

Age-associated changes in purinergic signaling and mechanoadaptation of bone cells

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Abstract

Mechanical loading in the form of exercise plays an essential role in maintaining bone health. However, the skeleton of aged individuals is unable to adapt to mechanical loads as evidenced in mice and human studies but little is known about the molecular mechanisms at the cellular level. When bone experiences loading, bone cells sense and transmit this information ultimately leading to the expression of genes that regulate overall bone mass changes. ATP is one of the earliest signals released on mechanical stimulus. ATP and its derivative, ADP act on purinergic receptors on bone cells and cause a cascade of events that ultimately regulate their activity. With age, there is an imbalance in bone-resorbing osteoclast and bone-forming osteoblast activity. It is also known that the ADP-sensing receptor $P2Y_{13}$ regulates osteoblast and osteoclast activity. However, little is known about the role of osteoclasts in mechanical loading and how aging affects mechanically induced signaling through ATP and ADP in bone cells. I hypothesize that ATP- and ADP-mediated early mechanical signaling changes with age in osteoblasts and osteoclasts, affecting the ability of bone to sense and adapt to mechanical load.

This thesis aims to examine: i) The principles underlying mechanosensitivity and mechanoresponsiveness of osteoclasts; ii) Aging-induced changes in the responses of osteoblasts and osteoclasts to reversible mechanical injury; iii) The characteristics of

signaling dynamics mediated through ADP receptor, P2Y₁₃.

Firstly, I investigated the mechanosensitivity and mechanotransduction in osteoclasts. Osteoclasts were subjected to in vitro mechanical stimulation and intracellular calcium signaling changes were recorded in stimulated (primary) and neighboring (secondary) cells. I found that osteoclasts respond to mechanical stimulus and can transmit this information to secondary cells as observed with rise in intracellular calcium signals. Severe injuries resulted in higher calcium transients in primary responders. Osteoclasts responded to fluid shear forces in an increasing stress-dependent manner and were able to repair the injury with time. Osteoclasts responded to both ATP and ADP in a concentration-dependent manner. Blocking the ATP-signaling P2X₇ receptor abolished secondary responders. Mildly injured osteoclasts exposed to ATP and ADP had similar calcium transients in both primary and secondary responders. However, severely injured osteoclasts had a higher fraction and amplitude of secondary responders in an ADP-rich environment thus highlighting the role of ADP in osteoclasts exposed to altered mechanical loading.

To understand the effect of age on the ability of bone cells to sense and transduce signals, I conducted in vitro mechanical stimulation experiments in osteoblasts or osteoclasts from young and old mice. I found that osteoblasts have decreased mechanotransduction with age whereas osteoclasts exhibited increased signaling trends. Osteoclasts from older mice displayed higher calcium transients on addition of ATP and ADP. The expression of the ADP-sensitive P2Y₁₃ receptor was altered in osteoclasts and osteoblasts with age.

To better understand P2Y₁₃-mediated signaling, I conducted a comprehensive meta-analysis of available studies on the P2Y₁₃ receptor. The primary objective was to assess

potency of various ligands of the P2Y₁₃ receptor. The secondary objective was to determine whether the response of P2Y₁₃ to ligands varies among different systems. P2Y₁₃ appeared to be the most sensitive ADP described to date. Thus, I identified distinct age-dependent changes in early mechanotransductive signaling in osteoblasts and osteoclasts, demonstrating that both cell types should be considered as active players in mechanoadaptation. Moreover, my research implicates ADP signaling through the P2Y₁₃ receptor in altered responsiveness of aging bone cells to mechanical stimulation, suggesting novel pharmacological targets for osteoporosis.

Résumé

La charge mécanique joue un rôle essentiel dans le maintien de la santé des os. Cependant, le squelette des individus âgés est incapable de s'adapter aux charges mécaniques, mais on en sait peu sur les mécanismes moléculaires. Lorsque l'os subit une charge, les cellules osseuses détectent et transmettent cette information, conduisant à l'expression de gènes qui régulent les modifications de la masse osseuse. L'ATP est l'un des premiers signaux émis lors d'un stimulus mécanique. L'ATP et l'ADP agissent sur les récepteurs purinergiques des cellules osseuses et provoquent une cascade d'événements qui régulent leur activité. Avec l'âge, il y a un déséquilibre dans l'activité des ostéoclastes, et des ostéoblastes. Le récepteur P2Y₁₃, sensible à l'ADP, régule l'activité de ces cellules. On sait peu de choses sur le rôle des ostéoclastes dans la charge mécanique et sur la manière dont le vieillissement affecte la signalisation induite mécaniquement via l'ATP et l'ADP dans les cellules osseuses. J'émetts l'hypothèse que la signalisation précoce médiée par l'ATP et l'ADP change avec l'âge, affectant la capacité des os à détecter et à s'adapter à la charge mécanique.

Cette thèse vise à examiner : i) Les principes de la mécanosensibilité/mécanoréactivité des ostéoclastes; ii) Les changements dus au vieillissement dans les réponses des cellules osseuses à des lésions; iii) la dynamique de signalisation médiée par le récepteur de

l'ADP P2Y₁₃.

Premièrement, j'ai étudié la mécanosensibilité et la mécanotransduction des ostéoclastes. Ceux-ci ont été soumis à une stimulation mécanique et des modifications de la signalisation du calcium ont été enregistrées dans les cellules stimulées (primaires) et voisines (secondaires). Les ostéoclastes répondent à un stimulus mécanique et transmettent cette information aux cellules secondaires, comme observé avec l'augmentation des signaux calciques. Des blessures graves ont entraîné des transferts de calcium plus élevés chez les cellules primaires. Les ostéoclastes ont réagi aux contraintes d'écoulement de manière croissante et dépendantes de la contrainte et ont pu réparer la blessure. Les ostéoclastes ont répondu à l'ATP et à l'ADP de manière dépendante de la concentration. Le blocage du récepteur de signalisation par l'ATP P2X₇ a aboli les répondeurs secondaires. Les ostéoclastes légèrement blessés exposés à l'ATP et à l'ADP présentaient des transferts calciques similaires chez les répondeurs primaires et secondaires. Cependant, les ostéoclastes gravement blessés présentaient une quantité de répondeurs secondaires plus élevées dans un environnement riche en ADP, soulignant ainsi le rôle de l'ADP dans les ostéoclastes exposés à une charge mécanique altérée.

Pour comprendre l'effet de l'âge, j'ai effectué des stimulations mécaniques in vitro sur des cellules osseuses de souris jeunes et âgées. J'ai découvert que les ostéoblastes présentaient une mécanotransduction diminuée avec l'âge, alors que les ostéoclastes présentaient des tendances de signalisation accrues. Les ostéoclastes de souris plus âgées présentaient des transferts de calcium plus élevés lors de l'ajout d'ATP et d'ADP. L'expression de P2Y₁₃ était altérée dans les ostéoclastes et les ostéoblastes avec l'âge. Pour mieux comprendre la signalisation médiée par P2Y₁₃, j'ai mené une méta-analyse

des études sur le récepteur P2Y₁₃. Les objectifs étaient 1) d'évaluer la puissance de divers ligands de P2Y₁₃ et 2) de déterminer si la réponse du P2Y₁₃ aux ligands varie selon différents systèmes. Ainsi, j'ai identifié des changements distincts dépendant de l'âge dans la signalisation mécanotransductive précoce dans les ostéoblastes et les ostéoclastes, démontrant qu'ils doivent être considérés comme des acteurs dans la mécanoadaptation. De plus, mes recherches impliquent la signalisation de l'ADP via le récepteur P2Y₁₃ dans la réactivité altérée des cellules osseuses vieillissantes, suggérant de nouvelles cibles pharmacologiques pour l'ostéoporose.

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Contribution

The contents of this thesis are original, and the distinct contributions to knowledge and the individual author contributions are summarized as follows:

Manuscript 1: *Extracellular ATP and its derivatives provide spatiotemporal guidance for*

bone adaptation to wide spectrum of physical forces

Authors: Chrisanne Dsouza, Mahmoud S. Moussa, Nicholas Mikolajewicz, and Svetlana V. Komarova

- **Originality:** In collaboration with my co-author(s), I wrote a review that summarizes the literature on mechanically stimulated ATP release and its metabolites, ADP, and adenosine in bone cells. I provide a qualitative and quantitative summary of the studies on mechanically stimulated bone cells. I also developed a conceptual model to explain the role of the complex network of the purinergic receptors and their ligands, ATP, ADP, and adenosine, and how they coordinate cellular responses to convey information about the level, degree, and proximity to the site of injury. This review article is published in Bone Reports.
- **Contribution of co-authors**

- **Chrisanne Dsouza:** Conceptualization, formal analysis, writing – original

draft, writing– review & editing, visualization.

- **Mahmoud S. Moussa:** Conceptualization, investigation, writing – original draft, writing – review & editing.
- **Nicholas Mikolajewicz:** Conceptualization, investigation, writing – original draft, writing – review & editing.
- **Svetlana V. Komarova:** Conceptualization, writing – review & editing, supervision, funding acquisition.

Manuscript 2: *Mechanosensitivity and mechanotransductive properties of osteoclasts*

Authors: Chrisanne Dsouza, and Svetlana V. Komarova

- **Originality:** I investigated the mechanosensitive and mechanotransductive properties of osteoclasts which are less thought of as mechanoadaptive cells. I showed that osteoclasts can sense and transduce mechanical forces to neighboring cells when subjected to local membrane deformation. I also found that they undergo reversible plasma membrane damage, which is an established mechanism of sensing forces that has previously been shown in osteoblasts and osteocytes. I showed that the P2X₇ receptor, as well as ADP-mediated signaling are important for osteoclast mechanotransduction. This article is published in the American Journal of Physiology- Cell Physiology.
- **Contribution of co-authors**
 - **Chrisanne Dsouza:** Conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures,

drafted manuscript, edited and revised manuscript, approved the final version of the manuscript

- **Svetlana V. Komarova:** Conceived and designed research, analyzed data, interpreted results of experiments, drafted manuscript, edited and revised manuscript, approved the final version of the manuscript

Manuscript 3: *Aging-associated changes in mechanotransduction of osteoblasts and osteoclasts*

Authors: Chrisanne Dsouza, and Svetlana V. Komarova

- **Originality:** I examined the age-related changes in bone mechanotransduction in osteoblasts and osteoclasts. I showed that osteoblasts become less mechanotransductive with age whereas osteoclasts display higher mechanotransductive properties. I also found that the expression levels of P2X₇ and P2Y₁₃ are down-regulated with age in osteoclasts. I also investigated sex-related differences in mechanotransduction with age and found that osteoblasts and osteoclasts from male mice were less mechanotransductive than female mice. This manuscript is in preparation for submission.
- **Contribution of co-authors**
 - **Chrisanne Dsouza:** Conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved the final version of the manuscript
 - **Svetlana V. Komarova:** Conceived and designed research, interpreted results

of experiments, drafted manuscript, edited and revised manuscript, approved the final version of the manuscript

Manuscript 4: *Characterization of Potency of the P2Y₁₃ Receptor Agonists: A Meta-Analysis*

Authors: Chrisanne Dsouza, and Svetlana V. Komarova

- **Originality:** I conducted a meta-analysis of existing studies on the P2Y₁₃ receptor. I provide a quantitative summary of the potency of different P2Y₁₃ receptor ligands. I also investigated how potency is affected in different signaling events, tissues, and physiological systems. This article is published in the International Journal of Molecular Sciences.
 - **Contribution of co-authors**
 - **Chrisanne Dsouza:** Conceptualization, methodology, formal analysis, data curation, writing
 - **Svetlana V. Komarova:** Conceptualization, methodology, formal analysis, writing, supervision, funding acquisition
-

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Abbreviations

- **ADA:** Adenosine deaminase
- **ADK:** Adenosine kinase
- **ADP:** Adenosine diphosphate
- **ADP β S:** Adenosine 5'-O-(2-thiodiphosphate)
- **ALP:** Alkaline phosphatase
- **AMP:** Adenosine monophosphate
- **AR:** Adenosine receptors
- **ATP:** Adenosine triphosphate
- **AUC:** Area under the curve
- **BMD:** Bone mass density
- **BMP:** Bone morphogenic proteins
- **BMU:** Bone multicellular unit
- **cAMP:** Cyclic adenosine monophosphate

- **cFMS:** Colony-stimulating factor 1
- **CI:** Confidence interval
- **CREB:** cAMP-responsive element binding protein
- **Cyto-5'NT:** cytosolic 5' nucleotidase
- **DAMP:** Danger-associated molecular pattern
- **Dkk1:** Dickkopf-1
- **Dlx5:** Distal-less homeobox 5
- **Dmp1:** Dentin matrix protein 1
- **EC₅₀:** Half maximal effective concentration
- **ECM:** Extracellular matrix
- **eN:** ecto-5'-nucleotidase
- **ENT:** Equilibrative nucleoside transporters
- **ERK:** Extracellular signal-regulated kinase
- **FBS:** Fetal bovine serum
- **FSS:** Fluid shear stress
- **FWHM:** Full width at half maximum
- **HK:** Hexokinase
- **IC₅₀:** Half maximal inhibitory concentration

- **IP₃**: Inositol trisphosphate
- **K_M**: Michaelis constant
- **LFF**: Laminar fluid flow
- **M-CSF**: Macrophage colony stimulating factor
- **MEPP**: Miniature end-plate potentials
- **MES**: Minimum effective strain
- **MMP**: Matrix metalloproteinase
- **MSC**: Mesenchymal stem cell
- **NO**: Nitric oxide
- **NTPDases**: Nucleoside triphosphate diphosphohydrolases
- **OB**: Osteoblast
- **OC**: Osteoclast
- **OPG**: Osteoprotegerin
- **Osx**: Osterix
- **PBS**: Phosphate-buffered saline
- **PEP**: Phosphoenolpyruvate
- **PG**: Prostaglandin
- **PGE2**: Prostaglandin E2

- **PK:** Pyruvate kinase
- **PKC:** Protein kinase C
- **PLC:** Phospholipase C
- **PMD:** Plasma membrane damage
- **PRISMA:** Preferred reporting items for systematic review and meta-analysis
- **RANKL:** Receptor activator of nuclear factor- $\kappa\beta$ ligand
- **Runx2:** Runt-related transcription factor 2
- **SAH:** S-adenosyl-L-homocysteine
- **SE:** Standard errors of the means
- **SNP:** Single nucleotide polymorphism
- **Sost:** Sclerostin
- **TB:** Trypan blue
- **TFF:** Turbulent fluid flow
- **tFSS:** Turbulent fluid flow shear stress
- **T_H:** Homogeneity threshold
- **TRAP:** Tartrate-resistant acid phosphatase
- **UDP:** Uridine diphosphate
- **UTP:** Uridine triphosphate

- **2MeSADP:** 2-methylthio-adenosine diphosphate
- **2MeSATP:** 2-methylthio-adenosine triphosphate
- **[Ca²⁺]_i:** Intracellular calcium
- **[³⁵S]GTPγS:** [³⁵S]guanosine-5'-O-(3-thiotriphosphate)
- **α-MEM:** Minimum Essential Medium-alpha

Preface

The bone is a dynamic organ that is constantly exposed to physical forces. These forces are important for the development and maintenance of musculoskeletal health. Exercise is known to be the most effective way of maintaining bone strength and mass. However, the aging skeleton is unable to benefit from exercise resulting in poor skeletal health and conditions such as osteoporosis (Morabia et al. [1998](#)). Aging is known to result in an imbalance of bone-forming osteoblast and bone-forming osteoclast activity (Almeida et al. [2013](#)). While osteoblasts and osteocytes are well-acknowledged for their role in mechanoadaptation, much less is known regarding the ability of osteoclasts to sense mechanical perturbations and to send the signal to other cells. *My first aim was to determine the mechanosensitivity and mechanotransductive characteristics of osteoclasts.*

In vivo studies in aging mice and humans have shown that the aging skeleton loses its ability to adapt to physical forces therefore leading to frailty and bone loss (Bassey et al. [1998](#); Srinivasan et al. [2012](#); Birkhold, Razi, et al. [2014](#); Holguin et al. [2014](#)). However, the understanding of the loss of mechanoadaptation with age at the cellular level is limited. Osteocytes in aging animals were previously shown to become resistant to loading-induced plasma membrane damage, thus losing mechanosensitivity (Hagan,

K. Yu, et al. 2020). However, it is unknown whether osteoblasts and osteoclasts also exhibit similar behavior. Additionally, changes in osteoclastic calcium responses with age have not been previously studied. ***My second aim was to determine aging-associated changes in the responses of osteoblasts and osteoclasts to mechanical loading.***

When bone cells experience mechanical loading, they release ATP and ADP, which act on receptors of the purinergic (P2) family consisting of seven P2X ligand-gated ion channels and eight P2Y G-protein-coupled receptors (I. R. Orriss, Burnstock, et al. 2010). P2-mediated signals lead to a rise in intracellular calcium ($[Ca^{2+}]_i$) signals and ultimately in the expression of genes that aid in the mechanoadaptation of bone. How aging affects ATP and ADP-mediated signaling in mechanically stimulated bone cells is not well-known. Interestingly, ADP-sensing P2Y receptors, P2Y₁, P2Y₁₂, and P2Y₁₃ that are expressed in bone cells (Kügelgen 2021), have been implicated in age-related bone loss (Alvarenga et al. 2010; Su et al. 2012; Biver et al. 2013; N. Wang, Rumney, et al. 2013). Among these receptors, P2Y₁₃ is the least studied. ***My third aim was to use knowledge synthesis approaches to examine the signaling dynamics of the ADP-sensing P2Y₁₃ receptor.***

My thesis consists of 6 chapters. I first provide a comprehensive overview of the literature on bone, mechanoadaptation, and aging in Chapter 1. In Chapter 2, I summarize the literature on the role of ATP and its metabolites and the function of purinergic receptors in bone mechanoadaptation. I also describe a conceptual model of how the complex system of ATP, ADP, and adenosine signaling convey information about the degree, proximity, and time from the event of mechanical loading. In Chapter 3, I investigate mechanically stimulated osteoclasts and determine their mechanosensitive

and mechanotransductive properties. I show that mechanically stimulated osteoclasts (primary responders) can also sense and transduce mechanical signals to neighbouring non-stimulated osteoclasts (secondary responders) and have increased calcium responses with increase in the severity of the injury. I also show that similar to osteoblasts and osteocytes, osteoclasts are also capable of repairing plasma membrane tears generated by mechanical forces. I demonstrate that the P2X₇ receptor as well as ADP-mediated signaling are important for osteoclast mechanotransduction. In Chapter 4, I examine age-related changes in the ability of osteoblasts and osteoclasts to sense mechanical stimulation in vitro and transduce signals to neighboring cells. I show that while injury levels and signaling are similar in young and old osteoblasts and osteoclasts, the propagation of mechanically-activated signals to neighbouring cells is differently altered in both cell types with age: while osteoblasts become less mechanotransductive with age, osteoclasts from older animals demonstrate higher mechanotransduction properties. The expression of P2X₇ and P2Y₁₃ were downregulated with age in osteoclasts while no differences were observed among young and old osteoblasts. Osteoblasts and osteoclasts from male mice were less mechanotransductive than female mice, thus highlighting sex differences in mechanotransduction of aging bone cells. Based on my findings in Chapter 3 on the role of ADP in osteoclast signaling, and in Chapter 4 on the downregulation of P2Y₁₃ in aging osteoclasts, my next step was to understand P2Y₁₃-mediated signaling. In Chapter 5, I conducted a comprehensive meta-analysis of existing studies on the P2Y₁₃ receptor. The primary objective of the meta-analysis was to determine the potency of P2Y₁₃ ligands. The secondary objective was to assess the differences in P2Y₁₃ signaling in different tissues and systems. The P2Y₁₃ receptor was identified to be the most sensitive

ADP receptor to date. In Chapter 6, I discuss and conclude the findings of my thesis.

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Chapter 1

Bone mechanoadaptation in the aging skeleton

1.1 Overview of the hierarchical structure of bone

The bone is a complex structure that supports numerous vital functions, such as locomotion, and protection of vital organs, and serves as a reservoir of calcium and phosphate in the body. It is a mineralized tissue whose extracellular matrix (ECM) is made up of inorganic matrix (50-70%), organic matrix (20-40%), water (5-10%), and lipids (<3%) (B. Clarke [2008](#)). The inorganic component is mainly composed of carbonated hydroxyapatite with small amounts of potassium, sodium, magnesium, and other elements. Type I collagen makes up 90% of the organic component, which also contains other collagen types, noncollagenous proteins, e.g. osteopontin, osteonectin, and bone morphogenic proteins (BMPs), and proteoglycans such as decorin and biglycan (Reznikov et al. [2014](#); Florencio-Silva et al. [2015](#)).

At the tissue level, the bone can be divided into cortical (compact) bone and trabecular (spongy or cancellous) bone. The cortical bone makes the outer shell of the structure and is dense and solid. At the microscopic level, the cortical bone is made up of osteons, which are concentric lamellae comprising a hypercalcified matrix containing mineralized collagen fibrils (B. Clarke [2008](#); Reznikov et al. [2014](#)). The osteon has a central canal (also known as the Haversian canal) that consists of blood vessels and nerves. The trabecular bone is made up of bony rods and plates in a honeycomb-like network that makes up the inner volume of bones and surrounds the bone marrow (B. Clarke [2008](#); Osterhoff et al. [2016](#)) (Fig [1.1](#)).

At the skeletal level, bones are categorized into long bones, e.g., femur and tibia; flat bones, e.g., skull and sternum; short bones e.g., carpal and tarsal; and irregular bones e.g., vertebrae and coccyx. Bones are formed by a process known as ossification, which includes two types: intramembranous and endochondral ossification. Intramembranous ossification, observed in flat bones, involves the direct transition of the mesenchymal cells to osteoblasts. Endochondral ossification involves an intermediate step where cartilage first forms the template, which is later replaced by bone. Long bones are formed through endochondral ossification (B. Clarke [2008](#); Breeland et al. [2024](#)).

1.2 Bone cells

Three main types of bone cells, osteocytes, osteoblasts, and osteoclasts (Fig [1.2](#)), play important roles in the growth and maintenance of skeletal health.

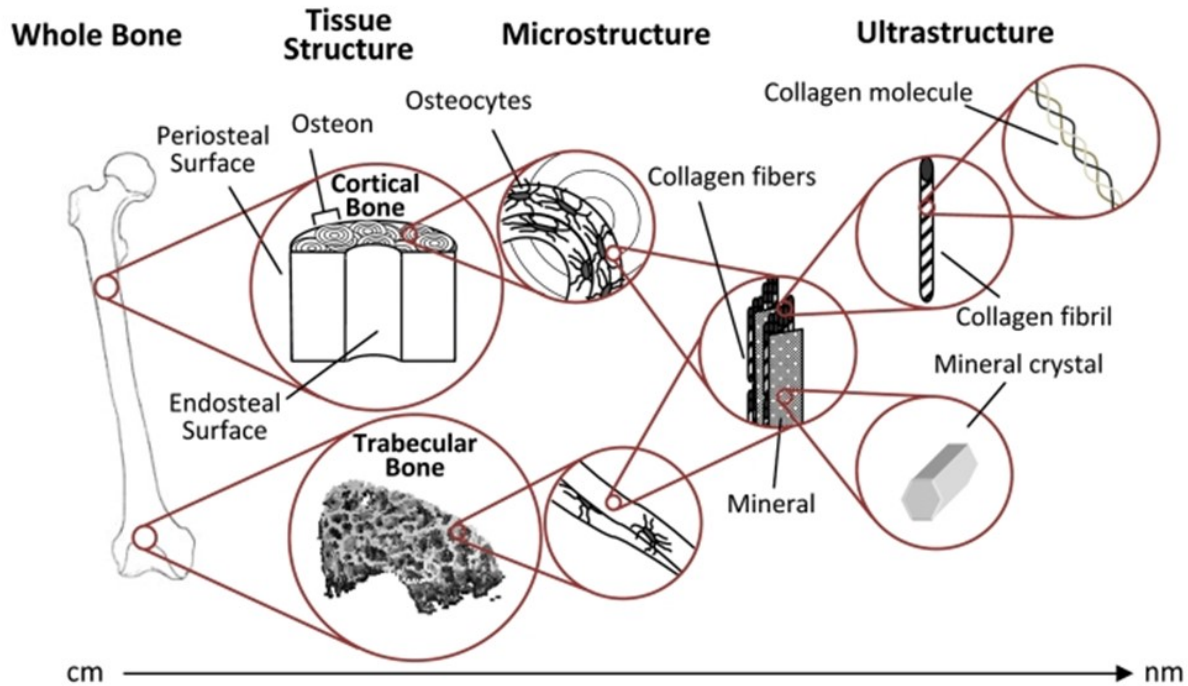


Figure 1.1: Hierarchy of bone. The figure illustrates the long bone and its components. The long bone consists of the solid cortical bone at the diaphysis and the spongy trabecular bone that can be found in the epiphysis and metaphysis. At the microscopic level, the cortical bone is made up of osteons, which are concentric circles made of lamellae and possess a highly mineralized outline that forms the cement line. The osteocytes reside here within their lacune. The Haversian canal runs through the centre of the concentric structure and provides blood supply. The lamellae are made up of collagen bundles organized in a twisted plywood arrangement. Extrafibrillar and intrafibrillar minerals, which contain non-collagenous proteins and enzymatic and non-enzymatic cross-links, can be found. The fibrils consist of type I collagen and inorganic hydroxyapatite crystals made up of calcium and phosphate ions (E. A. Zimmermann et al. 2015). Reproduced and adapted with permission from (Boskey et al. 2010).

1.2.1 Osteoblasts

Osteoblasts are cuboidal cells of mesenchymal origin mainly responsible for bone formation. They are present at the bone surface, laying down ECM and regulating mineralization. They have an average lifespan of 8 weeks in humans (Sommerfeldt et al. 2001; Long 2012). Osteoblast differentiation from the mesenchymal stem cells (MSC) is governed by “master transcriptional regulator” Runt-related transcription factor 2 (Runx2), as well as other important genes such as Distal-less homeobox 5 (Dlx5) and osterix (Osx) (Florencio-Silva et al. 2015; Rutkovskiy et al. 2016). Osteoblasts carry out bone

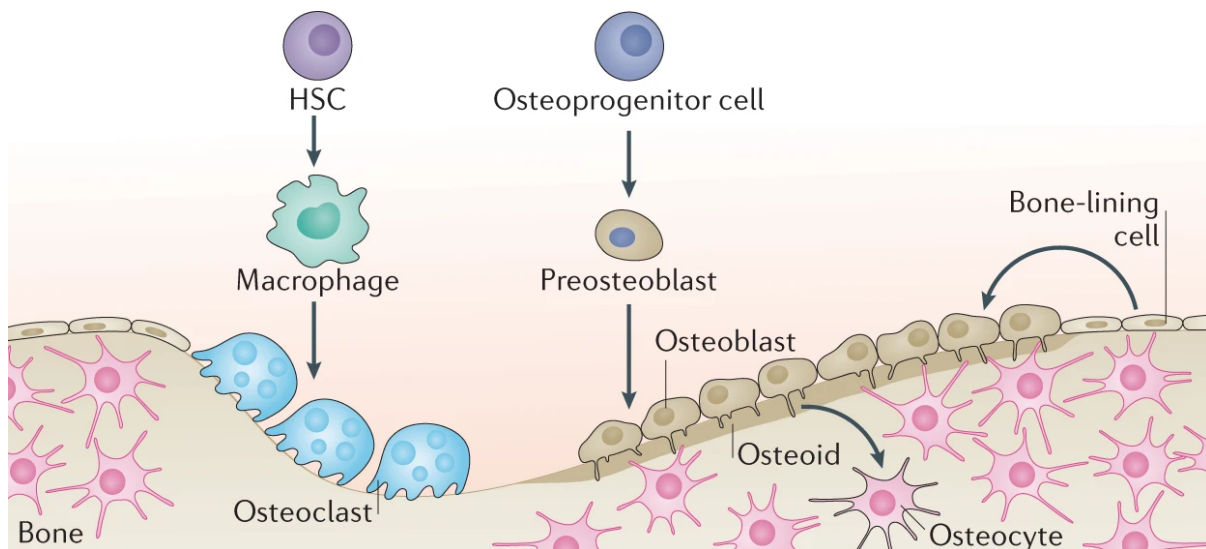


Figure 1.2: Bone cells. Bone comprises three cell types: osteoblasts, osteoclasts, and osteocytes. Osteoblasts are generated from the mesenchymal stem cell progenitor and are responsible for bone formation. Osteoclasts are derived from monocyte precursors of the hematopoietic stem cell lineage. Terminally differentiated osteoblasts mineralize and form osteocytes, the mechanosensory cells of bone. Reproduced with permission from Salhotra et al. [2020](#).

matrix synthesis firstly by organic matrix deposition where they secrete collagen (mainly collagen type I), non-collagenous proteins, such as osteocalcin, and proteoglycans to form the organic matrix known as osteoid (Florescio-Silva et al. [2015](#)). Osteoblasts lay approximately 0.5-1.5 μM of organic matrix per day (Sommerfeldt et al. [2001](#)). Matrix deposition is followed by mineralization, during which inorganic calcium and phosphate ions form hydroxyapatite crystals (Glimcher et al. [1998](#); Florescio-Silva et al. [2015](#)). Osteoblasts express key regulators of mineralization, such as alkaline phosphatase, and important factors, such as receptor activator of NF- κ B (RANKL) and osteoprotegerin (OPG), that regulate osteoclastogenesis (Rutkovskiy et al. [2016](#)). Mature osteoblasts either undergo apoptosis, become osteocytes incorporated in the bone matrix, or become bone-lining cells that are quiescent cells on the bone surface (Florescio-Silva et al. [2015](#)).

1.2.2 Osteoclasts

Osteoclasts are bone-resorbing multinucleated cells that have an average lifespan of 10-15 days (Sommerfeldt et al. [2001](#)). Osteoclasts are derived from cells of hematopoietic origin and require two important cytokines for osteoclast formation: macrophage colony-stimulating factor (M-CSF) and RANKL (B. Clarke [2008](#)). M-CSF binds to the cFMS (Colony-stimulating factor 1) receptor on osteoclasts and promotes the differentiation of hematopoietic stem cells into monocyte/macrophage lineage, stimulates proliferation, and increases the lifespan of the precursors. RANKL produced by osteoblasts, osteocytes, and osteoprogenitor cells binds to the RANK receptor and promotes the differentiation and fusion of mature osteoclasts (McDonald et al. [2021](#)). OPG also secreted by osteoblasts, acts as a decoy receptor for RANKL, thus regulating osteoclastogenesis.

When osteoclasts engage in bone resorption, they become polarized through cytoskeletal reorganization and form a specialized compartment (called Howship Lacuna) isolated by the sealing zone in which the osteoclast membrane is tethered to the bone via integrin $\alpha V\beta 3$. Underneath the cell, a ruffled border is formed, and an acidic environment (pH – 4.5) is created by the proton pump (H^+ -ATPase) and Cl^- channel. The acidic environment is important to facilitate the hydroxyapatite dissolution. Subsequent release of proteases, cathepsin K, matrix metalloproteinase 9 (MMP9), and matrix metalloproteinase 13 (MMP13) facilitates degradation of the organic matrix. The degraded fragments are endocytosed and transported in vesicles to the basolateral surface of the resorbing osteoclast, where they are released into the environment (Flores-Silva et al. [2015](#); Takayanagi [2018](#)).

1.2.3 Osteocytes

Osteocytes are terminally differentiated osteoblasts and are the most abundant bone cells. They make up to 90% of the bone cells and can live up to decades in the bone (Florencio-Silva, Sasso et al. 2015). Osteocytes have a cell body that resides in the lacunae and dendritic-like processes that are situated in canaliculi. The lacune and canaliculi are filled with interstitial fluid. The dendritic processes of neighboring osteocytes interconnect with each other to form a lacuna-canalicular network. The formation of osteocytes requires the expression of genes such as podoplanin/E11, dentin matrix protein 1 (Dmp1), and MMPs (Delgado-Calle et al. 2022). Osteocytes are known to be the main mechanosensors in bone cells. They sense and transduce mechanical signals that regulate osteoblasts and osteoclast activity. Important regulatory molecules produced by osteocytes include sclerostin (Sost) and Dickkopf-1 (Dkk1), which negatively regulate the Wnt/ β -catenin pathway that is important for bone formation and RANKL that can stimulate bone resorption (Bonewald 2010; Compton et al. 2014).

1.3 Bone modeling and remodeling

Bone adapts itself either through modeling or remodeling. In modeling, osteoblasts lay bone, and osteoclasts resorb bone on quiescent surfaces in different locations independent of each other's action. Bone modeling is responsible for modifying the shape and size of bone primarily during childhood but also continuously throughout life. (Allen et al. 2014; Isales et al. 2018; Bolamperti et al. 2022). On the other hand, bone remodeling involves coupling osteoblasts and osteoclasts as a basic multicellular unit (BMU), where resorption and formation occur in a coordinated manner. This process occurs in five

stages: 1) Activation- osteoclast precursors are recruited to the site and fuse to become mature osteoclasts; 2) Resorption- bone lining cells move away from the surface and mature osteoclasts resorb bone; 3) Transition phase (also known as the quiescent phase)- resorption is stopped and formation is initiated; 4) Formation- osteoblasts recruited to the site lay bone; and 5) Mineralization and quiescence- osteoblasts mineralize and become osteocytes. Bone remodeling occurs throughout life and is responsible for renewing/maintaining bone (Allen et al. 2014; Florencio-Silva et al. 2015; Arias et al. 2018; Isales et al. 2018)(Fig 1.3).

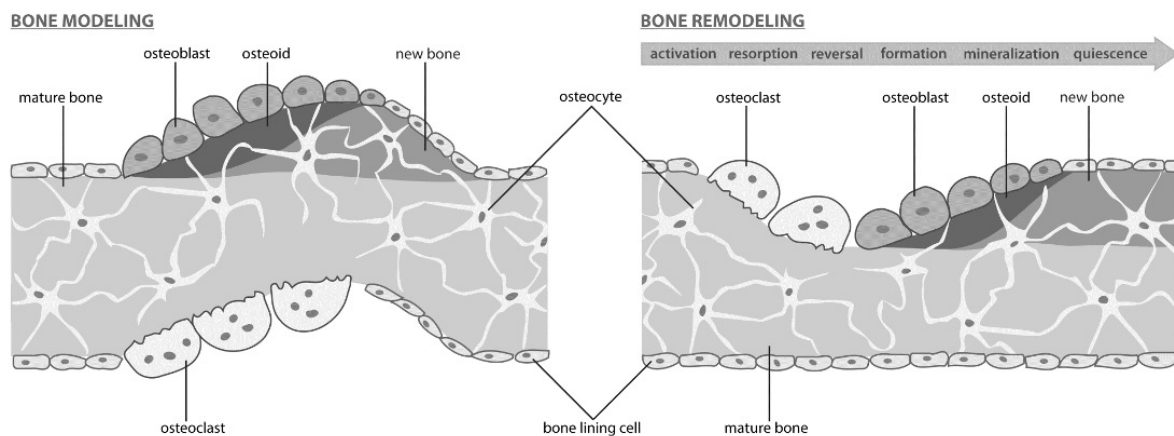


Figure 1.3: Bone growth and adaptation is regulated via modeling or remodeling. Bone modeling involves the independent action of osteoblasts and osteoclasts. In bone remodeling, the actions of osteoclasts and osteoblasts are coupled and form a BMU. The stages of bone remodeling involve activation and resorption by osteoclasts, reversal stage where osteoblasts are recruited, followed by laying down of osteoid (newly formed bone) by osteoblasts and ultimately mineralization and quiescence. Reproduced with permission from (Birkhold and Willie 2016).

1.4 Mechanobiology of bone

The bone is a dynamic organ that needs to maintain its structure and function to support daily activities. The level of exposure to mechanical forces affects bone mass and strength. Mechanical loading in the form of exercise is potentially the most effective way to increase or maintain bone mass (Marques et al. 2012). This can be observed in

tennis players whose playing arm has larger bones than the nonplaying arm (Jones et al. 1977). Unloading or a decrease in activity as experienced by astronauts or bed-ridden individuals, leads to a decrease in bone mass (Stavnichuk et al. 2020). When bone experiences mechanical forces, bone cells detect this stimulus and respond by (re)modeling the skeleton according to the load experienced. One of the earliest hypotheses on bone mechanoadaptation was by Julius Wolff in the 19th century, known as Wolff's law, which, in summary, states that the level of mechanical stresses determines the architecture of bone (Frost 1994). Later, Harold Frost proposed the mechanostat theory, which states that bones have a “minimum effective strain” (MES) or a set point for adaptation. If the load is above the set point (during exercise) it will result in bone formation and increase in bone strength. Conversely, if it is below the set point (in disuse), it will lead to resorption (Frost 1987). Over the years, many studies have shown how bone is influenced by mechanical loading. Intermittent loading to bone is more beneficial than static loading (Lanyon and C. T. Rubin 1984), and higher strains are more effective for bone formation (O'Connor et al. 1982; C. T. Rubin et al. 1985). Additionally, short bouts of loading with rest periods are better than continuous loading (Robling, Hinant, et al. 2002). Bone cells are also known to adapt to loads over time; hence, introducing new loading patterns is more beneficial (Lanyon, Goodship, et al. 1982; C. T. Rubin et al. 1984; Daly et al. 2018).

1.4.1 Mechanobiology at the cellular level

Understanding mechanobiology at the cellular level is essential for identifying how bone cells respond and adapt to stimuli. There are several methods of applying in vitro stimulation to bone cells, including fluid shear stress (Kapur et al. 2003; Kringelbach,

Aslan, Novak, Ellegaard, et al. 2015), local membrane deformation (Romanello and D'Andrea 2001; Lopez-Ayon et al. 2014), vibration (Lau et al. 2010; Steppe et al. 2020), and substrate stretching (H. S. Yu et al. 2016). Osteocytes and osteoblasts have been extensively studied as mechanoadaptors of bone; however, limited studies have been conducted on osteoclasts (Michael Delaine-Smith et al. 2015).

Mechanoadaptation of the skeleton occurs as a result of cellular processes where cells sense and transduce loads. Bone cells sense mechanical forces in a process known as mechanosensation (Klein-Nulend et al. 2013). Studies have identified various mechanosensors on bone cells responsible for sensing these forces, such as gap junctions, hemichannels, pannexins, voltage-gated ion channels, Piezo2 channels, and changes in the cytoskeleton (Scott et al. 2008; Qin et al. 2020). Plasma membrane damage (PMD) is another mechanism through which cells sense forces. These damages are reversible and are known to repair within a minute. Many cells in the body are known to undergo PMDs, including osteocytes and osteoblasts (Mikolajewicz, Mohammed, et al. 2018a; K. Yu et al. 2018; Hagan, Balayan, et al. 2021). Once a PMD occurs, cells release signaling molecules, e.g. ATP, that act on neighboring cells and convey spatiotemporal information regarding the injury level and distance from the injury site (Mikolajewicz, Sehayek, et al. 2019). Calcium signaling is commonly induced by mechanical stimulations and involves both an influx of extracellular Ca^{2+} and a release of Ca^{2+} from intracellular stores (Lopez-Ayon et al. 2014; Kringelbach, Aslan, Novak, Ellegaard, et al. 2015). Calcium signaling mediates the repair of PMD through vesicular exocytosis via the Ca^{2+} /phospholipase C/protein kinase C (Ca^{2+} /PLC/PKC) pathway. Particularly, $\text{PKC}\mu$ (also known as *Prkd1*) was identified as important for mediating the repair of the PMDs

(Mikolajewicz, Mohammed, et al. 2018a; Hagan, Balayan, et al. 2021) (Fig 1.4).

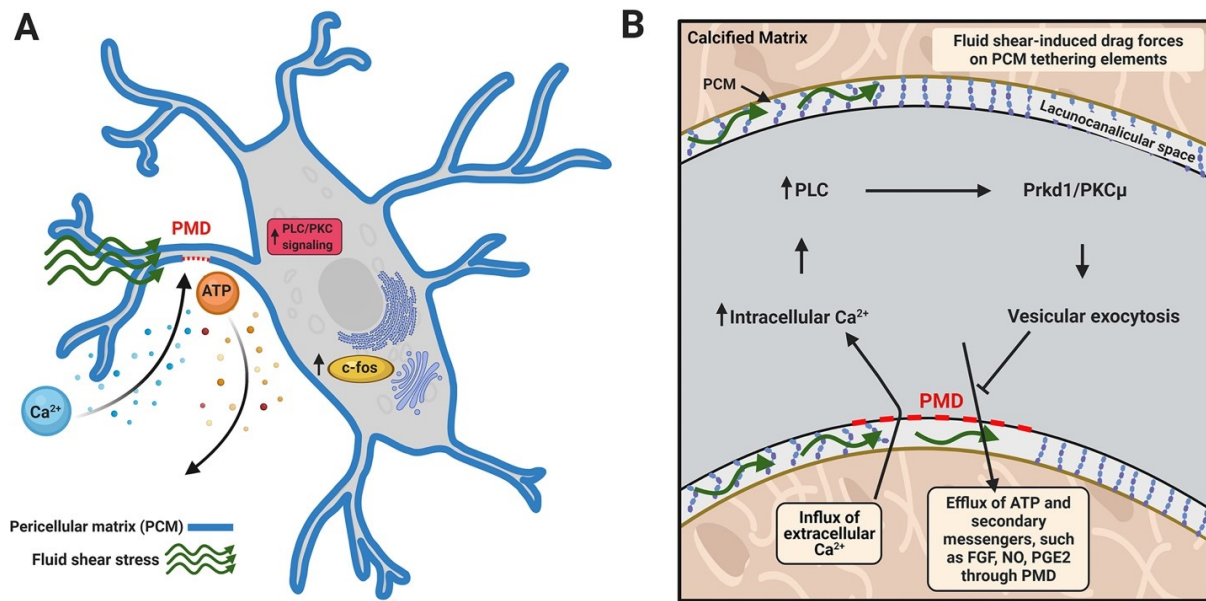


Figure 1.4: Mechanically stimulated cells undergo reversible plasma membrane damage (PMD). A. Osteocytes experience mechanical loads in the form of fluid shear forces, which result in PMDs, efflux of ATP, and influx of extracellular calcium. This results in the expression of downstream genes that are important for the mechanoadaptation of bone. B. PMD is reversible, and the repair occurs via Ca^{2+} /PLC/PKC mediated vesicular exocytosis to patch and reseal the membrane. Reproduced with permission from (Hagan, Balayan, et al. 2021)

After mechanosensation, the mechanical forces are transformed into biological signals in a process called mechanotransduction (Klein-Nulend et al. 2013). Upon experiencing forces, bone cells release ATP from the cell within seconds into the extracellular environment (Mikolajewicz, Mohammed, et al. 2018b). ATP acts as an extracellular signaling molecule and can be released via vesicular exocytosis, PMDs, or by direct transport (Burnstock and Verkhratsky 2012). A comprehensive literature review of ATP and ATP-mediated signaling is provided in Chapter 2. Within minutes after the stimulus, two other important factors required in mechanotransduction, prostaglandin E2 (PGE2) and nitric oxide (NO), are released. This is accompanied by the downregulation of Dkk1 and Sost (an inhibitor of the bone-forming Wnt/ β -catenin pathway) and upregulation of β -catenin-induced activity in osteocytes which are essential for bone matrix formation

(Robling, Castillo, et al. [2006](#); Robling and Turner [2009](#)). The signaling events involved in mechanosensing and mechanotransduction were actively studied in osteocytes (Hagan, Balayan, et al. [2021](#)) and osteoblasts (Dsouza, Moussa, et al. [2022](#)), however much less known about signaling events in osteoclasts. It is important to note that since osteoclasts are cells of hematopoietic origin, while osteoblasts and osteocytes are of mesenchymal, we can anticipate significant differences in the signaling machinery between these cell types.

1.5 The aging bone

Aging is associated with a reduction in bone mass, an increase in porosity, and poor bone health, ultimately leading to conditions such as osteoporosis. Women are more affected by aging-induced bone loss than men due to estrogen deficiency after menopause. Women lose 3.4-4.8% of BMD while men lose 0.2-3.6% BMD with age (Morabia et al. [1998](#); Smit et al. [2024](#)). At the cellular level, aging is associated with an imbalance in osteoblast and osteoclast activity. Aging results in increased osteoclast activity (Chung et al. [2014](#); Møller et al. [2020](#)) and decreased osteoblast activity (Rauner et al. [2008](#)) shifting the balance to more resorption than formation. Osteocyte number, connectivity, and function are known to be affected by aging (Tiede-Lewis et al. [2017](#)).

One of the reasons for poor bone health in aging individuals is the reduced ability of the skeleton to benefit from mechanical loading. Studies in mice and humans have shown that aging is associated with loss of mechanoadaptation affecting both the cortical and trabecular BMD (Bassey et al. [1998](#); Srinivasan et al. [2012](#); Willie, Birkhold, et al. [2013](#); Holguin et al. [2014](#); Gardinier et al. [2018](#)). Birkhold and colleagues conducted a

study where they subjected young (10 weeks), middle-aged (26 weeks), and old (78 weeks) mice to tibial loading and found that aging was associated with a decrease in bone formation rates with no change in resorption rates shifting to a net bone loss (Birkhold, Razi, et al. [2014](#)). Chermide-Scabbo and colleagues examined the transcriptional differences of young (20 weeks) and old (88 weeks) mice subjected to tibial loading over 5 days. They found that old mice had less transcriptional activation after exposure to mechanical forces. Aging resulted in reduced activation of pathways associated with proliferation and differentiation and exhibited diminished Wnt signaling (Chermide-Scabbo et al. [2020](#)).

At the cellular level, it has been shown that osteocytes have reduced Ca^{2+} signals with age and respond to stimuli with a delay (Hagan, K. Yu, et al. [2020](#); Morrell et al. [2020](#)). This reduced mechanosensitivity and mechanotransduction was explained by Hagan and colleagues, who showed that osteocytes become resistant to PMDs with age, thereby resulting in reduced mechanosensation and mechanotransduction of Ca^{2+} signals (Hagan, K. Yu, et al. [2020](#)). Osteoblasts from aging rats also showed that mechanically stimulated osteoblasts have lower basal calcium and reduced signals (Donahue et al. [2001](#)). While aging osteocytes and osteoblasts demonstrated alterations in the early mechanical signals, more studies are needed to understand the age-related changes in osteoclast signaling.

1.6 Conclusion

The mechanical properties of bone are impacted by its physical environment. Mechanical loading in the form of exercise is extremely beneficial to the skeleton. Mechanical

loading results in the activation of early mechanical signals that dictate bone adaptation. However, aging is associated with a reduced ability to sense and transduce physical forces. Aging also results in an imbalance in bone formation and bone resorption with a shift towards increased resorption and net bone loss. Osteocytes are well-studied in the context of aging and mechanoadaptation; however, studies on osteoblasts, especially in osteoclasts, are limited. Understanding the changes in early mechanical signals in osteoblasts and osteoclasts will be useful in potentially targeting these pathways to help rescue reduced mechanoadaptation and bone loss in older individuals.

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Chapter 2

Extracellular ATP and its derivatives provide spatiotemporal guidance for bone adaptation to wide spectrum of physical forces

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2.1 Abstract

ATP is a ubiquitous intracellular molecule critical for cellular bioenergetics. ATP is released in response to mechanical stimulation through vesicular release, small tears in cellular plasma membranes, or when cells are destroyed by traumatic forces. Extracellular ATP is degraded by ecto-ATPases to form ADP and eventually adenosine. ATP, ADP, and adenosine signal through purinergic receptors, including seven P2X ATP-gated cation channels, seven G-protein coupled P2Y receptors responsive to ATP and ADP, and four P1 receptors stimulated by adenosine. The goal of this review is to build a conceptual model of the role of different components of this complex system in coordinating cellular responses that are appropriate to the degree of mechanical stimulation, cell proximity to the location of mechanical injury, and time from the event. We propose that route and amount of ATP release depend on the scale of mechanical forces, ranging from vesicular release of small ATP boluses upon membrane deformation, to leakage of ATP through resealable plasma membrane tears, to spillage of cellular content due to destructive forces. Correspondingly, different P2 receptors responsive to ATP will be activated according to their affinity at the site of mechanical stimulation. ATP is a small molecule that readily diffuses through the environment, bringing the signal to the surrounding cells. ATP is also degraded to ADP which can stimulate a distinct set of P2 receptors. We propose that depending on the magnitude of mechanical forces and distance from the site of their application, ATP/ADP profiles will be different, allowing the relay of information about tissue level injury and proximity. Lastly, ADP is degraded to adenosine acting via its P1 receptors. The presence of large amounts of adenosine without ATP,

indicates that an active source of ATP release is no longer present, initiating the transition to the recovery phase. This model consolidates the knowledge regarding the individual components of the purinergic system into a conceptual framework of choreographed responses to physical forces.

2.2 Introduction

The human body constantly interacts with the physical world. Forces imposed externally, such as gravity and activity-generated, and forces imposed internally (due to blood flow, ventilation, etc.) are critical to the development, maintenance, and adaptation of various tissues in the body. Bone is known for its adaptive responses to mechanical loads (Willie, E. A. Zimmermann, et al. [2020](#)). Reduced forces, such as in microgravity (Stavnichuk et al. [2020](#)) and immobilized patients (Jo et al. [2015](#)), are associated with bone loss, whereas activity-related impact forces are conversely associated with bone gain (Kohrt et al. [2009](#)). While daily physical activity is critical for healthy bones (Burr et al. [1996](#); Fritton et al. [2000](#)), in extreme physical activity, such as in elite sports, bone strains can reach levels resulting in irreversible deformations and failure (Reilly et al. [1975](#); Lynch et al. [2014](#)). Eventually, external forces will reach magnitudes resulting in traumatic bone fracture; however, different individuals demonstrate significant variability in their susceptibility to fracture (Mikolajewicz, Bishop, et al. [2020](#)). These forces can be modeled in in vitro experiments by applying pressure, fluid shear stress (FSS), strain and compression to study the responses of individual cells (Michael Delaine-Smith et al. [2015](#)). Thus, bone tissue experiences a wide range of physical forces and can adapt accordingly to these different mechanical environments.

The architecture and mineral-collagen composition of the bone effectively transfers mechanical loads through the skeletal structure, so that mechanical perturbations are perceived by bone cells that mediate the adaptive response. Bone is a highly cellular organ, which is maintained throughout our adult lives by three main cell types: osteoclasts, cells that specialize in bone resorption; osteoblasts, cells that actively produce extracellular matrix that later mineralizes; and osteocytes, cells that are embedded in bone matrix and are considered the main mechanosensors and coordinators of bone adaptation (Willie, E. A. Zimmermann, et al. 2020). Together, these cells orchestrate the growth, modeling, and remodeling of bone, adapting the mineralized structure to systemic signals and mechanical stimuli.

Following mechanical stimulation, there is a temporal cascade of signaling events that ultimately manifests in cell- and tissue-level adaptations. Within seconds, fluid shears and substrate strains distort the extracellular matrix, integrin/focal adhesion complexes reorganize the cytoskeleton, and mechanosensitive channels are activated as the membrane yields to applied forces (Robling and Turner 2009). Transient intracellular free calcium ($[Ca^{2+}]_i$) elevations are among the earliest detectable events after cells are mechanically stimulated (Hung et al. 1995; Chen et al. 2000; Robling and Turner 2009). $[Ca^{2+}]_i$ signals exhibit various features such as transient or sustained signatures and single or multiple oscillatory peaks, which are believed to encode information (Semyanov 2019; Tian et al. 2020). On a similar timescale as the calcium response, seconds following mechanical stimulation, adenosine triphosphate (ATP) release is detected (Robling and Turner 2009; Mikolajewicz, Mohammed, et al. 2018a). Within minutes, nitric oxide (NO) is released into the extracellular space from osteoblasts and osteocytes (Mullender

et al. 2004), acting as a bone anabolic agent in low to moderate concentrations (Kapur et al. 2003; Wimalawansa 2010) and similarly prostaglandins (PG), lipid autocrine and paracrine mediators, are produced (Blackwell et al. 2010). Among these early mediators orchestrating bone adaptation to the mechanical stimulus, this review focuses on the mechanotransductive role of extracellular ATP, one of the earliest signals generated upon mechanical stimulation of bone cells.

Extracellular ATP is known to stimulate multiple receptors of the purinergic receptor family (Jacobson, Delicado, et al. 2020; Illes et al. 2021; Kügelgen 2021) and to undergo enzymatic degradation that leads to generation of metabolites, such as adenosine diphosphate (ADP) and adenosine (H. Zimmermann et al. 2012), which are also ligands for different subsets of purinergic receptors (Fredholm 2007; Jacobson, Delicado, et al. 2020; Illes et al. 2021; Kügelgen 2021). Here we build a conceptual model of how this complex system acts to convey the information regarding the degree, proximity to, and time from the event of mechanical force application.

2.3 ATP release is proportional to the amount of cellular damage

Mechanical forces applied to bone result in the release of ATP into the extracellular environment (Mikolajewicz, Mohammed, et al. 2018a). Osteoblasts have been demonstrated to release ATP in response to fluid shear stress (Pines et al. 2003; Genetos, Geist, et al. 2005; J. Li, Liu, et al. 2005; Y. Xing et al. 2011; Y. Li et al. 2013; N. Wang, Rumney, et al. 2013; Mikolajewicz, E. A. Zimmermann, et al. 2018), osmotic pressure (Pines et al. 2003; Romanello, Codognotto, et al. 2005) and ultrasonic stimulation (Hayton et al. 2005;

Alvarenga et al. 2010; Manaka et al. 2015). Osteocytes also release ATP in response to fluid shear (Genetos, Kephart, et al. 2007) and mechanical injury (Kringelbach, Aslan, Novak, Ellegaard, et al. 2015). ATP release from mechanically-stimulated osteoclasts was implicated in mechanotransduction (Jørgensen et al. 2002) and similarly, macrophages have been shown to release ATP in response to osmotic pressure (Burow et al. 2015). The amount of ATP released depends on the degree of membrane deformation and/or damage experienced by cells. This includes small boluses of ATP release during normal activity, microdamage induced ATP release from physical activity that prompts repair and bone adaptation, and overload-induced tissue damage and cell death that results in spillage of cellular contents, including ATP, that serves as a danger signal (Fig. 2.1).

Low-level mechanical stimulation is known to stimulate ATP release from bone cells. Osteoblasts (Buckley et al. 2001; Romanello, Pani, et al. 2001; Genetos, Geist, et al. 2005; I. R. Orriss, Knight, Utting, et al. 2009; Brandao-Burch et al. 2012), osteocytes (Kringelbach, Aslan, Novak, Ellegaard, et al. 2015) and osteoclasts (Brandao-Burch et al. 2012), were demonstrated in vitro to constitutively release low amounts of ATP, 1–25 amol/cell, to the extracellular environment (Table 2.1). For osteoblasts, the degree of ATP release was shown to depend on their proliferative and differentiation state (Brandao-Burch et al. 2012). ATP release from osteoblasts was shown to occur mainly through exocytosis of ATP-containing vesicles (Genetos, Geist, et al. 2005; Romanello, Codognotto, et al. 2005; I. R. Orriss, Knight, Utting, et al. 2009; Brandao-Burch et al. 2012). In osteocytes, vesicular release in addition to hemichannels (i.e., pannexins, connexins) has been implicated in ATP release (Kringelbach, Aslan, Novak, Ellegaard, et al. 2015; Seref-Ferlengez et al. 2016). When low-level physical forces, such as gentle perturbation of

the media, are applied to osteoblastic cells, ATP release increases (Romanello, Pani, et al. 2001; Genetos, Geist, et al. 2005; Romanello, Codognotto, et al. 2005; I. R. Orriss, Knight, Utting, et al. 2009; Kringelbach, Aslan, Novak, Ellegaard, et al. 2015). In several studies, the lack of membrane damage upon such stimulations was experimentally confirmed (Romanello, Pani, et al. 2001; Genetos, Geist, et al. 2005). Calcium-dependent vesicular release was strongly implicated in ATP release in these conditions (Genetos, Geist, et al. 2005; Romanello, Codognotto, et al. 2005; I. R. Orriss, Knight, Utting, et al. 2009; Kringelbach, Aslan, Novak, Ellegaard, et al. 2015). Thus, at low level of mechanical stimulation, bone cells release ATP mainly through vesicular exocytosis, in amounts proportional to the level of mechanical stimulation.

Physical forces that are large but not structurally damaging to the bone, such as those experienced during strenuous exercise, were shown to lead to micro-damage of the cells in the form of membrane tears, also called plasma membrane disruptions (PMD) (Terasaki et al. 1997; Mikolajewicz, E. A. Zimmermann, et al. 2018; K. Yu et al. 2018). These PMDs do not lead to cell death and are repairable via Ca^{2+} /PLC/PKC-dependent vesicular exocytosis which occurs within 60s of stimulus (Terasaki et al. 1997; Lopez-Ayon et al. 2014; Mikolajewicz, E. A. Zimmermann, et al. 2018; K. Yu et al. 2018). ATP is spilled through the PMDs in amounts proportional to the damage (Rumney et al. 2012; Mikolajewicz, E. A. Zimmermann, et al. 2018), however, cell repair, via vesicular exocytosis and membrane tension forces, limits the spillage of ATP, so that overall ATP release depends both on the degree of damage and the cell capacity for repair (Mikolajewicz, E. A. Zimmermann, et al. 2018; Mikolajewicz, Sehayek, et al. 2019; Hagan, K. Yu, et al. 2020). The amounts of ATP released through PMDs were estimated

to lead to a dose-dependent release of 21 ± 11 to 422 ± 97 amol ATP/cell, approaching 1/3 of total ATP content in the cell (Mikolajewicz, E. A. Zimmermann, et al. 2018). Thus, at intermediate ranges of force, ATP released is proportional to the stimulus applied but inversely proportional to the cell capacity for repair.

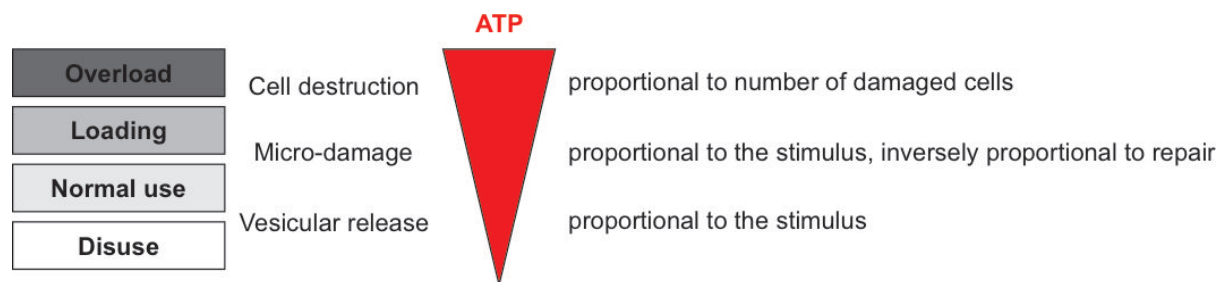


Figure 2.1: ATP release reflects the cell damaging capacity of physical forces. During normal use, low forces acting on bone result in vesicular release of ATP. With increase in forces during loading, such as exercise, bone cells undergo micro-damage in the form of reversible plasma membrane tears and spill intracellular ATP. In this case, amount of released ATP also depends on the cell ability to repair, which limits the spillage. Overload to the skeleton leading to tissue damage, results in the release of total cellular ATP content in amounts proportional to the number of destroyed cells.

Physical forces beyond the load-bearing capacity of the skeleton can occur in traumatic situations, or in high-intensity environments, as seen in athletes and military personnel. These forces result in tissue damage and bone fractures, which are associated with cellular destruction and spillage of cellular contents into the extracellular environment. ATP is present in the cell in mM concentrations (Ataullakhanov et al. 2002), therefore large amounts of ATP are released into the surrounding tissues, proportional to the number of irreversibly damaged cells (Kringelbach, Aslan, Novak, Schwarz, et al. 2014). The amount of ATP release due to high forces can be estimated to be between 50 and 500 amol ATP/cell (Table 2.1) to complete release of ATP content – 2.6–9.5 fmol ATP/cell (Mikolajewicz, E. A. Zimmermann, et al. 2018). Extracellular ATP has been implicated in fracture healing (Leung et al. 1989; Hayton et al. 2005; Manaka et al. 2015). ATP in large amounts also serves as a danger signal, or danger-associated molecular

Table 2.1: Estimated ATP release from bone cells. Given are examples of studies measuring ATP release from mechanically-stimulated bone cells, in which sufficient information was given to recalculate the values to the common scale.

Study	Cells	Stimulus	Cell damage	ATP release (mol/cell)
<i>Unstimulated release</i>				
(Romanello, Pani et al. 2001)	osteoblast	none	no	4.2E-18
(Genetos, Geist et al. 2005)	osteoblast	none	no	2.5E-17
(Romanello, Codognotto et al. 2005)	osteoblast	none	n/d	1.0E-18
<i>Low stimulation</i>				
(Genetos, Geist et al. 2005)	osteoblast	LFF	no	2.3E-16
(Wang, Rumney et al. 2013)	osteoblast	TFF	no	3.2E-17
(Romanello, Codognotto et al. 2005)	osteoblast	Hypotonic	n/d	4.9E-18
(Kringelbach, Aslan et al. 2015)	osteocyte	LFF low	n/d	1.7E-18
(Mikolajewicz, Zimmermann et al. 2018)	osteoblast	TFF low	low	2.1E-17
<i>High stimulation</i>				
(Kringelbach, Aslan et al. 2015)	osteocyte	LFF high	n/d	5.1E-17
(Mikolajewicz, Zimmermann et al. 2018)	osteoblast	TFF high	high	4.2E-16
(Mikolajewicz, Zimmermann et al. 2018)	osteoblast	Poke	yes	7.0E-17

pattern (DAMP) molecule, to the neighboring cells (Burnstock and Verkhratsky 2012), resulting in pain and inflammation (Inoue 2022). Thus, high levels of extracellular ATP reflect the damaging capacity of physical forces.

Overall, ATP release through different mechanisms engaged at different levels of mechanical stimulation allows to establish a signal that is proportionally and strongly increasing with an increase in the stimulus. Importantly, ATP release exhibits large increases in amplitude when significant physiologically damaging thresholds are reached, such as when force increases in size to damage a cell membrane and when it further

increases to irreversibly destroy the cells.

2.4 ATP diffusion and transformation to ADP indicates the proximity to the damaging event

When ATP is released from mechanically-stimulated or damaged cells, the contents of vesicles in case of vesicular release, or cytoplasm in case of membrane injury are also released into the extracellular environment. Generally, the intracellular ATP concentration is 10 times higher than that of ADP, and 100 times higher than the concentrations of AMP or adenosine (Ataullakhanov et al. [2002](#)). Therefore, initially ATP is the most abundant extracellular mediator that diffuses through the environment delivering information about the mechanical event to neighboring cells that did not directly experience the mechanical forces. As ATP diffuses, it is gradually degraded by ectonucleotidases, resulting in the simultaneous reduction of ATP- mediated signaling and production of other bio-active purines, mainly ADP and adenosine. Thus, spatiotemporal distance from the site of mechanical stimulation coincides with changes in the composition of signaling mediators (Fig. [2.1](#)). We propose that the combination of ATP and ADP-mediated signals communicate information about force proximity and magnitude to neighboring cells.

The fate of extracellular ATP is determined by the type and activity of the ATP degrading enzymes. In osteoblast cultures, the half-life of ATP has been measured to be between 5 and 20 min (I. R. Orriss, Knight, Utting, et al. [2009](#); N. Wang, Rumney, et al. [2013](#); Mikolajewicz, Sehayek, et al. [2019](#)). The key families of enzymes that degrade ATP include ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) that convert ATP to ADP and ADP to AMP; ectonucleotide pyrophosphatase/phosphodiesterases

(NPPs) that convert ATP to AMP, and ecto-5'-nucleotidase (eN) that degrades AMP to adenosine (H. Zimmermann et al. 2012; Giuliani et al. 2020)) (Table 2.2). Two other enzymes that degrade nucleotides are alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), highly expressed by osteoblasts and osteoclasts, respectively (H. Zimmermann et al. 2012). Several studies have examined the expression of purine nucleotide degrading enzymes on bone cells. Osteoblasts express NTPDase 1–6 (I. R. Orriss, Key, Hajjawi, et al. 2015; Yegutkin 2021), NPP1-3 (Vaingankar et al. 2004; I. R. Orriss, Key, Hajjawi, et al. 2015), and eN (Takedachi et al. 2012; I. R. Orriss, Key, Hajjawi, et al. 2015), as well as ALP that was strongly implicated in ATP degradation (Mikolajewicz, Sehayek, et al. 2019). Osteoclasts express NTPDase 1 and 3 (Hajjawi et al. 2014; Shih et al. 2019), NPP1 and 3 (Hajjawi et al. 2014), and eN (Shih et al. 2019). In osteocytes, few studies have examined ATP-degrading enzymes, however, osteocytes do degrade extracellular ATP and other nucleotides (Gibson et al. 1966), and express at least NPP1 (Hajjawi et al. 2014) and TRAP (Dallas et al. 2013; Solberg et al. 2014). Overall, the profile of nucleotides will depend on the affinity and efficacy of the specific nucleotidases present on cells neighboring the site experiencing mechanical forces. The cell composition at different sites can be complex and is not limited to bone cells, but also can include other tissues, such as bone marrow and vasculature. More studies are needed to characterize the extracellular metabolism of purine nucleotides and ligand availability for P2 receptor signaling in the complex in situ environment.

ATP released in response to mechanical stimulation diffuses through the extracellular environment (Jørgensen et al. 2002; Mikolajewicz, E. A. Zimmermann, et al. 2018; Mikolajewicz, Sehayek, et al. 2019). The distance covered by ATP will depend on

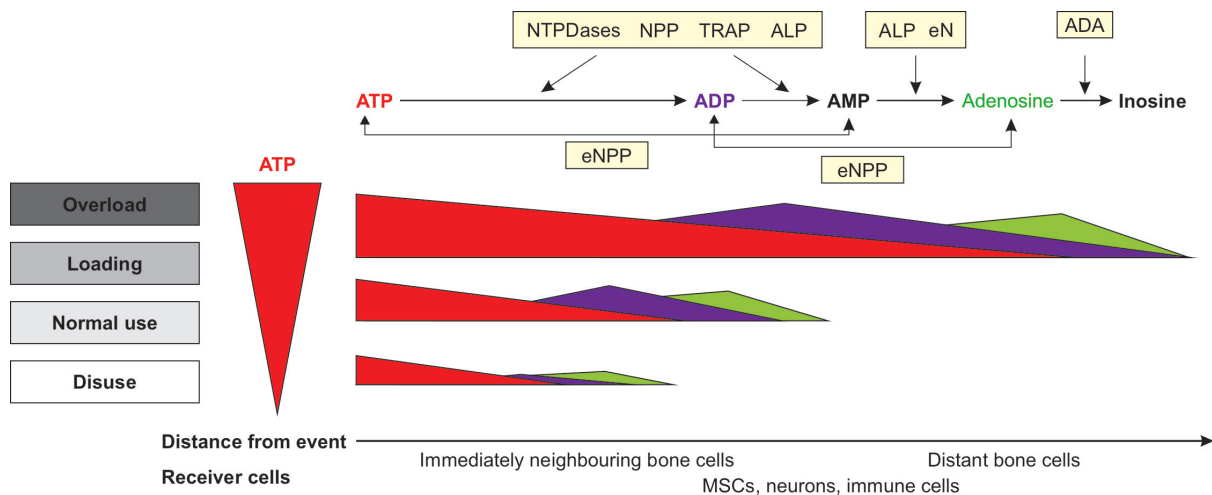


Figure 2.2: Proximity and strength of mechanical stimulus is encoded by variable purine compositions due to ATP diffusion and degradation. ATP released during mechanical stimulation diffuses into the environment, where it is degraded by ectonucleotidases into ADP and adenosine, which in turn continue to diffuse and undergo degradation. Depending on the distance from the source and on the magnitude of mechanical stimulation, receiver cells, including immediately neighboring and distant bone cells, as well as mesenchymal stem cells (MSCs), neurons and immune cells, will receive different combination of purinergic mediators.

the amount released, and thus the severity and extent of the mechanical stimulus. In addition, diffusion distance also depends on the geometry of the injury (Mikolajewicz, Sehayek, et al. 2019) and the constraints of the environment, as diffusion through bone tissue is slower than through fluids of the lacuna-canalicular network, bone marrow, or vasculature (Fernández-Seara et al. 2002). As ATP is actively degraded, ADP, AMP and adenosine are gradually generated and diffuse through the environment with their characteristic diffusion coefficients, thereby achieving faster diffusion due to lower molecular weight. Overall, cells that are near the site of mechanical stimulation will mainly receive ATP as the mechanotransductive signal (Fig. 2.2). If the magnitude of stimulation is low, ATP diffusion will quickly dissipate the signal, and ADP produced from ATP degradation will not be sufficiently high to stimulate an ADP-dependent P2 response, as ATP is an order of magnitude more potent in inducing cellular responses compared to ADP (Mikolajewicz, Sehayek, et al. 2019). However, in cases of injury

(Fig. 2.2), ATP diffusion and degradation will produce enough ADP to contribute to downstream signaling in neighboring cells at intermediate distances from the site of severe injury.

Thus, ATP and its degradation products provide spatiotemporal information about the degree of injury and the proximity of the receiver cell to the site of injury. In addition, the cellular composition of the environment through which purines diffuse has the potential to alter the signal arriving at distant neighbors, such as neurons and immune cells.

Table 2.2: Expression of ectonucleotidases degrading ATP and ADP in bone cells. The list of ectonucleotidases is based on (H. Zimmermann et al. 2012). Symbols indicate the following: +++: highly expressed; ++ moderately expressed; + expressed; – not expressed; ↓ (down arrow) expressed then decreased.

Family name	Protein name	Substrates	Osteoblasts	Osteoclasts	Osteocytes
Ecto-nucleoside triphosphate diphosphohydrolase	NTPDase1 (CD39)	ATP, ADP	+ (Orriss, Key et al. 2015)	+ (Hajjawi, MacRae et al. 2014, Shih, Liu et al. 2019)	+ (Hajjawi, MacRae et al. 2014)
	NTPDase2	ATP, less ADP	+ (Orriss, Key et al. 2015)	– (Hajjawi, MacRae et al. 2014)	
	NTPDase3	ATP, ADP	+ (Orriss, Key et al. 2015)	+ (Hajjawi, MacRae et al. 2014)	

	NTPDase4	Less ATP, ADP	+++ (Yegutkin 2008, Orriss, Key et al. 2015)		
	NTPDase5	ADP	+++ (Yegutkin 2008, Orriss, Key et al. 2015)		
Ecto-5'- nucleotidase	eN (CD73)	AMP	++ (Takedachi, Oohara et al. 2012, Orriss, Key et al. 2015)	++ (Shih, Liu et al. 2019)	++ (Gibson and Fullmer 1966)

Ecto-nucleotide pyrophosphatase /phosphodiesterase	NPP1	ATP, ADP	+++ (Vaingankar, Fitzpatrick et al. 2004, Orriss, Key et al. 2015)	+ (Hajjawi, MacRae et al. 2014)	
	NPP2	ATP, ADP	+ (Orriss, Key et al. 2015)	– (Hajjawi, MacRae et al. 2014)	
	NPP3	ATP, ADP	++ (Orriss, Key et al. 2015)	+↓ (Hajjawi, MacRae et al. 2014)	
Alkaline phosphatase	TNAP	ATP, ADP, AMP	++ (Orriss, Key et al. 2015)		
TRAP	TRAP	ATP, ADP	++ (Solberg, Brorson et al. 2014)	+++ (Oddie, Schenk et al. 2000, Kaunitz and Yamaguchi 2008)	++ (Dallas, Prideaux and Bonewald 2013, Solberg, Brorson et al. 2014)

2.5 P2 receptors form a complex signal integration network

When ATP and ADP reach the receiver cell, they act on the purinergic P2 receptor family. There are 7 ligand-gated P2X receptors (P2X₁₋₇) and 8 G-protein coupled P2Y receptors (P2Y_{1,2,4,6,11-14}). While P2X receptors are sensitive to ATP only, P2Y receptors exhibit a range of subtype-specific sensitivities to different purines and pyrimidines, including ATP, ADP, UTP, UDP, and UDP-glucose (Jacobson, Delicado, et al. 2020; Illes et al. 2021; Kügelgen 2021). P2Y receptors are further divided into two subgroups based on their sequence alignment and pharmacology. P2Y₁-like receptors comprising P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁, couple to G_q resulting in the activation of phospholipase C (PLC) (Jacobson, Costanzi, et al. 2013). The P2Y₁₁ receptor also couples to G_s, resulting in activation of cAMP. P2Y₁₂-like receptors that include P2Y₁₂, P2Y₁₃, and P2Y₁₄ couple to G_i, leading to inhibition of cAMP (Jacobson, Costanzi, et al. 2013). Bone cells have been shown to express all 15 P2 receptors by osteoblasts and osteoclasts of different origins and/or at different stages of their differentiation (I. R. Orriss, Burnstock, et al. 2010; Reyes et al. 2011).

The affinity for ATP varies widely among purinergic receptors, covering eight orders of magnitude of ATP concentrations (Grol et al. 2013; S. Xing et al. 2016). Of interest, most P2 receptors are tuned to exhibit maximal sensitivity to changes in [ATP] over a concentration range of 10 nM – 100 μ M [ATP], after which there is a single low-affinity receptor P2X₇ that is sensitive to changes in ATP in the mM range (Fig. 2.3). Importantly, many P2 receptors interact, either by directly forming heterodimers (Brown

et al. 2002; Nakata et al. 2005; Palygin et al. 2010), or indirectly through downstream signaling (Mikolajewicz, Smith, et al. 2021). One of the most prominent P2 downstream signals is a transient elevation of $[Ca^{2+}]_i$, which has a complex [ATP] dependence (Grol et al. 2013; Mikolajewicz, Smith, et al. 2021). The amplitude of $[Ca^{2+}]_i$ elevation has a non-monotonic dependence on [ATP], which was attributed to the interactions between high affinity and intermediate or low-affinity receptors (S. Xing et al. 2016; Mikolajewicz, Smith, et al. 2021). In addition, the duration of $[Ca^{2+}]_i$ elevation exhibits a sigmoidal [ATP] dependence, due to P2X₇ activation (Grol et al. 2013; S. Xing et al. 2016). Consequently, there are two ranges of [ATP] where P2 stimulation results in pronounced increases in the cumulative amount of $[Ca^{2+}]_i$ during the signaling event (i.e., the area under the calcium response): first at low [ATP], which can be considered a sensitivity threshold, and second at high [ATP] corresponding to tissue damage – an injury threshold (Grol et al. 2013; S. Xing et al. 2016). Moreover, P2-mediated responses to intermediate 0.1–10 μ M [ATP] exhibit highly oscillatory dynamics, that changes with an increase in [ATP] (Mikolajewicz, Smith, et al. 2021). Cyclic AMP (cAMP) is another prominent secondary messenger downstream of P2Y receptors, however, it has been less studied in the context of bone cell mechanotransduction. Thus, different P2 receptors interact to form complex concentration dependencies that i) are sensitive to a wide range of [ATP] and ii) modulate the downstream signaling signature and response.

Table 2.3: Roles of purinergic receptors in bone cells. Given are examples of demonstrated roles of purinergic receptors in the differentiation, function and survival of osteoblasts and osteoclasts, as well as the bone phenotype in knockout animals where available.

Type	Agonists	Osteoclasts	Osteoblasts	Knockout phenotype
X1	ATP		↓ mineralization (Orriss, Key et al. 2012)	
X2	ATP	↑ resorption (Morrison, Turin et al. 1998)	Transiently expressed during differentiation (Orriss, Knight et al. 2006)	
X3	ATP		Expressed (Orriss, Key et al. 2012, Zippel, Limbach et al. 2012)	
X4	ATP	↑ resorption (Naemsch, Weidema et al. 1999)	↑ mature osteoblasts (Orriss, Key et al. 2012)	

X5	ATP	↑ maturation /fusion (Kim, Walsh et al. 2017)	↑ proliferation (Nakamura, Uezono et al. 2000, Orriss, Knight et al. 2006), ↑ response to PDGF, IGF-1 (Kim, Walsh et al. 2017)	Protected from inflammation related bone loss (Kim, Walsh et al. 2017)
X6	ATP		Adipogenic lineage commitment (Zippel, Limbach et al. 2012)	

X7	ATP	↑ maturation /fusion (Agrawal, Buckley et al. 2010), ↑↓ resorption (Gartland, Buckley et al. 2003, Hazama, Qu et al. 2009, Wang, Agrawal et al. 2018) ↑↓ survival (Penolazzi, Bianchini et al. 2005, Miyazaki, Iwasawa et al. 2012)	↑ differentiation (Sun, Junger et al. 2013, Noronha-Matos, Coimbra et al. 2014), ↑↓ mineralization (Panupinthu, Zhao et al. 2007, Orriss, Key et al. 2012, Sun, Junger et al. 2013, Noronha-Matos, Coimbra et al. 2014), ↑↓ survival (Adinolfi, Amoroso and Giuliani 2012, Young, Chira et al. 2018)	Pfizer model (Ke, Qi et al. 2003): ↓ BMD, ↑ trabecular resorption, ↓ periosteal formation GSK model (Gartland, Buckley et al. 2003, Chessell, Hatcher et al. 2005): ↑ cortical thickness,
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Y1	ADP>ATP	↑ formation, ↑ resorption (Hoebertz, Meghji et al. 2001)	↑ proliferation (Alvarenga, Rodrigues et al. 2010, Rodrigues- Ribeiro, Alvarenga É et al. 2015), ↑ response to systemic factors (Bowler, Dixon et al. 1999, Buckley, Wagstaff et al. 2001)	↓ BMD, ↓ trabecular number (Orriss, Syberg et al. 2011)
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Y2	ATP = UTP	↑ resorption (Orriss, Guneri et al. 2017)	↑ proliferation (Katz, Ayala et al. 2011), ↑ Runx2 (Costessi, Pines et al. 2005), ↓ mineralization (Hoebertz, Mahendran et al. 2002)	↑ BMD (Orriss, Wang et al. 2011, Orriss, Guneri et al. 2017)
Y4	UTP > ATP	No effect (Jørgensen, Henriksen et al. 2002)	Interaction with P2Y2 (Orriss, Knight et al. 2006)	

Y6	UDP>>ATP	↑ formation, ↑ resorption (Orriss, Syberg et al. 2011), ↑ survival (Korcok, Raimundo et al. 2005)	↑ proliferation (Alvarenga, Rodrigues et al. 2010), ↑↓ differentiation (Orriss, Knight et al. 2006, Ayala-Peña, Scolaro and Santillán 2013)	↑ BMD (Orriss, Syberg et al. 2011)
Y11	ATP		Adipogenic lineage commitment (Zippel, Limbach et al. 2012)	

Y12	ADP>>ATP	↑ adhesion, ↑ resorption (Su, Floyd et al. 2012)	↑ proliferation (Syberg, Brandao-Burch et al. 2012) ↑↓ differentiation (Syberg, Brandao-Burch et al. 2012, Mediero, Wilder et al. 2016) ↑ survival (Syberg, Brandao-Burch et al. 2012)	Protected from bone loss related to arthritis, tumour growth and ovariectomy (Su, Floyd et al. 2012)
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Y13	ADP>ATP	↑ formation, ↓ RANKL/OPG by osteoblasts (Wang, Robaye et al. 2012)	Lineage commitment (Biver, Wang et al. 2013), ↑ differentiation (Wang, Robaye et al. 2012, Biver, Wang et al. 2013, Wang, Rumney et al. 2013)	Age- dependent ↓ bone turnover (Wang, Robaye et al. 2012, Biver, Wang et al. 2013, Wang, Robaye et al. 2014) ↑ osteogenic response to loading (Wang, Robaye et al. 2014)
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A1	Adenosine	↑ differentiation (Kara, Chitu et al. 2010, He and Cronstein 2012, He, Wilder and Cronstein 2013)	May favor MSC-adipocyte differentiation (Gharibi, Abraham et al. 2012)	↑ BMD (Kara, Doty et al. 2010)
A2A	Adenosine	↓ differentiation (Mediero, Kara et al. 2012)	↑ viability (Vincenzi, Targa et al. 2013), ↑ proliferation (Katebi, Soleimani and Cronstein 2009)	

A2B	Adenosine	↓ differentiation (He and Cronstein 2012)	↑ differentiation (Takedachi, Oohara et al. 2012, Trincavelli, Daniele et al. 2014)	↓ BMD (Carroll, Wigner et al. 2012), ↓ Tb.N, ↓ bone formation rate (Corciulo, Wilder and Cronstein 2016)
A3	Adenosine	↓ differentiation (Rath-Wolfson, Bar-Yehuda et al. 2006)	↑ proliferation (Costa, Barbosa et al. 2011)	

Given that P2 receptors function in a complex network rather than individually, it is difficult to decipher specific roles and identify distinct attributes of each P2 receptor (Table 2.3). Moreover, almost every mouse model harboring a P2 receptor knockout demonstrates an altered bone phenotype (I. Orriss et al. 2011). We have mapped the findings from knockout studies to specific aspects of osteoblast and osteoclast function (Fig. 2.3). Overall, it is evident that ATP is stimulatory for both osteoclast and osteoblast formation and function but is not strongly implicated in bone cell survival. When

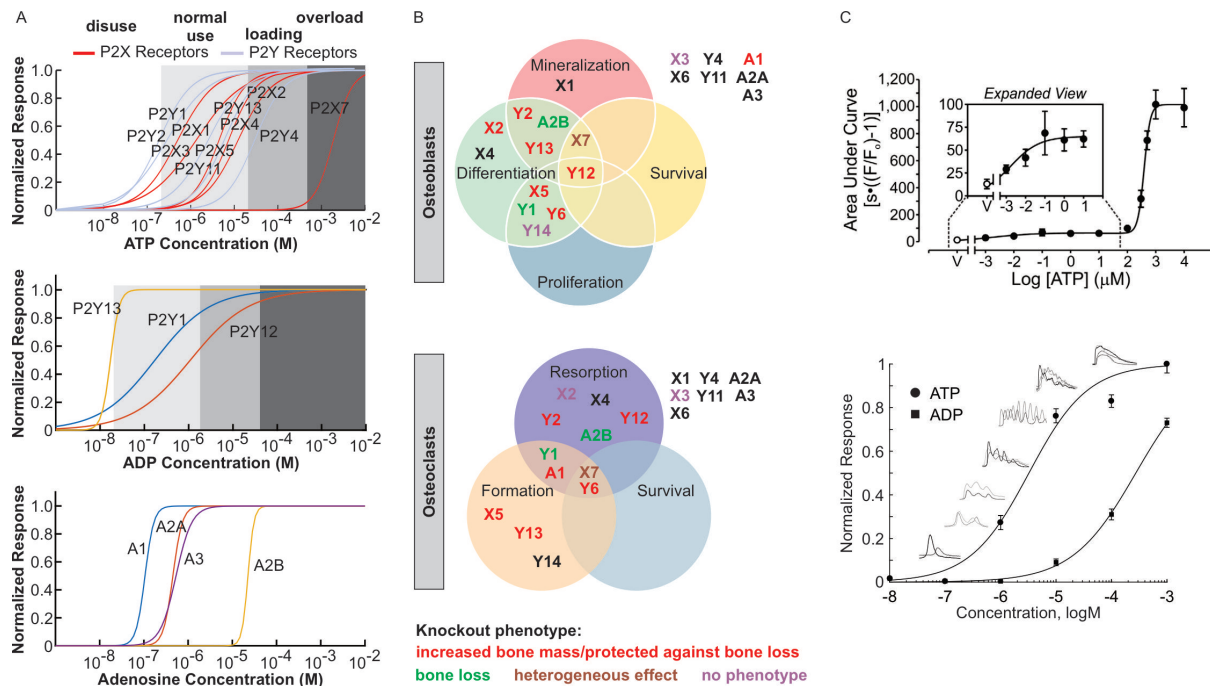


Figure 2.3: Complex signals generated by purinergic receptors. A) Concentration dependence curves for ATP-sensitive P2X and P2Y receptors (top, reproduced with modifications with permission from (S. Xing et al. 2016), ADP-sensitive P2Y receptors (middle, plotted based on data from ((Dsouza and Komarova 2021); (Ennion et al. 2004); (Léon et al. 1997)) and adenosine sensitive P1 receptors (bottom, plotted based on data from (Fredholm 2007)). Shaded areas represent approximate ranges of ATP (top) and ADP (middle) concentrations released during different levels of mechanical stimulation. B) Venn diagram depicting the role of purinergic receptors in osteoblast (top) and osteoclast (bottom) formation, activity and survival identified in knock-out studies ((Gartland et al. 2003; Ke et al. 2003; Chessell et al. 2005; I. Orriss et al. 2011; Su et al. 2012; N. Wang, Robaye, Agrawal, et al. 2012; Biver et al. 2013; N. Wang, Robaye, Gossiel, et al. 2014; H. Kim et al. 2017)). C) Examples of complex signaling features generated by P2 receptors. (Top) Area under the curve of calcium responses of osteoblasts to increasing concentration of ATP (Reproduced with permission: (Grol et al. 2013)) (Bottom) ATP and ADP dose dependence of the amplitude of osteoblast calcium responses. Inserts: prevalent patterns of calcium responses (adapted with permission from (Mikolajewicz, Sehayek, et al. 2019; Mikolajewicz, Smith, et al. 2021)).

individual receptors are deleted, such as P2Y₂ (I. R. Orriss, Utting, et al. 2007; I. R. Orriss, Guneri, et al. 2017), the phenotype of bone gain is commonly observed, suggesting that osteoclast regulation by ATP is critical for mechanotransductive bone adaptation. This highlights the importance of studies of osteoclast regulation by mechanical forces. While osteoclasts are generally not considered to be the main targets/ responders to loading, these cells are recognized as drivers of bone loss in response to unloading. Of interest, ADP receptors, P2Y₁, P2Y₁₂, and P2Y₁₃, have been more strongly implicated in bone formation, compared to ATP receptors, even though these receptors were shown to

be important for both osteoclasts and osteoblasts (I. Orriss et al. 2011; Su et al. 2012; N. Wang, Robaye, Agrawal, et al. 2012; Biver et al. 2013; N. Wang, Robaye, Gossiel, et al. 2014). P2Y₁ knockout (I. Orriss et al. 2011) and P2Y₁₃ (N. Wang, Robaye, Agrawal, et al. 2012) resulted in bone loss, and in some studies, a similar phenotype was observed after treating mice with P2Y₁₂-targeting drug clopidogrel (Syberg et al. 2012); however, conflicting results were seen in other studies, likely due to the combination of osteoclast- and osteoblast-mediated changes (Su et al. 2012; Mediero, Wilder, Reddy, et al. 2016). Nevertheless, these findings suggest that ADP-sensitive P2 receptors, all having EC₅₀ in the intermediate range of ATP/ADP values (Fig. 2.3), play a role in bone adaptation. Finally, the low-affinity ATP receptor P2X₇ has an important role in bone repair and has been referred to as the repair receptor (Jørgensen 2018). Mice deficient in P2X₇ had a significant delay in callus remodeling following osteotomy (J. Li, Meyer, et al. 2009). In humans, a study conducted on military personnel and elite athletes showed that loss of function single nucleotide polymorphism (SNP) in P2X₇ was associated with stress fractures, while gain of function SNP in P2X₇ was associated with reduced fracture occurrence (Varley et al. 2016). P2X₇ also plays a critical role in inflammation where it promotes pro-inflammatory factors, such as IL-6 and TNF (Di Virgilio et al. 2017), and in pain responses, where it acts as a positive mediator of pain (Hughes et al. 2007; Hansen et al. 2011).

Overall, we suggest that P2 receptors present on an individual cell interact at multiple levels to generate a concerted signal that integrates the information regarding ATP- and ADP- concentrations in the environment.

2.6 Termination of ATP-induced signals by adenosine

Extracellularly, adenosine is primarily formed as a result of ecto-5'-nucleotidase (eN) action on adenine nucleotides (Colgan et al. 2006; Fredholm 2014; Strazzulla et al. 2016), and secondarily to reactions catalyzed by NTPDase1, NPP-1 and ALP (Strazzulla et al. 2016). Adenosine is also produced intracellularly from AMP by soluble cytosolic 5'-nucleotidase (Cyto-5'NT) or used by adenosine kinase (ADK) to form AMP (Fredholm 2014), which then is involved in energy metabolism (Ataullakhanov et al. 2002). In addition, adenosine participates in homocysteine metabolism, where it can be produced together with L-homocysteine from S-adenosyl-L-homocysteine (SAH) by SAH hydrolase (Lee et al. 2001). Adenosine is degraded to inosine by adenosine deaminases (ADA) both intracellularly and extracellularly. Adenosine, as well as inosine, can be directly released from or taken up by the cells through equilibrative nucleoside transporters (ENTs), which strive to achieve equal intra- and extra-cellular nucleoside concentrations (Young et al. 2008; Strazzulla et al. 2016). Thus, adenosine in the extracellular environment can reflect both the degradation of extracellular purines as well as the state of cellular metabolism (Fig. 2.4A).

At resting state extracellular adenosine levels are generally in the nanomolar range with reports ranging from 1.4 to 2000 nM in human plasma (Ramakers et al. 2008), with a similarly wide range 0.8–2100 nM in the intracerebral extracellular space (Ballarin et al. 1991; Fredholm 2014; Mierden et al. 2018). Measuring endogenous adenosine concentrations can be technically difficult (Ramakers et al. 2008; Lofgren et al. 2018), and it was suggested that true physiological levels are likely at the lower side of the

measured spectrum, at 10–30 nM (Lofgren et al. 2018). In pathological states following tissue damage adenosine was shown to rise well above 1 μ M (10–1000-fold from basal levels under hypoxia) (Fredholm 2014; Yegutkin 2021). The characteristics of adenosine-processing enzymes suggest that at physiological concentrations adenosine is mostly used in cellular metabolism since ADK requires low concentrations of adenosine (Michaelis constant (K_M) for ADK is 40 nM (Spychala et al. 1996)). However, when adenosine levels increase, another adenosine processing enzyme, ADA becomes strongly involved, since it is activated at higher concentration of adenosine (K_M for ADA is more than 40 μ M (Spychala 2000; Yegutkin 2021), at least three orders of magnitude higher than that for ADK). Though local variations in adenosine levels were extensively studied in the brain and cardiovascular system, much less is known about the bone microenvironment.

Adenosine activates a family of G protein-coupled receptors known as P1 receptors or adenosine receptors (AR) (Ralevic et al. 1998). Four P1 receptors are currently recognized, A_1 , A_{2A} , A_{2B} , and A_3 . A_1 and A_3 act by inhibiting cyclic AMP (cAMP) production and decreasing protein kinase A activity, whereas both A_2 receptors stimulate cAMP (Sheth et al. 2014; Eisenstein et al. 2020). Similar to P2 receptors, P1 receptors cover a significant range of concentrations. A_1 and A_3 are activated by adenosine at low concentrations – 0.18 - 0.53 μ M (Fredholm et al. 2001; Fredholm 2007). A_{2A} has a slightly lower affinity and is activated by 0.56–0.95 μ M adenosine, while A_{2B} is only activated at very high adenosine levels, 16.2–64.1 μ M adenosine (Fredholm et al. 2001; Fredholm 2007). In bone, all four ARs were found to be expressed on mature osteoclast and osteoblasts as well as their precursors (Ham et al. 2012; Mediero and Cronstein 2013; Strazzulla et al. 2016). A_1 was reported to strongly stimulate osteoclastogenesis

(Kara, Chitu, et al. 2010; Kara, Doty, et al. 2010; He, Wilder, et al. 2013), while its role in osteoblast formation is less clear, even though it was shown to promote the osteogenic commitment of mesenchymal stem cells (D'Alimonte et al. 2013). A_3 was reported to inhibit osteoclastogenesis (Rath-Wolfson et al. 2006) and stimulate osteoblast differentiation (Evans et al. 2006; Costa et al. 2011). A_{2A} was shown to inhibit osteoclast differentiation and function (Mediero, Frenkel, et al. 2012; Mediero, Kara, et al. 2012), and to promote bone regeneration (Mediero, Wilder, Perez-Aso, et al. 2015). This receptor is also well established in vasculature as a modulator of vascular tone and blood flow (Tabrizchi et al. 2001; Khayat et al. 2017), therefore its angiogenic properties can also promote bone formation and help in the early stages of fracture healing (D. Wang et al. 2021). The low-affinity A_{2B} receptor has been implicated as the strong promoter of osteoblast differentiation (Carroll et al. 2012; Trincavelli et al. 2014). AR knockout animal models have been studied in A_1 and A_{2B} , where opposite phenotypes, bone gain and bone loss respectively, were found (Fig. 2.3). Thus, the adenosine receptors have a dynamic range of functions within the bone microenvironment and have diverse roles in bone homeostasis.

It is somewhat difficult to conceptualize the action of AR on bone cells. A_1 , A_{2A} , and A_3 receptors are activated in the same range of adenosine concentrations but produce distinct and contrary signals. A_1 and A_3 inhibit cAMP production, but A_{2A} stimulates cAMP. Although A_1 and A_3 both inhibit cAMP, A_1 stimulates while A_3 inhibits osteoclast differentiation. One potential explanation could be in the context of adenosine stimulation. Adenosine increases in the extracellular environment can occur due to two distinct processes – degradation of extracellular ATP or changes in cellular metabolism.

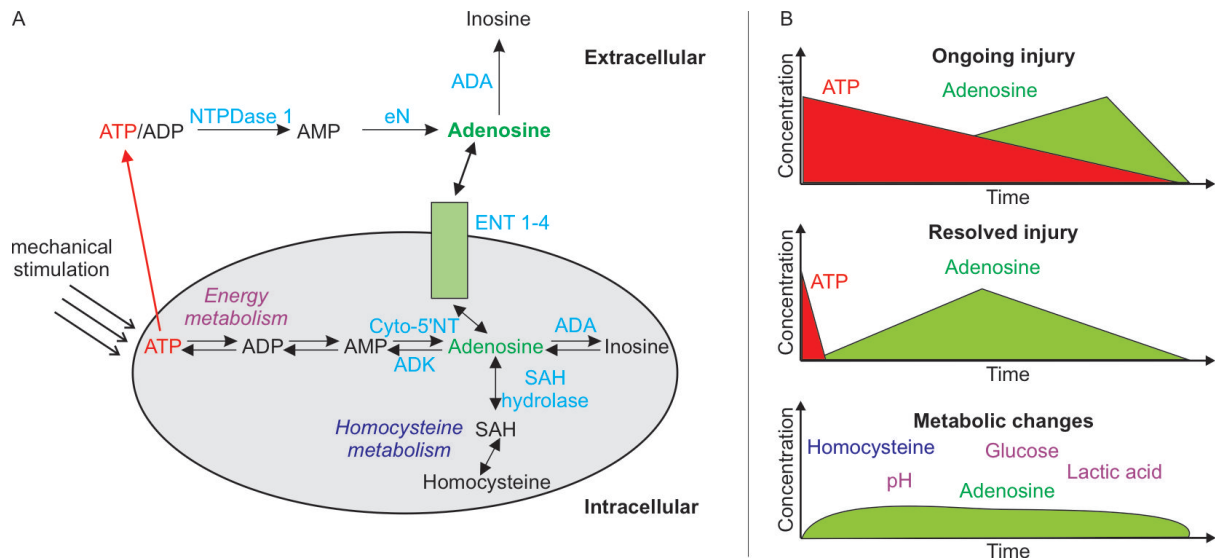


Figure 2.4: Adenosine signaling may differentiate between different mechanical and metabolic stressors. A) Extracellular adenosine can be produced through degradation of extracellular ATP, or through release of intracellular adenosine that is formed in the pathways of energy metabolism or homocysteine metabolism. ADK; Adenosine kinase, ADA; Adenosine deaminase, Cyto 5'-NT; cytosolic 5' nucleotidase, eN; Ecto-5'-nucleotidase, SAH; S-adenosyl-homocysteine. B) Adenosine profile as well as the presence of other metabolites reflect distinct states. Top: illustrated in the case of ongoing injury characterized by continuous presence of both ATP and adenosine. Middle: in the case of resolved injury, ATP signal stops, while adenosine produced from previously present ATP remains to signal the start of resolution phase. Bottom: the profile of adenosine released due to metabolic stresses likely has lower but more persistent profile compared to bolus production following mechanical stimulation.

Furthermore, in the context of cellular metabolism, adenosine levels can rise due to problems with bioenergetics or due to alterations in homocysteine metabolism, both of which are regulated independently. Thus, it is possible that these P1 receptors respond to adenosine in a context-dependent manner, allowing for cells to distinguish between mechanotransductive and bioenergetic mechanisms of adenosine elevation. For example, if adenosine increases due to the degradation of extracellular ATP, then activation of P1 receptors will be accompanied by activation of P2 receptors.

In contrast, if adenosine increases intracellularly due to deficiency in bioenergetics, then simultaneously other changes in the microenvironment will occur, such as changes in pH, oxygen, glucose, or lactate (Ataullakhanov et al. 2002; Tiedemann et al. 2020). Homocysteine regulation will be similarly linked to unique alterations in the respec-

tive metabolites. In this regard, adenosine receptors are known for the complexity of the regulatory layers, including the formation of homo- and heterodimers with other receptors; allosteric regulation, as well as biased agonism (Vecchio et al. 2018). A_{2A} receptor was recently shown to be strongly regulated by cations (Ye et al. 2018), and P1 and P2 receptors were shown to exhibit functional crosstalk (Morales et al. 2000). In the context of injury, adenosine was suggested to act as a termination signal with anti-inflammatory and pain-relieving capacities in some cases, while serving as a pro-inflammatory mediator in others (Blackburn et al. 2009; Antonioli et al. 2019; Jung et al. 2022). It is possible that these differences are related to the presence of ATP- and ADP-mediated signals: While the injury is ongoing, the simultaneous presence of ATP and adenosine are indicative of a serious and continuous problem that has lasted for a sufficient time for adenosine accumulation. In contrast, when the injury is terminated and ATP is no longer released in the environment, ADP and then adenosine become the last injury-related signals in the environment and therefore signal the beginning of the resolution of inflammation, pain, and active healing.

2.7 Conclusions

We present a conceptual model of concerted action of purinergic receptors providing information to the receiver cells regarding the degree of and proximity to the site experiencing physical forces. We suggest that ATP released in response to a variety of physical forces is proportional to their cell-damaging capacity. We propose that mechanically-stimulated ATP release acts as a signal to the neighboring non-wounded cells. The amplitude of ATP allows cells to distinguish between small non-damaging forces and

large detrimental forces. The total amount of ATP release reflects the magnitude of the force, while the stimulated cell's ability to repair limits further ATP release. The presence of an ADP component in the signal suggests proximity to the site where the large forces were applied, which is a positive signal to stimulate mechanoadaptation. Adenosine may indicate a serious ongoing issue when it occurs together with ATP, or it can serve as a termination signal if ATP is no longer present. Importantly, the properties of P2 and P1 receptors indicate that responses are not dichotomous, but rather graded, so that the information conveyed is complex and nuanced. The difficulty with interpreting knockout studies confirms that this system should be approached holistically, rather than as a sum of components. While we focused on the role of ATP-mediated signals in bone, this purinergic system is prevalent across a variety of cellular systems such as the brain, skeletal muscle, bladder, liver, and lungs, as well as other organs where mechanical forces are important in the regulation and maintenance of tissue health. The conceptual model presented in this manuscript consolidates the knowledge regarding the individual components of the purinergic system into a conceptual framework of choreographed responses to physical forces, intended to inform novel hypotheses for future studies.

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2.9 CRediT authorship contribution statement

Chrisanne Dsouza: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. Mahmoud S. Moussa: Conceptualization, Investigation,

Writing – original draft, Writing – review & editing. Nicholas Mikolajewicz: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. Svetlana V. Komarova: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

2.10 Declaration of competing interest

The authors of the manuscript “Extracellular ATP and its derivatives provide spatiotemporal guidance for bone adaptation to wide spectrum of physical forces”, Chrisanne Dsouza, Mahmoud S Moussa, Nicholas Mikolajewicz, and Svetlana V Komarova, state that they have no actual or potential conflicts of interest with regard to the submitted publication.

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Bridging Statement

The previous chapter, which is part of the literature review of my thesis, provided a summary of the studies on ATP, ADP, and adenosine and their role in bone mechanoadaptation. There are several studies on the role of purinergic receptors in bone; however, we still do not understand how these ligands and their receptors function together in a complex network. The review provided a quantitative summary of all literature on ATP release in mechanically stimulated bone cells. We proposed a conceptual model on the role of ATP, ADP, and adenosine in injury. We also provided a summary of the role of purinergic receptors in bone mechanoadaptation. Our review shows that osteocytes and osteoblasts are extensively studied for their mechanoadaptive properties, but osteoclasts are less studied. Osteoclasts are important in bone remodeling and are the first to appear at the site of stimulus. Along with osteocytes and osteoblasts, osteoclasts are subjected to mechanical forces in the environment. However, studies assessing the mechanosensitive and mechanotransductive properties of osteoclasts are limited. As the thesis aims to understand whether early mechanical signals are altered in aging osteoblasts and osteoclasts, it is first essential to characterize the mechanoadaptive properties of osteoclasts. Therefore, in this chapter, I investigated the mechanosensitive and mechanotransductive properties of osteoclasts.

Chapter 3

Mechanosensitivity and mechanotransductive properties of osteoclasts

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3.1 Abstract

Mechanical loading is essential for maintaining bone health. Here, we aimed to investigate the role of ATP and ADP in the mechanotransduction of bone-resorptive osteoclasts. Single osteoclast in primary cultures from 10 to 12-wk-old mice was mechanically stimulated by a gentle touch with a micropipette. Changes in cytosolic free calcium $[Ca^{2+}]_i$ were analyzed in Fura-2 loaded osteoclasts. The cell injury was assessed by analyzing the cellular Fura-2 loss and classified as severe or mild using k-means. Osteoclasts responded to mechanical stimuli with transient calcium elevation (primary responders) and transduced these signals to neighboring cells, which responded with delayed calcium elevations (secondary responders). Severely injured osteoclasts had higher calcium transients than mildly injured cells. Fluid shear stress similarly induced reversible cell injury in osteoclasts. Secondary responses were abolished by treatment with A-804598, a specific inhibitor of $P2X_7$, but not suramin, a broad P2 receptor blocker. Osteoclasts responded to ATP and ADP with concentration-dependent changes in $[Ca^{2+}]_i$. We performed osteoclast micropipette stimulation in the presence of phosphoenolpyruvate and pyruvate kinase which converted all ADP in solution to ATP, or with hexokinase converting all ATP to ADP. Osteoclasts with mild membrane injury demonstrated similar calcium responses in ATP and ADP-rich environments. However, when the mechanotransductive signal to severe osteoclast injury was converted to ADP, the fraction of secondary responders and their $[Ca^{2+}]_i$ amplitude was higher. This study suggests the importance of osteoclast mechanobiology and the role of ADP-mediated signaling in conditions of altered mechanical loading associated with bone loss.

3.2 New and Noteworthy

Osteoclasts are rarely considered as cells that participate in mechanical signaling in bone. We show that osteoclasts are capable of sensing and transmitting mechanical signals to neighboring cells. Mechanical stimulation commonly induces minor repairable membrane injury in osteoclasts. ATP and especially ADP were found to play important roles in the mechanoresponsiveness of osteoclasts. This study highlights the importance of osteoclast mechanobiology especially in conditions of altered mechanical loading associated with bone loss, such as in microgravity.

3.3 Introduction

Physical activity plays an important role in the maintenance of skeletal health. Mechanical forces are known to improve bone mass (1, 2), whereas inactivity is associated with bone loss (3). Bone adapts to mechanical loads through the process of bone remodeling where bone cells, namely osteocytes, osteoblasts, and osteoclasts play an important role. Osteocytes sense mechanical loads through shear forces generated by the interstitial fluid in the lacunocanalicular network. They relay these forces to osteoclasts that carry out bone resorption and osteoblasts that deposit bone, thus maintaining the process of bone remodeling. Osteoblasts are also known to be mechanosensory and mechanotransductive cells (4). Although osteoclasts were previously reported to respond to different types of mechanical forces (5-9), they are generally considered to be passive participants in mechanical sensing, and our understanding of the mechanisms of osteoclast mechanosensation and mechanotransduction is limited.

Osteoclasts are multinucleated cells uniquely capable of bone degradation. Osteoclasts

originate from the monocyte/ macrophage lineage of hematopoietic cells. Two main cytokines, M-CSF (macrophage colony-stimulating factor) and RANKL (receptor activator of nuclear factor- κ B ligand) are required for osteoclast formation (10). In the process of bone remodeling, osteoclasts are the first cells to appear and act on the damaged bone (11). Therefore, theoretically, it would be important for osteoclasts to have guidance to the location that has experienced mechanical failure. However, there is limited knowledge of the ability of osteoclasts to sense and react to mechanical perturbations.

When bone experiences physical forces, ATP is released into the environment, which acts on bone cells in an autocrine and paracrine manner. ATP and its derivatives, ADP and adenosine, act on bone cells in a spatiotemporal context relaying information about the distance and the extent of injury in the surrounding area (12). These mechanical signals act on a family of purinergic receptors, which include adenosine sensing P1 receptors and P2 receptors subdivided into 1) seven ligand-gated ion channels (P2X₁₋₇), which are sensitive to ATP and 2) eight G-protein coupled receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁₋₁₄), which respond to various ligands e.g., ATP, ADP, UTP, UDP, and UDP-glucose (13-15). These receptors are important for converting mechanical signals into biochemical signals in a process called mechanotransduction, leading to various biochemical outcomes, such as calcium transients, NO, and PGE2 activity, and ultimately the expression of genes that result in bone remodeling and maintenance (16). Numerous studies have highlighted the mechanotransductive pathways activated in osteocytes and osteoblasts upon the application of mechanical stimuli (17-21). Osteoclasts have been demonstrated to express multiple functional P2 receptors (12, 22), however, their roles in osteoclast mechano-adaptation are not fully understood.

The goal of this study was to examine the mechanisms of mechanosensitivity and mechanotransduction in mechanically stimulated osteoclasts. We used primary murine osteoclasts generated in vitro from bone marrow. We applied mechanical stimulus either to a single osteoclast through local membrane deformation using a micropipette or to osteoclast culture through fluid flow shear stress. We examined the degree of injury experienced by osteoclasts, the repair capacity of mechanically stimulated osteoclasts, and the role of ATP and ADP in the mechanotransduction of mechanical signals to the neighboring cells.

3.4 Materials and Methods

3.4.1 Reagents and Pharmacological Interventions

The following reagents and chemicals were used: tartrate-resistant acid phosphatase (TRAP) kit (Sigma No. 387 A), fast red violet salt (Sigma No. F3381), naphtol (Sigma No. N5000), N, N-dimethylformamide (Fisher Scientific No. BP1160), A 804598 (100 nM, P2X7 inhibitor, Tocris No. 4473), suramin (100 μ M, Cayman Chemicals No. 11126), adenosine 5'-triphosphate (Sigma-Aldrich No. A9187), adenosine 5'-diphosphate (Sigma-Aldrich No. A2754), quinacrine dihydrochloride (Sigma-Aldrich No. Q3251), Fura 2-AM (Invitrogen No. F1221), phosphoenolpyruvic acid (PEP; Sigma-Aldrich No. 860077), pyruvate kinase (PK) from rabbit muscle (Sigma-Aldrich No. P9136), hexokinase from *Saccharomyces cerevisiae* (Sigma-Aldrich No. H6380).

3.4.2 Cell Culture

All procedures received approval from McGill University's Animal Care Committee (protocol No. 7127) and were conducted in compliance with the ethical guidelines of the Canadian Council on Animal Care. Bone marrow cells were collected from the tibia and femur of 10–12-wk-old C57Bl/6J mice, as described previously (4). Osteoclasts were isolated and cultured from bone marrow cells, as described previously (23). Bone marrow cells were lysed with RBC lysis buffer (Sigma No. R57757) and plated with 50 $\mu\text{g}/\text{mL}$ M-CSF (PeproTech Inc. No. 300-25) for 24 h. The next day, nonadherent cells were collected and plated at 5×10^4 cells/ cm^2 in osteoclast medium: α -MEM (Gibco, No. 12561) supplemented with 10% FBS (Wisent No. 080-450), 1% sodium pyruvate (Wisent No. 600-110-EL), 1% penicillin-streptomycin (Wisent No. 450-201-EL), 50 $\mu\text{g}/\text{mL}$ M-CSF, and 50 $\mu\text{g}/\text{mL}$ RANKL (purified from clones, courtesy of Dr. M.F. Manolsson, University of Toronto). For phenotype characterization, mature osteoclasts (days 5–6) were fixed with 10% formalin (Fisher No. 23-245-685) and stained for tartrate-resistant acid phosphatase (TRAP; Sigma No. 387 A). Bone-marrow-depleted bones were used to obtain compact bone-derived osteoblast cultures, as described previously (4). Cells were plated at a density of 5,000/ cm^2 in osteoblast differentiation medium (α -MEM supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin-streptomycin, and 50 $\mu\text{g}/\text{mL}$ ascorbic acid) 2–3 days before experiments. Osteoblasts were also cultured for 28 days and stained for alkaline phosphatase activity, as described previously (4).

3.4.3 Intracellular Calcium Recording and Analysis

Cells were plated on glass-bottomed 35 mm dishes or 48-well plates (MatTek Corporation) and were loaded with a ratiometric calcium dye Fura 2-AM for 30 min. Cultures were washed with physiological solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 20 mM HEPES, pH 7.6, sterilized using a 0.22 μ m filter) and acclimatized for 10 min before experimentation. The cells were imaged at x40 magnification using an inverted fluorescence microscope (T2000, Nikon) with an imaging software (Volocity, Improvision), as described previously (4). The excitation wavelength was at 340 and 380 nm, and the emission wavelength was 510 nm, and the Fura 340/Fura 380 ratio was used as a readout of changes in $[Ca^{2+}]_i$.

3.4.4 Mechanical Stimulation Using a Micropipette

A single Fura-2 loaded osteoclast or osteoblast cell was mechanically stimulated with a glass micropipette made from glass capillary tubes (1.0 mm outer diameter, World Precision Instruments) using a Sutter pipette puller (Flaming/Brown, model P-87). The micropipette was attached to a FemtoJet microinjector NI2 (Eppendorf, Germany), positioned at a 45° angle from the horizontal plane at $\sim 10 \mu$ m from the cell membrane and moved at a speed of 250 μ m/s with a contact duration of 60 ms. The cells were recorded for baseline activity until 10 s, then stimulated with a micropipette and recorded for calcium activity until 150 s.

3.4.5 Analysis of Cell Damage Induced by Micropipette Stimulation

Cell injury was assessed through the loss of intensity of the Fura 340 nm channel during the time of recording (24). The data was processed as follows. The time course of changes in the Fura 340 channel intensity ($I_{340}(t)$) of the cell of interest and the cell-free region of the recording (background) were extracted. Background data were averaged over the recording time (B). In the Fura 340 channel the intensity during the period before the stimulation was isolated and averaged as baseline ($I_{340}(t_0)$). The normalized change in fluorescence intensity of the Fura 340 channel was then calculated as $\overline{I_{340}} = (I_{340}(t) - B) / (I_{340}(t_0) - B)$. The resulting curve started at 1 at baseline, then demonstrated a transient increase due to changes in calcium, and then declined to the value between 1 and 0, with 1 demonstrating the same intensity as before the stimulation and 0 complete loss of intensity which would be interpreted as loss of dye. To correct the curve for the transient increase due to calcium increase, we used the time course for Fura 340/Fura 380 calcium ratio changes ($Ca(t)$) of the cell, which we normalized to averaged baseline ($Ca(t_0)$) and scaled to 1 by identifying and averaging the maximum calcium ratio during the recording (Ca_{max}): $\overline{Ca(t)} = (Ca(t) - Ca(t_0)) / (Ca_{max} - Ca(t_0))$. Then we identified the maximum of normalized Fura 340 channel $\max(\overline{I_{340}})$ and scaled $\overline{Ca(t)}$ to the $\max(\overline{I_{340}})$: scaled $(\overline{Ca(t)}) = \overline{Ca(t)} \times (\max(\overline{I_{340}}) - 1)$, which was then subtracted from the normalized Fura 340 intensity to achieve the time course of Fura-2 dye loss ($F(t)$): $F(t) = \overline{I_{340}} - \text{scaled}(\overline{Ca(t)})$. Three parameters were extracted from each normalized dye loss curve: 1) the proportion of dye remaining by the end of the recording, 2) the rate of dye loss from 40 s to 90 s after stimulus, 3) and rate of continuous dye loss (from 90 s to 140 s after stimulus). These parameters were used as input for k-means clustering to

classify the cell injury as mild or severe injuries.

3.4.6 Turbulent Fluid Flow Shear Stress Mechanical Stimulation

Osteoclasts were subjected to turbulent fluid flow shear stress (tFSS) by displacing 50% of medium volume using a micropipette for n number of times (as indicated in experiments, for e.g., 5x tFSS denotes that 50% medium was displaced 5 times). Before each experiment, osteoclasts were loaded with 0.08% trypan blue dye, washed, and the number of dye-positive cells was counted. To assess shear stress-induced injury, osteoclasts were subjected to tFSS in the presence of 0.08% trypan blue dye in a physiological solution. The cells were washed with physiological solution, and the proportion of dye-positive cells was counted via microscopy at x20 magnification. To assess the repair of osteoclasts, cells were subjected to 20x tFSS, and trypan blue dye (at 0.08%, final concentration) was added at the n-th timepoint (indicated in the experiment) post-tFSS, and the proportion of dye-positive cells was counted via microscopy at x20 magnification.

3.4.7 ATP and ADP-Dependent Signaling

Since ATP and ADP can be transformed into each other, to specifically assess their roles we used enzymatic transformations either with 0.1 U/mL pyruvate kinase (PK) in the presence of excess, 100 μ M, phosphoenolpyruvate (PEP) which will transform all ADP to ATP, or with 0.1 U/mL hexokinase (HK) to transform all ATP to ADP. For concentration-dependence experiments, ATP and ADP powders were dissolved in physiological solution, and the reaction (ATP+PK+PEP or ADP+HK) was carried out at 37°C for 30 minutes, followed by heat inactivation at 95°C for 2 minutes. A range of ATP and ADP concentra-

tions were applied to Fura-2-loaded osteoclasts as described previously (25), and $[Ca^{2+}]_i$ responses were recorded. To study the roles of ATP and ADP in mechanotransduction, Fura-2 osteoclasts were bathed in physiological solution-containing either PK+PEP or HK for the duration of the experiment, whereas in control experiments osteoclasts were bathed in physiological solution.

3.4.8 Vesicle Release Kinetics

Osteoclasts were loaded with quinacrine dihydrochloride (10 μ M, 15 min, room temperature) and mechanically stimulated using a micropipette to visualize the release of vesicles. The cells were imaged at 435 excitation and 525 emission using x40 magnification, and data was analyzed, as described previously .

3.4.9 Statistical Analysis

Data are presented as means $\pm$ standard errors of the means (SE). Calcium recordings were exported to Excel, and the data was analyzed on GraphPad Prism 9. k-means clustering was performed on Python using NumPy, matplotlib, pandas, and sklearn. Outliers in data sets were identified using the ROUT method. Student's t test was carried out with Welch's correction. The normality of distribution was tested and when the data was not normally distributed it was log-transformed and tested using a t test with Welch's correction. One-way ANOVA test was carried out with Bonferroni correction. Fisher's exact test was carried out on categorical data.

3.5 Results

3.5.1 Osteoclasts Are Sensitive to Mechanical Stimuli

To assess osteoclast mechanosensitivity, we examined the calcium signals in mechanically stimulated osteoclasts. Primary murine osteoclasts were loaded with Fura 2-AM, a calcium indicator dye and a single cell was mechanically stimulated with a micropipette, and changes in $[Ca^{2+}]_i$ were recorded (Fig. 3.1 A). Membrane deformation resulted in a transient increase in $[Ca^{2+}]_i$ in the stimulated cell (primary responder) (Fig. 3.1, A and B). In addition, in 43% of cases, we also observed calcium elevations in the neighboring nonstimulated cells (secondary responders; Fig. 1, A and B). Primary responders displayed a more sustained calcium response, whereas the calcium responses of secondary responders were shorter and more oscillatory. We next investigated if osteoclasts are responsive to multiple stimulations by micropipette. Five minutes after the initial mechanical stimulation, we restimulated the same osteoclast and recorded changes in $[Ca^{2+}]_i$. Compared to the first stimulation, osteoclasts demonstrated a similar calcium response to the restimulation, although with a significantly lower area under the curve (AUC) ($P = 0.01$) and were capable of relaying a similar signal to neighboring secondary responders (Fig. 3.1, C and D). Thus, osteoclasts are sensitive to mechanical stimuli and can propagate mechanical signals to the secondary nonstimulated cells.

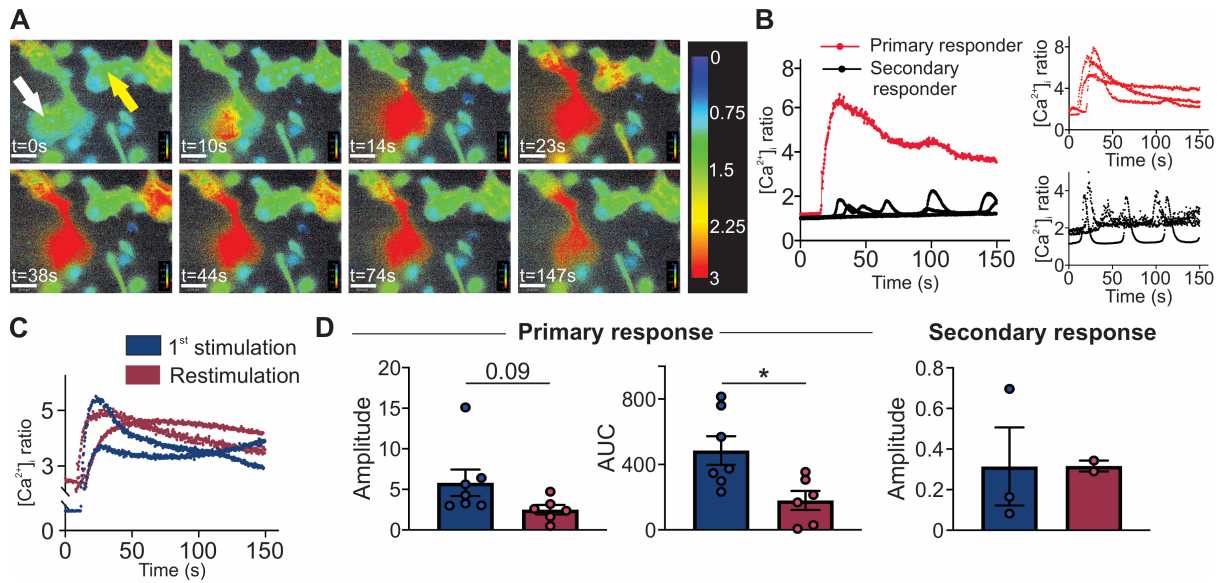


Figure 3.1: Osteoclasts are sensitive to mechanical stimuli. Osteoclasts cultured on glass bottom dishes were loaded with Fura 2-AM (calcium indicator dye), mechanically stimulated with a micropipette, and monitored for calcium changes. (A) micrographs were taken before (0 s), at the moment (10 s), and at indicated times after mechanical stimulation of osteoclast (white arrow, primary responder). The yellow arrow indicates a neighboring nonstimulated cell (secondary responder), which responds with a delayed increase in calcium. Scale bar: 32 μm , magnification: x40. (B), left: representative traces of calcium signal (Fura340/Fura380 ratio) in the primary (red) and secondary (black) osteoclasts induced by micropipette stimulation in a single experiment. Right: example of $[\text{Ca}^{2+}]_i$ traces of primary (top) and secondary (bottom) osteoclast responders. (C): calcium traces in osteoclasts stimulated for the first time (blue) and restimulated after 5 min (red). (D): calcium parameters, amplitude, and area under the curve (AUC), in primary and secondary responders in osteoclasts stimulated for the first and second time. Data are means $\pm$ SE, $n = 7$ stimulated osteoclasts/group, analyzed using Student's t test with Welch's correction, * $P < 0.05$.

3.5.2 Osteoclasts Are Less Sensitive to Mechanical Stimuli than Osteoblasts

To compare the mechanoresponsiveness of osteoclasts to that of established mechanosensory bone cells, osteoblasts, we applied local membrane deformation to a single primary osteoclast and osteoblast and characterized their calcium responses (Fig. 3.2). In osteoclasts, the primary response to mechanical stimuli had a significantly lower amplitude and AUC but longer duration (FWHM) compared with osteoblasts (Fig. 3.2B, top). Although both osteoclasts and osteoblasts were able to propagate the signal to the secondary responders, in osteoclast cultures, lower fraction of cells responded, and

secondary calcium transients had a significantly lower amplitude and AUC compared with osteoblast cultures (Fig. 3.2B, bottom). Thus, osteoblasts demonstrated higher mechanosensitivity and mechanotransduction compared with osteoclasts.

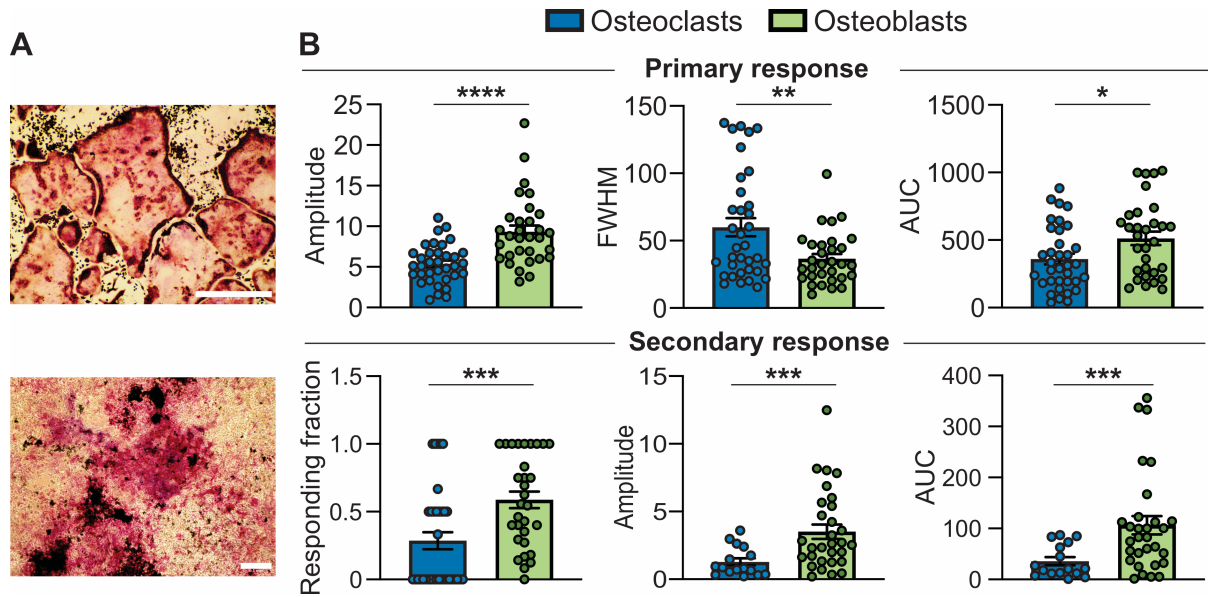


Figure 3.2: Comparing the response of osteoclasts and osteoblasts on mechanical stimulation. (A) representative images of TRAP-stained osteoclasts (top) and alkaline phosphatase-stained osteoblasts (bottom). Scale bar: 0.5 mm, magnification: osteoclasts: x10, osteoblasts: x4. (B) $[Ca^{2+}]_i$ response parameters, amplitude (arbitrary units), full-width half maximum (FWHM), and area under the curve (AUC), in primary (top) and secondary (bottom) responders to mechanical stimulation of osteoclasts (blue) and osteoblasts (green). Data are means $\pm$ SE, $n = 30\text{--}35$ stimulated cells/group, analyzed using Student's t test with Welch's correction, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. TRAP, tartrate-resistant acid phosphatase.

3.5.3 Osteoclast Micropipette Stimulation Generates a Reversible Plasma Membrane Damage

Mechanical stimulation results in reversible plasma membrane damage in osteoblasts (4) and osteocytes (17). We aimed to characterize the degree of injury experienced by osteoclasts upon mechanical stimulation. We used the decrease in fluorescence intensity of the Fura 340 channel as a measure of dye loss (24). The dye loss over time ($F(t)$) was calculated as described in Materials and Methods, and three parameters were extracted: 1) the proportion of dye remaining by the end of the recording, 2) the rate of dye loss from 40 s to 90 s after stimulus indicative of membrane injury, 3) and rate of continuous dye loss (from 90 s to 140 s) indicative of membrane repair. We

ran a k-means clustering ($k = 2$) on the extracted parameters and classified the dye loss curves into two categories: mild injury and severe injury (Fig. 3.3 A). In 79% of experiments, micropipette stimulation induced minimal dye loss, whereas, in 21% of recordings, cells demonstrated membrane injuries with a steep dye loss curve (Fig. 3.3, A and B). In severely injured osteoclasts, the rates of dye loss during both observation periods were significantly higher (Fig. 3.3C), and the proportion of dye remaining was significantly reduced (Fig. 3.3D) compared with those with minor injuries. Among the primary responders, severe injuries induced calcium responses with significantly higher amplitude, longer duration (higher FWHM), and higher AUC (Fig. 3.3E).

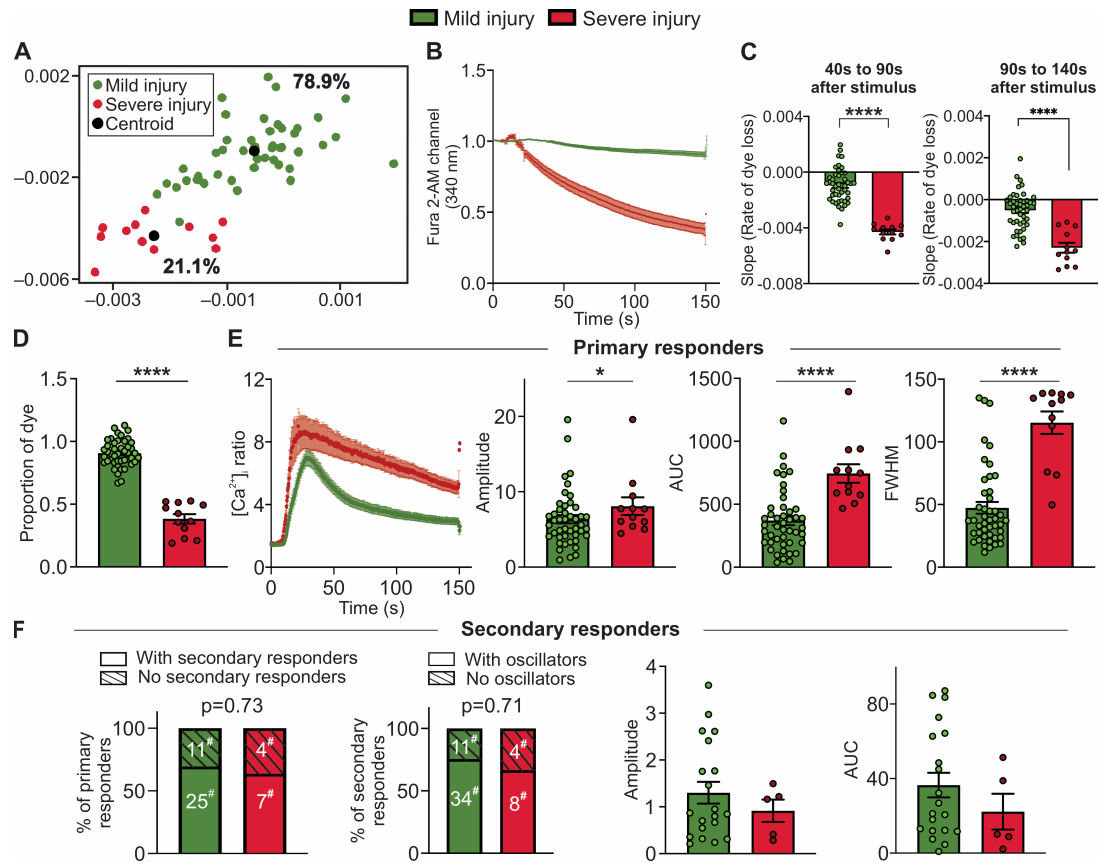


Figure 3.3: Classification of membrane injury in osteoclasts. The dye loss over time ($F(t)$) was calculated for each mechanically stimulated osteoclast, three parameters were extracted: the proportion of dye remaining by the end of the recording, the rate of dye loss from 40 s to 90 s after stimulus and rate of continuous dye loss from 90 s to 140 s and used as input for k-means clustering. (A) representative images of TRAP-stained osteoclasts (top) and alkaline phosphatase-stained osteoblasts (bottom). Scale bar: 0.5 mm, magnification: osteoclasts: x10, osteoblasts: x4. (B) $[Ca^{2+}]_i$ response parameters, amplitude (arbitrary units), full-width half maximum (FWHM), and area under the curve (AUC), in primary (top) and secondary (bottom) responders to mechanical stimulation of osteoclasts (blue) and osteoblasts (green). Data are means $\pm$ SE, $n = 30$ – 35 stimulated cells/group, analyzed using Student's t test with Welch's correction, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. TRAP, tartrate-resistant acid phosphatase.

When we compared secondary responders of mildly and severely injured osteoclasts, we found a similar fraction of cells with secondary responders, and among secondary responders, a similar number of oscillators, similar amplitude, or AUC of secondary responders (Fig. 3.3F). Thus, severe mechanical injury-induced higher and longer-lasting calcium transients in stimulated cells.

3.5.4 Osteoclast Shear Stress Generates Reversible Plasma Membrane Damages

Bone cells physiologically experience shear stress forces in their microenvironment. The turbulent fluid flow shear stress (tFSS) model has been previously utilized in osteocytes (18,26) and osteoblasts (4) and was shown to cause reversible membrane injury in these cells. We, therefore, examined if fluid flow shear induces repairable membrane injury in osteoclasts. We first added membrane impermeable dye, trypan blue (TB), to physiological solution bathing osteoclasts and performed tFSS by displacing 50% medium using a micropipette n times to simulate increasing shear stress. Osteoclasts that experienced shear stress took up the trypan blue dye, and the number of TB-positive cells significantly increased ($P = 0.023$ by ANOVA) with an increase in shear stress (Fig. 3.4, A and B). Next, we assessed whether osteoclasts could repair their membrane. Osteoclasts were subjected to 20x tFSS, and TB was added at various times (0 min–5 min) after mechanical stimulation (Fig. 3.4C). The proportion of TB-positive osteoclasts tended to decrease ($P = 0.089$ by ANOVA) with increase in time after mechanical stimulation (Fig. 3.4C). Thus, shear stress forces induced repairable membrane injuries in osteoclasts, which were proportional to the degree of mechanical stimulation.

3.5.5 P2X₇ Is Involved in Mechanotransduction between Primary and Secondary Osteoclasts

Next, we investigated the role of ATP and ADP, the primary mechanotransductive molecules in osteoblasts and osteocytes that act through P2 receptors (12), in relaying signals between the primary and secondary osteoclasts. We first used suramin, a broad

antagonist of purinergic P2 receptors (13), mechanically stimulated a single osteoclast and characterized the primary and secondary responses. In suramin-treated cultures, primary osteoclasts tended to have a higher amplitude ($P = 0.07$) and a significantly higher area under the curve of their calcium responses, and secondary osteoclasts tended to have a higher amplitude ($P = 0.07$) and significantly higher area under the curve compared with control cultures (Fig. 3.5, A and B). As suramin does not block all purinergic receptors effectively (13), and P2X₇, which is not blocked by suramin, was previously suggested to mediate cell-cell communications among osteoclasts (5), we used a P2X₇-specific antagonist, A 804598. Primary responders of the A 804598-treated group tended to have a lower amplitude ($P = 0.09$) and had significantly lower AUC (Fig. 3.5D, blue and pink). In addition, no secondary responders were observed in the A 804598-treated group (Fig. 3.5C, black graphs), suggesting that blocking P2X₇ activity affected the mechanically induced calcium responses in the primary responders and the ability of the primary responders to relay signals to secondary responders.

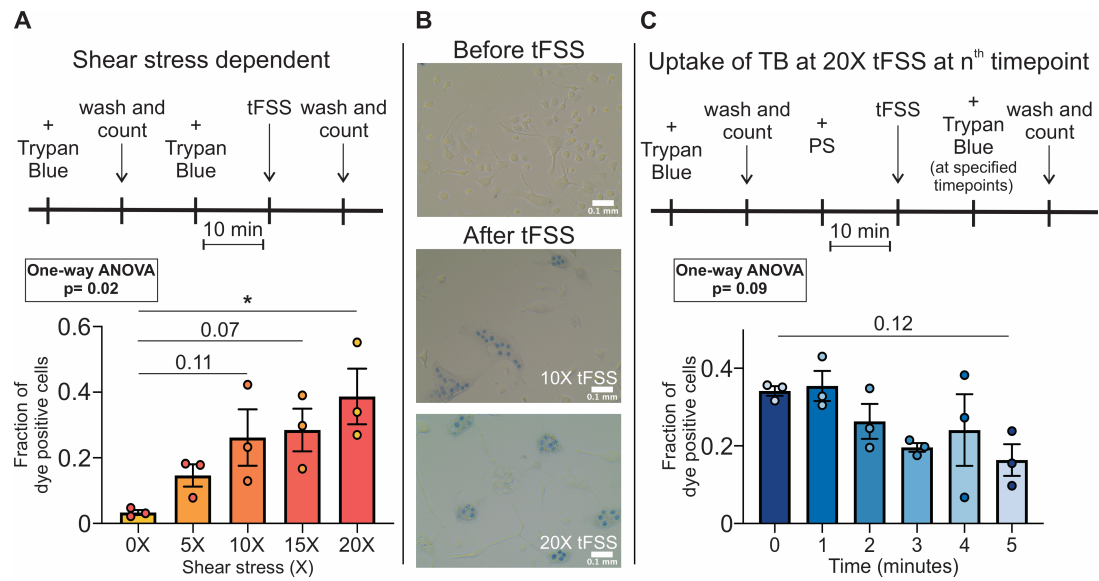


Figure 3.4: Osteoclasts are sensitive to shear stress forces. A: shear stress-dependent uptake of dye by osteoclasts was assessed as depicted on schematics (top) and the fraction of trypan blue dye-positive osteoclasts after increasing shear stress (0x to 20x) was analyzed (bottom). B: representative images demonstrating uptake of trypan blue dye by osteoclasts before tFSS (top) and after 10x and 20x tFSS (bottom). Scale bar: 0.1 mm, magnification: x20. C: uptake of dye added 0–5 min after shear stress at 20x was assessed as depicted on schematics (top), and the fraction of trypan blue dye-positive osteoclasts was analyzed (bottom). Data are means $\pm$ SE, $n = 3$ independent osteoclast cultures repeated thrice per experiment, analyzed using one-way ANOVA with Bonferroni post hoc, * $P < 0.05$.

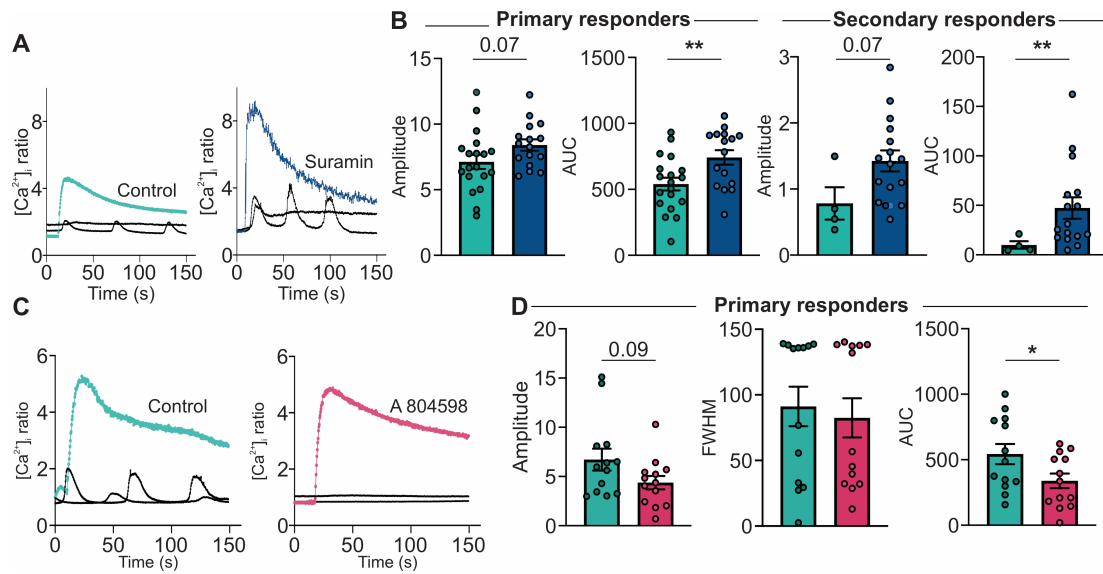


Figure 3.5: Effect of P2 inhibitors on osteoclast mechanotransduction. Osteoclasts were micropipette stimulated in physiological solution (control) or in the presence of nonspecific P2 receptor inhibitor suramin (100 μ M) or specific P2X₇ blocker A 804598 (100 nM). Representative traces of mechanically stimulated calcium responses in control and suramin-treated (A) and control and A 804598-treated (C) primary (colored) and secondary (black) responders. Calcium parameters, amplitude (arbitrary units), full-width half maximum (FWHM), and area under the curve (AUC) of primary and secondary responders in control and suramin-treated (B) and primary responders in control and A 804598-treated (D) cells. Data are means $\pm$ SE, $n = 16$ –19 stimulated osteoclasts/group for suramin experiments, $n = 13$ stimulated osteoclasts/group for A 804598 experiments, analyzed using Student's t test with Welch's correction, * $P < 0.05$, ** $P < 0.01$.

3.5.6 ATP and ADP Induce Concentration-Dependent Calcium Responses in Osteoclasts

We next characterized the concentration dependence for ATP and ADP effects on $[Ca^{2+}]_i$ responses in osteoclasts. Fura-2 loaded osteoclasts were imaged for 10 s, after which homogeneous ATP (1 μ M to 1 mM final concentrations) or ADP solutions (10 μ M to 1 mM final concentrations) were added to the bath (Fig. 3.6). Increasing concentrations of ATP (Fig. 3.6A) resulted in a significantly increased percentage of osteoclast responders, whereas the percentage of oscillators remained unaffected. The amplitude and the AUC of the calcium transients significantly increased with increasing concentrations of ATP (Fig. 3.6B). Increasing concentrations of ADP (Fig. 3.6C) similarly resulted in

a significantly higher percentage of responders and an increase in the percentage of oscillators in osteoclasts. The amplitude and AUC of ADP-induced calcium transients also increased with an increase in ADP concentrations (Fig. 3.6D). Taken together, osteoclasts respond to ATP and ADP with concentration-dependent changes in $[Ca^{2+}]_i$.

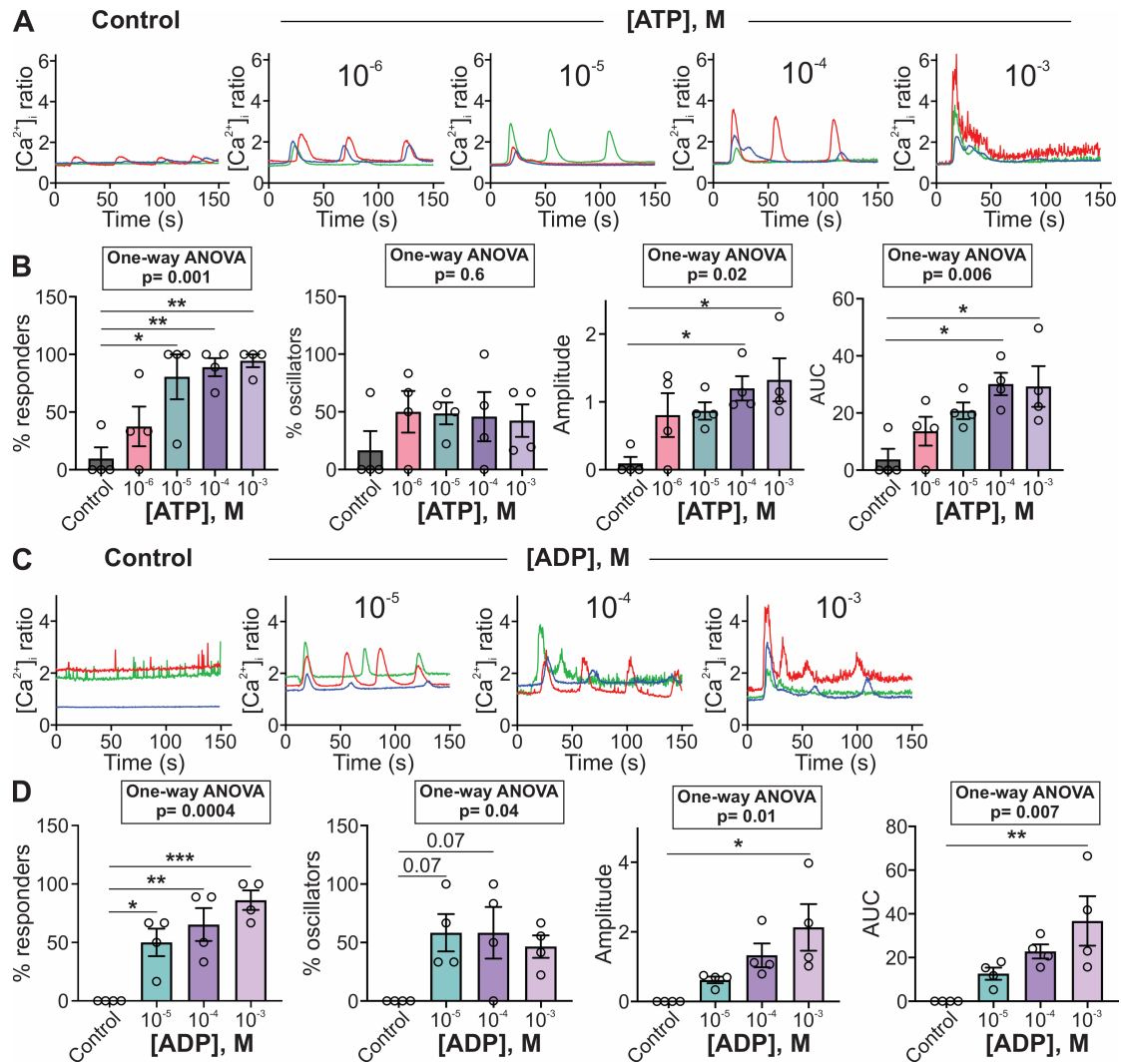


Figure 3.6: Concentration-dependence for osteoclasts responses to ATP and ADP. Calcium ratio traces in osteoclasts treated with physiological solution (PS) and increasing concentrations of ATP (A) or ADP (C). Percent responders (number of responding cells/total cell number in the field of view), percent oscillators (number of cells exhibiting oscillations/total number of responding cells), calcium response parameters, amplitude (arbitrary units), and area under the curve (AUC) for ATP-induced (B) and ADP-induced (D) responses. Data are means $\pm$ SE, n = 3 independent osteoclast cultures repeated thrice per experiment for ATP and ADP groups, analyzed using one-way ANOVA with Bonferroni post hoc, *P < 0.05, **P < 0.01, ***P < 0.001.

3.5.7 Mechanically Induced Vesicular Release from Osteoclasts

Vesicular release is an established mechanism of ATP release upon mechanical stimulation (27, 28). It has been shown that ATP is released through the vesicular route from osteoblasts and osteoclasts (29, 30) and through local membrane tears from osteoblasts and osteocytes (4, 17). We examined the release of vesicles stained with quinacrine dihydrochloride from osteoclasts mechanically stimulated using a micropipette. Mechanical stimulation resulted in the release of vesicles from the primary and secondary responders (Fig. 3.7A). Primary responders tended to have a higher and sustained release of vesicles than secondary responders (Fig. 3.7B), which resulted in a cumulative vesicular release of $8.1 \pm 4.3 \times 10^{-3}$ vesicles/ μm^2 from primary responders and $1 \pm 0.3 \times 10^{-3}$ vesicles/ μm^2 from secondary responders. From 36 stimulated osteoclasts, 12 did not show vesicular release from the secondary responders (Fig. 3.7C). We compared the net vesicular release from primary responders with and without secondary responders. We observed that primary responders with secondary responders tended to have a higher net vesicular release than the primary responders without secondary responders ($10.1 \pm 3.4 \times 10^{-3}$ vesicles/ μm^2 compared with $3.7 \pm 1.3 \times 10^{-3}$ vesicles/ μm^2 in primary responders without secondary responders; Fig. 3.7C). Thus, mechanical stimulation of osteoclasts results in continuous vesicle release both from the stimulated cells and to a smaller extent from the secondary cells.

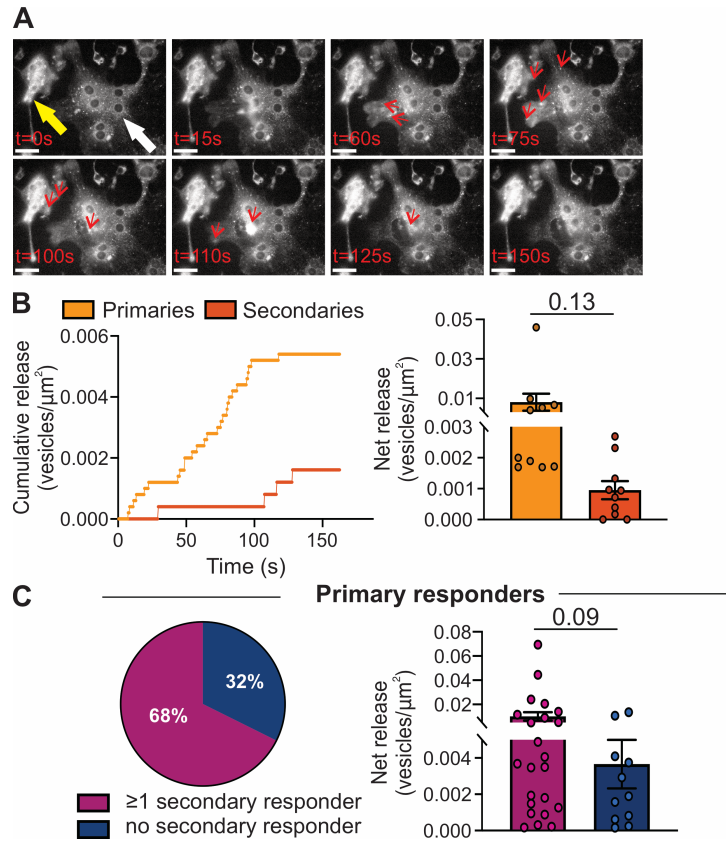


Figure 3.7: Vesicular release kinetics on mechanical stimulation in osteoclasts. Vesicles in osteoclasts were labeled with quinacrine. Osteoclasts were mechanically stimulated using a micropipette and vesicular release was analyzed in primary and secondary responders. A: micrographs were taken before (0 s), and at indicated times after mechanical stimulation of osteoclast (white arrow, primary responder, stimulated at 10 s-mark, yellow arrow: secondary responder, red arrows: vesicle release observed as sparks). Scale bar: 32 μm , magnification: x40. B, Left: representative traces of cumulative vesicular release after mechanical stimulation in primary and secondary responders. Right: net vesicle release from primary and secondary responders. C, Left: pie chart depicting the percentage of primary responders with and without secondary responders. Right: net vesicular release in primary responders with and without secondary responders. Data are means $\pm$ SE, n = 36 stimulated osteoclasts, analyzed using Student's t test with Welch's correction.

3.5.8 Role of ATP and ADP Osteoclast Mechanotransduction

Finally, we examined the role of ATP and ADP in osteoclast mechanotransduction at different injury levels. ATP released upon mechanical stimulation is broken down into ADP by ectonucleotidases present in bone cells (12, 31). To study the specific roles of ATP and ADP in mechanotransduction, we bathed osteoclasts in physiological solution containing either PK + PEP to convert all ADP to ATP, thus maintaining ATP as a main mechanotransductive signal, or HK to convert released ATP to ADP as the main

propagating signal. A single osteoclast was stimulated using a micropipette, and we examined signal propagation to secondary responders in control conditions (untreated cultures), or when the main propagating species was ATP (ATP-rich, in the presence of PK and PEP) or ADP (ADP-rich, in the presence of hexokinase; Fig. 3.8). We classified the Fura-2 dye loss in primary osteoclasts using the k means classification method ($k = 2$). Mild and severe injury groups had a similar number of cells in control, ATP-rich, and ADP-rich conditions. In the mild injury group, no apparent differences were observed in the primary (Fig. 3.8A) or secondary (Fig. 3.8B) responders in control, ATP-rich, and ADP-rich conditions. In the severely injured group, although no differences were observed in the primary responses (Fig. 3.8C), the secondary responders in an ADP-rich environment had a significantly higher amplitude and tended to have a higher probability of secondary events than in the ATP-rich conditions ($P = 0.09$; Fig. 3.8D). Therefore, osteoclasts responded differently when ATP or ADP were the main propagating species and demonstrated increased responses in an ADP-rich environment when the primary osteoclast experienced severe injury.

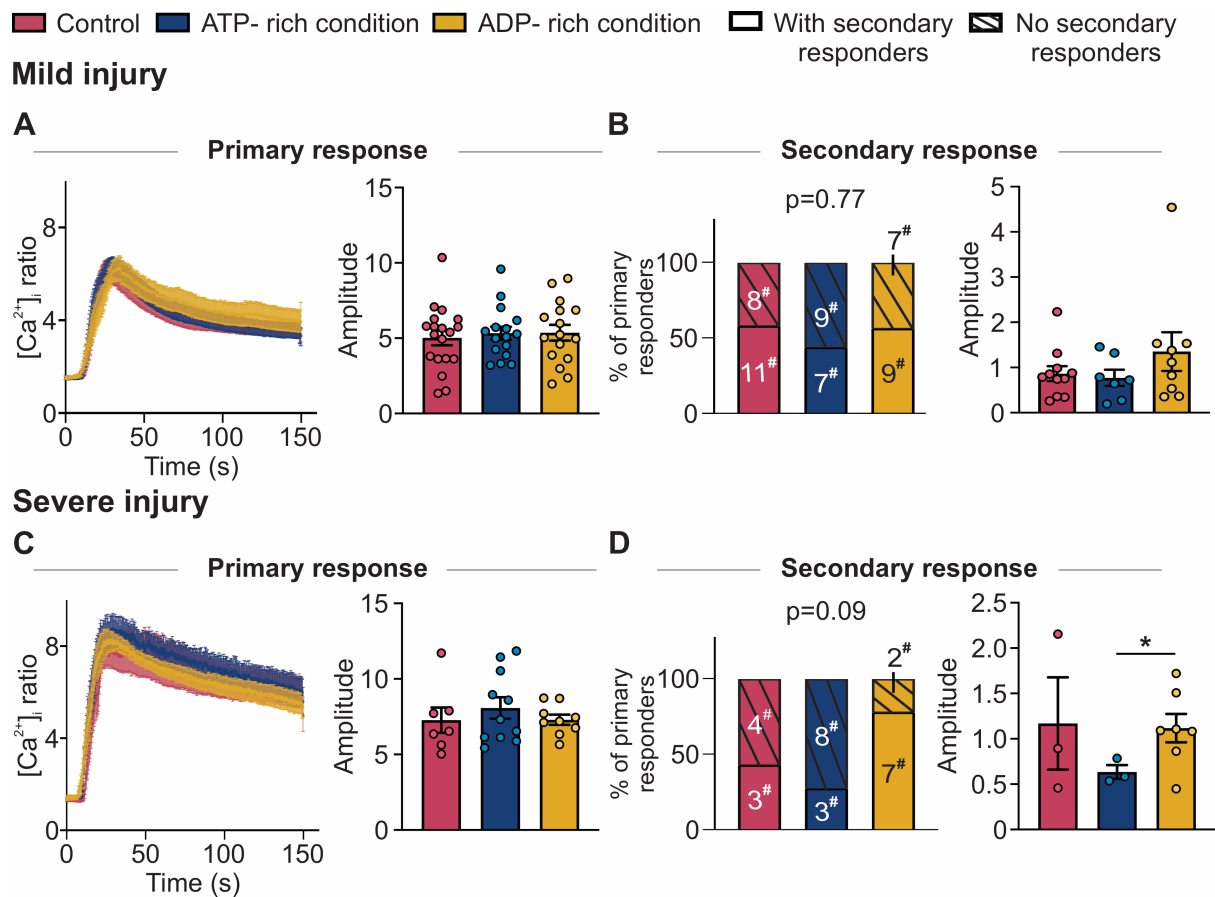


Figure 3.8: ATP and ADP-dependent mechanosensitivity in mild and severely injured osteoclasts. Osteoclasts were bathed in physiological solution without additions (control), with PEP + PK (ATP-rich condition) or with HK (ADP-rich condition), and a single osteoclast was mechanically stimulated using a micropipette. Primary responders were classified as mild injury (A and B) or severe injury (C and D) using k-means classification. Average calcium ratio (Fura340/Fura380) traces in primary responders of mild (A, line graphs) and severe (C, line graphs) injury groups. Amplitudes of primary responders (arbitrary units) in mild (A, bar graphs) and severe (C, bar graphs) injury groups. Percentage of primaries with mild (B, left bar graphs) or severe (D, left bar graphs) injury, with (open portion) and without (lined portion) secondary responses, analyzed using Fisher's exact test, #number of cells. (B and D, right bar graphs) Amplitude of secondary responders (arbitrary units) in mild (B) and severe (D) injury groups. Data are means $\pm$ SE, n = 25–27 stimulated osteoclasts/treatment group. Calcium data was analyzed using one-way ANOVA with Bonferroni post hoc. Significance between the groups was also analyzed using Student's t-test with Welch's correction, *P < 0.05.

3.6 Discussion

In this study, our main goal was to assess the mechano-sensitivity and mechanotransductive capacity of osteoclasts. We showed that 1) osteoclasts are sensitive to mechanical stimuli through local membrane deformation and can transduce signals to neighboring

cells; 2) osteoclasts commonly experience minor membrane injury upon mechanical stimulation by micropipette or by shear stress forces and are able to repair their membranes to avoid spillage of cellular contents; 3) ATP and ADP, likely released through microinjuries, participate in mechanotransduction among osteoclasts.

Bone remodeling is a continuous process performed by bone multicellular units (BMUs) and comprises the resorption of damaged bone by osteoclasts and the deposition of new bone by osteoblasts (32-34). Osteoclasts are the first cells to arrive at the site of damaged bone, hence, it would be important for osteoclasts to have an ability to sense and respond to mechanical signals. It is generally assumed that osteoclasts are guided by biochemical stimuli produced by osteocytes (35) however, several previous studies also noted the direct effect of mechanical stimulation on osteoclasts. Osteoclasts were shown to respond to membrane deformation (5), to fluid shear forces (7, 9), substrate stretching (8), and vibration (6). Some osteoclast parameters were shown to be negatively affected by mechanical loading and positively by unloading (9). In other cases, such as for substrate stretching, it was shown that osteoclast formation was inhibited by high-frequency cyclic substrate stretching (6, 36-38), but stimulated by low-frequency substrate stretching (8). Nevertheless, our data confirm that osteoclasts are mechanosensitive cells that respond to mechanical perturbations in their environment. We demonstrated that osteoclasts experience minor injury upon mechanical stimulation by micropipette or by shear stress forces and were able to repair their membranes to avoid spillage of cellular contents. Similar to osteoblasts and osteocytes that were shown to respond proportionally to increasing shear stress forces (4, 18), here we demonstrate that osteoclasts also respond proportionally to increasing mechanical forces, as shown by higher responses in severely

injured cells. Osteoblasts and osteocytes have been shown to repair plasma membrane damage generated by mechanical stimulus and thus limit ATP release (4, 17, 25). Similarly, we demonstrate that osteoclasts are also able to repair themselves to avoid spillage of cellular contents as demonstrated via a decrease in the fraction of trypan blue-positive cells with time. Taken together, osteoclasts are similar to other cells, in which plasma membrane damage was shown to occur in response to mechanical stimulation (39, 40), including osteoblasts and osteocytes (4, 17), skeletal muscle cell (41), skin (42), cardiac muscle (43), and lungs (43).

ATP is a key mechanotransductive signal (12). In osteoblasts, ATP release was shown to occur through membrane microinjuries (4, 28), as well as through vesicular release (4, 30). In osteoclasts, we observed vesicular release, however, the kinetics of this process was very variable and inconsistent with ATP release. In keeping, a previous study has found that blocking vesicular release in osteoclasts did not change the amount of released ATP (29). Of interest, the same study demonstrated that the low-affinity P2X₇ receptor was critical for ATP release from osteoclasts (29). These data are consistent with our finding that blocking the P2X₇ receptor prevented the propagation of mechanical signals to secondary responders. Similar to our observations, Jørgensen et al. (5) showed that mechanical stimulation of a single human osteoclast resulted in a rise in calcium signal in the stimulated cell and the neighboring nonstimulated cells, which was blocked by inhibition of P2X₇. P2X₇ is a low-affinity ATP receptor, and we have previously shown that even in more mechanotransductive cells such as osteoblasts, a single cell does not release sufficient ATP to stimulate P2X₇ on neighboring cells (25). It was previously suggested that P2X₇ may also interact with pannexin1, a hemichannel implicated in

mechanically induced ATP release as well as other molecules (4, 44), which may provide an explanation for the observed role of P2X₇ in our experiments. Alternatively, P2X₇ may interact with a high-affinity P2 receptor, as we demonstrated for P2Y₂/P2X₇ interactions (45), which may also explain its involvement in single-cell mechanotransduction. Another interesting observation from this study is the relative importance of ADP-mediated signals in osteoclast mechanotransduction. We have found that ADP induces prominent responses in osteoclasts and that transforming the propagating signal to ADP potentiated secondary responses. These data contrast with osteoblast-derived mechanotransductive signals, which are dominated by ATP, with ADP becoming important only in cases of large injuries (25). ADP is a strong ligand for three P2Y receptors, P2Y₁, P2Y₁₂, and P2Y₁₃, all of which have been identified on osteoclasts (22, 46, 47). Thus, our data suggests that the role of ADP-mediated signaling in mechanically induced osteoclasts needs to be further explored.

Thus, we show that osteoclasts are mechanoresponsive cells capable of sensing and transmitting mechanical signals to neighboring cells. Like other bone cells, they can sense membrane deformation and are affected by shear forces. Both ATP and ADP play a role in the mechanosensitivity of osteoclasts with ADP playing a more prominent role in severe injury. This study highlights the importance of understanding osteoclast mechanobiology and the role of ADP-mediated signaling in conditions of altered mechanical loading, especially those associated with bone loss, such as in microgravity (48) and in paralyzed and immobilized patients (49).

3.7 Data availability

The data are available on reasonable request.

3.8 Acknowledgments

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3.10 Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

3.11 Author contributions

C.D. and S.V.K. conceived and designed research; C.D. performed experiments; C.D. and S.V.K. analyzed data; C.D. and S.V.K. interpreted results of experiments; C.D. prepared figures; C.D. and S.V.K. drafted manuscript; C.D. and S.V.K. edited and revised manuscript; C.D. and S.V.K. approved the final version of the manuscript.

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Bridging Statement

In the previous chapter, I summarize the mechanosensitive and mechanotransductive characteristics of osteoclasts. I show that osteoclasts can sense and transduce signals to neighboring cells. They are also sensitive to fluid shear stress and undergo reversible PMDs. The P2X₇ receptor is essential for osteoclast mechanotransduction, and ADP-mediated signaling becomes more important in cases of severe injury to osteoclasts. Since we now also understand the mechanoadaptive properties of osteoclasts, the next step was to assess how these properties are affected in aging osteoblasts and osteoclasts. In this chapter, I compared early mechanical calcium signals among young and old osteoblasts and osteoclasts subjected to local membrane deformation. I also examined whether the level of injury and corresponding calcium signals were affected by age. I also investigated the expression of the P2 receptors previously identified to be important in osteoclast mechanoadaptation and aging as highlighted in Chapters [2](#) and [3](#). Finally, I investigated sex-related differences in calcium signaling in aging osteoblasts and osteoclasts.

Chapter 4

Aging-associated changes in mechanotransduction of osteoblasts and osteoclasts

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4.1 Abstract

Bone is a dynamic tissue and mechanical loading is important for its maintenance. However, the aging skeleton is unable to benefit from mechanical loading as it loses the ability to adapt to mechanical signals. Here we examined whether age-related changes in early mechanical signals are responsible for reduced mechanoadaptation in bone-forming osteoblasts (OB) and bone-resorbing osteoclasts (OC). OBs and OCs were differentiated from precursors obtained from young (10-12 weeks) and old (70-75 weeks) C57Bl/6 mice, loaded with the calcium-sensitive dye Fura-2, and a single cell was mechanically stimulated using a micropipette. Changes in cytosolic free calcium $[Ca^{2+}]_i$ in the mechanically stimulated (primary) cells were assessed as indicators of mechanosensitivity and in neighboring (secondary) cells as indicators of mechanotransduction. While OBs and OCs did not demonstrate age-related differences in mechanosensitivity, the mechanotransduction was affected both in aging OBs and OCs. Mechanically stimulated aging OBs exhibited deficient mechanotransduction, while OCs became better at propagating mechanical signals with age. The expression of P2X₇ and P2Y₁₃ decreased with age in OCs but was stable in OBs. Of interest, OBs and OCs from male animals were less mechanoresponsive than OBs and OCs from females. Thus, our study shows that aging is associated with an imbalance in mechanotransduction in OBs and OCs that would support an overall more resorptive phenotype with age. A better understanding of the mechanisms of age-associated changes in cellular mechanoadaptation will pave the way

to developing better exercise and pharmacological treatments for aging-associated bone loss.

4.2 Introduction

Bone mass and strength are affected by the level of exposure to mechanical forces, as highlighted by bone loss in astronauts and bed-ridden patients or overload experienced by athletes or military personnel (Willie, E. A. Zimmermann, et al. [2020](#)). Physical activity is important for the maintenance of bone mass and its quality. With age, a decrease in bone mass leads to conditions such as osteoporosis, which can be potentially ameliorated with exercise (Tatsuno et al. [2013](#)). However, studies in mice and humans have shown that aging results in a significant decrease in bone mechanoadaptation and remodeling (Bassey et al. [1998](#); Srinivasan et al. [2012](#); Willie, Birkhold, et al. [2013](#); Birkhold, Razi, et al. [2014](#); Holguin et al. [2014](#)). Aging also results in an imbalance between bone formation carried out by osteoblasts (OBs) and bone resorption by osteoclasts (OCs) (Møller et al. [2020](#); Rauner et al. [2008](#)). Age-dependent reduction in the number and declined function was also described for the osteocytes, the mechanosensory cells in the bone (Tiede-Lewis et al. [2017](#)). However, there are limited studies in understanding the mechanisms of age-associated loss of mechanoadaptation at the level of bone cells. When bone cells experience physical forces, ATP is released within seconds of mechanical stimulation (Dsouza, Moussa, et al. [2022](#)). ATP and its metabolite ADP act on the family of purinergic P2 receptors present on bone cells to induce mechanical signals such as a rise in intracellular calcium $[Ca^{2+}]_i$, and expression of genes that ultimately play a role in bone remodeling (Robling and Turner [2009](#)). P2 receptors are important players

in the remodeling and maintenance of skeletal health (I. Orriss et al. [2011](#); Dsouza, Moussa, et al. [2022](#)). Of interest, the ADP signaling P2Y₁₃ receptor has been implicated in age-related bone loss (N. Wang, Robaye, Agrawal, et al. [2012](#)). Osteocytes (K. Yu et al. [2018](#)), OBs (Mikolajewicz, Mohammed, et al. [2018a](#)), and OCs (Dsouza and Komarova [2024](#)) have been shown to respond to mechanical forces with an increase in calcium signaling, and reversible plasma membrane tears were strongly implicated in mechanotransduction (Mikolajewicz, Mohammed, et al. [2018a](#); K. Yu et al. [2018](#)). Aging osteocytes were also demonstrated to become resistant to plasma membrane tears, to lose responsiveness to stimulus, and to display reduced calcium transients, all resulting in poor mechanotransductive signaling (Hagan, K. Yu, et al. [2020](#); Morrell et al. [2020](#)). However, there are limited studies of the effect of aging on early mechanical signals in OBs and OCs. The goal of this study was to examine the changes with age in early signals induced by mechanical stimulation of OBs and OCs. We studied these changes in OBs and OCs from young (10-12 weeks old) and old (70-75 weeks) mice. Single OB or OC was mechanically stimulated in vitro via local membrane deformation. We studied cellular mechanosensitivity by examining the degree of injury and calcium signaling in the stimulated cells, and mechanotransduction by assessing ATP/ADP induced calcium signaling in neighboring cells.

4.3 Materials and Methods

4.3.1 Cell culture

All animal procedures were carried out as per the ethical guidelines of the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee

(protocol #7127). Primary OB and OC cultures were obtained from the tibia and femur of young (10-12 weeks old) and old (70-75 weeks old) C57Bl/6 male and female mice and cultured in α -MEM (Gibco, #12561), 10% FBS (Wisent #080-450), 1% sodium pyruvate (Wisent #600-110-EL) and 1% penicillin-streptomycin (Wisent #450-201-EL) as described previously (Dsouza and Komarova 2024). OBs were plated at a density of 5000 cells/cm² in OB differentiation medium containing 50 μ g/ml ascorbic acid (Sigma #A8960), and experiments were carried out on day 3. OCs were plated at 50,000 cells/cm² with culture medium containing 50 μ g/ml M-CSF (Peprotech Inc. #300-25) and 50 μ g/ml RANKL (from clones, courtesy: Dr. M.F. Manolson, University of Toronto).

4.3.2 OB and OC staining

OBs were cultured for 28 days in culture medium supplemented with ascorbic acid (50 μ g/ml) and β -glycerophosphate (4mM, Sigma #G5422-100G). To visualize calcium deposits, cells were fixed in formalin (10% buffered pH 7.4, 8 minutes), washed thrice with PBS, and thrice with 70% ethanol. OBs were stained with alizarin red staining solution (1% Alizarin Red (w/v) (Sigma #A5533) in Milli-Q H₂O) and incubated for 15 minutes at room temperature in the dark. Cells were then washed thrice with 50% ethanol and with excessive H₂O. The cells were finally rinsed with PBS and visualized under the microscope. OBs with calcium deposits were stained bright red. OBs were also stained for alkaline phosphatase activity as described previously (Mikolajewicz, E. A. Zimmermann, et al. 2018). To visualize collagen, OBs were fixed with ice-cold 70% ethanol for 1 hour at 4°C and washed twice with PBS. Cells were incubated with Sirius Red staining solution (Abcam Picro-Sirius Red Stain Kit #ab150681) for 1 hour at room temperature. Cells were washed twice with acetic acid solution and H₂O and visualized

under the microscope for collagen fibers (stained red). For OC cultures, mature cells (days 5-7) were fixed in 10% formalin and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma #387A) (Boraschi-Diaz et al. [2016](#)).

4.3.3 In vitro mechanical stimulation of a single cell

A single OB or OC was mechanically stimulated using a micropipette and visualized for intracellular calcium signals ($[Ca^{2+}]_i$) as described previously (Dsouza and Komarova [2024](#)). Briefly, cells were plated on 35 mm glass bottom dishes (MatTek Corporation) and loaded with Fura 2-AM, a ratiometric calcium dye, for 30 minutes. Cells were washed with physiological solution (130 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.6, filtered with 0.22 μ M filter) and were left to stand for 10 minutes before the experiment to avoid capturing signals due to washing. Cells were recorded for baseline activity (approx. 10 seconds), following which a single cell was gently stimulated with a glass micropipette (World Precision Instruments) using a FemtoJet microinjector NI2 (Eppendorf, Germany) and recorded until 150 seconds (Volocity, Quorum Technologies). Cells were excited at 340 nm and 380 nm, and emission was at 510 nm. The ratio of Fura 340/Fura 380 was used to analyze changes in $[Ca^{2+}]_i$.

4.3.4 Membrane injury assessment mechanically stimulated cells

Membrane injury of stimulated OBs or OCs was determined by analyzing the intensity of the Fura 340 channel over time. The data was exported and processed as described previously (Dsouza and Komarova [2024](#)). The injuries were classified as either mild or severe using an unsupervised k-means clustering method (Python).

4.3.5 RNA isolation and gene expression

RNA was isolated from mature OBs (Day 28) and OCs (Days 5-7) using the TRIzol reagent (Invitrogen #15596026) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368813). qPCR was carried out using the QuantStudio 7 Flex PCR System with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems #A25742). Housekeeping genes used were *Gapdh*: Forward- ACCCAGAA-GACTGTGGATGG, Reverse- CACATTGGGGGTAGGAACAC; *β-actin*: Forward- TGTTAC-CAACTGGGACGACA, Reverse- GGGGTGTTGAAGGTCTCAA. Genes tested were *P2x7*: Forward- ACAATGTGGAAGCGGACG, Reverse- TCAATGCACACAGTGGCCA; *P2y2*: Forward- GAAGAACTGGAGCAGGCGCT, Reverse- CCATTGCCCTGGACCTGATC; *P2y13*: Forward- CAGCTGAGTCTCTTCCAAAACAAA, Reverse- TGCATCCCAGTGGTGTGAT. A melt curve analysis was carried out to determine the dissociation data from the P2 gene amplicons. The data were normalized using the ddCt method. The target genes were normalized to the average housekeeping genes, *β-actin* and GAPDH.

4.3.6 Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Calcium data was analyzed on GraphPad Prism 10. Membrane injury data was analyzed in Excel, and k-means clustering was carried out using numpy, matplotlib, pandas, and sklearn in Python. Two-way ANOVA was carried out with Bonferroni correction posthoc. Student's t-test was performed with Welch correction. Fisher's exact test was carried out for categorical data.

4.4 Results

4.4.1 OBs and OCs characterization and experiment setup

To understand the changes in mechanosensitivity and mechanotransduction with age, we performed in vitro mechanical stimulation on OBs and OCs isolated from young (10-12 weeks old) and old (70-75 weeks old) mice. OBs were characterized using the alizarin red (calcium deposits), alkaline phosphatase (alkaline phosphatase activity), and Sirius red (collagen) staining while the OCs were characterized by the TRAP stain (Fig 4.1A). OBs from older mice displayed reduced calcium deposits as compared to OBs from young mice, while no differences between the age groups were observed in the OB alkaline phosphatase activity and collagen formation or in the OC TRAP staining. Mechanical stimulation of a single young or old OBs and OCs loaded with calcium-sensitive dye Fura2-AM was performed by gentle touch with a glass micropipette as described previously (Dsouza and Komarova 2024). Cells were recorded for 10s for baseline activity, mechanically stimulated at 10s, and were recorded until 150s to monitor the changes in intracellular calcium signals, $[Ca^{2+}]_i$, in the stimulated (primary responder) and the neighboring cell (secondary responder) (Fig 4.1B). The $[Ca^{2+}]_i$ responses were determined by the ratio of the Fura 340 and Fura 380 channels. The primary and secondary responders in young OBs and primary responders in young OCs overall displayed higher calcium transients than cells from older mice. (Fig 4.1C). We examined the expression of ATP and ADP receptors known for their important role in mechanotransduction, P2X₇ (J. Li, Liu, et al. 2005; Vieceilli et al. 2009), P2Y₂ (Mikolajewicz, Smith, et al. 2021), and P2Y₁₃ (N. Wang, Rumney, et al. 2013). While in

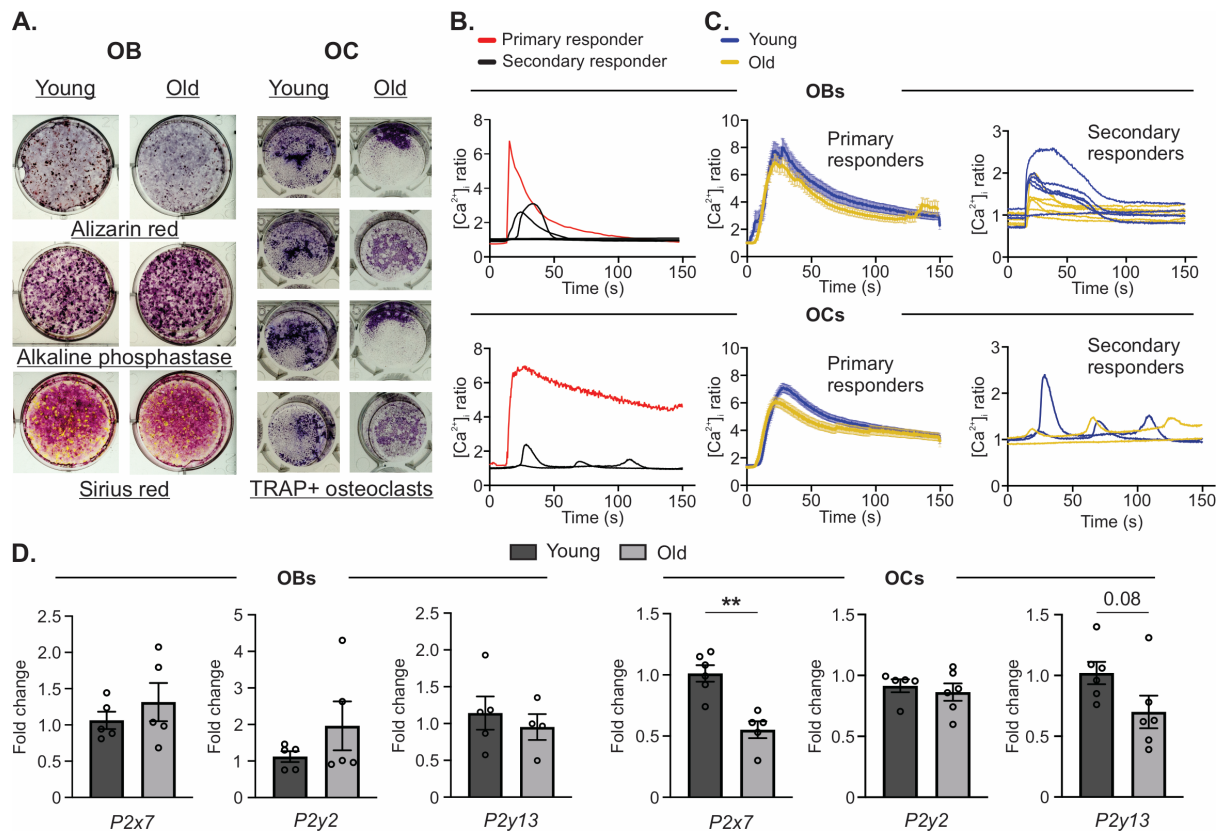


Figure 4.1: Calcium transients in in vitro mechanically stimulated OBs and OCs isolated from young (10-12 weeks) and old (70-75 weeks) mice. A. Representative images of young and old OBs (left) and OCs (right) stained for: (left) calcium deposition with alizarin red, alkaline phosphatase activity, and collagen deposition with Sirius red; (right): TRAP activity. B. Representative calcium responses (ratio Fura 340/Fura 380) of mechanically stimulated OBs (top) and OCs (bottom). Red curves: stimulated cells (primary responder), black curves: neighboring non-stimulated cells (secondary responder). C. Average calcium ratio traces of primary responders (left) and representative secondary responders (right) in young (blue) and old (yellow) OBs (top) and OCs (bottom). D. Fold change in expression of P2 genes, *P2x7*, *P2y2*, and *P2y13* in young and old OBs (left) and OCs (right). Data are mean $\pm$ SD, $n = 30-50$ cells for mechanical stimulation experiments and $n = 5-6$ cells/age group for gene expression studies

OB no age-related changes in these receptors were observed, OCs from older animals demonstrated reduced expression of *P2X₇* ($p = 0.001$) and *P2Y₁₃* ($p = 0.08$) (Fig. 4.1D).

4.4.2 Mechanically-induced membrane injury in OBs and OCs

We and others have shown that OCs (Dsouza and Komarova 2024), OBs (Mikolajewicz, Mohammed, et al. 2018a), and osteocytes (K. Yu et al. 2018) undergo reversible plasma membrane tears when exposed to mechanical forces. It is also known that osteocytes become more resilient to membrane tears with age (Hagan, K. Yu, et al. 2020), however,

it is not known whether OBs and OCs demonstrate a similar phenomenon with age. We stimulated Fura2-loaded OBs or OCs and extracted the Fura 340 channel recordings as an indicator of dye loss (Togo et al. 1999; Mikolajewicz, Mohammed, et al. 2018a; Dsouza and Komarova 2024) and processed as described before (Dsouza and Komarova 2024). We then used a k-means clustering to separate the injuries among each group into mild or severe injuries based on three parameters extracted from the dye loss curve, i) the proportion of dye remaining at the end of the experiment ii) the rate of dye loss from 40s to 90s after stimulus and iii) the rate of dye loss from 90s to 140s after stimulus (Fig 4.2A, D). The k-means clustering resulted in a separation of cells undergoing mild or severe injuries. Severely injured OBs and OCs had a significantly lower proportion of dye at the end (Fig 4.2B, E, left), and a steeper rate of dye loss throughout the experiment (Fig 4.2B, E *middle, right*). We identified no significant differences in the level of injury among young and old OBs and OCs (Fig 4.2C, F) suggesting that young and old OBs and OCs have similar resilience to injury.

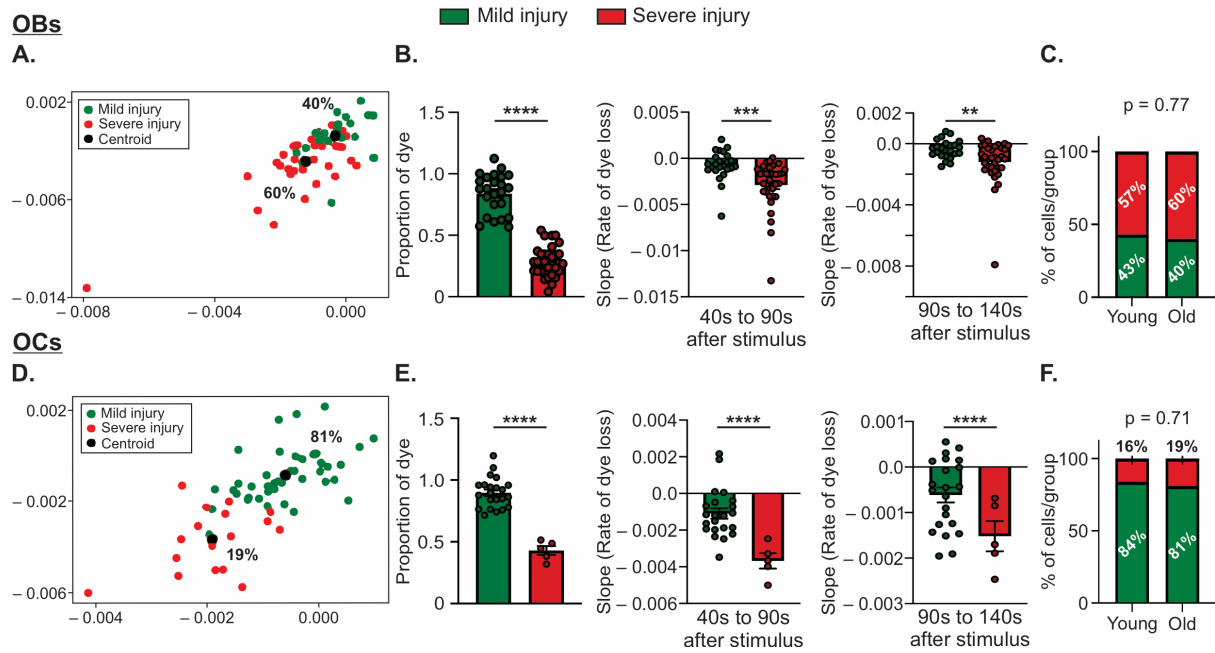


Figure 4.2: Membrane injury in mechanically stimulated young and old OBs and OCs. The Fura 340 dye loss curve was processed as described before (Dsouza and Komarova 2024), and three parameters i) dye loss at the end of the experiment ii) slope of the curve from 40s to 90s post-stimulus, and iii) slope of the curve from 90s to 140s post-stimulus were used to classify the injuries as mild (green) or severe (red). A and D. k-means cluster plots of old OBs (A) and OCs (D), black dots are the centroids of the mild and severe clusters. B and E. Average proportion of dye remaining at the end of the experiment (left), rate of dye loss from 40s to 90s after stimulus (*middle*), and rate of dye loss from 90s to 140s after stimulus (*right*) in primary responders among old OBs (B) and old OCs (E). C and F. Percentage of cells k-means classified as mildly or severely injured in young and old primary OBs (C) and OCs (F). Data are mean $\pm$ SEM, n (young OBs) = 31, n (old OBs) = 58, n (young OCs) = 51, n (old OCs) = 27 cells. Significance by Student t-test with Welch correction, **p < 0.01, ***p < 0.001, ****p < 0.0001 and by Fisher's test for categorical data (C, F).

4.4.3 Calcium signaling parameters in young and old OBs exhibiting mild and severe membrane injury

We next characterized the calcium parameters in mildly and severely injured young and old OBs. The calcium responses were higher in severely injured OBs from both young and old mice (Fig 4.3A). When the injury was mild, all young primary OBs were able to generate a secondary response, while 8% of older primary OBs did not generate a secondary response (p = 0.007). However, when the injury was severe, all older primary OBs generated a secondary response, whereas 6% of younger primary OBs could not

generate a secondary response ($p = 0.03$). In both young and old OBs, a significant increase was observed in the amplitude (2-way ANOVA, $p = 0.004$) and area under the curve (AUC) (2-way ANOVA, $p < 0.001$) of primary calcium responses with an increase in severity of injury (Fig 5.3B). Interestingly, the secondary responders demonstrated significant age-dependent differences (Fig. 4.3C). Older secondary OBs had significantly fewer oscillators than the younger OBs irrespective of the injury level (Fig. 4.3C left). In younger OBs, the calcium response parameters, amplitude, and AUC of secondary responders were significantly higher when the injury of the primary cell was severe. In contrast, in older OBs, no differences were observed in secondary calcium responses generated by mildly and severely injured primary cells. Moreover, secondary responses to severely injured young OBs were significantly higher in amplitude and AUC than secondary responses to severely injured old OBs (Fig. 4.3C *middle* and *right*). These data suggest that either the signal propagation from the primary to the neighboring cells or the general cell responsiveness to the propagating signals decreases with age in OBs.

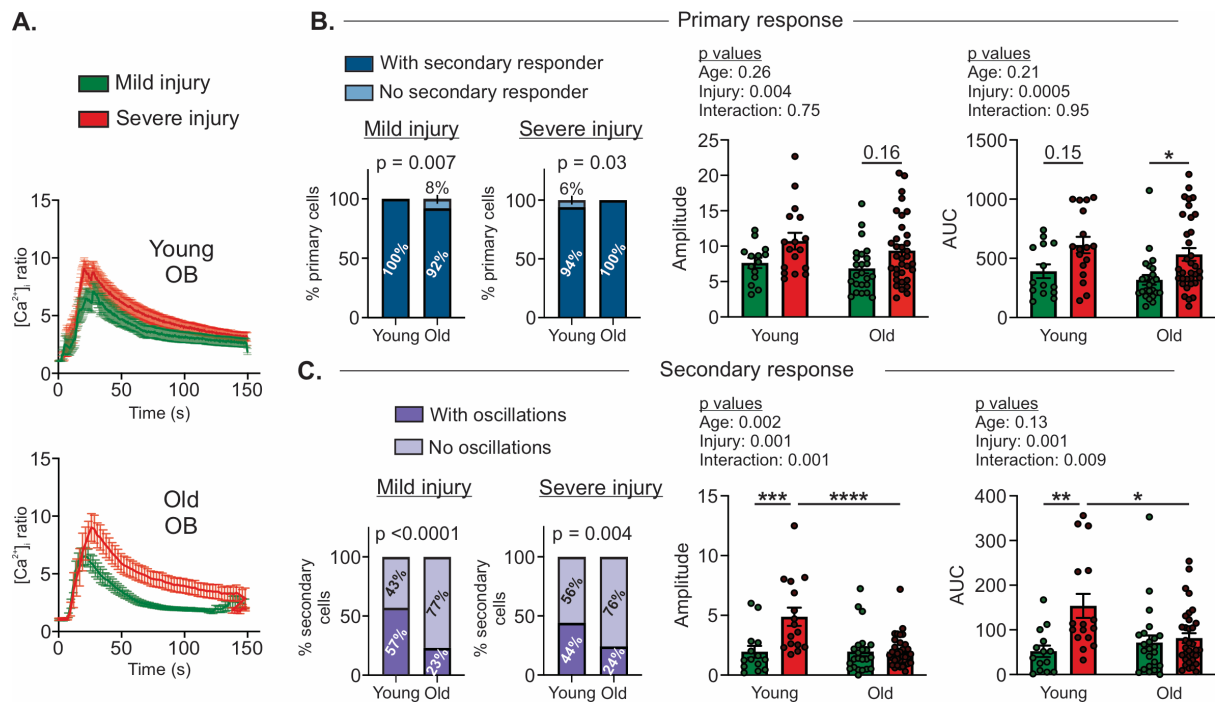


Figure 4.3: Calcium signaling parameters in young and old OBs exhibiting mild and severe membrane injury. Young and old primary OBs were divided by the level of injury, mild (green) and severe (red), and examined for changes in calcium signals with age and injury. A. Average calcium transients of primary responders of young (top) and old (bottom) OBs undergoing mild or severe injury. B. Calcium signaling in primary young and old OBs undergoing mild or severe injury. Percentage of primary responders that induce secondary responders (left, stacked bar graphs), amplitude (middle), and area under curve (AUC) (right). C. Calcium signaling in secondary young and old OBs. Percentage of secondary oscillators (left, stacked bar graph), amplitude (middle), and AUC (right). Data are mean $\pm$ SEM, n (young primary OBs) = 31, n (old primary OBs) = 58. Significance by 2-way ANOVA followed by Bonferroni correction post-hoc, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ or by Fisher's test for categorical data (B and C, left).

4.4.4 Calcium signaling parameters in young and old OCs exhibiting mild and severe membrane injury

We next carried out similar experiments in young and old OCs to determine the effect of membrane injury and age on calcium signaling. Similar to OBs, young and old OCs also exhibited increased calcium transients with increase in the injury severity (Fig. 4.4A). Primary young and old OCs that experienced mild injury, were similar in their ability to convey the signal to secondary responders. However, when the primary OCs experienced severe injury, only 38% of young OCs recruited secondary responders, while 80% of

older OCs were able to propagate a signal to the secondary responders ($p < 0.0001$) (Fig. 4.4B left). In primary young and old OCs, the amplitude and AUC of calcium transients significantly depended on the injury levels, however, no age-related differences were observed (Fig. 4.4B middle, right). Among the secondary responders, older OCs had more oscillators than younger OCs in the case of mild injuries ($p = 0.006$), and all secondary OCs displayed oscillations when the injury was severe (Fig. 4.4C left). No age or injury-related differences were observed in the calcium transients among secondary responders (Fig. 4.4C middle, right). Thus, with age, severely injured older OCs can signal better and recruit more neighboring cells than young OCs.

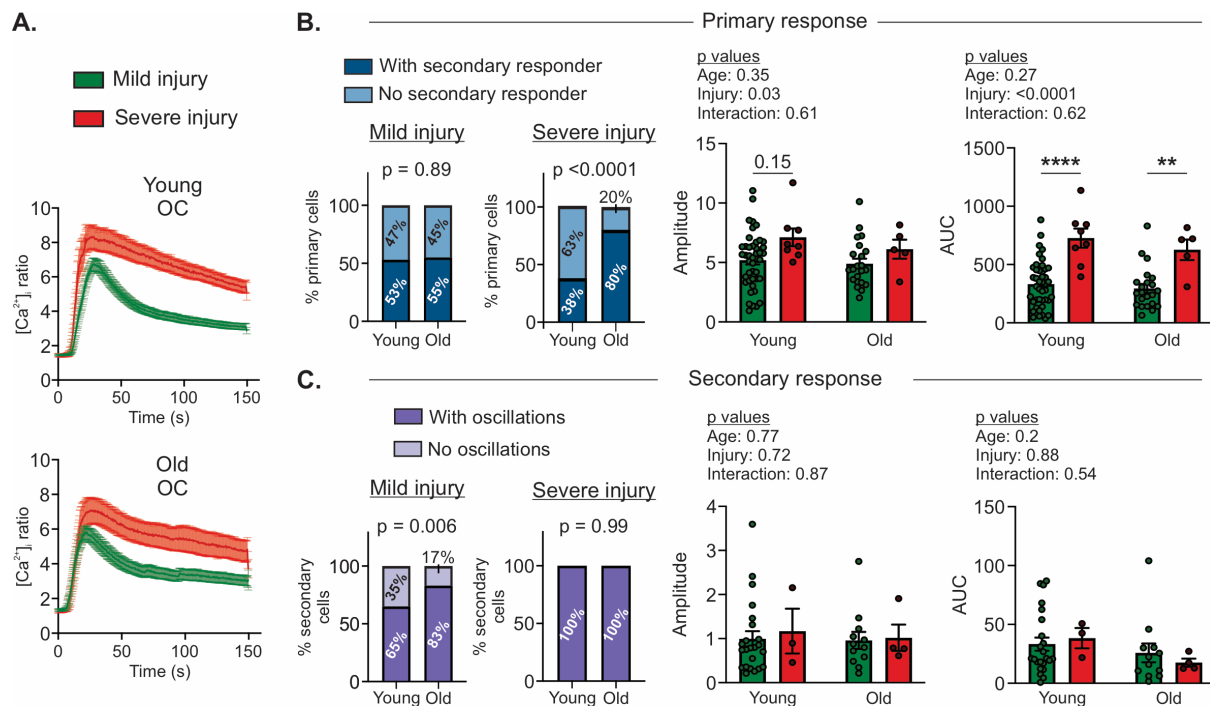


Figure 4.4: Calcium signaling parameters in young and old OCs exhibiting mild and severe membrane injury. Young and old primary OCs were divided by the level of injury, mild (green) and severe (red), and examined for changes in calcium signals with age and injury. A. Average calcium transients of primary responders of young (top) and old (bottom) OCs undergoing mild or severe injury. B. Calcium signaling in primary young and old OCs undergoing mild or severe injury. Percentage of primary responders that induce secondary responders (left, stacked bar graphs), amplitude (middle), and area under curve (AUC) (right). C. Calcium signaling in secondary young and old OCs. Percentage of secondary oscillators (left, stacked bar graph), amplitude (middle), and area under the curve (AUC). Data are mean $\pm$ SEM, n (young primary OCs) = 51, n (old primary OCs) = 27. Significance by 2-way ANOVA followed by Bonferroni correction post-hoc, ** $p < 0.01$, **** $p < 0.0001$ and by Fisher's test for categorical data (B and C, left).

4.4.5 Comparison of mechanically-induced calcium signals in aging OBs and OCs

We previously showed that OBs are more mechanosensitive and mechanotransductive than OCs (Dsouza and Komarova 2024). To determine whether these differences remain with age, we compared the $[Ca^{2+}]_i$ signals in mechanically stimulated OBs and OCs from older mice. Older primary OBs were able to generate significantly more secondary responders as compared to older primary OCs (Fig 4.5A left, $p < 0.0001$). The amplitude and AUC of primary responders in OBs were significantly higher than in OCs (Fig 4.5A). Secondary OCs showed significantly more oscillations than secondary OBs (Fig 4.5B left, $p < 0.0001$) but had significantly reduced amplitude and AUC of calcium responses than OBs (Fig 4.5B). Thus, aging OBs remain more mechanosensitive and mechanotransductive than OCs, however, the prevalence of oscillatory secondary responses is much higher in OCs than in OBs.

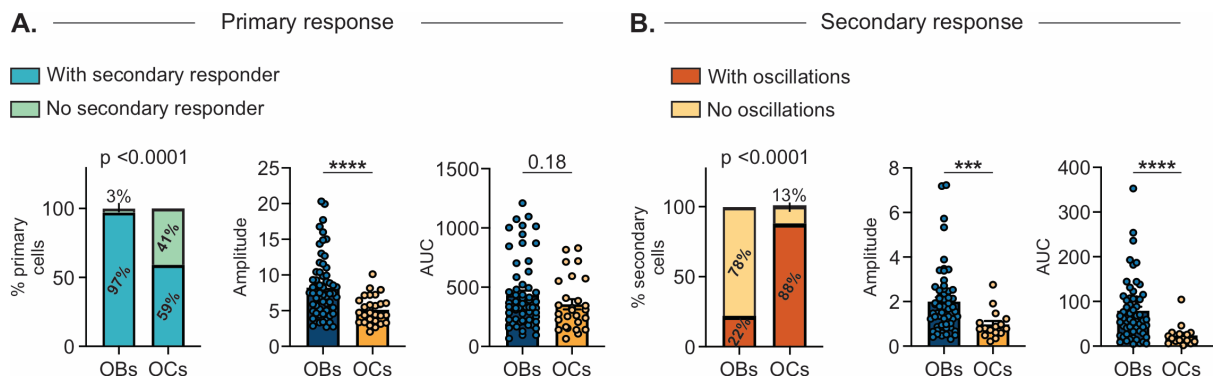


Figure 4.5: Comparison of in mechanically-induced calcium signaling in aging OBs and OCs. . A. Calcium signaling parameters in primary responders: percentage of primary cells with secondary responders (left, stacked bar graphs), amplitude (middle), and area under curve (AUC) (right). B. Calcium parameters in secondary responders: percentage of secondary responders displaying oscillations (left, stacked bar graphs), amplitude (middle), and area under the curve (right). Data are mean $\pm$ SEM, n (old OBs) = 60, n (old OCs) = 27. Significance by Student t-test with Welch correction, *** $p < 0.001$, **** $p < 0.0001$, and by Fisher's test for categorical data (A and B stacked bar graphs).

4.4.6 Contribution of sex to age-associated changes in mechanically-induced calcium signaling in OBs and OCs

We next assessed whether sex contributes to the age-related differences in mechanically-induced calcium signaling. The same data as in Figures 4.3 and 4.4 was analyzed by combining mechanically stimulated cells by age and sex while ignoring the injury level. OBs were able to generate a similar percentage of secondary responders independent of their age and sex (Fig. 4.6A *left*). Primary OBs from female mice displayed higher amplitude (2-way ANOVA, $p = 0.04$) and similar AUC of calcium responses compared to males (Fig. 4.6A *middle, right*). For the secondary responders, OBs from younger males had a significantly higher percentage of cells with oscillations compared to other groups (Fig. 4.6B *left*). No sex-related differences were observed among the secondary responders, but the amplitude was significantly reduced with age in both sex groups (Fig. 4.6B *middle*). For OCs, mechanically stimulated primary OCs from younger male mice showed a lower number of secondary responders ($p = 0.007$) (Fig. 4.7A *left*). No sex or age differences were observed in the amplitude or AUC among primary OC responders (Fig. 4.7A *middle, right*). For the secondary OC responders, no age or sex differences were observed among the percentages of oscillators and response amplitude and AUC (Fig 4.7B *left, middle*). Interestingly, OCs from females of both age groups tended to have higher secondary AUC than those from males (Fig 4.7B *right*, $p = 0.11$). Thus, sex-dependent differences were observed in primary responses for both OBs and OCs and in secondary OB responses.

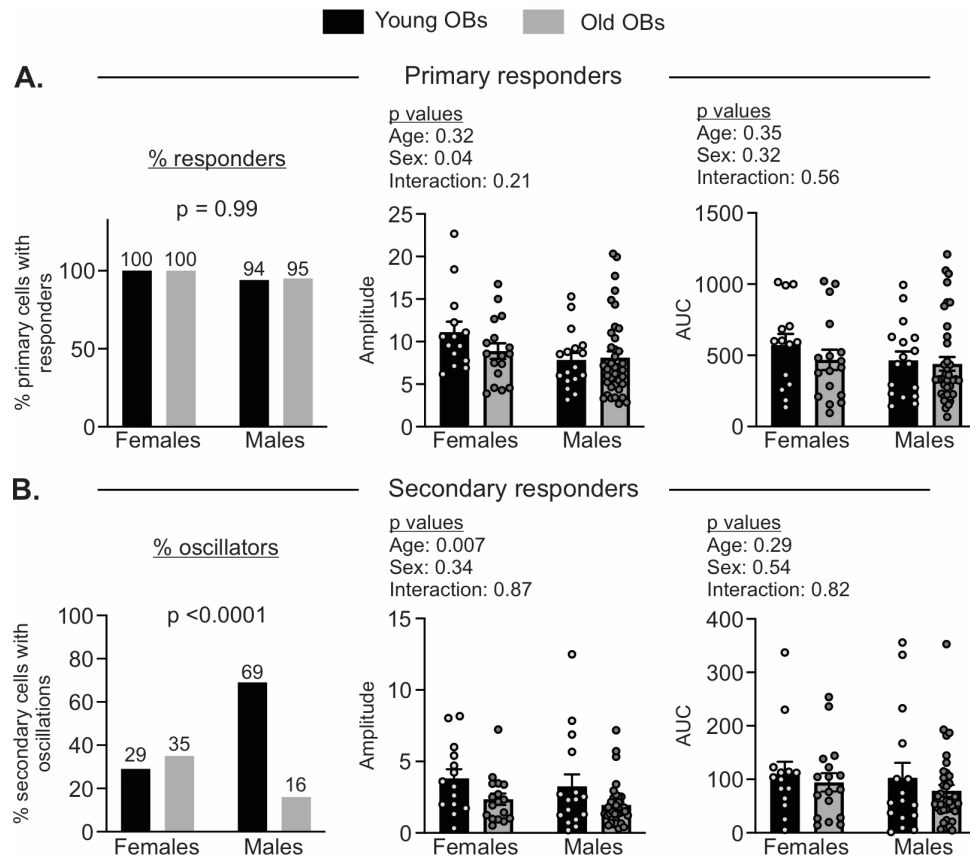


Figure 4.6: Sex and age-dependent changes in calcium signaling in mechanically stimulated OBs. A. Calcium signaling in primary OBs from young and old, female and male mice: percentage of primary cells with secondary responders (*left*), amplitude (*middle*), and area under curve (*right*). B. Calcium signaling in secondary OBs from young and old, female and male mice: percentage of secondary responders with oscillations (*left*), amplitude (*middle*), and area under curve (*right*). Data are mean $\pm$ SEM, n (young female OBs) = 14, n (old female OBs) = 17, n (young male OBs) = 17, n (old male OBs) = 39. Significance by 2-way ANOVA followed by Bonferroni correction post-hoc, and by Fisher's test for categorical data (A and B, *left*).

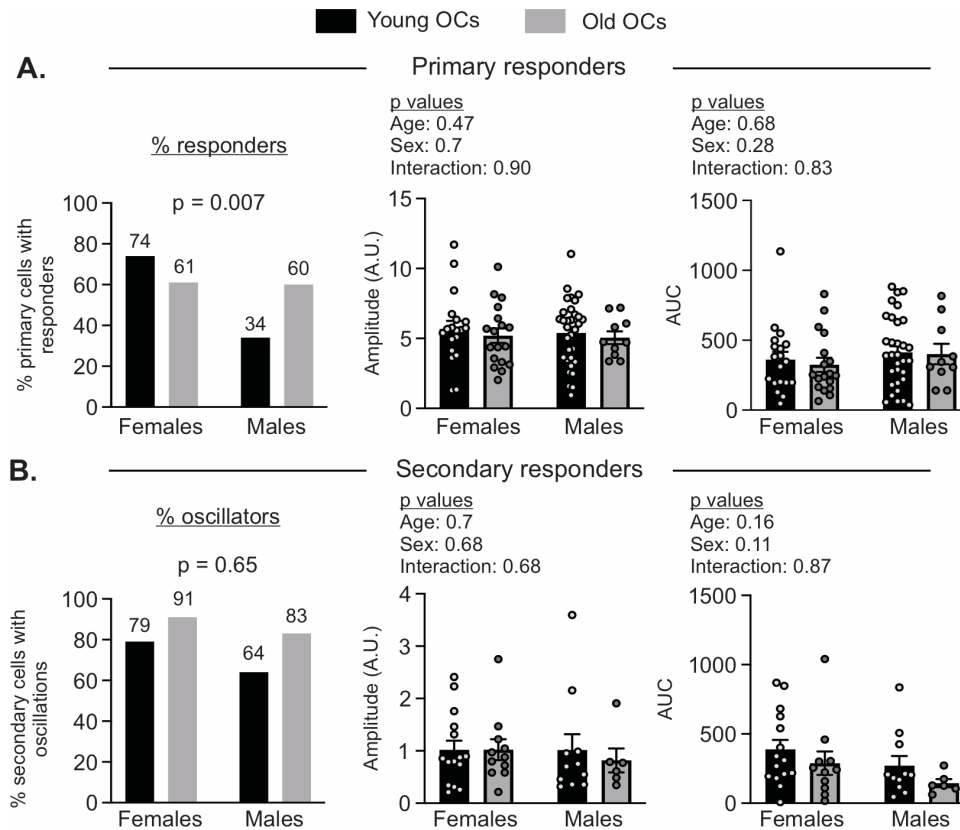


Figure 4.7: Sex and age-dependent changes in calcium signaling in mechanically stimulated OCs. A. Calcium signaling in primary OCs from young and old, female and male mice: percentage of primary cells with secondary responders (*left*), amplitude (*middle*), and area under curve (*right*). B. Calcium signaling in secondary OCs from young and old, female and male mice: percentage of secondary responders with oscillations (*left*), amplitude (*middle*), and area under the curve (*right*). Data are mean $\pm$ SEM, n (young female OCs) = 19, n (old female OCs) = 18, n (young male OCs) = 32, n (old male OCs) = 10. Significance by 2-way ANOVA followed by Bonferroni correction post-hoc or by Fisher's test for categorical data (A and B, *left*).

4.5 Discussion and Conclusion

In this study, we aimed to examine age-related changes in mechanosensitivity and mechanotransduction in OB and OC cultures. We show that injury levels and calcium signals in the mechanically stimulated cells were similar in OBs and OCs from young and old animals, suggesting that at the cellular level, mechanosensitivity is not affected by aging. In contrast, the propagation of mechanically-induced signal to the secondary cells was notably and differently affected by the aging of OBs and OCs. Specifically, mechan-

ically stimulated OBs from older animals demonstrated decreased signal propagation with age, while older OCs showed increased ability to signal to neighboring cells. While the expression of P2 receptors known for their involvement in mechanotransduction, P2X₇, P2Y₂, and P2Y₁₃ was stable in aging OBs, in OCs we observed downregulation of P2X₇ and P2Y₁₃ with age. Moreover, OBs and OCs from male animals were less mechanoresponsive, which for the first time, demonstrated a significant contribution of sex to cellular-level mechanical signaling. Taken together, our study demonstrates a significant dysregulation of mechanotransduction in aging OBs and OCs with a differential shift in age-related mechanically-induced signaling: a decrease in OBs and an increase in OCs.

Osteocytes and OBs are well-known mechanosensors and mechanotransducers in the bone (Romanello, Codognotto, et al. 2005; Genetos, Kephart, et al. 2007; N. Wang, Rumney, et al. 2013; Kringelbach, Aslan, Novak, Ellegaard, et al. 2015). Recently we showed that mechanically stimulated OCs are capable of sensing and transducing signals to neighboring cells proportionally to the level of injury (Dsouza and Komarova 2024). Several studies reported that osteocytes lose mechanosensitive and mechanotransductive properties with age (Hagan, K. Yu, et al. 2020; Morrell et al. 2020), in addition to a reduction in their number and connectivity ((Tiede-Lewis et al. 2017). AE Morrell and colleagues conducted ex vivo cyclical loading studies on mouse tibiae from young (~20 weeks) and old (~44 weeks) mice with Fluo-8 AM and examined changes in osteocytes [Ca²⁺]_i signaling in situ. They found that with age fewer osteocytes responded to mechanical loading and that the responses to stimulus were delayed (Morrell et al. 2020). Similarly, Hagan and colleagues showed in vivo and in vitro that aging osteocytes

become resistant to loading-induced plasma membrane tears and have decreased calcium signals (Hagan, K. Yu, et al. 2020). In contrast, our study suggests that aging OBs and OCs do not exhibit changes in the level of injury or calcium signaling induced by mechanical stimuli. A potential explanation for this could be that osteocytes are known to be long-lived in bone (up to 25 years) (Florencio-Silva et al. 2015) and therefore can undergo preferential selection for injury-resistant cells under repeated loading conditions (Hagan, K. Yu, et al. 2020). In contrast, OBs and OCs have a higher turnover, several months for OBs and several weeks for OCs; and in our experiments, OBs and OCs were differentiated in vitro from the precursor cells, thus limiting the possibility for the adaptation process similar to that described in osteocytes. Nevertheless, significant differences in mechanotransduction between OBs and OCs from young and old suggest age-related changes in OB and OC precursors.

We demonstrate that aging OBs exhibit deficient mechanotransduction while OCs become better at propagating mechanical signals with age. For OBs, our findings are similar to those by Donahue and colleagues (Donahue et al. 2001), who showed that OBs from old rats (~52 weeks) had lower basal $[Ca^{2+}]_i$ and decreased responsiveness to fluid flow compared to younger OBs (~16 weeks). OCs are rarely considered as active participants in mechanoadaptation, however, our study identified significant and opposite age-related changes in OCs compared to OBs. Aging is known to result in an imbalance in bone-forming OB and bone-resorbing OC activity thus shifting the remodeling to more resorption and eventually resulting in increased porosity and bone loss (Willie, E. A. Zimmermann, et al. 2020). In vivo, it has been shown that aging mice have a reduced ability to adapt to mechanical loads, which correlated with a reduction

in OB activity, while OC activity was sustained across the age groups (Birkhold, Razi, et al. 2014; Holguin et al. 2014). While both young (Dsouza and Komarova 2024) and aging OBs are more mechanosensitive and mechanotransductive than OCs, our study demonstrates the age-related shifts in single cell mechanotransduction in both OBs and OCs that would support a more resorptive phenotype with age.

We identified age-related changes in the expression of P2 receptors previously implicated in mechanical signaling, P2X₇ and P2Y₁₃. Of interest, the expression of both receptors was downregulated in OCs but not in OBs. P2X₇, an ATP-sensitive receptor that requires higher ATP concentration (in mM range) (S. Xing et al. 2016), was significantly downregulated in aging osteoclasts. P2X₇ was previously implicated in OB differentiation (D. Sun et al. 2013), mineralization (Panupinthu et al. 2007; I. R. Orriss, Key, Brandao-Burch, et al. 2012), and survival (Adinolfi et al. 2012), and in OC maturation/fusion (Agrawal et al. 2010), and resorption (Gartland et al. 2003; N. Wang, Agrawal, et al. 2018). Importantly, P2X₇ inhibitors abolished OC mechanotransduction in vitro (Jørgensen et al. 2002; Dsouza and Komarova 2024). P2Y₁₃, an ADP-sensitive receptor (Jørgensen et al. 2002; Dsouza and Komarova 2024), demonstrated a trend of decreased expression in old OCs. P2Y₁₃ was implicated in OB differentiation (N. Wang, Robaye, Agrawal, et al. 2012; Biver et al. 2013) and OC formation (N. Wang, Robaye, Agrawal, et al. 2012; N. Wang, Rumney, et al. 2013). P2Y₁₃ global knockout resulted in reduced bone volume and reduced number of osteoclast and osteoblast in mice. Additionally, P2Y₁₃ knockout mice were protected from age-related bone loss (N. Wang, Robaye, Agrawal, et al. 2012). Our previous study implicated ADP in osteoclast mechanotransduction (Dsouza and Komarova 2024), which is now corroborated by our

findings that changes in ADP receptor P2Y₁₃ are associated with the changes in osteoclast mechanotransduction. The signaling by diffusing ATP and ADP through the 15 receptors that can potentially be stimulated by these ligands is complex (Mikolajewicz, Sehayek, et al. 2019). We have proposed a conceptual model for the coordinated action of multiple receptors in transducing the spatiotemporal information regarding the mechanical injury to the neighboring cells (Dsouza, Moussa, et al. 2022). This study provides the first findings regarding the age-related changes in the mechanotransduction signaling by this system of receptors and ligands.

Taken together, our study demonstrates that aging is associated with changes in mechanotransduction by both OBs and OCs, which occurs in a manner that would be supportive for bone loss. Of note, we have also identified a novel contribution of sex to the single-cell mechanosensitivity and mechanotransduction in OBs and OCs. Sex is an important contributor to osteoporosis, with females being more susceptible to osteoporosis induced by the removal of sex hormones (such as postmenopausal osteoporosis) and to age-related osteoporosis (Manolagas 2010). While numerous studies examined the regulation of OB and OC differentiation and function by sex hormones (Khosla et al. 2012), we demonstrate a wider role of sex in influencing bone cell responses to mechanical forces. A better understanding of the mechanisms of age-associated loss of cellular mechanoadaptation will pave the way to developing better exercise and pharmacological treatments for aging-associated bone loss.

4.6 Data Availability

Data will be made available upon reasonable request.

4.7 Acknowledgements

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4.8 Grants

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4.9 Disclosures

The authors have nothing to disclose.

4.10 Author Contributions

C.D. and S.V.K. conceived and designed research; C.D. performed experiments; C.D. analyzed data; C.D. and S.V.K. interpreted results of experiments; C.D. prepared figures; C.D. and S.V.K. drafted the manuscript; C.D. and S.V.K. edited and revised the manuscript; C.D. and S.V.K. approved the final version of the manuscript.

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Bridging Statement

In Chapter 4, I show that the intracellular calcium signals released by the mechanical stimulus are altered in aging osteoblasts and osteoclasts. Osteoblasts have reduced signal propagation, whereas osteoclasts have an increased signaling ability with age. I also highlight sex-related differences in calcium signaling in aging osteoblasts and osteoclasts. In addition to these findings, I also found that the expression of the P2X₇ and P2Y₁₃ receptors was downregulated with age in osteoclasts. The studies in Chapter 3 show that ADP-mediated signaling is essential in osteoclasts in cases of altered loading, while Chapter 4 highlights the importance of ADP-mediated P2Y₁₃ signaling in aging. Taking these findings together, P2Y₁₃ may be a target for age-related bone loss. To summarize ADP-mediated P2Y₁₃ signaling, I conducted a meta-analysis of the literature on the P2Y₁₃ receptor in this chapter. My primary objective was to assess the potency of different ligands that act on the P2Y₁₃ receptor. My secondary objective was to distinguish whether P2Y₁₃ responses differ in different tissues and species.

Chapter 5

Characterization of Potency of the P2Y₁₃ Receptor Agonists: A Meta-Analysis

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5.1 Abstract

P2Y₁₃ is an ADP-stimulated G-protein coupled receptor implicated in many physiological processes, including neurotransmission, metabolism, pain, and bone homeostasis. Quantitative understanding of P2Y₁₃ activation dynamics is important for translational studies. We systematically identified PubMed annotated studies that characterized concentration-dependence of P2Y₁₃ responses to natural and synthetic agonists. Since the comparison of the efficacy (maximum response) is difficult for studies performed in different systems, we normalized the data and conducted a meta-analysis of EC₅₀ (concentration at half-maximum response) and Hill coefficient (slope) of P2Y₁₃-mediated responses to different agonists. For signaling events induced by heterologously expressed P2Y₁₃, EC₅₀ of ADP-like agonists was 17.2 nM (95% CI: 7.7–38.5), with Hills coefficient of 4.4 (95% CI: 3.3–5.4), while ATP-like agonists had EC₅₀ of 0.45 μ M (95% CI: 0.06–3.15). For functional responses of endogenously expressed P2Y₁₃, EC₅₀ of ADP-like agonists was 1.76 μ M (95% CI: 0.3–10.06). The EC₅₀ of ADP-like agonists was lower for the brain P2Y₁₃ than the blood P2Y₁₃. ADP-like agonists were also more potent for human P2Y₁₃ compared to rodent P2Y₁₃. Thus, P2Y₁₃ appears to be the most ADP-sensitive receptor characterized to date. The detailed understanding of tissue- and species-related differences in the P2Y₁₃ response to ADP will improve the selectivity and specificity of future pharmacological compounds.

5.2 Introduction

Purinergic signaling has gained importance as a therapeutic target since its introduction in the 1970s [1]. The family of purinergic receptors consists of two main groups,

P1 receptors that include four subtypes and P2 receptors that comprise of seven P2X ligand-gated ion channels and eight P2Y G protein-coupled receptors (GPCRs). P1 receptors respond to adenosine, whereas P2 receptors are activated by adenine and uridine nucleotides, including ATP, ADP, and UTP, and UDP [2]. Purinergic signaling is relevant to the functioning of almost all body systems [3]. ADP-mediated signaling is relatively less studied compared to ATP-mediated signaling; however, it is well known for its importance for thrombosis and hemostasis. Among three P2Y receptors that are selective for ADP- P2Y₁, P2Y₁₂, and P2Y₁₃ [4], P2Y₁ and P2Y₁₂ have been studied for their roles in platelet aggregation. P2Y₁ was shown to play an important role in the initial stages of platelet aggregation, while activation of P2Y₁₂ was demonstrated to play a critical role in regulating overall thrombosis [5,6]. The significance of P2Y₁₂ is highlighted by several successful drugs targeting the receptor, namely clopidogrel, prasugrel, ticagrelor, and cangrelor, which are clinically used to treat cardiovascular diseases [7,8,9]. However, some studies suggested that, in addition to P2Y₁₂, these drugs may also target the P2Y₁₃ receptor [10,11]. Although literature on the P2Y₁₃ receptor implicate it in various diseases [12], detailed characterization of this receptor in different tissues remains limited.

The P2Y₁₃ receptor was discovered in 2001 [13]. It is mainly found in the brain, spleen, heart, bone marrow cells, liver, and pancreas [14]. It is structurally similar to P2Y₁₂ and is primarily an ADP receptor [13,15]. Signaling through P2Y₁₃ involves coupling to G_i proteins to inhibit adenylyl cyclase [16]. Multiple functional outcomes of P2Y₁₃ activation have been described, such as cell proliferation, survival, and high-density lipoprotein endocytosis, as well as an important role in neuromuscular transmission,

neuroprotection, and neuronal differentiation [10,17,18,19]. In mice, P2Y₁₃ has been implicated in bone remodeling, where knockout of the receptor in mice resulted in reduced bone turnover [20]. To quantitatively summarize the existing literature on the P2Y₁₃ receptor, we conducted a systematic search of PubMed-indexed studies reporting a concentration-dependence of P2Y₁₃-mediated responses. Ideally, the receptor response to an agonist can be characterized by the efficacy (maximal response), affinity (the concentration at half-maximal response), and steepness (the Hill coefficient, the slope of the response). Experimentally, in each studied system, the concentration-dependence allows us to identify a system-specific maximum, EC₅₀, and the steepness. Since efficacy and affinity often correlate, it is difficult to dissociate them based on the EC₅₀. We assumed that, while the system-specific maximal responses are not directly comparable, the heterologously-expressed receptors demonstrate maximal efficacy, and therefore, EC₅₀ obtained in those systems is indicative of the agonist affinity. Therefore, our primary objective was to assess the potency (EC₅₀) of existing agonists to induce P2Y₁₃-mediated responses. Our secondary objective was to examine if P2Y₁₃ responses vary in different tissues and species.

5.3 Results

5.3.1 Summary of Studies

Since we were interested in full-length peer-reviewed quantitative studies focused on the P2Y₁₃ receptor, we used it as one of the inclusion criteria that the publication journal indexed in PubMed. The PubMed search performed on 14 September 2020 identified 153 articles focused on the P2Y₁₃ receptor. Upon screening by two co-authors, we selected 50

articles that focused on the P2Y₁₃ receptor for qualitative synthesis (Figure 5.1a). The P2Y₁₃ receptor was studied in various physiological systems (Figure 5.1b), with most of the studies conducted in the nervous (20 articles) and digestive (9 articles) systems, then the musculoskeletal and circulatory systems (6 and 3 articles, respectively). In total, 10 studies employed heterologous expression systems of the P2Y₁₃ receptor. Most studies focused on the P2Y₁₃ receptor of murine (22 articles), human, or rat origin (14 studies each). The full-text screening identified 19 articles that examined the concentration-dependence of P2Y₁₃-mediated responses. The concentration-dependences from one of the studies were in the linear range and therefore did not result in a good fit for the Hill curve, making it impossible to estimate the potency. Therefore, the meta-analysis was performed on 18 studies that mainly included the studies of the human, mouse, and rat P2Y₁₃ receptor in immune, nervous, musculoskeletal, and circulatory physiological systems (Table 5.1). The signaling and functional responses to stimulation of P2Y₁₃ using ADP, 2MeSADP, ADP β S, ATP, and 2MeSATP were examined in these studies (Table 5.1).

Table 5.1: Overview of selected papers for systematic review and meta-analysis. The search strategy is included in Table A1. Each paper was assigned a quality score based on a developed questionnaire (Table A2), where 14 points indicate a high-quality paper. The study names marked in “red” were conducted in a model system with heterologous P2Y₁₃ expression. ADP β S: adenosine 5'-O-(2-thiodiphosphate); CREB: cAMP-responsive element binding protein; 2MeSADP: 2-methylthio- adenosine diphosphate; MEPP: miniature end-plate potentials; ERK: extracellular signal-regulated kinase; IP₃: inositol trisphosphate; [³⁵S]GTP γ S: [³⁵S]guanosine-5'-O-(3-thiotriphosphate); 2MeSATP: 2-methylthio-adenosine triphosphate.

Author, Year	Physiological System	Species	Agonist	Outcome(s)	P2Y ₁₃ expression	Quality score
Zhang et al., 2019	Immune	Mouse	ADP	Viral RNA replication, Cell viability	Endogenous	13

Quintas et al., 2018	Nervous	Rat	ADP β S	Cell proliferation	Endogenous	12
Jacques et al., 2017	Sensory	Chick embryo	ADP	p-CREB	Endogenous	13
Guarracino et al., 2016	Musculo- skeletal	Mouse	2MeSADP, ADP, ATP	MEPP frequency	Endogenous	14
Björquist et al., 2016	Model system	Human	2MeSADP	Dynamic Mass Redistribution	Heterologous	13
Ortega et al., 2011	Nervous	Rat	2MeSADP	p-ERK	Endogenous	12
Gao et al., 2010	Immune	Rat	ADP	Hex release	Endogenous	14
Espada et al., 2010	Nervous Model system	Mouse	ADP 2MeSADP	Hmox1 induction	Endogenous Heterologous	14

Wang et al., 2005	Circulatory	Human	2MeSADP	ATP release, cAMP accumulation	Endogenous	14
Kim et al., 2005	Model system	Human	ADP	IP ₃ accumulation	Heterologous	12
Jacquet et al., 2005	Model system	Human	ADP	cAMP accumulation	Heterologous	14
Carrasquero et al., 2005	Nervous	Rat	2MeSADP, ADP	cAMP accumulation	Endogenous	14
Wirkner et al., 2004	Renal	Human	ADP, ADP β S, ATP	Inhibition of N-type calcium channels	Endogenous	12
Fumagalli et al., 2004	Model system	Rat	2MeSADP, ADP	[³⁵ S]GTP γ S binding	Heterologous	14
Chhatiwala et al., 2004	Model system	Human	2MeSADP	IP ₃ accumulation	Heterologous	14

Marteau et al., 2003	Model system	Human	2MeSADP, ADP, ADP β S, ATP, 2MeSATP	[³⁵ S]GTP γ S binding, IP ₃ accumulation	Heterologous	14
Zhang et al., 2002	Model system	Human	2MeSADP, ADP, ADP β S, 2MeSATP	Intracellular calcium, cAMP accumulation	Heterologous	14
Communi et al., 2001	Model system	Human	2MeSADP, ADP, ADP β S, ATP, 2MeSATP	IP ₃ accumulation, cAMP accumulation	Heterologous	14

5.3.2 P2Y₁₃-Mediated Signaling Events

We first focused on the responses of heterologously-expressed P2Y₁₃. Immediate signaling events studied, following P2Y₁₃ activation, were Ca²⁺/IP₃ release, cAMP inhibition, and [³⁵S]GTP γ S binding. From nine articles, we analyzed 22 datasets [10,11,13,14,15,16,27,28,30], each reporting changes in one of the signaling outcomes in response to one agonist. If within the same article, the response to an agonist was reported multiple times, so we averaged these data. For each concentration-dependence, the individual experimental points were extracted (Figure 5.2a), then if the baseline data were available, it was

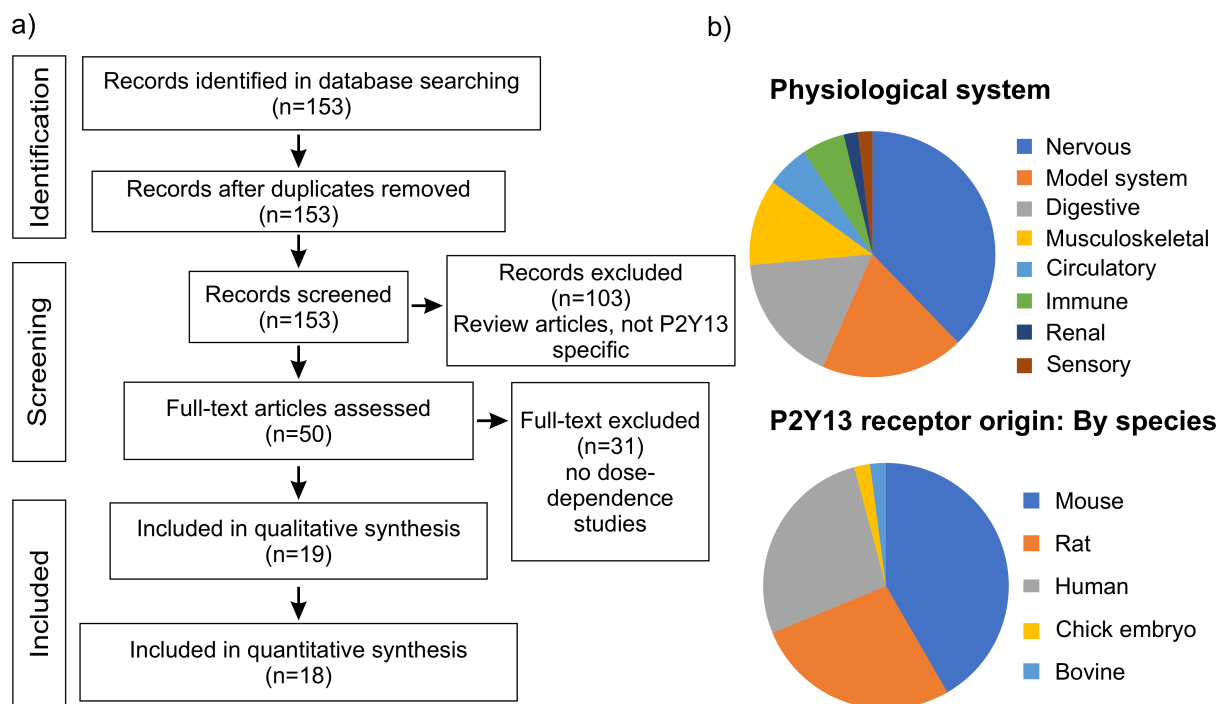


Figure 5.1: Screening and selection process. (a) Preferred reporting items for systematic reviews and meta-analyses (PRISMA), adapted. n indicates the number of studies (b) The pie chart indicates the distribution of P2Y₁₃ studies obtained at the full-text screening level (n = 50) in different species in terms of the receptor's origin and physiological systems. Studies performed in the heterologously-expressed P2Y₁₃ receptor are indicated as model systems

subtracted from the data and the maximal point was scaled as 100% (Figure 5.2b). The resulting data were fit with the Hills curve using the Monte-Carlo method [31], allowing us to estimate the main effect size of EC₅₀ values with the measure of its variance (SEM) (Figure 5.2c). When the dataset described P2Y₁₃-mediated inhibition, such as for cAMP, it was inversed before fitting with the Hills equation, and IC₅₀ ± SEM was identified. The forest plots represent the logEC₅₀/IC₅₀ ± SEM; however, in the text, we report the transformed values, EC₅₀/IC₅₀ ± SEM (Table A3). We analyzed the heterogeneity in the largest dataset of 12 concentration-dependencies for Ca²⁺/IP₃ response. The funnel plot analysis (Figure 5.2d) demonstrated that the study level effects were symmetrically distributed around the random effects estimate, but did not form a funnel, suggesting that the precision in these studies did not represent the more reliable estimate of the

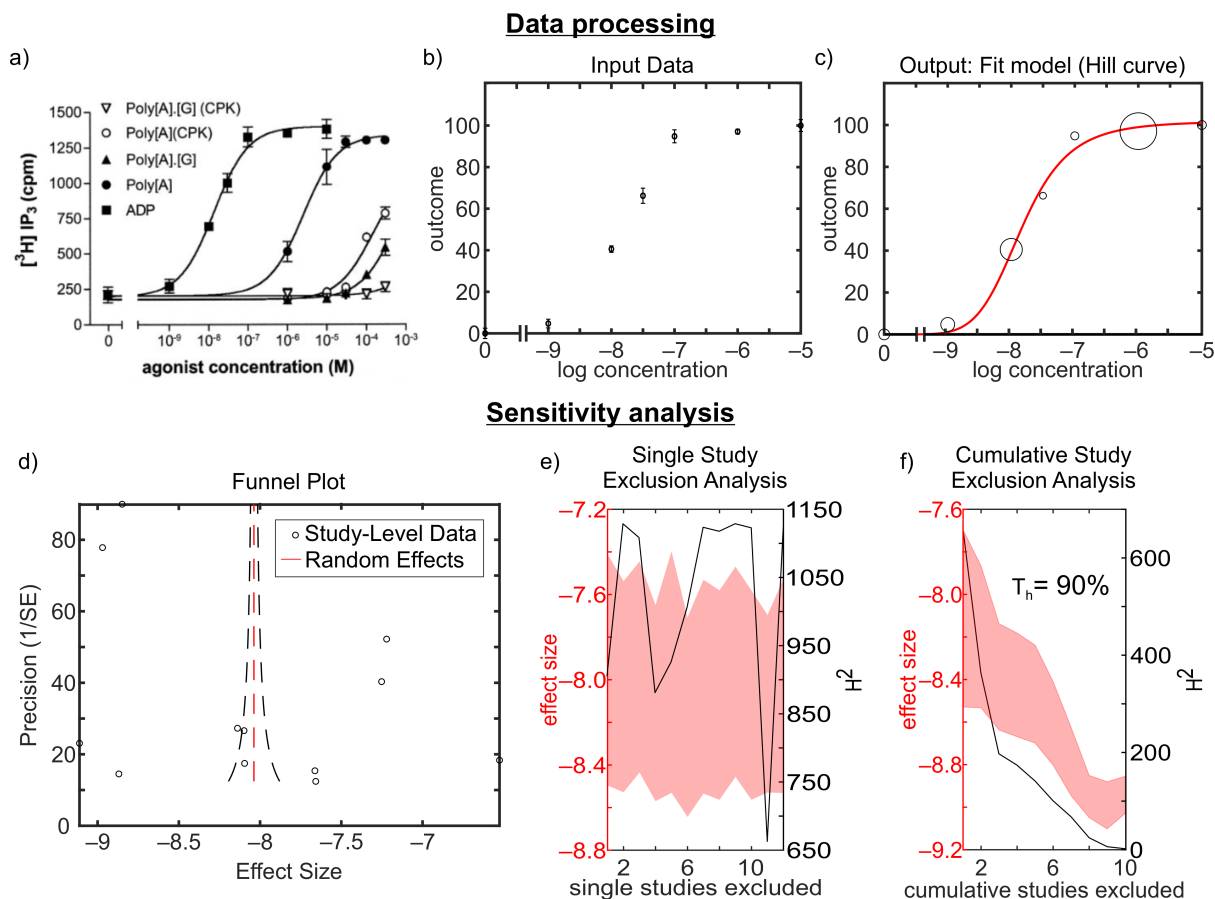


Figure 5.2: Data processing and sensitivity analysis in $\text{Ca}^{2+}/\text{IP}_3$ datasets. (a) Example of a concentration-dependence curve from a study (Marteau et al. 2003[#], reproduced with permission) that was used to extract P2Y_{13} -mediated responses. (b) Extracted dataset was used as an input to fit into the Hill function. (c) Final output of the dataset fit into the Hill curve after Monte-Carlo simulations. (d) Heterogeneity was analyzed in $\text{Ca}^{2+}/\text{IP}_3$ release dataset using a forest plot. Study-level ($\log_{10}$) effect sizes (black circles) were plotted against the precision (inverse standard error). The red dashed line represents the random effects estimate. (e) Single study and (f) cumulative study exclusion analysis was carried out to assess studies that contributed to heterogeneity. Red shaded region: 95% confidence interval; black line: H^2 statistic; T_h : homogeneity threshold [#] Reference: Marteau, F.; Le Poul, E.; Communi, D.; Communi, D.; Labouret, C.; Savi, P.; Boeynaems, J.M.; Gonzalez, N.S. Pharmacological characterization of the human P2Y_{13} receptor. *Mol. Pharmacol.* 2003, 64, 104–112, doi:10.1124/mol.64.1.104 [30].

effect size. Single study exclusion analysis (Figure 5.2e) identified three datasets that reduced heterogeneity; however, none of these studies affected the overall effect size. Cumulative study exclusion analysis (Figure 5.2f) demonstrated that 90% of studies needed to be excluded to obtain a homogenous dataset and that the effect size decreased (lower EC_{50}) with a decrease in heterogeneity.

Next, we compared the $\text{EC}_{50}/\text{IC}_{50}$ for P2Y_{13} -mediated responses to ADP and ADP-like

agonists for three signaling outcomes: $\text{Ca}^{2+}/\text{IP}_3$ release, cAMP inhibition, and $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (Figure 5.3, Table A3). $\text{Ca}^{2+}/\text{IP}_3$ release and cAMP inhibition required similar concentration of agonists with EC_{50} value of 9.2 nM (95% CI: 3.1–27.0) for $\text{Ca}^{2+}/\text{IP}_3$ release and IC_{50} of 12.3 nM (95% CI: 4.9–31.1) for cAMP inhibition. In contrast, EC_{50} for $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding was higher, at 136.88 nM (95% CI: 14.19–1320.78); however, the dataset for this parameter was relatively small. ADP and ADP-like agonists displayed distinct potencies in initiating different P2Y_{13} -mediated signaling events. 2MeSADP had a lower EC_{50} of 1.93 nM (95% CI: 1.18–3.16) than ADP (EC_{50} : 12.72, 95% CI: 3.0–54.5) in generating a $\text{Ca}^{2+}/\text{IP}_3$ response; however, for the cAMP inhibition, 2MeSADP IC_{50} was higher at 38.9 (95% CI: 29.1–52.0) compared to ADP, which had IC_{50} of 5.2 (95% CI: 1.1–25.8). In $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding, both 2MeSADP and ADP had wide confidence intervals, with the ADP and 2MeSADP subgroups being dominated by the Fumagalli et al. 2004 study [14]. Within studies that assessed both ADP and 2MeSADP-mediated responses within the same system, three studies reported higher potency for ADP compared to 2MeSADP [13,14,28], while two studies reported the reverse [15,30]. We also analyzed the Hills coefficient (steepness) of the agonist's concentration-dependent curves in the largest signaling dataset of $\text{Ca}^{2+}/\text{IP}_3$ release (Figure 5.4). The steepness was similar for all ADP and ADP-like agonists, with an overall steepness of $S = 4.4$ (95% CI: 3.3–5.4). Overall, when all signaling responses to all ADP-like agonists were combined, the EC_{50} value for P2Y_{13} -mediated signaling was 17.2 nM (95% CI: 7.69–38.48).

5.3.3 P2Y_{13} -Mediated Functional Events

Next, we examined functional responses reported to be mediated by P2Y_{13} (Figure 5.5). We analyzed 14 datasets from 9 articles [17,18,21,22,23,24,25,26,29] that described

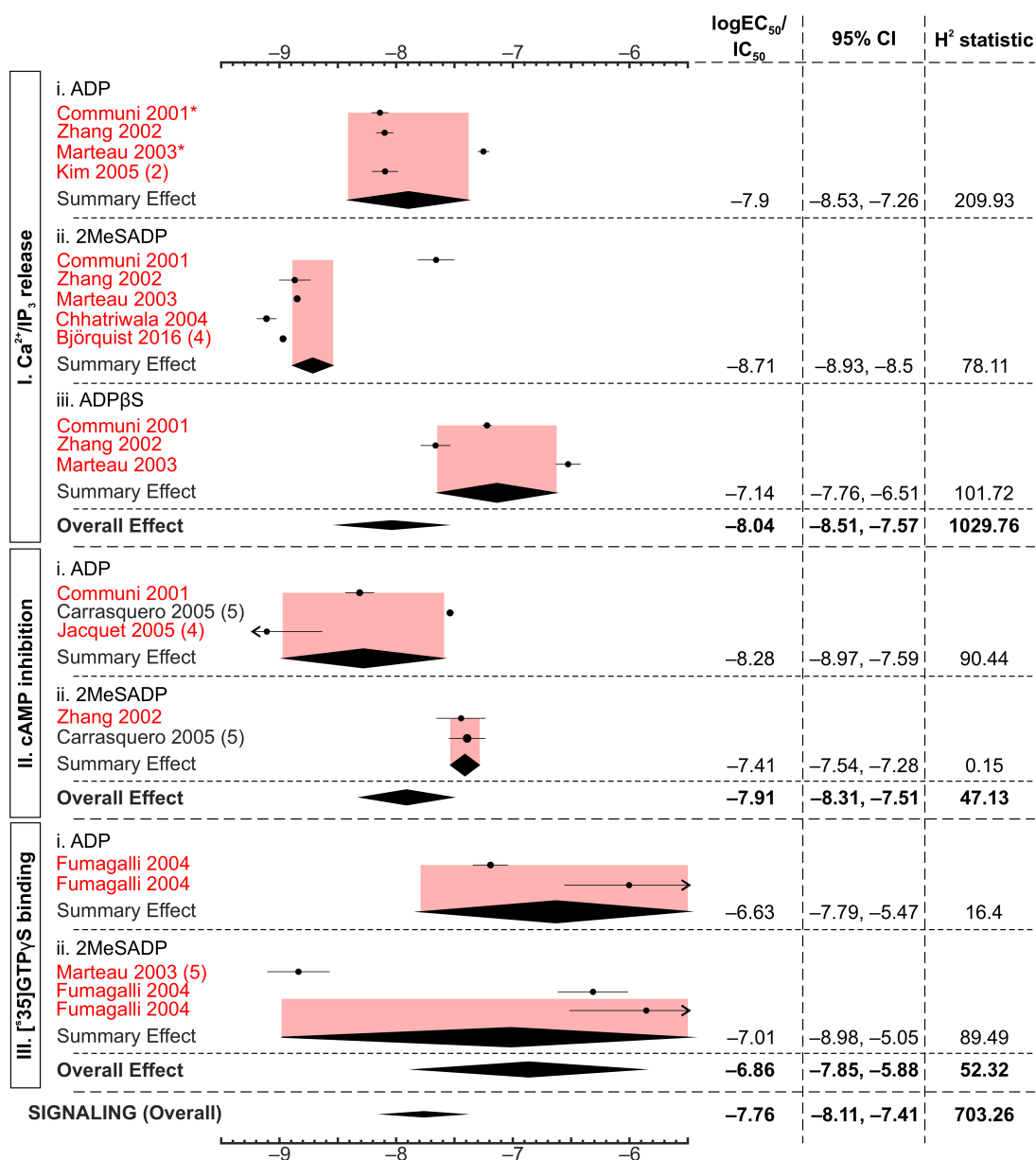


Figure 5.3: Signaling responses to ADP and ADP-like agonists in the P2Y₁₃ receptor. The forest plot represents the log₁₀ EC₅₀/IC₅₀ values of different ADP and ADP-like agonists to generate P2Y₁₃- mediated signaling outcomes. The black diamond represents the overall effect size, and the pink shaded region represents the 95% confidence interval. The majority of studies were conducted in heterologously-expressed P2Y₁₃ (marked in red), with the exception of the endogenously expressed P2Y₁₃ study in (Carrasquero et al. 2005). All the studies were of sample size, n = 3, unless otherwise stated in parentheses. The studies marked in * had multiple recordings of the same agonist and outcome in the individual study and were averaged, with (Communi et al. 2001), having 2 datasets and (Marteau et al. 2003) having 3 datasets.

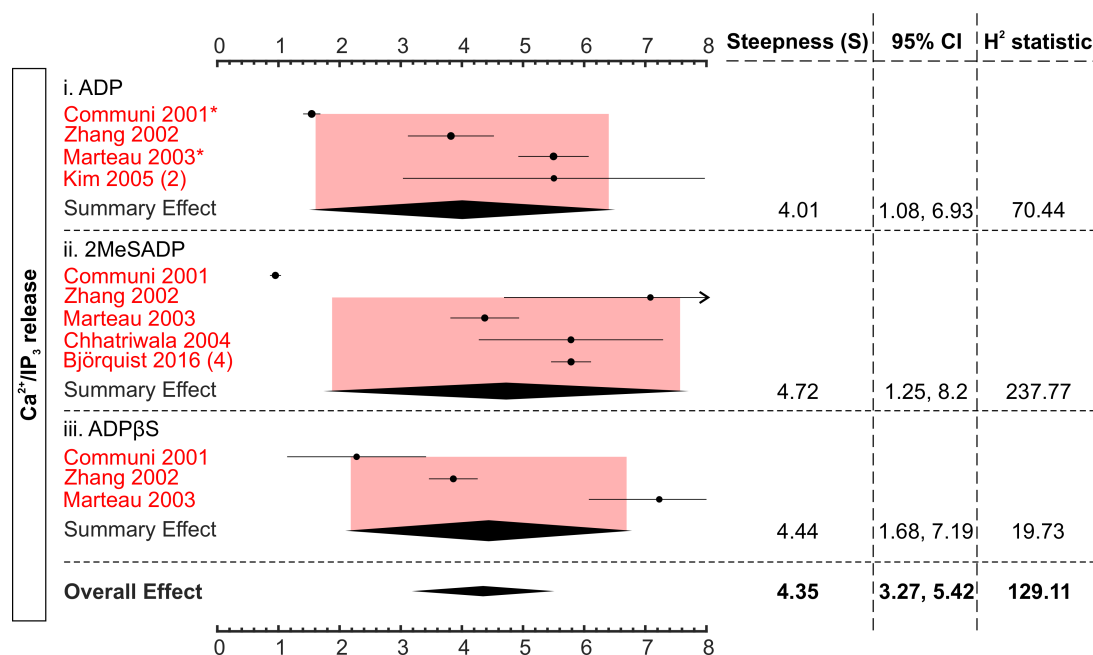


Figure 5.4: Steepness (S) in $\text{Ca}^{2+}/\text{IP}_3$ release dataset. The steepness of slope (S) was assessed in the largest signaling dataset, $\text{Ca}^{2+}/\text{IP}_3$ release. The black diamond represents the overall effect size, and the pink shaded region represents the 95% confidence interval. The study names marked in “red” were conducted in the heterologously-expressed P2Y_{13} receptor. All the studies were of sample size, $n = 3$, unless otherwise stated in parentheses. The studies marked in * had multiple datasets of the same agonist and outcome in the individual study and were averaged, with (Communi et al. 2001), having 2 datasets and (Marteau et al. 2003) having 3 datasets.

functional events consisting of intermediary cellular signaling, such as Hex release [25], calcium channel inhibition [29], phosphorylation of ERK 1/2 [24], and cellular proliferation [22]. In all but one dataset [17], the functional responses were examined in the systems with endogenously-expressed P2Y_{13} . The EC_{50} of ADP and ADP-like agonists-induced functional responses were many orders of magnitude higher than immediate signaling by heterologously-expressed P2Y_{13} . ADP and ADP-like agonist-mediated intermediary signaling and protein activity exhibited EC_{50} of $2.3 \mu\text{M}$ (95%CI: 0.1–143.4) and $0.1 \mu\text{M}$ (95% CI: 0.01–1.87), respectively, while cellular proliferation had an even higher EC_{50} of $46.7 \mu\text{M}$ (95% CI: 4.1–532.5). Comparison of different agonists was difficult because often only few datasets were available for each agonist. Nevertheless, for receptor-mediated intermediary signaling and protein phosphorylation,

2MeSADP demonstrated the lowest EC_{50} (Figure 5.5, Table A3), while ADP and ADP β S had relatively higher EC_{50} for each functional outcome (Figure 5.5, Table A3). Overall, when all functional responses to all ADP-like agonists were combined, the EC_{50} value for P2Y₁₃-mediated functional responses was 1.76 μ M (95% CI: 0.3–10.06).

5.3.4 ATP-Mediated P2Y₁₃ Signaling

We examined available data for ATP and 2MeSATP-induced P2Y₁₃-mediated responses (Figure 5.6). To be able to compare ATP-and ADP-mediated effects, we limited the dataset to studies that examined immediate signaling outcomes of heterologously-expressed P2Y₁₃ [15,30]. ATP and 2MeSATP had an overall EC_{50} of 0.45 μ M (95% CI: 0.06–3.15) to induce P2Y₁₃-mediated signaling, which was much weaker than ADP and ADP-like agonists that signal via P2Y₁₃ in the nanomolar range (Table A3).

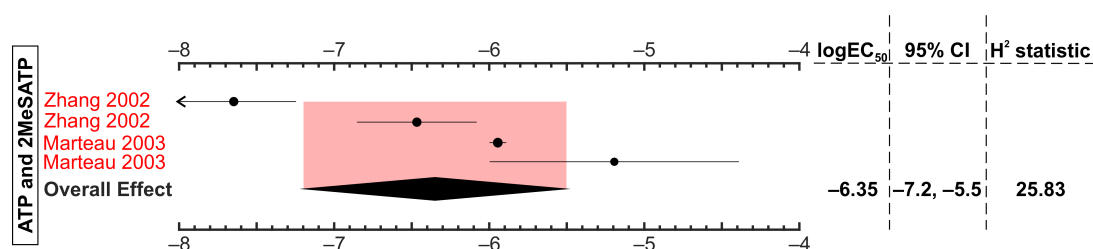


Figure 5.6: P2Y₁₃-mediated signaling outcomes with ATP and ATP-like agonists. The forest plot contains the $\log_{10} EC_{50}$ values of ATP and 2MeSATP in generating P2Y₁₃-mediated signaling outcomes. The black diamond represents the overall effect size, and the pink shaded region represents the 95% confidence interval. The study names marked in “red” were conducted in the heterologously- expressed P2Y₁₃ receptor. All the studies were of sample size, $n = 3$, unless otherwise stated in parentheses.

5.3.5 P2Y₁₃-Mediated Effects in Different Tissues

We next examined if the type of tissue studied affected the responses of endogenously-expressed P2Y₁₃ (Figure 5.7). In blood tissue, functional events were studied in 4 datasets across 3 articles [21,25,26] and the EC_{50} value was 17.9 μ M (95% CI: 0.8–426). In the brain, 5 datasets across 3 articles [17,22,24] examined functional responses and

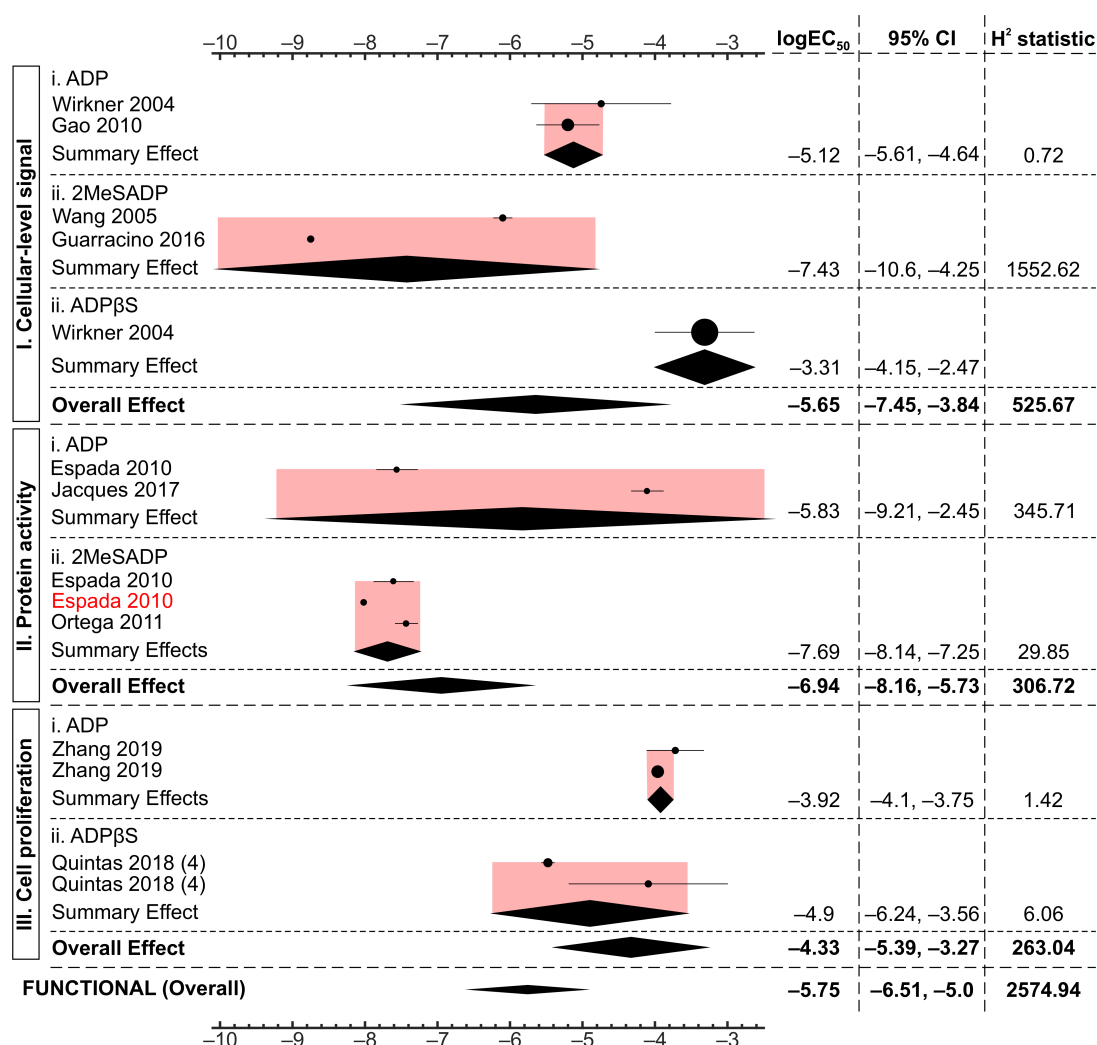


Figure 5.5: P2Y₁₃-mediated functional responses with ADP and ADP-like agonists. Forest plot of log₁₀ EC₅₀ values of different ADP and ADP-like agonists to generate P2Y₁₃-mediated functional outcomes. The black diamond represents the overall effect size, and the pink shaded region represents the 95% confidence interval. All studies were conducted in systems endogenously expressing the P2Y₁₃ receptor with the exception of the dataset marked in “red” from the (Espada et al. 2010) study, conducted in the heterologously-expressed P2Y₁₃ receptor. All the studies were of sample size, n = 3, unless otherwise stated in parentheses.

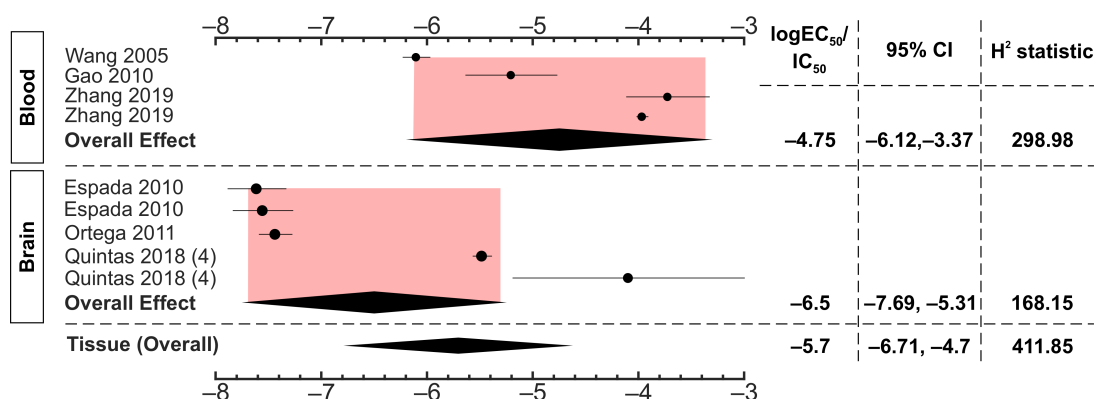


Figure 5.7: Subgroup analysis for endogenous P2Y₁₃ responses in tissues. The forest plot represents the log₁₀ EC₅₀ values in the blood and brain to generate a P2Y₁₃-mediated functional response. The black diamonds and the pink shaded region represent the overall effect size and the 95% confidence interval. All the studies were of sample size, n = 3, unless otherwise stated in parentheses.

the EC₅₀ value for ADP and ADP-like agonists was 0.3 μM (95%CI: 0.02–4.89). Thus, P2Y₁₃-mediated responses are noticeably different in different tissues.

5.3.6 Species Origin of the P2Y₁₃ Receptor

Finally, we assessed whether the potency of ADP and ADP-like agonists vary depending on the species origin of the P2Y₁₃ receptors (Figure 5.8). We analyzed 35 datasets divided into two major categories, humans with 19 datasets from 9 articles [10,11,13,15,16,26,27,29,30] and rodents that included 16 datasets from 8 articles on mouse and rat P2Y₁₃ [14,17,18,21,22,24,25,28]. In humans, signaling outcomes required nM levels of agonists with the order of potency of 2MeSADP > ADP > ADPβS, while in rodents, ADP and 2MeSADP had similar potency (Figure 5.8, Table A3). Overall, ADP and ADP-like agonists displayed a lower potency to induce signaling events in human P2Y₁₃, with EC₅₀ of 7.4 nM (95% CI: 2.9–18.8), compared to rodent P2Y₁₃, with EC₅₀ of 149.8 nM (95% CI: 64.3–348.8). In humans, functional responses could not be divided into agonist types due to a limited number of studies. In rodents, the EC₅₀ for P2Y₁₃-mediated functional responses was lower for 2MeSADP compared to ADP

and ADP β S (Figure 5.8, Table A3). Overall, the functional responses for rodent P2Y₁₃ displayed lower EC₅₀ than for human P2Y₁₃ (Figure 5.8, Table A3).

5.4 Discussion

We systematically identified 18 studies that assessed the concentration dependence of the P2Y₁₃ receptor and quantitatively summarized the EC₅₀ of P2Y₁₃ responses to different agonists. We found that ADP and ADP-like agonists are three orders of magnitude more potent than ATP and ATP-like agonists at the P2Y₁₃ receptor. 2MeSADP was more potent than ADP or ADP β S for immediate signaling events in pharmacological assays, in particular for human P2Y₁₃, but exhibited similar potency for physiological signaling events, such as cAMP inhibition. We found that much lower agonist concentrations are required to elicit immediate signaling events in heterologous expression systems, such as calcium and cAMP signaling, compared to functional outcomes in endogenous expression systems, such as protein phosphorylation and cell proliferation. Finally, our data suggest that ADP and ADP-like agonists have a lower EC₅₀ at the brain P2Y₁₃ compared to the blood P2Y₁₃ and an order of magnitude lower EC₅₀ at the human P2Y₁₃ compared to rodent P2Y₁₃. We provide the quantitative estimates for the potency of ADP, ATP, and their derivatives in stimulating P2Y₁₃-mediated responses. In heterologous systems, which we assumed provide a maximal efficacy for P2Y₁₃-mediated effects, our meta-analysis shows that 2MeSADP was more potent in a pharmacological assay that relies on activation of Ca²⁺/IP₃ release, while ADP was equivalent or more potent in cAMP inhibition, which is the physiological outcome of this Gi-coupled receptor [15]. Previous studies on the P2Y₁₃ receptor similarly concluded that, for P2Y₁₃, the difference

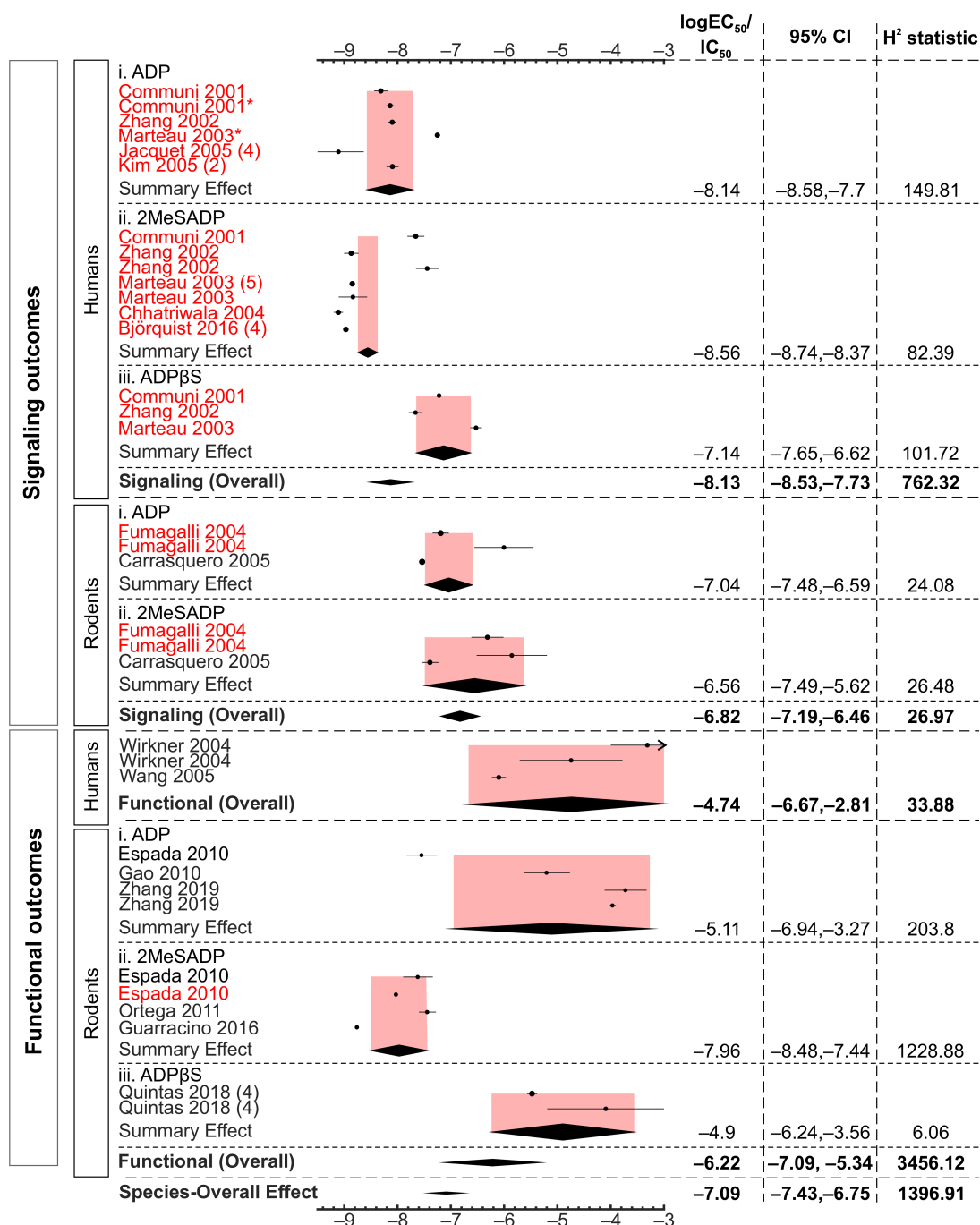


Figure 5.8: Subgroup analysis based on the species of the P2Y₁₃ receptor. The forest plot represents the log₁₀ EC₅₀ values in different P2Y₁₃ species (humans and rodents) to generate a P2Y₁₃-mediated response. The black diamonds and the pink shaded regions represent the overall effect size and the 95% confidence interval. The study names in “red” were conducted in the heterologously-expressed P2Y₁₃ receptor. All the studies were of sample size, n = 3, unless otherwise stated in parentheses. The studies marked in * had multiple datasets of the same agonist and outcome in the individual study and were averaged, with (Communi et al. 2001), having 2 datasets and (Marteau et al. 2003), having 3 datasets.

in potency between 2MeSADP and ADP depends on the experimental conditions and in most cases, is not as pronounced as for the P2Y₁₂ receptor [13,15,30]. In keeping with previous studies [15,29,30], our meta-analysis demonstrates that ATP and 2MeSATP act as weak agonists of the P2Y₁₃ receptor. The EC₅₀ for P2Y₁₃-mediated inhibition of cAMP by ADP was estimated as 5.24 nM (95% CI: 1.06–25.80), which confirms that ADP has a higher potency at the P2Y₁₃ receptor compared to other ADP-sensitive receptors, P2Y₁ and P2Y₁₂ [4].

We found that the reported EC₅₀ for ADP and ADP-like agonists of P2Y₁₃ was consistently higher (by 3 orders of magnitude) in the studies that focused on functional responses of the endogenously-expressed receptor, such as cellular proliferation and protein phosphorylation, compared to signaling outcomes of P2Y₁₃ activation in heterologous systems. There could be several potential explanations for this observation. First, it is important to note that the majority of the signaling or pharmacological studies used the heterologously-expressed P2Y₁₃ receptor, often co-transfected with chimeric G proteins, such as Gα/16, to isolate its responses from P2Y₁ and P2Y₁₂, while the endogenously expressed receptor was used in studies of functional outcomes. Since EC₅₀ is a complex function of the affinity for the receptor and its efficacy, which depends on the expression level [32], the apparent EC₅₀ would be expected to be lower for the exogenously-expressed receptor compared to the endogenously expressed receptor. However, the data quantifying the absolute amounts of receptors present in different systems are not available to validate this possibility. Second, the timing between signaling events measured immediately upon the addition of ligand and functional outcomes, which usually take hours or days, may lead to the differences in observed EC₅₀. It is possible

that a sustained agonist application is needed to observe similar EC_{50} in functional outcomes. Finally, it is possible that cooperativity with other receptors is required to induce a functional response through $P2Y_{13}$, since, naturally, ADP is presented not in isolation, but rather in combination with ATP [33]. Since no study investigated both signaling and functional events, future studies are required to uncover the mechanisms underlying these differences.

Our study suggests that $P2Y_{13}$ properties may depend on the tissue and species origin of the receptor. Specifically, the subgroup analysis that took into account agonist and outcome type suggested that ADP and its agonists are more potent at the brain $P2Y_{13}$ than the blood $P2Y_{13}$ and that ADP and ADP-like agonists are more potent at the human $P2Y_{13}$ receptor than the mouse $P2Y_{13}$ receptor. While these findings are potentially interesting, they require further confirmation, since subgroup analysis resulted in a relatively low number of datasets per condition. No study has directly compared $P2Y_{13}$ responses in different tissues, which is of potential interest when off-target tissue pharmacological side effects are considered. Moreover, the expression levels of $P2Y_{13}$ can differ in different tissues, resulting in variability in the apparent potency of its agonists. In regard to the species origin of the receptor, the original study by Zhang and co-authors [15] demonstrated that ADP and its analogs have higher potency at murine $P2Y_{13}$ compared to human $P2Y_{13}$, while we show that rodent receptors (mouse and rat) demonstrate higher EC_{50} compared to human $P2Y_{13}$ in signaling responses. Therefore, further comparative studies for receptors of murine, rat, and human origin are needed to identify their differences, which may translate to distinct in vivo responses to related pharmacological compounds in different species.

Several limitations of our study should be noted. The majority of the studies used the heterologously-expressed P2Y₁₃ receptor; therefore, we were limited in studying the endogenous response of the P2Y₁₃ receptor. Heterologous expression of a receptor may result in lower EC₅₀ values if the population of the P2Y₁₃ receptors is higher than in the native environment. Therefore, characterizing native P2Y₁₃-specific downstream signaling events, such as cAMP activity, remains important. However, it should be noted that, in the absence of specific P2Y₁₃ agonists, it is experimentally difficult to separate the P2Y₁₃-mediated responses from those mediated by other ADP-responsive P2 receptors, P2Y₁₂ and P2Y₁, since, in many cells, all three receptors can be expressed simultaneously [34,35,36]. Another limitation related to data availability was the small number of datasets available for some experimental conditions, making the subgroup analysis difficult. For example, studies on blood cells only had functional responses and no signaling responses and the analysis of the time dependence of P2Y₁₃ activation was not possible, since only two studies examined it. We also excluded one study from the meta-analysis because the concentration-dependence presented in that study was limited to the linear range, precluding us from extracting EC₅₀, which was our main meta-analytic outcome. Finally, we observed a high level of heterogeneity in the meta-analysis. While some heterogeneity was explained by the type of agonists, the type of outcome, and the cell origin, the remaining heterogeneity in several cases was still high. This may be due to the inherently different experimental conditions across studies and will be alleviated when this relatively new receptor is characterized more thoroughly.

In conclusion, our meta-analysis provides the EC₅₀ estimates with confidence intervals for different outcomes of P2Y₁₃-mediated responses. We have shown that the potency

of agonists on P2Y₁₃ differs according to the outcome, type of tissue, and the origin of species. These studies provide an important step towards quantitative characterization of different P2Y receptors. In the future, this will allow differentiating P2Y₁₃-mediated effects from those induced by P2Y₁₂ or P2Y₁. P2Y₁₂-targeting compounds, such as clopidogrel, ticagrelor, and cangrelor, are now in clinical practice [37,38], and it was previously suggested that these drugs may also interfere with P2Y₁₃ [10,30]. Therefore, the detailed understanding of different receptors will help in improving the selectivity and specificity of future pharmacological compounds.

5.5 Materials and Methods

This study implemented the preferred reporting items for systematic review and meta-analysis (PRISMA) checklist [39].

5.5.1 Software

EndNote X9 (Clarivate Analytics, Philadelphia, PA, USA) was used to import research articles from PubMed. Data extraction and meta-analysis were carried out using the META-LAB plug-in for MATLAB R2016b (The MathWorks Inc., Natick, MA, USA) . Interpolation and normalization of data were performed on GraphPad Prism 9 (SanDiego, CA, USA). Results obtained from the above-mentioned software were exported to Microsoft Excel for further characterization and analysis. The plots generated from META-LAB and Microsoft Excel were edited using CorelDraw Graphics Suite 2020 (Ottawa, ON, Canada).

5.5.2 Summary of Studies

The search strategy was developed such that the resulting studies contained concentration-dependence curves of the agonists to the P2Y₁₃ receptor (Table A1). The search was conducted on 3 July 2019 on PubMed and was updated on 14 September 2020, as the database covers an extensive list of high-quality articles, and the results were exported to EndNote. Reviews, editorials, letters, and conference abstracts were excluded and only papers in English were included. Abstracts were screened by two independent reviewers (C.D. and S.V.K.) to identify papers focusing on the P2Y₁₃ receptor and its function. Selected abstracts were further full text screened by the experiments conducted in the study. Only concentration-dependence studies of agonist(s) on the P2Y₁₃ receptor were included, while others were excluded. The selected studies were assessed for their quality using a questionnaire (Table A2).

5.5.3 Data Extraction

The figures from selected studies were provided as input to META-LAB's "Data Extraction" module, which facilitated the extraction of concentration-dependent experimental values and their reported variance. When provided, control or baseline experiments were also extracted. The sample size and the type of variance (standard error or standard deviation) were recorded. When the measure of variance was not reported, it was assumed to be a standard error. Studies with unreported sample size were set to 3. In cases of studies providing a range of sample size, the lowest number was selected.

5.5.4 Study Level Outcomes

Study level outcomes included (i) EC₅₀. The concentration of agonist required to achieve a half-maximum response and (ii) steepness of the slope (S) in the largest dataset in P2Y₁₃-mediated signaling responses (Ca²⁺/IP₃ release).

5.5.5 Scaling and Normalization of Data

Before fitting, the extracted data were scaled and normalized such that it ranged from 0–100% of output measurement:

$$A_{normalized} = \frac{A - \text{minimum value}}{\text{maximum value} - \text{minimum value}} \%$$

When baseline measurements were provided, concentration-dependent values were subtracted from the baseline prior to normalizing with the highest value to obtain measurements pertaining to the P2Y₁₃. When the baseline was absent, the lowest value was selected as the minimum response.

5.5.6 Fitting Dose-Dependency Curves in Hill Function

Using META-LAB's "Fit module", the normalized data were fit to the Hill equation with the Monte-Carlo error propagation method and iterated to 1000 times to obtain EC₅₀ and the steepness of the slope. The equation used:

$$Y = \frac{\beta_1 X^{\beta_3}}{\beta_2^{\beta_3} + X^{\beta_3}}$$

where β_1 is the maximum response, β_2 is the agonist's concentration required to induce half of its maximal response, and β_3 is the steepness of the slope.

5.5.7 Preparing Data for Meta-Analysis

Multiple independent measurements of the same agonist within studies were averaged for the analysis. Covariates were assigned to individual studies to conduct subgroup analysis.

5.5.8 Meta-Analysis

A random-effects model was used to pool study estimates, EC_{50} , and steepness (S) to generate a summary outcome $\hat{\theta}$:

$$\hat{\theta} = \frac{\sum_i^N (\theta_i \times w_i)}{\sum_i (w_i)},$$

where N refers to the number of datasets. The standard error computed for the summary effect:

$$se(\hat{\theta}) = \frac{1}{\sqrt{\sum_i^N (w_i)}}$$

The 95% CI was computed using a z-distribution:

$$CI = \pm z_{(1-\frac{\alpha}{2})},$$

where $\alpha=0.05$.

The weights w_i assigned to each study were calculated using an inverse variance ap-

proach:

$$w_i = \frac{1}{se(\theta_i)^2 + \tau^2}$$

The inter-study variance was predicted using the DerSimonian-Laird estimator:

$$\tau^2 = \frac{Q - (N - 1)}{c}$$

where Q is the heterogeneity statistic, N is the number of datasets, and c is the scaling factor.

5.5.9 Subgroup Analysis

Subgroup analysis was performed depending on (i) the type of tissue studied and (ii) the origin of the species of the studied P2Y₁₃ receptor.

5.5.10 Heterogeneity

The heterogeneity among data was quantified using the H^2 statistic:

$$H^2 = \frac{Q}{df}$$

where Q is the measure of total variation and df is the degrees of freedom. H^2 refers to the magnitude of heterogeneity in the data independent of the number of studies. Funnel plot, single study, and cumulative study exclusion analyses were performed to assess heterogeneity and publication bias. The funnel plot was generated with the inverse standard error, $1/se(\theta_i)$, against the individual study effect size θ_i . The homogeneity threshold, T_H , represents the percentage of studies that need to be removed to achieve

homogeneity.

5.6 Supplementary Materials

Supplementary materials can be found at

<https://www.mdpi.com/article/10.3390/ijms22073468/s1>.

5.7 Author Contributions

Conceptualization, C.D. and S.V.K.; methodology, C.D. and S.V.K.; formal analysis, C.D. and S.V.K.; data curation, C.D.; writing, C.D. and S.V.K.; supervision, S.V.K.; funding acquisition, S.V.K. All authors have read and agreed to the published version of the manuscript.

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5.9 Institutional Review Board Statement

Not applicable.

5.10 Informed Consent Statement

Not applicable.

5.11 Data Availability Statement

The data presented in this study are available on request from the corresponding author.

5.12 Acknowledgments

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5.13 Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Chapter 6

Discussion and Conclusion

The goal of this thesis was to investigate the changes in mechanotransduction and purinergic signaling in aging osteoblasts and osteoclasts. My first aim, described in Chapter 3, was to examine the mechanosensitivity and mechanotransduction of osteoclasts as they are less studied in the context of mechanotransduction. The second aim was to identify aging-associated changes in the response of osteoblasts and osteoclasts to mechanical loading, which is highlighted in Chapter 4. Finally, the last aim was to characterize the signaling dynamics of the ADP-mediated P2Y₁₃ receptor, which is described in Chapter 5. I will highlight and discuss the findings of my thesis in this chapter.

6.1 Osteoclasts as mechanosensitive and mechanotransductive cells

Bone health is critical for overall quality of life as it plays an essential role in locomotion, protection of internal organs, and metabolism. It also serves as a reservoir for mineral homeostasis (B. Clarke 2008). It is highly influenced by its physical environment, and

exercise is important for increasing and maintaining bone health (Kohrt et al. 2009; Marques et al. 2012). At the cellular level, bone adaptation is complex, requiring the coordination of bone cells to respond to these forces and adapt to maintain the structure and properties of bone. Osteocytes, the main mechanosensors in bone, sense the load and dictate osteoblasts to form bone and osteoclasts to resorb bone. When bone experiences mechanical forces, it adapts itself through a process known as remodeling where osteoblasts and osteoclasts are tightly coupled as a BMU, and their balanced activity helps renew and maintain bone (Allen et al. 2014). Osteocytes and osteoblasts have been studied extensively for their mechanosensitivity and mechanotransductive properties (Chapter 2). However, the mechanoadaptive properties of osteoclasts are less studied.

Osteoclasts play an important role in bone remodeling by resorbing old or damaged bone (Sommerfeldt et al. 2001). They are the first to appear at the site in the remodeling process (Allen et al. 2014). Therefore, it can be assumed that they possess some ability to sense loads and arrive at the site of bone subjected to forces. Previous studies have investigated the ability of osteoclasts to sense mechanical forces independently, either through local membrane deformation (Jørgensen et al. 2002), substrate stretching (Shafieyan et al. 2014), fluid shear forces (Morales-Jiménez et al. 2022), and vibration (Kennedy et al. 2021). In our study (Chapter 3), we also show that mechanically stimulated osteoclasts (primary responder) respond to the stimulus, displaying an increase in intracellular calcium $[Ca^{2+}]_i$ and conveying the information to neighboring osteoclasts (secondary responders). We also show that osteoblasts are more mechanosensitive and mechanotransductive than osteoclasts. Next, we separated primary responders based

on their injury level and showed that on severe injury, osteoclasts displayed increased $[Ca^{2+}]_i$. We also show that osteoclasts respond to increasing shear stress forces, which are more physiologically relevant forces in the bone. Thus, like osteocytes (Kringelbach, Aslan, Novak, Ellegaard, et al. 2015) and osteoblasts (Mikolajewicz, Mohammed, et al. 2018a), osteoclasts respond proportionally to mechanical stimuli.

Plasma membrane damage (PMD) is an important mechanism through which cells sense mechanical forces (Hagan, Balayan, et al. 2021). Bone cells, osteocytes and osteoblasts are known to undergo reversible PMDs in response to a stimulus (Mikolajewicz, Mohammed, et al. 2018a; K. Yu et al. 2018). PMDs also serve as one of the main routes of ATP release through which bone cells communicate with each other regarding the degree and level of stimulus (Chapter 2). Here we applied shear forces to osteocytes and added trypan blue dye at various time points post-stimulus. We found that with an increase in time, there was a decrease in the proportion of cells taking up the dye. Thus, for the first time, we show that osteoclasts also undergo reversible PMDs on mechanical stimulus. Together, these studies show that osteoclasts, indeed, are capable of sensing local strains independently in the environment.

6.2 Role of ATP and ADP-mediated signaling in mechanoadaptation

When bone cells experience forces, ATP is released within minutes into the environment. ATP and its metabolites such as ADP, act on the family of purinergic receptors to incite a cascade of downstream responses such as a rise in $[Ca^{2+}]_i$ and expression of genes that

regulate bone mechanoadaptation (Robling and Turner 2009; I. R. Orriss, Burnstock, et al. 2010). In Chapter 2, we built a conceptual model on how extracellular ATP and its metabolites ADP and adenosine participate in this complex process and possibly convey different information regarding the degree, severity, and stage of injury. We also highlight the role of different P2 receptors and propose how these receptors fit into this complex model.

The release of extracellular ATP is proportional to the level of stimulus and acts as a signal to neighboring bone cells conveying the proximity to the site of damage whereby cells near the site of stimulus will receive more ATP (Fig 2.1). We gathered studies on mechanically stimulated ATP release from bone cells and attempted to provide qualitative and quantitative information (Table 2.1). We found that, constitutively, bone cells release 1–25 amol/cell to the extracellular environment. At low levels of stimulus, bone cells are known to release ATP via vesicles (Burnstock and Verkhratsky 2012). On intermediate forces, cells release ATP via PMDs and were estimated to release 21 ± 11 to 422 ± 97 amol ATP/cell. At higher levels of force, the cell membrane ruptures, and the amount of ATP released can range between 50 and 500 amol ATP/cell.

ATP acts as a ligand on P2 receptors where the affinity for ATP ranges from 10 nM – 100 μ M (Grol et al. 2013; S. Xing et al. 2016). ATP-mediated P2 receptor signaling is important in regulating osteoblast and osteoclast formation and survival (Table 2.3). Modulating P2 activity results in either bone loss or bone gain phenotypes, highlighting the importance of studying these receptors in bone cells. We sought to identify the role of P2 receptors in mechanically stimulated osteoclast signaling (Chapter 3). We found that blocking the P2X₇ receptor abolished $[Ca^{2+}]_i$ signals in secondary responders

in agreement with another study (Jørgensen et al. [2002](#)). An interesting observation was that when we used suramin, a broad P2 receptor inhibitor (Illes et al. [2021](#)), we did not see abolished secondary responders but rather an increase in calcium signals. While our study shows that P2X₇, which is not blocked by suramin, is important for osteoclast mechanotransduction, there could also be other channels that are important in mechanically stimulated osteoclasts. One such example is anoctamin 1, which was recently implicated in regulating mechanical loading in osteoclasts, and deletion of this channel resulted in a blunted osteoclast response to mechanical loading (W. Sun et al. [2023](#)).

Extracellular ATP is degraded over time through the function of ectonucleotidases to form ADP (H. Zimmermann et al. [2012](#)). If the level of stimulus is low, ATP will be quickly degraded; thus, there will not be enough ADP to generate ADP-mediated P2 responses as ATP is an order of magnitude more potent than ADP in activating cellular responses (Mikolajewicz, Sehayek, et al. [2019](#)). However, in cases of severe injury, the amount of ATP degraded over time will generate considerable amounts of ADP to result in a downstream response. Hence, our model proposes that ADP becomes more relevant in sites of large injury and may be a positive signal for mechanoadaptation (Chapter [2](#)). In our study on osteoclast mechanobiology (Chapter [3](#)), we investigated the role of ATP and ADP in osteoclasts. We performed mechanical stimulation experiments on osteoclasts in either ATP or ADP-rich conditions. While ATP signals were necessary for mechanotransduction, we found that ADP signals become more critical in cases of severe injury as the $[Ca^{2+}]_i$ response to secondary responders was higher in ADP-rich conditions than in ATP-rich conditions. This is quite different from the mechanotransduction in

osteoblasts, where ATP-mediated signals are predominant (Mikolajewicz, Mohammed, et al. [2018a](#); Mikolajewicz, Sehayek, et al. [2019](#)).

Our study highlights the ability of osteoclasts to sense mechanical loads in the bone microenvironment. Further studies need to be carried out to determine how these findings fit in the context of bone remodeling. We show that osteoclasts are mechanosensitive and transduce signals to neighboring cells. Still, it would be of interest to understand how these signals play a role in the context of physical activity. Several studies have shown that changes in $[Ca^{2+}]_i$ signals in mature osteoclasts alter osteoclast-mediated bone resorption (Chambers et al. [1987](#); Malgaroli et al. [1989](#); Moonga et al. [1990](#); Kajiya [2012](#)). However, further research needs to be done to identify how the signaling dynamics of osteoclasts dictate their activity in an anabolic environment.

6.3 Mechanotransduction in aging osteoblasts and osteoclasts

Our next goal was to understand how mechanotransductive properties are affected in osteoblasts and osteoclasts with age. Aging is associated with an imbalance of osteoblasts and osteoclasts, where resorption by osteoclasts exceeds bone formation by osteoblasts with age (Møller et al. [2020](#)), resulting in decreased bone mass, altered mechanical properties, and increased risk for conditions such as osteoporosis (Almeida et al. [2013](#)). It has also been shown in vivo that the aging bone has a reduced ability to adapt to mechanical loads due to reduced osteoblast activity and sustained osteoclast activities with age (Birkhold, Razi, et al. [2014](#)). In Chapter 4, we aimed to identify whether changes in the osteoblast and osteoclast activity with age are due to changes in mechanosensation

and transduction.

It is known that osteocytes have reduced number, connectivity, and function with age (Tiede-Lewis et al. 2017). They also undergo fewer plasma membrane tears and have low $[Ca^{2+}]_i$ with age (Hagan, K. Yu, et al. 2020; Morrell et al. 2020). However, studies on aging osteoblasts and osteoclasts in this context are very limited. We found that the injury levels of osteoblasts and osteoclasts are similar in young and old mice and that their mechanosensitive properties are unaffected by age. However, osteoblasts and osteoclasts have altered mechanotransduction with age. We demonstrate that with age, primary osteoblasts had reduced signal propagation to secondary osteoblasts, while primary osteoclasts demonstrated an increased ability to propagate the signal to secondary osteoclasts. This shift in aging osteoblasts is consistent with a study on osteoblasts from aged rats where they showed that old osteoblasts had lower basal calcium and reduced responses to mechanical loads than young osteoblasts (Donahue et al. 2001). Here, for the first time, we report how aging osteoclasts behave in the context of mechanotransduction and identify unique alterations occurring in these cells.

We investigated age-related changes in P2 receptors important for osteoblast and osteoclast mechanotransduction. In Chapter 3, we show that the P2X₇ receptor is important for osteoclast mechanotransduction, and ADP-mediated signaling was found to be important in altered osteoclast loading conditions. In the context of aging, ADP-mediated P2Y₁₃ signaling has been linked to age-related bone loss where mice deficient in P2Y₁₃ had reduced bone volume and number of osteoblasts and osteoclasts. However, P2Y₁₃ deficient mice were protected from age-related bone loss (N. Wang, Robaye, Agrawal, et al. 2012). In our study, we found that the levels of P2X₇ and P2Y₁₃ were downregulated

with age in osteoclasts, while no differences were observed in osteoblasts with respect to age.

We highlight the importance of sex in age-related loss of mechanotransduction. It is known that sex plays an important role in bone loss with age (Isales et al. 2018). Here, we found that osteoblasts and osteoclasts from male mice were less mechanoresponsive than osteoblasts and osteoclasts from aging female mice. Thus, our study shows that aging results in a change in mechanotransduction in osteoblasts and osteoclasts and that sex plays an important role in the activity of aging osteoblasts and osteoclasts.

Further studies need to investigate the molecular mechanisms underlying age-related changes in the mechanoadaptation of bone cells. It would be important to characterize the ATP and ADP-induced signaling in aging osteoblasts and osteoclasts and examine whether and how these specific receptors affect the mechanosensitive and mechanotransductive properties of these cells. Our studies show that ADP-mediated signaling is important in osteoclasts (Chapter 3) and that the ADP-sensing P2Y₁₃ is downregulated in osteoclasts with age (Chapter 4) strongly suggesting that P2Y₁₃ may be linked with altered osteoclast mechanotransduction with age. Future studies should investigate whether pharmacologically altering ADP-mediated signaling in osteoclasts can rescue bone loss and improve physical activity outcomes.

6.4 P2Y₁₃ mediated signaling: meta-analysis

Our findings that ADP-mediated P2Y₁₃ signaling is important in osteoclast mechanotransduction (Chapters 3, 4) as well as prior studies (N. Wang, Robaye, Agrawal, et al. 2012; N. Wang, Rumney, et al. 2013) have identified the P2Y₁₃ receptor to be a target for

age-related bone loss, suggesting that P2Y₁₃ may serve as a drug target, especially for age-related bone loss where osteoclast activity is higher than osteoblast activity. However, there is a gap in knowledge consolidation of P2Y₁₃-mediated signaling. We quantitatively summarized the literature on the P2Y₁₃ receptor by conducting a meta-analysis on PubMed-indexed studies on the concentration dependence of P2Y₁₃-mediated responses (Chapter 5). Our primary objective was to assess the potency (EC₅₀) to generate a P2Y₁₃-mediated response. Our secondary objective was to examine if P2Y₁₃ responses vary in different tissues and species.

We identified 18 studies that assessed the concentration dependence of P2Y₁₃. We found that ADP and ADP-like agonists are more potent than ATP and ATP-like agonists at the P2Y₁₃ receptor. P2Y₁₃ is a G_i-linked receptor that inhibits cAMP activity (Mariya et al. 2004). We found that ADP has a higher potency at P2Y₁₃ compared to other ADP-sensitive receptors, P2Y₁ and P2Y₁₂, making the P2Y₁₃ receptor the most sensitive ADP receptor characterized to date. We also found that ADP and ADP-like agonists had higher EC₅₀ for functional events such as cell proliferation and protein phosphorylation than for immediate signaling events, e.g. Ca²⁺/IP₃, cAMP inhibition. These findings are essential to distinguish the differences in the potency of ligands to generate these P2Y₁₃-mediated responses as they become important, especially in cases of targeting the receptor pharmacologically to modulate changes either in immediate signaling or functional responses.

For our secondary objective, we identified the differences in P2Y₁₃-mediated signaling in different tissues and systems. We observed that the EC₅₀ of ADP-like agonists was lower for P2Y₁₃ expressed in the brain than the P2Y₁₃ originating in blood. ADP-like

agonists were also more potent for human P2Y₁₃ compared to rodent P2Y₁₃. Although P2Y₁₃ is not well studied across all systems and tissues, we were able to provide a general summary of the activity of this receptor in response to different ligands leading to immediate signaling events or functional responses. In the future, more studies need to be carried out on the P2Y₁₃ receptor in different systems, especially the bone, to understand the pathophysiological role of this receptor.

6.5 Conclusions

This thesis identifies osteoclasts as important players in mechanosensation and mechanoadaptation. We demonstrate that osteoclasts can sense and transduce signals to neighboring cells and that the P2X₇ receptor plays an important role in this process. We have also identified the role of ADP in osteoclast mechanotransduction in altered loading conditions. With age, expression of P2X₇ and ADP-mediated P2Y₁₃ was downregulated in osteoclasts with no differences in osteoblasts. This was accompanied by decreased osteoblast mechanotransduction and increased osteoclast signal propagation with age. For the first time, we identified sex as an important contributor to the mechanotransduction of isolated bone cells. Finally, we investigated P2Y₁₃-mediated signaling and identified it as the most sensitive ADP receptor. These findings provide the groundwork for designing targets for age-related bone loss conditions such as osteoporosis in conjunction with physical activity to help older people benefit from exercise and have healthier bones.

6.6 References

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Appendix

Appendix A Supplementary Material for Chapter 5

Supplementary material for **Characterization of Potency of the P2Y₁₃ Receptor Agonists: A Meta-Analysis**, published in the International Journal of Molecular Sciences.

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Table A1: Search strategy for the P2Y₁₃ meta-analysis

Search strategy:	
Database	PubMed
Search date	initial: July 03, 2019; updated: September 14, 2020
Search strategy	("P2RY13" OR "GPCR1" OR "GPR86" OR "GPR94" OR "P2Y13" OR "SP174" OR "purinergic receptor P2Y13" OR "53829" OR "74191") AND ("ATP" OR "adenosine triphosphate" OR "ADP" OR "adenosine diphosphate" OR "UTP" OR "uridine triphosphate" OR "UDP" OR "uridine diphosphate" OR "[33P]2MeSADP" OR "2MeSADP" OR "2MeSATP" OR "ADPβS" OR "ATPγS" OR "cangrelor" OR "Ap4A" OR "MRS2603" OR "MRS2211" OR "reactive blue-2" OR "suramin" OR "2MeSAMP" OR "PPADS")
Eligibility criteria	P2Y ₁₃ response to P2Y ₁₃ -specific ligands
Initial hits	153
Eligible	50
Potential datasets	18

Table A2: Quality assessment questionnaire for the articles included in the meta-analysis. 14 points indicates a high-quality article

Quality checklist (maximum 14 points):	
1	Whether the study focused specifically the P2Y ₁₃ (receptor overexpression or specific agonist) (1) or other general agonists were added which can generate a response through other P2 receptors (0)
2	Whether the study reported the following: (1 point each, maximum of 8): dose dependence curves, agonist/antagonist addition, reporting concentration of ligand, expression of receptor, type of assay, outcome, sample size, origin of cells (in vivo, in vitro, etc.)
3	Whether the study reported control data (1) or normalized to control (1)
4	Whether data was presented in a graph (1) or tabular form (0)
5	When only EC ₅₀ was reported (0), or a dose dependency curve was provided (1)
6	Whether the paper reported what was the measure of central tendency (e.g. mean, median) or spread (e.g. SD or SE) (1) or whether it was unclear (0)
7	Contains detailed information on whether cells were exposed to particular treatments (1) (CP/CPK, other antagonists etc.) or if some aspects were unclear (e.g. duration, concentration, application) (0)

Table A3: Study level effect sizes (EC_{50}/IC_{50}) of the reported outcomes and subgroups in P2Y₁₃-mediated events, and steepness (S) in the largest dataset of signaling outcomes (Ca²⁺/IP₃ release).

Outcome/Subgroup			Agonist	log ₁₀ EC ₅₀	log ₁₀ low CI	log ₁₀ high CI	EC ₅₀ (nM)	low CI (nM)	high CI (nM)	I ² statistic	H ² statistic
Signaling	Ca ²⁺ /IP ₃ release	ADP	-7.90	-8.53	-7.26	12.72	2.97	54.51	99.52	209.93	
		2MeSADP	-8.71	-8.93	-8.50	1.93	1.18	3.16	98.72	78.11	
		ADPbS	-7.14	-7.76	-6.51	73.13	17.32	308.80	99.02	101.72	
		Overall	-8.04	-8.51	-7.57	9.16	3.11	27.02	99.90	1029.76	
	cAMP accumulation	ADP	-8.28	-8.97	-7.59	5.24	1.06	25.80	98.89	90.44	
		2MeSADP	-7.41	-7.54	-7.28	38.89	29.07	52.02	0.00	0.15	
		Overall	-7.91	-8.31	-7.51	12.28	4.86	31.05	97.88	47.13	
	[³⁵ S]GTPγS binding	ADP	-6.63	-7.79	-5.47	235.32	16.18	3423.30	93.90	16.40	
		2MeSADP	-7.01	-8.98	-5.05	96.81	1.04	9011.04	98.88	89.49	
		Overall	-6.86	-7.85	-5.88	136.88	14.19	1320.78	98.09	52.32	
Signaling-overall			-7.76	-8.11	-7.41	17.20	7.69	38.48	99.86	703.26	
Functional	Cell signaling	ADP	-5.12	-5.61	-4.64	7513.29	2461.66	22931.50	0.00	0.72	
		2MeSADP	-7.43	-10.60	-4.25	37.53	0.03	55684.46	99.94	1552.62	
		ADPbS	-3.31	-4.15	-2.47	488136.91	70971.48	3357371.85			
		Overall	-5.65	-7.45	-3.84	2262.78	35.69	143448.44	99.81	525.67	
	Protein activity	ADP	-5.83	-9.21	-2.45	1492.11	0.62	3548133.89	99.71	345.71	
		2MeSADP	-7.69	-8.14	-7.25	20.28	7.32	56.16	96.65	29.85	
		Overall	-6.94	-8.16	-5.73	113.96	6.95	1867.86	99.67	306.72	
	Cell proliferation	ADP	-3.92	-4.10	-3.75	119363.63	79775.26	178597.66	29.55	1.42	
		ADPbS	-4.90	-6.24	-3.56	12671.85	580.59	276574.84	83.51	6.06	
		Overall	-4.33	-5.39	-3.27	46654.64	4087.79	532477.02	99.62	263.04	
Functional-overall			-5.75	-6.51	-5.00	1761.12	308.23	10062.54	99.96	2574.94	
Signaling	ATP and ATP like agonists	Overall	-6.35	-7.20	-5.50	447.04	63.45	3149.52	96.13	25.83	
Functional	Blood	Overall	-4.75	-6.12	-3.37	17877.10	750.03	426104.28	99.67	298.98	
Functional	Brain	Overall	-6.50	-7.69	-5.31	316.84	20.53	4890.88	99.41	168.15	
Tissue-overall			-5.70	-6.71	-4.70	1973.73	193.68	20113.95	99.76	411.85	
Signaling	Humans	ADP	-8.14	-8.58	-7.70	7.24	2.65	19.76	99.33	149.81	
		2MeSADP	-8.56	-8.74	-8.37	2.77	1.80	4.26	98.79	82.39	
		ADPbS	-7.14	-7.65	-6.62	73.13	22.49	237.83	99.02	101.72	
		Overall	-8.13	-8.53	-7.73	7.42	2.93	18.81	99.87	762.32	
	Rodents	ADP	-7.04	-7.48	-6.59	91.70	32.85	256.00	95.84	24.05	
		2MeSADP	-6.56	-7.49	-5.62	278.41	32.54	2382.00	96.22	26.48	
Functional	Humans	Overall	-6.82	-7.19	-6.46	149.79	64.32	348.83	96.29	26.97	
		Overall	-4.74	-6.67	-2.81	18319.60	215.79	1555272.96	97.05	33.88	
		ADP	-5.11	-6.94	-3.27	7783.95	113.86	532164.25	99.51	203.80	
		2MeSADP	-7.96	-8.48	-7.44	10.94	3.31	36.18	99.92	1228.88	
	Rodents	ADPbS	-4.90	-6.24	-3.56	12671.85	580.59	276574.84	83.51	6.06	
		Overall	-6.22	-7.09	-5.34	609.26	81.66	4545.48	99.97	3456.12	
Species-overall			-7.09	-7.43	-6.75	80.94	36.96	177.26	99.93	1396.91	

Outcome	Agonist	Steepness (N)	low CI	high CI	I2 statistic	H2 statistic
Ca ²⁺ /IP ₃ release	ADP	4.01	1.08	6.93	98.58	70.44
	2MeSADP	4.72	1.25	8.20	99.58	237.77
	ADPbS	4.44	1.68	7.19	94.93	19.73
	Overall	4.35	3.27	5.42	99.23	129.11

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Article Title	P2 receptor networks regulate signaling duration over a wide dynamic range of ATP concentrations.	Publication Type	Journal
		Start Page	3615
		End Page	3626
Author/Editor	COMPANY OF BIOLOGISTS.	Issue	Pt 16
		Volume	126
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		Expected Presentation Date	2024-04-15
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