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The Interactions Between Glucose and Bone-forming Osteoblasts

Milja Arponen

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To my family

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ABSTRACT

Bone is an active tissue that undergoes constant remodelling. Bone-forming osteoblasts use various energy sources to meet their energy demand, and one of the main energy sources of the cells is glucose. Glucose is transferred into the cell via passive transportation through the protein family of glucose transporters. In disorders of glucose metabolism, such as type 2 diabetes, bone metabolism is disturbed, and patients with type 2 diabetes have an increased risk of fragility fractures. Diabetes is characterized by elevated blood glucose levels, hyperglycaemia, and long-term hyperglycaemia impairs the functions of osteoblasts. The mechanisms responsible for these changes are complicated and not yet fully understood.

In this thesis, multiple glucose transporters were shown to be expressed and have unique functions in rat osteoblasts and their precursors, mesenchymal stromal cells, *in vitro*. Further, short- and long-term exposures to hyperglycaemia were shown to have different responses in the osteoblast transcriptome. Long-term hyperglycaemia decreased the proliferation of osteoblasts, whereas short-term exposure to hyperglycaemia increased the expression of genes related to osteoblast differentiation and function. In addition, a new recombinant antibody-based immunoassay was developed to measure osteoblast-specific, non-carboxylated form of osteocalcin in human blood samples. Negative associations between type 2 diabetes, blood glucose levels and bone formation were demonstrated with this assay.

In summary, osteoblasts rely on several glucose transporters to ensure sufficient energy, and long-term exposure to high glucose decreases the functions of osteoblasts. A proper balance in glucose metabolism is necessary for proper bone formation.

KEYWORDS: osteoblast, glucose metabolism, energy metabolism, glucose, type 2 diabetes, hyperglycaemia, mesenchymal stromal cell

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TIIVISTELMÄ

Luu on aktiivinen kudos, joka käy läpi jatkuvaa uudismuodostusta. Luuta muodostavat osteoblastit käyttävät luunmuodostuksessa eri energianlähteitä ja yksi pääenergianlähteistä on glukoosi. Glukoosi siirtyy soluun passiivisesti glukooosinkuljettajaproteiinien kautta. Glukoosiaineenvaihdunnan sairauksissa, kuten tyypin 2 diabeteksessa, luun metabolia on häiriintynyt ja diabeetikoilla on lisääntynyt luunmurtumisriski. Diabeteksessa veren glukoosipitoisuus on kohonnut ja pitkäaikaisen kohonneen verensokerin eli hyperglykemian onkin todettu heikentävän osteoblastien toimintaa. Näiden muutosten taustalla olevat mekanismit ovat monimutkaisia, eikä niitä vielä täysin tunneta.

Tässä väitöskirjassa osoitettiin rotan osteoblastien ja niiden esiasteiden, mesenkymaalisten stroomasolujen, käyttävän useita glukoosinkuljettajaproteiineja turvaamaan glukoosinsaantinsa ja kullakin kuljettajaproteiinilla olevan oma merkityksensä solun toiminnassa. Lisäksi osoitettiin saman osteoblastisolumallin avulla ja RNA-sekvensointia hyödyntämällä, kuinka lyhyt- ja pitkäaikaisella hyperglykemialla on erilainen vaikutus osteoblastien geeni-ilmentymiseen. Pitkäaikainen altistus heikensi osteoblastien kasvua, kun taas lyhytaikainen altistus puolestaan lisäsi osteoblastien erilaistumiseen ja toimintaan liittyvien geenien ilmentymistä. Lisäksi väitöskirjassa kehitettiin uusi vasta-aineisiin perustuva määritysmenetelmä erään osteoblastiperäisen proteiinin, ei-karboksyloidun osteokalsiinin, mittaamiseksi ihmisten verinäytteistä. Määrityksen avulla todettiin tyypin 2 diabeteksella sekä veren glukoosipitoisuudella olevan negatiivinen yhteys luun muodostuksen kanssa.

Yhteenvetona voidaan todeta glukoosin olevan osteoblastien toiminnalle elintärkeää, mutta pitkäaikainen, liian korkea glukoosipitoisuus kasvuelin-ympäristössä heikentää osteoblastien toimintaa. Glukoosinaineenvaihdunnan tasapaino onkin välttämätöntä normaalin luun muodostumisen turvaamiseksi.

AVAINSANAT: osteoblasti, energiametabolia, glukoosimetabolia, tyypin 2 diabetes, glukoosi, hyperglykemia, mesenkymaalinen stroomasolu

Table of Contents

Abbreviations

AGE	Advanced glycation end product
αKG	α-ketoglutarate
ALP	Alkaline phosphatase
ATP	Adenosine 5'triphosphate
BLC	Bone lining cell
BMAT	Bone marrow adipose tissue
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
BTM	Bone turnover marker
BSP	Bone sialoprotein
COL1	Type 1 collagen
CTX-1	C-terminal telopeptide of type 1 collagen
DMP-1	Dentin matrix acidic phosphoprotein 1
ECM	Extracellular matrix
ETC	Electron transport chain
FGF	Fibroblast growth factor
GSH	Glutathione
GLUT	Glucose transporter
HbA1c	Glycated haemoglobin
HIF-1α	Hypoxia inducible factor 1 alpha
HSC	Hematopoietic stem cell
IGF-1	Insulin-like growth factor 1
IR	Insulin receptor
M-CSF	Macrophage colony stimulating factor
MEPE	Matrix extracellular phosphoglycoprotein
MSC	Mesenchymal stromal cell
mTORC	Mammalian target of rapamycin complex
NADH	Nicotinamide adenine dinucleotide
OC	Osteocalcin

OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix
OXPHOS	Oxidative phosphorylation
PHEX	Phosphate regulating endopeptidase X-linked
P1NP	Amino-terminal propeptide of type 1 collagen
PTH	Parathyroid hormone
RAGE	Receptor for advanced glycation end products
RANKL	Receptor activator of NF-kB ligand
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
ucOC	Uncarboxylated osteocalcin
SOST	Sclerostin
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor beta
tOC	Total osteocalcin
TRACP	Tartrate-resistant acid phosphatase
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Wnt	Wingless-related integration site

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Milja Arponen**, Niki Jalava, Nicko Widjaja, Kaisa K Ivaska. Glucose transporters GLUT1, GLUT3, and GLUT4 have different effects on osteoblast proliferation and metabolism. *Front Physiol.* 2022 Nov 29;13:1035516

II Niki Jalava, **Milja Arponen**, Nicko Widjaja, Terhi J. Heino, Kaisa K. Ivaska. Short- and long-term exposure to high glucose induces unique transcriptional changes in osteoblasts *in vitro*. Manuscript.

III **Milja Arponen**, Eeva-Christine Brockmann, Riku Kiviranta, Urpo Lamminmäki, Kaisa K Ivaska. Recombinant Antibodies with Unique Specificities Allow for Sensitive and Specific Detection of Uncarboxylated Osteocalcin in Human Circulation. *Calcif Tissue Int*. 2020 Dec;107(6):529–542

1 Introduction

Bones were considered as inert structures that protect our vital organs, enable movement with other musculoskeletal tissues and act as a mineral reservoir. However, bone is a very active tissue, and it is in a constant state of remodelling where old bone is degraded, and new bone is formed to repair microdamage and maintain calcium homeostasis (Clarke, 2008; Manolagas, 2000). Bone formation is performed by osteoblasts which proliferate upon maturation and produce and secrete proteins and minerals. To produce all the components of the bone extracellular matrix, osteoblasts need substantial amount of energy. The energy metabolism of osteoblasts is a complicated process with complementing pathways to fulfil the energy requirement of the cells (Chlebek & Rosen, 2023).

Type 2 diabetes (T2D) is a glucose metabolism disorder characterized by elevated plasma glucose levels, i.e. hyperglycaemia. T2D, specifically if uncontrolled, can cause multiple adverse events in several organs but also skeletal complications such as increased risk of fragility fractures (Ferrari et al., 2018). Controversially, patients with T2D have higher bone mineral density than healthy subjects (Vestergaard, 2007), suggesting a disturbance in bone formation. However, the mechanisms behind the changes in the skeleton are complex and further characterization of the molecular mechanisms behind the changes is needed.

The aim of this thesis was to study the relationship between glucose and osteoblasts, with the focus on how osteoblasts utilize glucose and to understand the changes in osteoblasts induced by hyperglycaemia. To achieve this, we first studied the expression and relevance of glucose transporters in rat osteoblasts *in vitro*. In the same model, we examined the transcriptional changes induced by exposure to hyperglycaemia. Finally, we developed a method to detect osteoblast-specific uncarboxylated osteocalcin in human blood samples to further study the link between high glucose and osteoblasts.

2 Review of the Literature

2.1 Bone – an active tissue

Bone is one of the most extensive tissues in the human body comprising of approximately 10% of the body weight. The skeleton enables movement with muscles, provides protection to vital organs and is the source of haematopoiesis. In addition, bone is a living tissue that undergoes constant remodelling where old bone is replaced with new bone. During remodelling, minerals, such as calcium, are both stored and released from bone; thus, bone participates in the mineral homeostasis (Clarke, 2008). The following chapter introduces bone structure, bone cells and bone remodelling.

2.1.1 Structure and composition

Bone is a dense tissue with a defined microarchitecture. Bones come in many shapes and sizes with unique functions: flat bones of the skull and rib bones protect our organs, while irregular bones have variable shape, such as spine and the sacrum. Short bones in the wrists and ankles enable fine movements and long bones provide support, movement, and site for haematopoiesis (Clarke, 2008).

The structure and morphology of the long bone is presented in Figure 1. The ends of the long bones are called epiphyses, and the middle part is diaphysis. Between epiphysis and diaphysis is metaphysis, which contains the growth plate. Structurally bone is divided into cortical (80%) and trabecular (20%) bone. Cortical bone is dense and tough and forms the outer layer of long bones. The outer surface of the cortical bone is called the periosteum, and it contains nerves and blood vessels while the inner surface is called the endosteum which has higher remodelling activity than periosteum (Clarke, 2008; Isojima & Sims, 2021). Cortical bone is constructed of circular-shaped osteons, which form the structural and functional units. Osteons consist of bone extracellular matrix and bone cells, osteocytes, embedded in the bone matrix, which are connected through a lacuno-canalicular network. In the middle of the osteons are Haversian canals containing nerve and blood vessels, which are connected through osteons via Volkmann canals. Osteons can be divided into primary and secondary osteons according to their maturity (Chang & Liu, 2022;

Clarke, 2008). Cortical bone lays on top of trabecular bone although the cortical bone thickness can vary depending on the bone site. Trabecular, also called cancellous bone, has a large surface area due to its honeycomb-like structure. It is less dense and without a similar osteon structure as cortical bone. Trabecular bone consists of a network of lamellar bone plates stacked with lesser parallel orientation forming the porous texture. Trabecular bone consists of packets that are semilunar in shape forming layers of lamellae. Nutrients of the trabecular bone are taken in passively from the bone marrow (Clarke, 2008; Oftadeh et al., 2015; Osterhoff et al., 2016).

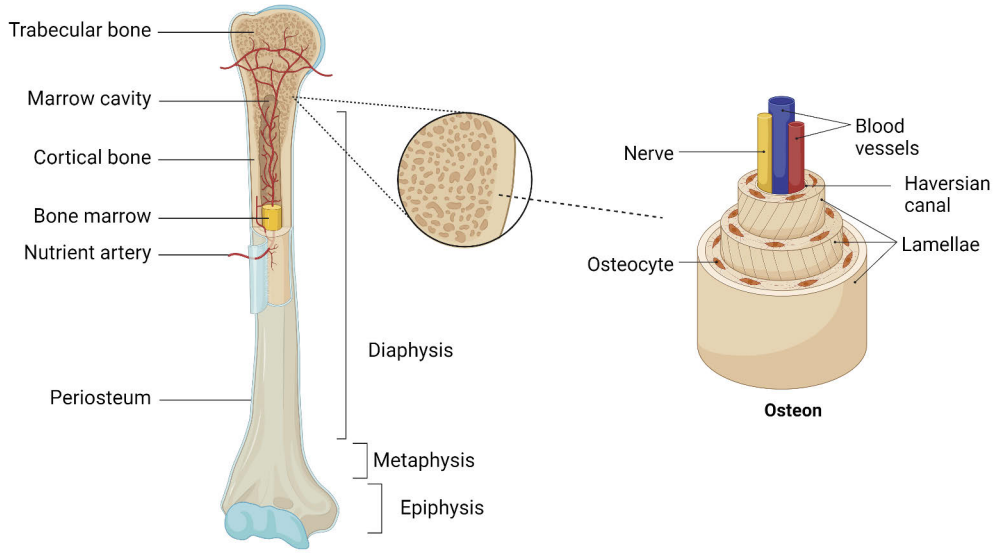

Figure 1. Structure and microarchitecture of the long bone. The ends of the long bones are called epiphyses; next is the metaphysis containing the growth plate, and the middle part is the diaphysis. The diaphysis contains a marrow cavity where bone marrow lays. Cortical bone is dense and covers cancellous trabecular bone. The outer surface of the cortical bone is the periosteum. Cortical bone consists of osteons, which are defined as circular structures consisting of extracellular matrix and osteocytes. In the middle is the Haversian canal, containing nerve and blood vessels. Osteons are surrounded by concentric rings of extracellular matrix called lamellae. Created with Biorender.

Bone marrow contains multiple cell types and is a protein and nutrient-rich microenvironment. In the bone marrow, there are two distinct stem cell populations relevant to bone biology: hematopoietic stem cells (HSCs) and skeletal stem cells, also called mesenchymal stromal cells. HSCs reside in the HSC niche and provide the origins for e.g erythrocytes and osteoclasts. Skeletal stromal cells, on the other hand, are the origins of many cells, which are introduced further in chapter 2.1.2.1. Bone marrow is heavily vascularized for oxygen supply, nutrient exchange, red

blood cell release, and to release and receive factors for interaction with other tissues of the body (Bianco, 2011; J. Chen et al., 2020).

Bone is constituted of bone extracellular matrix (ECM) which can be divided to inorganic (50–70%) and organic (20–40%) matrix. In addition, it contains water (5–10%) and lipids (<3%). However, the composition of the ECM depends on the sex, age, health conditions and structure of the bone. For example, trabecular bone has lower calcium content and higher water content compared to cortical bone (Clarke, 2008; X. Lin et al., 2020). Inorganic material consists largely of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which gives the matrix its toughness and stores minerals. The organic component of ECM is further divided into collagenous and noncollagenous proteins. Collagenous proteins cover over 90% of the ECM proteins, of which type 1 collagen (COL1) is the most abundant. COL1 is a large protein encoded by *COL1A1* and *COL1A2* -genes. The proteins form a triple helical structure in which individual chains are connected via cross-links involving lysine or hydroxylysine (Schlesinger et al., 2020; Viguet-Carrin et al., 2006). Hydroxyapatite and COL1 form the basis of bone ECM and the formation of collagen fibrils enables other ECM proteins to attach and regulate the amount and size of hydroxyapatite accumulation and, thus, bone stiffness. Noncollagenous proteins consist of various proteins that are classified into proteoglycans (e.g. decorin and keratocan), gamma-carboxyglutamic acid-containing proteins (e.g. osteocalcin (OC) and periostin), glycoproteins (e.g. osteonectin), and small integrin-binding ligand N-linked glycoproteins (e.g. bone sialoprotein (BSP), osteopontin (OPN), dentin matrix acidic phosphoprotein 1 (DMP-1) or matrix extracellular phosphoglycoprotein (MEPE)). When released from the matrix upon resorption, proteins can regulate the functions of cells in their surroundings or be taken into circulation and are involved in whole-body homeostasis (X. Lin et al., 2020).

2.1.2 Bone cells

The maintenance of bone tissue is performed by bone cells which come in two lineages: bone-forming osteoblast lineage and bone-resorbing osteoclast lineage.

2.1.2.1 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs), sometimes referred to as skeletal stem cells, were discovered by Friedenstein and colleagues in the 1970s as they found fibroblast colonies from guinea-pig bone marrow that were inducible to osteogenesis *in vitro* (Friedenstein et al., 1970). Years later, Arnold Caplan named the cells mesenchymal stem cells due to the cells having stem-like properties (Caplan, 1991). However, as the cells lack certain distinct characteristics of stem cells, such as self-renewal,

Horwitz and colleagues later suggested them to be called mesenchymal stromal cells (Horwitz et al., 2005).

MSCs are spindle-shaped and can be isolated from multiple tissues, including bone marrow, adipose tissue (Zuk et al., 2002), dental pulp (Gronthos et al., 2000) and various neonatal tissues (Fukuchi et al., 2004; O. K. Lee et al., 2004). With altering extracellular stimuli and growth factors, MSCs can be differentiated *in vitro* into multiple cell types of mesenchymal origin, such as adipocytes, osteoblasts, and chondrocytes (Pittenger et al., 1999), but also other cell types, such as myocytes (Gang et al., 2004), independent of the origin of the cells. However, the differentiation potential can vary (Kozlowska et al., 2019), and recent studies using single-cell RNA sequencing have identified subpopulations of MSCs, which prefer to differentiate into precise lineage while the other populations perish (Hou et al., 2021; S. Zhang, Wang, et al., 2021). The heterogeneous nature of MSCs causes problems with data reproducibility.

Heterogeneity and multiple origins of MSCs have led to the International Society for Cellular Therapy to propose of three minimal criteria for the definition of (human) MSCs (Dominici et al., 2006) that should be addressed in *in vitro* work. In addition to these definitions, in 2019, the committee suggested adding the origin of the MSCs in the abbreviation, with bone marrow-derived MSCs being BM-MSCs (Viswanathan et al., 2019).

- adherence to plastic
- specific surface antigen expression: positive for CD105, CD73 and CD50 and lack of CD45, CD34, CD14, CD79
- multipotent differentiation potential to osteoblast, adipocyte and chondroblast lineages *in vitro*

The three criteria can be used when working with MSCs despite species' origin, although the surface antigen expression profiles can vary, perhaps due to specificity or a lack of antibodies. Human MSC surface antigens along with mouse are the most characterized while other species, such as rat, remains largely to be identified. Furthermore, the antigen expression profiles can vary between the tissue origins of the MSCs (Uder et al., 2018).

MSCs are relatively easy to obtain from different tissues and due to their ability to differentiate into multiple tissues, interest using them for cell-based therapies is growing. The cells hold relatively well their characteristics during freeze-thaw cycles (T. Lee et al., 2023). However, differentiation potential and characteristics may alter while passaging (Ghazanfari et al., 2017). MSCs have been investigated for use in regenerative medicine, e.g. for bone, heart, liver, kidney, and wound regeneration (Margiana et al., 2022).

2.1.2.2 Osteoblasts

Osteoblasts are cubic-shaped cells that are responsible for bone formation. They were discovered in the 1920s as cells that "laid on the bone" (Keith, 1927). Osteoblasts originate from the bone marrow MSCs and their differentiation is a complicated process involving several transcription factors and external components. The master regulator of osteoblast differentiation is transcription factor runt-related transcription factor 2 (Runx2 or Cbfa1) (Ducy et al., 1997). The lack of *Runx2* gene expression in transgenic mice led to nearly non-existent ossification and homogenous mutation to death at birth (Komori et al., 1997). Runx2 upstreams transcription factor osterix (Osx or Sp7) (K. Nakashima et al., 2002) and both factors are required for osteoblast-specific gene activation and, thus, osteoblast differentiation. In addition, Akiama and colleagues showed in tracing studies in mice that before Runx2 activation, transcription factor SRY-box transcription factor 9 (*Sox9*) distinguishes both osteoblast and chondrocyte precursors from MSCs (Akiyama et al., 2005). Both Runx2 and Osx are early transcription factors of MSCs commitment to osteoblasts and after commitment, in the later stage of the differentiation, activating transcription factor 4 enhances the production of osteoblast-specific genes in mice (Xiao et al., 2005; Yang & Karsenty, 2004).

Osteoblast differentiation is regulated by multiple pathways and extracellular stimuli which activate transcription factor Runx2. The pathways include Hedgehog, Wingless-related integration site (Wnt) pathway, bone morphogenic protein (BMP), fibroblast growth factor (FGF) and Notch signalling pathways (Long, 2012). In mice studies, the Wnt-signalling pathway has been identified as crucial to osteoblast differentiation since enhancing Wnt/beta-catenin pathways increased the osteoblast differentiation (Day et al., 2005), and the lack of the signalling can change preosteoblasts to adipocyte lineage (L. Song et al., 2012). In addition to molecular pathways, systemic endocrine signals affect the cells of osteoblast lineage, such as leptin, parathyroid hormone (PTH), growth and sex hormones, and insulin, but also environmental changes, such as mechanical stimulus and hypoxia (Long, 2012).

During osteoblast differentiation, osteoblastogenesis, the cells exhibit distinct functional phases along with specific gene and protein expression patterns (Figure 2). After Runx2 activation, MSCs commit to osteoblast lineage and become osteoblast progenitors. Osteoblast progenitors start to form proteins and enzymes involved in ECM production. These proteins include mainly COL1 and alkaline phosphatase (ALP) but also lesser amounts of OPN and fibronectin (Ahmad et al., 2018; Alves et al., 2010; Stein & Lian, 1993). ALP is an enzyme which increases phosphate concentration around the cells and induces the formation of hydroxyapatites (Vimalraj, 2020). Osteoblast progenitors start to mature and proliferate excessively before further commitment to immature osteoblasts. After the proliferation phase, immature osteoblasts slow down proliferation and during the

maturation phase begin producing growing amounts of ECM-related proteins, such as ALP, COL1, BSP and smaller amounts of OC as well as mineral components. BSP is needed for the synthesis of ECM, and it can also promote osteoblast differentiation (X. Lin et al., 2020). Mature osteoblasts exhibit a cuboid-shape and they do not proliferate. Mature osteoblasts support mineralization and form ECM. Proteins produced by mature osteoblasts include large amounts of OC, OPN and phosphate-regulating endopeptidase homolog X-linked (PHEX). COL1 production is decreased (Ahmad et al., 2018; Alos & Ecarot, 2005; Khayal et al., 2018). OC is the most abundant noncollagenous protein in bone ECM. It is a small protein which is post-translationally carboxylated. The carboxylated form has a high affinity for Ca^{2+}, and it binds to hydroxyapatite in ECM. If OC remains uncarboxylated, it is unable to bind to hydroxyapatite and is secreted to circulation (Hauschka et al., 1989).

In addition to producing ECM-related proteins, osteoblasts communicate with other bone cells. Osteoblasts secrete soluble factors to maintain bone resorption. To promote osteoclastogenesis, osteoblasts secrete macrophage colony stimulating-factor (M-CSF) (Lacey et al., 1994) and receptor activation of NF-kB ligand (RANKL) (J. Li et al., 2000) into their surroundings. To reduce bone resorption, osteoblasts secrete osteoprotegerin (OPG), which inhibits osteoclastogenesis (Cawley et al., 2020; Yasuda et al., 1998).

The osteoblast life cycle is approximately three months (Manolagas, 2000). After fulfilling their duties to build bone, mature osteoblasts have three possible fates: they may undergo apoptosis, become bone-lining cells (BLC), or they may further mature into osteocytes (discussed in chapter 2.1.2.4) (Manolagas, 2000). The mechanisms related to which path osteoblasts are driven into are not completely understood. One of the suggested mechanisms is mechanical loading, and one recent study proposes that mechanical unloading can accelerate the transformation of mature osteoblasts into BLCs in the early stages of bone loss *in vivo* (Ram Hong et al., 2020).

Bone formation can be estimated from blood circulation by measuring proteins secreted by osteoblasts. These include bone-specific isoform of ALP, the amino-terminal propeptide of type 1 procollagen (P1NP), and OC (Seibel, 2005). Although OC is considered a marker for bone formation, it is also released from the ECM during resorption (Ivaska et al., 2004; Vasikaran et al., 2011).

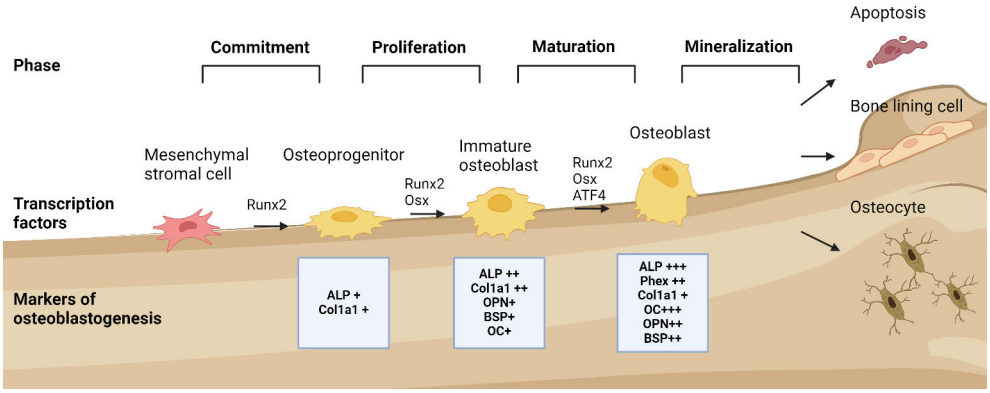

Figure 2. Phases of osteoblast differentiation. Osteoblasts have different phases during their differentiation: In the commitment phase, Runx2 activates MSCs to become osteoprogenitors which start to produce COL1 and ALP. In the proliferation phase, osteoprogenitors start to proliferate and become premature osteoblasts. Premature osteoblasts produce growing amounts of ALP and COL1 and lesser amounts of other proteins such as OPN, BSP and OC. After the proliferation phase, in the maturation phase the cells do not proliferate, exhibit their cuboid form and produce proteins, such as an excessive amount of OC but a lesser amount of COL1 and minerals to their surroundings. Finally, osteoblasts either further differentiate into osteocytes, become bone-lining cells, or undergo apoptosis. Abbreviations: ALP, alkaline phosphatase; ATF, activating transcription factor 4; BSP, Bone sialoprotein; MSC, mesenchymal stromal cell; OC, osteocalcin; OPN, osteopontin. Created with BioRender.

2.1.2.3 Osteoclasts

Bone resorption is the task of osteoclasts. Osteoclasts are large, multinucleated cells that are traditionally thought to be derived from the HSCs from the bone marrow niche, and specifically from the mononuclear cells (Udagawa et al., 1990). Osteoclasts can fuse with other multinucleai or apoptotic osteoclasts and form a heterogeneous osteoclast population to adapt quickly to resorption demands (Jansen et al., 2012; Søe et al., 2015).

The differentiation of osteoclasts, osteoclastogenesis, is initiated mainly by two key transcription factors: the monocyte or macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL). Osteoclastogenesis begins when M-CSF initiates the differentiation by activating and initiating the proliferation of the colony of monocytes to become preosteoclasts. After commitment, transcription factor RANKL binds to its receptor RANK and determines the fate of the preosteoclasts as they start to fuse and form multinucleated cells and thus mature into functional osteoclasts (Kong et al., 1999). RANKL is mainly produced by stromal osteoblast lineage (Fumoto et al., 2014; T. Nakashima et al., 2011), and it is shown to be fundamental to osteoclast maturation. Mature osteoclasts are defined by the production of an enzyme called tartrate-resistant acid phosphatase 5b (TRACP), which is used to evaluate the maturity of osteoclasts *in vitro*.

During resorption, osteoclasts form a tight junction called a “sealing zone” to the bone matrix. The sealing zone reduces the leakage of protons and proteases but also enables the lowering of pH in resorption lacuna to dissolve hydroxyapatite. Osteoclasts produce proteases to digest organic ECM. Very few proteases can digest the dense structure of COL1 fibrillin, and these proteases tend to belong to cysteine proteinases, such as cathepsin B, K or L, or matrix metalloproteinases, such as MMP-1, -8 and -13. Cathepsins need low pH to activate and dissolve ECM, and matrix metalloproteinases finalize the degradation to prepare the resorption area for subsequent bone formation (Everts et al., 2022). Another abundant enzyme is TRACP, which has various functions, such as dephosphorylation of OPN and other phosphoproteins (Everts et al., 2022). It is also expressed in intracellular vesicles which transport the degraded components to osteoclast surroundings (Vääräniemi et al., 2004). Originally, osteoclasts were thought to resorb the bone in one place and, after resorption, move and begin the resorption cycle at another location. However, recent findings suggest that osteoclasts resorb the bone, both staying still and moving across the bone (Søe & Delaissé, 2017).

Osteoclasts communicate with osteoblasts by enhancing osteoblastogenesis by secreting sphingosine 1 phosphate, which enhances bone formation and increases the migration and survival of the osteoblasts (Lotinun et al., 2013; Pederson et al., 2008). In addition, osteoclasts secrete leukaemia inhibitory factor, which suppresses the expression of sclerostin in osteocytes, resulting in increased osteoblastogenesis (Koide et al., 2017).

Osteoclast lifespan is evaluated to be from a couple of weeks (Manolagas, 2000) to even multiple months (Jacome-Galarza et al., 2019) depending on the origin of the cells. Osteoclasts are suggested to mainly undergo apoptosis but recently McDonald and colleagues presented an alternative fate in mice *in vivo*. Osteoclasts could recycle themselves by fission into smaller and more motile daughter cells, osteomorphs. Osteomorphs can later fuse to form functional osteoclasts (McDonald et al., 2021)

After resorption, the resorbed material can be absorbed by other cell types or secreted to the bloodstream, and it can be measured as an indicator of bone resorption. One of these is the degradation product of type 1 collagen, C-terminal-crosslinked telopeptides of type 1 collagen (CTX-1), which is widely used to evaluate bone resorption rate *in vivo* (Rosen et al., 2000).

2.1.2.4 Osteocytes

Osteocytes are the most abundant cell type in the bone, consisting of approximately 80% of bone cells. Osteocytes differentiate from the MSC lineage when osteoblasts get embedded in the ECM and mature into osteocytes. Morphologically osteocytes

are flat-shaped and have dendrites that form a lacuno-canalicular network which they use to communicate with each other. Through the network, osteocytes regulate other bone cells and are considered the master regulators of bone homeostasis (Bonewald, 2007). Osteocytes are mechanosensing cells. However, the mechanisms behind it are not completely understood. Possible mechanisms include fluid-flow stimuli in the lacuno-canalicular network, cell-matrix connections and mechanosensory ion channels as suggested by *in vitro* and *in vivo* studies. Mechanosensing plays critical role in the ability of bone to adapt to changes. (Qin et al., 2020)

Osteocytes maintain bone homeostasis and regulate bone formation and resorption. Ablation of osteoblasts in mice leads to severe bone loss (Ding et al., 2022; Kwon et al., 2012; Tatsumi et al., 2007), enhancement of osteoclastogenesis (Ding et al., 2022) and increased cellular senescence in bone tissue (Ding et al., 2022; Tatsumi et al., 2007). Mature osteocytes regulate osteoclastogenesis as they are a major source of RANKL, and in murine models, lack of RANKL specifically in osteocytes preserves cortical bone mass in aging mice (H. N. Kim et al., 2020) and both rescues and increases cancellous bone mass (Xiong et al., 2015; Zimmerman et al., 2018). In addition to RANKL, osteocytes produce OPG, a decoy receptor for RANKL, to regulate osteoclastogenesis (Kramer et al., 2010). Osteocytes inhibit bone formation via secretion of sclerostin (SOST), encoded by gene *Sost*, and transgenic mice with overexpression of SOST developed severe osteopenia (Poole et al., 2005; Winkler et al., 2003). SOST is a BMP antagonist which binds to LRP5/6 and thus inhibits Wnt-mediated osteoblast differentiation (Marini et al., 2023; Y. Wu et al., 2017). Human anti-sclerostin antibody, Romosozumab, was accepted for clinical use for osteoporosis treatment a few years ago (Marini et al., 2023).

Other proteins secreted by osteocytes include MEPE, DMP-1, PHEX and FGF23. MEPE, DMP-1 and PHEX are crucial for the proper mineralization of the bone. In addition, MEPE and DMP-1 control the production of FGF23, which contributes to phosphate metabolism by increasing renal phosphate excretion and thus lowering serum phosphate levels (Bonewald, 2007; Michigami, 2022; Youlten et al., 2021)

Osteocytes are one of the long living cells in mammals with an estimated lifespan of over decades (Bonewald, 2007; Manolagas, 2000). When osteocytes undergo apoptosis, they leave empty lacunae behind.

2.1.3 Bone remodelling

In bone remodelling, old and damaged bone is replaced with new bone without changing the bone's shape. Bone remodelling is not to be confused with bone modelling, in which bones are shaped through outside factors and the activities of osteoblasts and osteoclasts are not necessarily coupled. Remodelling occurs constantly, and approximately 10% of the skeleton is renewed yearly. Bone

remodelling is affected by multiple factors such as hormonal, environmental, and nutritional factors and when in imbalance, remodelling can cause skeletal defects such as osteoporosis (Manolagas, 2000).

In the 1960s Frost identified a group of cells that moved on mammalian compact bone and introduced the concept of bone remodelling (H. M. Frost, 1965). Remodelling occurs in a basic multicellular unit (BMU) which consists of osteoclasts, osteoblasts, bone lining cells and their precursors (Figure 3), of which all function in synergy. For example, osteoblasts and osteoclasts use coupling signals to regulate their maturation and, thus, maintain the balance of formation and resorption (Manolagas, 2000). BMUs are shaped differently in trabecular and cortical bone. In cortical bone, BMUs drill holes with "cutting cones" consisting of osteoclasts, while in trabecular bone BMUs move on the surface of the bone trabeculae. Trabecular bone is being renewed more efficiently and rapidly than cortical bone due to its larger surface area (Eriksen, 2010). Both trabecular and cortical bone BMUs are made of osteoclasts which resorb the bone, the reversal zone with several cell populations and lastly, the closing zone with osteoblasts and osteocytes (Bolamperti et al., 2022). In trabecular BMU there are also canopy cells which isolate the BMU from the surroundings (Kristensen et al., 2014). Canopy cells are bone marrow envelope cells which are suggested to be of bone lining cell origin. During the formation of BMU, the bone marrow envelope cells lift from the surface of the trabecular bone and form a layer of canopy cells called bone marrow envelope (Andersen et al., 2023) and along with BMU and small vascular capillaries, they form bone remodelling compartment (BRC) (Hauge et al., 2001). Bone remodelling can be divided into five phases: activation, resorption, reversal, formation, and determination phase.

Activation phase starts with the recruitment of osteoclast precursors, HSCs, and mononuclear cells, from the circulation or surroundings to start osteoclastogenesis. This occurs when RANKL is released to the site. One of the sources is apoptotic osteocytes, e.g. as a response to microfracture (Gu et al., 2005; Kennedy et al., 2014) or activation of osteoblasts and BLCs. After differentiation, mature osteoclasts adhere to the bone surface and resorb the bone during the resorption phase. At the end of the resorption phase, many factors induce osteoclasts to undergo apoptosis to prevent excessive bone loss. During resorption, osteoclasts release coupling factors from the ECM and one of the cytokines released, transforming growth factor-β (TGF-β), has been shown to induce apoptosis in osteoclasts (Houde et al., 2009). At the reversal phase, the resorbed area is covered with mononucleated reversal cells (Lassen et al., 2017). The cells seem to have an osteoblastic phenotype (Abdelgawad et al., 2016) but their purpose, as well as the reversal phase in general, are not yet completely understood. Lassen and colleagues provided insight that in cortical bone BMUs, resorption and reversal phases are partially overlapping, and the resorption is finally terminated

by the recruitment and density of osteoprogenitor cells (Lassen et al., 2017). Next, in the formation phase, factors released upon resorption from the bone matrix, such as BMPs, insulin-like growth factor 1 (IGF-1), FGFs and TGF-β, recruit osteoblasts to form bone to complete the remodelling (Tang et al., 2009). First, osteoblasts produce an organic matrix and build up the ECM. After the formation of an uncalcified osteoid, the matrix is further mineralized to complete the hardness of the bone. After the mineralization period, osteoblasts mature into osteocytes or BLCs or go through apoptosis completing the remodelling process. The process of remodelling is estimated to last approximately 6–9 months, with mineralization accounting for the longest period (Manolagas, 2000).

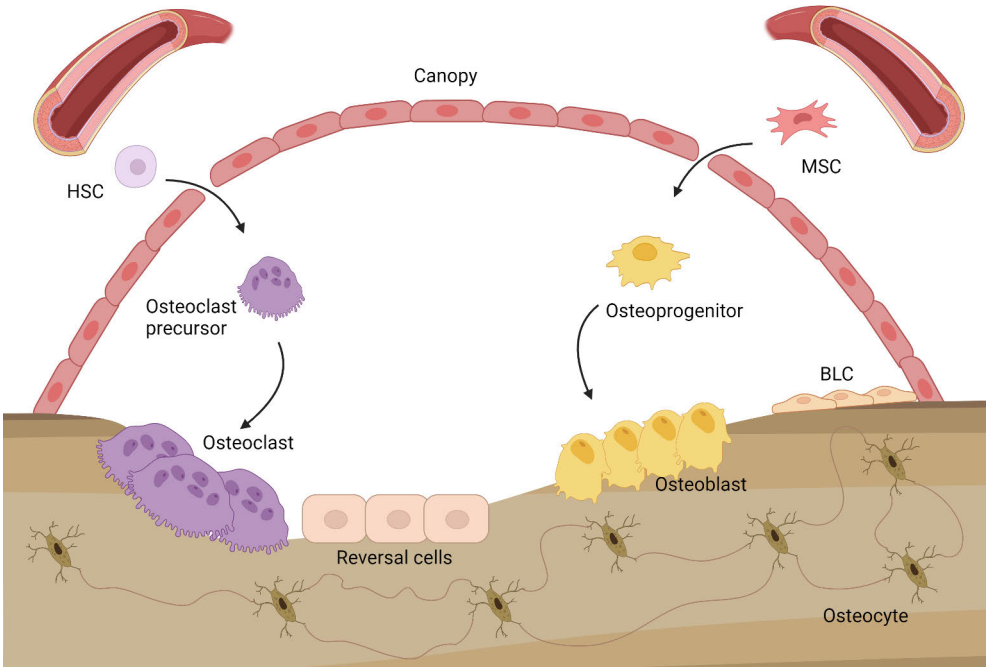

Figure 3. Schematic and simplified presentation of BRC in trabecular bone. At the beginning of the bone remodelling cycle, canopy cells form a tight envelope and isolate the surface area and the cells from their surroundings. In the resorption phase, osteoclast precursors are recruited from the surroundings to resorb bone. In the reversal phase, the reversal cells cover the resorbed area and further recruit osteoprogenitors that differentiate into osteoblasts and start the formation phase, where osteoblasts form new bone. At the determination phase, osteoblasts either mature into osteocytes or become BLC and the BRC is completed. Abbreviations: BLC, bone lining cell; BRC, bone remodelling compartment; HSC, hematopoietic stem cell; MSC, mesenchymal stromal cell. Created with BioRender. Modified from Sims & Martin, 2014.

2.2 Energy metabolism of osteoblasts

Preosteoblasts proliferate and mature osteoblasts produce proteins and ECM to their surroundings. Thus, osteoblasts have a high energy requirement. The primary sources of energy for mammalian cells are glucose, amino acids and fatty acids, which are transported through the cell membrane via membrane transporters and then used to produce adenosine 5'triphosphate (ATP) through metabolic pathways (Figure 4). The most efficient way for the cell to produce ATP is oxidative phosphorylation (OXPHOS) which occurs in the mitochondria.

The energy metabolism of osteoblast lineage is a complex system with overlapping and complementary mechanisms to ensure maintaining of osteoblast energy needs. The following chapter summarizes how osteoblast lineage uses glucose, amino acids, and fatty acids as energy sources.

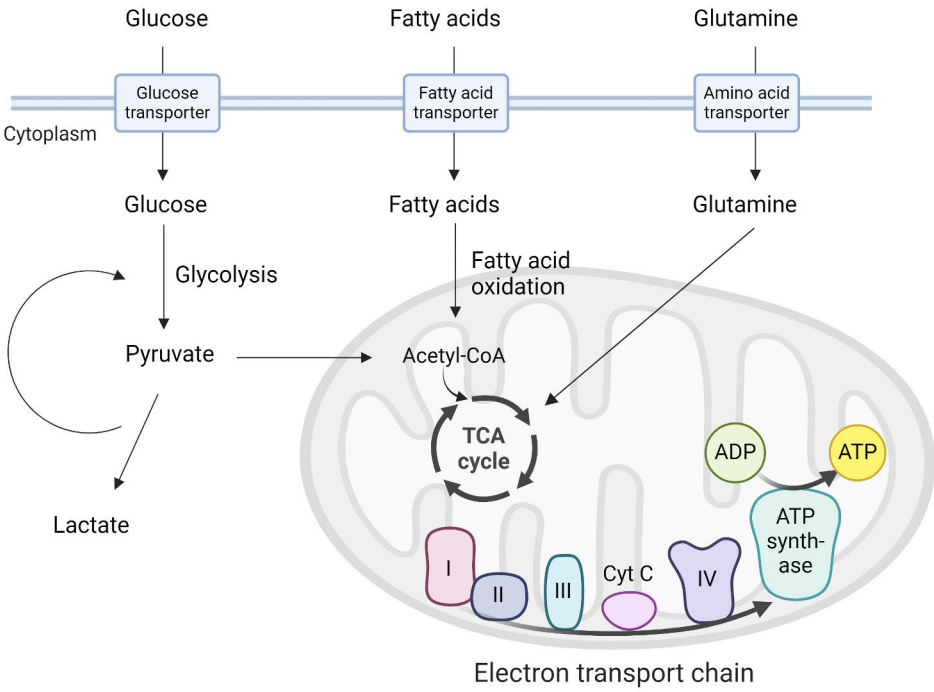

Figure 4. Simplified presentation of energy metabolism of the cell. Glucose, fatty acids and glutamine are transported through the cell membrane via their respective transporters. Glucose and fatty acids are metabolized to intermediate metabolites, which can be further converted to acetyl-CoA and enter the TCA cycle. Glutamine can enter the TCA cycle through α-ketoglutarate. The metabolites produced in the TCA cycle can be further oxidized in the electronic transport chain and finally converted into ATP in the ATP synthase unit. Abbreviations: TCA, tricarboxylic acid. Created with BioRender. Modified from Chlebek & Rosen, 2023.

2.2.1 Mitochondria and osteoblasts

Mitochondria are the power houses of the cell. After intake, glucose and fatty acids are metabolized to intermediate metabolites, which are then further converted to acetyl coenzyme A (acetyl-CoA) through metabolic pathways. Acetyl-CoA can further enter the tricarboxylic acid (TCA) cycle in the matrix of mitochondria, where it is primarily oxidized to nicotinamide adenine dinucleotide (NADH) and carbon dioxide (CO_2). Conversely, glutamine, enters the TCA cycle through anaplerosis which is mediated by α-ketoglutarate (αKG). Metabolites of the TCA cycle can exit the cycle and be used in bioprocesses in the cell. The metabolites can be measured and used to evaluate the energy consumption of the cell. One TCA cycle produces one guanosine-5'-triphosphate, three NADH and one flavine adenine dinucleotide ($FADH_2$) in which the latter two transfer electrons to the electron transport chain (ETC) located in the inner membrane of mitochondria. ETC has protein complexes I-IV in which electrons pass in a series of redox reactions leading to ATP synthase complex (sometimes referred to as complex V) which produces ATP. Complex I consist of clusters of NADH dehydrogenases, flavin mononucleotide and iron-sulfur. In complex I, NADH is oxidized to NAD+. Complex II is also known as succinate dehydrogenase, due to succinate from the TCA cycle, can enter the ETC in the complex II. Complex III is also known as cytochrome c reductase. It is made up of cytochrome b, Rieske subunits, and cytochrome c proteins. Complex IV oxidizes cytochrome c, resulting in water and intermembrane proton gradient. Protons from the reactions are transferred to intermembrane space in mitochondria and in the ATP synthase complex, proton gradient is used to generate ATP from ADP and Pi, completing the process of OXPHOS. For example, when glucose is completely oxidated via OXPHOS, it is estimated to yield up to 38 ATP molecules per glucose molecule (Martínez-Reyes & Chandel, 2020; Nolfi-Donegan et al., 2020).

Electron leakage from complex IV of ETC generates reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). The level of ROS are controlled by antioxidants, such as glutathione peroxidase, catalase and other antioxidants, e.g., vitamin E. Antioxidants can convert ROS to oxygen and water. Accumulation of intracellular ROS in cells causes oxidative stress, which damages DNA and other biomolecules, such as proteins and lipids, and may impair cellular function and cellular senescence. These protein alterations due to ROS can also be reversed by antioxidant defence proteins such as glutaredoxins and thioredoxins (Sheppard et al., 2022).

Osteoblasts contain a large number of mitochondria. As osteoblast need more energy to proliferate and produce components, the number and size of mitochondria has been shown to be increased during the differentiation of osteoblasts (C. T. Chen et al., 2008; J. Gao et al., 2018; Komarova et al., 2000). Although Shum et al. did not observe a similar change in the amount of mitochondria, they observed a

redistribution of mitochondria during osteoblast differentiation of human bone-derived MSCs (Shum et al., 2016). In addition, an increase in OXPHOS has been observed (Komarova et al., 2000; Müller et al., 2020) and a change in the mitochondrial membrane potential (Komarova et al., 2000) to possibly produce more effectively energy. Pathways regulating mitochondrial functions in osteoblasts include the Wnt pathway, and treatment with Wnt3a increased mitochondrial biogenesis during the differentiation of murine cell line C3H10T1/2 (An et al., 2010). Wnt3a and, also BMP2, stimulated OXPHOS through phosphorylation of Akt in both ST2 and MC3T3-E1 cell lines (Smith & Eliseev, 2021). Interestingly, although the number of mitochondria and the level of OXPHOS increase during osteoblast differentiation, which could lead to elevated ROS production, the amount of ROS has been shown to decrease, suggesting that osteoblasts can adapt to the altered energy production (C. T. Chen et al., 2008; J. Gao et al., 2018). Mitochondrial dysfunction has been shown to lead to bone loss in mice *in vivo* studies (Dobson et al., 2020; Sautchuk et al., 2023) and disturbance in OXPHOS to impaired bone formation *in vivo* (C. Lin et al., 2022), highlighting the importance of constant energy production in osteoblast.

2.2.2 Glucose metabolism

Glucose is metabolized through three main pathways: glycolysis, hexosamine biosynthetic pathway and pentose phosphate pathway. The most studied pathway to metabolize glucose to pyruvate in osteoblasts is glycolysis. Glycolysis occurs in the cell cytosol, and during glycolysis, glucose is converted into two pyruvate molecules. Under anaerobic conditions, pyruvate can be converted to lactate and in aerobic conditions, pyruvate can be converted to acetyl-CoA which can further enter the TCA cycle and undergo OXPHOS and produce a large amount of ATP (Figure 4). Although anaerobic glycolysis yields only two ATP molecules per glucose, it is faster compared to OXPHOS and, thus, it can result in a larger amount of ATP in a shorter time if glucose is available (Lunt & Vander Heiden, 2011).

Glucose is considered the main energy source for osteoblasts. Glucose and other hexoses are taken up through the cell membrane via passive transport facilitated by proteins called glucose transporters (GLUTs). The *SLC2A* gene family encodes GLUTs and currently, there are 14 known GLUTs found in humans (Mueckler & Thorens, 2013) with GLUT1 being the first to be found and characterized (Mueckler et al., 1985). GLUTs have unique affinities to their substrates, different sensitivities to external stimuli, such as insulin or hypoxia, and unique expression patterns in different tissues (Joost & Thorens, 2001; Mueckler & Thorens, 2013). In bone cells, Hahn and others were the first to show passive transport of glucose and hexoses using bone explants (Hahn et al., 1988) and after the discovery of GLUTs, GLUT1

and GLUT3 were first identified in UMR-106 rat osteosarcoma cell line by Thomas and colleagues (Thomas et al., 1996). Since then, multiple GLUTs have been found in both rodent osteoblastic cell lines (Pacicca et al., 2019; Thomas et al., 1996; Zoidis et al., 2011) and primary cells (Z. Li et al., 2016; Ohnishi et al., 2020; Wei et al., 2015). In humans, GLUT1 has been reported in human osteosarcoma cell lines MG-63, Saos-2 and U-2 OS (Cifuentes et al., 2011) and GLUT1, GLUT3 and GLUT9 in the intervertebral discs (Richardson et al., 2008). The relevance of GLUTs in osteoblasts is poorly known and GLUT1 remains the most studied glucose transporter. Deletion of GLUT1 in osteoblast lineage in mice leads to impaired bone formation both *in vivo* (Wei et al 2015) and *in vitro* (Chen et al 2019). Wei and colleagues established that glucose uptake, mostly via GLUT1, had a positive effect on osteoblast differentiation through inhibiting AMP-dependent protein kinase (AMPK) dependent proteasomal degradation of Runx2 in a transgenic mouse model (Figure 5) (Wei et al 2015). Li et al. found similar results as the deletion of Mst1/2 kinases in mouse osteoblasts *in vivo* resulted in a loss of GLUT1-expression, which led to AMPK-dependent proteasomal degradation of Runx2 (W. Li et al., 2018). In addition, a study by Li and colleagues found that GLUT4 is responsible for insulin-dependent glucose uptake of the osteoblasts and further, the ablation of GLUT4 in osteoblasts and osteocytes of mice did not impact on the bone architecture *in vivo* but decreased the osteoblast differentiation at the later stage *in vitro* (X. Li et al 2016).

Glycolysis is suggested to be the primary source of energy for the osteoblasts and their precursors. Already, the very first studies showed that bone cells use glucose and glycolysis as their energy substrate by measuring lactate in the culture medium of cultured bone chips (Borle et al., 1960), and multiple recent studies have coupled glycolysis with bone formation (S. Y. Lee et al., 2018; W. C. Lee et al., 2020; Regan et al., 2014). It has been estimated that osteoblasts obtain 75–80% of their energy through glycolysis (W. C. Lee et al., 2020; Misra et al., 2021), and the importance of glycolysis in osteoblasts has been widely investigated in *in vitro* -models. Glycolysis as a source for ATP has been studied in murine osteoblast-like cell lines (Guntur et al., 2014, 2018; Tencerova et al., 2019), primary murine osteoblasts (Komarova et al., 2000; Regan et al., 2014) and in primary human osteoblast lineage (C. T. Chen et al., 2008; Shum et al., 2016). A lower rate of glycolysis has been associated with cellular senescence (Sun et al., 2022) and ageing (Nandy et al., 2023) in bone marrow MSCs, further establishing the importance of glycolysis to osteoblast lineage.

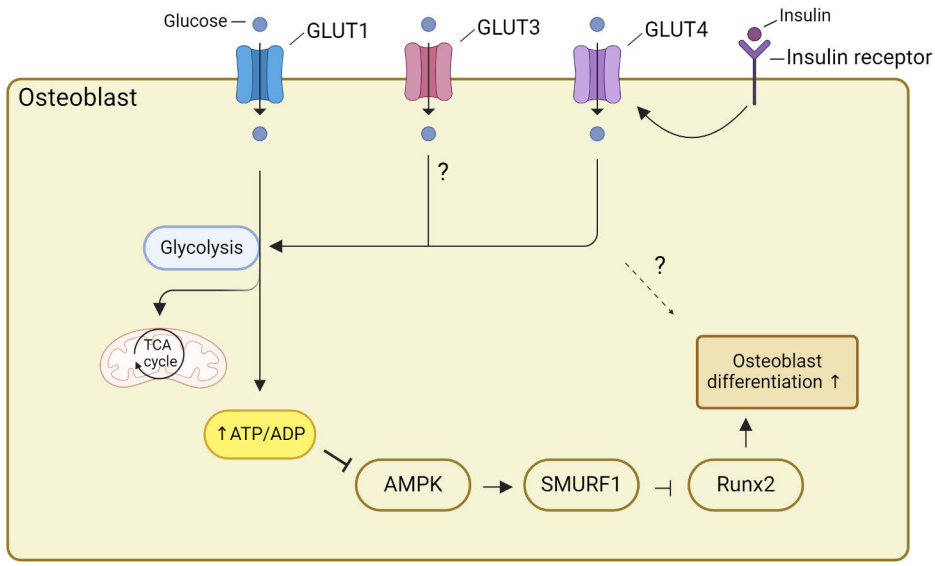

Figure 5. Glucose transporters 1, -3 and -4 in osteoblasts. GLUT1-mediated glucose uptake increases energy levels, which inhibits AMP-activated protein kinase activity and further affects the levels of Runx2, resulting in enhanced osteoblast differentiation and bone formation. GLUT4 is responsible for the insulin-mediated glucose uptake of osteoblasts. GLUT3 is expressed by osteoblasts but its relevance is not yet established. Abbreviations: GLUT, glucose transporter; TCA, tricarboxylic acid. Modified from Dirckx et al., 2019.

During the differentiation of osteoblasts, cells use both glycolysis and OXPHOS (Misra et al., 2021; Schilling et al., 2022) to meet their energy demands, but it is suggested that the main pathway for energy production shifts during osteoblast differentiation. However, there is still some disagreement regarding the shift: some studies suggest that the undifferentiated cells use glycolysis and differentiated cells rely more on OXPHOS (X. Gao et al., 2023; Shum et al., 2016; Smith & Eliseev, 2021) but others studies suggest that glycolysis is the main pathway in differentiated osteoblasts (Guntur et al., 2018; W. C. Lee et al., 2020; Misra et al., 2021). Conflicting results could be due to differences in the experimental models. For example, different sites of the skeleton could have altered metabolic response during differentiation: ST2 cell line, originally isolated from the long bones of the mouse, responded to osteoinduction faster compared to MC3T3-E1 which originates from the calvaria of the mouse. This resulted in ST2 cells reaching their maximal level of oxygen consumption rate in an hour while in MC3T3-E1, it took 12 hours, as measured by Agilent Seahorse XZ (Smith & Eliseev, 2021).

Many pathways have been shown to regulate glycolysis in osteoblasts. Multiple members of the Wnt-protein family stimulate glycolysis in osteoblasts. Induction with Wnt3a for 24 hours resulted in enhanced glycolysis as measured by increased

extracellular lactate levels and enhanced protein levels of glycolytic regulators in the ST2 cell line. Activation of the mammalian target of rapamycin complex 2 (mTORC2) via the Wnt-LRP5 -pathway was suggested as a mechanism. In addition, an increase in lactate levels was also observed by induction with Wnt10b (Esen et al., 2013). Lactate production was also increased in osteoblasts from transgenic mice with overexpression of Wnt7b in osteoblasts (H. Chen et al., 2019). Another pathway to increase glycolysis in osteoblasts is the PTH pathway. Acute 24-hour treatment with PTH increased glycolysis as measured by increased extracellular acidification rate but had no effect on the oxygen consumption rate, representing OXPHOS, in undifferentiated MC3T3-E1 cells (DeMambro et al., 2023). The same increase in the glycolysis pathway in the MC3T3-E1 cell line was observed by Esen et al by additionally measuring radioactive metabolites from glucose containing ^{14}C. Activation of the IGF-1-mTORC2 -signalling pathway by PTH was suggested as a mechanism (Esen et al., 2015). Hypoxia via the Hif-1α pathway, is closely related to maintaining glycolysis and bone formation (Regan et al., 2014; Shum et al., 2016). Dirckx et al. demonstrated that suppression of the Hif-1α pathway via deletion of Von Hippel Lindau protein resulted in mice with higher bone mass and increased glucose uptake and glycolysis *in vitro* (Dirckx et al., 2018). In addition to osteoblasts, a recent study connected mechanical loading and osteocytes with cell energy metabolism in both osteocyte-like mouse cell line MLO-Y4 and in an *in vivo* mouse model. Unloading of the bone led to activation of the Hif-1α/PDK1 pathway, which increased glycolysis and glutamate intake and further impaired the bone recovery after unloading. Bone recovery was accelerated when glycolysis was inhibited by blockage of PDK1 or glutamine supplementations were controlled (X. Liu et al., 2023).

The bone microenvironment is most likely relatively hypoxic (Stegen & Carmeliet, 2019). Multiple studies with murine models suggest that osteoblasts convert glucose to ATP via glycolysis even though there would be oxygen available, a process also known as the Warburg effect (Esen et al., 2013; Yang et al., 2021), which is also used e.g. by cancer cells for energy (Warburg et al., 1927). Although aerobic glycolysis yields less ATP than complete oxidation of glucose via OXPHOS, the ATP production with this pathway is estimated to be 10-100 times faster than OXPHOS. In addition, it produces components, such as nucleotides and proteins, as by-products which can be used as building blocks for the cell during proliferation and protein production (Liberti & Locasale, 2016; Lunt & Vander Heiden, 2011). The relevance of aerobic glycolysis in osteoblasts remains to be investigated, but stimulation of glycolysis (Dirckx et al., 2018; Esen et al., 2015; L. Zhang et al., 2023) have been shown to preserve bone anabolism and formation in multiple murine models.

2.2.3 Amino acid metabolism

Amino acids are used as molecular components in protein synthesis and are essential in the formation of enzymes and other biomolecules. Amino acids can also be used as an energy source, but are also needed in other metabolic pathways, such as anaplerosis, or coenzymes (Chandel, 2021a). The most extensively studied amino acid in osteoblasts and bone is glutamine. Glutamine is transported through the cell membrane via multiple different transporters found in the families of slc1, slc6, slc7, and slc38 (Bhutia & Ganapathy, 2016). In osteoblast lineage, slc1a5, a Na+-dependent neutral amino acid exchanger, is thought to be the primary glutamine transporter as it is expressed in calvarial cells, bone marrow-derived MSCs and differentiated osteoblasts (Sharma et al., 2021; Stegen et al., 2021). It is also capable of transporting other amino acids such as asparagine, alanine, serine, and threonine. Another essential glutamine transporter in osteoblasts is slc7a7 (Shen et al., 2021).

After uptake, intracellular glutamine metabolism is initiated by glutaminase enzymes. The enzyme converts glutamine to glutamate, which is involved in many pathways of the cell. For example, glutamate, along with amino acids glycine and cysteine, are synthesized to glutathione (GSH), an enzymatic cellular antioxidant, and thus is crucial for maintaining cellular redox balance. Glutamate is further deaminated by glutamate dehydrogenase into αKG, which enters the TCA cycle and can be used to produce energy (Chandel, 2021a).

Several studies suggest that glutamine is required for osteoblast differentiation and mineralization in murine cells (Karner et al., 2015; Stegen et al., 2021; Y. Yu et al., 2019). Glutamine has also been identified as an energy source for osteoblasts (Biltz et al., 1983; Karner et al., 2015). A study with radioactively labelled glutamine showed an increase in both glutamine consumption and metabolites released from the TCA cycle during osteoblast differentiation in both ST2 cells and primary mouse bone-derived MSCs, indicating glutamine is fulfilling the energy needs of osteoblasts (Y. Yu et al., 2019)

Pathways that regulate glutamine metabolism in osteoblasts include the Wnt signalling pathway. Wnt3a increases mTORC1 leading to increased glutaminase and thus entering glutamine into the TCA cycle in mouse bone marrow stromal cell line ST2 (Karner et al., 2015). In addition, Stegen and colleagues found that PTH treatment increases glutamine intake of the cells, possibly due to increasing glutamine transporters, such as *slc1a5* mRNA expression, and induces glutamine catabolism in Osx-positive osteoprogenitors *in vitro* (Stegen et al., 2021).

Proline is another amino acid that has recently been under investigation. Proline is a non-essential amino acid that can be generated from glutamine if proline is not available. Osteoblasts require a large amount of proline in the synthesis of proline-rich proteins, such as Runx2, OC and particularly COL1 (Shen et al., 2022). Proline is transported mainly to osteoblasts via the slc38a2 transporter, and the deletion of

the transporter in *Osx*-expressing cells led to impaired osteoblast differentiation and bone development in mice (Shen et al., 2022). However, the role of proline in osteoblast energy homeostasis in detail remains to be elucidated.

2.2.4 Fatty acid metabolism

Fatty acids are a major source of energy to cells as complete beta-oxidation of fatty acids yields more ATP than complete oxidation of glucose. In addition, fatty acid oxidation leads to small molecules for cells to use in other molecular processes. Cells can either synthesize fatty acids by themselves or they can be taken in from their surroundings via transporters (Chandel, 2021b). Kim et al. eliminated intracellular lipolysis by the deletion of adipose triglyceride lipase in osteocalcin-positive cells in mice *in vivo.* The cells were fully functional *in vitro,* and there were no changes in bone structure *in vivo,* suggesting that osteoblasts use extracellular fatty acids (S. P. Kim et al., 2017). Uptake of (long-chain) fatty acids is mediated by a variety of fatty acid transporters, e.g. slc27a1-6, plasma membrane fatty acid-binding proteins (Fabp) and fatty acid translocase CD36 (Kazantzis & Stahl, 2012). In osteoblasts, the role of fatty acid transporters is poorly understood. CD36 is expressed in mouse and human osteoblasts (Brodeur et al., 2008), and CD36 null mice have exhibited lower bone mass than wild type (Kevorkova et al., 2013). Fatty acid oxidation occurs in the mitochondrial matrix, and generates acetyl-CoA, NADH and $FADH_2$, which are then used to produce ATP via the TCA cycle and OXPHOS (Chandel, 2021b).

Compared to glucose and amino acid metabolism, fatty acid metabolism in osteoblasts is less studied. One early study suggested that fatty acid oxidation was used as an energy source in rat calvarial osteoblasts (Adamek et al., 1987). Niemeier et al. demonstrated bone uptake of postprandial lipoproteins using labelled chylomicrons in mice *in vivo* (Niemeier et al., 2008). In another study, Bartelt et al. showed in *in vivo* tracer study with radiolabelled fatty acids, ^{3}H-linoleic acid and ^{14}C-palmitate, that fatty acids are taken up in skeleton in mice (Bartelt et al., 2017). van Gastel and colleagues measured high levels of OXPHOS along with high palmitate oxidation but lower oxidation of glucose in primary osteoblasts obtained from mice calvaria with Agilent Seahorse, indicating that the cells a have higher rate of fatty acid oxidation (van Gastel et al., 2020).

Recent work from Alekos et al. showed that PTH enhances both fatty acid uptake and oxidation in bone marrow-derived MSCs and calvarial osteoblasts and suggested that this could be one of the mechanisms behind PTH-induced bone formation, at least in mice (Alekos et al., 2023). The loss of carnitine palmitoyltransferase 1a (van Gastel et al., 2020) or carnitine palmitoyltransferase 2 (S. P. Kim et al., 2017), enzymes, which are involved in fatty acid oxidation, in cells of osteoblast lineage led to impaired osteoblast differentiation and impaired bone formation in mice,

respectively. Furthermore, genetic ablation of *Lrp5* in mice osteoblasts and osteocytes lower bone mass, but also changes in energy metabolism of the cells compared to wild type mice. The change was linked to the Wnt/Lrp5 pathway, which regulated fatty acid utilization in osteoblasts (Frey et al., 2015). Together, fatty acids are required for proper osteoblast function.

2.3 Type 2 diabetes and bone formation

Diabetes is a group of diseases characterized by elevated blood glucose levels, hyperglycaemia, due to disruptions of insulin-mediated cellular glucose uptake as well as defects in insulin secretion. The most common forms of diabetes include type 1 and type 2 diabetes (T1D, T2D, respectively) which differ in their pathophysiology. T1D is an autoimmune disease that results from the destruction of beta cells in the islets of Langerhans in the pancreas and, hence, is characterized by insulin deficiency. Insulin deficiency results in hyperglycaemia which is treated with life-long injections of insulin. T2D, on the other hand, is a metabolic disorder in which peripheral tissues do not respond to insulin, a condition called insulin resistance. Due to insulin resistance, the pancreas produces increasing amounts of insulin and thus, in T2D, both hyperglycaemia and hyperinsulinemia may be present. The overproduction of insulin may lead to exhaustion of the pancreas and, finally, destruction of beta cells at the later and severe stages of the disease, causing hypoinsulinemia. (Banday et al., 2020; International Diabetes Federation, 2021).

Currently, more than 400 million people in the world have diabetes and T2D accounts for approximately 80% of cases, making T2D a worldwide health problem. The incidence of T2D is steadily growing due to aging population, sedentary lifestyle and increasing prevalence of obesity (L. Chen et al., 2012; International Diabetes Federation, 2021). The most known adverse effects of T2D include, e.g. cardiovascular disease (atherosclerosis), nephropathy, neuropathy, and retinopathy, but bone is increasingly recognized as a target for diabetic complications. Although the skeletal effects of diabetes have long been known based on epidemiological observations (Levin et al., 1976), the recent research have focused on elucidating the mechanisms responsible for these changes. This chapter focuses on T2D associated-changes on the bone and how hyperglycaemia affects on the bone-forming cells.

2.3.1 Alterations in bone metabolism and microarchitecture

In clinical studies, patients with T2D have been shown to have a lower bone turnover rate (Hygum et al., 2017; Vavanikunnel et al., 2022), an elevated risk for fragility fractures (Ferrari et al., 2018) as well as altered bone quality (Farr & Khosla, 2016). Several meta-analyses conclude that T2D patients have an increased risk of fracture,

specifically in the wrist (Dou et al., 2021; Moayeri et al., 2017), ankle (Dou et al., 2021) and hip areas (Dou et al., 2021; Ha et al., 2021; Vestergaard, 2007), with hip fracture risk being higher in female patients with T2D (Vilaca et al., 2020). Controversially, it has been reported in several studies (Di Monaco et al., 2024; Shu et al., 2012) and meta-analyses (Walle et al., 2022) that the bone mineral density (BMD) is either normal or even higher in patients with T2D when compared to non-diabetic controls. The phenomenon was already reported in the 1990s by Krakauer et al. (Krakauer et al., 1995). The higher BMD could also be due to increased body mass index (BMI), as patients with T2D tend to have higher BMI.

Several factors have been associated with a higher risk of fracture. These include younger onset of diabetes, disease duration and the use of insulin (Vilaca et al., 2020). Moreover, the incidence of fractures was higher in those with poor glycaemic control (Hidayat et al., 2021). The fracture risk might also be elevated in the course of disease duration due to other adverse events, such as dizziness and hypoglycaemia (low blood glucose) (Hidayat et al., 2021), resulting in increased risk for falls and, therefore, fractures.

Patients with T2D have altered bone metabolism as measured by bone turnover markers (BTMs) from blood samples, although there are some conflicting data. Bone resorption marker CTX-1 was found to be lower in patients with T2D diagnosis when compared to controls (Hygum et al., 2017; Reyes-García et al., 2013; A. Sheu et al., 2023). The lower levels in circulation were also detected for another resorption marker, TRACP (Hygum et al., 2017; Reyes-García et al., 2013). The same trend has been shown with bone formation markers as levels of OC (Hygum et al., 2017; A. Sheu et al., 2023; Starup-Linde et al., 2014) and P1NP (Hygum et al., 2017; Starup-Linde et al., 2014, 2016) were lower in patients with T2D. However, some studies found no differences between patients with T2D and control subjects when OC (Reyes-García et al., 2013), bone-specific ALP (Reyes-García et al., 2013) or P1NP were measured (A. Sheu et al., 2023). The conflicting results can be due to ethnicity, sample size and methods used. Although BTMs are lower in T2D patients compared to controls, biomarkers might not be able to predict the fracture risk in T2D patients. Napoli and colleagues performed a 9-year follow-up study, where they measured P1NP, CTX-1 and OC in a cohort of elderly patients with or without T2D, and found that although markers were lower in patients with T2D, there was no correlation between the levels and incidence of fracture risk. Interestingly, all the BTMs analysed were increased during follow-up and modestly associated with fracture risk in controls (Napoli et al., 2020). Similarly, Meier et al. found that BTMs do not correlate with fracture risk, but glycated haemoglobin (HbA1c), an indicator of long-term glucose balance in diabetics, could be used to estimate the risk of fracture in patients with T2D (Meier et al., 2023). In addition to BTMs, SOST levels have been shown to be higher in patients with T2D (Ardawi et al., 2013; Gaudio et

al., 2012; Gennari et al., 2012) which could indicate lower bone formation due to the inhibition of osteoblasts and thus bone formation by SOST.

T2D patients have normal to high BMD, but decreased bone material strength (Farr et al., 2014; Nilsson et al., 2017) and the quality and microarchitecture of both cortical and trabecular bone are altered. Measurements by magnetic resonance imaging (MRI) and/or high-resolution peripheral quantitative computer tomography suggest that the cortical bone thickness is more narrow (Shanbhogue, Hansen, et al., 2016) while its porosity is increased in both pore size and amounts in patients with T2D (Heilmeier et al., 2021; Patsch et al., 2013; Shanbhogue, Hansen, et al., 2016). Furthermore, cortical porosity was positively correlated with an increased risk of fracture in patients with T2D (Heilmeier et al., 2016). de Waard et al. reported that adult patients with T2D who had poor cortical bone quality also had poor glycaemic control, measured by HbA1c (de Waard et al., 2018).

In trabecular bone, multiple studies and meta-analyses suggest that the BMD is either normal or increased, but the bone strength is compromised (Burghardt et al., 2010; Walle et al., 2022), suggesting alterations in the material quality and microarchitecture of trabecular bone. Poor glycaemic control (Dhaliwal et al., 2014), HbA1c levels, or microvascular disease (Shanbhogue, Hansen, et al., 2016) have been associated with worse bone adverse events. In addition, alterations in bone microarchitecture could occur already in the early onset stage of the diabetes (Heilmeier et al., 2016). In conclusion, BMD of the trabecular bone seems to be normal or elevated but compromised and cortical bone to be more porous and narrower, resulting in poor bone quality.

In addition to changes in bone microarchitecture, there are also indications of an increase in bone marrow adipose tissue (BMAT) in subjects with T2D. BMAT has been identified recently as a metabolically active endocrine tissue distinct from other adipose tissues (Pham et al., 2020). An increase in BMAT was negatively correlated with BMD and positively with levels of HbA1c in the vertebra of men with T2D (Y. Sheu et al., 2017) and similar results were found with postmenopausal women with T2D (Baum et al., 2012). Furthermore, poor glycaemic control was associated with hypertrophy and hyperplasia of the BMAT in premenopausal women with T2D (Andrade et al., 2021). An increase in BMAT is also reported in murine *in vivo* models of T2D (Figeac et al., 2022; Picke et al., 2016).

2.3.2 Hyperglycaemia

T2D is characterized by elevated plasma glucose levels, i.e. hyperglycaemia. Normal fasting plasma glucose concentrations are under 6.1 mM, fasting plasma glucose concentration between 6.1 and 6.9 mM is considered prediabetic, and fasting plasma glucose 7.0 mM or higher is considered diabetic. In poorly controlled or untreated

diabetes, plasma glucose levels can rise temporarily over 20 mM, and over 11 mM for even a longer period (International Diabetes Federation, 2021).

Hyperglycaemia has been studied widely as a potential cause of diabetic adverse events in osteoblasts. In clinical studies, fasting plasma glucose is negatively correlated with BTMs (Starup-Linde et al., 2016), and HbA1c levels correlate negatively with bone formation markers (H. Li et al., 2021). Decreased bone turnover, measured by BTMs, and specifically decreased bone formation measured with both BTMs and imaging, has been shown in *in vivo* study with murine T2D model with prolonged hyperglycaemia (F. Song et al., 2023). Although reduced cortical thickness and increased porosity are observed in murine models of T2D, trabecular BMD is often also decreased, and the increased trabecular BMD seen in humans is not observed in reported murine *in vivo* models.

In vitro studies of glucose excess have had somewhat contradictory results. One of the early studies with human osteosarcoma cell line MG63 found that seven days of exposure to up to 49.5 mM high glucose decreased the viability of the cells in a concentration-dependent fashion when compared to either cells grown in normal glucose concentration (5.5 mM glucose) or equivalent concentration of mannitol, which is used as a control for osmolarity (Terada et al., 1998). The decrease in proliferation was also observed in several studies using osteoblast-like MC3T3-E1 cells, which were cultured over seven days at 20–25 mM glucose concentration (Dong et al., 2017; Medeiros & Wallace, 2022; Takeno et al., 2021). In addition to decreased proliferation, culturing MC3T3-E1 cells in high glucose (20–25 mM) decreased the expression levels of osteoblast-specific genes, such as *Runx2*, *Col1a1* and *Alpl* (Botolin & McCabe, 2006; Cunha et al., 2014; Dong et al., 2017; Takeno et al., 2021) but also decreased mineralization of the cells (Dong et al., 2017). The decrease in proliferation and mineralization capability of osteoblasts has also been detected in primary osteoblasts obtained from human fetuses (Shahen et al., 2023). Impairment of osteoblastogenesis due to hyperglycaemic conditions has been reported in murine embryonic stem cells (Dienelt & zur Nieden, 2011) and rat bone marrow-derived MSCs (Huang et al., 2019). Hyperglycaemia may also divert the bone marrow derived MSCs towards adipogenic lineage instead of osteogenic lineage (Wang et al., 2014), which could contribute to the elevated BMAT but also impaired bone formation.

Lower than 20 mM but still physiologically high glucose concentrations of 15–15.5 mM were reported to increase proliferation (Balint et al., 2001; Z. Liu et al., 2015) and differentiation, measured by gene expressions of *Runx2, Osx and Bglap* (Z. Liu et al., 2015), of MC3T3-E1 cells. Although the proliferation of the cells was enhanced, the mineralization capability was impaired through alterations in calcium intake of the cells (Balint et al., 2001). In human umbilical cord-derived MSCs differentiated to osteoblasts, 25 mM glucose exposure up to 14 days enhanced the

ALP activity, mineralization, and expression of *ALPL, COL1A1, OPN, RUNX2* and *BGLAP*. However, high glucose induced the apoptosis of the MSC via downregulation of the Akt-Sirt1-TWIST pathway (Ren et al., 2022). In another study with primary human alveolar bone-derived cells, 14 days of 24 mM high glucose exposure enhanced the mineralization of the cells, but decreased the ALP activity as compared to cells grown in normal glucose environment (García-Hernández et al., 2012).

The molecular mechanisms responsible for the changes induced by hyperglycaemia are still under investigation. Exposure to hyperglycaemia of 25 mM suppressed the Wnt pathway by decreasing Wnt3a protein expression, but also by inducing β-catenin degradation in MC3T3-E1 cells (Lõpez-Herradõn et al., 2013). Alterations in the Wnt pathway have recently been reported in human bone samples from the hips of women with T2D (Leanza et al., 2024). Further, the inhibition or alteration of the Wnt pathway can also lead to increased adipogenesis of MSCs, which partly explains the increase in BMAT (Keats et al., 2014; Ross et al., 2000). In addition, exposure to 25 mM high glucose upregulated Notch2 protein expression while decreasing osteoblastogenesis of rat bone marrow-derived MSCs when cultured for two or seven days (Huang et al., 2019). Upregulation of mRNA expression levels of PTH receptor and both *Rankl* and *Opg*, which are activated by PTH, were observed when MC3T3-E1 cells were exposed to 30 mM high glucose for 24 hours (Cunha et al., 2014). Upregulation of RANKL and OPG could enhance osteoclastogenesis and, therefore, leads to an imbalance of bone remodelling by favouring osteoclastogenesis. Exposure to 16.5 mM high glucose for seven days decreases the proliferation and migration of primary rat bone marrow-derived MSCs through activation of glycogen synthase kinase-3β (GSK3β) and suppression of β-catenin (B. Zhang et al., 2016). Chen and colleagues observed similar results in MSCs obtained from mice (Y. Chen et al., 2021). Interestingly, the time of the exposure could also affect whether glucose has anabolic effects, as Takeno et al. reported that exposure to either 16.5 mM or 27.5 mM high glucose at the early stage of differentiation promoted mineralization of MC3T3-E1 cells via increasing the mRNA expression of *Bmp4* and increasing phosphorylation of Smad1/5/8 (Takeno et al., 2021).

The contradictory results of glucose exposure on osteoblasts can partly be explained by differences in study models such as time of exposure, glucose concentration and origin of the cells.

2.3.3 Advanced glycation end products

Advanced glycation end products (AGEs) are glycated proteins and other macromolecules due to nonenzymatic glycation. Prolonged exposure to glucose,

damaged proteins and oxidative stress may lead to the formation of AGEs. AGEs are accumulated in ageing or in diseases such as diabetes. They are a heterogeneous group of molecules with some tissue-specific tendencies: in erythrocytes, glycated haemoglobin is called HbA1c, and e.g. pentosidine and carboxymethyl lysine are known to crosslink to collagen. AGEs bind to their receptor (Receptor for Advanced Glycation Endproducts, RAGE) on the cell membrane, which regulates multiple transcription factors, such as nuclear factor kappa B (NF-kB) and those regulated by the mitogen-activated protein kinase (MAPK) signalling pathway (Prasad et al., 2019, Yamamoto & Sugimoto 2016).

AGEs have been associated with higher fracture risk in patients with T2D. HbA1c has been associated with higher fracture risk, as described above. A higher concentration of carboxy-methyl-lysine in circulation was associated with an increased risk of fractures in patients with T2D (Dhaliwal et al., 2022), and another study found that a higher concentration of pentosidine in urine was associated with an increased vertebral fracture prevalence (Schwartz et al., 2009). Further, the accumulation of AGEs as measured by skin autofluorescence was negatively correlated with bone material strength index as measured by microindentation (Furst et al., 2016; Samakkarnthai et al., 2020). Levels of pentosidine in bone biopsies from male patients with T2D were elevated compared to non-diabetic controls (Oren et al., 2011). Other studies showed an accumulation of AGEs in trabecular bone biopsies of either patients with T2D (Sihota et al., 2021) or female patients with T2D (Piccoli et al., 2020) compared to non-diabetic controls. Both studies found a positive correlation with AGEs and impaired bone microarchitecture. In rat models of T2D, accumulation of AGEs in bone has been detected compared to control rats (Campbell et al., 2016; Saito et al., 2006).

AGEs have been considered one of the key mechanisms of bone weakening. AGEs form crosslinks in collagen altering the quality and mechanical properties of collagen, making it more brittle (Howard et al., 1996; Poundarik et al., 2015). In addition, AGEs might impair the functions of bone cells via multiple pathways mediated by activation of RAGE. When human osteoblasts obtained from trabeculae were incubated with pentosidine, it decreased the mRNA expression levels of *ALPL* and *COL1A1,* but also decreased the mineralization capability of cells (Sanguineti et al., 2008). A decrease in differentiation could be due to increased expression of TGF-β after AGE binding to RAGE, as observed in ST2 cells (Notsu et al., 2014). Moreover, incubation of human foetal osteoblasts with AGE activated the Raf/MEK/ERK signalling pathway, a component in the MAPK pathway, through RAGE, resulting in autophagy (Meng et al., 2015). Overexpression of RAGE in MC3T3-E1 cells decreased the proliferation of the cells via suppression of Wnt, PI3K and ERK signalling pathways (G. Li et al., 2012). AGEs can also lead to metabolic changes in the cells as exposure to AGEs induced apoptosis of MC3T3-

E1 cells through mitochondrial dysfunction and increased oxidative stress mediated by activation of RAGE (Mao et al., 2018).

2.3.4 Metabolic changes

Mitochondrial dysfunction is reported in osteoporosis (Yan et al., 2023) but also in T2D, as multiple other tissues, including adipose, myocardium and skeletal muscle tissues, can have mitochondrial dysfunction (Pinti et al., 2019). In osteoblast-like MC3T3-E1 cells, hyperglycaemia induced a decrease in mitochondrial biogenesis (Pahwa et al., 2020) and caused a decrease in mitochondrial matrix content and a shift in the metabolism (Medeiros & Wallace, 2022), suggesting that hyperglycaemia can alter mitochondrial functions.

As discussed earlier, osteoblasts prefer glycolysis as their main source of energy. Song et al. found that T2D suppresses both glycolysis and TCA cycle-mediated energy production in the murine T2D model, which resulted in declined bone mass. Restoration of glycolysis by either metformin, an antidiabetic drug, or by other pathways restored bone phenotype (F. Song et al., 2023). The same research group showed that impairment in glycolysis in osteoblasts resulted in a diabetic osteopenia in the mouse model for T1D (Akita mice) (Ji et al., 2023).

Hyperglycaemia induces the accumulation of ROS and increased oxidative stress, which are observed in diabetic bone (Hamada et al., 2009). At a cellular level, oxidative stress has been suggested to accelerate cellular senescence in cells of osteoblast lineage, resulting in reduced proliferation and differentiation of the cells (Aswamenakul et al., 2020; Tencerova et al., 2019). In MC3T3-E1 cells, the accumulation of ROS has been shown to promote apoptosis and autophagy (Bartolomé et al., 2013; P. Zhang et al., 2020).

2.3.5 Insulin resistance

T2D is characterized by insulin resistance in peripheral tissues, such as muscle and adipose tissue, but whether bone tissue can also become insulin resistant is still under investigation. Insulin binds to its receptor, insulin receptor (IR), on the cell membrane activating the insulin signalling pathway. Following the binding of insulin to IR, IR tyrosine kinase is activated leading to the activation of the PI3K/AKT pathway. Activated AKT leads to downstream signalling of multiple pathways, including forkhead family box O, mTORC1 pathways, GSK3β and RabGAP TBC1 domain family member 4, which mediates the translocation of GLUT4 containing intracellular vesicles to the cell membrane (Haeusler et al., 2018; White & Kahn, 2021). In insulin resistance, binding of insulin to IR can be unaffected but the downstream signalling pathways are impaired which results in,

e.g. impaired GLUT4-mediated glucose uptake and, thus, hyperglycaemia. The molecular mechanisms behind insulin resistance are not completely understood. However, the effects may vary in different tissues and cell types (Könner & Brüning, 2012; Saltiel, 2021).

The effect of insulin on bone has had contradictory results in both human and animal studies. Abrahamsen et al. reported that insulin sensitivity was negatively associated with BMD in non-diabetic men (Abrahamsen et al., 2000), and Stolk et al. found a positive correlation between insulin levels and BMD (Stolk et al., 1996). In line with the older studies, a correlation was reported between higher insulin resistance, measured with homeostatic model assessment for insulin resistance, and higher BMD in non-diabetic older adults (Napoli et al., 2019) and with volumetric BMD in non-diabetic women (Shanbhogue et al., 2016). These studies suggest that insulin resistance may increase BMD. However, Sheu and colleagues found that low bone turnover was associated with insulin resistance in men (A. Sheu et al., 2023). Acutely, as measured during hyperinsulinaemic euglycaemic clamp, insulin has been reported to suppress CTX-1 after a four-hour infusion (Ivaska et al., 2015). However, there was no association between insulin sensitivity and BTM in a large cohort of adult males (M. Frost et al., 2018).

Insulin might have an anabolic effect on bone as two separate transgenic mouse studies with deletion of IR in osteoblast lineage observed reduced bone formation in mice *in vivo* (Ferron et al., 2010; Fulzele et al., 2010). However, a transgenic mouse model with GLUT4 ablation in osteoblasts and osteocytes exhibited normal bone microarchitecture. In the same study, insulin enhanced the glucose uptake of calvarial osteoblasts, and the ablation of GLUT4 resulted in impaired osteoblastogenesis and mineralization *in vitro* (Z. Li et al., 2016). Studies of insulin-mediated glucose uptake of osteoblasts are inconclusive as an early study also found that insulin enhances glucose uptake in rat calvarial osteoblasts *in vitro* (Hahn et al., 1988), but Wei et al. found no effect on glucose uptake of mouse calvarial osteoblasts *in vitro* or in total bone *in vivo* (Wei et al., 2015) Furthermore, in MC3T3-E1 cells there was no change in glucose uptake after adding insulin to cells *in vitro*, but the cell line was found to be absent of GLUT4 which could explain the lack of response (Cunha et al., 2014). Wei et al. further established that primary osteoblasts obtained from the calvaria of transgenic mice could become insulin resistant through degradation of IR, which occurred by free saturated fatty acid-induced upregulation of *Smurf1* expression (Wei et al., 2014). In human cells, Tencerova and colleagues either blocked or silenced IR in bone marrow-derived MSCs from obese subjects which led to a shift in the cell metabolism and enhanced osteoblast differentiation as measured by an increase in ALP production *in vitro* (Tencerova et al., 2019). Thus, further studies in human cells are called for.

3 Aims

Glucose metabolism in bone-forming osteoblasts is a complicated system and is incompletely understood. In addition, excess glucose in hyperglycaemia impairs osteoblast bone formation through various pathways and a wider perspective of the transcriptional changes induced by hyperglycaemia is needed. The aim of this PhD project was to study the relationship between glucose and osteoblasts, focusing on how osteoblasts take up glucose and to characterize the changes in osteoblasts induced by hyperglycaemia.

The specific aims were:

1. To get insight into the molecular mechanisms of glucose uptake via glucose transporters in osteoblasts and their precursors *in vitro*

2. To study the effect of short- and long-term hyperglycaemia on rat osteoblasts *in vitro*

3. To develop a method to measure ucOC in human blood samples and establish a link between osteoblasts and hyperglycaemia *in vivo*

4 Materials and Methods

4.1 Permits and ethical statements

Animal experimentation for *in vitro* -studies was approved by the local review committee of the Central Animal Laboratory, University of Turku (Turku, Finland).

The human study was performed in line with the principles of the Declaration of Helsinki and approved by the Local Ethics Committee, University of Turku (1/2017). Informed consent was obtained from all voluntary donors included in the study. For plasma samples obtained from Auria Biobank (Turku, Finland), written informed consent was obtained from the donors by the Biobank.

4.2 *In vitro* studies

4.2.1 Extraction of rat BMSCs and differentiation to osteoblasts (I, II, III)

Primary bone marrow stromal cells (BMSCs) were isolated from the femurs and tibias of 3–4-week-old female Sprague-Dawley rats. Epiphyses were removed, and bone marrow was extracted either by flushing with a needle (**I, III**) or by centrifugation (**II**). BMSCs were enriched by plastic adherence for 48 h, and adherent cells were expanded for 4-6 days in extraction medium. After expansion, BMSCs were detached by trypsinization and used for experiments as described below. Osteoblast differentiation was introduced with osteoblast differentiation medium containing ascorbic acid and Na-β-glycerophosphate. To characterize the differentiation of BMSCs to osteoblasts (**I**), we plated BMSCs into six-well plates (80 000 cells/well), differentiated them for 12 days and collected samples for RNA extraction (chapter 4.2.2) every two days. BMSCs (160 000 cells/well) cultured for 24 hours in basal medium were used as an undifferentiated control. Medium components, reagents and concentrations are summarized in Table 1.

Table 1. Contents of the mediums used in the studies. All components were diluted in Mem alpha (Gibco, Thermo Scientific).

Reagent	Extraction medium	Basal medium	Osteoblast differentiation medium	Manufacturer
Fetal bovine serum, certified, USA	15%	10%	10%	Gibco, Thermo Fisher Scientific, Inc.
Amphotericin B	2.5 µg/ml	-	-	Gibco, Thermo Fisher Scientific, Inc.
Penisillin-Streptomycin	100 µg/ml/100 units	100 µg/ml/100 units	100 µg/ml/100 units	Gibco, Thermo Fisher Scientific, Inc.
GlutaMAX™	2 mM	2 mM	2 mM	Gibco, Thermo Fisher Scientific, Inc.
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	10 nM	10 nM	10 nM	Gibco, Thermo Fisher Scientific, Inc.
Dexamethasone	10−8 M	-	-	Sigma
L-ascorbic acid 2-phosphate	-	-	70 µg/ml	Sigma
Beta glycerol phosphate disodium salt pentahydrate	-	-	10 mM	Fluka BioChemika

4.2.1.1 siRNA transfections (I)

siRNA constructs for silencing GLUT1 (siGLUT1, SR503164), GLUT3 (siGLUT3, SR502308), GLUT4 (siGLUT4, SR515187) and universal scramble control (Control, SR30004), were purchased from OriGene Technologies and siTRAN 2.0 (Origene) was used as transfection reagent. Transfections were done according to the manufacturer's instructions. Briefly, BMSCs were seeded on six-well plates (150 000 cells/well), at 48 well plates (18 000 cells/well) or at glass coverslips (30 000 cells/well) in basal medium. After 24 hours, the medium was changed to basal medium containing 10 nM of individual siRNA and transfection reagent. A fluorescently labelled siRNA duplex (Trilencer-27, 10 nM, OriGene) was used as a transfection control and to evaluate transfection efficiency. After 18 hours, the medium was changed to osteoblast differentiation medium and transfected cells were cultured for a maximum of six days. Study design is presented in Figure 6.

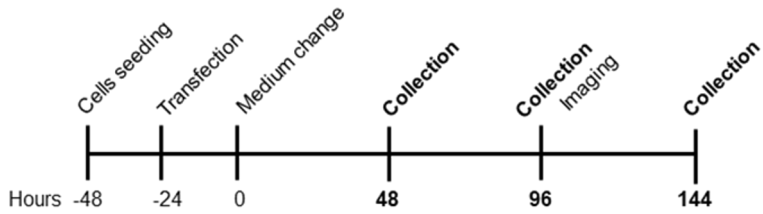

Figure 6. Study design of Study I. From original publication I.

4.2.1.2 Exposure to hyperglycaemia (II)

Hyperglycaemic (HG) cell culture medium was prepared by adding D-glucose (Sigma) to the osteoblast differentiation medium (glucose concentration of 5.5 mM) to achieve a final glucose concentration of 25 mM. Osteoblast differentiation medium containing 5.5 mM glucose was defined as normoglycaemic (NG) conditions. BMSCs were seeded on six-well plates (40 000 cells/well) or 24-well plates (10 000 cells/well) and differentiated to osteoblasts for 10 days and exposed to hyperglycaemia for short-term (24 or 72 hours) or long-term (10 days) prior to sample collection on day 10. Mediums were changed on days four and seven. All three exposure times had their own controls with the same days of medium change. Study design is shown in Figure 7.

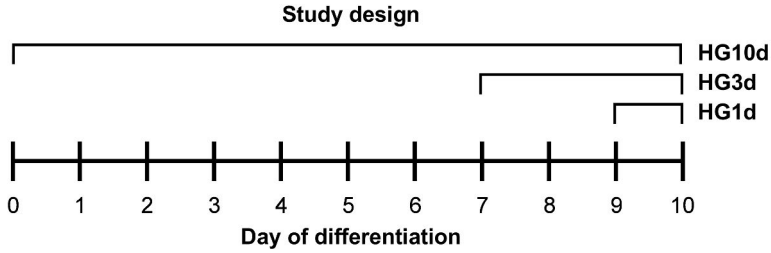

Figure 7. Study design of Study II. From original publication II.

4.2.1.3 Induction of osteocalcin production (III)

BMSCs were seeded and cultured on either a 24-well microwell plate with glass cover slip (4 000 cells/well) or on six-well plate (80 000 cells/well) in osteoblast differentiation medium for nine days. To induce osteocalcin expression and secretion, the culture medium was changed on days three and six, and 25 µM 1,25$(OH)_2$ dihydroxy vitamin D_3 (Vitamin D, Sigma) was added to osteoblast differentiation medium. To inhibit the carboxylation of osteocalcin, 25 µM warfarin (Sigma) was added to osteoblast differentiation medium. Medium samples were collected upon medium change until day 9 and stored at -20°C for further analysis.

On day 9, osteoblasts differentiated on glass coverslips were fixed with 4% PFA and stored at +5°C.

4.2.2 Real-time quantitative PCR (I, II)

For real-time quantitative PCR (RT-PCR), cells were grown on 6 well plates. Gene expressions of the following genes (Table 2) were analysed. Primers were purchased either from Oligomer or Integrated DNA Technologies. Total RNA was isolated according to the manufacturer's instructions with either RNeasy Mini RNA Isolation Kit (Qiagen) or Macherey-Nagel™ Nucleospin™ RNA plus kit (Macherey-Nagel™). The concentration and purity of isolated RNA was analysed with either NanoDropND1000 or NanoDrop1 devices (NanoDrop Technologies). 0.5–1 µg of RNA was reverse transcribed to cDNA using 5 µM Oligo-dT mRNA-primer (NewEngland BioLabs) and Maxima RT enzyme (Thermo Fisher) according to the manufacturer's instructions. Dynamo HS SYBR green (Thermo Fisher) with 100 ng of cDNA was used for quantitative RT-qPCR and analysed using CFX96 RealTime system C1000 Thermal cycler (BioRad) with annealing temperatures optimized separately for each primer pair (table 2). Data was analysed by -ΔΔCT-method (Livak & Schmittgen, 2001) and mRNA expression of Cyclophilin B (*Ppib*) was used to normalize the data (Pachot et al., 2004).

Table 2. Sequences and annealing temperatures of primers used for RT-qPCR used in the study.

Primer	Gene id	Sequence	Temperature (℃)
Alpl	NM_013059.1	F-GCAGGATCGGAACGTCAAT R-GGGTCTTTCTCTTTCTCTGGCA	60
Bglap	NM_013414.1	F-TGAGTCTGACAAAGCCTTCATGT R-GAAGCCAATGTGGTCCGCTA	61
Col1a1	NM_053304.1	F- CACTGCAAGAACAGCGTAGC R- AGT TCCGGTGTGACTCGTG	59
Fgf23	NM_130754.1	F- AGGATGCTGGCTCCGTAGTG R- CGGGCTGAAGTGATACGATCCA	62
Mepe	NM_024142.1	F- GAAGGTGAACGACACCAGAGAG R- GGCTCAGGCTTCACAGATGC	59
Phex	NM_013004.2	F- GGGAACAGAATACCCTCGATCTC R- CACAGACCACCACGGATCAA	62
Ppib	NM_022536.1	F-ACCTGTAGGACGAGTGACCT R-GCTCTTTCCTCCTGTGCCAT	60
Runx2	NM_053470.2	F-CGCCTCACAAACAACCACAG R-TGCAGCCTTAAATATTACTGCATGG	60
slc2a1	NM_138827.1	F- GCCGCTTCATCATTGGAGTG R- GAGTCTAAGCCGAACACCTGG	58
slc2a2	NM_012879.2	F- CCGGCACATGCTCTCATCAT R- CTGAGGCCAGCAATCTGACTA	58
slc2a3	NM_017102.2	F- GATCCTTGTGGCTCAGGTCT R- ATCTCCGTCGCTTGGTCTTC	58
slc2a4	NM_012751.1	F- CGCGGCCTCCTATGAGATAC R- ACTCCAAACCCAACACCTGG	58
slc38a2	NM_181090.3	F- CATCTTCGGGTTCATTGGTGC R- CATCACGACTACGCCACTCA	60
Sost	NM_030584.2	F- GCCTTCGTTGCTGTGGAGAG R- TGTACTCGGACACGTCTTTGGT	61
Txnip	NM_001008767.2	F- CCATTCTGGGCTGCAACATC R- TATAGCAAGGTGGAGCTTCTGG	61

4.2.3 RNA-sequencing (I, II)

RNA-sequencing was done for both siRNA-silenced (**I**) and hyperglycaemia treated (**II**) cells. Cells were treated as described above with the following modifications. For siRNA silencing, BMSCs were plated in six-well plates (200 000 cells/well), transfected with siRNAs as described and cultured in osteoblast differentiation

medium for 48 hours. Total RNA was extracted at the end of the culture and RNA samples were stored at -80°C.

mRNA library preparation, RNA-seq, and bioinformatic analysis were obtained from Novogene, Ltd. (United Kingdom). First, the RNA quality and integrity were evaluated with BioAnalyser Agilent 2300 system. After validation, the samples were prepared via poly-A enrichment -technology to build a mRNA library suitable for RNA-seq. Then samples were sequenced with the Illumina NovaSeq platform (Illumina NovaSeq 6000 sequencing system) using a paired-end 150 bp strategy and 20 M reads per sample. For quality control, the raw reads were filtered for error rate, GC content, and read quality and reads that did not meet the criteria were filtered. For the read alignment, the HISAT2 algorithm was used to map the reads to the *Rattus norvegicus* reference genome (Rnor_6.0). Alignment data was used for the calculation of gene expression levels based on read counts. Differential expression analysis was performed using the DESeq2 R-package (Anders & Huber, 2010). The resulting p-values were adjusted for false discovery rate using the Benjamini–Hochberg multiple testing adjustment procedure (Benjamini & Hochberg, 1995).

Functional analysis of differentially expressed (DE) genes was done by common pathway enrichment analysis referencing to the Gene Ontology database using clusterProfiler software (G. Yu et al., 2012) (Study **I**) or metascape online tool (Zhou et al., 2019) (Study **II**) of five genes overlap and 3.0 enrichment score.

4.2.4 Cell proliferation and viability (I, II)

Cell proliferation and viability was assessed for cells with siRNA treatments (48-well plate, **I**) or hyperglycaemia exposures (24-well plates, **II**). Cell proliferation was estimated with IncuCyte® S3 Live-Cell Analysis System (Sartorius). Cells were imaged at 2h (**I**) or 12h (**II**) intervals, and cell confluency (%) was assessed at each timepoint with user-defined settings. Cell viability was assessed at the end of the culture using either (**I**) alamarBlue® Cell Viability Reagent (Thermo Fisher) at day five or (**II**) both alamarBlue® and Calcein staining (Thermo Fisher) at day 10 according to the manufacturer's instructions.

4.2.5 Immunofluorescence (I, II, III)

BMSCs were seeded on 24-well plates on glass coverslips (4 000 cells/well (**III**) or 10 000 cells/well (**I**, **II**) and let to attach for 24 hours before fixation. To stain differentiated osteoblasts, the cells were differentiated on coverslips in osteoblast differentiation medium for either four or six (**I**), or nine (**III**) days prior to fixation. Cells were fixed with 4% paraformaldehyde for a maximum of 20 min, permeabilized with 0.05% Triton X-100 for 5 min on ice and blocked with 10%

normal goat serum (Abcam) for 60 min at RT. Primary antibodies (Table 3) were incubated overnight at +4°C. Secondary antibodies (Table 4) were incubated for 60 min at RT in darkness. Antibodies were diluted in 3% w/v bovine serum albumin in PBS containing Tween-20. Cells were washed with 0.1% Tween-20 in PBS three times between incubations. Samples were mounted on microscopic slides with Vectashield Antifade containing 1.5 µg/ml DAPI (Vector Laboratories). Cells were imaged with Zeiss Axio Imager 1 (Zeiss), with standardized settings maintained throughout each experiment.

Table 3. Primary antibodies used in the study.

ANTIBODY	DILUTION OR CONCENTRATION	MANUFACTURER	CAT. NO
Rabbit Pab to CD44	0.2 µg/ml	Abcam	ab157107
Rabbit Pab to CD45	10 µg/ml	Merck	SAB4502541
Mouse Mab to CD90	1 µg/ml	Abcam	ab225
Mouse Mab to GLUT1	1:1000	Abcam	ab40084
Rabbit Pab to GLUT3	6.8 µg/ml	Abcam	ab41525
Rabbit Pab to GLUT4	1:1000	Abcam	ab654
Mouse Mab to OC	1 µg/ml	HyTest	4OC8, clone 2H9cc
Human Fab-AP to ucOC*	5 µg/ml	in house	clone -13

*alkaline phosphatase (AP)

Table 4. Secondary antibodies used in the study.

ANTIBODY	DILUTION OR CONCENTRATION	MANUFACTURER	CAT. NO
Alexa Fluor® 488-conjugated goat anti-rabbit IgG	2 µg/ ml	Abcam	ab150077
Alexa Fluor® 594-conjugated goat anti-mouse IgG	2 µg/ ml	Abcam	ab150116
Rabbit Anti-AP	5 µg/ ml	LifeSpan Bioscience Inc	LS-C59288

4.2.6 Evaluation of osteoblast differentiation (I, II)

Enzymatic activity of ALP in cell lysates was measured at the endpoints of cell cultures (**I**, **II**). Cells were collected in lysis buffer (50 mM Tris-HCl, 0.1% Triton X-100, 0.9% NaCl, pH 7.6), and ALP activity was measured using 0.15 nM PNPP (0.1 M Tris, 1 mM MgCl2, pH 10) as the substrate. After 45 min incubation, 1 M NaOH was used to stop the enzymatic reaction and absorbance was measured at 405 nm using EnSight Multimode Plate Reader (PerkinElmer/Revvitty). Total protein concentration was measured using Bradford protein assay (Biorad) according to the manufacturer's instruction. ALP activity is presented as A405 (absorbance units, AU per protein (mg/ml). In addition, ALP activity was also visualized by histochemical staining (**II**). After 4% PFA fixation ALP activity was detected with Alkaline Phosphatase Leukocyte Kit (86-R, Sigma-Aldrich) according to the manufacturer's instructions.

Mineralization was assessed with von Kossa staining (**II**). First, the cells were fixed with 4% PFA and washed five times with water. 2% $AgNO_3$ (Fisher Scientific) was added to the cells and incubated for 60 min at RT under a 20W table lamp. Staining was completed by adding 2,5% $Na_2S_2O_3$ (JT Baker) for 5 minutes. The wells were washed with water and air dried before imaging (HP ScanJet G4010, Hewlett-Packard Company)

4.2.7 Detection of cell organelles and structures (II)

CellROX Green fluorescent probe (Thermo Fischer) was used to assess intracellular oxidative stress according to the manufacturer's protocol. Briefly, 5 µM of CellROX was diluted in serum-free medium (37°C). Solution was added on the cells and incubated for 30 min at 37°C, 5% CO_2. Cells were then fixed with 4% PFA, and 5 µg/ml Hoechst 33258 (Thermo Fischer) was used to stain the nuclei.

MitoTracker Orange CMTMRos (Thermo Fischer) fluorochrome probe was used to stain the mitochondria. 250 nM of MitoTracker Orange was diluted in serum-free medium (37°C) and added to the cells. After 30 min of incubation at 37°C, 5% CO_2, cells were fixed with 4% PFA and 5 µg/ml Hoechst 33258 was used to stain the nuclei.

LysoTracker, Red DND-99 reagent (Thermo Fischer) was used to stain acidic compartments within the cell and estimate lysosome quantity. The cells were stained with 50nM LysoTracker and 5µg/ml Hoechst 33258 in 37°C serum-free medium. Incubation was done for 30 min at 37°C, 5% CO_2.

Cells were imaged immediately using EVOS fluorescent microscope system (Thermo Fischer) under user-defined settings.

4.3 Development of detection method for uncarboxylated osteocalcin (III)

4.3.1 Human plasma samples (III)

Human blood samples were obtained either from healthy volunteers or Auria Biobank, Turku, Finland.

Fasting blood samples (10–12h) from 14 healthy volunteers (7 males and 7 females, age 22–48 years, mean 28.1±6.5 years) were collected in four different collection tubes (Vacuette®, Greiner): serum (with or without gel), (lithium) heparin plasma or EDTA plasma (with EDTA K2). Separation of serum or plasma was done by centrifugation (2 500G and 2 000G, respectively), and samples were aliquoted and stored at -80°C. To evaluate the effect of samples processing on stability of uncarboxylated osteocalcin (ucOC), we collected serum and plasma samples from three volunteers, incubated aliquots at +4°C, RT (+22°C) or -20°C. The effect of repeated freeze-thaw cycles (5 times) was also tested. After incubations, all samples were stored at −80°C for further analysis. A reference sample was stored at -80°C immediately after sample collection.

Human EDTA plasma samples were obtained from Auria Biobank. Plasma samples were collected from volunteers during routine blood sample collections in health care centres or similar conditions. Inclusion criteria for samples were as follows: fasting plasma sample available (overnight fast), result of fasting plasma glucose measurement, and sample stored at −80°C within six hours from sampling. Exclusion criteria were as follows: any cancer, fracture within one year from sampling or the use of bone-active medication (bisphosphonates, denosumab, teriparatide). We categorised the samples into three age- and sex-matched subgroups: patients with confirmed T2D diagnosis and no limitation on fasting plasma glucose level (T2D, N=49, ICD10 E11), controls without T2D and fasting plasma glucose within normal range (Con-NG, N=46) and lastly, controls without confirmed T2D diagnosis but elevated fasting plasma glucose level to represent possible undiagnosed subjects (Con-HG, fasting plasma glucose over 7 mmol/L, N=29). Samples were analysed for ucOC, as described below, total osteocalcin (tOC, methods in Study **II**, Supplemental data) and N-terminal propeptides of type I collagen (IDS-iSYS Intact PINP, IDS, UK).

4.3.2 Selection of ucOC-specific binders

The selection for the ucOC-specific binders was made in collaboration with the Institute of Biotechnology, University of Turku. Briefly, synthetic human scFv antibody libraries scFvM (diversity of 6 × 109) and scFvP (diversity of 1 × 1010),

were used, and ucOC-specific binders were selected with phage display technique (Brockmann et al., 2011; Huovinen et al., 2013). For ucOC, the detection was done for three rounds for positive selection against biotinylated ucOC (Peptide 2.0) on paramagnetic streptavidin beads using cOC (Sigma) as a free blocker in the solution. For OC, detection was done for both bio-ucOC and bio-cOC (without blocker) in different panning rounds to isolate binders recognizing both forms of osteocalcin. Trypsin was used to elute bound phages, which were used to infect *E. Coli* XL1-Blue cells, and VCSM13 helper phage was used to produce phage stocks. Individual clones were isolated from the enriched libraries and produced as scFv fused with bacterial alkaline phosphatase (scFv-AP) and further verified for binding to bio-ucOC.

Based on the binging properties to the ucOC, the best clones were identified and targeted for mutagenesis and further panning to improve the binding of the antibodies. In order to produce the mutagenesis libraries, sequence and length variation were introduced in CDR loops L1, L3, H1, and H2 using oligonucleotide-directed mutagenesis (Huovinen et al., 2012). Two rounds of panning against ucOC were done for the constructed scFv phage libraries, followed by screening of individual clones for positive binding to ucOC and negative for cOC. Selected candidates were cloned in pLK06H vector (Huovinen et al., 2013) and produced in *E. Coli* as soluble scFv-AP proteins.

Next, selected scFc clones were converted to more stable IgG1,κ Fab fragments. Synthetic human Fab genes were purchased from GeneArt (Thermo Scientific). Cloning was performed to SfiI sites into vector pLK06FT, similar to the vector pLK06H (Brockmann et al., 2011), resulting in Fab as a fusion protein with bacterial and His6 and a FLAG tag. Both vectors (scFv pLK06H and Fab pLK06FT) were further transformed into *E. Coli* BL21 cells for antibody production. Production of the antibodies was done in shake flask culture (V=200 ml), and antibody expressions were induced for 10 h with isopropyl β- D -1-thiogalactopyranoside (Sigma) at 22–26°C. After cell collection, antibodies were purified with NiNTA affinity chromatography (HisPur, Thermo Scientific) according to the manufacturer´s instructions. Protein concentrations were assessed with NanoDropND1000.

4.3.3 Antibody characterization

Monoclonal MAb-2H9 against human osteocalcin (Käkönen et al., 2000) and polyclonal anti-Escherichia coli alkaline phosphatase (anti-AP, LS-C59288, LifeSpan Bioscience Inc) were used in the assays. Human full-length ucOC peptide (1–49, Glu at positions 17, 21 and 24; C23–C29 bridge) was purchased from Peptide 2.0 and human full-length cOC peptide (1–49, Gla at positions 17, 21 and 24; C23–C29 bridge) from Sigma-Aldrich. Biotinylation of the antibodies or peptides was

done with EZ-Link NHS-SS-PEG4-Biotin (Thermo Scientific) according to the manufacturer's instructions and labelling with N1 europium chelate (Eu) as previously described (Eriksson et al., 2003).

Streptavidin-coated microtiter wells (SA-plate), Buffer Solution RED (BSR), Wash Solution, and Europium Fluorescence Intensifier (EFI) were all purchased from Uniogen (Turku, Finland). All assay experiments were done in BSR, washing the wells four times with wash solution and incubations at RT in a shaker if not stated otherwise. Time-resolved fluorescence (TR-FIA) was enhanced by 200 µl EFI and measured with Victor 1240 Multilabel Counter (PerkinElmer/Revvitty)

To characterize novel recombinant antibodies, we determined their thermostability, binding affinity (EC50 value) and their binding site on human ucOC. Thermostability was evaluated by heating the antibodies in the presence of 5 mM dithiothreitol (Thermo Scientific) at various temperatures ranging from 40 to 71°C for 60 min and measuring the immunoreactivity afterwards. EC50 values were determined by coating the SA-wells with 1.4 pm of biotinylated human ucOC peptide (bio-ucOC) and incubated with different concentrations of recombinant antibodies together with Eu-anti-AP or Eu-labelled MAb-2H9. To determine the binding site of the antibodies, we designed custom-made BioTides™ biotinylated synthetic peptides (JPT Peptide Technologies) with a length of 22 amino acids and offset of one amino acid that covered the entire osteocalcin sequence. In addition, we determined the binding of the Fab-APs at the carboxylation site by designing three peptides, which had only one carboxylation (Gla) at position 17 or 21 or 24, or carboxylation at all three carboxylation sites. Briefly, the SA-plates were coated with the designed peptides, and incubated with Fab-AP antibodies, and Eu-anti-AP was used for detection by TR-FIA measurement. To elucidate whether the Fab-APs have cross-reactivity to rat osteocalcin and also detect bioactive ucOC (in addition to synthetic peptides), we measured culture mediums of vitamin D and warfarin-treated rat osteoblasts (described above) with the assay.

4.3.4 Assay characterization

After antibody characterization, we developed a two-site immunoassay. First, biotinylated MAb-2H9 (200 ng/well) was used to coat the SA-plates. Then, 100µl of standard or sample was added with 100 µl of BSR containing 5mM EDTA, and incubated for 60 min. Next, Fab-AP and Eu-antiAP (100 ng/well) were added to the wells and incubated for 90 min. Finally, EFI was added for 5 min and TR-FIA was measured. The total volume of the assay was 200 µl, and as a calibrator, we used human ucOC peptide diluted in BSR with concentration varying from 0.11 to 9 ng/ml. Signals were converted to logarithmic values for the preparation of a standard curve, which was used to evaluate ucOC concentration in unknown samples.

We optimized the assay for Fab-AP concentration (from 50 to 365 ng/well), incubation time (from 30 to 120 min) and assay volumes (from 50 to 200 µl). For assay/antibody performance, we characterized cross-reactivity to carboxylated osteocalcin, and determined the assay linearity and the effect of assay matrix by making serial dilutions from samples with 8 ng/ml of ucOC peptide in either biological sample or BSR. Determination of within-run and between-run variability, the lowest limit of detection (LOD) and the lowest limit of quantification (LOQ) were assessed. Comparison to a commercial ELISA assay against ucOC (Glu-OC EIA kit, Takara Bio Inc) was done with serum (N = 20), heparin plasma (N = 20) and EDTA plasma (N = 20) samples, according to the manufacturer's instructions.

4.4 Statistical analyses (I, II, III)

All *in vitro* experiments were repeated two or three times using freshly isolated cells and representative data and images are presented.

Data are presented as means with standard deviations (**I**, **II**. **III**) or as medians with interquartile range (**III**) unless otherwise stated. Comparisons between groups were tested with a two-tailed Student's t-test or analysis of variance (ANOVA) with either Dunnet's or Tukey's post hoc tests (normally distributed) or Kruskal–Wallis test (nonparametric). When comparing ranks, a Mann-Whitney U test was used.

P values < 0.05 were considered statistically significant and are indicated with asterisks as * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$. Statistical analyses were performed using GraphPad Prism software (8.1.2 (**I**, **III**) or 9 (**II**) GraphPad Software) or SPSS, version 25 (**III**, SPSS,Inc).

5 Results

5.1 Glucose transporters GLUT1, GLUT3 and GLUT4 are needed in osteoblasts (I)

5.1.1 Preosteoblasts express multiple glucose transporters

First, we validated the *in vitro* osteoblast culture model used in this study. We found the BMSCs to be positive for mesenchymal markers CD44 and CD90 and negative for hematopoietic marker CD45 (**I**, Figure 1A). After six days of osteoblast differentiation, the cells were found to be positive for osteoblast markers, such as osteocalcin, at both mRNA (**I**, Figure 1D) and protein (**I**, Figure 1E) level, confirming osteoblast differentiation.

BMSCs and osteoblasts use glucose as a source of energy. We evaluated the expressions of twelve members of the GLUT family, GLUT1-GLUT12, in the transcriptome of rat preosteoblasts. We detected expressions of all other glucose transporters except for GLUT2 and GLUT7 (**I**, Figure 2A). GLUT11 was also absent, as rodents lack the gene for GLUT11 (Scheepers et al., 2005). Next, we focused on GLUT1, GLUT3 and GLUT4, which, together with GLUT2, belong to the class I glucose transporters. mRNA expression levels for GLUT3 were relatively stable through the 12-day differentiation, while both GLUT1 and GLUT4 mRNA levels decreased by up to 80–90%, respectively ($p<0.0001$) (**I**, Figure 2). We then used immunostaining to confirm the presence of the GLUT1, GLUT3 and GLUT4 proteins. Changes in mRNA levels were not directly translated to protein level as there were no clear differences in GLUT protein levels between BMSCs and osteoblasts differentiated for six days (**I**, Figure 2).

5.1.2 Silencing of GLUT1, GLUT3 or GLUT4 results in different outcomes in osteoblasts

To further characterize the relevance of GLUT1, GLUT3 and GLUT4, we used siRNA -technology to silence *slc2a1*, *slc2a3* or *slc2a4* individually in BMSCs and examined the effects of silencing during six days of osteoblast differentiation. The universal scramble construct was used as a control. The silencing was effective, and

48 hours after transfection, the mRNA level of GLUT1 was suppressed by 77%, GLUT3 by 97% and GLUT4 by 91% (**I**, Figure 3B, F and J, respectively). Six days after transfection, mRNA levels were still downregulated by approximately 50%. The silencing was highly specific for each Class I glucose transporter examined, and there were only moderate changes in mRNA levels of other GLUTs (**I**, Figure 3B-J). We further evaluated the protein levels in silenced cells by immunostaining, and GLUT1 seemed to be less abundant 96 hours after transfection, while no clear changes observed in the protein levels of GLUT3 or GLUT4 (**I**, Figure 3K).

We then evaluated whether the downregulation of GLUTs influences the growth or differentiation of the osteoblasts. First, we assessed the proliferation of the cells after transfection by IncuCyte imaging. Surprisingly, silencing of GLUT4 decreased (area under the curve, $p<0.0001$) while silencing of GLUT3 increased the proliferation of the cells ($p<0.0001$) when compared to cells treated with scramble control. Silencing of GLUT1 did not have an apparent effect on cell proliferation (**I**, Figure 4A). The viability of the cells was assessed with alamarBlue method on day five post-transfection. Silencing of GLUT4 decreased the viability of the cells ($p=0.0001$), while silencing of GLUT3 or GLUT1 had no significant effect (**I**, Figure 4B). Silencing of GLUT4 resulted in decreased differentiation of the cells, as measured by decreased ALP protein activity (70%, $p < 0.0001$) (**I**, Figure 4C) and relative expression of OCN mRNA ($p=0.029$) (**I**, Figure 4D). Silencing of GLUT1 or GLUT3 had no effects on the differentiation of the cells.

5.1.3 Silencing of GLUT1, GLUT3 or GLUT4 results in unique transcriptional changes

To further elucidate the global changes and molecular mechanisms caused by the silencing of GLUT1, GLUT3 or GLUT4, we performed bulk RNA sequencing from samples grown in osteoblast differentiation medium for 48-hour post-transfection. All samples with silencing were compared to scramble control.

Silencing of GLUT1 resulted in fewer changes in the transcriptome, with 2104 differently expressed (DE) genes (595 DE genes after multiple comparison), compared to silencing of either GLUT3 (3290 DE genes and 1653 after multiple comparison) or GLUT4 (3049 DE genes and 1573 after multiple comparison) (**I**, Figure 5A). The silencing of GLUT1 resulted in 718 unique DEs, silencing of GLUT3 in 1556, and silencing of GLUT4 in 1454.

We further analysed statistically significant DE genes with common pathway enrichment analysis utilizing the Gene Ontology annotations. Enriched pathways are categorized by biological processes (BPs), cellular components (CCs), and molecular functions (MFs). The top 10 pathways changed by silencing are listed in Study **I**, Supplementary Figure 2.

In GLUT1-silenced preosteoblasts, we observed an upregulation of transcripts related to carboxylic acid and small molecule metabolic processes (e.g. Pyruvate kinase (*Pkm*), Glutamine dehydroxylase 1 (*Glud1*), cationic amino acid transporter *slc7a1*) but also in genes related to lipase activity and ECM production (e.g. Decorin (*Dcn*), hyaluronan synthase 2 (*Has2*)). Pathways related to the ribosome and its structural components were downregulated (**I**, Figure 5C).

Silencing of GLUT3 in preosteoblasts led to downregulation of multiple pathways related to biosynthetic pathways. These included pathways related to protein translation and carbohydrate metabolism. There was also suppression in genes encoding proteins of ETC in the mitochondrial inner membrane, including 15 out of 42 subunits of NADH dehydrogenase (e.g. *Ndufa4*), 5 out of 27 subunits of cytochrome oxidase (e.g. *Cox6a1*) and 10 out of 19 subunits of ATP synthase (e.g. *Atp5e*) (**I**, Figure 5D).

In the GLUT4-silenced preosteoblasts, we observed a suppression of metabolic pathways related to carbohydrate derivative biosynthesis and oxidoreductase activity (e.g. HMG-CoA reductase and hydroxyacyl-CoA dehydrogenase, *Hadh*). However, also downregulation of genes encoding ECM components, such as genes related to collagen synthesis (prolyl hydroxylases), suggest impaired ECM production and support the observed decrease in proliferation and differentiation of the cells. There were also changes in cell signalling pathways: binding of IGF (*Igfbp3*, *-4*, *-6* and *-7*) and guanyl nucleotide and GTP (e.g. guanylate cyclase 1, *Gucy1a3* and *Gucy1b3*). (**I**, Figure 5E).

Taken together, BMSCs and osteoblasts rely on multiple glucose transporters to ensure their supply of glucose. A delicate balance of classical GLUTs 1, -3 and 4 are needed for proper osteoblast differentiation.

5.2 Long- and short-term exposure to hyperglycaemia results in different responses in osteoblasts (II)

5.2.1 Long-term hyperglycaemia affects the proliferation and viability of the osteoblasts

Next, we used the same *in vitro* -model to characterize the effects induced by hyperglycaemia on bone forming osteoblasts. We differentiated BMSCs to osteoblasts for 10 days and exposed the osteoblasts to a hyperglycaemic environment before collection as follows:10 days (HG10d) to represent long-term exposure or for either the last 24 h (HG1d) or the last 72 h (HG3d) to short-term exposure. 25 mM glucose concentration was used as a hyperglycaemic environment, and 5.5 mM glucose was used as a control.

Long-term exposure to hyperglycaemia impaired osteoblast differentiation (**II**, Figure 2B) and reduced the viability by approximately 40% (**II**, Figure 2C) compared to control ($p<0.0001$, $p=0.0014$, respectively). In addition, long-term exposure seemed to have a minor effect on the mineralization capability without affecting the differentiation (**II**, Figure 2D). Shorter exposure time to hyperglycaemia did not have a significant effect on osteoblast proliferation or viability.

5.2.2 Hyperglycaemia induces modest changes in the transcriptome of osteoblasts

To further examine the changes in osteoblasts induced by hyperglycaemia, we performed bulk RNA-sequencing at the endpoint of the culture and compared the changes in the osteoblast transcriptome upon short- and long-term exposure to high glucose compared to cells grown in a normoglycaemic environment. Short-term exposure of 24 hours resulted in 1706 DEGs (294 after p-value adjustment) and of 72 hours of glucose exposure in 1342 DEGs (121 after adjustment of p-value) when compared to their normoglycaemic control cells. Long-term exposure of 10 days to hyperglycaemia induced the most changes in the transcriptome, with 1932 DEGs (407 significant after adjustment of p-value) (**II**, Figure 3A). Overall, the changes induced by hyperglycaemia were modest and the fold-changes were relatively low (median fold change 0.86 for downregulation and 1.13 for upregulation).

5.2.3 Common glucose-responsive genes related to energy metabolism

The three glucose exposure times evaluated in this study resulted in only 89 common DEGs without p-adjustment (**II**, Figure 5C). Interestingly, most of the common genes were related to energy metabolism. We observed downregulation of four mitochondrially-encoded electron transport chain genes (*Mt-co2*, *Mt-cox3*, *Mt-nd4l*, *Mt-atp6*) in both short-term exposures, but they were upregulated by long-term exposure to glucose (**II**, Figure 5C). However, the staining of mitochondria did not reveal any major differences observed by visual estimation between the three exposure groups (**II**, Supplementary Figure 1). Upregulation of proline transporter *slc38a2* was seen in response to all three glucose exposures, with the upregulation at HG10d also verified with qPCR ($p=0.016$) (**II**, Figure 5C).

5.2.4 Short-term hyperglycaemia (24 hours) upregulates pathways related to ECM and ossification

We next examined the changes upon 24 hours of glucose exposure induced in osteoblasts. The short-term exposure time resulted in predominantly upregulated DEGs (**II**, Figure 3C). Enrichment analysis revealed that pathways related to ECM organization and ossification were changed (**II**, Figure 4A). Genes changed in the ECM pathway included several collagens (e.g. *Col1a1, Col3a1*) and genes responsible for post-translational modifications of collagens, such as several hydroxylases. Interestingly, genes involved in the ossification pathway, including *Alpl*, *Mepe*, *Phex*, *Sp7*/*Osterix*, *Sost*, and *Bglap,* were all upregulated and the upregulation was further verified with qPCR (*Phex* ($p=0.0004$), *Alpl* ($p=0.014$), *Sost* ($p<0.0001$), *Mepe* ($p=0.0019$) and *Bglap* ($p<0.0001$)) (**II**, Figure 5A). The ALP activity of the cells was also enhanced by 24 h exposure to hyperglycaemia (**II**, Figure 5A)

Glucose exposure of 72 hours resulted in both up and downregulated genes (**II,** Figure 3D). Moreover, the changes in the pathways after 72 hours of glucose exposure were modest and less statistically significant compared to other exposure times (**II**, Figure 4B). Transcriptomic analysis revealed changes in, e.g., amino acid transporters (*Slc38a4*, *Slc6a9*), ECM interactions and lysosome activity.

5.2.5 Long-term hyperglycaemia (10 days) increases the intracellular reactive oxygen species

Upon long-term exposure to glucose, DEGs were mostly downregulated compared to normoglycaemic control (**II**, Figure 3E). Enrichment analysis of DEGs revealed responses in the pathways related to ROS, chemical stress and ECM organization (**II**, Figure 4C). Regarding the ROS pathway, genes related to ROS response, such as glutathione peroxidases (*Gpx3, Gpx1*) and superoxide dismutase (*Sod3*), were upregulated. In addition, thioredoxin-interacting protein (*Txnip*), which inhibits the antioxidant thioredoxin (Choi & Park, 2023), was the most upregulated gene in the entire RNA-seq analysis of long-term glucose exposure. Upregulation was also verified by qPCR ($p=0.0002$) (**II**, Figure 5B). The accumulation of intracellular ROS was also observed in cells treated with 10 days of hyperglycaemia with the help of the CellROX probe, which becomes fluorescent upon oxidation by ROS (**II**, Figure 5B).

The downregulated pathway in the GO analysis included lysosome pathway (**II**, Figure 4C), which interestingly had all 42 genes downregulated, including *Lamp1*, and genes encoding multiple cathepsins. Despite the profound downregulation at the transcriptional level, we observed enhanced cytoplasmic staining of acidic intracellular vesicles when we used LysoTracker fluorescent staining (**II**, Figure 5B).

ECM organization pathway was also changed in the GO analysis (**II**, Figure 4C). Most of the genes related to this pathway were downregulated by long-term exposure to hyperglycaemia. The changed genes included genes e.g. related to post-translational modification of collagen, such as hydroxylase. Only three of the genes belonging to this ontology were upregulated: lysyl oxidase (*Lox*), hyaluronan synthase 2 (*Has2*), and biglycan (*Bgn*).

Although we observed an increase in the transcript of proline transporter *slc38a2*, several other amino acid transporters, such as *slc1a5*, *slc7a8*, *slc7a5*, *slc7a15*, and *slc43a2,* were downregulated.

In summary, both short- and long-term exposures to high-glucose environment induce unique transcriptional changes in osteoblasts. Short-term hyperglycaemia has a stimulatory effect on the transcriptome of osteoblasts, while long-term exposure impairs their functions.

5.3 Elevated plasma glucose levels and type 2 diabetes are associated with lower uncarboxylated osteocalcin levels in humans (III)

5.3.1 Antibody characterization

Lastly, we examined the association between osteoblast-derived osteocalcin and plasma glucose levels in humans. We developed a two-site immunoassay to detect human uncarboxylated osteocalcin (ucOC) in blood samples. After initial characterization, we selected clone scFv-AP13 from the synthetic human scFv antibody libraries by phage-display technique. To enhance the abilities of the recombinant antibody, we used secondary libraries built from the scFv-AP13 by rerandomizing either CDR-L1, -L3, H1 and/or H2 loops, and isolated additional clones scFv-AP2, scFv-AP16 and scFv-AP19. All antibodies shared an identical CDR-H3 but had variations in sequence and loop length in loops CDR-H2 (scFv-AP2 and scFv-AP19), and CDR-H1 (scFv-AP16). We further aimed to improve the stability of the antibodies by converting them into Fab-fragments. Measuring of thermostability (Tm, value at which 50% of the immunoactivity was lost) of both scFv-AP and Fab-AP-recombinant antibodies confirmed the improved stability of Fab fragments over scFv fragments, as the durability to heat was enhanced in all the antibodies when converted to Fab-fragments (**III**, Figure 1). We continued with the Fab-AP clones.

We determined the binding site of the Fab-AP clones and the specificity to carboxylation at positions 17, 21 or 24 with the help of biotinylated peptides. The binding sites of the Fab-AP -clones varied slightly by a couple of amino acids, the

binding site for Fab-AP13 being on residues Val10-Leu25 of osteocalcin (**III**, Figure 2A). However, regarding the carboxylation site, all four clones required Glu17 to be uncarboxylated. In addition, Fab-AP13 and Fab-AP2 needed uncarboxylation at Glu21. None of the antibodies could bind if the OC-peptide was carboxylated at all three sites (**III**, Figure 2B). Thus, our antibodies were confirmed to be specific for uncarboxylated osteocalcin.

5.3.2 Immunoassay characterization

We used biotinylated MAb-2H9 as a capture with the amount of 200 ng per well. As a detecting antibody, the Fab-APs were used in the following concentrations, which were determined by initial characterization:100 ng per well for Fab-AP13, -2 and -19 and 50 ng per well for Fab-AP16. Europium-labelled antiAP (Eu-antiAP, 100 ng per well) was used as a tracer to measure time-resolved fluorometry in the immunoassay. The schematic presentation of the assay is presented in Figure 8.

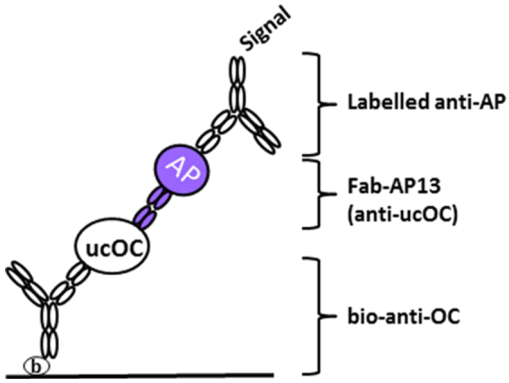

Figure 8. Schematic presentation of immunoassay to detect human ucOC.

We continued to examine the properties of the Fab clones and tested the signal-to-noise ratio (**III**, Figure 2E) and EC50 value (**III**, Figure 2F) in which FabAP-13 performed the most efficiently. Next, we tested whether the immunoassay with Fab-AP13 measures biosynthetic ucOC by measuring culture mediums collected from rat osteoblasts cultured *in vitro*. We cultured rat osteoblasts and stimulated the expression and secretion of osteocalcin with vitamin D. To inhibit the carboxylation of osteocalcin, we treated the cells with warfarin (**III**, Figure 3A). We further used Fab-AP13 and MAb-2H9, the antibodies, to detect biosynthetic ucOC and tOC, respectively, using immunofluorescence (**III**, Figure 3B).

We compared the antibody performance in different sample matrices. We obtained serum, heparin plasma and EDTA plasma samples from the same

individuals and measured ucOC levels with all Fab-AP -clones. All clones were able to detect endogenous ucOC in blood samples; in all of them, the measured ucOC concentrations were higher in plasma samples than in serum samples (**III**, Figure 3C-F). Immunoassays based on different Fab clones performed slightly differently. When compared to Fab-AP13 (set as reference), Fab-AP16 performed relatively similarly, with no difference in serum, 1.3-fold in heparin plasma and 1.1-fold in EDTA plasma samples, respectively. Use of Fab-AP2, on the other hand, resulted in 1.5-fold, 1.3-fold, and 1.5-fold higher ucOC levels, respectively, while Fab-AP19 appeared to underestimate ucOC levels (0.6-fold, 0.8-fold, and 0.8-fold, respectively) (**III**, Figure 3G).

Based on the epitope, thermostability, and performance with peptide and blood samples, we continued with Fab-AP13 for the final immunoassay optimisation. The summary of the optimisation is found in Table 5. Cross-reactivity to carboxylated osteocalcin was 2.3% at 3 ng/ml, 4.3% at 9 ng/ml and 0.2% at 1000 ng/ml excess of cOC peptide in assay buffer RED (**III**, Figure 4B). When ucOC peptide was spiked in either serum or plasma, the assay was linear up to dilution factor 0.125, which equals 0.87 ng/ml and 0.83 ng/ml ucOC, respectively. Acceptance value was determined to be of 20% deviation from the calculated value (**III**, Figure 4C).

Next, we evaluated how the sample handling and storage affects the stability of ucOC and the immunoreactivity of our assay. Serum and plasma samples were collected from three individuals and stored at RT, + 4°C or -20°C at various times. The control sample was transferred at -80°C immediately after sampling. We set the cut-off for acceptable loss of immunoreactivity to 20%. In serum samples, the significant loss of immunoreactivity (below 80% of control) was observed between 2 and 4 h at RT and after 6 h but before 24 h at + 4 °C In plasma, the corresponding times were between 6 h and 24 h at RT and 1 and 2 weeks at + 4°C. There was no loss in immunoreactivity when samples were stored at –20°C within the time period analysed (**III**, Figure 4F) nor with five freeze–thaw cycles (data not shown). Thus, collection of plasma samples and storing them at -20°C can be used in further studies.

We further compared the Fab-AP13-based immunoassay to a commercial Glu-OC EIA kit (Takara Inc.). In a parallel analysis of 60 blood samples, our Fab-AP13 immunoassay exhibited a strong correlation with the results obtained by the Glu-OC EIA kit ($r = 0.57$, $p < 0.001$, $N = 60$) (**III**, Figure 4G), although the average levels of ucOC measured using the commercial Glu-OC EIA kit were approximately 6.5 times higher than those obtained with our immunoassay.

Table 5. Summary of assay characteristics.

PARAMETER	VALUE
Lowest limit of detection (LOD)	0.18 ng/ml
Between-run variability	8.5%
Within-run variability	6.1%
Total variability	10.7%

5.3.3 Plasma ucOC levels associate with plasma glucose levels

Next, we used our immunoassay to measure the levels of uncarboxylated osteocalcin in fasting plasma samples in subjects with T2D, subjects with elevated fasting plasma glucose but no T2D diagnosis and age- and sex-matched normoglycaemic controls. In addition to osteocalcin and plasma glucose levels, we measured the levels of P1NP as a marker of bone formation. Table 6 summarizes the main characteristics of study subjects; more comprehensive table can be found in Study **III**, Table 1. Interestingly, ucOC levels were lower both in subjects that had hyperglycaemia (Con-HG, median ucOC 0.58 ng/ml, p=0.008) and patients with T2D diagnosis (T2D, median 0.68 ng/mL, p=0.015) than in normoglycaemic controls (Con-NG, median 1.01 ng/ml). Further, ucOC and tOC levels were both negatively correlated with fasting plasma glucose levels (r=-0.24, p=0.007; r=-0.27, p=0.003, respectively) (**III**, Figure 5D and E). Surprisingly, although P1NP levels were also lower in both Con-HG group (median 31.3 ng/ml, p=0.026) and T2D group (median 36.0 ng/ml, p=0.026) compared to Con-NG group (median 44.0 ng/ml) (**III**, Figure 5C), there was no correlation between fasting plasma glucose levels (r=-0.12, p=0.19) (**III**, Figure 5F).

Taken together, we developed a highly sensitive and well-characterized immunoassay for the detection of uncarboxylated osteocalcin in human blood. We further showed that the level of hyperglycaemia and the level of uncarboxylated osteocalcin produced by osteoblasts have a negative association in humans *in vivo.*

Table 6. Characteristics of study subjects (N=124). EDTA plasma samples were obtained from normoglycaemic (NG) and hyperglycaemic (HG) subjects and patients with type 2 diabetes (T2D). Data are presented as mean with SD (age) or median with interquartile range (fP-Glucose, ucOC, tOC and PINP) or number of subjects (gender). Modified from original publication III. More comprehensive table can be found in study **III**, Table 1.

	CON-NG	CON-HG	T2D	P VALUE
N	46	29	49	
M/F(N)	23/23	13/16	24/25	0.90
AGE (YEARS)	66.3 (9.6)	64.5 (9.1)	65.7 (9.6)	0.70
FP-GLUCOSE (MMOL/L)	5.50 (5.3–5.8)	7.70 (7.2–8.7)	8.00 (7.1–9.1)	**<0.001**
UCOC (NG/ML)	1.01 (0.72–1.80)	0.58 (0.40–1.47)	0.68 (0.32–1.21)	**0.015**
TOC (NG/ML)	3.90 (2.31–7.38)	2.01 (1.15–4.20)	2.11 (1.02–3.70)	**0.001**
PINP (NG/ML)	44.0 (34.0–63.2)	31.3 (24.2–57.1)	36.0 (26.7–52.9)	**0.036**

* One-way ANOVA (for fP-Glucose, ucOC, tOC and PINP after log-transformation) or $\chi 2$ test were used to test differences across the groups and statistically significant differences are highlighted

6 Discussion

6.1 GLUT1, GLUT3 and GLUT4 in osteoblasts (I)

Glucose is the essential energy source of osteoblasts. However, both inhibition of glucose uptake (Takeno et al., 2019) and excess (Botolin & McCabe, 2006) of glucose leads to decreased function of the osteoblasts, suggesting that proper glucose availability is essential for the cells. Interestingly, although bone is one of the largest organs in humans, its role in glucose disposal is yet to be established. Positron emission tomography studies with labelled glucose have shown that cells in bone marrow (Ojala et al., 2020) and also bone (Lu et al., 2023; Zoch et al., 2016) can take up glucose. Mouse *in vivo* study using radiolabelled glucose suggest that up to 20% of circulating glucose is taken up by bones (F. Song et al., 2023), highlighting the importance of bone in whole-body glucose uptake.

Glucose transporter GLUT1 has been suggested to be the most fundamental GLUT in osteoblast lineage, and its ablation resulted in impaired bone formation (Wei et al., 2015). Our results indicated that GLUT1 is the most abundantly expressed in the BMSCs compared to GLUT3 and GLUT4, which could be explained by its high expression in foetal tissue and other stem cells (Maurer et al., 2006; Mueckler & Thorens, 2013). We silenced GLUT1 in BMSCs and did not observe a significant effect on the proliferation or differentiation of the osteoblasts during the time studied. Transcriptome analysis of the GLUT1 silenced cells aligned with our cellular observations as it revealed less profound changes in gene expression compared to either GLUT3 or GLUT4 silenced osteoprogenitors. GLUT1 has been speculated to be responsible for the basal glucose uptake of many cell types (Pragallapati & Manyam, 2019), which could partly explain the lack of significant changes upon silencing of GLUT1. Further supporting our results, Takeno et al. reported no effect on the mineralization when GLUT1 was silenced in bone marrow-derived stromal derived ST2-cell line. However, they observed a slight improvement in osteoblastogenesis, which was not present in our study (Takeno et al., 2019). Chen and colleagues reported that depletion of GLUT1 in osteoblast lineage of one-month-old mice had no effect on bone formation after a week of the depletion *in vivo*. However, they observed reduced mineralization of the GLUT1-depleted osteoblasts obtained from the mice *in vitro* (H. Chen et al., 2019). As there are conflicting

reports, there is still a need to investigate whether GLUT1 participates in the basal glucose uptake along with other glucose transporters in osteoblasts.

The function of GLUT3 in osteoblasts is far less understood. We observed a stable expression of GLUT3 during 12 days of osteoblast differentiation, which was previously reported also in mouse calvarial osteoblasts (Z. Li et al., 2016). GLUT3 has a higher affinity to glucose than GLUT1 or GLUT4, and is expressed abundantly in cells that require large amounts of glucose, such as brain, sperm, and cancer cells (Simpson et al., 2008). This could explain the high expression levels of GLUT3 in osteoblasts, which require high amounts of energy for bone formation. However, the silencing of GLUT3 in BMSCs and preosteoblasts unexpectedly resulted in the accelerated proliferation of the cells. We observed a redistribution of the remaining GLUT3 proteins in the silenced cells. In the transcriptome analysis of the GLUT3 silenced cells, we observed an upregulation of protein localization pathways, which is in line with the observation of redistribution of the proteins. In addition, changes in the cellular distribution of GLUT3 have been reported in liver cells upon activation and change in the energy requirement of the cell (Defries et al., 2016). We observed downregulation of genes related to mitochondrial electron chain transport complexes, including 15 out of 42 *Ndufs* encoding complex 1, and also genes from other complexes (*Cox*). Downregulation of the *NDUF*-genes, although not all significant, was observed during osteoblastogenesis of human MSCs *in vitro*. Shum and colleagues additionally observed elevated OXPHOS as measured with Agilent Seahorse and redistribution of mitochondria during the osteoblast differentiation (Shum et al., 2016). It would be interesting to study further the energy metabolism and the possible alternations in mitochondrial functions of the GLUT3 silenced osteoblasts.

mRNA levels of GLUT4 were significantly lower compared to mRNA levels of GLUT1 or GLUT3, and the expression level of GLUT4 further decreased during osteoblast differentiation. Our observation was contradictory to Li and colleagues, who observed upregulation of GLUT4 during differentiation of mouse calvarial osteoblasts. This could be due to differences in the origin of the cells. However, we both show that GLUT4 ablation (Z. Li et al., 2016) or silencing of GLUT4 (in our study) decreases the proliferation and differentiation of the preosteoblasts. We observed changes in the IGF signalling pathway in response to GLUT4 silencing in our RNA-seq analysis, which could provide one possible explanation for the decreased proliferation and differentiation. In addition, transcriptome analysis suggested that silencing of GLUT4 resulted in the downregulation of fatty acid metabolism (*Hadh*, *Idh2*), but upregulation of several adipogenesis-related genes (such as *Fabp4, Adipoq,* data not shown). As adipocytes and osteoblasts derive from BMSCs, it would be tempting to speculate and study further whether the downregulation of GLUT4 affects the preferred lineage of the cell. GLUT4 is

responsible for the insulin-mediated glucose uptake, which was thus disturbed due to silencing of GLUT4. Although it is not yet established whether bone cells can become insulin resistant, this could provide insight into the possible mechanisms of putative insulin resistance in osteoblasts. However, the silencing of insulin receptors in human MSCs led to enhanced osteoblastogenesis (Tencerova et al., 2019). As insulin receptors are upstream of GLUT4 regulative pathway, it could be that the effect of insulin receptor silencing and GLUT4 silencing are partially different and need further characterization.

Regarding RNA interference, it must be acknowledged that it does not result in total ablation of the protein. The half-life of glucose transporters has been shown to vary depending on glucose availability (McMahon & Frost, 1995) and as a response to metabolic stress (Khayat et al., 1998). Further, although silencing of each GLUT was highly specific, we observed modest, possibly compensatory, upregulations in the mRNA levels of other GLUTs post-transfection, which could indicate compensatory mechanisms to ensure glucose uptake.

In addition to GLUT1, GLUT3 and GLUT4, we observed expression of multiple GLUTs in the transcriptome of preosteoblasts. Out of 12 GLUTs, preosteoblasts lacked gene transcripts of *slc2a2*, *slc2a7* and *slc2a11*. Others have also found the expressions of multiple glucose transporters, such as GLUT1, GLUT3, GLUT4, GLUT5 and GLUT9, in the osteoblast lineage (Cifuentes et al., 2011; Ohnishi et al., 2020; Richardson et al., 2008; Thomas et al., 1996; Wei et al., 2015; Zoidis et al., 2011). We silenced effectively GLUT1, GLUT3 or GLUT4 in preosteoblasts, and although silencing of each glucose transporter had a different cellular response, the cells endured the silencing and were still alive, suggesting that multiple GLUTs are involved in the glucose uptake of the osteoblast lineage to ensure sufficient glucose supply. We and others have shown that GLUT1, GLUT3 and GLUT4 are all important to glucose uptake of osteoblasts, but further investigation is needed to evaluate the role of other GLUTs and other hexoses, such as fructose, for osteoblast energy metabolism.

6.2 Effects of hyperglycaemia on osteoblasts is dependent on the duration of the exposure (II)

Osteoblasts differentiate and proliferate less when exposed for the long term to a hyperglycaemic environment (Balint et al., 2001; Z. Liu et al., 2015; Takeno et al., 2021) but the effects of short-term exposure are far less known. We exposed the osteoblasts to 25mM high glucose environment for 24 or 72 hours for short-term analysis and 10 days for long-term and evaluated the global changes with RNA sequencing. Constant exposure to hyperglycaemia for 10 days decreased the

proliferation and cell viability of the osteoblasts, while shorter exposure of 24 hours or 72 hours did not have an apparent effect on the proliferation and cell viability.

The three exposure times resulted in unique changes in transcriptomes. Short-term exposure resulted in more upregulated than downregulated differentially expressed genes. Upregulation was observed in the pathways related to bone formation, ECM production and bone matrix mineralization. We confirmed the upregulation of *Phex*, *Mepe, Sost* and *Bglap* via qPCR, and ALP with both mRNA expression and protein activity, suggesting an enhancing effect of high glucose on the osteoblasts. A few studies have previously investigated the effect of short-term glucose exposure on osteoblasts and the results are variable. 24-hour exposure to a 30 mM high glucose environment increased *Col1a1* but decreased *Alpl* expression in osteoblast-like MC3T3-E1 cell-line (Cunha et al., 2014). In the same cell line, 48 hours exposure to a 30 mM glucose environment resulted in increased *Alpl* expression (Botolin & McCabe, 2006). In our study, the upregulation of ossification-related pathways observed after 24 hours was not present when osteoblasts were grown in high glucose for 72 hours, suggesting the enhancing effect of hyperglycaemia is time-dependent. Takeno et al. reported that exposure to hyperglycaemia for 72 hours at an early stage of the osteoblast differentiation resulted in enhanced mineralization at the endpoint in MC3T3-E1 cells (Takeno et al., 2021). It is tempting to speculate, whether the fluctuation of glucose levels could cause enhanced mineralization and, thus, the elevated bone mineral density observed in patients with type 2 diabetes. However, this calls for further investigations both *in vitro* and *in vivo*.

Long-term exposure of 10 days to hyperglycaemia resulted in more downregulated than upregulated genes in the osteoblast transcriptome, which is in line with the observation of impairment in cell viability. One of the mechanisms suggested for the impaired cellular functions is impaired intracellular redox homeostasis (Hamada et al., 2009), and hyperglycaemia has been previously shown to increase ROS production in human adipose-derived stem cells (Cheng et al., 2016) and also in osteoblast-like MC3T3-E1 cells (Zhen et al., 2010). We observed an increased expression of genes within ROS-related ontology pathways and an accumulation of intracellular ROS. To further strengthen our observation, *Txnip*, a gene encoding TXNIP, which is a protein that inhibits the antioxidant function of thioredoxin, was the most significantly upregulated gene in the entire dataset. Previously, Li et al. reported upregulation of *TXNIP* due to exposure to hyperglycaemia in human immortalized MSCs (Y. M. Li et al., 2007). In human osteoblasts, silencing of *TXNIP* resulted in increased osteoblast differentiation (Lekva et al., 2013) and in a rat model of T2D, TXNIP was elevated in bones with decreased bone quality (Liang et al., 2019), further supporting that increased levels of TXNIP and ROS are associated with impaired bone formation.

We further identified 89 genes that were altered in all three hyperglycaemia exposure times. Mitochondria has 13 genes encoding proteins in mitochondrial DNA (mtDNA), and four were changed in 24-hour exposure, nine in 72 hours and eight in 10 days of hyperglycaemia exposure. Interestingly, expression levels were downregulated in both 24- and 72-hour exposure times and upregulated in long-term exposure. Hyperglycaemia causes mitochondrial dysfunction in 3T3-L1 adipocytes (C. L. Gao et al., 2010), but also in human adipose tissue-derived MSCs (Abu-El-Rub et al., 2023) and osteoblast-like MC3T3-E1 cells (Medeiros & Wallace, 2022; Pahwa et al., 2020). We did not observe any notable differences in the distribution or quantity of mitochondria, with fluorescent staining suggesting changes in the mitochondrial energy metabolism rather than mitochondrial number. Further, we observed downregulation of lysosomal degradation pathways in both long-term hyperglycaemia and 72 hours of hyperglycaemia exposure. Surprisingly, we observed an increase in acidic intracellular vesicles in fluorescent stainings. Dysfunction of lysosomes has been observed in T2D-related adverse events, such as diabetic cardiomyopathy (Kobayashi et al., 2023). Hyperglycaemia induces apoptosis of MC3T3-E1 cells, which could provide a possible explanation (P. Zhang et al., 2020). Further, osteoblasts are rich in acidic vesicles as ECM components are transferred through vesicles to their surroundings (Iwayama et al., 2019; Nabavi et al., 2008), which explains the high amount of vesicles, but the elevated amount induced by hyperglycaemia needs further investigation.

Amino acid transporter *slc38a2* was upregulated in all datasets. Slc38a2 transports proline into osteoblasts during osteoblast differentiation (Shen et al., 2022), which is used to produce proline-rich proteins, such as collagens and osteocalcin. Excess glucose could increase proline uptake of osteoblasts and, thus, protein synthesis. However, in the long-term hyperglycaemia, which presented a decrease in the mineralization, the function of slc38a2 must be further explored. In the long-term hyperglycaemia dataset, we observed downregulation of multiple amino acid transporters, and s*lc1a5* was among them. Slc1a5 prefer glutamine as its substrate, and recently, ablation of slc1a5 in osteoblast lineage in mice was shown to impair differentiation of osteoblasts (Sharma et al., 2021). An imbalance of amino acids and their intake might affect the osteoblast differentiation and proliferation.

6.3 Association between bone-derived ucOC and hyperglycaemia (III)

We examined the association of osteoblast-derived protein, uncarboxylated osteocalcin, to demonstrate the link between hyperglycaemia and type 2 diabetes in humans *in vivo*. First, we developed recombinant antibodies against human ucOC with single-framework antibody phage libraries and developed and characterized an

immunoassay. The research group had previously been working with osteocalcin (Ivaska et al., 2005) and the uncarboxylated form has been identified to have a possible endocrine role in glucose and lipid metabolism (N. K. Lee et al., 2007). Briefly, Lee et al. observed increased body weight and impaired glucose metabolism in osteocalcin deficient mice (N. K. Lee et al., 2007). Later, the ucOC form of osteocalcin was shown to have metabolic effects by enhancing the secretion of insulin and adiponectin but also improving glucose uptake in skeletal muscle (Ferron et al., 2008; N. K. Lee et al., 2007; X. Lin et al., 2018) in mice. However, more recently developed osteocalcin-deficient mouse models (Diegel et al., 2020; Moriishi et al., 2020), and also in mouse models with postnatal IR deletion in osteoprogenitors (Fowlkes et al., 2020), have not been able to emulate the endocrine effect of ucOC. In humans, osteocalcin has been shown to associate with circulating glucose levels, insulin sensitivity, and adiponectin concentration in cross-sectional and observational studies (Fernández-Real et al., 2009; Gravenstein et al., 2011; Pittas et al., 2009).

We developed a highly specific immunoassay against the ucOC form of osteocalcin. We used single-framework antibody phage libraries to construct four specific recombinant antibodies, Fab-AP13, Fab-AP2, Fab-AP16 and Fab-AP19, which performed uniquely. After a thorough evaluation of the antibodies, Fab-AP13 was selected for the final immunoassay characterization based on the thermostability of the antibody, specificity to ucOC and performance in different sample matrices. The assay based on Fab-AP13 was found to be sensitive (LOD 0.18 ng/ml) and reproducible as shown by between-run variability (8.5%), within-run variability (6.1%) and total variability (10.7%). As the immunoreactivity of the spiked serum and plasma samples was approximately 10 % lower than expected, there might be degrading proteases or serum and plasma matrices interfering slightly with the binding of ucOC to recombinant antibody. The assay was specific to ucOC as only minor cross-reactivity to cOC (4.3% at 9 ng/ml and 0.2% at the excess of 1000 ng/ml cOC-peptide) was detected. Human osteocalcin is carboxylated at three sites, and we evaluated that the immunoassay detects fully uncarboxylated ucOC but also partially carboxylated with uncarboxylations at positions Glu17 and Glu21 and carboxylation at position Gla24. Fab-AP13-based immunoassay detects mid-molecular region Val10-Leu25; hence, it measures both full-length ucOC, but also fractions containing the region. In addition to human ucOC, the assay also detects rat osteocalcin as there is a difference of only two amino acids in the binding site of Fab-AP13 (Hauschka et al., 1989). The immunoassay uses time-resolved fluorescence as a detection method, which enhances the sensitivity of the assay. The advantages of the recombinant antibodies are that they can be both produced in large quantities in *E.Coli* and they are reproducible compared to for example polyclonal

antibodies. Further, the recombinant antibodies can be used in other applications, such as immunofluorescence staining's.

We measured blood samples collected from a cohort of adult subjects with either normal fasting glucose or elevated fasting glucose but no confirmed T2D and patients with T2D. The group of subjects with elevated plasma glucose of over 7.0 mmol/l was chosen to represent undiagnosed T2D patients, as fasting plasma glucose of over 7 mmol/l is considered as diabetic. Patients with T2D, as well as subjects with elevated fasting plasma glucose levels, had lower circulating concentrations of both ucOC and total OC, compared to the normoglycaemic control group. This has been previously reported for total OC (Im et al., 2008). There was a negative correlation between ucOC and fasting plasma glucose in subjects with elevated glucose levels but not in T2D patients. However, total OC levels were similarly associated, and thus, in this study, measuring the ucOC form of osteocalcin did not provide additional value over measuring total OC. We additionally measured PINP levels to evaluate bone formation. In our cohort, we did not observe a similar correlation to fasting plasma glucose in PINP, although the levels of PINP were lower in subjects with hyperglycaemia and patients with T2D when compared to controls. Plasma glucose could be specifically associated with osteocalcin protein, especially ucOC form, instead of bone formation rate in general, suggesting a possible link with osteocalcin and hyperglycaemia. In addition to the biosynthesis in osteoblasts, osteocalcin is also released during bone resorption (Ivaska et al., 2004), which could explain the observed difference between osteocalcin and PINP. To evaluate the relationship between uncarboxylated osteocalcin, glucose metabolism and T2D in humans, larger cohorts are clearly needed. Nevertheless, our results are in line with previous studies and indicate that hyperglycaemia is associated with suppressed bone formation *in vivo*.

6.4 Limitations of the study

This thesis study comes with some limitations. Although this thesis used a well-characterized *in vitro* model for cell culture experiments, the cross-species variation has to be addressed, as rat cells might not behave similarly to human cells. However, rat osteoblasts share many similarities with human osteoblasts, e.g. response to vitamin D_3 and organization of the osteocalcin gene, which are both different from human in mouse osteoblasts (Usda et al., 2013). Nevertheless, although the *in vitro* -model used was well characterized and repeatable, further validation with human osteoblasts is required.

RNA sequencing is a valuable tool that reveals differences in the gene expressions and provides an overall view of the changes induced by various treatments in cells. The studies in this thesis investigating the effects of GLUT

silencing or hyperglycaemia on osteoblasts were conducted at a transcriptional level, and whether the changes observed are translated to a post-transcriptional level, remains to be validated. Indeed, additional evaluation of the changes with proteomics would provide a better and more comprehensive understanding of the responses induced by the treatments. Further, we did not measure the direct effect of silencing of individual GLUTs on glucose uptake of the cells. However, after silencing of GLUT1, GLUT3 or GLUT4, preosteoblasts were viable, indicating complementary mechanisms, and thus, we examined and focused on the transcriptional changes. It also must be noted that in the second study (**II**), we observed global changes in transcriptome induced by hyperglycaemia on osteoblasts *in vitro*. However, *in vivo*, T2D is a metabolic disorder with variables other than hyperglycaemia, e.g. hyperinsulinemia and possible medications, that can also affect the cells.

This thesis focused on bone formation, but bone resorption is also an energy-consuming process (Da et al., 2021) and affected in T2D *in vivo* (Picke et al., 2019). As osteoblasts and osteoclasts are coupled and bone resorption precedes bone formation, impairment of bone resorption due to hyperglycaemia (Tanaka et al., 2017; Wittrant et al., 2008) could lead to impaired bone formation as well. It would be interesting to evaluate the effects of hyperglycaemia in co-cultures of bone cells and whether hyperglycaemia affects the signalling between osteoblasts, osteoclasts and even with osteocytes.

6.5 Future perspectives

Glucose is transported to cells via GLUTs, and it would be reasonable to speculate that the cells could control the intake of glucose via glucose transporters. Pacicca and colleagues reported that exposure to 25 mM hyperglycaemia for 14–35 days decreased while exposure to 2.5 mM hypoglycaemia upregulated the mRNA expressions of GLUT1 and GLUT3 compared to control (10 mM glucose) in mouse osteocyte-like IDG-SW3 cells (Pacicca et al., 2019). We did not observe any statistically significant changes in the expression of glucose transporter expressions in the transcriptome in any of the glucose exposure times observed (data not shown). Longer exposure times to hyperglycaemia than those used in our study, over 10 days, are needed to detect changes in the expression of GLUTs, but whether such exposures would be physiologically relevant is debatable. However, it would be interesting to challenge the cells silenced for glucose transporters in either hypo- or hyperglycaemic environments to elucidate the possible compensatory mechanisms. As silencing of GLUT4 in osteoblasts could partly mimic the effect of insulin resistance in osteoblasts, adding a hyperglycaemic environment on such cells could better recapitulate the environment of bone cells in T2D.

In the present study, we showed that silencing of glucose transporters resulted in changes in gene expressions of genes related to energy metabolism. As our study was transcriptional, we were unable to link changes in transcriptome to cellular responses. Further validation of changes in energy metabolism and functions of mitochondria would provide valuable information and potential new therapeutical aspects for rescuing bone cell function. The research group of F. Long demonstrated that both T1D (Ji et al., 2023) and T2D (F. Song et al., 2023) are associated with decreased bone quality in mice, which is caused by impaired glucose metabolism as both glycolysis and OXPHOS were decreased in osteoblasts. By enhancing glycolysis in osteoblasts *in vivo* via multiple experimental approaches, they were able to rescue the bone quality of diabetic mice. Thus, the enhancement of glycolysis or glucose metabolism in osteoblasts could serve as a new potential therapeutical target for osteoporosis.

Interestingly, one gene was changed in most of the RNAseq datasets: *Txnip*. TXNIP expression is negatively associated with bone formation in rats (Liang et al., 2019), which is in line with our observation. We observed an upregulation of *Txnip* in transcriptomes of long-term hyperglycaemia and silencing of GLUT4 (data not shown), which both led to reduced proliferation and differentiation of osteoblasts. Downregulation of *Txnip* was observed in short-term hyperglycaemia of 24 hours, in silencing of GLUT1 and GLUT3 (data not shown). TXNIP promotes oxidative stress, and its expression is elevated in hyperglycaemic or diabetic conditions (Choi and Park 2023; Thielen and Shalev 2018) but is also involved in regulation of glucose uptake and metabolism. Glucose uptake of human adipocytes was enhanced by reduced TXNIP levels, and elevated expression levels of TXNIP decreased glucose uptake (Parikh et al., 2007). Further, TXNIP regulates intracellular movement of GLUT1-containing vesicles and mRNA expression of GLUT1 (Qualls-Histed et al., 2023; N. Wu et al., 2013) but also mediates basal endocytosis of GLUT4 *in vivo* (Waldhart et al., 2017). Interestingly, Kover et al. showed that treatment with osteocalcin reduces glucose-induced stress and preserves rat pancreatic beta-cells under hyperglycaemic culturing conditions by reducing the glucose-elevated TXNIP expression in beta cells (Kover et al., 2015). TXNIP has recently gained interest as a possible drug candidate for T2D due to its inhibition having a preserving effect on pancreatic cells (A. Li et al., 2023; Thielen et al., 2020). The molecular mechanisms of TXNIP in bone cells are not fully understood, and it would be intriguing to study whether inhibition of TXNIP would also function as a possible drug target for preserving bone.

7 Conclusions

Based on the original studies and discussion presented in this thesis, the following conclusions can be made:

1. GLUT1, -3 and -4 are expressed during the differentiation of rat osteoblasts *in vitro*. Glucose uptake in osteoblast lineage rely on several glucose transporters.
2. Long-term exposure to hyperglycaemia impairs rat osteoblast function *in vitro*, most likely via mechanisms involving an imbalance in intracellular oxidative stress.
3. Short-term exposure to hyperglycaemia has a stimulatory effect on genes and pathways related to bone matrix production and mineralization in rat osteoblasts *in vitro.*
4. An immunoassay based on the recombinant antibody Fab-AP was developed to create a reliable and specific method to evaluate the circulating levels of uncarboxylated osteocalcin in humans.
5. Uncarboxylated osteocalcin levels in plasma are negatively associated with fasting plasma glucose levels in humans *in vivo.*

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