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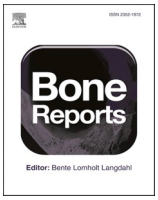

# Oxidative phosphorylation in bone cells

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ABSTRACT

The role of energy metabolism in bone cells is an active field of investigation. Bone cells are metabolically very active and require high levels of energy in the form of adenosine triphosphate (ATP) to support their function. ATP is generated in the cytosol via glycolysis coupled with lactic acid fermentation and in the mitochondria via oxidative phosphorylation (OXPHOS). OXPHOS is the final convergent metabolic pathway for all oxidative steps of dietary nutrients catabolism. The formation of ATP is driven by an electrochemical gradient that forms across the mitochondrial inner membrane through to the activity of the electron transport chain (ETC) complexes and requires the presence of oxygen as the final electron acceptor. The current literature supports a model in which glycolysis is the main source of energy in undifferentiated mesenchymal progenitors and terminally differentiated osteoblasts, whereas OXPHOS appears relevant in an intermediate stage of differentiation of those cells. Conversely, osteoclasts progressively increase OXPHOS during differentiation until they become multinucleated and mitochondrial-rich terminal differentiated cells. Despite the abundance of mitochondria, mature osteoclasts are considered ATP-depleted, and the availability of ATP is a critical factor that regulates the low survival capacity of these cells, which rapidly undergo death by apoptosis. In addition to ATP, bioenergetic metabolism generates reactive oxygen species (ROS) and intermediate metabolites that regulate a variety of cellular functions, including epigenetics changes of genomic DNA and histones. This review will briefly discuss the role of OXPHOS and the cross-talks OXPHOS-glycolysis in the differentiation process of bone cells.

## 1. Introduction

Energy metabolism is essential for cell and tissue differentiation, homeostasis, and repair. Once thought to be a mere consequence of the state of the cell, energy metabolism is now known to control tissue differentiation and function through the production of adenosine triphosphate (ATP), the accumulation of reactive oxygen species (ROS), and the synthesis of intermediate metabolites that regulate a variety of cellular functions, including epigenetics changes of genomic DNA and histones (Gatie and Kelly, 2018). Along those lines, the role of energy metabolism in bone cells is an active field of investigation.

Bone cells are metabolically very active and require high levels of ATP to support their function. Osteoblasts, the bone forming cells, secrete and mineralize a collagen type I enriched matrix (Long, 2011). Osteoclasts, the bone resorbing cells, secrete proteases and generate $H^+$ to create an acidic environment that dissolves the mineral and allows those proteases to properly resorb the bone matrix (Boyle et al., 2003). Osteocytes, which are embedded in the bone matrix, act as mechanical sensors and are a source of hormones such as Fgf23 (Sitara et al., 2004).

Given the constant energy demand of bone cells to perform their activity, it is intuitive that any perturbation in bioenergetic processes can affect their differentiation and function (Fig. 1). ATP is generated in the cytosol via glycolysis coupled with lactic acid fermentation and in the mitochondria via oxidative phosphorylation (OXPHOS). Across many tissues, both during development and in adulthood, the general consensus is that the glycolytic metabolism is the main source of ATP in undifferentiated progenitors, whereas, in terminally differentiated cells, OXPHOS overcomes glycolysis (Pattappa et al., 2011; Tsogtbaatar et al., 2020). For bone cells, the picture appears to be more complex. Glycolysis is the cytoplasmic pathway that breaks down glucose into pyruvate. This process takes place regardless of the presence of oxygen, generates a net synthesis of 2 molecules of ATP per glucose, and is the preliminary step of both OXPHOS and lactic acid fermentation. The latter occurs in the cytoplasm with the aim of regenerating $NAD^+$ to maintain the glycolytic flux. Authors often refer to the combination of glycolysis and lactic acid fermentation as “anaerobic glycolysis” because oxygen is not

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required. Otto Warburg, in early 1900, discovered that tumor cells consume more glucose than the other cell types and that glucose is mainly converted into lactate through fermentation rather than oxidized in the OXPHOS pathway. This phenomenon was named "aerobic glycolysis" because, different from the lactic acid fermentation that occurs in response to oxygen limitation, aerobic glycolysis generates high levels of lactate in presence of normal oxygen tension. Although aerobic glycolysis is considered a hallmark of cancer cells, it is not exclusive to cancer only, but is a general feature of proliferative cells (Vander Heiden et al., 2009). For simplicity, as stated by other authors, we will use the term "glycolysis" in its broadest meaning, i.e. the combination of glycolysis and lactic fermentation, whether they occur in the presence or in the absence of oxygen.

In this review, we discuss data from the recent literature emphasizing the role of mitochondria and OXPHOS in osteoblasts, osteocytes, and osteoclasts and how these processes are essential in promoting proper bone tissue development and function.

## 2. Mitochondrial bioenergetics

Mitochondria generate most of the energy needed for cells to function optimally. OXPHOS takes place in the mitochondria and is the final convergent metabolic path for all the oxidative steps of carbohydrates, amino acids, and fatty acids catabolism (Fig. 2). Its main purpose is to couple the oxidation of NADH, $FADH_2$, succinate, and other primary electron donors to ATP synthesis. The mitochondrial structure is a major component in shaping up the function of OXPHOS since it determines the sub-compartmental division that allows the creation of a proton-motive force driving ATP synthesis. These double-membrane-bound organelles present an inner matrix at a pH of 7.9–8 and an intermembrane space at a pH of 6.9–7.2, separated by the inner membrane. The outer membrane completes the structure and separates this cell compartment from the rest of the cytoplasm. The inner and outer membranes differ mainly in their selective permeability to molecules of different sizes. The outer membrane, with 26–36 kDa porins that function as voltage-dependent, anion-selective channels, is permeable to ions and small molecules, including NADH and ATP, while proteins and larger molecules cannot pass through porins and need to be imported by translocases. The inner membrane is impermeable to molecules and ions of any size, which can only penetrate through selective protein channels. OXPHOS occurs in the inner membrane through the activity of the electron transport chain (ETC) complexes incorporated into the membrane along with ATP synthase. The spatial organization of the internal membrane is essential to the correct functioning of OXPHOS. It is equipped with cristae which are convolutions of the membrane itself that extend into the mitochondrial matrix. This conformation helps store $10^4$ ETC complexes and an equal amount of ATP synthases.

The Krebs cycle, also known as the tricarboxylic acid cycle (TCA) or citric acid cycle (Krebs and Johnson, 1937), is the major metabolic pathway of energy production in cells providing most of the reduced

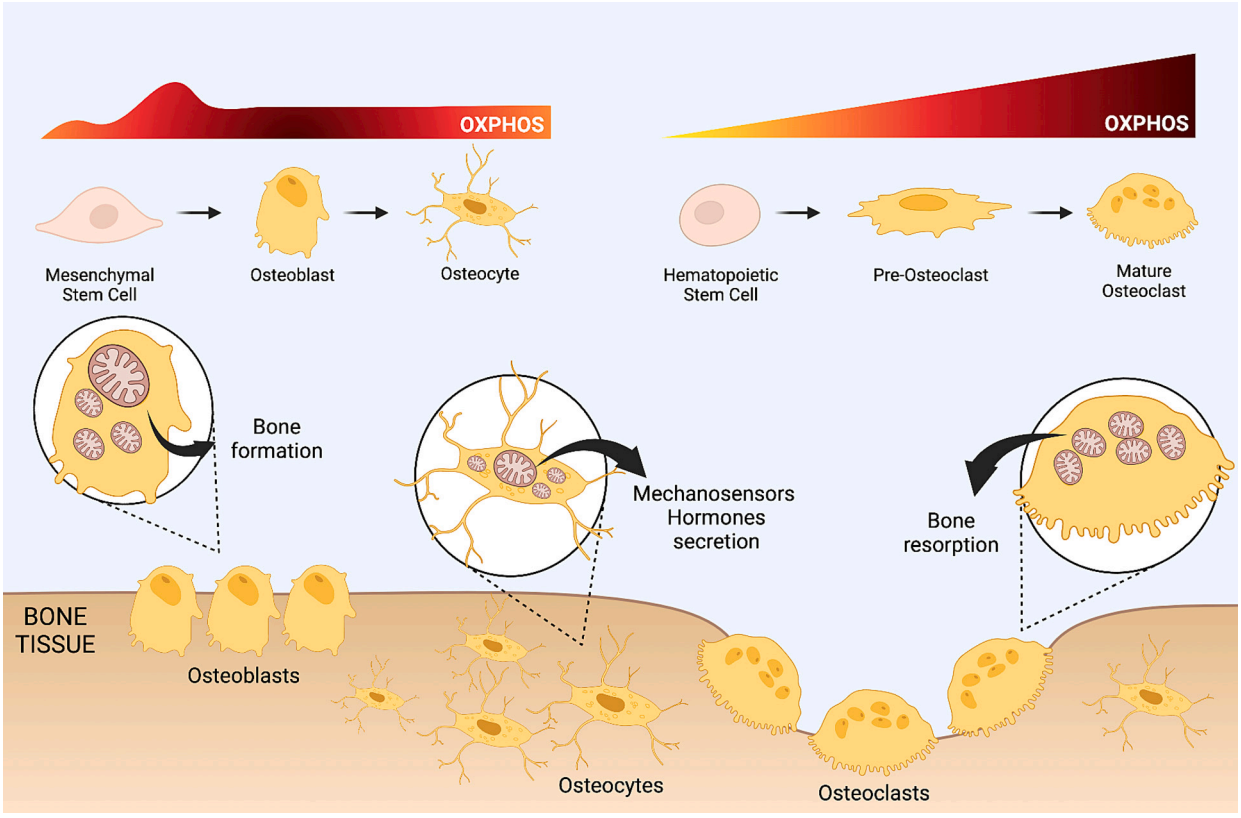
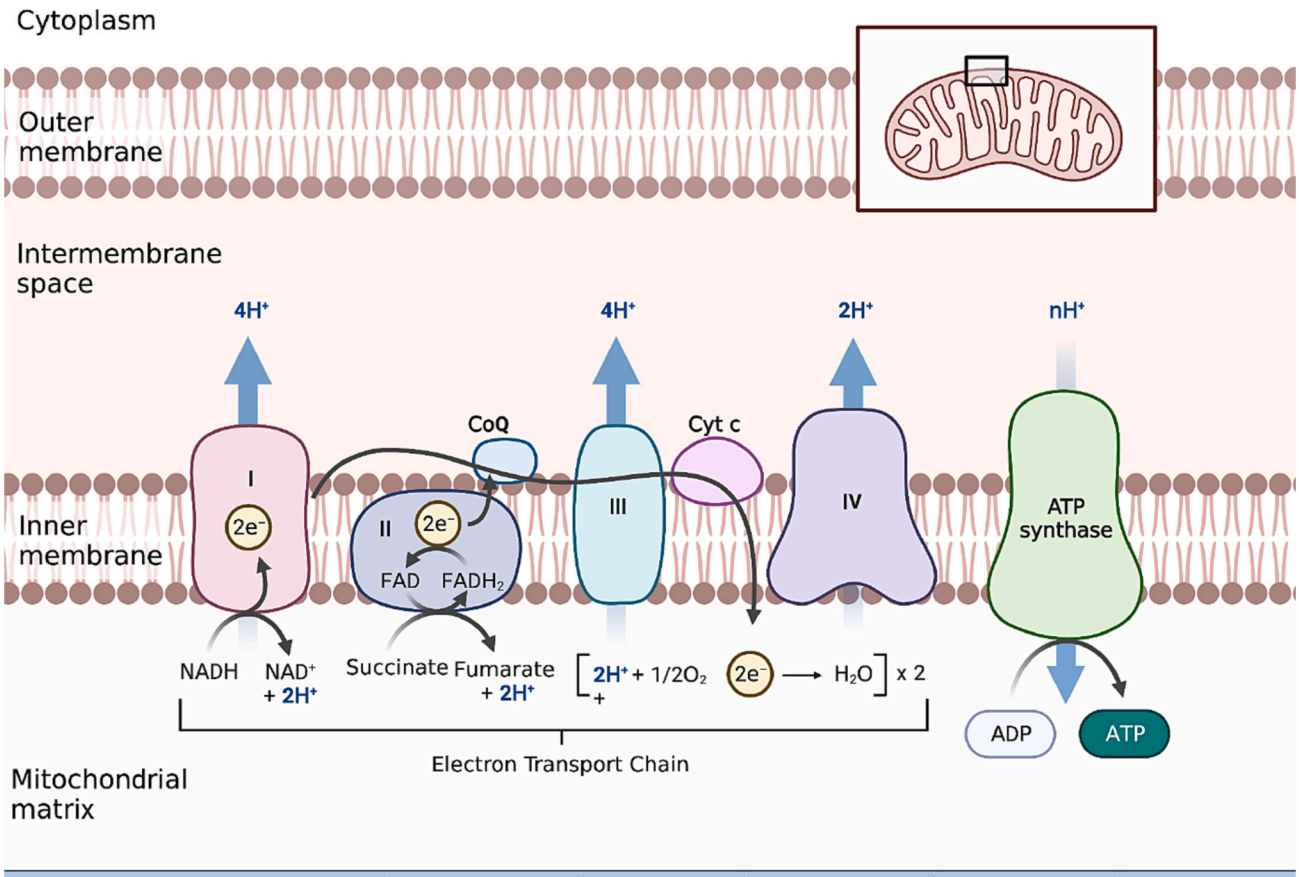



**Fig. 1.** Role of oxidative phosphorylation (OXPHOS) in bone cells bioenergetics.
Osteoblasts are the cells responsible for bone formation. Osteoblasts originate from mesenchymal stem cells and undergo a process of terminal differentiation to generate osteocytes. Osteocytes are mechanical sensors and produce hormones involved in a variety of biological functions. Osteoclasts originate from hematopoietic progenitors of the monocyte lineage and are highly specialized multinucleated cells responsible for the resorption of mineralized bone matrix. Bone cells are metabolically highly active and require high levels of energy in the form of adenosine triphosphate (ATP) to support their function. The role of OXPHOS in the energy metabolism of bone cells varies during the different stages of differentiation. In undifferentiated mesenchymal progenitors and terminally differentiated osteoblasts, OXPHOS plays a marginal role as a source of ATP, while it prevails over glycolysis during a limited phase of the differentiation process. In osteoclasts, the contribution of OXPHOS in energy metabolism increases progressively during differentiation and is accompanied by an increase in the number of mitochondria. However, the greater availability of mitochondria in mature osteoclasts is not necessarily associated with a higher supply of ATP, which, on the contrary, seems to be low in those cells. The low availability of ATP plays an essential role in the susceptibility of mature osteoclasts to undergo apoptosis.




| Complex | I | II | III | IV | V |
|---|---|---|---|---|---|
| mtDNA | 7 | 0 | 1 | 3 | 2 |
| nDNA | 39 | 4 | 10 | 10 | 14 |

**Fig. 2.** Oxidative phosphorylation (OXPHOS) overview.
OXPHOS takes place in the mitochondria and is the final convergent metabolic pathway for all oxidative phases of dietary nutrients. OXPHOS occurs in the inner mitochondrial membrane through the activity of electron transport chain (ETC) complexes incorporated into the membrane together with ATP synthase. The complexes use the potential energy generated via ETC to move protons across the inner membrane. The electrochemical gradient formed across the membrane is thus a combination of two elements: one is the difference in proton concentration between the two regions separated by the inner membrane and acquired through active pumping of Complexes I, III, and IV, while the other element results from the segregation of charges when a proton moves across the membrane without a counter ion. The energy potential stored in the electrochemical gradient is spent on multiple mitochondrial functions, most notably synthesizing and releasing ATP within the mitochondrial matrix. The generation of the electrochemical gradient is due to the coordinated activity of all mitochondrial complexes. For this reason, the mitochondrial genetic heritage is preserved, in most mammals, in the maternal DNA. Mitochondria originate from genes encoded in both genomic (nDNA) and mitochondrial DNA (mtDNA). In particular, 13 core subunits of OXPHOS are encoded by mtDNA, while the remaining subunits are encoded by nDNA.

cofactors, such as NADH and $FADH_2$, that will be oxidized by the ETC complexes to produce energy. It was originally described as "*a cyclic sequence of eight biochemical reactions that catabolize nutrients to release intermediates and reducing equivalents as cellular currencies to meet metabolic demands for energy and biosynthesis*" (Krebs and Johnson, 1937). It is a highly conserved pathway in mammalian cells that occurs in the mitochondrial matrix in ways that appear similar across cell types, although the metabolic requirements may differ in each cell (Metallo and Vander Heiden, 2013). Acetyl-CoA is considered the "initiator" of the process, which must be replenished at each round of the cycle. During the first step, acetyl-CoA and oxaloacetate, a compound carrying 4 carbons (C), form citrate (6C) by citrate synthase. Citrate is converted to its isomer isocitrate and then dehydrogenated and decarboxylated to form alpha-ketoglutarate (5C). During this reaction, a molecule of $CO_2$ is released, and NAD+ is converted into NADH. Alpha-ketoglutarate undergoes oxidative decarboxylation to form succinyl-CoA (4C) by alpha-ketoglutarate dehydrogenase resulting in the release of another $CO_2$ and conversion of additional NAD+ to NADH. Then succinyl-CoA synthetase forms succinate from succinyl-CoA, which is subsequently oxidized to fumarate by succinate dehydrogenase. In the process, FAD is converted to $FADH_2$. Fumarase, in the presence of $H_2O$, converts fumarate to malate, which is then dehydrogenated to form oxaloacetate with the formation of another NADH. Finally, oxaloacetate combines with another acetyl CoA by malate dehydrogenase and starts a new cycle (Krebs and Johnson, 1937).

It should be mentioned that alongside this well-known TCA model, a variant of the TCA cycle has recently been characterized by Arnold et al. (Arnold et al., 2022). In this non-canonical TCA, citrate is exported out of the mitochondria to the cytosol by citrate/malate antiporter SLC25A1 and then cleaved by ATP citrate lyase into cytosolic acetyl-CoA, used for biosynthetic and acetylation reactions, and oxaloacetate, utilized to regenerate NAD+ and produce malate, which is then imported into

mitochondria to complete one turn of the TCA cycle without the loss of $CO_2$. The authors observed that the non-canonical TCA is necessary for changes in cellular state, and they demonstrated in mice and embryonic stem cells that blocking the non-canonical TCA cycle prevents cells from exiting pluripotency, underlining the role of TCA in cell fate as well as in energy metabolism (Arnold et al., 2022).

The ETC is the other cell component that sets Oxphos along with the TCA cycle. ETC complexes contain multiple polypeptides and various prosthetic groups; their main function is to carry electrons (e-) from respiratory coenzymes to oxygen, and they are functionally coupled by cytochrome *c* and coenzyme Q. In detail, e- are transferred from NADH and succinate to ubiquinone through Complex I (NADH-ubiquinone oxidoreductase) and Complex II (succinate-ubiquinone oxidoreductase), respectively. Ubiquinone collects e- from complexes I and II and other FAD-containing enzymes that catalyze redox reactions and transfer free e- to coenzyme Q. Complex III (ubiquinol-ferricitochrome c oxidoreductase) oxidizes ubiquinone and transfers e- to cytochrome *c*, which in turn throws e- to complex IV and finally to molecular oxygen to generate $H_2O$. Cytochrome c is not part of any multienzyme complex but rather moves freely through the external side of the inner membrane collecting e- from the different complexes. Complex V (oligomycin-sensitive ATP synthase) utilizes the energy potential generated by the flux of e- for the synthesis of ATP (Walker, 2013). The complexes use the energy potential generated via ETC to move protons across the inner membrane. The electrochemical gradient formed throughout the membrane is thus a combination of two elements: one is the difference in concentration of protons between the two regions separated by the inner membrane and gained through the active pumping of Complexes I, III, and IV, while the other element results from the segregation of charges when a proton moves through the membrane without a counter ion. The energy potential stored in the electrochemical gradient is spent for multiple mitochondrial functions, particularly synthesizing and releasing ATP within the mitochondrial matrix. The efficiency with which the proton gradient can generate ATP is called “coupling efficiency” and depends on the efficiency of the proton flux to reach the ATP synthase rather than being dissipated by complexes I, III, and IV. This will also determine the amount of energy intended for ATP generation versus energy dissipated as heat. The generation of the electrochemical gradient is due to the coordinated activity of all the mitochondrial complexes. If a complex acts independently of the others, it would cause an imbalance that compromises the activity of all the other complexes and, consequently, the production of energy. It is, therefore, necessary that the mitochondrial complexes have a single evolutionary origin. Mitochondria originate from genes encoded in both genomic and mitochondrial DNA (mtDNA). MtDNA encodes 13 core OXPHOS subunits, 22 transfer RNA, and 12S and 16S ribosomal RNA genes, while genomic DNA (nDNA) encodes the remaining 80 % of the OXPHOS genes. In most animals, all the mitochondrial genetic heritage is conserved in the maternal DNA. Therefore, mtDNA-derived genes are transcribed as a single unit avoiding recombination problems that would affect the mitochondrial activity and, ultimately, the production of energy (Wallace et al., 2010). Mutations of mtDNA are much more frequent than those of genomic DNA, likely due to the proximity of mtDNA to ROS production. The appearance of mtDNA mutations results in a condition called “heteroplasmy”, characterized by a mixed population of normal and mutated DNA. This situation can evolve into a “homoplasmy” when the mtDNA is segregated so distinct populations with only mutated or only normal mtDNA are generated. Cellular dysfunction occurs if the accumulation of mutated mtDNA outnumbers the presence of normal mtDNA (threshold effect). This impairs the ability of the mitochondria to produce ATP, resulting in the onset of pathological conditions such as neurodegenerative diseases, cancer, and aging (Wallace, 2005).

Of note, mitochondrial ROS are a major product of ETC. The role of ROS in skeletal biology is extensively discussed in a review by Almeida and colleagues as part of this Bone Reports Special Issue on Metabolism and Bone.

## 3. Mitochondriopathies and bone

Mitochondrial diseases, due to either sporadic or hereditary mutations of genes located in the genomic or mtDNA or exogenous factors, often have a chronic, slow, and progressive course with multi-organ involvement. The typical onset is at birth, although they can manifest later in adulthood. Tissues that have a high energy demand, such as brain, muscle, heart, and the endocrine system, are the most affected, although any organ can be involved. As a result, mitochondrial diseases classically manifest with blindness, deafness, dementia, heart, and muscle disorders. Furthermore, given that mitochondria are essential to metabolize dietary nutrients like carbohydrates and fatty acids, their dysfunction contributes to the development of metabolic diseases like cardiovascular disease, diabetes, obesity, and cancer (Wallace, 2005). Finally, the role of mitochondria in aging is well documented. The free radical theory of aging postulated more than 40 years ago, suggests that the accumulation of cellular damage from the mitochondrial ROS is a major contributor to the aging rate (Harman, 1956). Since then, an increasing number of evidence have been collected in different animal models, which confirmed that mitochondrial dysfunction contributes to the aging decline through multiple mechanisms such as accumulation of mtDNA mutations, oxidative damage, reduction in the number and efficiency of mitochondria, and dysregulation of energy metabolism (Amorim et al., 2022).

The degree of skeletal involvement in mitochondrial diseases is variable. However, most patients show some degree of poor bone health, and dramatic cases of bone involvement have been reported (Tzoufi et al., 2013). Skeletal disorders often result from malnutrition and immobility. In addition, mitochondropathies-related endocrine disorders, such as diabetes, hypoparathyroidism, growth hormone deficiency, and hypogonadism, adversely affect bones, as do other conditions that increase the risk of osteopenia and osteoporosis, such as renal tubular acidosis and liver dysfunction.

On the other hand, a direct pathophysiological connection between bone and mitochondrial diseases has never been clearly established, and our knowledge is often limited to the study of clinical cases. Dobson et al. investigated the potential impact of mtDNA mutations on bone biology using $PolgA^{mut/mut}$ mitochondrial ‘mutator’ mouse, which carries a mutated, proofread-deficient form of nucleus-encoded mtDNA polymerase (Polg). They observed an increased bone loss in $PolgA^{mut/mut}$ mice compared to the wild type, which was associated to a defect in bone matrix mineralization and an increase in osteoclast activity in vitro (Dobson et al., 2020). This study demonstrates that mitochondrial dysfunction severely impairs osteogenesis through a bone cell-directed mechanism, although the possibility that the phenotype is due to non-autonomous cellular effects of the Polg mutation on osteoblasts or osteoclasts should be considered. Noticeably, mitochondrial dysfunction due to defective Polg has also been implicated in the pathogenesis of premature aging and frailty observed in patients with HIV, likely caused by antiretroviral drugs that inhibit Polg (Desquilbet et al., 2007). Therefore, in mitochondrial diseases, the possibility that bone impairment may occur through a direct mechanism, as well as being the result of systemic complications, should be considered. A comprehensive elucidation of the bioenergetic processes in bone cells could help in the study and treatment of bone diseases in mitochondropathies.

## 4. Mitochondrial bioenergetics in bone cells

### 4.1. Osteoblast lineage

Osteoblasts, which originate from mesenchymal progenitors, are the primary bone building cells (Long, 2011). They have special metabolic requirements due to their role in bone matrix synthesis, secretion, and mineralization. In conditions associated with unbalanced energy metabolism, such as aging, diabetes, and anorexia nervosa, we often witness the development of osteopenia/osteoporosis, which results in fragility

fractures (Manolagas, 2000). In animal models of caloric restriction, a reduction in bone mass due to a lower rate of bone formation has also been reported (Devlin et al., 2010). This may depend on the inability of the osteoblasts to maintain an efficient function in the presence of insufficient ATP availability. Osteoblast bioenergetics is still poorly understood. Several studies demonstrated that metabolic requirements in osteoblasts change over time according to the differentiation stage (Shum et al., 2016).

Glucose is considered the primary fuel for osteoblastic cells (Zoch et al., 2016). Glucose, in addition to providing energy in the form of ATP, also provides a carbon source for synthesizing bone-building block molecules like intermediates in the hexose phosphate or in the hexosamine biosynthesis pathways (Bouché et al., 2004).

Glutamine is another actor that plays a critical metabolic role in the osteoblast lineage. It is a carbon and nitrogen source for synthesizing amino acids, tricarboxylic acid (TCA) components, and nucleotides. The enzyme glutaminase is responsible for synthesizing glutamic acid from glutamine, which is then deaminated into α-ketoglutarate to enter the TCA cycle. Conditional deletion of glutaminase in Prx-1(+) skeletal progenitors impairs osteoblast specification, differentiation, and bone formation (Yu et al., 2019). However, since glutaminase inhibition does not alter energy metabolism in skeletal progenitors, it has been hypothesized that the primary role of glutamine is to provide a substrate for biosynthetic demands rather than being an energy substrate (Yu et al., 2019). The transcription factor hypoxia-inducible factor 1α (Hif-1α) induces glutaminase expression that mediates the glutamine-dependent glutathione biosynthesis, which helps preserving the redox balance during oxidative stress or nutrient deficiency in bone. Hif-1α-mediated glutathione production has been critical for enabling the survival of implanted osteoblast precursors in a bone regeneration mouse model (Stegen et al., 2016).

Finally, among the energy sources, fatty acids should be mentioned. After entering the mitochondria via carnitine-mediated transport, fatty acids are degraded via β-oxidation and enter the TCA cycle to serve ATP production in OXPHOS. It has been shown that the induction of fatty acid β-oxidation by Wnt-Lrp5 signaling is required for optimal differentiation of osteoblastic cells (Frey et al., 2015).

For a more extensive discussion of the energy sources in bone cells, please, refer to Long et al. for glucose metabolism (Long, 2022), Devignes et al. for glutamine and amino acid metabolism (Devignes et al., 2022), Kim et al. for fatty acids metabolism (Kim et al., 2017).

### 4.2. Osteoblast differentiation

The general assessment that stem cells rely on glycolysis, whereas OXPHOS is activated in differentiated/mature cells, (Pattappa et al., 2011; Tsogtbaatar et al., 2020), may not be valid for osteoblastic cells. One of the first studies on cells of the osteoblast lineage was conducted by Komarova et al.: the authors showed that glycolysis is the main energetic pathway in both undifferentiated mesenchymal progenitors and terminally differentiated osteoblasts, while OXPHOS is relevant between days 3 and 7 of differentiation. After day 7 and during bone nodule formation, an increase in ATP content was reported that was associated with a switch from OXPHOS to glycolysis (Komarova et al., 2000). After this original report, the field has subsequently expanded. However, despite exponential progress in recent years, studying energy metabolism, particularly mitochondria in bone cells, remains challenging. Shum et al. in 2016 highlighted a series of technical as well as methodological difficulties that had characterized previous studies on mesenchymal progenitor bioenergetics and have led to misinterpretation of some of the findings (Chen et al., 2008; Guntur et al., 2014; Pattappa et al., 2011; Esen et al., 2013). In particular, among others, the notion that osteoblasts of different embryological origin might have different metabolic requirements is often not taken into consideration; oxygen consumption rates unrelated to OXPHOS are not studied; the use of pyruvate in the culture medium, which directly fuels the TCA, may remove a potential obstacle to the activation of OXPHOS and thus eliminate any putative difference between undifferentiated and differentiated cells. Another significant downside highlighted by Shum and colleagues is that cell culture studies of mesenchymal progenitors are often conducted using supraphysiological levels of glucose and glutamine in the culture media, which could impact cellular energy metabolism (Shum et al., 2016). In light of those conflicting data, Shum and colleagues systematically investigated the bioenergetics of mesenchymal progenitors during differentiation using bioenergetic profiling and transcriptomics in vitro (Shum et al., 2016). They observed that mesenchymal progenitor cells activate OXPHOS during osteogenic differentiation but maintain levels of glycolysis similar to undifferentiated cells (Shum et al., 2016). More recently, Lin et al. corroborated the importance of OXPHOS in osteoblast differentiation in vivo. Targeted deletion of Evolutionarily Conserved Signaling Intermediate in Toll pathways (ECSIT), a core subunit of mitochondrial complex I, in skeletal progenitors, resulted in bone deformities, frailty, and fragility fractures in mice. Those defects were associated with the development of muscle atrophy through a TGF-β1 mediated mechanism underlining the importance of skeletal tissue-muscle tissue cross-talk (Lin et al., 2022). In another recent publication, Tournaire et al. elegantly demonstrated that following pharmacological inhibition of the ETC Complex III, skeletal stem and progenitor cells activate the glycolytic pathway. They observed that this phenomenon is associated with an increase in intermediate metabolites such as succinate and hydroxyglutarate, which can induce epigenetic changes of genomic DNA through inhibition of the Ten-eleven enzyme (TET). This allows progenitor cells to proliferate and maintain self-renewal potential by bypassing the ETC blockade (Tournaire et al., 2022). This study shows that cellular energy metabolism can shape cell differentiation and function by modulating epigenetic changes of genomic DNA.

Multiple ligands that have a critical role in osteoblast differentiation have been reported to control energetic metabolism. Parathyroid hormone (PTH), which promotes the differentiation of bone marrow stromal cells into osteoblasts and inhibits their adipogenic differentiation (Fan et al., 2017), has been shown to stimulate glycolysis by promoting glucose entry into the cells, glucose metabolism, and lactate production during osteoblast differentiation. In a parallel series of studies, PTH has been shown to inhibit glucose oxidation in the TCA cycle and, at the same time, increase oxygen consumption, which is probably due to the utilization of other substrates, such as glutamine and fatty acids, rather than glucose (Esen et al., 2015). Lastly, PTH stimulates the adipolysis of adipocytes and the transfer of free fatty acids from adipocytes to osteoblast lineage cells, resulting in reduced bone marrow adipose tissue (Fan et al., 2017).

Wnt signaling regulates multiple biological processes and plays an essential role during development. Wnt3a, Wnt10b, and Wnt7b have been shown to promote osteoblast differentiation through the regulation of energy metabolism. Wnt3a augments glycolysis by upregulating critical glycolytic enzymes such as pyruvate dehydrogenase kinase 1, LDHA, and the glucose transporter Glut1 (Esen et al., 2013). This metabolic regulation is independent of β-catenin but requires LRP5 and is mediated by mTORC2-AKT signaling. Notably, the Wnt-dependent increase of glucose transport was associated with increased lactate production and extracellular acidification, whereas oxygen consumption rate (OCR) was not affected, confirming that the increased glucose promoted by the Wnt signal fuels the glycolytic pathway rather than being metabolized in OXPHOS. Similarly to Wnt3a, Wnt10b augments glucose metabolism in ST2 cells (Esen et al., 2013). Moreover, Wnt7b overexpression during embryo development appears to promote bone formation by increasing Glut1 levels and glucose consumption in osteoblastic lineage (Chen et al., 2019).

It is well established that transcription factor Hif-1α increases glycolysis and suppresses mitochondrial respiration (Kim et al., 2006). Reagan et al. demonstrated that stabilization of Hif-1α in osteoblast precursors of postnatal mice markedly increases osteoblast number and

bone mass. This appeared to be a direct effect of the Hif-1α-mediated up-regulation of glycolysis rather than being secondary to the stimulation of angiogenesis, an event which is also driven by the stabilization of Hif-1α. It was found that bone mass gain promoted by Hif-1α was likely due to increased glycolytic activity over mitochondrial respiration as the pharmacological inhibition of pyruvate dehydrogenase kinase 1 (PDK1), one of the downstream targets of Hif-1α and a key enzyme in the formation of acetyl-CoA from pyruvate, reversed Hif-1α-driven bone formation in vivo (Regan et al., 2014).

### 4.3. Osteocytes

Osteocytes are the most abundant cells in bones. It has been calculated that, on average, the adult human skeleton contains about 42 billion osteocytes (Buenzli and Sims, 2015). They derive from osteoblasts that have reached terminal differentiation and are enclosed within niches in the mineralized bone matrix. These cells are mechano-sensor, play a key regulatory role in the communication between osteoblasts and osteoclasts, and coordinate the adaptive responses of the skeleton to mechanical loading in order to ensure bone homeostasis (Dallas et al., 2013). They are also source of hormones such as Fgf23 (Sitara et al., 2004). Little is known about the energy metabolism of these cells. The peculiar hypoxic environment to which osteocytes are exposed within the skeletal matrix likely influences the availability of substrates and the choice of preferential pathways for energy production. Since these cells live in a hypoxic environment (Frikha-Benayed et al., 2016), one could speculate that anaerobic glycolysis is the main pathway supporting osteocyte survival and function. However, to date, there are not sufficient data in the literature to support this hypothesis.

Like other bone cells, mitochondria are essential for multiple cellular processes in osteocytes. However, due to their unique location, mitochondrial activity in osteocytes is closely related to oxygen availability, which is relatively low in the bone marrow niche (9–32 mmHg) (Spencer et al., 2014). Liu et al., for the first time, extensively studied the role of mitochondrial energy metabolism in osteocytes in growth hormone receptor null (GHRKO) mice. These mice show severe skeletal impairment, despite their increased longevity. They found that conditional deletion of GHR was detrimental to the mitochondrial function of osteocytes. In vivo multiphoton microscopy revealed significant reductions in mitochondrial membrane potential and reduced mitochondrial volumetric density. This was accompanied by reduced levels of ATP, NADH, and OCR and increased levels of cytoplasmic ROS in GHRKO osteocytes in vitro (Liu et al., 2019). In a separate study using multiphoton microscopy, mouse cortical osteocytes were studied in situ under normoxic conditions and following extreme hypoxic stress (post-mortem) to assess the redox status and distribution of total and active mitochondria (Frikha-Benayed et al., 2016). A difference in mitochondrial content and activity was found based on the location of osteocytes in the bone cortex. Osteocytes in the periosteum preferred oxidative metabolism as indicated by high expression of mtDNA and ETC enzymes and low lactate levels, while osteocytes with dysfunctional mitochondria were often found in the endosteum.

By using high-resolution live-cell imaging, it has also been shown that healthy osteocytes transfer mitochondria across the dendritic network to stressed osteocytes with non-functioning mitochondria (Gao et al., 2019). This phenomenon, called “mitochondrial transfer” is a known mechanism in cancer cells where it plays a crucial role in the regulation of tissue homeostasis and resistance to cancer chemotherapy. Given the vulnerability of osteocytes to stresses such as hypoxia and mechanical overloading, as well as their long lifespan, it has been hypothesized that mitochondrial transfer may provide protection against excessive cell damage and avoid subsequent permanent dysfunction of the “imprisoned” osteocytes in the bone matrix. While not directly related to energy metabolism, this study demonstrates the importance of mitochondria for osteocyte function and for regulating bone homeostasis.

### 4.4. Osteoclast lineage

Osteoclasts are highly specialized multinucleated cells responsible for the resorption of mineralized bone matrix, and their activity profoundly impacts skeletal health. Despite the common misconception that the skeleton is immutable, it's actually an ever-evolving organ in which the activities of osteoblasts and osteoclasts dictate mass and shape. An imbalance between bone formation and bone resorption in favor of the latter is the underlying cause of osteopenia/osteoporosis, leading to bone fragility and risk of bone fractures. Over time, the biological regulation of osteoclasts has been extensively researched to develop anti-resorptive drugs. We will discuss below what is known regarding the metabolic and bioenergetic requirements during osteoclastogenesis.

### 4.5. Osteoclast differentiation

Differentiation of monocyte lineage cells into osteoclasts ensures the formation of mature cells capable of bone resorption (Teitelbaum, 2000). This process is subject to multifactor regulation and is accompanied by increased mitochondria, cellular respiration, and ATP production (Karner and Long, 2018). Two indispensable stimuli dictate the differentiation of monocyte lineage into osteoclast lineage: the macrophage colony-stimulating factor (M-CSF) and the nuclear factor kappa B ligand receptor activator (RANKL), a member of the tumor necrosis factor superfamily (Warde, 2011). M-CSF in osteoclast precursors promotes survival and proliferation, while in mature cells, promotes resorptive activities such as diffusion, motility, and cytoskeletal organization. Its role has been studied in op/op mice that lack a functional form of M-CSF and therefore develop an osteoclast-deficient form of osteopetrosis (Umeda et al., 1996). RANKL is widely expressed on the surface of osteoblasts and secreted by those cells. It activates RANK on osteoclastic cells. RANK activation has been associated with mitochondrial biogenesis in osteoclasts (Bae et al., 2017). RANKL-induced osteoclastic differentiation has also been shown to correlate with LDH expression and activity (Ahn et al., 2016).

Glucose is the primary energy substrate during osteoclastogenesis. Osteoclasts significantly increase glucose uptake during differentiation due to the activity of Glut1 and Glut3 expressed on the surface of osteoclast precursors. In particular, Glut1 has a critical role since the deletion of Glut1 in osteoclast progenitors has been shown to reduce osteoclast differentiation (Li et al., 2020). Increased expression of Glut1, along with enzymes involved in glycolysis, has been observed during osteoclastogenesis.

Osteoclast differentiation is also accompanied by overexpression of enzymes of TCA and OXPHOS, increased number of mitochondria, and increased OCR. The importance of OXPHOS in osteoclastogenesis has been extensively studied. For example, deletion of the mitochondrial transcription factor A (Tfam) in mature osteoclasts leads to decreased intracellular ATP levels and accelerated apoptosis (Miyazaki et al., 2012). The ubiquinone iron-sulfur oxidoreductase protein 4 (Ndufs4) is an essential component of mitochondrial complex I and, thus, mitochondrial biogenesis during osteoclastic differentiation. It has been demonstrated that Ndufs4 deficiency inhibits osteoclastogenesis by suppressing the activity of mitochondrial complex I, which leads to a decrease in bone resorption and an increase of bone mass (Jin et al., 2014).

Although OXPHOS is the preferred bioenergetic pathway for supporting osteoclast differentiation, glycolysis has also been shown to be critical during osteoclastogenesis, and both glycolysis and OXPHOS increase during osteoclast differentiation. Along those lines, the knockdown of Hif-1α, which is a major driver of glycolysis, and glucose deprivation inhibit osteoclast bone resorption function and suppress Glut1 and glycolytic enzymes expression (Ikeda and Takeshita, 2016).

Augmented lactate production associated with increased extracellular acidification has been observed during osteoclastogenesis.

Furthermore, an increase in the expression of proton-linked monocarboxylate transporters (MCTs) was also reported (Imai et al., 2019). Those proteins remove the excess lactate from cells, reducing intracellular toxicity and increasing local acidosis, thus promoting bone resorption. Conversely, it has been shown that the deletion of LDH in vitro is associated with low glucose consumption, lactate accumulation in culture media, and reduced extracellular acidification. This causes a reduction in metabolic activity that leads to dysfunctional osteoclast formation, secondary to defective osteoclast precursor fusion, and transcription factor NFATc1 down-regulation, as shown by Ahn and colleagues (Ahn et al., 2016).

Malate dehydrogenase (MDH) is a component of the malate/aspartate shuttle across the mitochondrial membrane and catalyzes the NAD/NADH-dependent malate and oxaloacetate conversion that is crucial for NADH recycling. RANKL upregulates the expression of the cytosolic form of MDH (MDH1) during osteoclastogenesis. The knockdown of MDH1 causes a reduction in ATP production due to reduced shuttle activity and, therefore, the inability to drive NADH recycling. Furthermore, it also causes the reduction of the expression of crucial transcription factors involved in osteoclast formation, such as c-FOS and NFATc1 (Oh et al., 2016).

### 4.6. Mature osteoclasts

Osteoclasts are highly differentiated cells characterized by two opposing tendencies: a low ability to survive and an efficient ability to perform energy-demanding functions such as bone resorption (Boyle et al., 2003). Changes in metabolic pathways have been observed during osteoclast differentiation and maturation. While being mitochondria-rich cells, mature osteoclasts have been defined as “ATP-depleted” compared to less differentiated precursors. They have lower levels of intracellular ATP and fewer copies of mtDNA, despite having a higher number of mitochondria (An et al., 2014). Intracellular ATP plays a critical role in osteoclast apoptosis and bone resorption. Low levels of intracellular ATP can trigger cytochrome *c* release, accelerating apoptosis. Conversely, higher intracellular ATP concentrations promote osteoclast survival (Hazama et al., 2009). This hypothesis was confirmed following the severe ATP depletion due to Tfam conditional deletion (cKO), which caused an acceleration of osteoclast apoptosis in mice (Miyazaki et al., 2012). On the other hand, Tfam cKO ATP-depleted osteoclasts showed greater bone resorption activity despite increased apoptosis. This phenomenon suggests the possible role of mitochondrial dysfunction in bone resorption responsible for age-related osteoporosis (Miyazaki et al., 2012). Quantitative proteomics and mRNA profiling data analysis identified mitochondrial changes and signaling pathways alterations during mature osteoclasts development. In mature cells, changes in protein expression have been observed, resulting in the redirection of energy flow from basic cellular functions to bone resorption. Changes in cellular organization and differences in cytoskeletal protein abundance were also observed, suggesting that bone resorptive activity requires cytoskeletal remodeling (An et al., 2014). RANKL prolongs life of both osteoclast progenitors and mature osteoclasts. However, osteoclast survival is contrasted by the activation of cell death pathways that outnumber the pro-survival signal induced by RANKL.

## 5. Conclusion

Although the literature on bone bioenergetics is already quite extensive, and promising in vivo and in vitro studies are on the way, more clarity is needed on the bioenergetic mechanisms that drive bone development and bone function. In particular, studies conducted to date have not yet fully clarified the energetic metabolism and bone cell differentiation connection. If the metabolic changes that occur during differentiation are not only a mere consequence of the differentiation process but actively contribute to the differentiation process itself, then what are the molecular mechanisms through which energy metabolism influences cell differentiation? Is it simply a matter of ATP or, alternatively, ROS and intermediate metabolites play a key role? The solution to those questions is fundamental and could have an important impact in treating bone pathologies and beyond. Defects in OXPHOS machinery are the root cause of mitochondrial dysfunction and occur as a state of energy deficiency that fails to meet cellular needs. Mitochondrial dysfunctions have been linked to skeletal disorders, including impaired bone strength, decreased bone density, and premature bone aging leading to osteoporosis and fragility fractures. However, the pathogenesis of skeletal disorders in mitochondrial diseases has not yet been elucidated. The understanding of the molecular mechanisms regulating mitochondrial bioenergetics and OXPHOS in bone development could have translational value to understanding the bone dysfunctions associated with mitochondrial diseases, facilitate the diagnostic approach, and open up perspectives for future, potentially causal therapies.

## CRediT authorship contribution statement

**Elena Sabini:** Conceptualization, Writing – original draft, Writing – review & editing. **Lorenzo Arboit:** Validation. **Mohd Parvez Khan:** Validation. **Giulia Lanzolla:** Validation. **Ernestina Schipani:** Conceptualization, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Data availability

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

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