Metabolic reprogramming of osteoclasts represents a therapeutic target during the treatment of osteoporosis

Medizinische Klinik 3, Rheumatologie und Immunologie

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Der Medizinischen Fakultät

der Friedrich-Alexander-Universität

Erlangen-Nürnberg

zur

Erlangung des Doktorgrades Dr. med.

vorgelegt von

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Als Dissertation genehmigt

von der Medizinischen Fakultät

der Friedrich-Alexander-Universität Erlangen-Nürnberg

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Tag der mündlichen Prüfung: 26.07.2022

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1. Zusammenfassung

1.1. Hintergrund und Ziele

Knochen ist ein aktives Gewebe, welches einen ständigen Umbauprozess durchläuft. Dieser wird zu gleichem Anteil von den knochenbildenden Osteoblasten und knochenabbauenden Osteoklasten bewältigt. Mit zunehmendem Alter oder als Folge von Krankheiten wird das Gleichgewicht zugunsten der Osteoklasten verschoben. Folglich überwiegt der Knochenabbau und das Frakturrisiko steigt an. Ansatzpunkte für neue Therapien sind zum einem die Reduktion der Osteoklastenanzahl oder die Hemmung ihrer Aktivität. Die Differenzierung und die Aktivität des Osteoklasten sind energetisch aufwändige und hochkomplexe Prozesse. Aktuelle Forschungsarbeiten deuten darauf hin, dass der Osteoklast im Laufe seiner Entwicklung einen metabolischen Wandel vollzieht. Aus methodisch-technischen Gründen blieb das metabolische Profil des aktiven, knochenresorbierenden Osteoklasten aber weitestgehend unerforscht.

Das Ziel dieser Arbeit ist es, den gesamten Metabolismus des Osteoklasten besser zu verstehen, um daraus neue Strategien für Therapien zu entwickeln. Um dies zu erreichen, wird zunächst ein neues Medium etabliert, welches feinste Knochenfragmente enthält. Dieses induziert eine Differenzierung und weiter eine Aktivierung der Zellen, um so zwischen einem ruhenden und einem aktiven Osteoklasten in verschiedenen Versuchsmodellen zu unterscheiden.

1.2. Methoden

Zur Herstellung des Knochenpulvermediums werden Rinderknochen fein zermahlen. Für die Anwendung in der Zellkultur wird das Pulver gelöst und in mehreren Schritten sterilisiert. Die aus Mausknochenmark isolierten Stammzellen werden entweder mit Osteoklastenmedium oder Knochenpulvermedium inkubiert. Auf diese Weise werden zwei Gruppen, die der ruhenden und die der aktivierten Osteoklasten unterschieden

und in weiteren Experimenten auf metabolischer Ebene verglichen. Dafür kommen eine extrazelluläre Durchfluss-Analyse mit dem sogenannten „Seahorse“, ein Metabolom und die Messung von Stoffwechselprodukten im Überstand der Zellkulturschalen zum Einsatz. In Osteoplatten, die eine dünne Knochenschicht enthalten, wird die resorptive Aktivität der Zellen gemessen. Anschließend werden die für die Resorption als relevant identifizierten Stoffwechselwege sowie deren Metabolite als Therapieversuch im Osteoporose-Mausmodell geblockt.

1.3. Beobachtungen und Ergebnisse

Im Gegensatz zu ruhenden Osteoklasten zeichnen sich aktivierte Osteoklasten durch eine gleichzeitige Steigerung der Glykolyseaktivität und der mitochondrialen Atmung aus. Unsere metabolischen Analysen zeigen einen Anstieg an Metaboliten der Glykolyse und des Krebszyklus in aktivierten Osteoklasten. In Übereinstimmung mit dem aktuellen Stand der Wissenschaft stellen wir fest, dass die oxidative Phosphorylierung für die Osteoklastendifferenzierung wesentlich ist. Durch die Hemmung des mitochondrialen Komplexes I mit Rotenone wurde die Fähigkeit der Osteoklasten zur Differenzierung und Fusion signifikant verringert, die knochenresorbierende Aktivität aber nur mäßig beeinflusst. Im Gegensatz dazu, blockierte die Hemmung der Glykolyse durch 2-Desoxy-D-Glukose (2dG) effizient die Knochenresorption, zeigte aber keinen Einfluss auf die Osteoklastendifferenzierung. Die funktionelle Hemmung der Osteoklasten durch 2-dG konnte durch die Zufuhr von Pyruvat oder Laktat behoben werden. Wir erzielten eine wirksame Verbesserung des durch Ovariektomie verursachten Knochenabbaus bei Mäusen, die mit einem Glykolyse- oder Laktatinhibitor behandelt wurden.

1.4. Praktische Schlussfolgerungen

Die erhobenen Daten machen deutlich, dass der Osteoklast während seiner Entwicklung einer spezifischen metabolischen Adaptierung unterliegt, welche seine spätere Aktivität bestimmt. Für seine vollständige Funktion im Knochenabbau zeigt er spezielle energetische und metabolische Bedürfnisse. Die aerobe Glykolyse sowie deren Endprodukt Laktat sind entscheidend für seine resorptiven Fähigkeiten. Unsere Erkenntnisse ermöglichen damit einen neuen therapeutischen Ansatz in der Behandlung von Erkrankungen, die mit einer erhöhten Osteoklastenaktivität und damit reduzierten Knochendichte einhergehen.

2. Relevance of the article to the scientific field of research

2.1. Introduction

2.1.1. Bone tissue in formation and remodeling

Bone is an active tissue, that undergoes a constant process of remodeling to cope with various requirements. In addition to its function as the internal framework of the body, it contributes the haematopoietic system and provides a crucial niche for cells of the innate and adaptive immune system, such as neutrophils, B- and plasma-cells (Arron and Choi 2000). Bone tissue is composed of different cell types and mineralised matrix, whose main components are collagen fibrils and hydroxyapatite crystals. It serves as a reservoir for calcium and phosphorus and maintains the body's mineral homeostasis (House et al. 1997). It occurs in two macroscopically distinct forms, as cortical and trabecular bone (Adler 2000). With its dense and mostly calcified matrix, cortical bone makes up to 80% of the bone mass. It builds the surrounding of our bones and provides mechanical strength. Contrary, trabecular bone represents 20% of bone mass, but constitutes a major part of the surface, which can be found inside the long bones. It is less dense and more elastic with a higher rate of bone turnover. It helps with mechanically support and provides the initial supplies in minerals (Hadjidakis and Androulakis 2006). Throughout lifetime, bone is changing its structure, particularly in trabecular bones, such as vertebrae. During growth, bone must be constantly remodeled to achieve its definitive shape. In this process, immature woven bone is gradually replaced by lamellar bone. However, continuous remodeling takes place in the adult skeleton. The constant reconstruction serves different purposes. It prevents material fatigue, repairs micro-damage and allows the tissue to adapt to mechanical stresses. The bone remodeling process is kept in balance by bone building osteoblasts and bone destructing osteoclasts. It starts with the quiescence phase, where preosteoclasts arrive from the endosteal sinus. They migrate and differentiate along the bone surface to become multinucleated and functional cells (Hadjidakis and Androulakis 2006). Activated osteoclasts anchor themselves to the bone surface and dissolve the matrix with the help of an acidic environment in the resorption pit. During reversal, unclassified macrophages dispose the apoptotic remnants. At the same time, calcium and growth factors are released, which further induce and activate the preosteoblast population. This is followed by a column of osteoblasts that lay down the

first osteoid lamella, followed by a second, thus walling in the first generation, who then become osteocytes. This cycle is referred to as mineralisation.

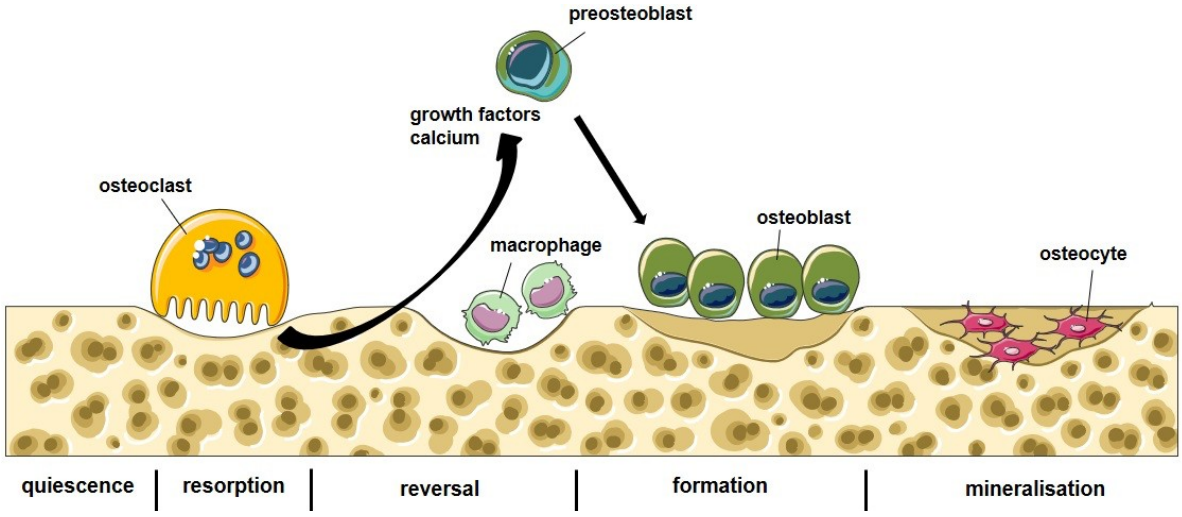

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Figure 4 | Bone remodeling: During resorption, bone is degraded by osteoclasts following reversal, where macrophages clear apoptotic fragments. Released growth factors and calcium activate osteoblasts and promote bone formation. Finally, osteoblasts turn into the mineralization state and wall themselves to become osteocytes.

Remodeling is a highly dynamic process, where a total annual turnover of up to 10% of the skeletal mass is renewed every year (Lüllmann-Rauch 2012). The regulation of bone remodeling is achieved systemically or locally by hormones and proteins secreted by hematopoietic bone marrow cells and bone cells. Systemic bone regulation is important for calcium homeostasis. Parathyroid hormone is the most important regulator increasing renal calcium reabsorption and calcitriol induction. The continuous secretion stimulates bone resorption, whereas given intermittently, it stimulates bone formation (Kim et al. 2003). In addition, vitamin D3 has anabolic effects on bone. Local regulation of bone remodeling is dependent on a finely tuned interplay between osteoclasts and osteoblasts. Although, these two cell types appear to be adversaries, their functions are interrelated in many ways. They connect through diffusible factors, cell-cell contact and cell-bone matrix interaction. Their communication occurs in a basic multicellular unit (BMU) at the initiation of bone remodeling (Matsuo and Irie 2008). In various diseases, bone resorption outweighs bone formation leading to a decreased bone mass, skeletal fragility and diseases like osteoporosis. As a therapeutically approach, bone resorption can be regulated

systemically by hormone replacement or locally by reducing osteoclast number and/or their bone resorbing activity.

2.1.2. Differentiation and function of osteoclasts

Osteoclasts are unique cells, whose activity dictate skeletal mass. They originate by the differentiation and fusion of hematopoietic precursors of the monocyte lineage (Massey and Flanagan 1999). Osteoclastogenesis includes several steps of proliferation and differentiation into mononuclear pre-osteoclasts followed by fusion into mature multinucleated cells up to 100 mm in diameter and the final step of activation for bone resorption (Zaidi 2007). Osteoclast differentiation starts with the expression of the macrophage colony-stimulating factor receptor (c-Fms). Activation of c-Fms by macrophage colony-stimulation factor (M-CSF) leads to proliferation of bone marrow-derived macrophages (BMMs), which are osteoclast precursors (Zaidi 2007; Kelley et al. 1999). Subsequently, binding of receptor activator of NF-KB ligand (RANKL) to its receptor RANK causes tumour necrosis factor receptor-associated factor 6 (TRAF6), a key adaptor protein (Kobayashi et al. 2001), and triggers a downstream signalling cascade. This involves the activation of NF-KB, AP-1 and mitogen-activated protein kinases (MAPKs) (Iotsova et al. 1997; Matsumoto et al. 2000; Wagner 2002). The functioning of osteoclasts consists in the steps of dissolution of calcium compounds by an acidic environment, secretion of lysosomal enzymes for the purpose of decomposition of organic matrix and endocytosis of fragments. Therefore, mature osteoclasts adhere firmly to the bone surface at the sealing zone with their ruffled border (Yoriko Indo 2013). This resorptive face forms a dense pleat, which increases the surface area of the plasma membrane. The folded membrane is derived from the endosome-lysosome apparatus and is the location of the H^+ATPase, which pumps protons into the space between the membrane and bone surface called Howship-lacuna. This space, where a pH of 4,5 is achieved, is closed off from the surrounding area by integrins, which anchor tightly to the surface. The acidic environment gradually dissolves the bone matrix and the demineralized organic components are degraded by the lysosomal protease, Cathepsin K. The products of bone degradation are endocytosed by the osteoclast and transported to and released at the cell's anti-resorptive surface (Teitelbaum 2000; Nesbitt and Horton 1997). After two weeks of activity the osteoclast perishes by apoptosis.

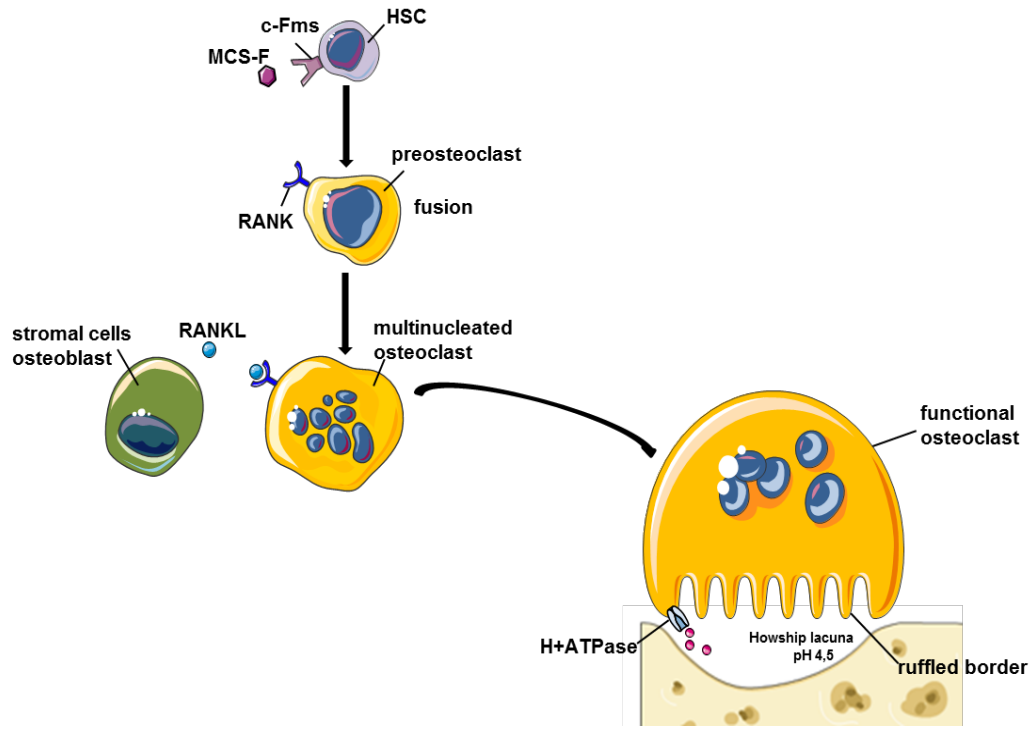

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Figure 5 | Osteoclast differentiation and function: M-CSF/c-Fms signalling induces haematopoietic stem cell (HSC) differentiation. Binding of RANKL to its receptor induces further fusion and activation of osteoclasts. Consequently, the functional osteoclast attaches to the bone surface with his ruffled border and releases protons and enzymes to start bone resorption.

Several mechanisms are known to interfere with the steps of osteoclast differentiation and activity leading to an increase of their number or over-activation and furthermore pathological bone loss. During inflammation, local cytokines, particularly tumour necrosis factor-α (TNF-α), induce the expression of RANKL and M-CSF resulting in enhanced osteolysis (Wei et al. 2005; Lin et al. 2013). Moreover, RANKL activates nuclear factor of activated T-cells c1 (NFATc1), a master regulator of osteoclast development (Takayanagi 2007). Another important regulator of osteoclastogenesis is osteoprotegerin (OPG), a protein secreted by osteoblasts and many other immune cells, which works as a soluble receptor for RANKL. It inhibits the final steps of osteoclast differentiation and induces early apoptosis. Most hormones and cytokines that affect bone remodeling interfere with the RANKL/OPG pathway resulting in a decrease of bone mass (Simonet et al. 1997). Estrogen has been identified as a major regulator of bone metabolism. It increases OPG formation and inhibits osteoclast formation meaning a positive effect on bone balance in both sexes (Khosla, Oursler, and Monroe 2012).

2.1.3. Osteoporosis and therapeutic options

In a balanced cycle of remodeling, bone breakup is equivalent to bone buildup. As we age or as a result of diseases, this balanced act becomes tipped in favor of osteoclasts. Then, bone resorption exceeds bone formation rendering bones brittle and fractures (Rodan and Martin 2000). Osteoporosis is a systemic skeletal disorder characterized by low bone density, with bad microarchitecture and a high risk of fractures. It is the most frequent bone disease of the old patient. The most common cause is estrogen deficiency. Two categories of osteoporosis have been identified, which are primary and secondary osteoporosis. Primary osteoporosis is either postmenopausal throughout a lack of estrogen, classified as type 1, or the senile type 2, related to men and women at the age of 70 and older. Secondary forms can be caused by endocrinology, malabsorption, medications like glucocorticoids or are associated with diseases like rheumatoid arthritis (Herold 2020).

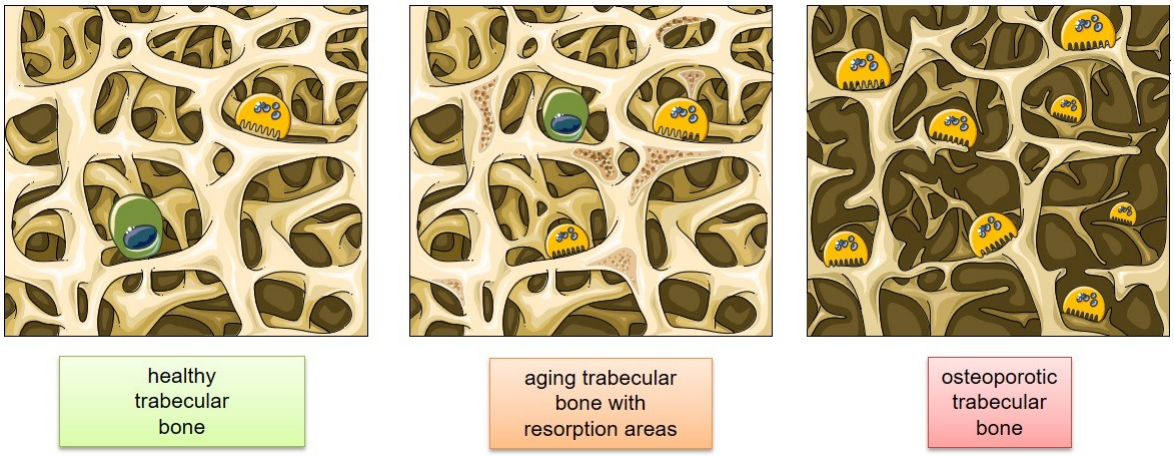

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Figure 6 | Development of osteoporosis: Healthy bone is characterized by a balanced cycle of remodeling performed by osteoblasts and osteoclasts. Further aging or diseases lead to an imbalanced process and the development of osteoporosis demonstrated by an increase of osteoclast number and activity.

The standard method for diagnosing osteoporosis is dual-energy x-ray absorptiometry (DXA), which measures the bone mineral density (Tu et al. 2018). According to the World Health Organization (WHO) criteria, osteoporosis is classified as a bone mineral density (BMD) that ranks 2,5 standard deviation below the mean for healthy subjects referred as the T-score (Butz et al. 1994). Up to 50% of women and 20% of men older than 70 years are suffering from low bone density and are confronted with a high risk

of fractures with the consequences of disability, mortality and costs (Coughlan and Dockery 2014). Due to change in demographics, osteoporosis is a huge problem of our future health system. However, therapeutic options for treatment are limited. Supportive therapy, like mobilization and building strength in muscles, supportive gadgets for walking and optimizing levels of calcium and vitamin D can help. Yet, hormonal replacement is the only causal therapy and is just available for some sub-forms of osteoporosis. Class A drugs have been consistently shown to have positive fracture-lowering effects. These include bisphosphonates, selective estrogen receptor modulators (SERM) and human monoclonal antibody of RANKL, Denosumab.

Bisphosphonates are the most common and have been used since the 1960s. They have been extensively studied and updated and are available in multiple formulations. They develop their effect by cumulating in the bone matrix and continuous release during osteoclastic bone resorption. They interfere with cellular processes and lead to early apoptosis (Shaw and Bishop 2005). Due to their accumulation, they have been found to remain in the bone matrix for years after the end of therapy negatively affecting bone metabolism (Rauch et al. 2006). They are also reported to be associated with a complication called osteonecrosis of the jaw (Lim and Bolster 2015). Besides increasing concern about their long-term safety, bisphosphonates are the first line therapy to many diseases associated with low bone density.

SERMs are a class of compounds that interact with estrogen receptors as agonist or antagonists. Raloxifen, specifically designed for postmenopausal osteoporosis, has proven to be a highly versatile drug for treatment (Gennari, Merlotti, and Nuti 2010). Nevertheless, SERMs are less potent on the skeleton than estrogen and their specific effect remain uncertain. Additionally, they are increasingly abandoned due to many side effects including vaginal bleeding, hot flashes, venous thromboembolism, strokes and cancer (Prestwood and Raisz 2002).

Latest studies of Denosumab revealed a significant decrease in bone resorption without penetration of the bone matrix making them an better alternative to bisphosphonates for long term therapy (Kearns, Khosla, and Kostenuik 2008). However, some clinical studies report a significant risk of hypocalcemia and a loss of bone mineral density over time (Iseri et al. 2018).

Little progress has been made since the discovery of Denosumab and the management of osteoporosis is still challenging. Osteoporosis induced fractures reflect a high economic burden, increased morbidity and mortality, that urgently need further

improvements in prevention strategies. There is mounting evidence for the importance of understanding the cross talk between metabolic control and signaling pathways to find new potential targets for therapies. Despite years of research, the complex connection between metabolism and cell function remains an exciting field of investigation.

2.1.4. Metabolic reprogramming during osteoclastogenesis

Except its stiff structure, bone is a metabolically active tissue that undergoes continuous changes throughout life. In this dynamic tissue a link between metabolism regulation and cell differentiation was already described before (Lemma et al. 2016). Within the basic process of cell energy metabolism, glycolysis is used as an upstream path to further steps in the mitochondria. There, the Krebs cycle and the oxidative phosphorylation rapidly process the end product of glycolysis, pyruvate, to CO_2. Together, these are highly efficient pathways for energy generation in form of adenosine triphosphate (ATP). For the complete oxidation of glucose, oxygen is required as the final electron acceptor. When oxygen is limiting, cells can redirect their metabolism away from oxidative phosphorylation. Under such anaerobic conditions pyruvate is converted to lactate and on its metabolic way it creates products allowing glycolysis and energy supply to continue. However, tumour cells and other fast proliferating cells dramatically increase the rate of glucose uptake and lactate production, even in the presents of oxygen. This process is known since the 1920s and described as the Warburg effect (Liberti and Locasale 2016). However, mitochondria remain functional and some oxidative phosphorylation continues. Nevertheless, per unit of glucose aerobics glycolysis is less efficient in the generation of ATP compared to oxidative phosphorylation leaving the question why these cells undergo such metabolic adaptions.

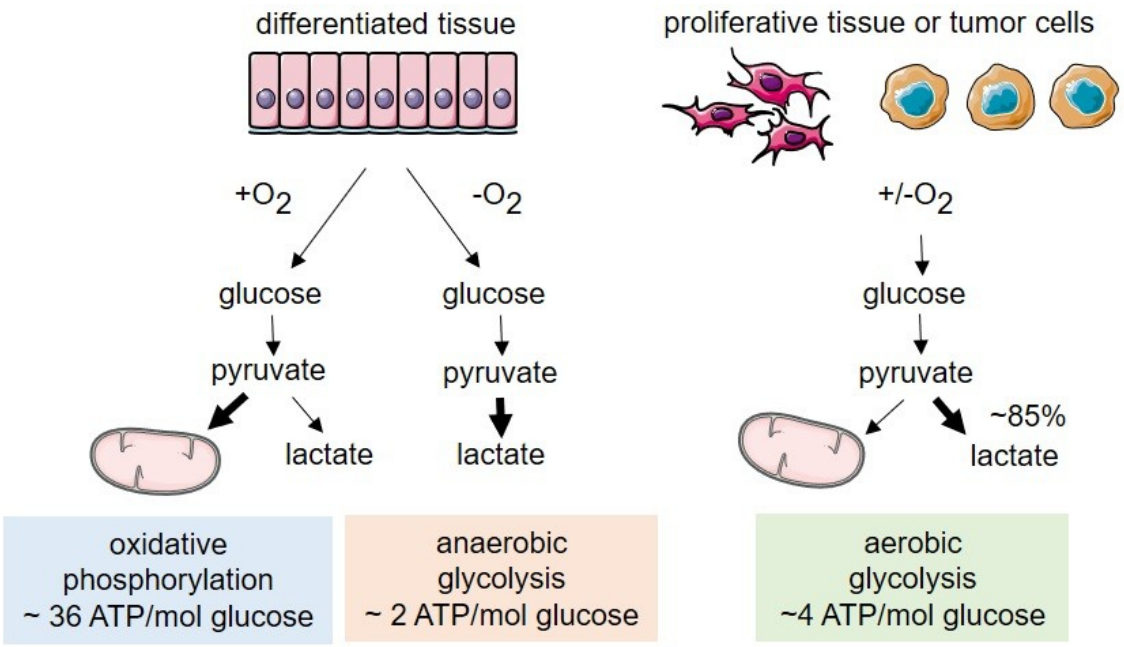

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Figure 7 | Schematic representation of oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis: In the presence of oxygen differentiated tissue metabolizes glucose via glycolysis and oxidative phosphorylation. Without oxygen cells redirect pyruvate away from oxidative phosphorylation by generating lactate via anaerobic glycolysis. Regardless of oxygen, fast proliferating tissue or tumor cells convert glucose to lactate described as aerobic glycolysis and the Warburg effect.

A growing number of data points to the reciprocal influence of cellular metabolic processes and the function of innate and adaptive immune cells. Macrophages showed to undergo defined metabolic reprogramming in response to various activation stimuli. Classically activated, pro-inflammatory macrophages increase their glycolytic activity and turn off their mitochondrial respiration, whereas anti-inflammatory, alternatively activated macrophages exhibit increased mitochondrial respiration (Van den Bossche, O'Neill, and Menon 2017). These contrasting metabolic adaptations of each macrophage subset appear to support different activation and differentiation programs promoting either the onset or resolution of inflammation. For the osteoclasts, as mononuclear phagocyte-derived cells, multiple steps of bone resorption, including osteoclast differentiation, migration, fusion, actin-reorganisation and bone degradation require high metabolic activity. Recent data indicate, that osteoclasts remain on oxidative phosphorylation as their primarily energy source to enhance differentiation,

where these cells show an increase in mitochondrial respiration (Divakaruni and Brand 2011). Moreover, Lemma et al. compared immature cells to fully differentiated osteoclasts and identified oxidative phosphorylation as the main bioenergetic source during osteoclast formation. That was demonstrated by a substantial increase of mitochondria in number and size and enrichment of cristae. Differentiated osteoclasts also revealed higher levels of enzymes of the electron transport chain and a higher mitochondrial oxygen consumption rate (Lemma et al. 2016). More recently, osteoclasts were described to actively reprogram their metabolism using aerobic glycolysis for bone resorbing activity. The expression of the key enzymes of glycolytic genes, such as hexokinase, phosphofructokinase and pyruvatekinase were found to be progressively increased toward the final maturation stage of osteoclast differentiation (Indo et al. 2013). The glycolytic enzyme aldolase was discovered to interact with some subunits of the V-ATPase in the osteoclasts ruffled border, where protons are pumped across and are used to create the acidic environment for bone resorption (Lu et al. 2004). Additionally, Miyazaki et al. reported an inverse correlation between osteoclast survival and activity through the intra- and extracellular ATP concentrations, where less intracellular ATP increased bone resorbing activity (Miyazaki et al. 2012).

To sum up the latest founding's, the processes of osteoclast differentiation and bone resorption are fast and energy intense. The osteoclast seems to adapt the metabolism according to its cellular needs relying on oxidative phosphorylation to induce differentiation and switching to aerobic glycolysis for bone resorption. During periods of high metabolic activity, reactive oxygen species accumulate and induce apoptosis to help limit bone resorption according to the motto „live fast, die young“ (Arnett and Orriss 2018). Beside all the progress, the mechanisms of osteoclast metabolism and the in vitro and in vivo consequences of these events are still missing. Not at least because of technical challenges of studying active bone resorbing cells.

To uncover the holistic osteoclast metabolism and its consequences on osteoclast function we established a new medium containing bone fragments. This allows us to distinguish between a fully differentiated but resting osteoclast (rOC) and an activated bone resorbing osteoclast (aOC). We further used extracellular flux assays and compared metabolic changes between monocytes, rOCs and aOCs. In contrast to many other immune cells aOCs are characterized by a simultaneous increase in glycolysis activity and mitochondrial respiration compared to rOCs (Fig. 1A-D). Further

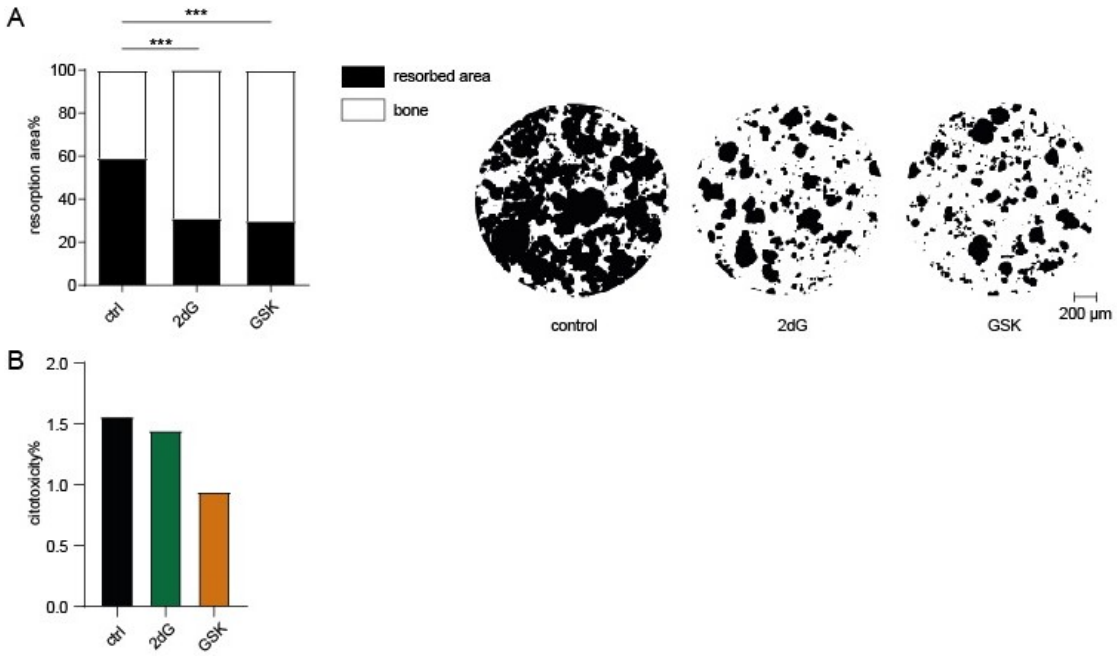
metabolic profiling showed an increase of metabolites of both, glycolysis and the Krebs cycle in aOCs (Fig. 1E). Consistent with the current state of science we found oxidative phosphorylation to be essential for osteoclast differentiation. That was shown by the functional inhibition of the mitochondrial complex I via rotenone, which significantly reduced their ability to fuse and differentiate, but only moderately effected bone resorbing activity (Fig. 2A, B). In contrast, inhibition of glycolysis by 2-desoxy-D-glucose (2dG) efficiently blocked bone resorption, but did not affect osteoclast differentiation (Fig. 2A, B). We identified aerobic glycolysis as a crucial regulator for bone resorbing activity. In accordance with a key role of the glycolytic pathway during osteoclast-mediated bone resorption in vitro we observed effective amelioration of ovariectomy-induced bone loss in mice that received 2-dG (Fig. 3A-D). Taken together, bone resorption considerably increases the metabolic and energetic demands of osteoclasts and points to the need for a supply of specific and essential metabolites derived from glycolysis and/or mitochondrial respiration.

2.1.5. Aerobic glycolysis derived lactate as crucial regulator of bone resorption

Since the discovery of the reliance on aerobic glycolysis of fast proliferating cells, such as cancer cells and innate and adaptive immune cells, much effort has been made to develop new potential targets for therapy of various diseases. The main enzyme regulating aerobic glycolysis is lactate dehydrogenase-A (LDH-A), which catalyses the conversion of glycolytic derived pyruvate to lactate. For a long period of time, lactate was considered as a dead-end waste product of glycolysis. At present, its role has shifted and most of the evidence represents lactate as an important intermediary in numerous metabolic processes. Current research showed, that LDH-A inhibition suppressed metabolic plasticity and caused quiescence of tumor growth in vitro and in vivo (Oshima et al. 2020). Another lactate dehydrogenase inhibitor suppressed tumor growth through apoptotic cell death by the production of reactive oxygen species (Kim et al. 2019). However, sufficient LDH-A inhibitor options for new fields of application are lacking.

During RANKL induced osteoclastogenesis an up-regulation of LDH-A was already discovered (Ahn et al. 2016). In our research we found gene expression levels of LDH-A significantly up-regulated in aOCs. Contrary, lactate dehydrogenase-B (LDH-B) working as an opponent to LDH-A by converting lactate back to pyruvate, was significantly decreased during bone resorbing activity (Fig.1G). Although rOCs and aOCs substantially consumed glucose, lactate production was only significantly

elevated in aOCs (Fig. 1F). Functional inhibition of glycolysis by 2-dG could be rescued by supplementation of either, pyruvate or lactate (Fig. 2C). Additionally, our supplementary figure showed blocking of osteoclast bone resorbing activity by a small molecular inhibitor of lactate dehydrogenase-A (LDH-A), GSK2837808A, similar to 2dG (suppl. Fig. A). We also proved that these effects were not caused by cytotoxic effects (suppl. Fig. B.). Suggesting a key function of lactate during osteoclast-mediated bone resorption in vitro, we observed significant improvements of ovariectomy-induced bone loss in mice that were treated with GSK (Fig. 3E-G).



Supplementary figure | GSK blocked bone resorbing activity similar to 2dG in vitro: (A) Osteoclasts were generated on bone resorption plates for 5 days and stimulated with 2dG (10µM) or GSK (1µM). The percentage of the resorbed area was quantified via Photoshop and pictures show representative images of resorption pit formation. (B) Supernatants of osteoclast resorption pits were taken, and cytotoxicity was measured and related to supernatants of medium only.

On its metabolic way, lactate creates a lot of metabolites important for nucleotide synthesis in fast proliferating cells (Vander Heiden, Cantley, and Thompson 2009). Furthermore, lactate can be a significant component to increase protons meaning a huge impact on pH levels (Gladden 2004). The dissolution of bone matrix is achieved by creating an acidic environment in the osteoclast resorption pit reaching pH levels of 4,5. It is therefore a likely possibility, that lactate and its concomitant in acidosis might

be important in the bone resorbing process. The finding that aerobic glycolysis and its metabolite lactate play a key role in osteoclast activity have opened up new possibilities for the treatment of bone-related diseases such as osteoporosis and rheumatoid arthritis.

2.2. Explanation to main material and methods

2.2.1. Evaluation of osteoclastogenesis by TRAP staining

Previous data showed an active metabolic reprogramming during osteoclastogenesis. To comprehend the metabolic adaption, we unraveled the metabolic profiles at different stages during osteoclast differentiation and activity. Osteoclast differentiation can be analyzed through their fusion by staining. Therefore, we isolated primary bone marrow cells from wild type mice and cultured them in medium containing M-CSF to generate preosteoclasts. After 24 hours, we transferred non-adherent cells and incubated them in medium containing M-CSF and RANKL. As described before, these specific factors induce osteoclast differentiation, fusion and the expression of genes, which typify the osteoclast lineage, such as tartrate resistance acid phosphatase (TRAP) (Boyle, Simonet, and Lacey 2003). TRAP is an iron containing enzyme and osteoclasts show an intense staining towards it (Hayman 2008). It has proven to be the standard method for detection of osteoclasts. It is available as a kit and the staining results can be easily detected by light microscopy (Filgueira 2004). After four to six days of incubation cells are fully differentiated and fused.

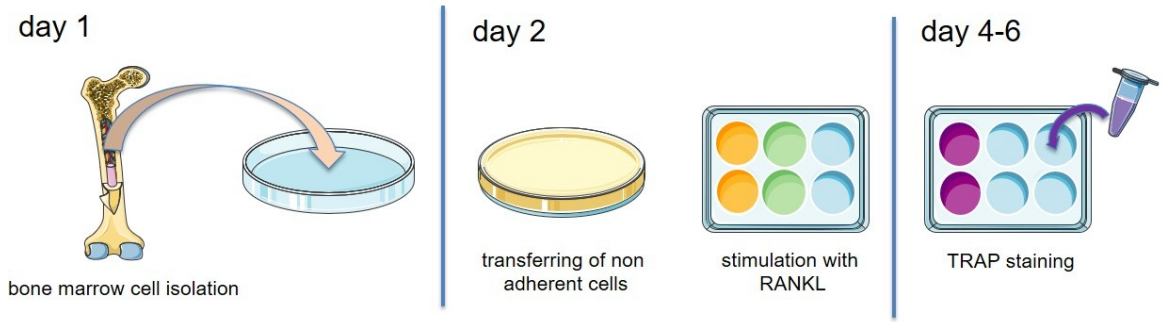


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Figure 8 | TRAP staining: Bone marrow stem cells were isolated and incubated in medium containing M-CSF. On the next day, non-adherent cells were transferred and osteoclastogenesis was induced via RANKL. After four to six days of incubation, cells were stained with TRAP.

For the quantification of the osteoclast differentiation, we took representative photos of the stained cells using a Zeiss Axioskop 2 microscope. We identified a fully differentiated and multinucleated osteoclast as having three or more nuclei per cell. For final detection of the fusion index, the absolute number of cells with the same number of nuclei were set in ratio with the total number of cells per setting. The resulting cumulative frequency in percentage defined osteoclast differentiation by fusion efficiency.

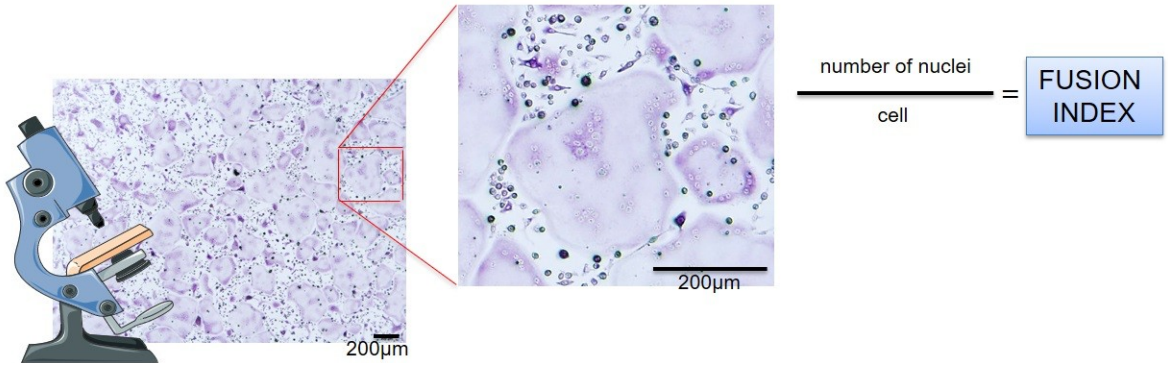

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Figure 9 | Fusion index: Representative pictures of TRAP-stained osteoclasts were taken and the number of nuclei per cell were counted to calculate the fusion efficiency.

2.2.2. Production of bone powder medium

Due to technical circumstances, analysis of the metabolic adaption during bone resorbing activity have been missing. Therefore, we established a variation of the cell culture medium containing bone powder to stimulate osteoclast function allowing us to distinguish between a group of resting and active osteoclasts and uncover the differences between their metabolic profiles. For the medium, bovine bone was crushed into fine fragments until it was suitable for pipetting. For cell culture application, the powder was dissolved in fetal calf serum (FCS) and sterilized by heat inactivation and UV light treatment.

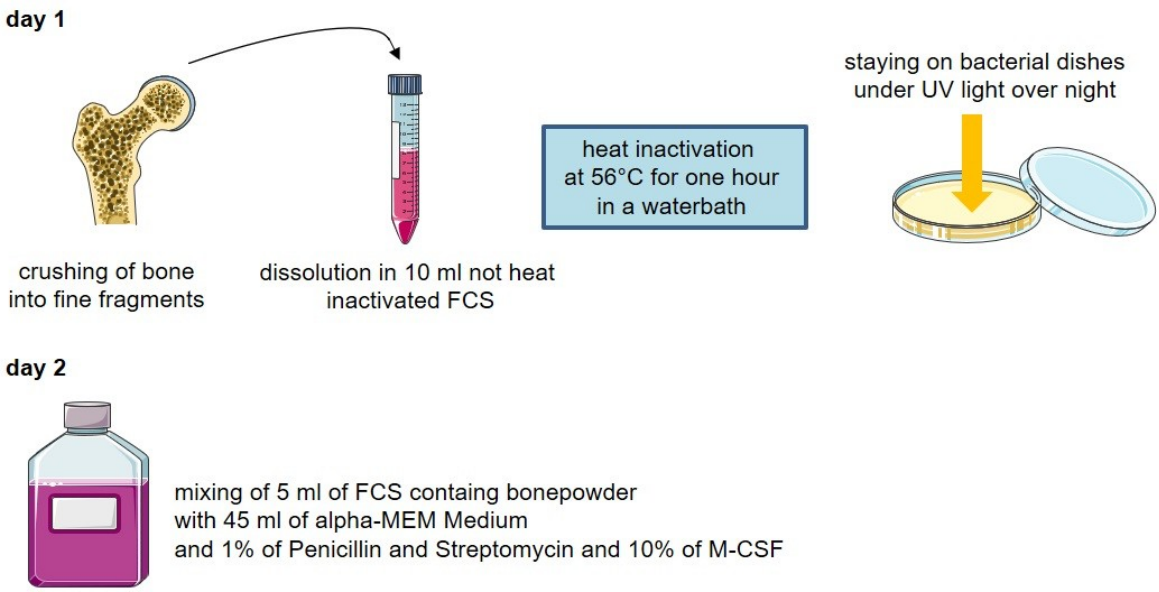

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Figure 10 | Production of bone powder medium: On the first day, bone was crushed into fine fragments and dissolved in FCS. For further cell culture usage, serval steps of sterilization like heat inactivation and UV light treatment followed. On the next day, FCS containing bone powder was mixed with alpha-minimum-essential-medium (MEM) and further factors necessary for osteoclast differentiation.

The application of bone powder did not affect fusion capacity, osteoclast number or the expression of differentiation marker (Fig. 1A, B). With further staining and experiments we were now able to reveal differences in the metabolic profiles of rOCs and aOCs.

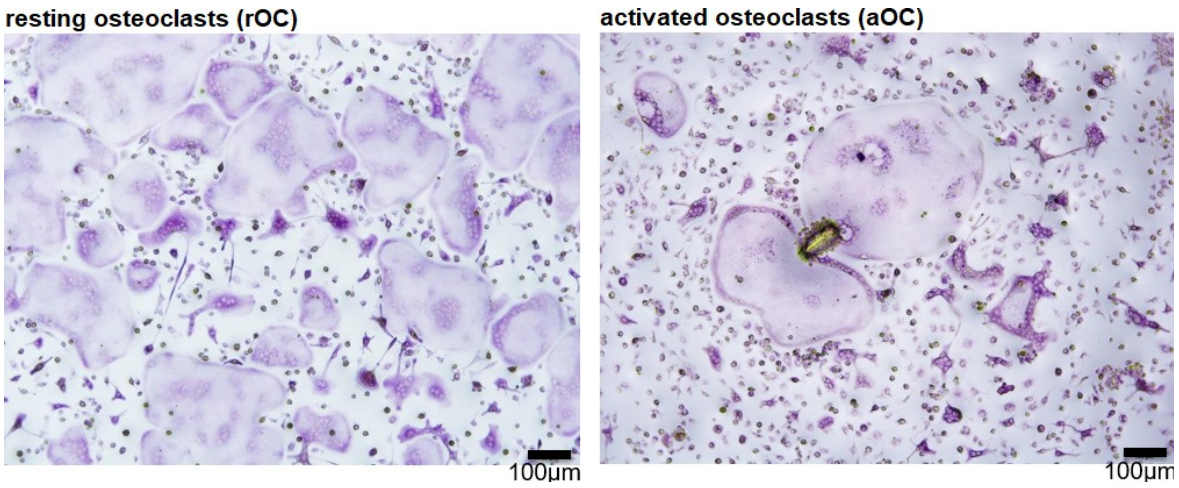

Figure 11 | Bone powder medium: Representative images of TRAP-stained osteoclasts with differentiation medium referred to as rOCs and bone powder medium representing aOCs.

2.2.3. Seahorse analysis for live measurements of cell metabolism

Efficient and accurate evaluation of the metabolic profile is crucial for understanding pathologies as well as facilitating novel therapeutic developments. The bone powder medium in addition to the seahorse assay allows real time cell measurements and provide a clear window into critical cell functions. Seahorse XF Flux Analyzer measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells in a multiway plate. Through compound addition it interferes with key cellular functions, such as mitochondrial respiration and glycolysis providing a systemically metabolic view of the cells. To prepare for the assay, we isolated mouse bone marrow derived cells and cultured them in growth medium, growth medium in addition with RANKL, or bone powder medium and RANKL for 48 hours. Through this, we defined three different groups of cells including monocytes, rOCs and aOCs.

The glycolysis stress test measures the ECAR, which indicates the glycolic function of the cell. For this assay, the cells are periodically incubated with medium free of glucose or pyruvate and the ECAR is measured. The first injection is glucose, which stimulates the glycolytic pathway and produces protons. These protons differ into the medium causing a rapid increase in ECAR. This response is reported as the rate of glycolysis under basal conditions. The second injection is the inhibitor oligomycin, which blocks complex V of the respiratory chain and forces the cell to shift from oxidative phosphorylation to glycolysis revealing the cells maximum glycolysis capacity. The last inhibitor is 2-dG, a glucose analog which blocks the first enzyme of glycolysis, hexokinase. This proves, that the ECAR produced in this experiment is due to glycolysis. Finally, glycolytic reserve is calculated by the difference between glycolytic capacity and glycolytic rate (Agilent user guide kit, glycolysis stress test, 2019).

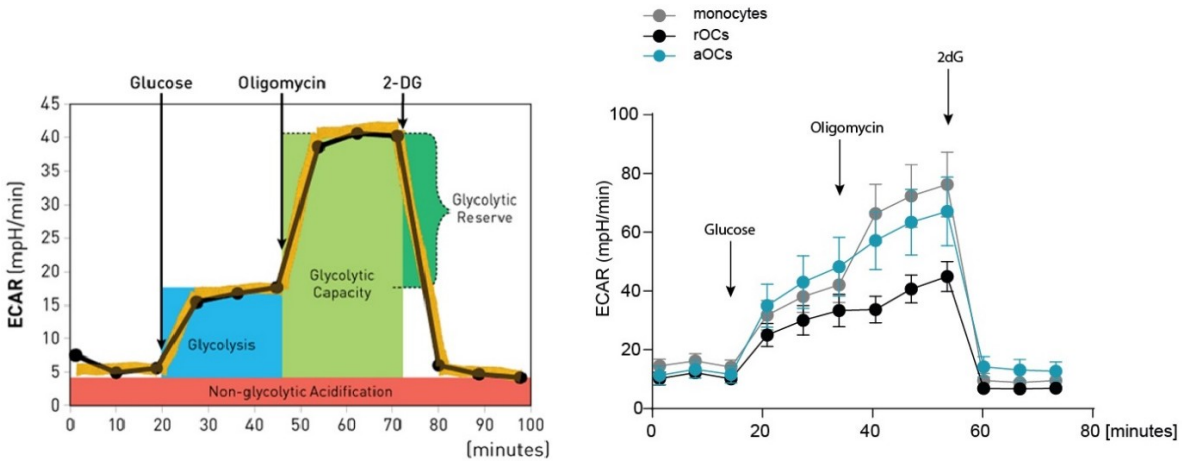

Figure 12 | Glycolytic stress test: Extracellular acidification rate (ECAR) including glycolysis, glycolytic capacity and glycolytic reserve. Left graph shows ECAR according to the manufacture description from Agilent (Agilent user guide kit, glycolysis stress test, 2019). Right graph shows measured ECAR rates from monocytes, rOCs and aOCs after 48 hours.

Within the mitochondrial stress test, OCR is measured before and after the addition of several inhibitors. Firstly, baseline cellular OCR is measured, from which non-mitochondrial respiration is distracted to generate basal respiration. The initial inhibitor of the mitochondrial stress test is oligomycin. Thereby, ATP associated respiration and proton leak respiration can be calculated. Following, carbonyl cyanide-p-trifluorometho-xyphenyl-hydrazon, FCCP, a protonophore, is added to collapse the inner membrane gradient. Now, the cell is forced to its maximal function rate and maximal respiratory capacity. Finally, antimycin A and rotenone, inhibitor of complex III or I, are given to shut down electron transport chain function leading to the non-mitochondrial respiration. Mitochondrial reserve capacity can be calculated by substructure basal respiration from maximal respiratory capacity (Agilent user guide kit, mito stress test, 2019).

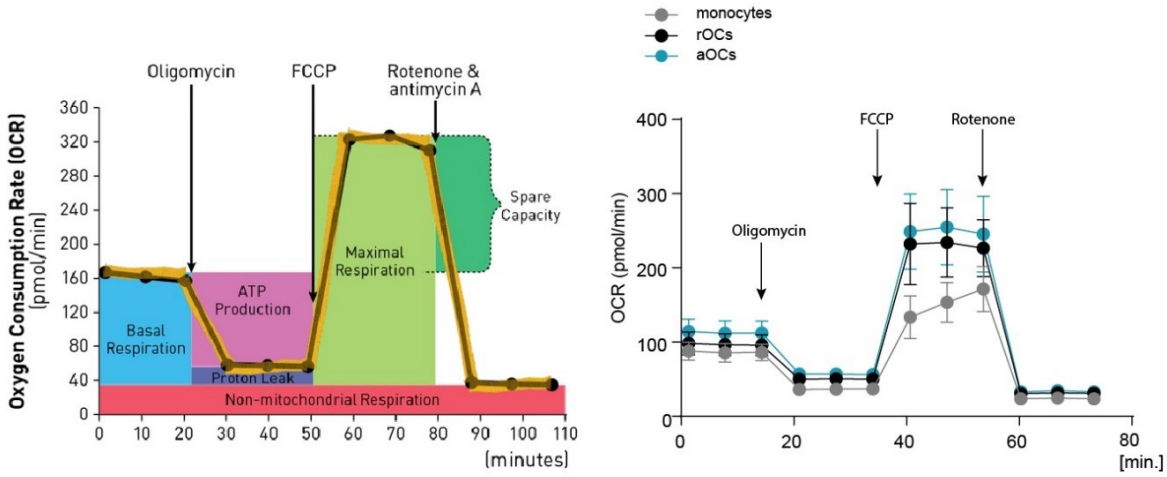

Figure 13 | Mitochondrial stress test: Oxygen consumption rate (OCR) including basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity. Left graph shows OCR according to the manufacture description from Agilent (Agilent user guide kit, mito stress test, 2019). Right graph shows measured OCR rates from monocytes, rOCs and aOCs after 48 hours.

2.3. Additional results and methods

2.3.1. Effect of glycolysis and lactate inhibition on other cells and bone parameters

Osteoporosis affects about 200 million people worldwide. The treatment of bone related diseases is still challenging and further studies are important to establish new prevention strategies (Yousefzadeh et al. 2020). To evaluate the potential of the glycolytic pathway and its metabolite lactate as therapeutic targets for the treatment of osteoporosis, we decided to study the effect of blocking both in a mouse model of ovariectomy induced osteoporosis. The ovariectomy (OVX) mimics estrogen-deficiency bone loss and shows clinical manifestation of postmenopausal osteoporosis.

Our data represents blocking of glycolysis and lactate with 2dG and GSK as an efficient strategy to interfere with pathological bone loss. Both treatments revealed significantly increased bone mass in osteoporotic mice, but also slightly decreased bone density in healthy mice, classified as the sham group (Fig. 3C, D, F, G). Albeit, the underlying reasons need to be explored, blocking glycolysis likely affects other cell types, such as osteoblasts, which are equally important for bone homeostasis. Osteoblasts were

shown to use glucose as a major nutrient and glycolysis is suggested to be essential for bone formation (Esen and Long 2014). Moreover, metabolites derived from glycolysis were recently found to be critical for the structure of the apatite nanocrystals providing stability and strength (Costello et al. 2012). Osteoblasts were also shown to evenly require mitochondrial respiration and fatty acids for the mineralization process (Müller et al. 2020). Increasing studies highlight the relevance of understanding the connection between metabolic regulation and signaling pathways. However, the complex link between metabolism and cell function in bone homeostasis remain uncertain.

In our osteoporosis mice model, we analyzed the effect of glycolysis and lactate inhibition on osteoblasts and further bone parameters. The number of osteoblasts on tibial bone sections seemed slightly lowered in mice treated with GSK compared to the untreated osteoporotic group (Fig. 14A). Interestingly, the mineral apposition rate, which represents bone formation, was highest in OVX mice treated with 2dG (Fig. 14B). To further determine differences in bone remodeling rates, bone formation and resorption markers were measured in blood plasma samples. Type 1 collagen is the predominate connective tissue in tendon, ligaments and bone, which exerts metabolic and inflammatory activity. Six weeks after start of treatment, procollagen type 1 levels in serum were significantly higher in the OVX group compared to the sham group and could be efficiently lowered by 2dG treatment (Fig. 14C). Moreover, excessive collagen type 1 degradation is part of several diseases associated with pathological bone loss like rheumatoid arthritis (Cremer, Rosloniec, and Kang 1998). After three weeks, collagen type 1 degradation was highest in mice suffering from osteoporosis group and could be effectively lowered with 2dG treatment (Fig. 14D). Although, this tendency disappeared after six weeks, and collagen type 1 degradation seemed highest in the sham group treated with 2dG. These results suggest that aerobic glycolysis and lactate not only regulate osteoclastic bone resorption, but also influence osteoblast function and thus bone formation.

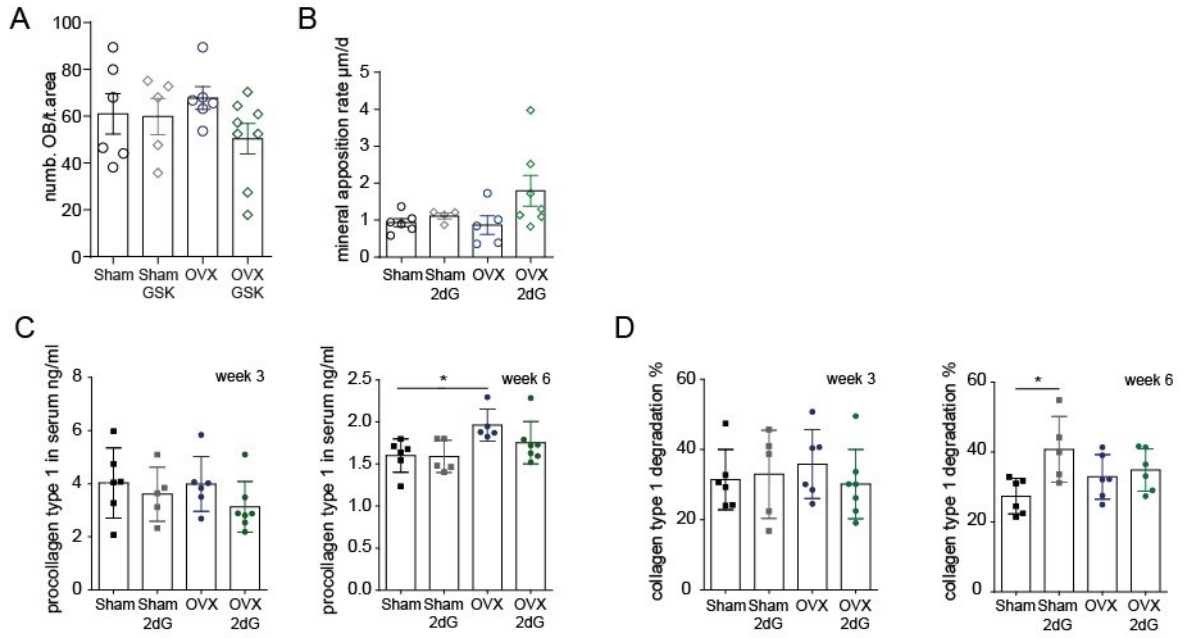

Figure 14 | Effect of glycolysis and lactate inhibition on other bone cells and bone parameters: (A) The number of osteoblasts according to the total area of tibia plateau were counted on methacrylate-embedded undecalcified sections of tibial bones in toluidinblue staining. (B) Calcein was injected 7 and 2 days before killing. Calcein accumulation in bone was analyzed with the osteomeasure software and microscope and shown as the mineral apposition rate in µm/d. (C) PINP EIA from blood. Type 1 procollagen in Serum from 2dG treated mice after three and six weeks showing the bone formation rate. Blood serum was collected from vena facialis. (D) Ratlaps ELISA from blood. Collagen type 1 degradation after three weeks and six weeks of 2dG treatment. Blood was collected from vena facialis.

Taken together, our data identified aerobic glycolysis and its metabolite lactate as crucial regulators of osteoclast activity. Although, the entire influence on bone homeostasis is not fully understood, their blocking represents a potential therapeutic intervention in diseases characterized by accelerated osteoclast-mediated bone loss such as osteoporosis and rheumatoid arthritis.

3.2.2. Histomorphometry

We performed all histomorphometric analyses with the OsteoMeasure Analysis System (Osteometrics©) to determine osteoclast and osteoblast parameters. Tibial and vertebral bones were fixed overnight in 4% formalin and then decalcified in “Teitle Buffer” (14% EDTA (Sigma)) at 4°C (the pH was adjusted to 7.2 by adding ammonium

hydroxide (Sigma) until the bones were pliable. Serial paraffin sections (2µm) from bones. Histomorphometry for osteoblasts and bone formation parameters was performed on methacrylate-embedded undecalcified sections of tibial and vertebral bones. The quantification of osteoblasts numbers was performed on Toluidinblue stained sections. For assessment of bone formation mice were injected with 5 mg/kg body weight of green fluorescent Calcein (Sigma) 7 and 2 days before killing. Mineral apposition rate (MAR) was measured in methacrylate sections of tibiae by measuring the double labelled fluorescent lines, which represents the newly formed bone, using the OsteoMeasure software (Osteometrics©) with a wavelength of 495 nm.

3.2.3. ELISA

For enzyme-linked immunosorbent assay (ELISA), blood was harvested by puncture of vena facialis after 3 weeks and 6 weeks of treatment. Serum was separated from blood by centrifugation. The serum of week 3 was stored at -80 C until assayed. To determine difference in bone remodeling rates, bone formation and resorption markers were measured in plasma samples. This was done using a sandwich ELISA osteocalcin kit (Mouse Osteocalcin EIA Kit; Biomedical Technologies Inc, Stoughton, MA, USA) and a C-terminal telopoptides of type I collagen ELISA kit (RatLaps TM, IDS, Boldon, UK) respectively, and performed according to manufacturer's instructions.

3.2.4. Cytotoxicity assay

Cytotoxicity assay was performed with CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega as a measurement of lactate dehydrogenase (LDH) release into the supernatant according to the manufactors protocol. During experimental treatment, supernatant samples were cleared from non-adherent cells and transferred to a 96 plate and an equal volume of CytoTox 96® reagent is added to each well and incubated for 30 minutes. Stop solution is added and the absorbance signal is measured at 490nm in a plate reader. Results were calculated by subtracting the average values of the culture medium background from all values of experimental wells to compute percent cytotoxicity.

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3. Original paper

JuleTaubmann, Brenda Krishnacoumar, Christina Böhm, Maria Faas, Dorothea I. H. Müller, Susanne Adam, Cornelia Stoll, Martin Böttcher, Dimitrios Mougiakakos, Uwe Sonnewald, Jörg Hofmann, Georg Schett, Gerhard Krönke & Carina Scholtysek

„Metabolic reprogramming of osteoclasts represents a therapeutic target during the treatment of osteoporosis“, nature, scientific reports, Nov. 2020

doi: 10.1038/s41598-020-77892-4

https://pubmed.ncbi.nlm.nih.gov/33273570/

4. Attachement

4.1. List of abbreviations

2dG	2-desoxy-D-glucose
α-MEM	alpha-minimum-essential-medium
µCT	micro–computed tomography
aOC	active osteoclast
AP-1	activating protein 1
ATP	adenosine triphosphate
BMD	bone mineral density
BMDM	bone marrow derived monocyte
BMM	bone marrow-derived macrophage
BV/TV	bone-volume/total-volume
Cit	citrate
CO_2	carbon dioxide
Ctrl	control
c-Fms	macrophage colony-stimulating factor receptor
DMSO	dimethylsulfoxid
DXA	dual-energy x-ray absorptiometry
ECAR	extracellular acidification rate
EDTA	ethylendiamintetraacetat
ELISA	enzyme-linked immunosorbent assay
FCCP	carbonyl cyanide-p-trifluorometho-xyphenyl-hydrazon
G6P	glucose 6-phosphate
glut1	glucose transporter 1
GSK2837808	selective lactate dehydrogenase-A inhibitor
H^+ATPase	proton adenosine-triphosphatase
HK2	hexokinase 2
HSC	haematopoietic stem cell
KX	ketamine-xylazine
LDH	lactate dehydrogenase
LDH-A	lactate dehydrogenase-A
LDH-B	lactate dehydrogenase-B
MAPK	mitogen-activated protein kinase

MCS-F	macrophage colony-stimulating factor
mRNA	messenger ribonucleic acid
NAD^+	nicotinamid-adenin-dinukleotid
NFATc1	nuclear factor of activated T-cells c1
O_2	oxygen
OC	osteoclast
OCP	precursor cells of osteoclast
OCR	oxygen consumption rate
OPG	osteoprotegerin
OVX	ovariectomy
pdk4	pyruvate dehydrogenase lipoamide kinase isozyme 4
PINP EIA	N-terminal propeptide of type I procollagen enzyme immunoassay
RANK	receptor activator NF- κB
RANKL	receptor activator NF- κB ligand
rOC	resting osteoclast
SERM	selective estrogen receptor modulators
SRC	spare respiratory capacity
Tb.N	trabecular number
Tb.Sp.	trabecular separation
Tb.Th.	trabecular thickness
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor- α
TRAF 6	receptor-associated factor 6
TRAP	tartrate resistance acid phosphatase
UV light	ultraviolet light
V-ATPase	vacuolar type adenosine-triphosphatase
WHO	World Health Organization
XF analyzer	extracellular flux analyzer

5. List of publications

Müller DIH, Stoll C, Palumbo-Zerr K, Böhm C, Krishnacoumar B, Ipseiz N, Taubmann J, Zimmermann M, Böttcher M, Mougiakakos D, Tuckermann J, Djouad F, Schett G, Scholtysek C, Krönke G. PPARδ-mediated mitochondrial rewiring of osteoblasts determines bone mass. Sci Rep. 2020 May 21;10(1):8428. doi: 10.1038/s41598-020-65305-5. PMID: 32439961; PMCID: PMC7242479.

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6. Acknowledgement

This work was supported by the IZKF Erlangen (J.T.), the Deutsche Forschungsgemeinschaft (FG 2886 „PANDORA“ to G.K., D.M. and G.S. as well as the CRC1181 to G.K. And G.S.). Additional support was provided by the Emerging Field Initiative (EFI) of the Friedrich-Alexander University Erlangen-Nürnberg (FAU) and the STAEDLER Stiftung (EFI_VERBUND_MED_05_MIRACLE to G.K., U.S. and J.H.) and the Bundesministerium für Bildung und Forschung (BMBF) (METARTHROS to G.K. And G.S.) and the European Union (Horizon 2020 ERC-2014-StG 640087 – SOS to G.K. And Horizon 2020 ERC-2018-SyG nanoSCOPE as well as RTCure to G.S.).

I would like to thank everyone who contributed at various levels to the creation and completion of this work.

First and foremost, I would like to thank Prof. Dr. med. Georg Schett, who, as director of the department of Internal Medicine 3, Rheumatology and Immunology, at the Friedrich-Alexander University Erlangen-Nürnberg, made it possible for me to write my dissertation at his department.

I would like to thank Prof. Dr. med. Gerhard Krönke in particular. As part of his working group, I got the opportunity to do this experimental work. He was always available for questions and problems and supported me. Due to the very pleasant working atmosphere and the interesting inspirations, it was a great honor to be able to do the work under his supervision.

Many thanks to Dr. rer. nat. Carina Scherbel, who always supported me with advice and action and accompanied me through the entire time with her excellent supervision.

Special thanks to Brenda Krishnacoumar, who always helped me with advice and support during the experimental design, performance and evaluation.

Further thanks to Cornelia Stoll, who showed me the experimental procedures and working protocols in the laboratory.

I also thank Dana Weidner for excellent technical assistance and Monica Pascul for helping with performing the in vivo experiments.

I would also like to thank the other members of the working group for always being available to answer my questions about the experimental setup and evaluation.

A special thanks to my parents, family and friends, who always helped me during all stages of my studies and during the preparation of this thesis by their interest and support.