphometrically for the Osteoblasts Surface over Bone Surface (Ob.S/BS%). **(B)** RNA was extracted from the femurs of the same mice and real time RT-PCR was performed using primer pairs specific for the osteoblast markers Runx2 and Alp, normalized by the housekeeping gene Gapdh. Data are the mean $\pm$ SD of 3-5 mice per group (unpaired two-tailed Student's t test). n.s., not significant.

Statistical comparison of siRNA-treated ADO2 mice with SCR-treated wildtype mice showed that treatment regimen 2 improved but did not return all variables tested to the wildtype level (**Figure 1B-J**). Therefore, at variance with the outcome observed in young mice ⁶, 4 weeks of treatment did not fully rescue the bone phenotype in adulthood.

To assess whether this lack of phenotypic full rescue was a matter of treatment duration, we applied treatment regimen 3 (**Figure 3A**), which was identical to treatment regimen 2 but prolonged for 12 weeks. Secondary aim of this experiment was also to evaluate treatment safety over a long term. μ CT imaging showed a trabecular bone phenotype of siRNA-treated ADO2 mice indistinguishable from that of SCR-treated wildtype mice (**Figure 3B**), suggesting a full phenotypic rescue. Measurements of bone trabecular variables (**Figure 3C-F**) and histomorphometric analysis of osteoclast variables (**Figure 3G-H**) confirmed a return to the wildtype structural and cellular phenotype. Consistently, the eroded bone surface returned to normal (**Figure 3I**). Furthermore, the bone quality parameters were also rescued to wildtype levels, achieving statistical significance compared to SCR-treated ADO2 mice (**Figure 3J,K**).

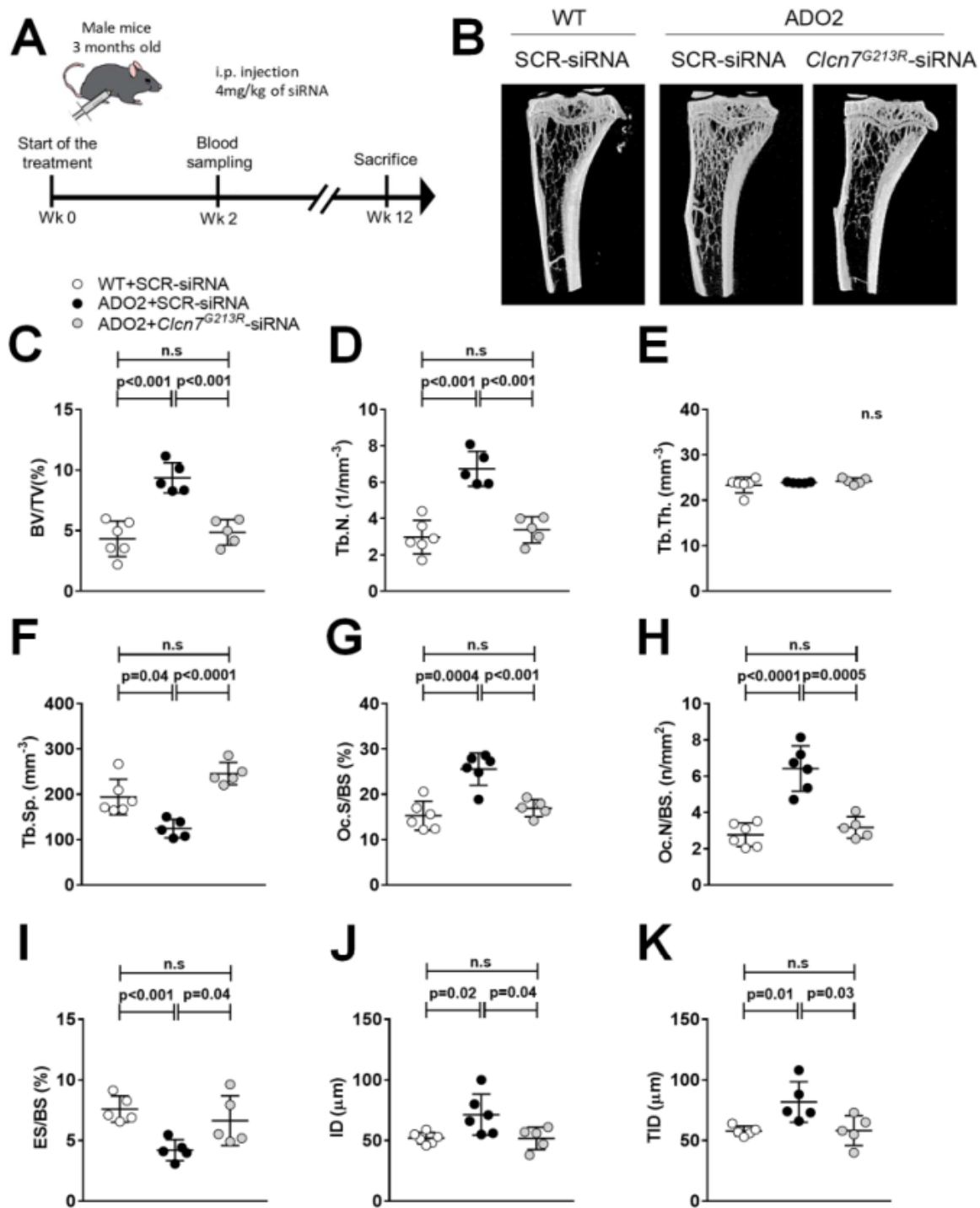


Figure 3 - Bone phenotype of adult male mice treated intraperitoneally for 12 weeks. (A) Three months old wildtype (WT) and ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 12 weeks. After 2 weeks, blood was sampled from live mice. At the end of the experiment, mice were culled, hind-limb bones isolated and analyzed. (B) Representative μ CT 3D reconstructions of proximal tibias. (C) Morphometric analysis of trabecular Bone Volume over total Tissue Volume (BV/TV), (D) Trabecular Number (Tb.N), (E) Trabecular Thickness (Tb.Th) and (F) Trabecular Separation (Tb.Sp). (G) After μ CT scanning, the same bones were embedded in plastic,

sectioned, stained for TRAcP enzymatic activity and evaluated histomorphometrically for Osteoclast Surface over Bone Surface (Oc.S/BS%), **(H)** Osteoclast Number over Bone Surface (Oc.N/BS) and **(I)** Eroded Surface over Bone Surface. **(J)** Micro-indentation was performed on the contralateral femurs to assess bone quality by measuring the first cycle Indentation Distance (ID) and **(K)** the Total Indentation Distance (TID). Pictures are representative, and data are the mean $\pm$ SD of 5-6 mice per group (unpaired two-tailed Student's t test). n.s., not significant.

ADO2 mice did not display cortical bone alterations compared to wildtype mice at any age and the cortical variables were not affected by the treatment even after 12 weeks **(Figure 4A-C)**. Once again, the osteoblast variables were unremarkable **(Figure 4D-E)**, as well as the serum calcium and phosphorus levels **(Figure 4F-G)**.

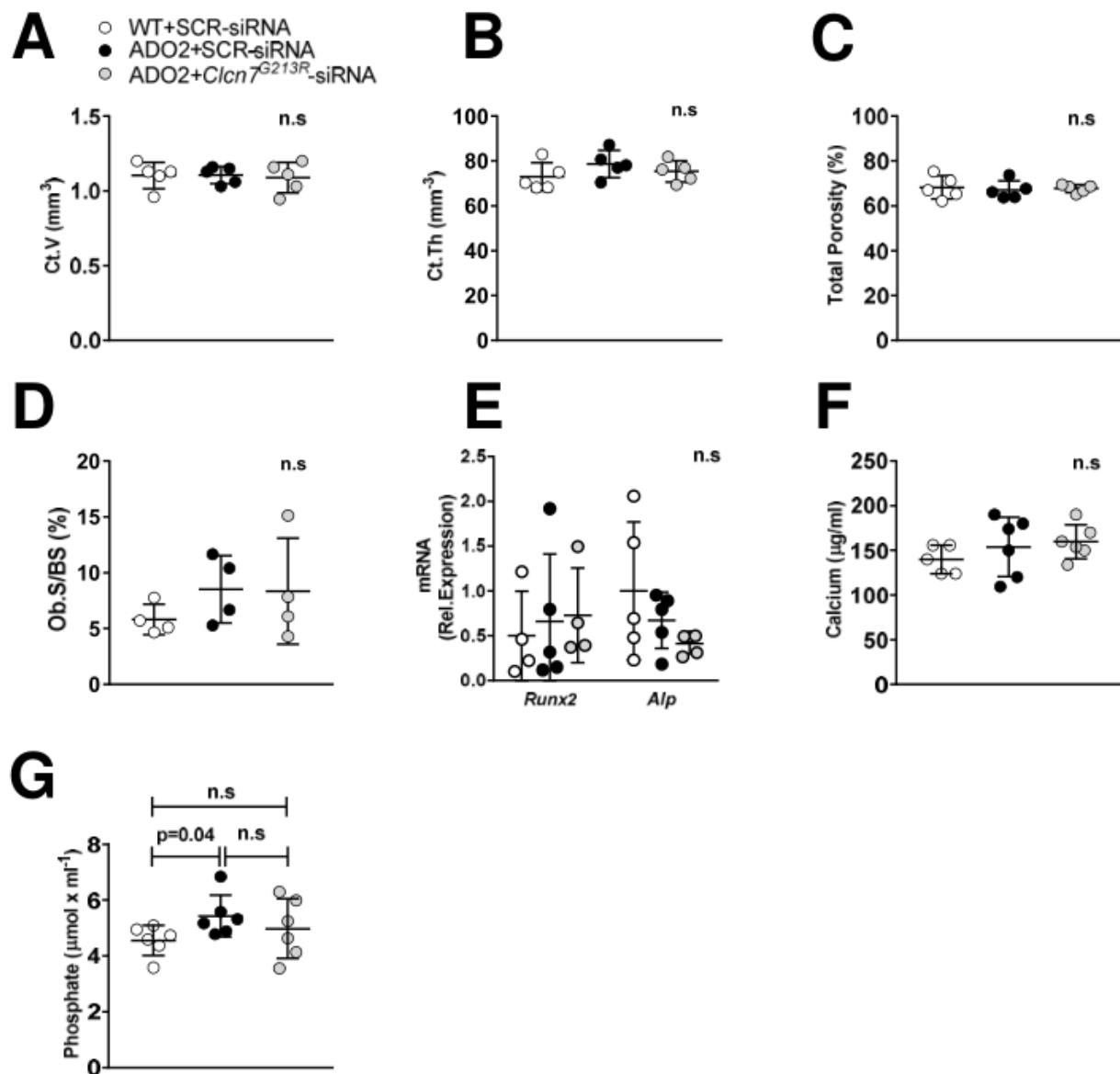


Figure 4 - Cortical bone, osteoblast and calcium/phosphorous metabolism in adult male mice treated intraperitoneally for 12 weeks. Three months old wildtype (WT) and ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or Clcn7 G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 12 weeks. At the end of the experiments mice were culled, hind-limb bones were isolated, and μ CT was performed on proximal tibias to evaluate **(A)** Cortical Volume (Ct.V), **(B)** Cortical Thickness (Ct.Th) and **(C)** total porosity of the cortical bone. **(D)** After μ CT scanning, the same bones were embedded in polymethyl-methacrylate, sectioned, stained with toluidine blue evaluated histomorphometrically for the Osteoblast Surface over Bone Surface (Ob.BS). **(E)** RNA was isolated from femurs of treated mice and the expression levels of the osteoblast markers Runx2 and Alp were evaluated by real time RT-PCR and normalized by Gapdh. **(F)** Serum levels of calcium and **(G)** phosphate were quantified in sera harvested from the same mice at end-point. Data are the mean $\pm$ SD of 4-6 mice per group (unpaired two-tailed Student's t test). n.s., not significant.

As mentioned above, these mice were carefully investigated for treatment safety. Gross anatomic evaluation of visceral organs unveiled no changes in liver and kidney size (**Figure 5A**) and weight (**Figure 5B,C**). Histopathology showed no obvious structural alterations of these organs (**Figure 5D**). Serologic analysis showed no significant changes of the kidney biomarkers, uric acid (**Figure 5E**) and urea (**Figure 5F**) and the liver biomarker, Glutamic-Pyruvic Transaminase (GPT) (**Figure 5G**), while pancreatic amylase was reduced (**Figure 5H**). However, this latter change is not reported to have pathologic relevance. Interestingly, the muscle biomarker, Creatine Kinase (CK) (**Figure 5I**), typically increased in ADO2 mice and humans, returned within the normal range, although differences between the siRNA- and SCR-treated groups did not achieve statistical significance, likely due to the high variability of this serologic parameter in SCR-treated mice, which reflects the situation in patients²⁵. The treatment did not induce pro-inflammatory responses (Figure 5J), nor did it cause changes in the expression of closely related mRNAs, *Clcn3* and *Clcn5* (**Figure 5K,L**).

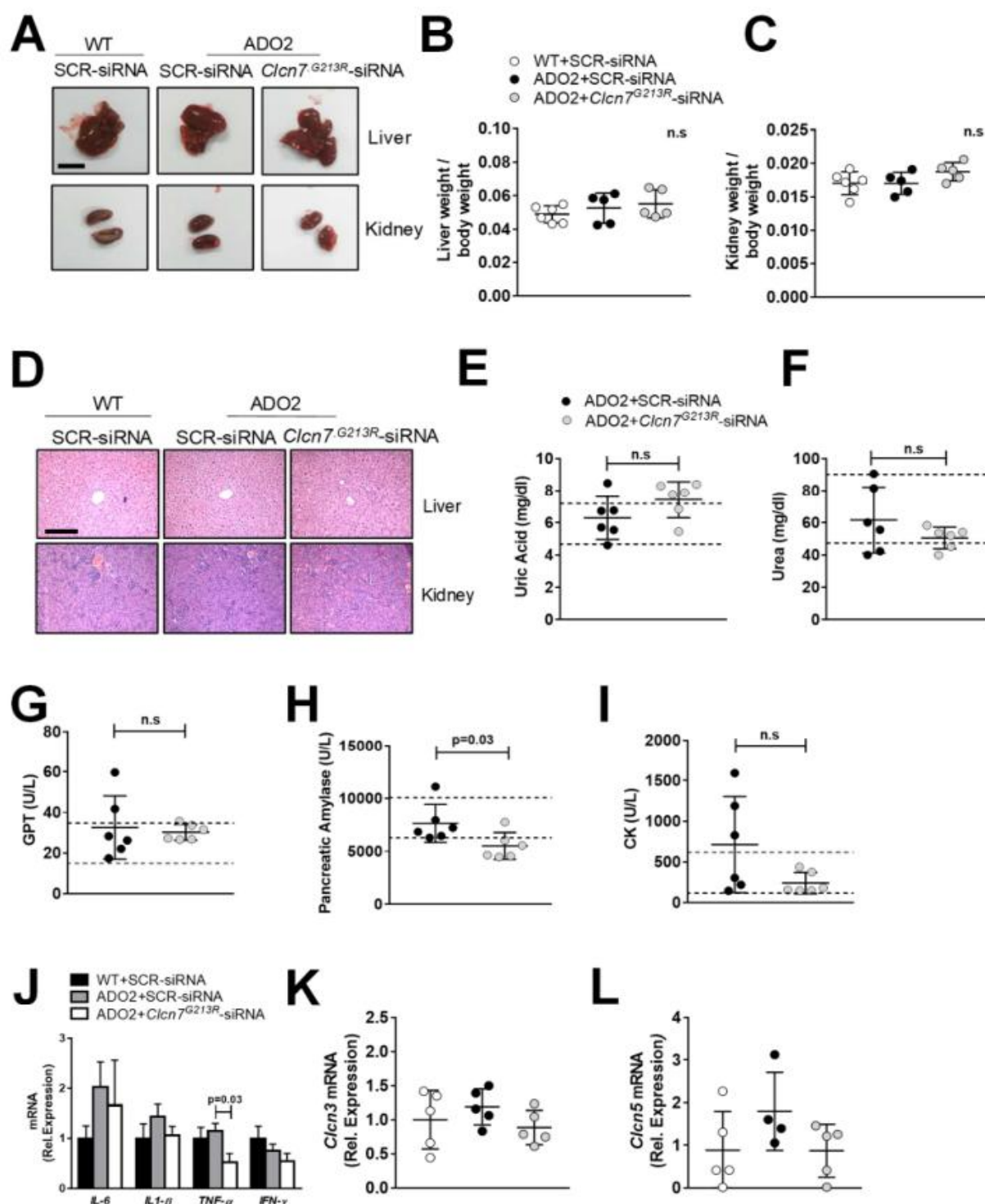


Figure 5 - Pathologic evaluation of adult male mice treated intraperitoneally for 12 weeks. Mice described in Figure 2 were subjected to pathologic evaluation of vital organs. (A) Livers and kidneys were isolated and analyzed by gross observation. (B) Liver weight over body weight. (C) Kidney weight over body weight. (D) Histopathological evaluation of livers and kidneys by haematoxylin-eosin staining. Sera collected from the same mice were analyzed by the Reflotron® method for the indicated biomarkers of (E,F) kidney, (G) liver and (H) pancreas diseases, and (I) for the ADO2 biomarker Creatine Kinase (CK). Normal value ranges are between the two dotted lines. (J) RNA isolated from the femurs was subjected to real time RT-PCR for the

transcriptional expression of the indicated pro-inflammatory cytokines and (K) of the chloride channels *Clcn3* and (L) *Clcn5*. *Gapdh* was used to normalize the gene expression. Scale bar = 1 cm in (A) and 100 μ m in (D). Pictures are representative, and data are the mean $\pm$ SD of 5-6 mice per group (unpaired two-tailed Student's t test). n.s., not significant. GPT: glutamic-pyruvic transaminase.

These results complement the previous observations 6 obtained with treatment regimen 1 in young mice lasting 4 weeks, confirmed in this study also in adult mice (**Figure 6A-D**). No changes in serological variables were observed also in wildtype mice treated with SCR-siRNA versus untreated mice (**Figure 6E-G**), confirming the safety of the formulation independent of the specificity of the siRNA.

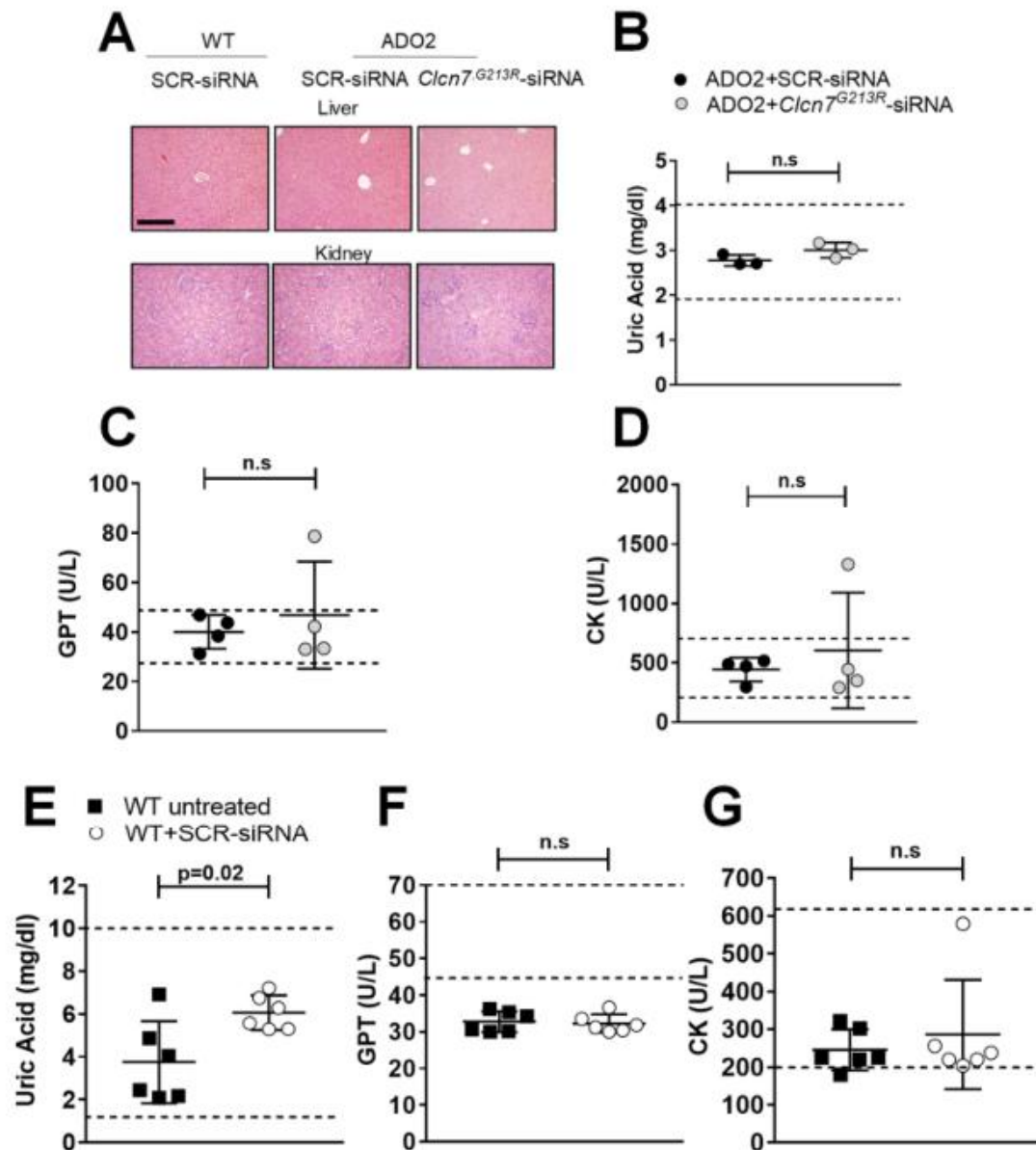


Figure 6 - Pathologic evaluation of adult male mice treated intraperitoneally for 4 weeks. Three months old wildtype (WT) and ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA jetPEI® conjugate, 3 times a week for 4 weeks. At the end of the experiment, mice were culled and vital organs subjected to pathological evaluation. **(A)** Haematoxylin-eosin staining of livers and kidneys (Bar = 100 μ m). **(B)** Sera collected from the same mice were analyzed by the Reflotron method for the indicated biomarkers of kidney and **(C)** liver disease, and **(D)** for the ADO2 biomarker Creatin Kinase (CK). **(E)** Three months old WT male mice were treated intraperitoneally with 4 mg/kg of SCR-sticky siRNA jetPEI® conjugate, 3 times a week for 4 weeks. Sera were collected and evaluated for kidney and **(F)** liver disease and for CK. Normal values ranges are between the two dotted lines (2). Pictures are representative, and data are the mean $\pm$ SD of 5-6 mice per group (unpaired two-tailed Student's t test). n.s., not significant. GPT: Glutamic-Pyruvic Transaminase.

To investigate if the treatment was effective also at older age, we subjected 6 months old mice to treatment regimen 4 (4 mg/kg of Clcn7 G213R -siRNA or SCR-siRNA 3 times a week for 2 weeks in old mice). This was identical to treatment regimens 1 and 2 except for the duration (**Table 2**) and (**Figure 7A**). In fact, to reduce the number of animals, in agreement with the 3Rs principle for the use of laboratory animals, we decided to provide a proof of principle investigating the serum CTX level after two weeks of treatment in a limited number of mice/group. Results showed 1.6-fold increase of this bone resorption biomarker (**Figure 7B**), complemented by a 47% reduction of Clcn7 G213R mutant mRNA expression in the femurs (**Figure 7C**) of siRNA- versus SCR-treated ADO2 mice. As expected, this short-term duration of treatment was not sufficient to alter the bone structural parameters of 6 months old ADO2 mice, as shown in (**Figure 7D-H**).

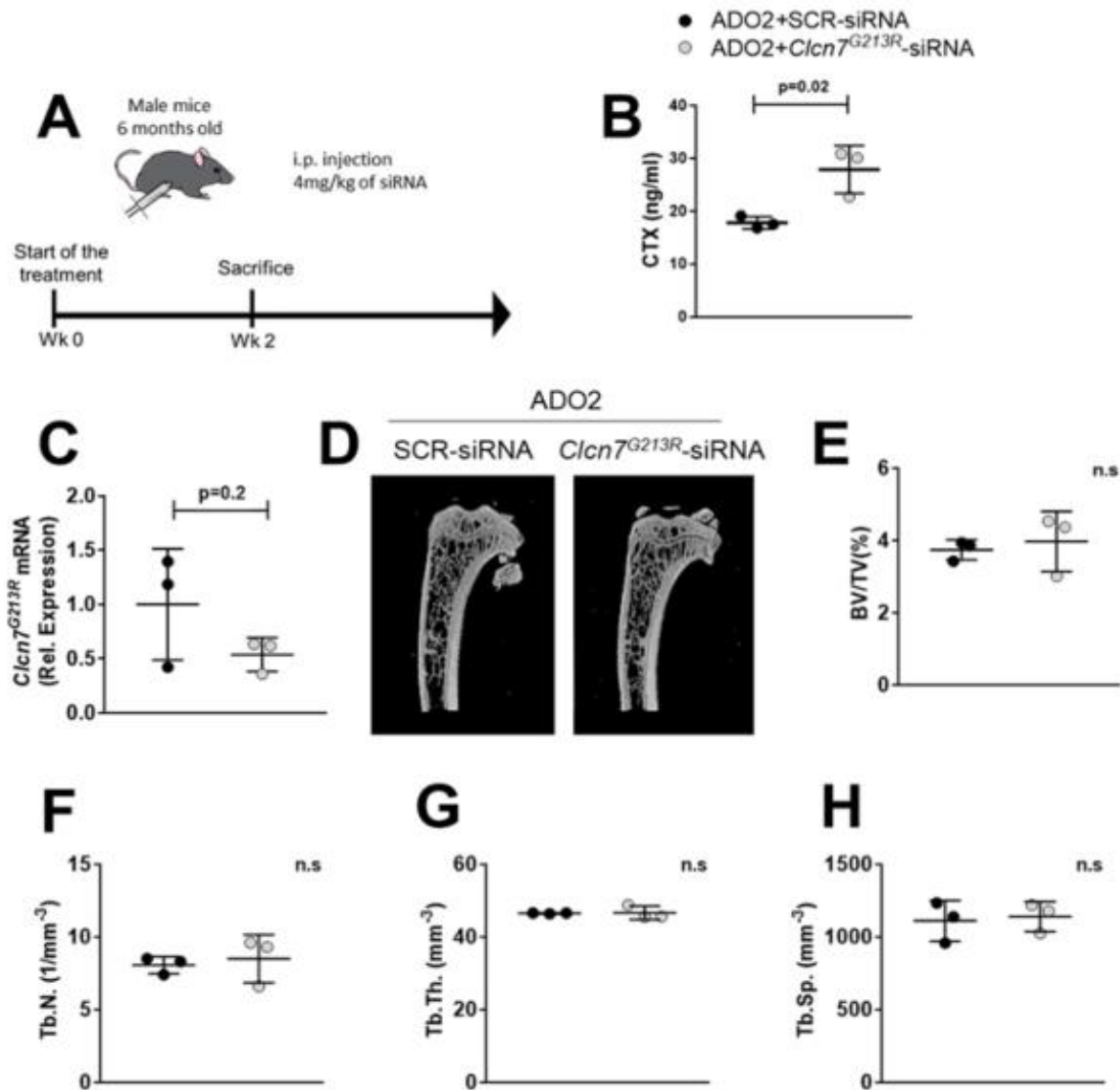


Figure 7 - Phenotype of ageing ADO2 male mice treated for 2 weeks. (A) Six months old ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or Clcn7 G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 2 weeks. At the end of the experiment, mice were culled, sera were collected and hind-limb bones were isolated and analyzed. (B) Serum levels of CTX. (C) Clcn7 G213R mRNA expression in femurs. (D) Representative μ CT 3D reconstructions of proximal tibias. (E) Morphometric evaluation of trabecular Bone Volume over total Tissue Volume (BV/TV), (F) Trabecular Number (Tb.N), (G) Trabecular Thickness (Tb.Th) and (H) Trabecular Separation (Tb.Sp). Pictures are representative, and data are the mean $\pm$ SD of 3 mice per group (proof of principle experiment) (unpaired two-tailed Student's t test). n.s., not significant.

4.2 ADO2 siRNA efficacy in female mice

Because the gender difference of bone mass at baseline prevents to pool males and female data, all previous treatments were performed in males. However, given that ADO2 equally affects males and females, we set to demonstrate whether the siRNA treatment rescued the ADO2 phenotype of female mice as well. We used the shorter fully effective treatment to conform to the 3Rs principle by reducing the number of animals. To this end, we subjected 10 days old female mice to treatment regimen 1, lasting 4 weeks (**Figure 8A**), and observed 1.5-fold increase of serum CTX (measured after 2 weeks from the first injection) (**Table 3**) and 78% reduction of Clcn7 G213R (measured at end- point) (**Table 4**) in siRNA- versus SCR-treated ADO2 mice. siRNA-treated ADO2 mice showed a full rescue of the trabecular bone variables and bone quality parameters, which returned to levels comparable to the SCR-treated wildtype mice (**Figure 8B-H**), in line with the results obtained in young males.

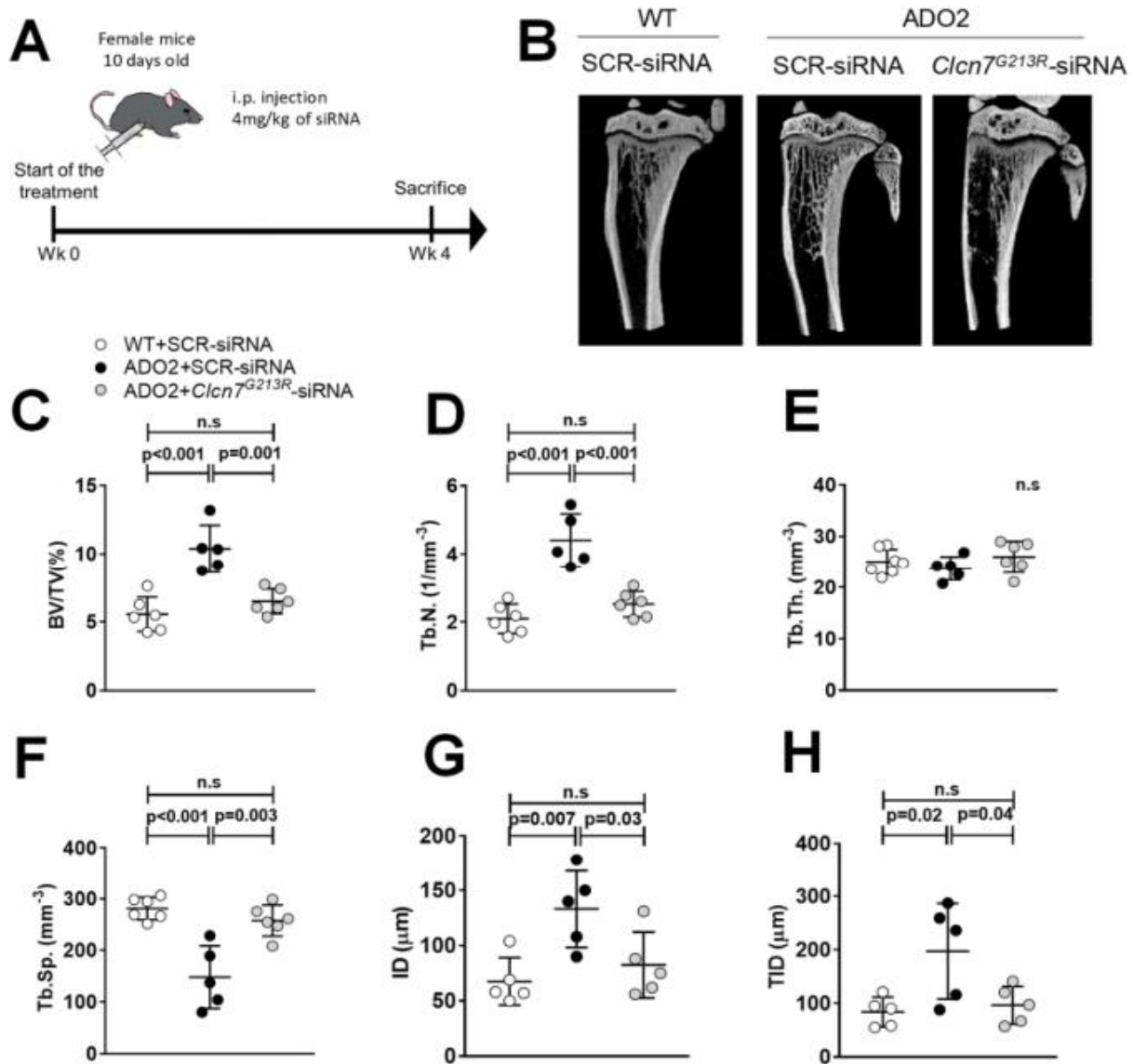


Figure 8 - Bone phenotype of young female mice treated intraperitoneally for 4 weeks. (A) Ten days old wildtype (WT) and ADO2 female mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 4 weeks. At the end of the experiments mice were culled and their bone phenotype analyzed. (B) Representative μ CT 3D reconstructions of proximal tibias. (C) Morphometric analysis of trabecular Bone Volume over total Tissue Volume (BV/TV), (D) Trabecular Number (Tb.N), (E) Trabecular Thickness (Tb.Th) and (F) Trabecular Separation (Tb.Sp). (G) Micro-indentation performed on the contralateral femurs to assess bone quality measuring the first cycle Indentation Distance (ID) and (H) the Total Indentation Distance (TID). Pictures are representative, and data are the mean $\pm$ SD of 5-6 mice per group (unpaired two-tailed Student's t test). n.s., not significant.

Since bone turnover in females is strongly affected by the estrogen status, we also performed a proof of principle experiment to demonstrate whether the effect was apparent also in sexually mature mice. To this purpose, we subjected 3 months old female ADO2 mice to treatment regimen 4 (4 mg/kg of Clcn7 G213R -siRNA or SCR-siRNA 3 times a week for 2 weeks) (**Table 2**) and, at the end of the experiment, we observed a significant increase of CTX in the sera (**Table 3**) and a reduction of Clcn7 G213R expression in the femurs (**Table 4**) of Clcn7 G213R -siRNA- treated mice versus SCR-siRNA treated mice, thus suggesting efficacy of the treatment independent on the sexual maturity of the mice. As expected given the short-term treatment regimen, the bone structural parameters were not modified in Clcn7 G213R -siRNA treated ADO2 mice (**Figure 9A-F**).

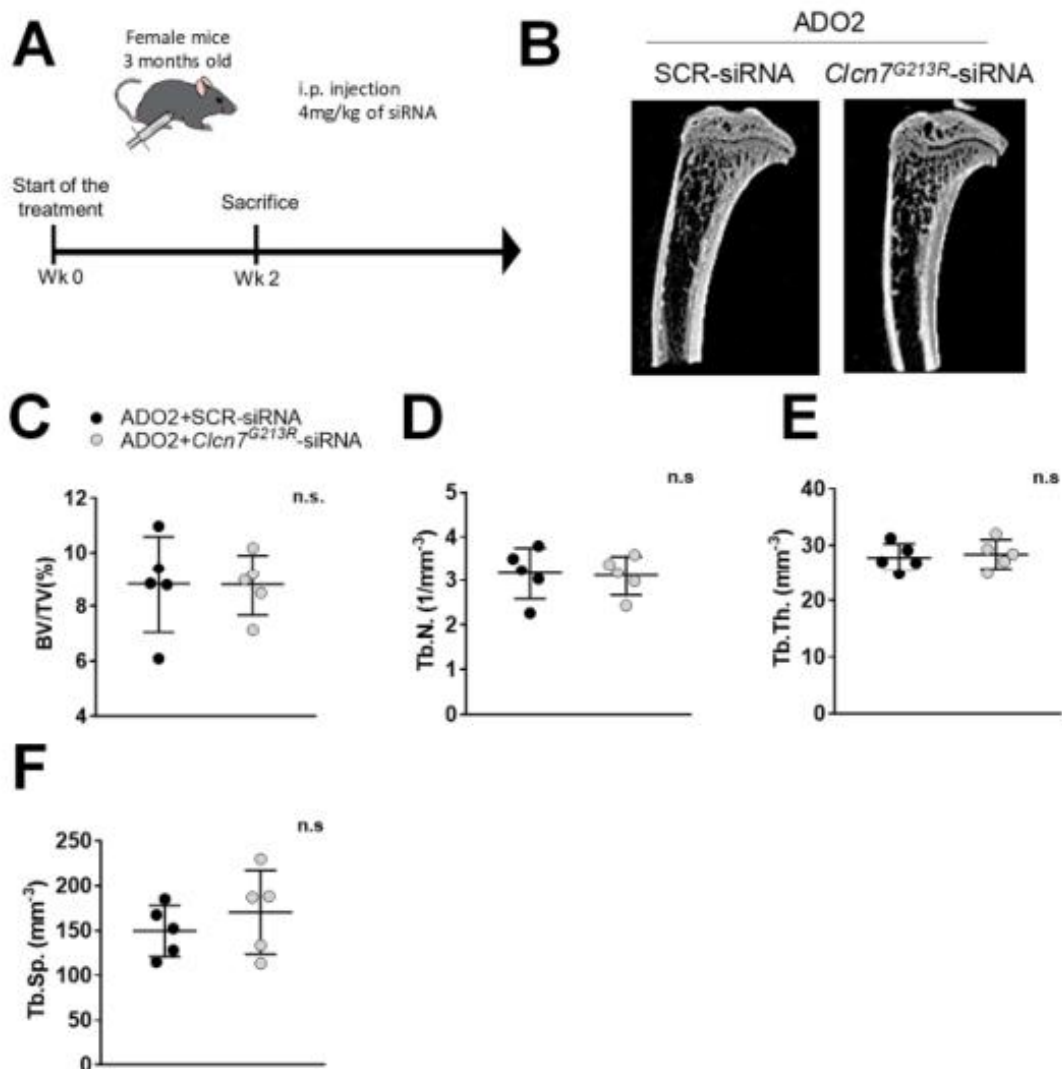


Figure 9 - Phenotype of adult ADO2 female mice treated for 2 weeks. (A) Three months old ADO2 female mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or Clcn7 G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 2 weeks. At the end of the experiment, mice were culled, hind-limb bones were

isolated and analyzed. **(B)** Representative μ CT 3D reconstructions of proximal tibias. **(C)** Morphometric evaluation of trabecular Bone Volume over total Tissue Volume (BV/TV), **(D)** Trabecular Number (Tb.N), **(E)** Trabecular Thickness (Tb.Th) and **(F)** Trabecular Separation (Tb.Sp). Pictures are representative, and data are the mean $\pm$ SD of 3 mice per group (proof of principle experiment) (unpaired two-tailed Student's t test). n.s., not significant.

4.3 Treatment discontinuation

To investigate whether the effect of our Clcn7 G213R -siRNA formulation was persistent, we treated 10-day-old male ADO2 mice with treatment regimen 1 (4 mg/kg of Clcn7 G213R -siRNA or SCR-siRNA 3 times a week) for 4 weeks. Mice were then randomly divided in two groups, one group was sacrificed at this stage, whilst in the other group the treatment was discontinued, and the mice were sacrificed 4 weeks later. In this circumstance, we observed that treatment discontinuation returned the tibia trabecular bone variables to those observed in ADO2 mice treated with SCR-siRNA (**Figure 10A-H**), suggesting that the effect of the Clcn7 G213R -siRNA was reversible.

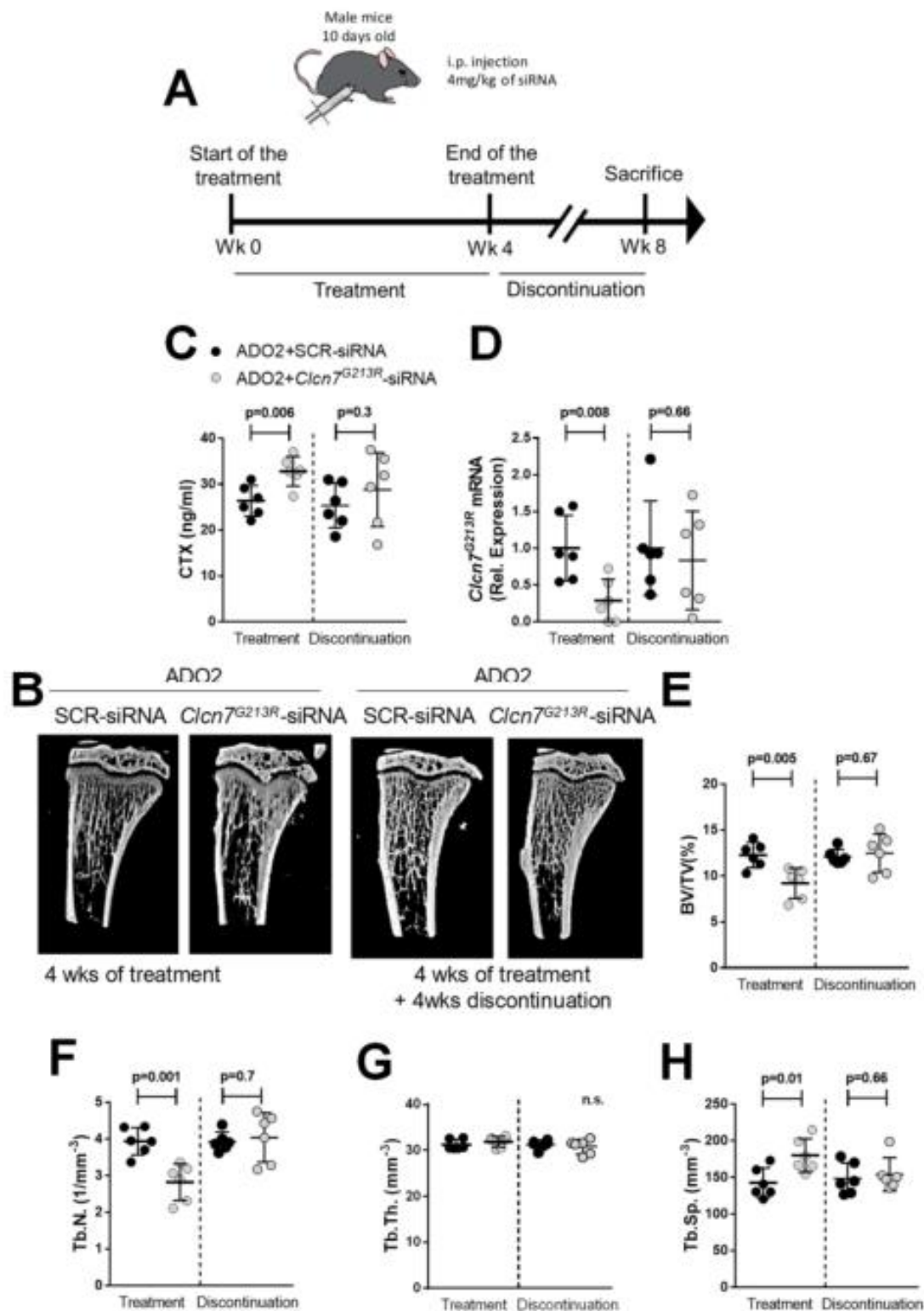


Figure 10 - Treatment discontinuation. (A) Ten days old ADO2 male mice were treated intraperitoneally with 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 4 weeks. At the end of the treatment mice were randomly separated in two groups. In one group mice were culled and the bone phenotype analyzed at this stage. In the other group the treatment was discontinued, and the mice were culled and analyzed 4 weeks later. (B) Serum level of CTX at end-points. (C) *Clcn7* G213R

mRNA expression in femurs at end-points. **(D)** Representative μ CT 3D reconstructions of proximal tibias. **(E)** Morphometric analysis of trabecular Bone Volume over total Tissue Volume (BV/TV), **(F)** Trabecular Number (Tb.N), **(G)** Trabecular Thickness (Tb.Th) and **(H)** Trabecular Separation (Tb.Sp). Pictures are representative, and data are the mean $\pm$ SD of 6 mice per group (unpaired two-tailed Student's t test). n.s., not significant.

4.4 ADO2 siRNA efficacy in translational setting

Next steps were aimed at improving the translational impact of this experimental therapy, predicting future preclinical development and clinical application. To this purpose, we evaluated whether subcutaneous (s.c.) administration could have a similar effect on the bone phenotype as the i.p. injection. To test this hypothesis, we first performed a pilot study on a small number of male animals (according to the 3R principles for the use of laboratory animals) to identify the time of effect in 10 days old ADO2 male mice. We observed that a single s.c. injection of 4mg/Kg siRNA (**Figure 11A**) reduced the *Clcn7* G213R mutant mRNA in femurs within 72 hours (**Figure 11B**). Therefore, we treated 10 days old male mice with treatment regimen 5, identical to regimen 1 but administered s.c. (**Figure 11C**) and observed that the outcome of this strategy overlapped the outcome of treatment regimen 1 performed in young mice by i.p. injection (**Figure 11D-J, Table 2**).

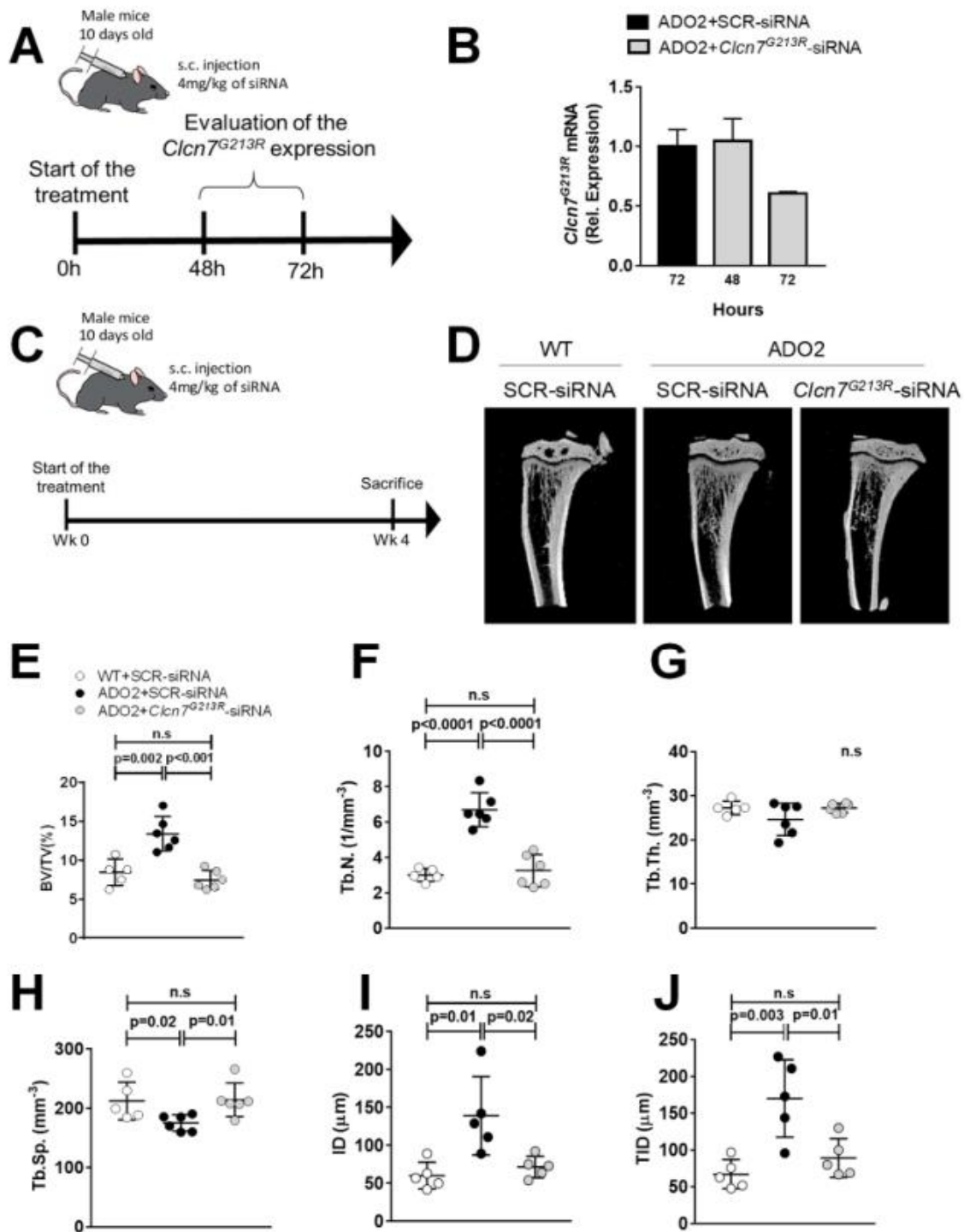


Figure 11 - Bone phenotype of young male mice treated subcutaneously for 4 weeks. (A) Ten days old ADO2 male mice were treated subcutaneously with a single injection of 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA/jetPEI® conjugate and culled at the indicated times, to evaluate the kinetics of *Clcn7* G213R downregulation, in a pilot experiment with 2 mice per group. (B) *Clcn7* G213R gene expression evaluated by real time RT-PCR on RNA isolated from the femurs, normalized by Gapdh. (C) Ten days old wildtype (WT) and ADO2 male mice were treated subcutaneously (s.c.) with 4 mg/kg of scrambled- (SCR) or *Clcn7* G213R -sticky siRNA/jetPEI® conjugate, 3 times a week for 4 weeks. At the end of the experiments,

mice were culled and their bone phenotype analyzed. **(D)** Representative μ CT 3D reconstructions of proximal tibias. **(E)** Morphometric analysis of trabecular Bone Volume over total Tissue Volume (BV/TV), **(F)** Trabecular Number (Tb.N), **(G)** Trabecular Thickness (Tb.Th) and **(H)** Trabecular Separation (Tb.Sp). **(I)** Micro-indentation performed on the contralateral femurs to assess bone quality measuring the first cycle Indentation Distance (ID) and **(J)** the Total Indentation Distance (TID). Pictures are representative, and data are the mean $\pm$ SD of 5-6 mice per group (unpaired two-tailed Student's *t* test). Data in **(B)** are the mean $\pm$ SD of 2 mice for group, with no statistics. n.s., not significant.

On the same line, we evaluated whether reliable end-point parameters could be identified in view of potential clinical application. We have already demonstrated that the serum level of the bone resorption biomarker, CTX, is consistently increased by the treatment (**Table 3**). Indeed, bone resorption biomarkers are already constantly used in clinical trials to assess the efficacy of anti-osteoporosis treatment, therefore their use as end-points could be extended to our therapy. However, to demonstrate the specific efficacy on the silencing of the of *Clcn7* G213R mutant mRNA during the progress of the treatment, we isolated peripheral blood mononuclear cells from 3 months old live mice treated with our siRNA for two weeks and performed RT-PCR on the extracted RNA using our primer pairs highly specific for the mutant transcript 6 (**Table 1**). We confirmed the reliability of this strategy, which allowed us to detect 50% reduction of *Clcn7* G213R mutant mRNA expression in siRNA- versus SCR-treated ADO2 mice (**Table 5**). Given the ease of blood sampling in humans, we believe that these results open the door to the determination of serum CTX and peripheral blood mononuclear cell *Clcn7* G213R silencing as end-points in future Phase I/II clinical trials in ADO2 patients.

Table 5. *Clcn7*^{G213R} gene expression in peripheral blood mononuclear cells of live mice after 2 weeks of treatment.

Age	n	Gender	Route	<i>Clcn7</i> ^{G213R} relative expression ¹			p-value
				WT SCR	ADO2 SCR (1) ²	ADO2 siRNA (2) ²	
3 m	4	Male	i.p.	0	1 $\pm$ 0.22	0.51 $\pm$ 0.33	0.05

m: months; i.p.: intraperitoneal injection; SCR: scrambled siRNA; siRNA: *Clcn7*^{G213R}-siRNA. ¹Primer pairs specific for *Clcn7*^{G213R} (Table 1) that do not detect WT *Clcn7*⁶. ²Identify groups for statistics reported in the last column. Data are the mean $\pm$ SD (unpaired two-tailed Student's *t* test).

5. Discussion

siRNA therapy represents one of the new frontiers for the treatment of genetic diseases, and several studies are now investigating the medication in clinical trials²⁶. Currently, there is a surge of interest to optimize the formulations and make siRNAs available for patients^{26,27}.

The treatment of autosomal dominant diseases is a challenge for many reasons, including the targeting of the tissues involved and the lack of specific drugs that could affect the altered pathway.

ADO2 is paradigmatic of this category of genetic diseases and, so far, there is no treatment that can mitigate the debilitating outcome in patients. In fact, ADO2 patients rely only on palliative treatments that can alleviate their symptoms but do not affect the principal pathogenic cause of the altered phenotype^{17,28}. The osteoclast lineage, which is mostly impaired in ADO2, has a hematogenous origin^{29,30}. However, hematopoietic stem cell transplantation, which is successfully employed to treat patients affected by the lethal autosomal recessive forms of the disease, is not an option for ADO2, given that this disease only rarely reduces the life expectancy of patients¹⁸ and that the transplant procedure itself is at high risk of failure due to complications that are life-threatening³¹. Our siRNA therapy instead has many advantages over the palliative treatments since it i) effectively targets the mutant gene, ii) saves the normal gene creating a condition of pseudo-haplosufficiency, iii) completely rescues the phenotype, iv) is apparently harmless, v) works at any age (although only three mice/group were investigated at 6 months of age), vi) is effective in both genders, vii) can be administered by s.c. injections and viii) improves parameters measurable in serum and in blood cells. The effect of the therapy is reversible, suggesting that the administered siRNA is not accumulated in the body, thus reducing the risk of over-engagement of the RISC multiprotein complex, which degrades the target mRNAs through both the siRNA and the miRNA pathways. In fact, holding persistently the RISC complex could reduce its function in the physiological siRNA/miRNA processing, thus impairing the normal gene silencing that contributes to the cellular homeostasis³².

Our work suggests that the therapy could be developed for the clinical use. The safety studies seem to rule out any major concern of development of serious side effects, an essential requirement to progress toward preclinical regulatory toxicity studies in another rodent species and in a non-human primate species³³. This, in turn, is mandatory for obtaining the approval from public health authorities for the transition to the Phase I/II clinical trials. Furthermore, given the low prevalence of ADO2 in the general population (1:20000 born live), the therapy could receive the Orphan Drug Designation for the cure of a rare disease.

One limitation of this study could be related to the formulation of the siRNA. The in-vivo JetPEI® used in our experiments is a clinically tested vehicle that in our hands proved to be safe and efficient in mice. This is not a targeted vehicle, which could be an advantage for the treatment of a systemic disease. This is exactly the case of ADO2 that in primis is a bone anomaly spread out in the skeleton and in secundis does not affect only the osteoclasts but also a variety of other cells in other organs. In fact, detailed studies demonstrated that the Clcn7 is expressed in several mouse and human tissues and cells ^{6,8}, thus suggesting that a targeted delivery to the osteoclasts is probably not desirable. Notably, there are several ongoing studies aimed at identifying new effective and safe vehicles for systemic siRNA delivery, which include polymer nano-vectors, such as modified PEI (polyethylenimine derivatives), viral-like nanoparticles, lipoprotein particles and others ^{26,27}. Associated with improved siRNA stability and bioavailability ^{26,27}, we can predict that future developments could bring about new siRNA formulations that could make the therapy available for the treatment of various diseases, including ADO2 and other systemic autosomal dominant conditions. Our siRNA therapy is protected with the patent application PCT/IB2015/053730 (publication number WO2015177743 A1), thus opening the door to the generation of Good Manufacturing Practice (GMP)-siRNA formulations and to toxicology studies, which are required to move the treatment to Phase I/II clinical trials.

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Chapter 3: “Novel hybrid silicon-lipid nanoparticles deliver a siRNA to cure autosomal dominant osteopetrosis in mice. Implications for gene therapy in humans.”

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1. Abstract

Rare skeletal diseases are still in need of proper clinically available transfection agents as the major challenge for first-in-human translation relates to intrinsic difficulty intargeting bone without exacerbating any inherent toxicity due to used vector. SiSaf's sil-iron stabilized hybrid lipid nanoparticles (sshLNPs) constitute next-generation non-viral vectors able to retain the integrity and stability of constructs and to accommodate considerable pay-loads of biologicals, without requiring cold-chain storage. sshLNP was complexed with a small interfering RNA (siRNA) specifically designed against the human CLCN7 G215R mRNA. When tested via single intraperitoneal injection in pre-puberal autosomal dominant osteopetrosis type 2 (ADO2) mice, carrying a heterozygous mutation of the Clcn7 gene (Clcn7 G213R), sshLNP, this significantly downregulated the Clcn7 G213R related mRNA levels in femurs at 48 h. Confirmatory results were observed at 2 weeks and 4 weeks after treatments (3 intraperitoneal injections/week), with rescue of the bone phenotype and demonstrating safety. The pre-clinical results will enable advanced pre-clinical development of RNA-based therapy for orphan and genetic skeletal disorders by safely and effectively delivering biologicals of interest to cure human systemic conditions.

2. Introduction

Osteopetrosis, a rare bone genetic disorder characterized by high bone density, is known for encircling a group of altered skeletal phenotypes ascribable to an impairment of osteoclast activity. The autosomal dominant form of the disease (autosomal dominant osteopetrosis type 2 [ADO2]) is caused by single allele dominant negative mutations of the *CLCN7* gene, encoding the ClC-7 chloride transporter indispensable for the mechanism of bone resorption.¹ Although expert opinion-based guidelines for management of ADO2 have been published² and a promising small interfering RNA (siRNA)-based approach has been recently identified,^{3,4} there is still an unmet need for developing suitable clinical therapies addressing bone gene-associated disorders, without resorting to bone marrow transplantation or the administration of off-label drugs².

The treatment, as well as the prophylactic interventions, of genetic disorders and other pathologies such as cancer potentially require the introduction of nucleic acid specimens to a living organism to re-balance a clinical condition, immunize a subject, or ameliorate the effect of a chronic disease. Transfection of genetic material will usually occur through two main classes of vectors: viral and non-viral.

Currently, only six replicating-incompetent viral vectors have been authorized.^{5,6} However, they still present several shortcomings, such as vaccine-induced immune thrombocytopenia and thrombosis.⁶ Furthermore, subjects previously exposed to a viral vector can potentially raise an immune response against it, a phenomenon called “anti-vector immunity.”⁷

Current viral strategies are based on adenoviruses (AVs), adeno-associated viruses (AAVs), and lentiviruses (LVs). Despite their successes in preclinical settings, many challenges still limit these approaches from attaining their full potential at the clinical level. Exogenous infections can occur in some instances with AV, while the majority of adverse events seem to be related to viral reactivation.⁸ AAV limitations in clinical use are related to their immunogenicity or restricted gene packaging ability,^{9,10} while LV show a high risk of insertional mutagenesis, mitigated but not fully eliminated by self-inactivating LV.¹¹

Non-viral gene delivery systems are based on nanometric particles interacting with the negatively charged phosphate backbone of the nucleic acid and cationic lipids, peptides or other compounds,¹² which shield nucleic acid from degradation.¹³ Various other cationic lipids combine a synthetic cationic lipid and a neutral lipid with cholesterol to generate a lipid nanoparticle (LNP) delivery system. They protect RNA up to a certain degree from degradation.^{14–16} However, LNPs are susceptible

to degradation over time ^{17,18} and raise concerns for safety and stability, inducing acute immune responses, liver accumulation or poor active targeting/selectivity. ¹⁹ Finally, polyethylenimines (PEIs) are highly cationic polymers that can result in toxicity, which precludes their use in clinical treatments. ^{7,18}

Although there has been a significant increase in demand for non-viral vector delivery systems in the last few years, several limitations persist. One of the most challenging is the safe targeting of skeletal tissue, for which no technology as such is clinically available to address bone diseases.

SiSaf Ltd has developed a biocompatible and biodegradable silicon-based drug delivery system for ocular gene therapy. ²⁰ Using a similar approach, the company has developed a novel non-viral vector composed of mesoporous silicon nanoparticles functionalized with specific cationic moieties, neutral lipids, and a fraction of pegylated (PEG) lipids dispersed in an aqueous environment containing non-reducing disaccharide and amino acids for targeting bone tissue. ²⁰ In this work, leading stabilized hybrid LNP (sshLNP) prototypes have been evaluated in vivo on an ADO2 mouse model harboring a CLCN7 G215R heterozygous mutation, representing a genetic bone disorder characterized by high bone mass and bone fragility. ²¹ We identified an approach by complexing the siRNA with sshLNP technology for an effective and safe long-term treatment with multiple dose regimens, thus being a key future prospect for skeletal gene therapy-based treatments. This mutation is caused by a G-A transition at human DNA position 21543 and at mouse DNA position 14365. It is the most frequent in-human ADO2 and leads to the G215R amino acid substitution in humans and G213R in mice. ²¹ Our siRNA recognizes and complements with similar efficacy and specificity both the human and mouse mutant mRNAs, ³ allowing us to test it in cells of both species and in ADO2 knock-in mice.^{3,4}

3. Material and Methods

3.1 Preparation and characterization of silicon nanoparticles

Silicon nanoparticles (Si-NPs) were sourced as silicon nano-powder, purity R98% (American Elements, non-doped material; PorousSilicon, doped material). These were assessed in preliminary studies and determined to be suitable for use, using procedures and techniques previously described.^{14–16,32} Si-NPs were preliminarily subjected to methanol rinsing and then as low evaporation technique, until dryness reached. Afterward, the Si-NPs were analyzed for determining silicon content, available surface area, particle size, and residual solvent before further formulation process. ICP-MS analysis was performed to determine the concentration of ²⁸Si in the Si-NPs as described in.²⁰

The surface area was determined by subjecting particles to gas sorption using Quantachrome Nova 2200e and values were calculated applying the BET theory. Before the measurements, the samples were degassed at 350° C overnight. To visually examine structure and size of Si-NPs, particles were suspended at approximately 0.01% in deionized water and a drop of sample was dried at reduced temperature under vacuum over a carbon-coated copper grid for analysis via TEM. Accelerating voltage of 100 kV under FEI Talos L120C G2 TEM was then applied for observing samples.

3.2 Preparation of sshLNP formulations

The sshLNP formulations were prepared with various compositions, using rotary evaporation technique followed by extrusion process. All chemicals used in the preparation were purchased from Sigma-Aldrich unless stated otherwise.

Formulations were prepared starting with a lipid thin film generated by means of solvent evaporation and hydrated with Si-NPs aqueous suspension. Specifically, required amounts of lipids were transferred into around bottom flask and the solvent was carefully evaporated using rotary evaporator with a water bath set at 40 ° C under vacuum. The thin lipid film was then hydrated using hydrating suspension containing the required amounts of silicon and water dispersible excipients in a water bath set at 60 °C (amounts stated in the results section). The sshLNP were rested in the fridge (4°C) overnight and were then extruded through polycarbonate membrane filters of the pore

sizes 0.4 mm and 0.1 mm (10 times each) at 60 °C (Avanti Polar Lipid Extruder, equipped with Whatman polycarbonate filters). Prepared samples were stored at standard refrigerated conditions until usage.

SiS12 sshLNP was used in formulations for in vivo biodistribution/pharmacokinetics experiments and for in vitro tests on RAW264.7 cells were labeled with 10% w/w of 18:1 PE-CF (Avanti Polar Lipids).

3.3 Residual solvent analysis for sshLNP

sshLNP formulations were analyzed for residual solvents content as previously described.²⁰ For headspace gas chromatography, 1 mL of the sample was weighed accurately into a headspace vial (weight of the 1 mL is recorded and critical for concentration calculations) and the sample vial was capped with a magnetic lid. The analysis was performed using Shimadzu GC-2030 connected to a flame ionization detector (FID) and an AOC-6000 autosampler, with a Phenomenex Zebron ZB-624plus 30 m x 0.32 mm x 1.80 mm column. Autosampler and headspace conditions were: incubation 80 °C for 20 min, syringe 90°C, 250 rpm, pre-purge time 30 s, and injection flow rate 10 mL/min. Injector conditions were: hydrogen as the gas carrier, 140°C, split ratio 5:1, and carrier gas linear speed 35 cm/s. Oven temperature was: baseline at 40 °C for 12 min, then ramp/rate 16.7°C/min for 12 min, and then hold at 240 °C for 12 min. FID temperature was 250 °C, nitrogen flow 30 mL/min, and air flow 400 mL/min. For blank measurement, deionized water was used, and standard curves for methanol (75–4,500 ppm) quantification were prepared from reference material purchased from Sigma Aldrich (United States Pharmacopeia [USP] Residual Solvent Class 2 – Methanol Reference Standard), properly diluted with deionized water for reaching desired concentrations. The limit of methanol in any sample must be below 3,000 ppm to fulfill USP method 467 criteria.

3.4 Silicon assessment for sshLNP

sshLNP were analyzed for silicon content post filtration by means of Shimadzu ICPE-9820 plasma atomic emission spectrometer. Briefly, empty sshLNP samples were vortexed to ensure homogeneity, and an aliquot of 10 mL was transferred to a 15-mL polypropylene tube and sonicated for 1.5 h. All samples were prepared and analyzed in triplicate, with imposing a software input of 1 for dilution

rate. Concentration of silicon in samples was measured against a calibration curve generated by diluting the Periodic Table Mix 1 for ICP certified standard (Sigma Aldrich, UK) with type 1 ultrapure water, with standards prepared as per dilutions. Parameters setting for ICPE-9820 were as follows: carrier gas 0.7 L/min, auxiliary gas 0.6 L/min, plasma gas 10 L/min, radiofrequency power 1.2 kW, exposure time 30 s, view direction axial, element/Wv = Si/251.61 nm, solvent/sample rinse time 30–60 s, and peristaltic pump ranging from 20 to 60 rpm.

3.5 Complexation of siRNA to sshLNP

In this study, two different siRNAs were used: standard ADO2 siRNA with dTdT overhang and ADO2 siRNA with dAdT overhang, which served as a positive control (Table 1). The selected siRNA was complexed with commercial preclinical transfection agent, in-vivoJetPEI (JetPEI), as previously described.^{3,4} Both siRNAs were designed as 21-mers with a central 19-bp duplex region and above specified dinucleotide overhangs on each 3' end (Table S7). These were chemically synthesized and annealed by Horizon Discovery. For nucleic acid complexation with sshLNP formulations, siRNA dissolved in nuclease-free water was added to the aqueous suspension of nanoparticles and incubated at room temperature for 60 min before further analysis. To examine the complexation ratio, siRNA was mixed with sshLNP at different w/w ratios and analyzed by gel electrophoresis.

3.6 Gel retardation assay

To assess the formation of complexes between the siRNA and sshLNP, nanoparticles suspension in nuclease-free water was combined with siRNA at various loading ratios (i.e., 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, or 1:20 w/w of siRNA to sshLNP) and analyzed by electrophoresis on a 1% agarose gel (E-Gel agarose gels [1%]; Invitrogen, ThermoFisher Scientific) for 7 min at 100 V. Equal amount of 150 ng siRNA was loaded into each well. Gels were run and visualized using E-Gel Power Snap Electrophoresis Device and camera (Invitrogen, ThermoFisher Scientific). As marker, E-Gel 1Kb plus Express DNA ladder (Invitrogen, ThermoFisher Scientific) was used.

3.7 Dynamic light scattering measurement

Mean hydrodynamic particle size, polydispersity index (PDI) and surface charge (zeta-potential) of siRNA complexes and empty sshLNP were determined using ZetaSizer Nano ZS (Malvern P Analytical). For measuring the particle size and PDI, 20 mL of each sample was mixed with 980 mL nuclease free water and placed inside a single use DLS cuvette (DT0012, Malvern). Each sample was measured four times and the average of the last three runs is reported. For measuring the zeta potential, 150 mL of each sample was diluted either with 850 mL nuclease-free water to give a total of 1,000 mL and filled inside a folded capillary cell (DTS1070, Malvern) using a 1-mL graded syringe, avoiding introducing any bubbles in the capillary, to reach yield concentration in a range of 100–400 kcps. Measurements were performed at 25° C in triplicate and reported as mean $\pm$ SD. Instrumental settings were: absorption = 0.001, refractive index = 1.33, dispersant = water, viscosity = 0.8872 at 25 °C, and time of equilibration 120 = sec.

3.8 Accessible siRNA assay

To evaluate the amount of accessible siRNA within the complexes, the Quant-iT RiboGreen RNA reagent from Invitrogen (ThermoFisher) was used, according to the manufacturer's guideline. For analysis, Varioskan LUX multimode plate reader (ThermoFisher) was used at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The test was performed in 1xTris-EDTA (TE)-buffer (10 mM Tris-HCl and 1 mM EDTA).

3.9 Cell culture

The AD293 human embryonic kidney cells (Life Technologies) were cultured in DMEM (ThermoFisher) enriched with 10% fetal bovine serum (ThermoFisher). All cell cultures were incubated at 37 °C in the presence of 5% CO₂ and passaged following standard laboratory protocols.

3.10 Cell transfection efficiency assessed by fluorescence measurement

For this assessment, sshLNP were complexed to a fluorescent commercially available model siRNA, siGLO Green Transfection Indicator (Dharmacon, Horizon). AD293 cells were seeded at 7×10^3

Cells per well in a 96-wellplate and grown overnight in a cell incubator at 37°C with 5% CO₂. Transfection experiments were performed 24 h later. The siGLO samples (kept at different storage conditions after preparation, specifically 4°C, room temperature and 37°C over 0 and 24h) were diluted in OptiMEM reduced-serum cell culture medium (ThermoFisher) and added to the cells in n=6 replicates at final 100 nM siGLO concentration (10 pmol–133 ng per 100 mL growth medium per well). Medium only (untransfected control for background fluorescence) and 10 pmol siGLO complexed with 0.2 mL LPF 2000 transfection reagent (Invitrogen) as a positive control, were prepared alongside at same 100 nM final concentration. After 24 h of incubation at standard culture conditions, the cells were washed with PBS (Sigma Aldrich) and stained with Hoechst 33342 fluorescent nuclear dye (NucBlue Live reagent, ThermoFisher Scientific) diluted in FluoroBrite DMEM medium (ThermoFisher). The fluorescence intensity of siGLO was measured at Ex/Em wavelength 485/520nm using well-scanning mode on Varioskan LUX multimode platereader (ThermoFisher). To standardize siGLOFI data, the number of cells per well was assessed by nuclear fluorescence measurement at Ex/Em wavelength 360/460 nm, and the value of siGLO/Hoechst fluorescence intensity was calculated.

3.11 Imaging of transfected cells by fluorescence microscopy

AD293 cells were seeded at 5×10^4 cells per well on a 24-well plate with coverslips and grown in a cell incubator at 37° C with 5% CO₂ overnight. Transfection experiments were performed 24 h later. The siGLO samples were diluted in OptiMEM reduced-serum cell culture medium (ThermoFisher) and added to the cells in duplicates at final 100 nM siGLO concentration (50 pmol–665 ng per 500 mL growth medium per well). As a positive control, 50 pmol siGLO was complexed with 1 mL LPF and used for transfection at same 100 nM final siGLO concentration. After 24 h of incubation at standard culture conditions, the cells were washed with PBS (Sigma Aldrich), fixed with 4% paraformaldehyde (ThermoFisher) in PBS, and counterstained with DAPI in UltraCruz Aqueous Mounting Medium (Santa Cruz Biotechnology). Imaging was performed using Zeiss Model

AxioScope A.1 LED Fluorescent Illumination Microscope equipped with DAPI (365 nm) and GFP (470/40 nm) filter and Zeiss AxioCam MRc digital camera (Carl Zeiss).

3.12 Animals

The *Clcn7* G213R/WT ADO2 mouse model (*Mus Musculus*, C57/BL6 background) has been described by Alam et al., 2013,²¹ and was previously used by Capulli et al.³ and Maurizi et al.⁴ All in vivo experiments were conducted in compliance with applicable national and international guidelines and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 4.03.2014, n.26, Gazzetta Ufficiale della Repubblica Italiana) and were approved by the Italian Ministry of Health (approval n.112/2020-PR). The study was performed according to the Animal Research Reporting of In Vivo Experiments (ARRIVE, **TableS8**) guidelines, with n = 5 mice/group upon randomization. Mice were injected i.p. with 50–100µL of the solution containing the siRNA-ssLNP complex at doses and frequencies described in the text and in the figure legends. At the end of treatments, mice were humanely sacrificed by CO₂ inhalation after anesthesia.

3.13 Isolation of PBMCs and serological tests

Blood sampling was performed in the morning in fasted mice, according to Christgau.³³ Buffy-coat mononuclear cells were isolated from diluted blood (1:1 in Hanks' solution) layered over Histopaque 1077 (Sigma Aldrich) solution and centrifuged at 400 x g for 30 min. Sera were used to measure levels of the bone resorption marker CTX by ELISA kit (IDS), following the manufacturers' instructions. Serum biomarkers of liver (alanine aminotransferase and alkaline phosphatase) and kidney (urea) diseases were measured using Reflotron strips (Roche).

3.14 Real time RT-PCR

Total RNA was extracted from tissues using Trizol (ThermoFisher) according to the manufacturer's instructions. RNA was quantified by NanoDrop (ThermoFisher) and quality checked by 1% agarose

gel run. One microgram of RNA was reverse transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher). Real-time PCR reaction was performed loading 0.1 mg of cDNA using the Luna Universal qPCR Master Mix (NewEngland Biolabs). Gene expression data were represented as relative expression over control, normalized by Gapdh. Primer sequences and PCR conditions are listed in **Table S2**.

3.15 μ CT analysis

Tibias were fixed in 4% paraformaldehyde for 48 h and scanned, by mCT SkyScan 1174, with a 6.-mm resolution using an X-ray voltage of 50 kV. The Skyscan Nrecon software was used to reconstruct the images using a modified Feldkamp algorithm.³⁴ Three-dimensional analysis was carried out with a Marching Cubes type model with a rendered surface.³⁵ The trabecular bone parameters were calculated on 550 consecutive slides starting from 100 mm below the growth plate. Pratt's algorithm was used to take two-dimensional measurements. Threshold values were applied for segmenting trabecular bone, and structural variables were determined according to Buxsein et al.³⁶

3.16 Indentation tests

Mechanical tests were performed on the midshaft of the femurs using the Reference Point Indentation technique by Biodent. Bones were kept in ice-cold PBS during the tests to maintain tissue hydration. Tests were performed using 5–10 indentation cycles at 2 Hz with a force of 2 N. The indentation distance and total indentation distance were calculated for each test.

3.17 Statistical analyses

Statistical analyses were performed in line with the recommendations of experimental design and analysis in Pharmacology.³⁷ All results are presented as mean $\pm$ SD of $n = 3$ experiments per cell preparations or $n=5$ mice/group. Statistical analyses were carried out by the Prismby GraphPad v7.0, software, using Student's t test or multiple comparisons one-way ANOVA. The statistical methods are indicated in the figure and table legends, and the p values threshold was 0.05.

4. Results

4.1 sshLNP characterization

sshLNP characterization showed results in line with previous findings, reporting purity of more than 99% with other metal traces of less than 1% of total mass (evaluated by inductively coupled plasma-mass spectrometry [ICP-MS]), a spherical shape, and an average particle size of less than 100 nm (assessed by transmission electron microscopy [TEM]) and a mean surface area of approximately 34 m²/g (determined by gas absorption and calculated through the Brunauer-Emmett-Teller [BET] theory).²⁰ The surface charge of sshLNP was determined to be -39.0 ± 1.3 mV and residual organicsolvent analysis reported methanol levels below maximum International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use limits (3,000 ppm) for both sshLNP and formulated silicon-based couriers.²⁰

4.2 Characterization of the sshLNP delivery system

Seven sshLNP prototypes were formulated through surface functionalization of silicon nanoparticles with a variety of cationic moieties, additional helper and neutral lipids, and excipients commonly used for nucleic acid delivery intended for human applications, as shown in **Table S1**. SiS B sshLNP was formulated using the same ratios and composition of excipients as for SiS 2 sshLNP, with replacing nondoped silicon with doped silicon and by using siRNA with dAdT overhangs. All remaining prototypes were formulated with non doped silicon and complexed with siRNA having dTdT overhangs. Zeta potential and binding efficiency of sshLNP prototypes were tested at different ratios. Selected leading sshLNP prototypes reported surface charge, hydrodynamic size and PDI, as stated in **Table 1**. Zetapotential analysis on lead sshLNP prototypes revealed expected trend and inversion of surface charge based on ratio complexation siRNA:sshLNP (**Table 1**). For sshLNP siRNA complexes tested in vivo, the amount of accessible RNA determined via Ribogreen assay reported as $78.1\% \pm 1.7\%$, $44.3\% \pm 1.9\%$, and $5.1\% \pm 2.2\%$ for 4 mg/kg, 2 mg/kg, and 0.2 mg/kg siRNA doses, respectively.

Table 1. Surface charge assessment at different ratios sshLNP-siRNA

Sample	sshLNP:siRNA	Size	PDI	ZP (mV)
sshLNP SiS + siRNA	20:1	194.8 ± 3.1	0.23 ± 0.03	+47.61 ± 1.13
sshLNP SiS + siRNA	12:1	142.7 ± 1.23	0.15 ± 0.01	+47.20 ± 0.35
sshLNP SiS + siRNA	8:1	144.0 ± 1.69	0.17 ± 0.02	+43.27 ± 0.75
sshLNP SiS + siRNA	6:1	143.3 ± 1.99	0.16 ± 0.02	−29.59 ± 1.06
sshLNP SiS + siRNA	4:1	143.7 ± 1.15	0.15 ± 0.01	−31.90 ± 0.79
sshLNP SiS + siRNA	2:1	147.2 ± 1.08	0.16 ± 0.02	−43.80 ± 0.74
Naked siRNA	–	–	–	−22.0 ± 0.30
Empty sshLNP	–	98.33 ± 0.54	0.08 ± 0.01	+55.43 ± 1.38
Si-NPs	–	–	–	−39.0 ± 0.41
Si-NPs doped	–	–	–	−27.5 ± 0.36

Zeta potential of siRNA, empty sshLNP, and Si-NPs are equally reported.

4.3 Short-term in vivo treatments

For the in vivo experiments, it was first evaluated whether we could downregulate the *Clcn7* ^{G213R}-mutant gene in our target organ, the bone, in a short-term experiment. To this end, 10-day-old ADO2 male mice were treated for 48h with a single intraperitoneal (i.p.) injection of the selected sshLNP loaded with 4mg/kg of *CLCN7* ^{G215R}-siRNA. Each subject weighing 10 g received a dose containing 40 mg siRNA formulated within the sshLNP or ssLNP as such (empty sshLNP). At 48 h after injection, mice were sacrificed and RNA extracted from femurs was subjected to real time RT-PCR using primer pairs specific for the *Clcn7* ^{G213R}, not recognizing the WT mRNA (**TableS2**). ³ Results showed that the sshLNP formulations, siRNA-SiS 2 and siRNA-SiS B, significantly downregulated the expression of the mutant *Clcn7* ^{G213R} mRNA in the femurs of ADO2 mice (**Figure 1A**).

In addition, no adverse events or side effects were observed in all ADO2 mice treated with the sshLNP formulations. The treatment was well tolerated, and no topical reactions were observed at the injection site. After treatment, mice walking, feeding, and behavior were normal. No sign of suffering or weight loss was observed. At sacrifice, gross evaluation of visceral organs, including liver, kidney, spleen, and lung, did not reveal overt alterations of the organ size and macroscopic morphology. No mice died during the treatment. Overall, all the SiS sshLNP formulations tested in this short-term protocol were safe without overt side effects evidently impacting the health of the treated ADO2 mice.

To further evaluate the effect of adding a positive charge to silicon through a doping process on retaining transfection efficiency, SiS 2 and SiS B sshLNP were selected as leading candidates, renamed SiS12 and SiS13 sshLNP, respectively, and further tested on a 2-week dose regimen, with 3 i.p. injections per week after being both complexed with CLCN7^{G215R} siRNA having dTdT overhang. The results obtained from the gene expression analysis showed that both siRNA-SiS12 and siRNA-SiS13 candidates significantly downregulated the expression of the mutant Clcn7^{G213R} mRNA in the femurs (**Figures 1B and 1C**) of ADO2 mice, compared with the empty SiS12, while a prominent effect on downregulating the expression of the mutant gene in peripheral blood mononuclear cells (PBMCs) was highlighted for siRNA-SiS12 (**Figure 1D**) over the siRNA-SiS13 (**Figure 1E**). The evaluation of the serum bone resorption marker, collagen type 1 C-telopeptide (CTX), unveiled a trend of increase in mice treated with both siRNA-SiS12 and siRNA-SiS13 vs. empty SiS12 and SiS13, respectively (**Figures 1F and 1G**).

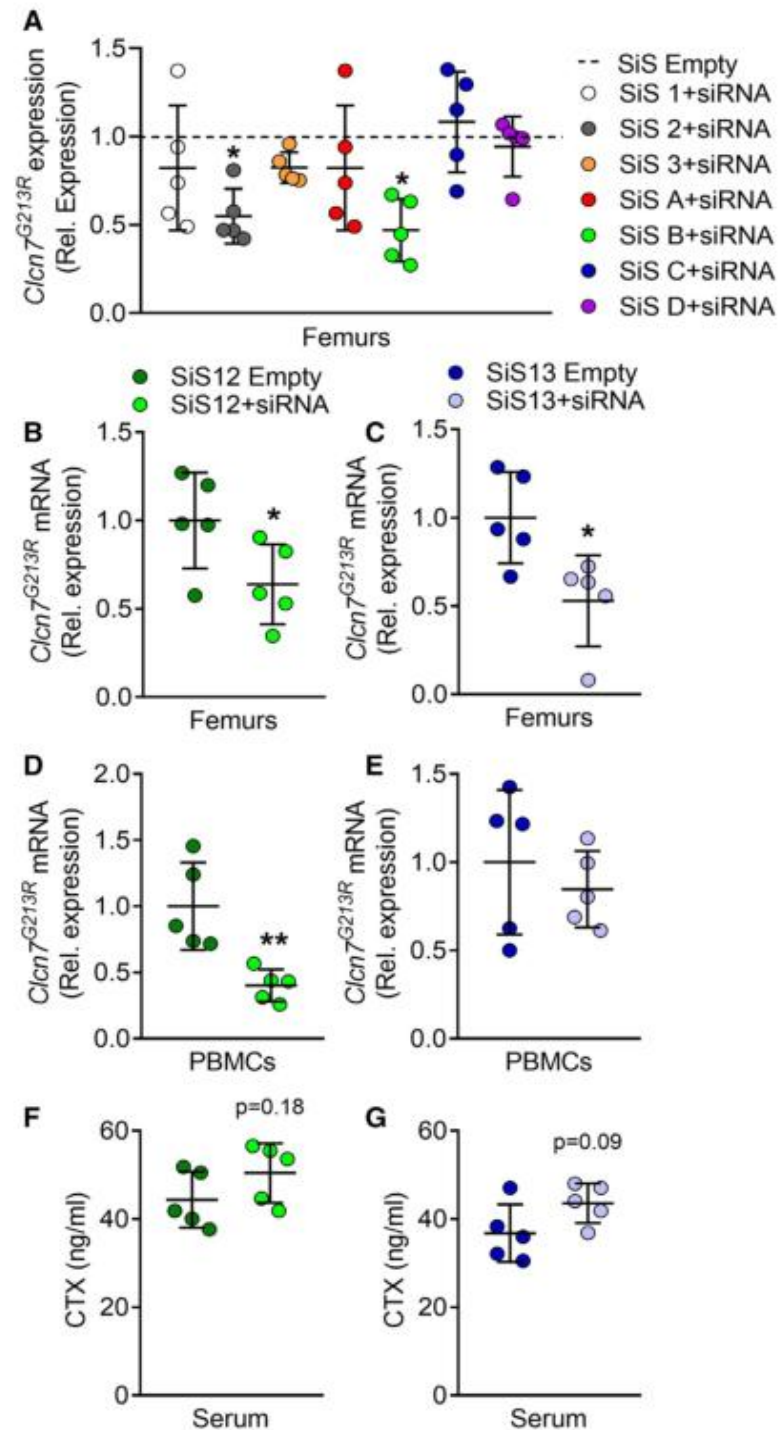


Figure 1. *Clcn7* G213R gene expression and serum CTX analysis in ADO2 mice treated with SiS sshLNP formulations. Ten-day-old ADO2 male mice were treated i.p. with the indicated SiS sshLNP loaded with 4 mg/kg CLCN7 G215R -siRNA or empty. (A) Mice were treated for 48 h and real time RT-PCR was performed using a specific primer pair for the mutant *Clcn7* G213R (Table S2), normalized by *Gapdh*. (B and C) Mice were treated for 2 weeks and real time RT-PCR for the mutant *Clcn7* G213R was performed in femurs and (D and E) in PBMCs normalized by *Gapdh*. (E and F) Evaluation of serum CTX in ADO2 mice treated for 2 weeks. Data represent the mean $\pm$ SD of $n = 5$ ADO2 mice per group. Student's *t* test vs. the SiS empty sshLNP group. * $p \leq 0.05$; ** $p < 0.01$.

No adverse events or side effects were observed in mice treated with the siRNA-SiS12 and siRNA-SiS13 formulations. The multiple dose administration regimen was well tolerated, and no change of behavioral stereotypes or variation in food intake were observed. The gross evaluation of visceral organs (liver, kidneys, spleen, brain, heart, and lungs) did not reveal alterations of size, weight (Figures 2 and 2B), or macroscopic morphology when compared with untreated wild-type (WT) mouse organs. No deaths were reported during treatment.

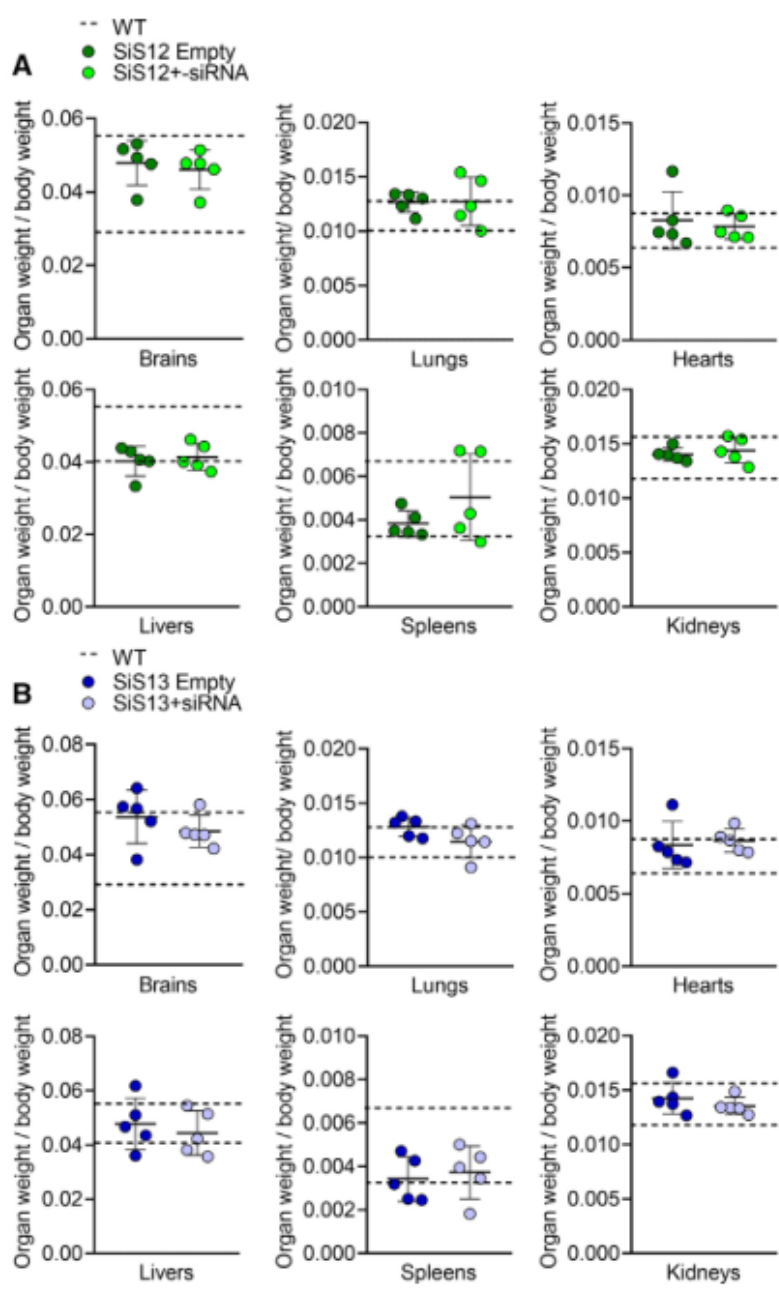


Figure 2. Treatment with SiS12 and Sis13 formulations for 2 weeks. Ten-day-old ADO2 male mice were treated i.p. 3 times a week for 2 weeks with SiS12 or SiS13 sshLNP complexed with 4 mg/kg of CLCN7

G215R -siRNA or as such (empty sshLNP). (**A and B**) At the end of the experiment, the weights of the indicated organs were taken to assess the presence of macroscopic alterations. For each mouse, the organs' weight was normalized for the body weight. An untreated WT group was examined for comparison. Data are the mean $\pm$ SD of $n = 5$ mice per group. Student's t test.

Although both sshLNPs were shown to be effective in downregulating the expression of mutated gene in target tissue, based on these results and intending to obtain a circulating biomarker to be potentially used for in vivo monitoring of treatment progression for this particular study, the siRNA-SiS12 formulation was selected to be further investigated.

4.4 *In vitro* stability of SiS12 sshLNP and cellular uptake

Before performing the complete in vivo tests, we evaluated the stability and the uptake efficiency of SiS12 sshLNP in a series of in vitro studies. First, to track stability by imaging, we incubated the AD293 cells for 24 h with our test nanoparticles loaded with the siGLO green, fluorescent indicator.²² As a reference transfection reagent, we used lipofectamine (LPF). Over a treatment time of 24 h with freshly prepared complexes, we observed that naked siGLO was not internalized by cells, while the siGLO+LPF complex showed very low transfection efficiency. siGLO+LNPs were instead internalized by cells with medium efficiency, while siGLO+fresh LPF was transfected with higher efficiency. Importantly, siGLO+SiS12 sshLNP showed the highest internalization potential compared with the other conditions.

When siGLO complexes were incubated for 24 h at 4°C, room temperature (22°C) (Figure S1C), or 37°C before being administered to cells, siGLO+SiS12 sshLNP retained stability in all conditions, followed by naked siGLO+fresh LPF and siGLO+LNP (Table S4). In contrast, siGLO+LPF complex remained inefficient.

The stability of the formulation at 4 °C, 22 °C and 37°C was confirmed in the mouse osteoclast precursor cell line, RAW264.7 cells, incubated with 100 nM CLCN7 G215R siRNA complexed with SiS12 labeled with the a green fluorescent tag, containing 18:1 of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-carboxyfluorescein (PE-CF) at 10% of the total amount of DOPE used for formulating the Bio-Courier (SiS12-18:PE-CF10%) (**Figure 3A**). Furthermore, the uptake in this cell line was dose dependent (**Figure 3B**) and increased with time, reaching a plateau after 24h of

incubation (**Figure 3C**), further confirming stability. Taken together, these results underline the potential of this formulation to represent a stable product suitable for safe storage.

4.5 Bone and bone marrow cell uptake

To address which cell types in the bone take up the siRNA-SiS12 formulation, we flushed out bone marrow cells from femurs and adherent cells from non-adherent cells. Then we evaluated the green fluorescence by fluorometry on 500,000 cells per group, observing that non-adherent cells, known to be enriched in the hematopoietic cell component, which includes the osteoclast lineage, showed twice as much fluorescence compared with adherent cells, the latter of which is known to be enriched in the stromal cell component, which includes the osteogenic lineage (**Figure 3D**). One million labeled non-adherent cells were then sorted by fluorescence-activated cell sorting for size and granularity to distinguish the populations enriched in lymphocytes, monocytes, and granulocytes (**Figures 3E and 3F**). Among them, the highest fluorescence was observed in the monocyte-enriched fraction (**Figure 3G**), known to include the osteoclast precursors, representing our *in vivo* target bone cells to treat osteopetrosis.

4.6 Biodistribution and pharmacokinetics

To evaluate the biodistribution and pharmacokinetics of the siRNA-SiS12 formulation, we used 4mg/kg CLCN7^{G215R} siRNA in the complex with the SiS12 tagged with 18:1PE-CF10%. Four-week-old WT mice were injected i.p. once with the formulation and sacrificed after 6, 24, and 48 h. After sacrifice, organs were collected and freshly analyzed to detect the green fluorescence of the SiS12 sshLNP. Results showed a modest fluorescence in serum at 6 h, with a subsequent decline, suggesting a rapid transit in the circulation. In contrast, the fluorescence increased over time in the other organs, reaching the maximum at 24h followed by a plateau (**Figure 3H**). Femurs, representing our target bone segments in this experiment, showed a mean fluorescence uptake at 48h of 13% of the administered dose. Fluorescence was also observed in liver, kidneys, lungs, and spleen (**Figure 3H**).

To evaluate the impact of the route of administration on biodistribution and pharmacokinetics, we also treated the mice by subcutaneous injection with the same protocol as for the i.p. administration,

observing similar results, with femur uptake at 48 h of approximately 19% of the administered dose (**Figure 3I**).

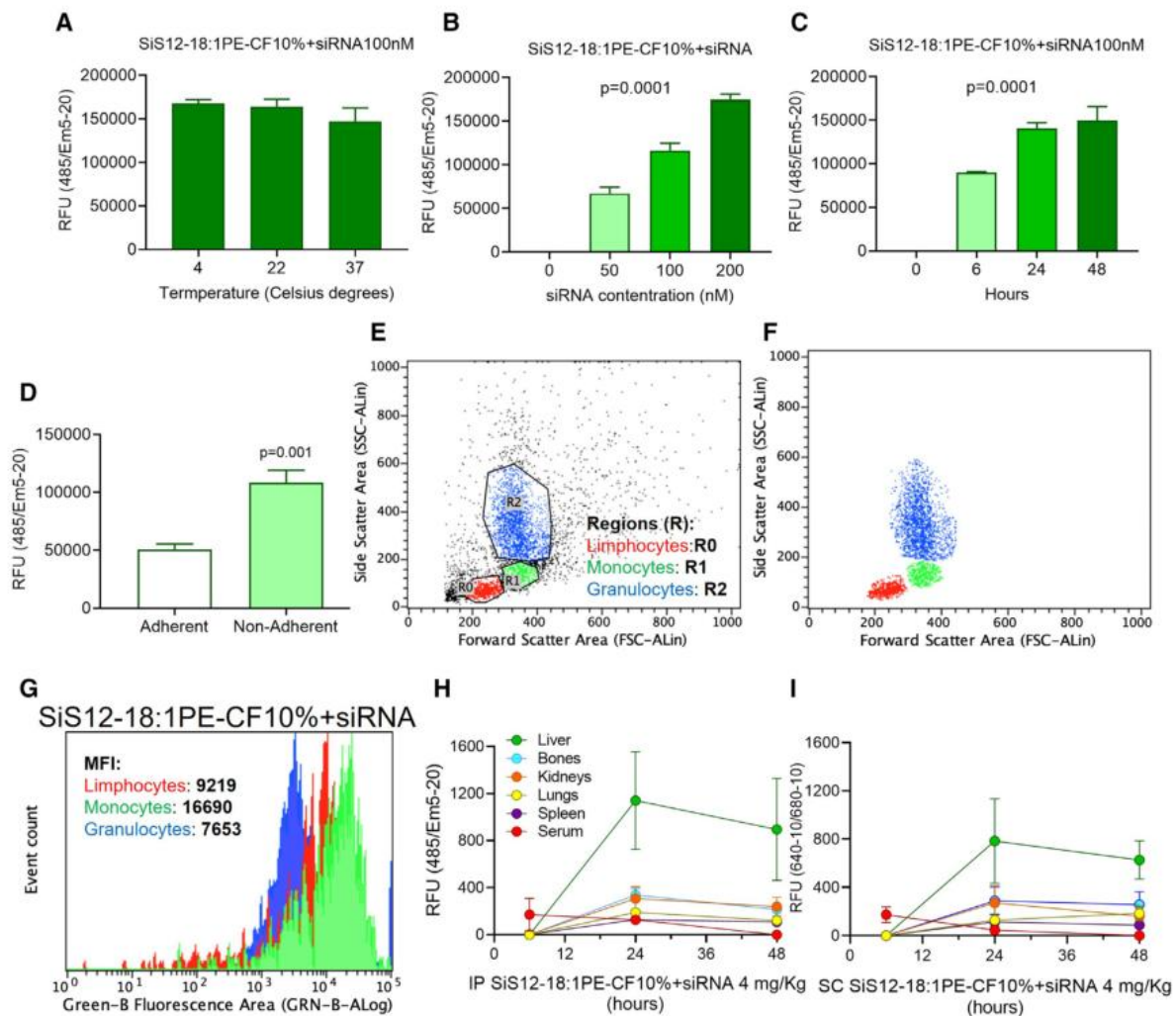


Figure 3. Cellular uptake, biodistribution, and pharmacokinetics. RAW264.7 cells were incubated with SiS12-18:1PE-CF10%+siRNA at the indicated (A) temperatures (time: 24 h), (B) concentrations (time: 24 h) and (C) times and evaluated for cellular uptake by fluorometry. (D) Cells were flushed out from femurs and allowed to adhere to culture dish for 12 min to separate the adherent fraction (enriched in stromal cells) from the non-adherent fraction (enriched in hematopoietic cells). Five hundred thousand cells per fraction were then incubated with SiS12-18:1PE-CF10%+siRNA for 3 h, washed, and evaluated by fluorometry to detect the green fluorescence. (E) One million of labeled non-adherent cells were sorted by fluorescence-activated cell sorting (FACS) by size and granularity to distinguish the fractions enriched in lymphocytes, monocytes and granulocytes. (F) Image in (E) cleaned from the background to better visualize the three fractions. (G) Mean fluorescence intensity (MFI) in the cell fractions shown in (G). (H) Four-week-old ADO2 mice were injected i.p. and (I) subcutaneously with 4 mg/kg ADO2 siRNA formulated with SiS12-18:PE-CF10%. Mice were sacrificed at the indicated time points, and organs were harvested and analyzed by fluorometry to detect

green fluorescence. Results are the mean $\pm$ SD of 3 in vitro experiments or 5 mice per group. Statistics: (B and C) multiple comparison ANOVA; (F) Student's t test.

4.7 Long-term in vivo experiments

Having demonstrated the efficacy and tolerability of the siRNA-SiS12 formulation in short-term in vivo experiments, and efficacy and stability in vitro tests, we performed in vivo long-term experiments to evaluate the effect of siRNA-SiS12 on the bone phenotype of 10-day-old ADO2 male mice treated i.p. 3 times a week for 4 consecutive weeks. WT mice treated with saline were used as control to compare the phenotype of the ADO2 mice with normal reference values. Mice treated with the CLCN7^{G215R}-siRNA naked or combined with the commercially available PEI derivative, in-vivo JetPEI^{3,4} were used as negative and positive controls, respectively. At the end of the experiment, mice were sacrificed, and bones, organs, and blood were harvested for analysis.

4.8 Bone morphometry and quality

Micro computed tomography (mCT) analysis carried out on proximal tibias revealed that ADO2 mice treated with SiS12 sshLNP combined with 4 and 2 mg/kg of CLCN7^{G215R}-siRNA showed a rescue of the of 4 mg/kg siRNA-SiS12 formulation was comparable with that of the positive control formulation at the same dose of siRNA. No effect on trabecular bone structural variables was observed with 0.2 mg/kg CLCN7^{G215R}-siRNA combined with SiS12 sshLNP or with 4 mg/kg naked CLCN7^{G215R}-siRNA (**Figures 4A–4E**) compared with ADO2 mice treated with empty SiS12.

At the same time, bone quality analysis was carried out using the micro indentation apparatus Biodent. The indentation test on the midshaft of the femurs harvested from the treated mice was performed for calculating the indentation distance and the total indentation distance. These parameters are inversely correlated with bone strength and quality. The results revealed that the indentation distance (**Figure 4F**) and the total indentation idstance (**Figure 4G**) were significantly lower in the ADO2 mice treated with the SiS12 sshLNP combined with 4 and 2 mg/kg of CLCN7^{G215R}-siRNA compared with the SiS12 empty sshLNP, indicating an increase in bone strength that was rescued to the WT level. Of note, the effect of 4 mg/kg siRNA-SiS12 formulation was comparable with that of the positive control at the same dose of siRNA. No effect on bone quality was observed with 0.2 mg/kg CLCN7^{G215R}-

siRNA combined with the SiS12 sshLNP, or with 4 mg/kg naked CLCN7^{G215R}-siRNA, compared with ADO2 mice treated with SiS12 sshLNP empty for the considered route of administration (Figures 4F and 4G).

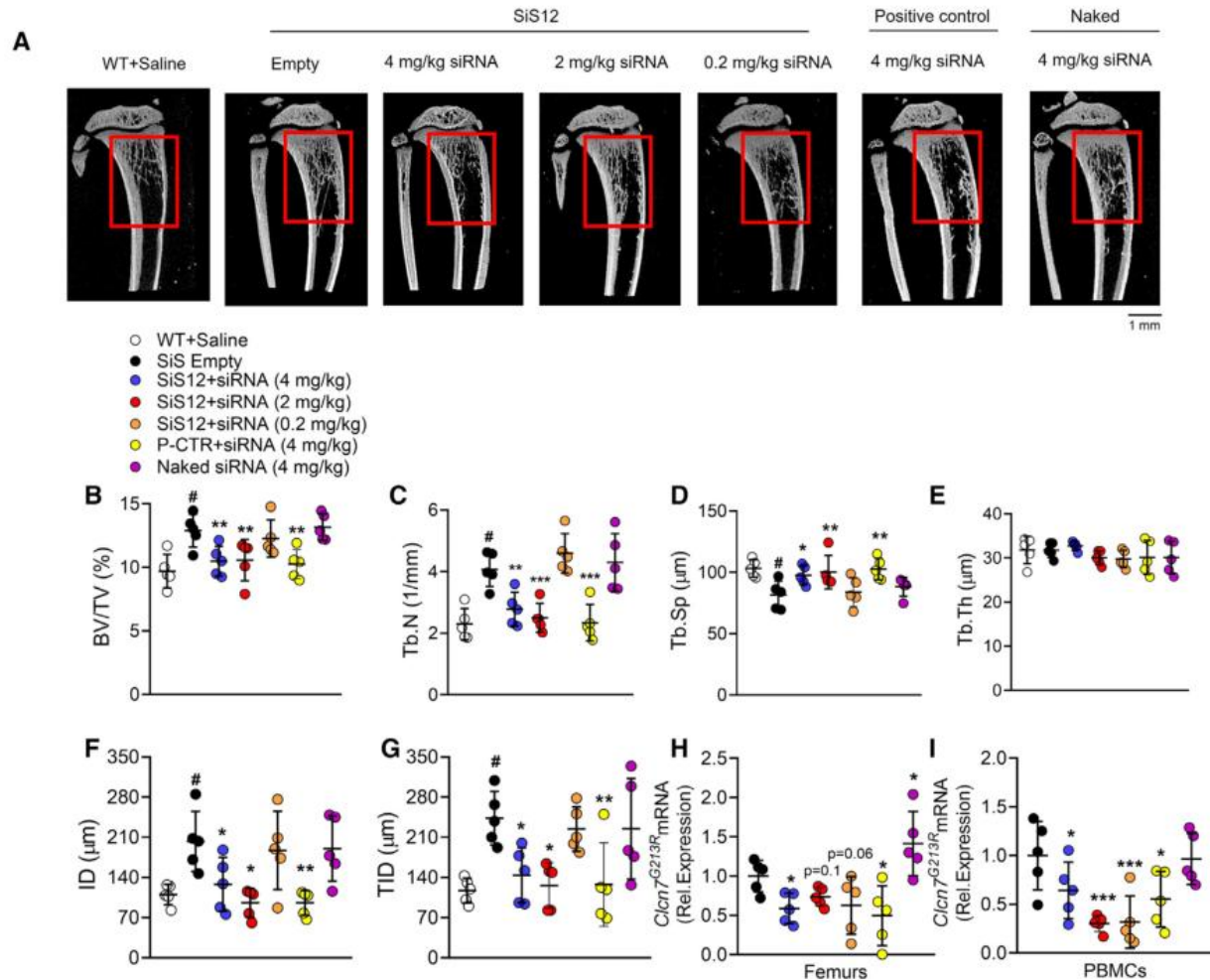


Figure 4. Bone phenotype analysis. Ten-day-old ADO2 male mice were treated with the indicated siRNA and siRNA-SiS sshLNP Porpositive control (P-CTR) complexes and doses, 3 times a week for 4 weeks. At the end of the experiment, mice were sacrificed, and tibias and femurs were collected and analyzed. (A) Representative mCT reconstructions of proximal tibias. Red squares indicate the region of interest subjected to the analysis. (B) Morphometric analysis of bone volume over total volume (BV/TV%) and (C) trabecular number (Tb.N), (D) separation (Tb.Sp), and (E) thickness (Tb.Th) assessed by mCT. (F) Indentation distance (ID) and (G) total indentation distance (TID) measured in femur midshafts by indentation test using the Biodent device. (H) Downregulation of the mutant *Clcn7*^{G213R} in femurs and (I) PBMCs, normalized by *Gapdh*. Results are (A) representative or (B–I) the mean ± SD of n = 5 mice per group. Multiple comparison

ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. the SiS empty sshLNP group; # $p < 0.01$ vs. WT + Saline group.

4.9 Clcn7 G213R gene downregulation in bone and PBMCs

RNA was isolated from femurs and PBMCs of ADO2-treated mice and the downregulation of the mutant Clcn7 ^{G213R} mRNA was evaluated by real time RT-PCR using primers specific for the mutant Clcn7 ^{G213R}.³ The results in femurs showed a significant downregulation of the mutant Clcn7 G213R in ADO2 mice treated with 4 mg/kg CLCN7 ^{G215R} -siRNA combined with the SiS12 sshLNP or with the positive control compared with ADO2 mice treated with empty SiS12 sshLNP (**Figure 4H**). Moreover, a trend of reduction was also observed in ADO2 mice treated with 2 and 0.2 mg/kg CLCN7 ^{G215R} -siRNA combined with the SiS12 sshLNP, while the administration of naked CLCN7 ^{G215R} -siRNA significantly increased the Clcn7 ^{G213R} gene expression in the femurs of ADO2, compared with empty SiS12 sshLNP (**Figure 4H**).

In the PBMCs isolated from ADO2 mice treated with SiS12 sshLNP combined with 4, 2, and 0.2 mg/kg of CLCN7 ^{G215R} -siRNA we observed a significant downregulation of the mutant Clcn7 ^{G213R} compared with the empty SiS12 sshLNP (**Figure 4I**). Similar Clcn7 ^{G213R} downregulation was also observed in the PBMCs of ADO2 mice treated with 4 mg/kg CLCN7 ^{G215R} -siRNA combined with the positive control (**Figure 4I**).

4.10 Safety analysis

No obvious adverse events or side effects were observed in all ADO2 mice treated with the naked CLCN7 ^{G215R} -siRNA and siRNA-SiS12 formulations. The treatment was well tolerated, and no topical reactions were observed at the injection site. After treatments, mice locomotion, food intake, and behavior were normal. No sign of suffering or weight loss was observed in ADO2 mice treated with the naked siRNA and the siRNA-SiS12 formulations compared with WT mice treated with saline (**Figure 5A**). At sacrifice, body weight and size, and weight of treated ADO2 visceral organs, including liver, kidneys, spleen, brain, heart, and lungs, were not altered compared with WT mice (**Figures 5B–5H**). No mice died during the treatment (**Figures 5B–5H**). Of note, ADO2 mice treated with 4 mg/kg of CLCN7 ^{G215R} -siRNA combined with the positive control showed a lower body

weight gain during the treatment and, at sacrifice, the body weight was significantly lower compared with empty SiS12 sshLNP-treated ADO2 mice and WT mice (**Figures 5A and 5B**). In contrast, brain, lung, and kidney weights, normalized by body weights, were higher than control (**Figures 5C, 5D, and 5H**), suggesting a possible toxic effect of the positive control confirming previous observations.²³ Finally, serological disease biomarkers of liver (alanine aminotransferase and alkaline phosphatase) (**Figure 5I**) and kidney (urea) (**Figure 5K**) were unchanged in all treated ADO2 mice compared with the WT controls.

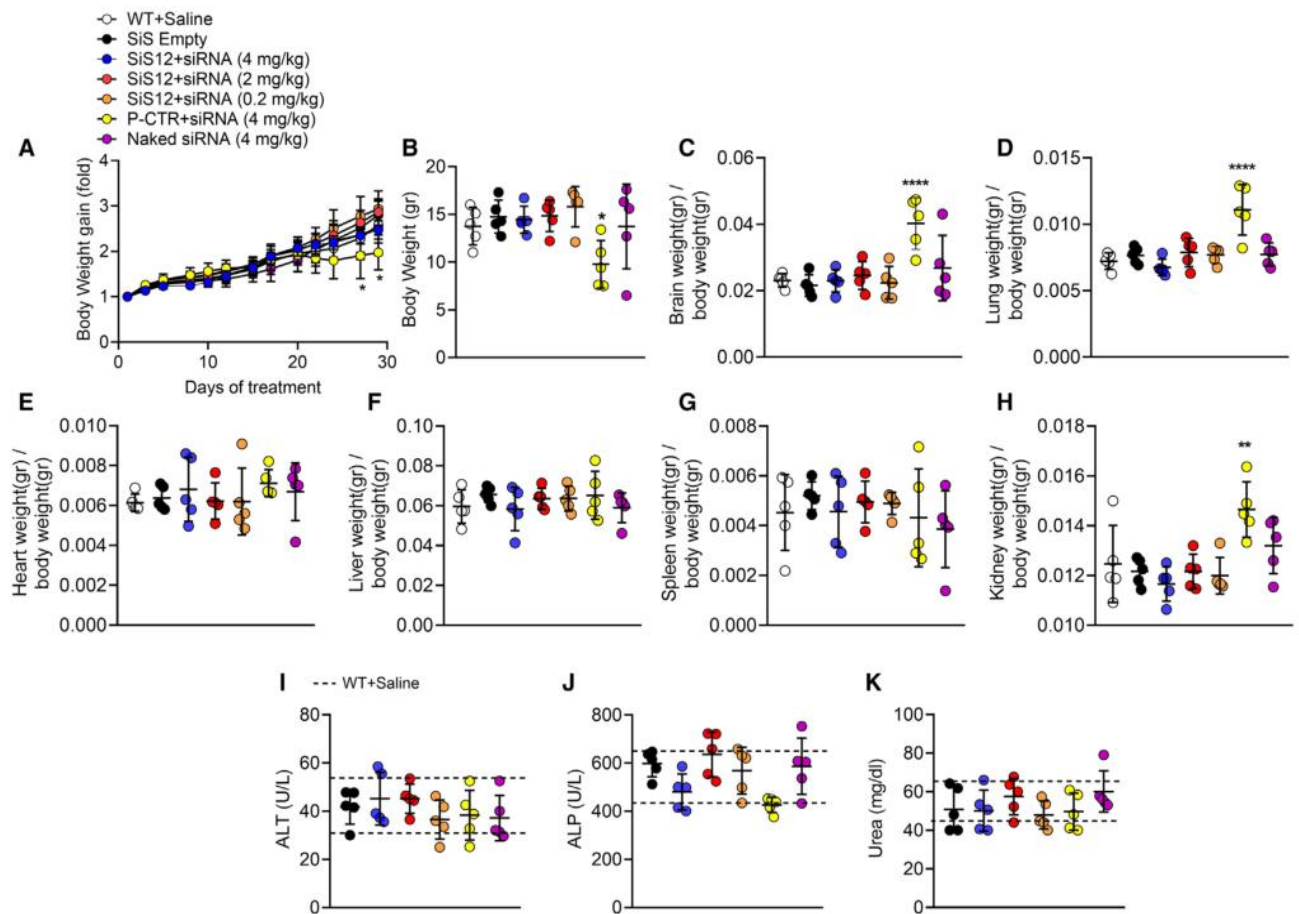


Figure 5. Safety analysis. Ten-day-old ADO2 male mice were treated with the indicated siRNA and siRNA-SiSsshLNP or positive control (P-CTR) complexes and doses, 3 times a week for 4 weeks. (**A**) Body weights measured at the indicated time points and their gain calculated normalizing the body weights measured during the treatment with the body weights at time=0 (pre-treatment). (**B**) Body weights and weights of (**C**) brains, (**D**) lungs, (**E**) hearts, (**F**) livers, (**G**) spleens, and (**H**) kidneys assessed at the end of the experiment. (**I and J**) Sera collected from the same mice were analyzed by the Reflotron method for the indicated biomarkers of liver and (**K**) kidney diseases. Results are the mean $\pm$ SD of $n = 5$ mice per group. Multiple comparison ANOVA vs. the WT + Saline group. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

The siRNA-SiS12 formulations did not significantly alter the transcriptional expression in femurs of the inflammatory cytokines *Tnf- α* , *Il-1 β* , *Il-6*, and *Ifn- γ* (**Figures 6A–6D**) and of the closely related chloride transporters, *Clcn3* and *Clcn5* (**Figures 6E and 6F**), nor were significant differences observed in the serum levels of the inflammatory cytokines tumor necrosis factor (TNF)- α and IL-6 (**Figures 6G and 6H**) and of the antibodies against PEG liposome products vs. WT-saline mice (**Figure 6H**).

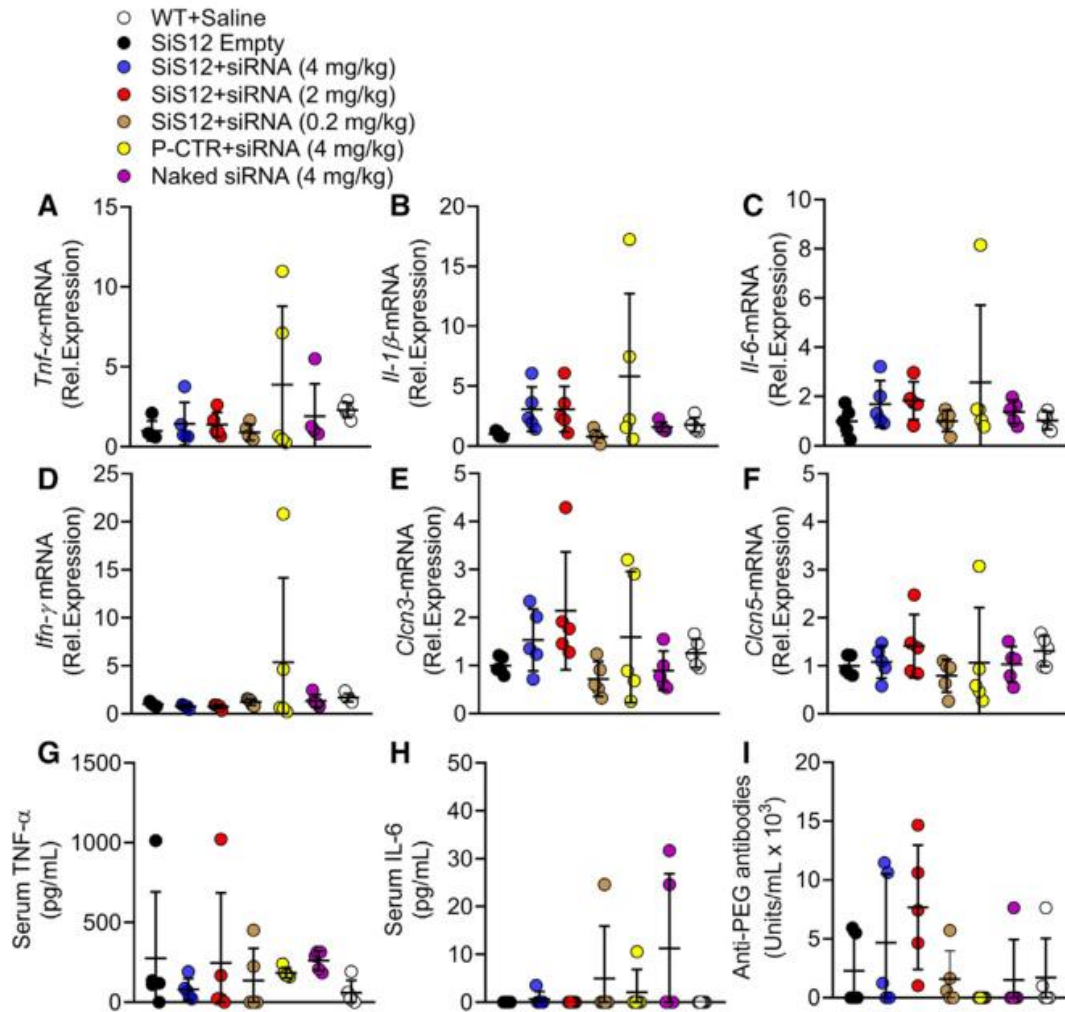


Figure 6. Expression of inflammatory cytokines, chloride transporters and anti-PEG antibodies. Ten day-old ADO2 male mice were treated with the indicated siRNA and siRNA-SiS sshLNP or positive control (P-CTR) complexes and doses, 3 times a week for 4 weeks. (A) Real-time RT-PCR of the inflammatory cytokines *Tnf- α* , (B) *IL-1 β* , (C), *IL-6*, and (D) *INF- γ* expressed in femurs, normalized by *Gapdh*. (E) Real time RT-PCR of the chloride transporters *Clcn3* and (F) *Clcn5* expressed in femurs, normalized by *Gapdh*. (G) Serum levels of *TNF- α* , (H) *IL-6* and (I) anti-PEG antibodies. Results are the mean $\pm$ SD of $n = 5$ mice per group. MC-ANOVA vs. the WT+saline group. Differences are statistically not significant.

5. Discussion

In this study, a siRNA therapy has been optimized to treat an autosomal dominant bone disease (ADO2) characterized by the impaired activity of the chloride/proton antiporter type 7, essential for charge balancing the environment of the resorption lacuna excavated by osteoclasts during the process of bone resorption.¹ Autosomal dominant diseases are ideal candidates for siRNA therapy, provided that the curative siRNAs are properly designed to complement the mutant site of the affected heterozygous mRNA without affecting the WT mRNA. Indeed, this is the case for our therapy, for which we have identified a highly efficient and specific siRNA, complementing the mRNA encoded by the ADO2 mutant *CLCN7* gene.

In this study, we provided evidence that a formulation of our ADO2 siRNA complexed with sshLNPs proved safe and effective in downregulating the expression of the mutant *Clcn7*^{G213R} mRNA in ADO2 mice, rescuing the ADO2 bone mass to the WT level. Importantly, the formulation also improved bone quality, which is degraded in ADO2 patients, causing numerous atraumatic fractures that are difficult to heal.²⁴ The relevance of these observations is several folds, since these results open an avenue to the exploitation of this discovery for a first-in-human clinical approach.

From a technological perspective, several considerations emerged from our study. Chemically modified siRNAs are currently approved for therapy.²⁵ Of note, we did not increase siRNA stability by chemical modifications because they are reported to be associated with toxicity, immune stimulation, and off-target effects.²⁶ Moreover, an increased stability of the siRNA is known to increase resistance to cleavage, which induces altered response to the RISC complex in both short- and long-term dose regimens. To avoid these adverse events, we developed the SiS sshLNP technology that protects the efficacy of siRNA without need for chemical modifications.

Using the sshLNP technology, we performed in vivo screening of many *CLCN7* G215R siRNA-SiS sshLNP complexes from a formulative perspective. Comparing SiS 2 and SiS 3 sshLNP, although cationic lipid DOTAP is present and the total amount of lipids is unvaried between the two, the change of helper moieties affected the transfection efficiency, leading to the absence of statistical significance between treated and control mice for the SiS 3 group. Furthermore, the presence of PEI groups, integrated to helper lipids only (SiS C) or in the presence of DOTAP (SiS D), did not sustain the transfection in the presence of siRNA dTdT overhangs for the intended sequence. This outcome resembles the lack of efficacy of other non-viral vectors constituted by polycationic PEI groups, complexed with siRNA possessing alternative overhangs with respect to sticky 3' overhangs dAdT.²⁷

Substitution of positive charge (SiS A, SiS C vs. SiS 2) or alteration of helper lipid composition (SiS 3 vs. SiS 2) had a similar impact on the efficiency of transfection. In comparison, the identified leading candidates, SiS 2 and SiS B, formulated with dTdT overhangs or dAdT overhangs, retained transfection efficiency.

Our short-term (48 h) and medium-term (2 weeks) *in vivo* experiments using the SiS 2 (renamed SiS12) and SiS B (renamed SiS13) sshLNP allowed us to refine the selection of the formulations, taking the SiS12 as lead sshLNP prototype to be investigated in long-term (4 weeks) experiments. To this end, we first tested the stability *in vitro* of the siRNA-SiS12 formulation, noting no significant impairment upon 24 h incubation of the complex at 4 °C, room temperature (22 °C), and 37°C. The uptake was dose and time dependent and remained stable for 48 h both *in vitro* and *in vivo*. Furthermore, the formulation was biodistributed *in vivo* systemically in bones and other organs, representing a strength for a potential use in humans, given that the *CLCN7* gene is expressed in many organs, proved to alter organ function when mutated, and to be susceptible in ADO2 to down-regulation and phenotypic rescue when targeted by our siRNA.²⁸

Then, we administered 0.2 to 4 mg/kg siRNA-SiS12 complex in ADO2 mice for a complete phenotypic evaluation in comparison to the commercially available positive control, with which the efficacy of the *CLCN7* ^{G215R} -siRNA was assessed in our previous studies.^{3,4} This treatment proved to be effective, with a dose-dependent improvement in bone structural variables and bone quality. The maximal effect was observed already at 2 mg/kg siRNA dose, which is one-half of the effective dose of the reference positive control formulation.³ The *CLCN7* ^{G215R} -siRNA-SiS12 complex downregulated the mutant *Clcn7* ^{G213R} gene in femurs and PBMCs, including circulating mononuclear osteoclast precursors.

Importantly, in contrast with the *CLCN7* ^{G215R} -siRNA-positive control complex, no toxic effects were observed injecting the *CLCN7* ^{G215R} -siRNA-SiS12 complex. No topical reactions were noticed, while walking ability, food intake, and body weight gain were unchanged compared with WT mice treated with saline and with ADO2 mice treated with naked siRNA or SiS12 empty sshLNP. Similar unchanged weights were observed in all single visceral organs investigated in treated mice vs. controls. In contrast, the weight of brain, lung, and kidney, normalized to body weight, was higher in mice treated with the *CLCN7* ^{G215R} -siRNA-positive control complex. Furthermore, the *CLCN7* ^{G215R} - siRNA-SiS12 complex did not exert pro-inflammatory effect, as demonstrated by the unchanged expression of *Tnf-α*, *IL-1β*, *IL-6*, and *INF-γ* and their serum proteins when detectable. No changes were also observed in the mRNA expression of the *Clcn7* closely related chloride transporters, *Clcn3* and *Clcn5*, suggesting high specificity for the *Clcn7* mRNA and lack of off-target's events. Finally,

anti-PEG antibodies were detectable in the serum of our control WT mice, and their levels were not statistically different vs. the treated groups. This result is consistent with,^{29,30} who detected anti-PEG antibodies in healthy control mice without specific exposure to PEG. Altogether, these results suggest that the CLCN7^{G215R}-siRNA-SiS12 complex is effective, safe, and stable in our murine model of ADO2, suggesting potential for first in human translation to the ADO2 patients.

In conclusion, genetic skeletal disorders are still challenging to be treated because of the lack of tissue specificity reported for most drug delivery systems with respect to bone targeting.³¹ We here demonstrated that a siRNA-based approach targeting the CLCN7^{G215R} mRNA, when vehiculated through SiSaf's sshLNPs, is able to significantly downregulate the Clcn7^{G213R} expression in femurs. The complexed sshLNP leading prototype induced a significant downregulation of the mutant mRNA in PBMCs of treated ADO2 subjects, thus providing an additional translational circulating marker for ADO2 treatment toward clinical development. Further analyses revealed no adverse alterations in treated mice, demonstrating excellent tolerability and achievement of intended therapeutic outcome. Reported results have significant translational impact on orphan and rare bone disease therapies and open the path to human trials using sshLNP technology with RNA.

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