DIPLOMA THESIS

Effects of Immobilization on Bone Physiology and Metabolism

Submitted by
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for the Academic Degree of

Doktor der gesamten Heilkunde

(Dr. med. univ.)

at the

Medical University of Graz

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Graz, November 2014

Statutory Declaration

I hereby declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz, November 2014 ***Kurt Weber eh.***

ACKNOWLEDGEMENTS

First of all, I would like to express my thank to my supervisors Ass. Prof. Priv. Doz. Dr. med. Nandu Goswami and Dr. med. Lukas Holzer, who gave me the opportunity to write on a subject of my personal interest. Thank you, for your excellent guidance, support and patience throughout this thesis and for introducing me to scientific writing.

Further, my thanks goes to Prof. Dr. Patrick De Boever, who not only provided all the necessary data but also gave fundamental advices on how to cope with this special subject. Thank you a lot!

Secondly, my utmost gratitude goes to my parents, Christa and Kurt Weber, who gave me the opportunity to take and finish my studies by supporting and encouraging me in any circumstances of my life. Thank you very much indeed!

ABSTRACT

Introduction: Bone is underlying a constant process of remodelling, adapting to inner and outer mechanical forces. Physical loading is an essential determinant for the maintenance of a physiological bone quality. Bone is known to response fast in terms of mechanical unloading. As a result, deconditioning with a loss of bone mass and reduction of bone density will set in quickly. Bone metabolism is not fully understood on a molecular biological level. Therefore, this diploma thesis will provide an overview of bone biology and physiology. Further, the effects of a 21 days bed rest trial on the molecular mechanisms of bone metabolism will be investigated.

Methodology: 12 study subjects were exposed to bed rest for 21 days with a 6° head-down-tilt position. 3 groups were set up, respectively: Bed rest (BR), bed rest plus exercise (BR + EX), bed rest plus exercise plus diet (BR + EX + Diet). Each of the 12 volunteers acted as their own control and changed their allocated group during each campaign. Due to cost issues, only 3 subjects from each group of the first campaign (n=4) were chosen randomly for investigation of the molecular mechanisms associated with bed rest, with and without exercise and nutrition interventions. Blood samples were taken before and at campaign end. Total mRNA was extracted to generate cRNA, which was hybridized on microarray slides. Biological analysis and interpretation were performed with GeneSpring and Ingenuity Pathway Analysis (IPA).

Results: Using IPA a list of 233 genes related to "*Skeletal and muscular system development and function"* was constructed, which mapped back to 409 probes. After applying a threshold of FC = 1,5 15 genes of the BR, 33 genes of the BR + EX and 16 genes of the BR + EX + Diet group were sorted out. For a better biological understanding and interpretation, the 15 genes of the BR group were compared to those of the treatment groups (BR + EX, BR + EX + Diet) based on their expression values and polarity.

Discussion: We identified differences in gene expression changes based on the type of intervention using the innovative technique of transcriptomics. Differentially expressed genes in the BR group are reported to play an important role in bone metabolism and mechanotransduction. We conclude that microarray analysis of blood samples is an appropriate technique to investigate early biological changes due to bed rest on a molecular level.

ZUSAMMENFASSUNG

Einleitung: Der menschliche Knochen unterliegt im Rahmen des „Bone remodelling“ einem ständigen Umbau. Er besitzt die Fähigkeit entsprechend äußerer und innerer mechanischer Einflüsse zu adaptieren. Regelmäßige physikalische Belastung ist ein essentieller Faktor für die Erhaltung einer physiologischen Knochenqualität. Im Gegensatz dazu, kommt es durch Entlastungsphasen schon nach kurzer Zeit zu messbaren Veränderungen im Knochenstoffwechsel. Die molekularbiologischen Grundlagen dieser Veränderungen sind bis heute weitestgehend unklar. Die vorliegende Diplomarbeit gibt einen Überblick über die Biologie und Physiologie des Knochens, sowie dessen Metabolismus. Im speziellen wird eine Immobilisationsstudie hinsichtlich molekularbiologischer Veränderungen des Knochenstoffwechsels analysiert.

Material und Methoden: 12 Probanden nahmen an einer 21-tägigen Immobilisationsstudie teil und wurden in 3 Gruppen zu jeweils 4 Personen aufgeteilt: Bettruhe (BR), Bettruhe mit Trainingseinheiten (BR+EX) und Bettruhe mit Training und spezieller Diät (BR+EX+Diet). Aus Kostengründen wurden nur 3 Personen von den ursprünglichen 4, aus jeder Gruppe der ersten Kampagne ausgewählt und untersucht. Blutproben wurden am Anfang und Ende der Studie gewonnen. Die gesamte mRNA wurde extrahiert und durch Anwendung von Mikrochip DNA Technologie auf Veränderungen im Transkriptom analysiert. Gene Spring und Ingenuity Pathway Analysis (IPA) wurden zur biologischen Interpretation verwendet.

Ergebnisse: Mittels IPA konnten 15 Gene in der BR Gruppe, 33 Gene in der BR+EX Gruppe und 16 Gene in der BR+EX+Diet Gruppe identifiziert werden, die sich in ihrer Expression mindestens um den Faktor 1,5 unterschieden. Für ein besseres biologisches Verständnis und Interpretation wurden die 16 Gene der BR Gruppe mit jenen der Behandlungsgruppen (BR+EX, BR+EX+Diet) verglichen.

Diskussion: Anhand der Transkriptom Analyse konnten Unterschiede in der Genexpression abhängig vom Behandlungstyp festgestellt werden. Die 16 unterschiedlich exprimierten Gene der BR Gruppe spielen eine wichtige Rolle im Knochenstoffwechsel und in der Mechanotransduktion. Die Microarray DNA Analyse von Blutproben stellt eine geeignete Methode dar, um frühe biologische Veränderungen aufgrund von Immobilisation festzuhalten.

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ABBREVATIONS

AGEs	Advanced glycation end products
ALP	Alkaline phosphatase
BDC	Baseline data collection
BH-FDR	Benjamini-Hochberg false discovery rate
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BMU	Bone multi-cellular unit
BR	Bed rest
BSAP	Bone sialoprotein
BTM	Biochemical bone turnover markers
CNV	Copy number variations
DMP-I	Dentin matrix acidic phosphoprotein I
DXA	Dual-energy X-ray absorptiometry
ECM	Extracellular matrix
ESA	European Space Agency
EX	Exercise
FC	Fold change
FGF-23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptor
FRAX	Fracture risk assessment
GO	Gene Ontology
HA	Hyaluronic acid

HDT	Head down tilt
HGP	Human Genome Project
IGF	Insulin-like growth factor
IPA	Ingenuity Pathway Analysis
LRP	Low density lipoprotein receptor-related protein
M-CSF	Macrophage colony-stimulating factor
MEDES	Institute for Space Medicine and Physiology
MNX	Medium Duration Nutrition and Vibration Exercise
MSC	Mesenchymal stem cells
NCPs	Noncollagenous proteins
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
OPG	Osteoprotegerin
OPN	Osteopontin
OSX	Osterix
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PTHLH	Parathyroid hormone-like hormone
QCT	Quantitative computed tomography
R	Recovery
RANKL	Receptor activator of NF-kb ligand
RGD	Arginylglycylaspartic acid
SD	Standard deviation
sFRP	Secreted frizzled-related protein
SNPs	Single nucleotide polymorphisms
TGF-β	Transforming growth factor ß

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I INTRODUCTION

1 BONE MORPHOLOGY AND FUNCTION

The shape of the human body is basically determined by the skeleton. The adult human skeleton has a total number of 206 bones. This excludes sesamoid bones except for the patella and accessory bones. It can be divided in the axial and appendicular skeleton. The axial skeleton consists of 74 bones comprising the vertebral column, skull, ribs, the hyoid bone and sternum. The appendicular skeleton includes the limbs with a total sum of 126 bones. Additionally 6 bones are termed auditory ossicles. Conjoined with cartilage, ligaments, intervertebral discs and joints bone tissue is the main part building the *passive human mobile apparatus*. The *active mobile apparatus* is composed of muscles, fascias, tendons, their synovial sheets and bursas. Together these two compartments form the musculoskeletal system (1).

1.1 Function

The musculoskeletal system provides several functionally and dynamically tasks which are combined tightly. The bones of the skeleton are giving form and structure to the human body, protect internal organs, amplify acoustic signals in the middle ear plus enable locomotion by providing levers and sites of attachment for muscles. Furthermore, bone is a highly active metabolic tissue including mineral homeostasis, contribution in acid base balance as a second line defence against metabolic acidosis and is a reservoir for growth factors and cytokines (2). In recent years it has been identified as an endocrine organ which is involved in energy and phosphate metabolism by secreting fibroblast growth factor 23 (FGF-23) and osteocalcin (3). Moreover, undercarboxylated osteocalcin has an influence on beta-cell proliferation in the pancreas enhancing insulin secretion. It also affects adipocytes outside of the bone to produce adiponectin helping to lower insulin resistance (4). The huge surface is able to adsorb toxins and heavy metals, limiting adverse effects on other organs (2). Finally it is building the environment for haematopoiesis in the bone marrow cavity and provides an energy store (5).

1.2 The Shapes of Bones

Generally there are five categories of bones: long, short, irregular, pneumatic and flat bones. Form and structure are genetically determined but also influenced by mechanical forces and gravity.

Long bones are located in the limbs, they consist of a shaft or diaphysis and two thickened extremities, the epiphysis. In between the diaphysis and the epiphysis lies the metaphysis (growth plate). During childhood and adolescence the metaphysis is the zone of longitudinal growth. The diaphysis is cylindrically shaped and has a massive corticalis, which is thicker in the middle and thins out towards the epiphysis. Covering a space in the middle, the medullary cavity, it provides the environment for haematopoiesis during development and is filled with some cancellous tissue and fat in the adult skeleton. The epiphysis is usually broad in order to provide muscle attachment and articulation. Cortical bone is thin, covering cancellous bone, which is formed in a trabecular meshwork. Influenced by pressure, tension, muscle-stress and the body weight, an internal structure is formed, where plates and bars are crossing at right angles, in order to gain a maximum in strength at a minimum of weight.

Short bones have a thin cortical bone, covering cancellous tissue filled with bone marrow. Particularly found in places of high pressure and limited movement, such as the tarsus and carpus. *Flat bones* consist of a thin inner and outer layer of corticalis, mainly to serve as protection and as a large muscle attachment site. *Irregular bones* are those, difficult to match a scheme above such as the vertebrae. *Pneumatic bones* are aeriferous and are covered by a mucous membrane, they communicate with the environment by the nasopharyngeal zone (6).

Figure 1 Internal structure of the human femur (7)

1.3 Composition

The chemical composition of bone tissue by weight accounts about 65% mineral, 20-25% organic material, 10% water and 2% lipids. To a certain amount water is bound to collagen fibers, the remainder is free to flow in the canalicular system and bone vessels. 90% of the organic compartment is basically collagen type I with smaller amounts of collagen type III and collagen type V. Noncollagenous proteins (NCPs) build the remaining 10% and contribute in mineralization, embryogenesis, development, regulation of size and formation of collagen fibrils and adhesion processes. Hydroxyapatite is basically built by calcium-, phosphate- and hydroxyl ions with marks of magnesium, acid phosphate and carbonate (8).

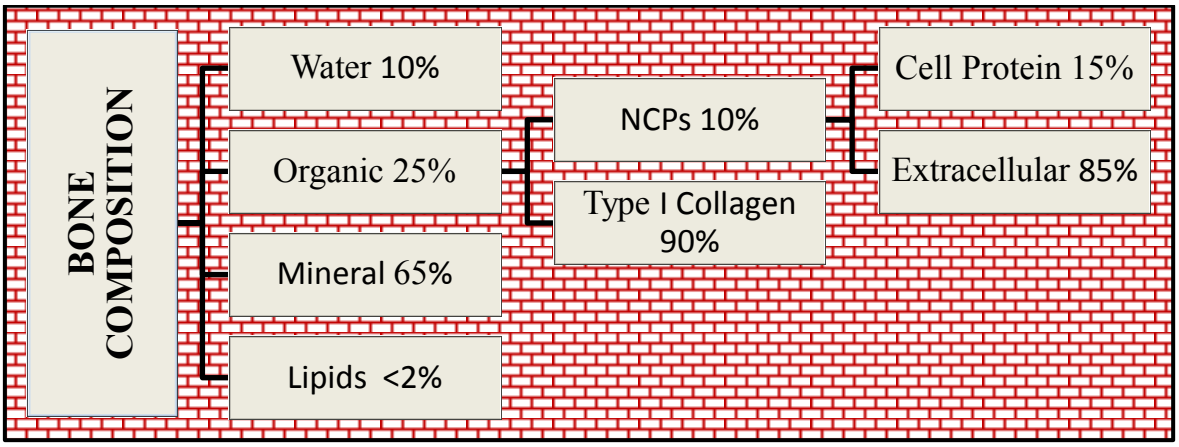

Figure 2 Schematic overview of bone composition

1.4 Structure and Organization

To accomplish bone specific functional aims, bone tissue is organized in a hierarchical way, from a molecular level to macroscopic structures.

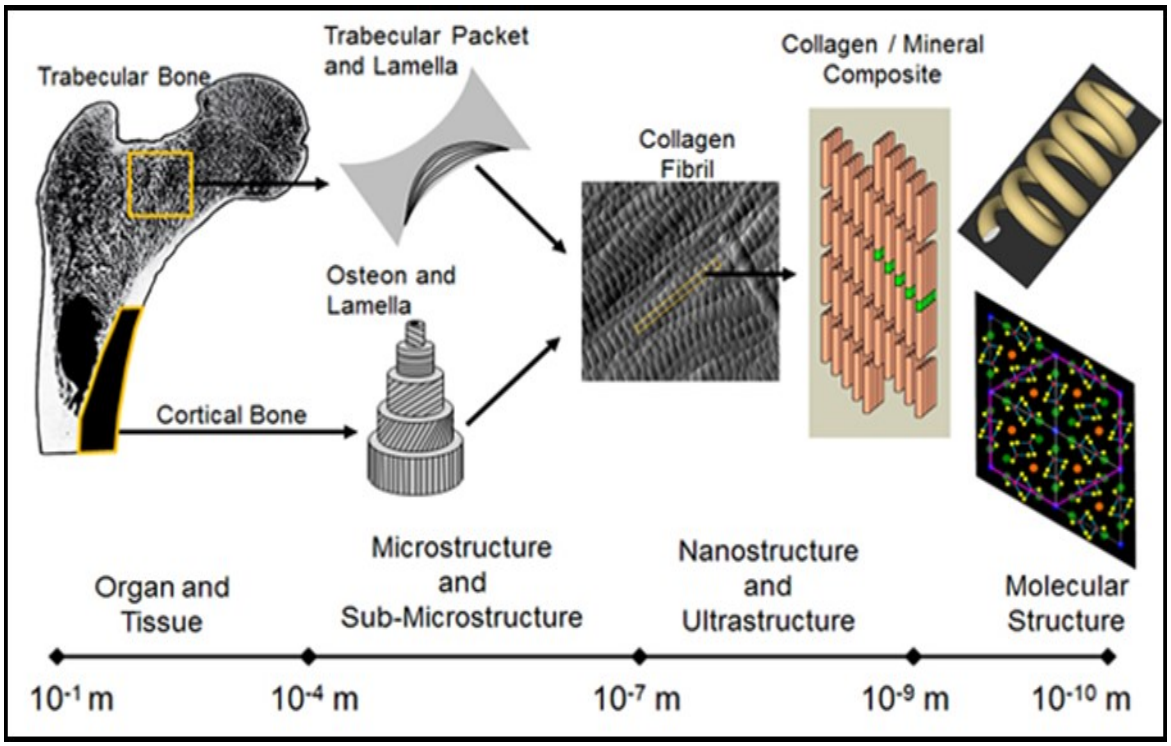

Figure 3 Hierarchical arrangement of bone structure (9)

1.4.1 Nanostructural organization

1.4.1.1 Collagen

On a molecular level, collagen fibers are built by collagen molecules, reaching a diameter of 15 – 130nm and 10um in length. They are built by two alpha-I chains and one alpha-II chain, forming a triple helix which is mineralized with carbonated apatite within and between the fibrils. This composition provides stiffness and load bearing strength according to the mineral portion and elasticity and resilience by the collagen fibers. Collagen molecules do aggregate lateral and in a longitudinal way. Condition for building a triple helix is a high amount of amino acids, namely glycine, proline and hydroxyproline. These amino acids are repeated constantly, forming a ring with the backbone of the chain (8). Hydroxyproline is essential for binding water molecules, thus providing stability to the triple helix by supporting the helix with a sheath of water molecules and hydrogen bonds. The connection of collagen fibrils by different kinds of cross-links leads to a high tensile strength and has basic effects on the mechanical properties of bone tissue. It is achieved by crosslinked lysine and hydroxylysyl, formed through an enzymatic pathway by the enzyme lysyl oxidase. Other bonds are formed by nonenzymatic glycation, leading to advanced glycation end products (AGEs) and the

aggregation of arginine, ribose and lysine in a nonenzymatic pathway. AGE accumulation over years is known to cause increased bone fragility and is especially found in people suffering from diabetes mellitus. In the extracellular matrix AGE accumulation leads to an altered regulation of bone forming and resorbing cells, which has a negative effect on bone quality, supposable leading to fractures (10).

Microscopically the organization of the collagen fibrils is highly related to its functional requirements and changes due to growth rate and location where it is formed. Longitudinal formation is found in areas under tension, whereas transverse arrangement is predominant in portions which are under compression (10)

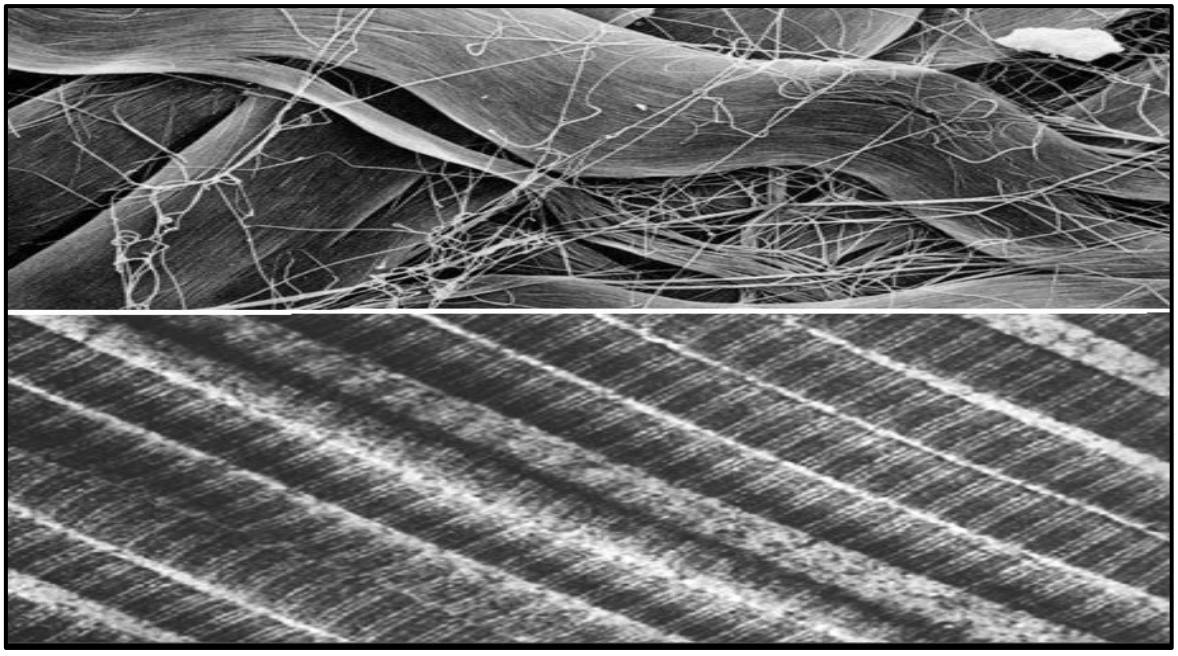

Figure 4 Microscopic image of collagen fibers (11)

1.4.1.2 Mineral

The main mineral component of bone is crystalline hydroxyapatite [$Ca_{10}(PO_4)6(OH)_2$], which makes up about the half of the mass and nearly a quarter of adult bone volume, with small amounts of magnesium, acid phosphate and carbonate. These crystals nucleate along the pores, longitudinally between the fibrils and within the ends of collagen fibrils, called hole zones. Initial deposition starts with amorphous calcium phosphate, derived from the blood plasma, and matures through several stages to form hydroxyapatite, resulting in a highly organized composition of collagen and hydroxyapatite. Maturation of bone matrix is associated with expression of noncollagenous proteins, such as osteopontin, bone sialoprotein, osteocalcin and

alkaline phosphatase. These proteins are considered to help regulating the size and amount of hydroxyapatite crystals to attain an ordered deposition, by binding calcium and phosphate. Extracellular matrix vesicles contain proteins, calcium, inorganic phosphate and acidic phospholipids. These vesicles serve as a nucleation promoter by building a protected microenvironment, where calcium and phosphate concentrations can rise to an adequate level to precipitate crystal formation. Known mineralization promoters are dentin matrix protein I (DMP1) and bone sialoprotein. The mineralization process is regulated by bone alkaline phosphatase (ALPL) by increasing local phosphorus concentrations, removing phosphate containing inhibitors of crystal growth or can change phosphor proteins in a way to act as a nucleation core. Vitamin D is indirectly involved in mineralization. It is maintaining a sufficient serum level of calcium and phosphorus by increasing intestinal absorption of calcium and phosphorus. Furthermore, it is also involved in osteoblast differentiation and expression of bone specific alkaline phosphatase, Osteonectin, Osteoprotegerin (OPG), Osteocalcin and other cytokines (10,12,13).

1.4.1.3 Noncollagenous extracellular matrix proteins

Noncollagenous proteins can be divided in exogenously and endogenously derived proteins. Exogenously derived proteins represent about 25% of the total protein amount, including serum albumin, α2-HS-glycoprotein, growth factors and a broad variety of others contributing in mineralization and cell activity. Endogenous proteins are synthesized and secreted by osteoblasts and can be categorized in the following large classes:

- Proteoglycans
- Glycoproteins
- Proteins of the small integrin-binding ligand N-linked glycoprotein (SIBLING)
- Osteocalcin
- Osteonectin

Table 1 **Schematic overview of noncollagenous extracellular matrix proteins (10)**

PROTEOGLYCANS AND GLYCOSAMINOGLYCANS	
Heparan sulphate	• Produced by osteoclasts and osteoblasts • Plays important roles in cell-cell interactions
Hyaluronan	• Nonsulfated glycosaminoglycan • Hyaluronan in periosteum, endostium and around cells • CD44 is the cell surface hyaluronan receptor and plays a role in development
Small leucine-rich proteoglycans	• Provide structural organization in bone
Biglycan	• Found in pericellular location undergoing morphological delineation • Up regulated in osteoblasts and found in osteocytes • May act as shear sensors • Binds collagen and TGF-β
Decorin	• First appears in preosteoblasts and is down regulated in more terminal osteoblastic cells • Binds to collagen and TGF-β plus regulates the diameter of fibrils • Inhibition of fibronectin attachment to cells
Fibromodulin	• Binds to distinct regions of collagen fibers • Binds TGF-β
Osteoadherin	• Contains RGD sequence • Function unknown
Versican	• CS-containing PG found in osteoid • May act as a space holder for future bone areas
GLYCOPROTEINS	
Alkaline phosphatase	• Carries calcium • Involved in the inhibition of mineral deposition • Loss of function leads to hypophosphatasia • Bone formation marker • Nonspecific and bone-specific forms (BSAP)
Fibronectin	• Important for bone formation during onset stages

	• Binding of cells via a RGD - independent process • Important for cell proliferation
Thrombospondin	• Role in development- found in early stages of bone formation(MSCs and chondrocytes during cartilage development) • Anti-angiogenic
Vitronectin	• Involved in cell attachment and spreading; shows specificity for osteopontin
SIBLING FAMILY OF GLYCOPROTEINS	
Bone sialoprotein	• Limited expression pattern • Marker of late differentiation phases and onset phase of mineralization
Dentin matrix acidic phosphoprotein I (DMP-I)	• Osteocytes plus osteoblasts expression • Has affinity for hydroxyapatite and the N-terminus of type I collagen • Part of mineralization process
Matrix extracellular phosphoglycoprotein	• Osteocytes plus osteoblasts expression • Involved in the regulation of mineralization • Negative regulation of osteoblastic activity
Osteopontin	• Secreted by bone cells in onset stages of osteogenesis • Promotes adhesion of different tissues • Inhibits mineral formation and crystal growth
OTHER IMPORTANT NONCOLLAGENOUS PROTEINS	
Osteocalcin	• Enhances calcium binding, controls mineral deposition • Expressed by osteoblasts and osteocytes • Bone remodelling marker • Overexpressed in cancer and some autoimmune diseases
Osteonectin	• Binds to collagen, HA and Vitronectin • Located at sites of mineral deposition (possible nucleator) • May play role in osteoblast proliferation

1.4.2 Microstructural organization

The microstructural architecture of bone is closely related to its function and the way of its deposition. Most bone show a lamellar pattern, which means that collagen fibers and mineral are organized in sheets building circumferential bands. Two lamellas are

disconnected by an interlamellar layer and each lamella shows an altering orientation of the parallel aligned collagen fibrils. This construction is similar to plywood and results in a high mechanical strength.

1.4.2.1 Woven bone

Woven bone is architecturally not based on a lamellar pattern. Collagen fibers are incidentally organized according to its rapid formation and mineralization. Thus, resulting in lower load bearing capability and weakness. Usually woven bone is built directly on mesenchyme tissue de novo without any intermediate stages, also known as intramembranous ossification. Woven bone is found in all fetal bones and as a repair tissue after fractures, which is replaced by lamellar bone throughout development. Woven bone also occurs in the pars petrosa of the skull, during inflammation processes and under conditions of high mechanical loads due to not fully adapted bone.

1.4.2.2 Primary bone

Primary bone is defined as bone formed in spaces where no bone has existed before. There are two different types of primary bone: primary lamellar bone and primary osteons. Alongside their common definition they all have different mechanical and physiological properties. Primary lamellar bone is principally built on the periosteal and trabeculae surface and covers the interior of the bone marrow cavity. It is built of sheets of lamellas, poorly vascularized, dense, solid and stands for high resistance.

By infilling vascular channels (Haversian canal) primary osteons are formed. It is a system built of concentric lamellar sheets, carpeting the surface of the channels in a layer by layer altering orientation until a small canal remains containing a neurovascular bundle. Primary osteons are smaller than secondary ones with a smaller number of lamellas and might appear during periods of rapid growth.

1.4.2.3 Secondary bone

Whenever pre-existing bone is resorbed and replaced by new bone it is called secondary bone. This difference is important because primary bone only requires formation, whereas secondary bone is formed in a coordinated interaction of resorbing and rebuilding. The main principle of lamellar bone is the Osteon or the Haversian system. The Osteons are circular or oval and measure a diameter of 100-400 um, are 1-10mm long and build a branching network. They consist of 5-20 layers of collagen

fibers surrounding one Haversian canal in a concentric, layer by layer, altering orientation similar to plywood resulting in a high mechanical strength. Each osteon is clearly separated from the surrounding matrix by a cement line. This is the zone where osteoclast resorption ends and bone formation starts. Osteons are pervaded by blood vessel canals in a transverse direction (Volkmann´s canals) to ensure communication amongst all osteocytes by diffusion from the Haversian canals. On the outer and inner surfaces lamellas are not organised in osteons but fully cover the whole circumference (14).

1.4.3 Macroscopic organization

From a macroscopic point of view bones are built by four components: the *periosteum* which covers the outer surface and contains a high amount of blood vessels, nerve fibers, osteoblasts and osteoclasts. It consists of two layers – a stratum fibrosum on the outside made of tense collagen fibers and a stratum osteogenicum that covers the corticalis directly. It conveys tendons and ligaments to the corticalis (sharpey´s fibers), nourishes and protects the bone. Moreover, it is important for appositional growth and bone regeneration. The *endostium* covers all inner bone surface inclusive of blood vessels canals (Volkmann´s canals) and is made of a continuous cell layers (lining cells) including stem cells, osteoprogenitor cells, osteoblasts and osteoclasts. It is essential for regeneration and remodelling (14).

The **cortical bone** (**compacta**) of lamellar bones is composed of osteons. It is the main component of long and short bones shafts. By the high number of Haversian canals cortical bone is porous to an amount of 3-5% which is increasing in age or throughout pathological conditions.

Trabecular bone (**spongiosa**) is a honeycomb like network of trabecular small rods and plates interstratified in the medullar cavity. Influenced by pressure, tension, muscle-stress and the body weight an internal structure is formed where plates and bars are crossing at right angles, in order to gain a maximum in strength at a minimum of weight. Lamellas are organized parallel to the trabecular surface. This special design ensures a high mechanical support. Trabecular bone is mainly localized in the weight bearing areas of long bones to transfer load to the cortical bone and in the axial skeleton. The amount in the human skeleton is 80% compact and 20% trabecular bone (2, 19).

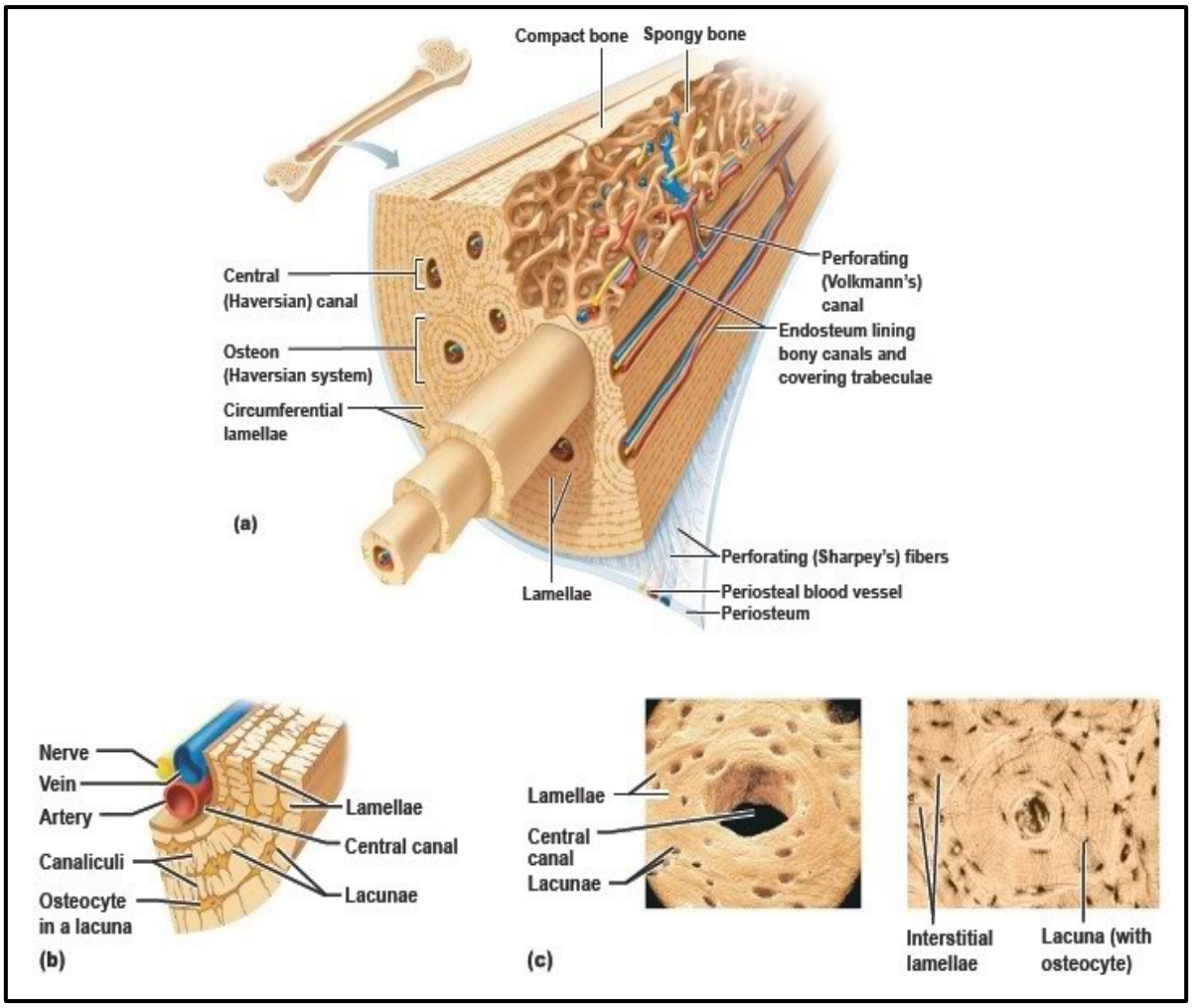

Figure 5 Microstructure of long bones (15)

1.5 Cell Types

Bone consists of three main cell types: support cells, termed osteoblasts plus osteocytes, and remodelling cells, called osteoclasts.

Osteoblasts derive from mesenchymal stem cells, influenced by local growth-factors, hormones and the Wnt - catenin pathway. They differentiate into osteoprogenitor cells, preosteoblasts and finally into adult osteoblasts. Mature osteoblast build new bone matrix by synthesizing collagen fibers, osteocalcin and the proteoglycans of ground substance. They control bone mineralization and keep up osteoclast balance by secreting local cytokines and growth factors. During the process of ossification, osteoblasts can get trapped between two lamellas and turn into **osteocytes** covered by mineralized bone matrix. The bodies of osteocytes are situated in small lacunas from where a netlike labyrinth of canals is sent out filled with osteocyte-processes,

interstitial fluid and collagen fibers. This syncytial network ensures metabolic and electrical communication amongst all osteocytes by gap-junctions and their supply per diffusion. For this reason osteocytes are thought to operate as mechanosensors transducing mechanical stress signals into biological activity. Proteins involved in this process are prostaglandin E2, cyclooxygenase 2, various kinases, Runx2 and nitrous oxide. Further, they delegate osteoblasts and osteoclasts where and when bone matrix has to be formed or eliminated by expressing bone matrix proteins such as osteocalcin, galectin 3 and CD44. Osteoblasts have receptors for parathyroid hormone and oestrogen. Their activity on bone is also influenced by physical activity, hormones and growth factors.

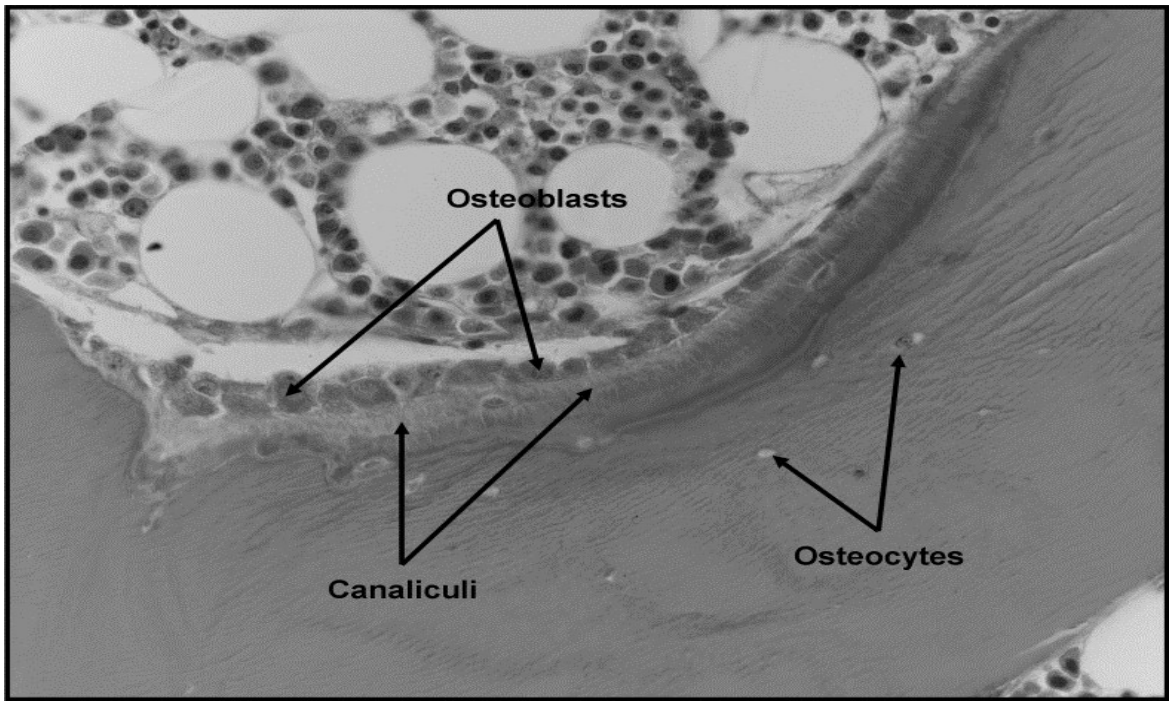

Figure 6 Microscopic image of osteoblasts and osteocytes (16)

Osteoclasts are big cells with multiple nuclei, which are specialized in resorbing bone due to mechanically forces or microdamaged areas. They derive from mononuclear precursor cells of the monocyte-macrophage lineage. For osteoclast activation RANKL and macrophage - CSF (M-CSF) cytokines are essential, which are secreted by osteoblasts and marrow stromal cells. RANKL is important for osteoclast activation, whereas M-CSF is necessary for cell proliferation, differentiation and survival. OPG binds RANKL and inhibits its effect on the RANK receptor. The principle of operation is as follows: dissolving calcium connections by acid; secretion of lysosome enzymes (Cathepsin K) to dissolve the organic matrix and endocytosis of matrix fragments.

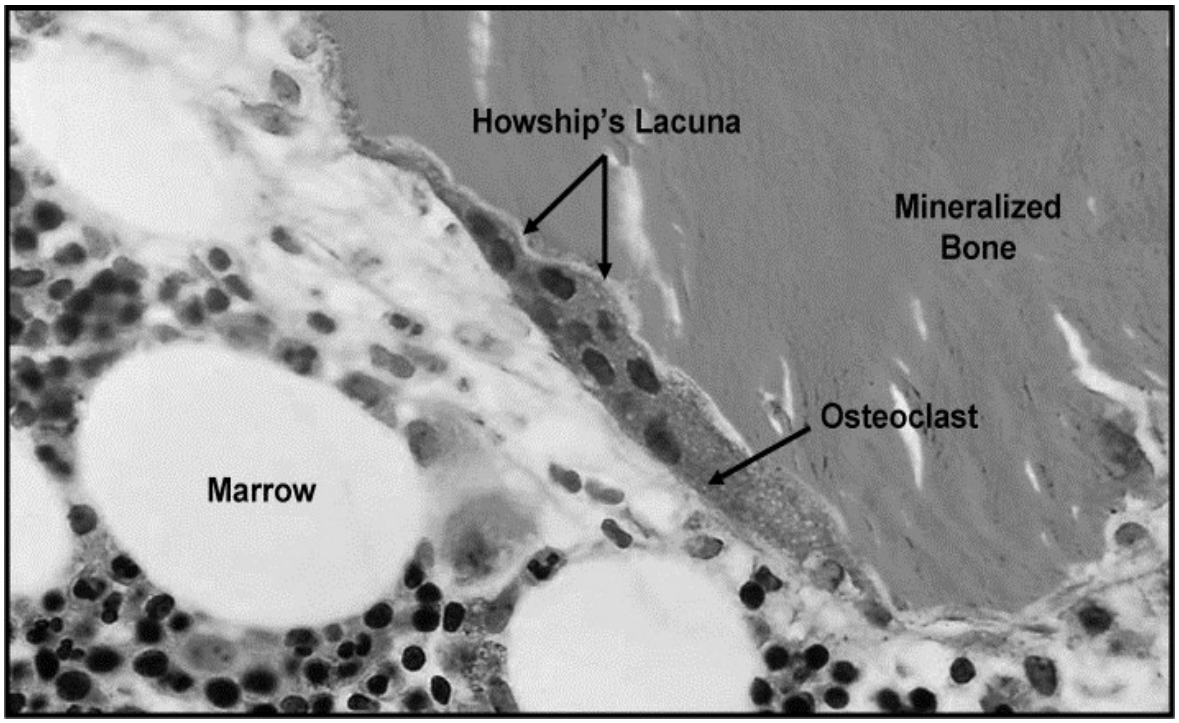

Figure 7 Microscopic image of osteoclasts (17)

1.6 Modelling

Bone modelling is defined as a process of bone formation by osteoblasts, namely formation modelling, or as bone resorption by osteoclasts, called resorptive modelling. Modelling dominates throughout childhood and growth and is less prominent in adulthood. Longitudinal growth is closely associated with endochondral ossification to gain bone length. Due to changing mechanically strains bone is removed on the periosteal surface and added on the endocortical surface to keep up the proper shape of the bone. The main difference to bone remodelling is that the process of formation and resorption runs uncoupled, occurs unsequentially and is locally independent. From a global point of view, resorptive modelling and formation modelling occur simultaneously but on different sites, which implies a coordinated process in time. The main goal of modelling is to gain bone mass and to maintain or alter bone shape, induced by changing mechanical forces. Local tissue strain is the main signal for bone modelling; if a certain threshold is exceeded, formation modelling is induced; if the strain is too low, resorption modelling is initiated. Bone modelling also plays an important role in longitudinal growth, radial growth and bone axis changes, called bone drift. Bone modelling always takes place on preexisting bone surface, an important point to distinguish from endochondral and intramembranous ossification (10,12,13).

1.7 Remodelling

Bone underlies a constant process of remodelling throughout life, in order to maintain bone strength and mineral homeostasis. At any time about 10000 remodelling sites can be detected in the skeleton, with a ratio of formation to resorption equals 4:1 (18). This process is characterized by an in time and space coordinated activation of osteoclasts, removing old bone and osteoblasts, building new bone in discrete packets. Numerous local factors such as growthfactors or cytokines, hormones and physical activity may lead to bone remodelling. Targeted remodelling is triggered by an accumulation of microdamaged areas and through osteocyte apoptosis. Whereas, stochastic remodelling occurs site - unspecific, especially in trabecular bone, to keep up mineral homeostasis. It is also involved in reshaping bone during growth, reparation processes after fractures and as a response to mechanical loading.

The remodelling cycle can be subdivided in five stages: activation, resorption, reversal, formation and quiescence phase. Each cycle takes about 4 – 6 months to complete in which the resorption stage by osteoclasts only lasts for approximately 3 – 6 weeks. It is estimated that approximately 5 – 10% of total bone is renewed each year. Whereas trabecular remodelling accounts for 25% each year cortical remodelling is estimated at 2 – 3% per year (19). Bone balance is the difference between new built bone and the amount of resorbed bone during a remodelling cycle in one bone multi-cellular unit (BMU). A BMU is formed by a group of osteoblasts, osteoclasts and their associated blood vessels. From the third decade of life bone balance is getting slightly negative, considering the whole bone mass and increases to even higher negative levels in pre- and postmenopausal women, elderly or under pathological conditions. A high rate of bone turnover also leads to a deficiency of bone strength because of the much longer ossification phase in each BMU at each remodelling site and the unfilled pores which have to be reorganized during formation and mineralisation stage.

Every remodelling cycle starts with the **activation phase**. During this phase mononuclear monocyte - macrophage osteoclast precursors are recruited to the bone surface and get activated. The interaction of osteoclastic and osteoblastic precursors is initiated. Now cells of the osteoclastic lineage differentiate, migrate and fuse to multinucleated mature osteoclasts. These cells are now able to stick to the surface of the bone, in order to dissolve mineralized bone by secreting hydrogen ions and

Cathepsin K, initiating the **resorption phase**. Throughout this phase irregular cavities, namely Howship´s lacunae, in trabecular bone occur. Considering cortical bone the equivalent is called Haversian canal. During bone resorption several growth factors are released. These include IGF - I (insulin - like growth factor I), IGF-II (insulin - like growth factor II), TGF - β (transforming growth factor ß) and PDGF (platelet – derived growth factor). In the **reversal stage** osteoblasts precursors are recruited from the mesenchymal stem linage and differentiate into mature osteoblast. They start to produce new osteoid by laying down an unmineralized organic matrix, which is called **formation phase** and is finally mineralized during **mineralization stage** in two steps. First calcium and phosphate ions bind to the new formed osteoid, which makes up to 70% of the mineral content. In a second step mineral crystals are added and mature over a period of up to one year.

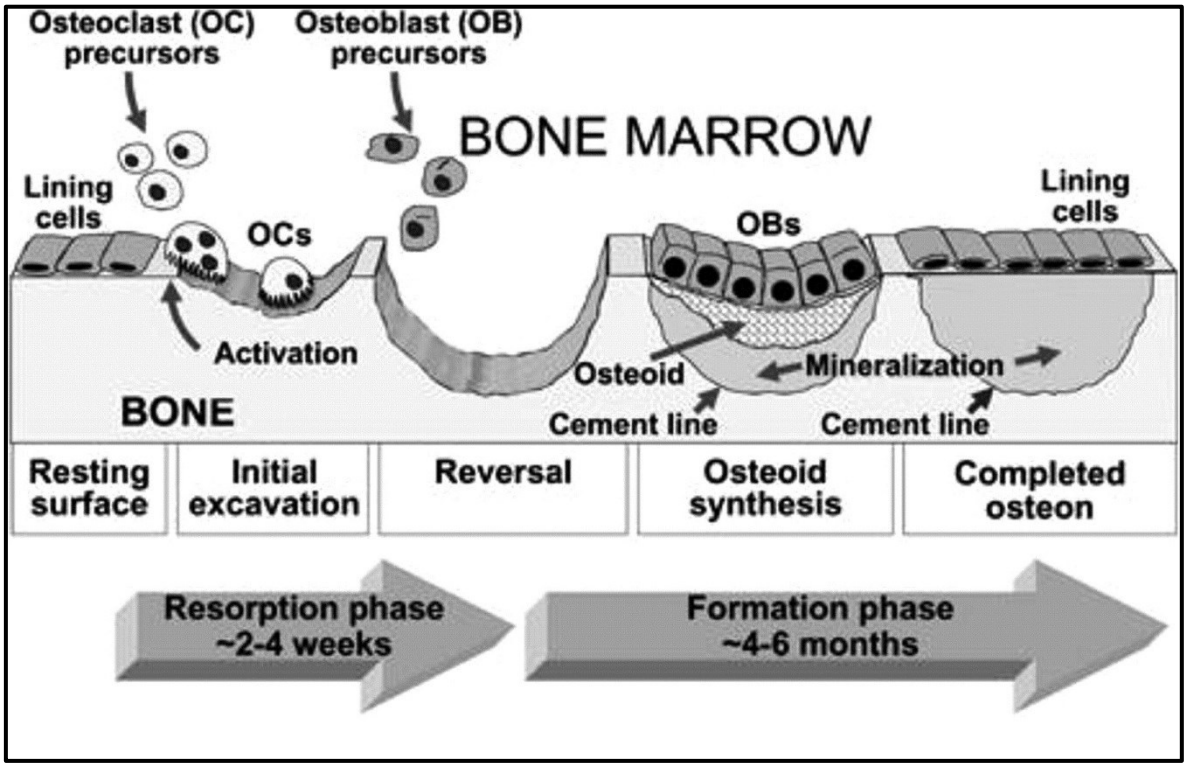

Figure 8 Schematic overview of bone remodelling and timeline(20)

1.8 The Strength of Bone

During lifetime bone is constantly adapting to its environment and physiological demands to preserve a healthy, functioning tissue (12).

Bone mass proliferates constantly throughout childhood to reach its peak between the age of 18 - 35 years and is well balanced with minimal changes in bone mass. From

then on bone mass is decreasing constantly. Generally peak bone mass levels are genetically determined but are also affected through specific conduct, such as diet, physical activity, pharmaceuticals and lifestyle (21). Approximately 0,5% - 1% of total bone mass is lost per year from the fourth decade of life. This value adds up about tenfold after menopause and stabilizes at a value of 1% – 3% per year.

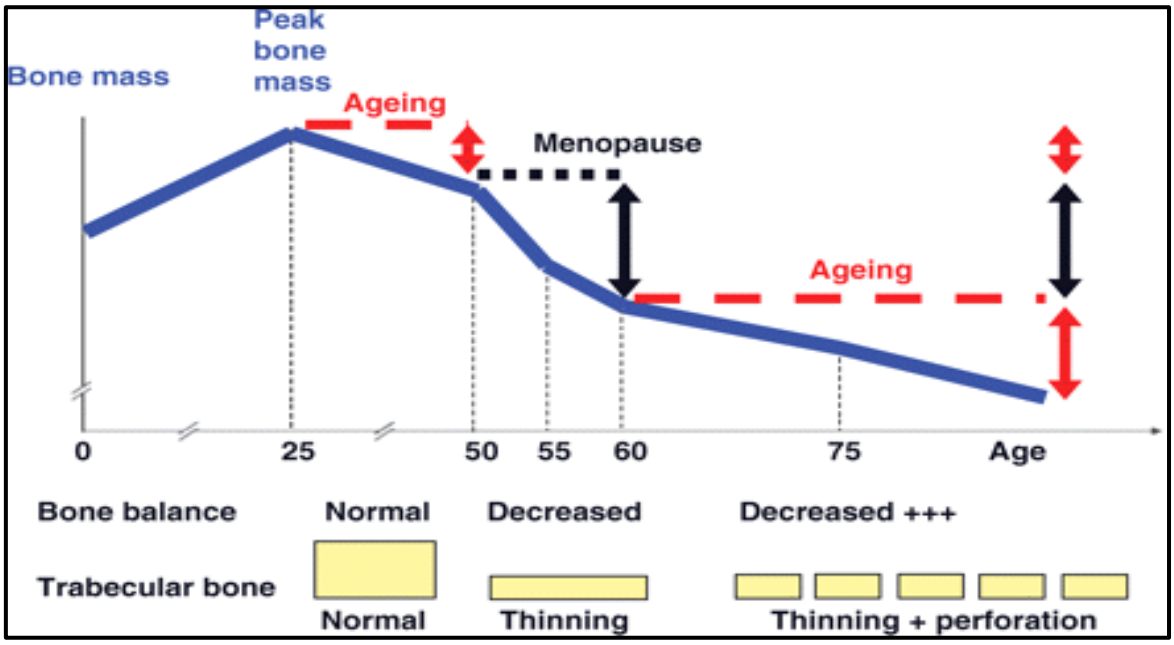

Figure 9 Timeline of peak bone mass (22)

The strength of bone is based on bone mass, its geometry and bone quality. Bone mass is estimated to account for 50 – 70% of bone strength (12). In general, larger bones are stronger than smaller ones. A larger diameter increases bone strength by the radius raised to the fourth power (23).

The quality of bone is determined by four physiological and structural parameters: the rate of bone turnover, trabecular architecture, material properties and accumulation of microdamaged areas (10). These components are independent of bone mineral density in their contribution to bone strength.

The rate of bone turnover is a result of constant remodelling. At any given time, approximately 10% of the skeleton is underlying a process of remodelling (21). The outcome is a temporary deficit in bone mass due to a formation – resorption ratio of 4:1 but it also affects the trabecular architecture with major impact on trabecular strength. A faster rate of turnover implies a higher transient deficit. Furthermore resorption cavities can be the origin of new microcracks and decrease bone strength as well.

Besides the effect of bone loss by resorption cavities alone, a high turnover rate thins out connections between trabecular rods and plates leading to disconnections or perforations. This process contributes more in bone fragility than an equal loss of bone mass from all trabecular surfaces would have. Bone mineralization is vital for bone strength and stiffness. On the other hand highly mineralized bone is more likely to crush, e.g. osteopetrosis. The amount of collagen type I and the way it is cross-linked also affects bone stiffness and strength, e.g. collagen defects in osteogenesis imperfecta. As well as accumulation of nonenzymatic cross-links can demagnify the diameter of collagen fibrils. Moreover a low bone turnover rate caused by increased damage accumulation by repeatedly loading or induced by a decrease of bone repair, also results in a decrement of bone strength.

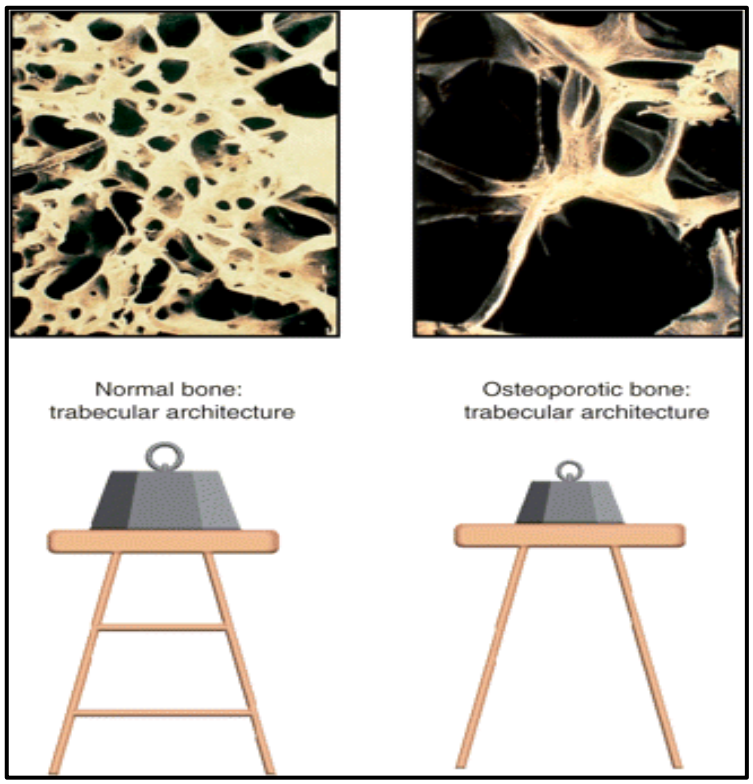

Figure 10 Trabecular architecture: osteoporosis vs. normal bone (24)

2 LOCAL REGULATION OF BONE CELLS

Bone mass is kept in a tightly regulated balance by a coordinated cross talk between osteoblasts, osteocytes, osteoclasts and their corresponding progenitors. Bone cells and cells of the bone marrow secret local factors to trigger intercellular signaling and thereby modulate bone formation or resorption. Proliferation, differentiation, function and survivability are also controlled via locally produced cytokines and growth factors. Additionally, systemic influences of hormones (e.g. PTH, sex steroids, glucocorticoids, Vitamin D) nutrition and mechanical forces contribute to bone homeostasis.

2.1 Osteoblastogenesis

Mesenchymal stem cells are adult stem cells showing a high proliferative and multilineage differentiation potential, containing adipose tissue, bone, cartilage, tendon, muscle plus stroma and are indispensable for bone homeostasis. Under the influence of biochemical, physical (e.g. pulsed electromagnetic fields (PEMF)) mechanical forces (e.g. fluid shear stress, hydrostatic pressure) MSCs differentiate into specific cell lineages. The commitment as well as the differentiation of MSCs towards the osteogenic cell line depends on certain key factors such as RUNX 2, distal-less homeobox 5 (DLX 5) and bone morphogenetic protein 2 (BMP 2). Simultaneously, the differentiation to the adipogenic cell linage is inhibited, especially by RUNX 2. After commitment, the differentiation into preosteoblasts is initialized by RUNX 2, DLX 5, P2Y4, P2Y14 and msh homeobox homologue 2 (MSX 2) and other markers of osteoblasts like type I collagen, osteopontin (OPN) and alkaline phosphatase (ALP). The differentiation of preosteoblasts into immature osteoblasts is established by OSX (Osterix), RUNX 2 and β - catenin. Preosteoblasts express bone sialoprotein, OPN and bone matrix protein. Terminal maturation into mature osteoblasts is initiated by OSX which induces the expression of osteocalcin. Further, RUNX 2 inhibits the maturation of osteoblasts in later stages and OPN expression is also reduced, while P2X5, collagen type I, osteocalcin and alkaline phosphatase is increased (25).

Some of the factors mentioned above show an appositional regulation pattern concerning the commitment of MSCs to either the adipogenic or osteogenic lineage and therefore might be considered as key factors. Differentiation into osteogenic lineage by WNT10b, RUNX 2 and bone morphogenetic proteins (BMPs) are inhibiting adipocyte

differentiation. Secreted frizzled – related proteins (sFRPs) were shown to enhance adipogenesis but inhibit osteogenic differentiation. Delta like 1 factor inhibits adipogenesis and enhances osteogenesis by WNT and NF – kB signaling. Active Rho A differentiates MSCs into osteoblasts. Transforming growth factor - ß inhibits osteocyte differentiation. Platelet – derived growth factor (PDGF) and fibroblast growth factors (FGF) are involved in the differentiation of adipogenic, chondrogenic and osteogenic linages. Physico – mechanical factors also have an influence on MSCs fate: A soft extracellular matrix structural geometry triggers adipogenic differentiation, whereas osteoblast differentiation is raised by a stiff scaffold. Finally, oxygen, pH, ionic strength and the shapes of the cell and the cytoskeleton control differentiation of MSCs (26).

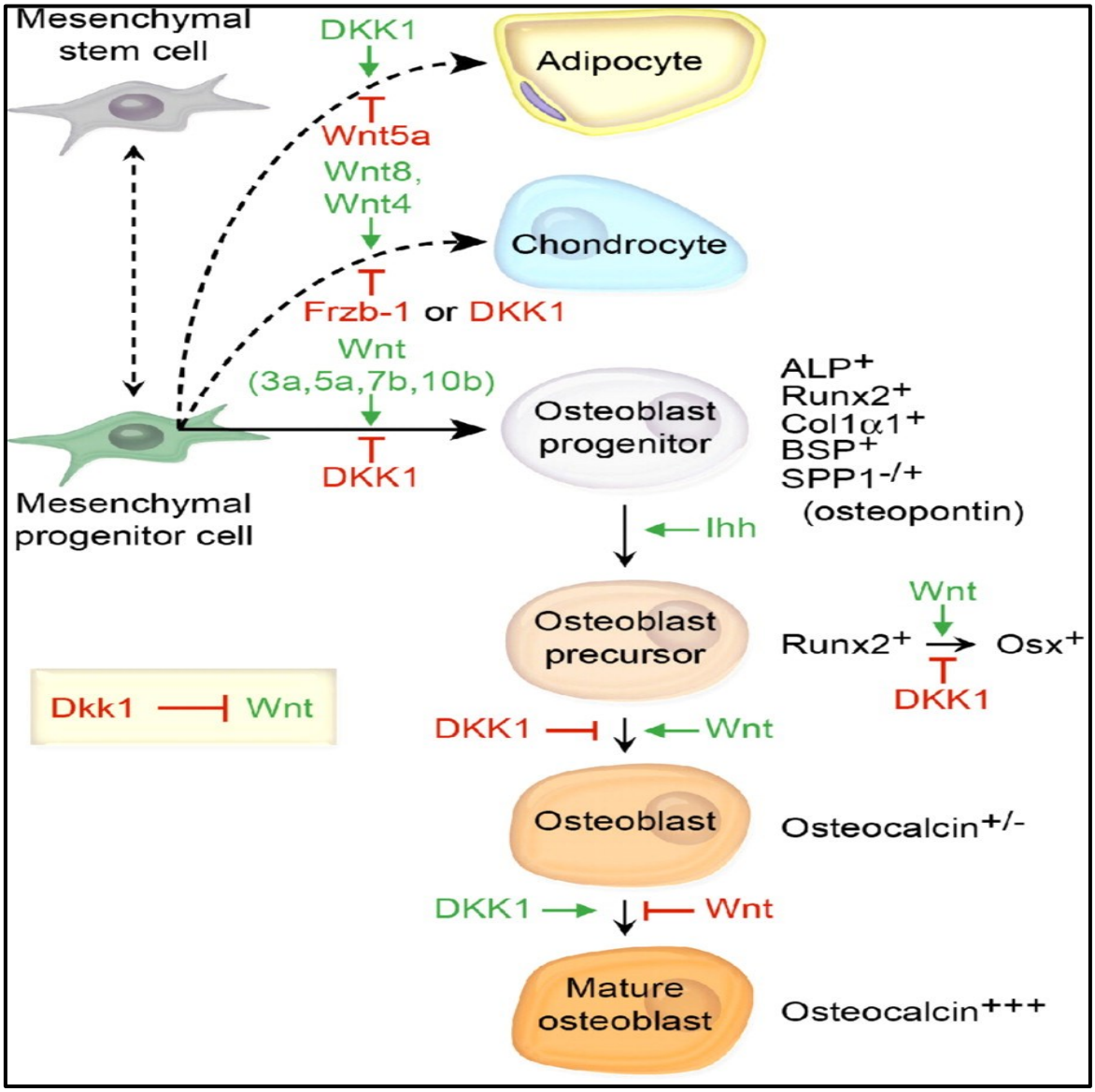

Figure 11 From MSCs to the osteogenic cell line (27)

2.2 Osteoclastogenesis

Osteoclasts derive from multipotent hematopoietic stem cells (HSCs) which are commited to the monocyte – macgrophage linage by the transcription factor PU 1 and the macrophage-colony stimulating factor (M – CSF). PU 1 enhances the up – regulation of the M – CSF receptor, namely c – Fms (28). M – CSF stimulates the survival, differentiation and proliferation of mononuclear phagocytic cells, plus enhances the migration of mature osteoclasts. This molecule is encoded by the CSF1 gene and secreted by osteocytes, osteoblast precursors and mature osteoblasts. The corresponding receptor is CSF-1R /c-Fms, which is expressed by mononuclear phagocyte progenitor cells, mono-macrophages and osteoclasts. Receptor–ligand action initiates the association of phospoinositide 3 – kinase and SRC with c-Fms and leads to the activation of two pathways: mitogen – activated protein kinase (MAPK) and Akt, which both activate Cyclin D. Additionally, RANK transcription is promoted and precursors are fully committed to the osteoclast linage (18).

The interaction of RANKL and its receptor RANK needs co – stimulation from OSCAR (osteoclast associated receptor) and TREM 2 (Triggering receptor expressed on myeloid cells 2). Through activation, TRAF 6 is recruited by RANK and MAP kinases and NF–kB is activated. Simultaneously, immunoreceptor tyrosine based activation motif (ITAM) – harbouring adapters FcRγ (Fc receptor related gene γ) and DAP 12 (DNAX activation protein of 12 kDa) activate Syk kinases, which in turn invoke phospholipase C^{γ} (PLCγ) releasing calcium from intracellular space. The release of calcium recruits the calcium / calmodulin activated phosphatase Calcineurin, which leads to dephosphorylation of the transcription factor NFAT 2. After nuclear translocation and binding to consensus DNA sequences, NFAT 2 conjoined with c – fos amplifies osteoclast specific genes (29).

Attachment of mature osteoclasts to the bone surface is initiated by the coaction of RGD motifs and integrin $\alpha_v\beta_3$, which leads to the formation of c–src kinases, Syk and Vav – 3 (guanine nucleotid binding factor) complexes causing adhesion. Upon adhesion, the osteoclast gets polarized: the bone facing ventral membrane is put into a ruffled border, containing all the secretory activity. Actin remodelling is accomplished by the forming of molecular complexes, which are rapidly dissociated and reformed (30).

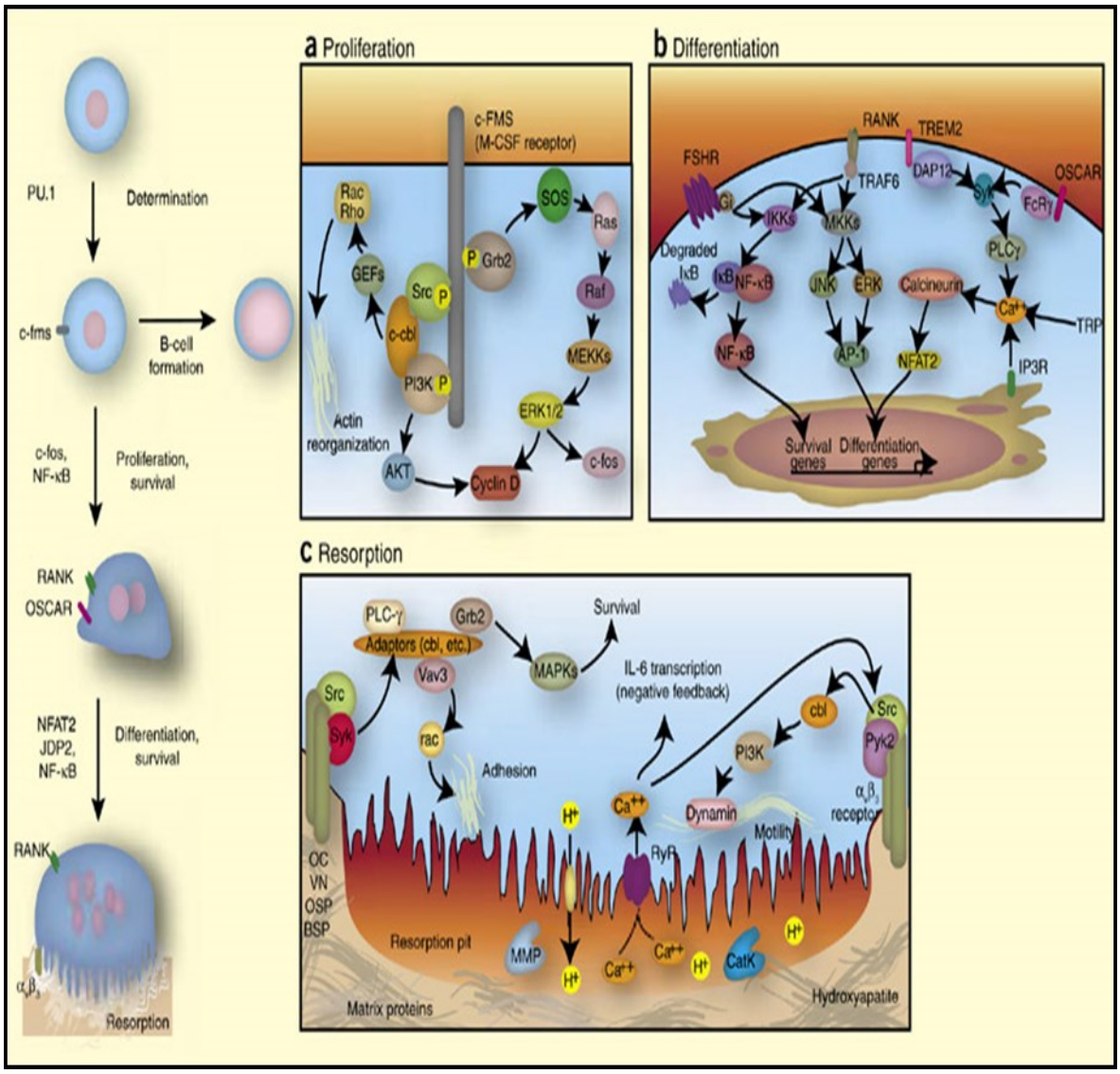

Figure 12 Schematic overview of osteoclastogenesis (30)

2.3 Important Signaling Pathways

2.3.1 RANKL/RANK/OPG system

The RANKL/RANK/OPG system is vital for bone resorption and osteoclast differentiation. It is composed of the two receptors, namely RANK and Osteoprotegerin (OPG) and the receptor activator of nuclear factor κB-ligand (RANKL). OPG and RANKL are secreted by osteocytes, osteoblasts and stromal cells. RANK is a transmembrane protein expressed in osteoclast precursors. Binding of RANK and RANKL activates the transcription factors NF-$_k$B, nuclear factor of activated T–cells (NFATc1) and c–FOS which promote the differentiation of mononuclear precursors

into active multinucleated osteoclasts. Moreover, RANK signaling promotes osteoclast activity and survival. OPG acts as a protector from excessive bone resorption by binding and neutralizing RANKL. Therefore the ratio of RANKL and OPG is a well-established indicator for bone resorption (18).

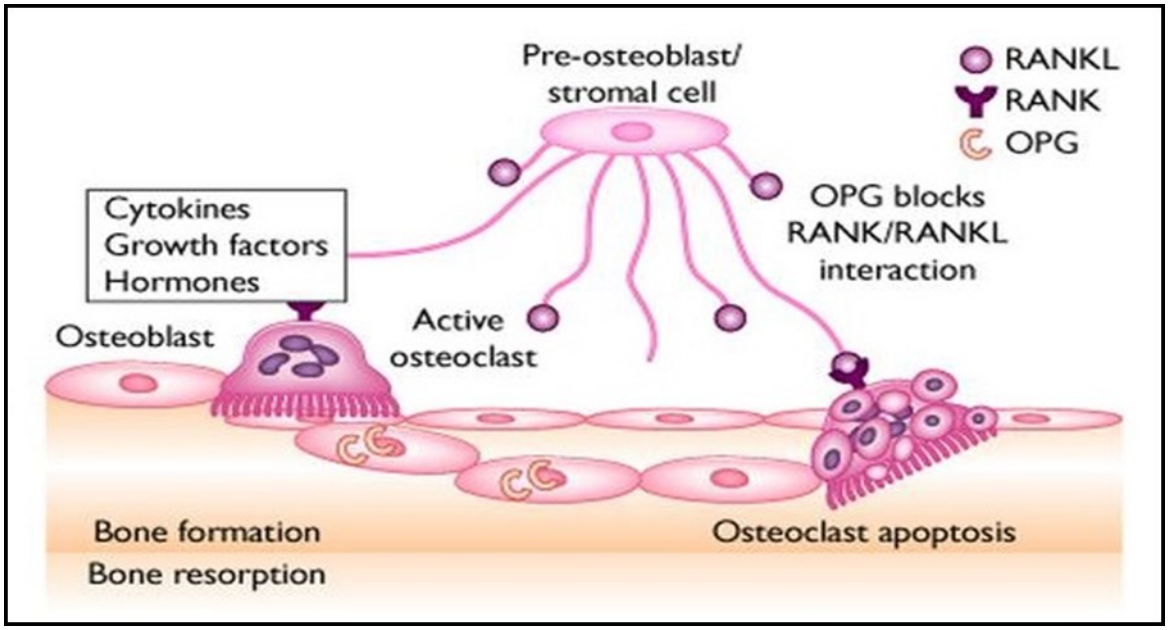

Figure 13 Illustration of the RANKL – RANK – OPG System (31)

2.3.2 WNT Signaling Pathways

WNTs are molecules of the secreted cysteine-rich glycoproteins family. WNTs are involved in cell differentiation and proliferation, they also contribute to cell apoptosis. There are three major pathways: WNT Ca^{2+}, canonical (ß – catenin – dependent) and non – canonical (planar cell polarity) pathway. Inactively, ß – catenin is attached to a so called "multiprotein ß – catenin destruction complex", which consists of Axin, glycogen synthase kinase 3ß (GSK 3ß) and adenomatous polyposis coli (APC) and leads to a proteosomal degradation of ß – catenin. The initiation of the WNT signaling cascade is established upon binding to one of the members of the frizzled (FZD) family receptors and low density lipoprotein receptor protein 5 (LRP5) or LRP6 (LRP 6). Upon binding, Axin connects to LRP5 or LRP6, leaving the destruction complex and allowing the accumulation and thereby the translocation of ß – catenin into the nucleus. This step leads to an association with lymphoid enhancer factor (LEF) and T-cell factor (TCF) to initiate target gene expression (32). The most important WNT antagonists are DKKs (Dickkopf), Sclerostin (Sost) and secreted frizzled-related proteins.

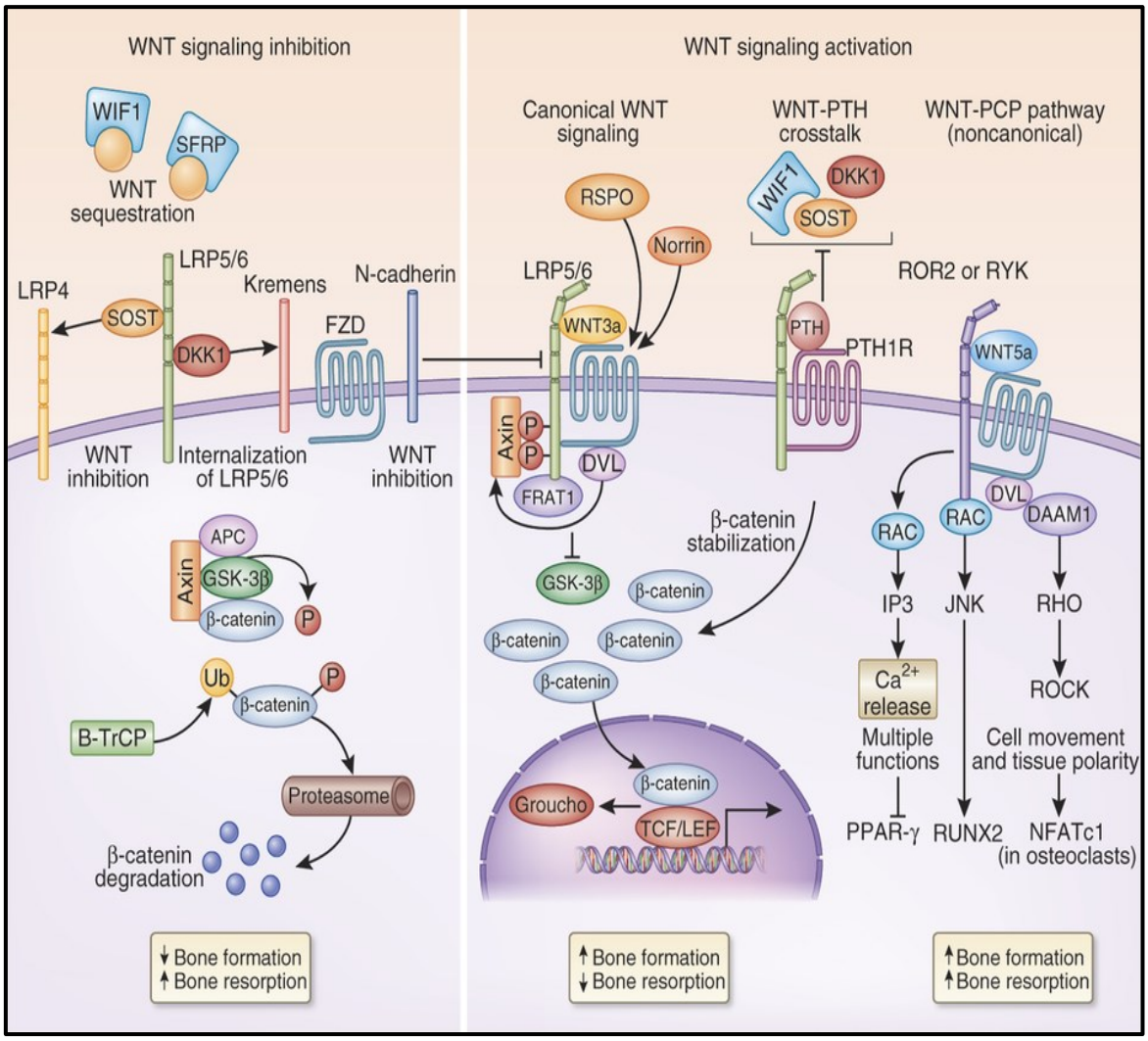

Figure 14 Schematic overview of WNT signaling pathways (33)

The canonical WNT – signaling pathway is by now the best studied. It affects the complete osteoblastic lineage by enhancing the commitment and differentiation of mesenchymal stem cells (MSCs). Simultaneously, the adipocytic and chondrocytic lineages are repressed. Indirectly, osteoclastogenesis and the resorption of bone is also decreased by secretion of Osteoprotegerin (OPG). On the other hand, there is proving that WNT signaling positively affects osteoclasts and their precursors, which might be the result of an autocrine loop. Briefly, WNT – signaling is enhancing bone formation by increasing osteoblast differentiation and maintaining MSCs and osteoblast precursors. Simultaneously, osteoclast proliferation and differentiation are tightly controlled leading to a slightly positive bone net balance. Furthermore, there is a complicated cross talk at the level of ligands, agonists, receptors and antagonists with other signaling pathways (eg. BMPs, TGFß, FGF, PTH) contributing to bone homeostasis.

3 MECHANICAL SKELETAL ADAPTION

Though it seems dead and not very responsive, bone is one of the most adaptive and vital tissues in the body. It is a highly regulated, multifunctional tissue representing an extraordinary biomaterial. Due to its mechanically efficient structure bone has the same strength as cast iron, though being light as wood (34). Its outstanding biomechanical properties have been discovered almost 200 years ago, by observing the alignment of trabecular bone in the femoral neck which seemed to follow mathematical and engineering rules. This idea was picked up by Julius Wolff, a German anatomist and surgeon, who refined this theory as follows:

"Alterations of the internal architecture clearly observed and following mathematical rules, as well as secondary alterations of the external form of the bones following the same mathematical rules, occur as a consequence of primary changes in shape and stressing or in the stress of the bones (35) *."*

This means bone tissue responds to mechanical stress by placing or replacing bone where it is needed to keep up its strength and integrity.

By 1900 functions of bone cells were already known but only at a cellular-level: osteoblasts produce and osteoclasts resorb bone. Until 1960 these bone effector cells were thought to be only affected by nonmechanical factors. In 1964 Harold M. Frost and Prof. W.S.S. Jee gave birth to the Utah paradigm to lift bone physiology from cellular-level to tissue-level functions (36). This paradigm is discussed controversial until today but the introduction of the mechanostat, which means how bone cells react upon a certain amount of strain, is a proven part of bone physiology up to date (37) .

A lot of experiments have shown that bone reacts specifically to a certain threshold of strain: if the mechanical stimulus is too low, bone will be resorbed; if the mechanical signal exceeds a certain threshold, bone mass will be added in such a way, that the mechanical stress in that particular location will be reduced to normal range (38). This effect can obviously be observed in high impact sports (e.g. volleyball, tennis) where the dominant arm exceeds higher bone mass and BMD compared to the non – dominant arm, on the opposite, a blaster for 5 weeks will reveal the negative effects of impaired mechanical loading. Alongside these two extreme conditions there also exists a physiological window, in which bone net balance is almost kept at a constant level

(Figure 15). However, massive amounts of strain or repeatedly applied overstrain might lead to increased microdamage or even fracture.

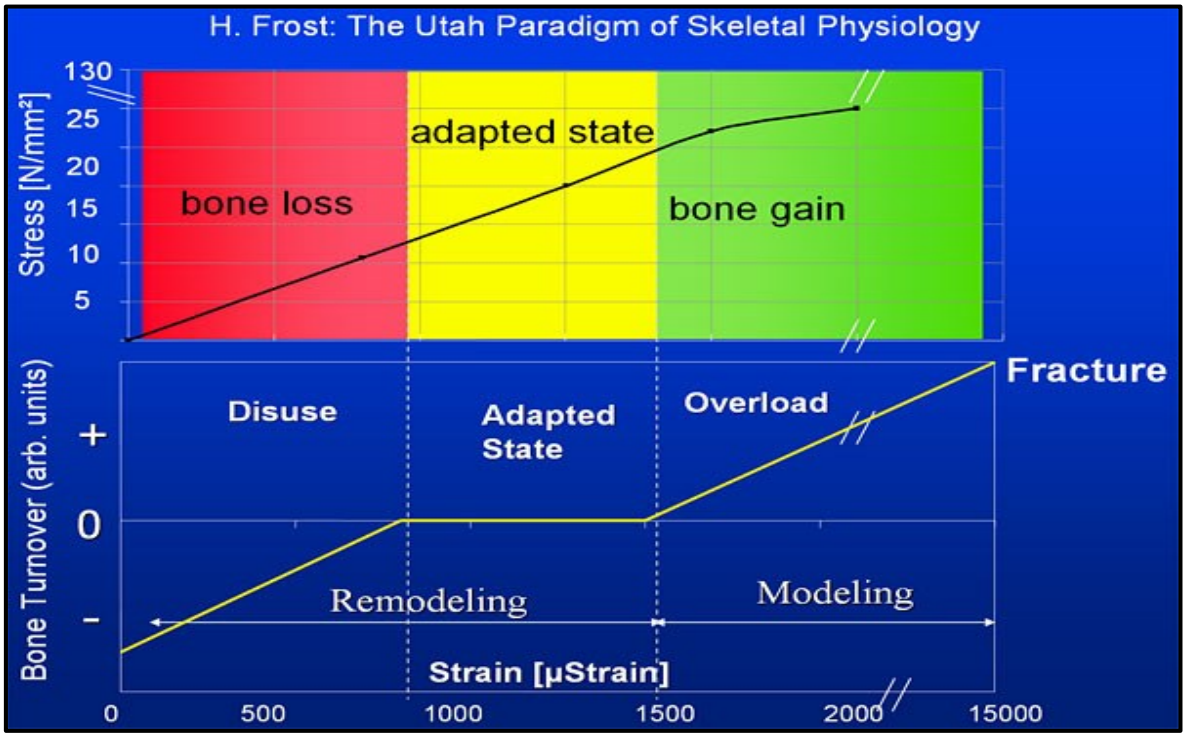

Figure 15 The Utah paradigm (39)

Besides strain magnitude, also strain rate, frequency, distribution and duration play an important role in skeletal mechanical adaption. In an overview, bone responds best to dynamically loads of short duration, with a high rate in preferably several shorter intervals with up to 4-8 hours of break due to cell saturation (40).

In addition to physical properties, the main question is how physical forces are transduced into intracellular signals. Many studies suggest that osteocytes are the putative **mechanosensors** in bone and play a vital role in detecting mechanical forces and translate them into intracellular signals to regulate mechanical skeletal adaption. This theory is reasonable, since osteocytes are the most dominant cell type in the human skeleton and represent about 80 - 90% of all bone cells (41–43). Furthermore, osteocytes are connected to each other by their cell processes throughout a canaliculi system via gap junctions to ensure fast communication. This syncytial network enables metabolic and electrical communication within osteocytes and cells on the bone surfaces. The dendritic like processes are surrounded by an extracellular space filled with fluid, which is pushed upon mechanical loading. Secondly, osteocytes are long lived cells whereas osteoblasts and osteoclasts only appear transient, in low number

and various locations. This suggests that osteocytes are well capable to locate areas of mechanical stress or microdamaged zones and to detect circulating hormones or cytokines.

Mechanical loading evokes different kind of stressors like shear, compression or tensile stress. This leads to deformation in the extracellular matrix (ECM) and shear stress or tension in tethering elements. Mechanical stimulation is mandatory for cell proliferation, differentiation, regeneration and maintenance from embryonic development to the elderly and plays an important role in certain pathological conditions

It has been suggested that osteocytes are able to sense mechanical stress via changes in fluid flow and thereby induced shear stress because of the fluid´s viscosity. Cell process attachments are connected to the canalicular walls via integrins and the glycocalix which get distorted on the dragging forces (Figure 16) (44) .

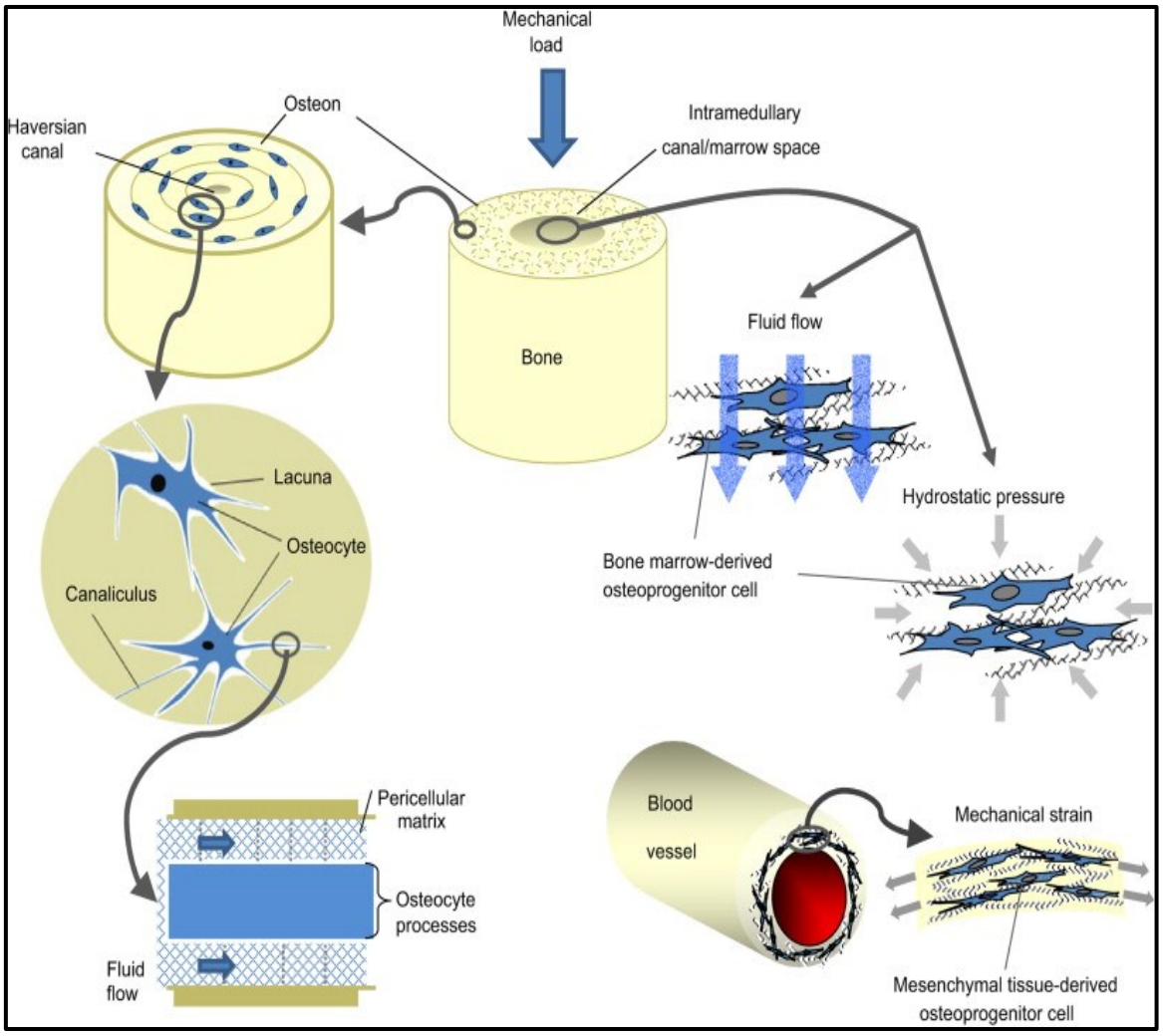

Figure 16 Mechanosensing due to dynamic fluid flow shear stress (45)

New studies show that the primary cilia a single, immotile organelle extending the surface of osteocytes might play an important role in mechanosensing. They appear in almost every mammalian cell and have been associated with several diseases (e.g. polycystic kidney disease). Due to their omnipresence, defects can lead to multiple systemic disorders. In the skeletal system they are involved in ossification disorders and congenital defects. Moreover, they seem to participate in proliferation and differentiation of bone cells (46).

Bone cells have three different types of **mechanoreceptors**:

Ion channels: transverse the plasma membrane by building pores, enabling ion flux based on different electrochemical concentrations. Ion channels are known to play numerous roles in mechanosensitive tissues all over the body. Ion channels are activated by voltage changes (electrochemical), mechanical forces or biochemical ligands (18).

Cell Adhesion and the cytoskeleton: bone cells are connected to the ECM via membrane spanning proteins and capable of detecting and binding extracellular matrix peptide sequences. The cytoskeleton provides structural and load bearing function inside the cell. Whereas a signaling function organizes and binds enzymatically active proteins. Integrins (e.g. actinfilaments) are linked to the cytoskeletal framework by adaptor proteins (e.g. FAK, NMP4, p130cas). It has been proven that besides changes in mechanical properties, mechanical stimulation leads to specific changes in the nucleus, implicating a direct linkage of mechanical stimuli to nuclear changes. Secondly, mechanical loading leads to signal transduction changes in integrins, co inducing adaptor and signaling proteins, also known as focal adhesions, resulting in the activation of several downstream signaling pathways. Adaptor proteins can also be found in the nucleus under certain conditions, which implies that mechanical loading can be transduced by molecules to the nucleus. This is another possibility of mechanotransduction besides the direct linkage of loading to the nucleus (40)

G – protein related molecules: G protein coupled receptors are cell surface receptors and are activated by a variety of ligands, such as fatty acids, hormones, local cytokines amino acids, small peptides, neurotransmitters and others. Fluid shear stress also activates these receptors and is therefore the third possible way of mechanosensitive receptor signaling in bone cells (47).

4 PATHOPHYSIOLOGY

4.1 Osteoporosis

Osteoporosis is defined as a systemic, skeletal disease with a multifactorial aetiology, leading to a loss of bone mass and impairing the microarchitecture of bone. Poor bone quality and quantity may lead to fractures, which represent the main clinical consequence. "*According to the report of the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA) in 2010 twenty-two million women and 5.5 million men were estimated to suffer from osteoporosis in the European Union. 3.5 million new fragility fractures were sustained, comprising 610,000 hip fractures, 520,000 vertebral fractures, 560,000 forearm fractures and 1,800,000 other fractures. Associated fractures are a major public and individual health concern because of related morbidity and disability, decreased quality of life and mortality. Every three seconds someone in the European Union has a fracture as a result of osteoporosis. The economic burden of incident and prior fragility fractures was estimated at € 37 billion. Incident fractures represented 66 % of this cost, long-term fracture care 29 % and pharmacological prevention 5 %. Previous and incident fractures also accounted for 1,180,000 quality-adjusted life years lost during 2010. The costs are expected to increase by 25 % in 2025. The majority of individuals who have sustained an osteoporosis-related fracture or who are at high risk of fracture are untreated and the number of patients on treatment is declining* (48)*".*

Generally, osteoporosis is classified into primary osteoporosis, including Type I (postmenopausal osteoporosis) and Type II (senile osteoporosis), which are mainly associated with decreased levels of oestrogen and aging. Secondary Osteoporosis is caused by various exogenous factors: such as medical conditions (e.g. hyperparathyroidism), pharmaceuticals (e.g. steroids) and lifestyle conditions (e.g. smoking) (49). Alongside with smoking, high alcohol consume and low calcium diet, immobilization is one of the major risk factors causing a poor bone stock, leading to osteopenia and osteoporosis.

Disuse osteoporosis is defined as a reduced bone mass relating to bone volume, while the percentage of mineral to collagen stays the same. The loss of bone mass refers to a loss of mechanical stress. Such skeletal unloading occurs under neurological or

muscular disorders, more frequently after a spinal cord injury, under conditions of prolonged bed rest and during spaceflight (50). Loss of mechanical stress leads to metabolic changes in osteoblasts and osteoclasts activity, inducing a larger bone turnover rate, elevated bone absorption and lower bone formation (51). Hence, immobilization may induce a loss of bone mass from 1% - 3% in one month in load bearing structures, like the femoral neck or vertebrae (52). Other results from cross-sectional studies showed a dramatic reduction in bone mass up to 20% in the distal femur after spinal cord injury (53) and a decrement in bone mineral density of 43% in the distal femur compared to the control-group (54).

Whereas primary osteoporosis is well treatable, disuse osteoporosis is lacking effective countermeasures up to date. This is due to a different aetiology, pathophysiology and therefore, resultant pathology.

5 EVALUATION OF BONE STRUCTURES

The silent and mostly unrecognized progression of bone resorbing diseases, as well as the continually loss of bone mass during lifetime, often puts fractures in the first place to give a hint at presumably underlying pathophysiological processes in the patient´s history. Fractures occur whenever external physical loads exceed the whole bone´s strength. The resistivity of bone to fractures is characterized by the total amount of bone, microarchitecture of cortical and trabecular bone and material properties.

Besides a detailed medical history and evaluation of individual risk factors, skeletal imaging and laboratory are mandatory.

5.1 Anamnesis and Risk Factors

A thoroughly executed medical history is the first step towards a diagnosis; along all risk factors special focus should be put on previous fractures. The WHO developed a risk analysing tool called FRAX (fracture risk assessment). This tool combines individual risk-factors and clinical-factors, such as BMD. Based on these factors a risk-score is evaluated to assess the probability of hip fractures and other osteoporotic fractures (e.g.: forearm, shoulder, spine) over a period of 10 years (55). It is based on well-studied population-cohorts from Australia, Asia, Europe and North America with the objective to obtain a more individual diagnosis and treatment finding, in opposition to single based BMD decision making.

Table 2 Major plus minor risk factors for a poor bone stock

Major plus minor risk factors for a poor bone stock	
• Low BMD	• Premature menopause
• Age	• Primary/secondary hypogonadism
• Female gender	• Excessive alcohol abuse
• Caucasian, Asiatic ethnicity	• Deficient vitamin D, diminished sun exposure
• Loss in body height	• Low calcium intake
• Low body weight	• High turnover rate of bone
• Earlier fragility fracture	• Long immobilization
• Family history of hip fractures	• Neuromuscular diseases
• Smoking	• Rheumatoid arthritis
• Glucocorticoid therapy	• Restrictions in ability to see

5.2 Skeletal Imaging

Conventional radiography is still the gold standard to confirm and categorize fractures. Due to its high availability, the small amount of radiation exposure, short time of measurement and moderate costs it is also used to get a picture of gross morphologic alterations and the course of a disease. For example, in comparing previous X-rays in consideration of the vertebral body height. Poor bone stock, i.e. BMD, is the major risk factor causing fractures. On a given conventional X-ray picture density changes have to be in a range of approximately 20 - 40% to be visual detectable, which has made this method obsolete in clinical practice (56).

To evaluate the risk for an osteoporotic fracture and to quantitatively assess bone macrostructure **Dual-energy X-ray absorptiometry (DXA)** is the standard procedure. DXA exposes the tissue to two different types of energy sources, one diminished by soft tissue and fat the other by bone. Based on these results BMC, the area of bone and BMD are calculated. The results are also reported in T-scores and Z-scores (Figure 17). The T-score stands for the count of standard deviation in excess or underneath the mean BMD, considering healthy adults, aged 30, having the same gender plus ethnicity. The Z-score additionally compares age matched results with a matched population. Important to mention is that DXA calculates BMD in a two-dimensional manner, giving results in g/cm^2 and is neglecting the three-dimensional aspect of bone geometry. Therefore larger bones are more likely to be presented with a higher BMD than smaller ones, despite of the real BMD. Furthermore a differentiation between cortical and trabecular bone is not possible and the origin of actual bone loss remains hidden.

As mentioned before BMD is a strong indicator for bone fragility and fracture risk, however it lacks efficiency to represent the whole bone´s strength in some cases. About 50% of all fractures are found in persons not suffering from osteoporosis by means of BMD testing and the majority of women diagnosed with osteoporosis do not have a fracture (57). As a matter of consequence, quality parameters such as bone microarchitecture, accumulation of micro damaged areas, material properties and the rate of bone turnover should be considered carefully.

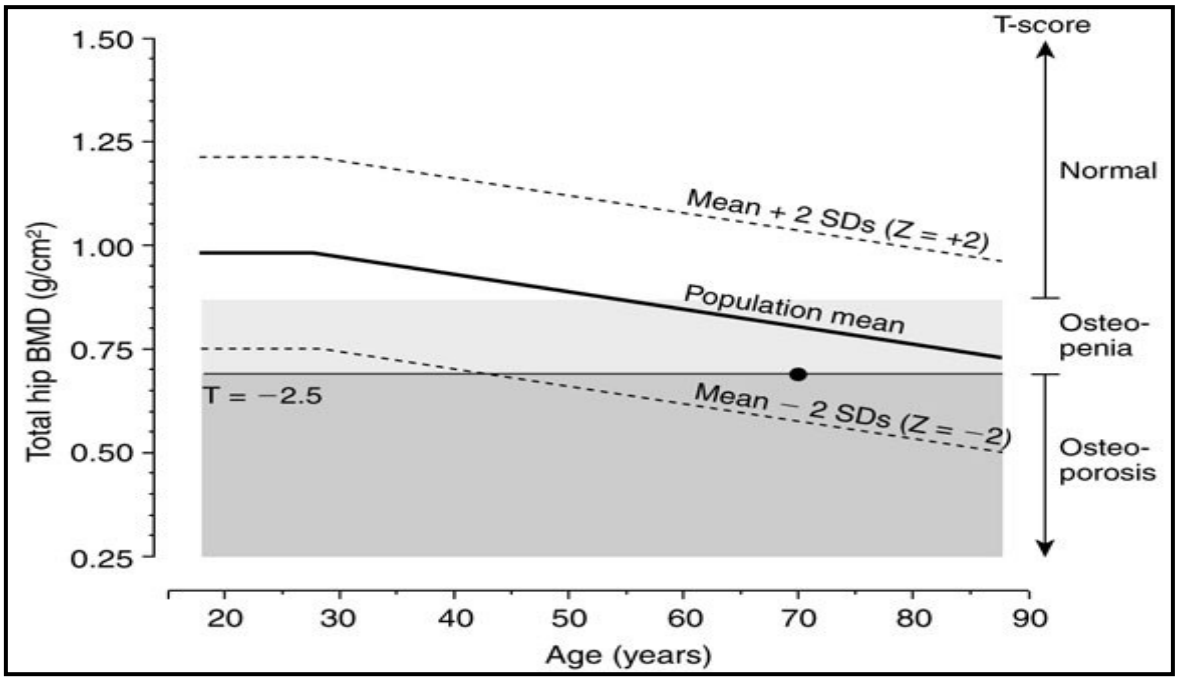

Figure 17 T- and Z - Score in evaluation of BMD (58)

Quantitative computed tomography (QCT) and **peripheral quantitative computed tomography (pQCT)** are also in use to measure bone densitometry in vivo. Secondary to BMD, measured in g/m^3, it can distinguish areas of trabecular bone from those of cortical bone, providing an additional insight to the three - dimensional architecture. This imaging technique is used to observe morphological changes due to aging (59), to observe the effects of pharmaceuticals (60), mechanical unloading of the femur (61) and the correlation of bone structure plus strength of the femur and hip fractures (62).

For ex – vivo examination of bone microarchitecture and morphology **micro computed tomography (µCT)** has become an established standard. By using X - ray attenuation data from multiple viewing angles a three dimensional representation of the spatial distribution of material density is reconstructed. This technique provides several advantages compared to stereological models: (63)

- Direct three dimensional measurement of trabecular morphology, such as trabecular thickness and separation
- Analysis of larger volume of interest
- Faster measurement throughput than typical histologic analysis
- It is non-destructive, thus samples can be used subsequently in other assays
- Estimation of bone tissue mineralization can be performed

Bone microarchitecture can be measured by **high resolution computed tomography**, **high resolution MR** and **micro MR** (64). Though these new technological approaches show very promising results, there is still no non-invasive technique to assess bone material properties. **Finite element analysis** considers structural information combined with a virtual simulation of heterogeneous material properties and the respond under different circumstances of load application. Due to the excessive amount of technology involved, such techniques might become clinical relevant with further technological advance.

5.3 Biochemical Markers

Biochemical bone turnover markers (BTM) are direct or indirect elements of bone metabolism which reflect changes in bone formation and resorption processes and can be measured by the collection of urine or blood samples. In addition to skeletal imaging, biochemical markers have shown to be very useful in terms of estimating bone-net-balance and turnover rate because of their fast responsiveness: days to weeks, compared to months to years in BMD changes. Thus, providing early information on metabolically changes, they are widely used in planning and monitoring drug therapies. Furthermore high levels of biochemical bone turnover markers correspond to fracture risk independently from bone mineral density (65).

The lack of an international standard complicates the evaluation of BTM in clinical practice because of their high individual variability, different types of used analytes and insufficient control. Therefore the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommend to use only one marker for bone formation: *serum procollagen type I N propeptide* (s-PINP) and one marker for bone resorption: *serum C-terminal telopeptide of type I collagen* (s-CTX) as reference analytes in clinical studies (66).

The aim of future investigation will be to consolidate this high sophisticated data under aspects of identifying patients at highest risk for fractures and to implement a suitable clinical method to improve the measurement of the patient´s individual risk to fractures. This would eventually help to reduce costs in osteoporosis treatment and to improve the patient's compliance.

6 IMMOBILIZATION

In a medical aspect, immobilization is defined as rendering a person or a body part incapable of moving and as the act of quiet, prolonged rest in bed as a medical treatment or the fixation of body parts to promote healing processes (67).

Bed rest, the most frequently form of immobilization, is a commonly described medical treatment and has a long historical tradition. Although it is often associated with a mild, healing process and as a recovering period this belief is very delusive. Even Hippocrates, back in 450 B.C., warned people of protracted bed rest due to loss of bone, muscle and tooth. (68). Bed rest was rarely prescribed and accepted as a medical treatment until the 18th century because of the very need to work hard for a living (69). However, in the 19th century it became a very well established treatment, especially in the fields of psychiatry but also in everyday clinical practice, e.g.: 4 weeks of bed rest due to myocardial infarction, 21 days past hernia surgery and 14 days past childbirth (70). Opinions started to shift during World War 2, due to the fact that injured soldiers were forced to get out of bed rather quickly because of limited capacities. Nonetheless, they recovered even quicker than bed rest ridden comparisons. At the same time the beginning of space flight set focus on the physiology behind prolonged bed rest and weightlessness.

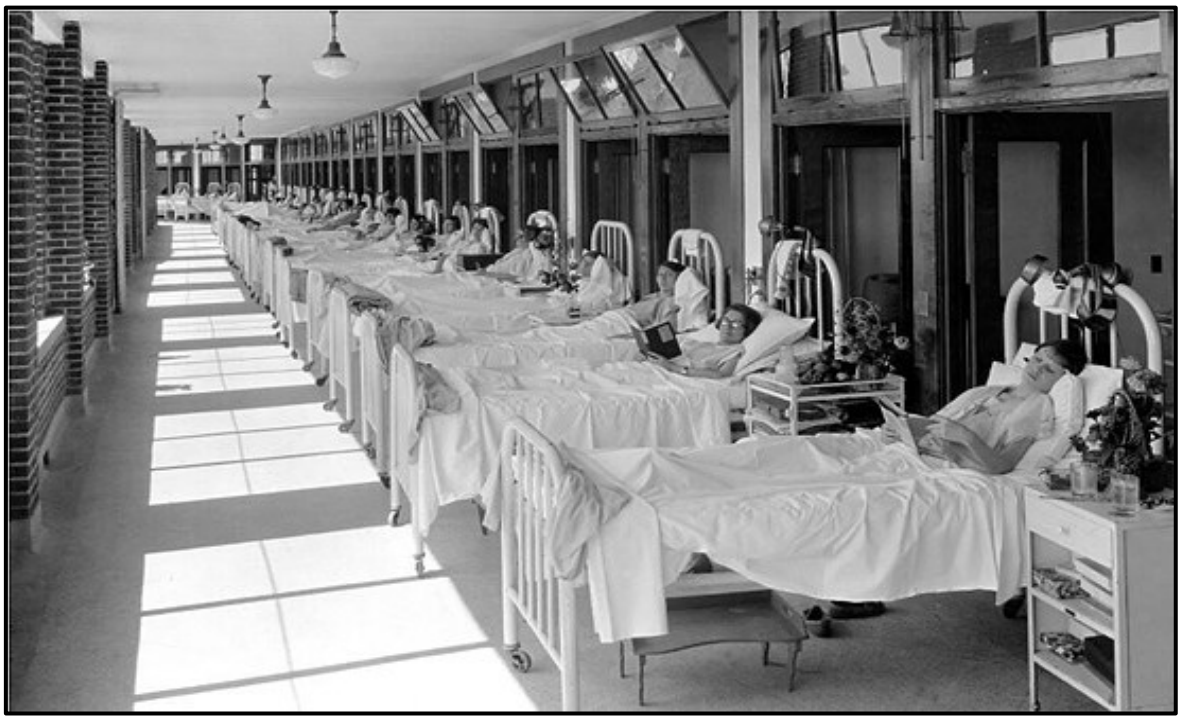

Figure 18 Waverly Hills Tuberculosis Sanatorium, 1926 (71)

Since the human body relies on moving and functioning in an upward position; various problems will set in very quickly after a short time of deconditioning or even habitual sleeping for more than 9 hours a day (72). To mention a few, problems which will arise are as follows: muscle atrophy, loss of bone, cerebral fluid shifts; changes of cardiovascular, endocrine, cognitive and neurovestibular function (73).

Hence, many studies have been investigating the impact of bed rest as primary treatment or prophylactically after medical interventions. A systemic literature review conducted by Allen et al. on 39 randomized controlled trials of bed rest as therapeutic intervention sums up the results which were published between 1966 and 1998, representing a collective of 5777 patients and 17 different conditions (74). Bed rest performed after a medical intervention did not show any significance in the outcome improvement in 24 trials but 8 worsened significantly in the following cases: cardiac catheterisation, lumbar puncture, radiculography and spinal anaesthesia. 15 trials investigated bed rest as first line treatment and showed no significantly improved outcomes, whereas nine got worse significantly for the following conditions: labour, myocardial infarction, proteinuric hypertension during pregnancy, acute low back pain and acute infectious hepatitis (75). On that account bed rest should be considered very critically as a medical treatment and should not be taken as beneficial without clinical trials. Quite the contrary, bed rest should be minimized to its possible limits and early physical activity and ambulation should be directed more generously.

6.1 Bed Rest as a Model for Microgravity

Since the beginning of human space flight researchers and astronauts are facing several problems the whole human body is subjected to in a micro gravitational environment. As mankind evolved under the influence of constant gravitation on earth, microgravity opposes a new surroundings the body has to adapt to in a slowly, gradual manner. Table 4 gives a schematic overview of the most obvious physiological effects due to micro gravity, possible risks and potential countermeasures.

Time in space is very limited and so is the number of astronauts. Therefore, bed rest studies have become the model of choice to simulate microgravity in humans on earth. By putting subjects in a 6° head down tilt position for a certain period of time, micro gravitational conditions, especially fluid shifts to the upper body part can be simulated. Of course these results are important for space programs but also hold great

opportunities for the investigation of human biology and physiology in a controlled environment. For example, the results from a 90 – day male bed rest study concerning a metabolic protocol on fatty acid oxidation, suggests Mediterranean diets for recumbent patients. Another surprising result from a 60 - day female bed rest study was a special designed nutritional supplement to prevent muscle atrophy and eventually turned out to maintain cardiac muscle mass, which might be useful for new therapy concepts in cardiac diseases (76).

Table 3 Physiological effects of microgravity exposure and countermeasure (77)

System	Effect	Risk	Current CM	Potential CM
Bone	Loss of bone mineral density (1 - 2 % per month) in lower extremities with elevated blood calcium levels	Increased risk of bone fracture Calcification of soft tissues and kidney stone formation	Exercise Dietary vitamin D, K and calcium Penguin suit	Bisphosphonates (antiresorptive) Parathyroid hormone (anabolic) 30 Hz vibration plate Artificial gravity
Muscle	Calf muscle volume ↓13% Peak power ↓ 32% 10- 17% shift from slow type I fibers to faster type II fibers	Insufficient muscle strength to perform emergency egress and post-landing EVA	Exercise Penguin suit Electro-stimulation of muscle	Amino acid supplements Artificial gravity
Cardiovascular	Venous fluid shift to upper body Plasma volume ↓ 17% Total blood volume ↓ 10% Decreased vasoconstrictor response	Orthostatic intolerance when upright upon return to gravity, increasing fall risk	Exercise Saline loading prior to re-entry Penguin suit Braslet Chibas (lower body negative pressure)	Midodrine (vasopressor antihypotensive) Artificial gravity
Sensorimotor	Space motion sickness Deconditioning of reflexes Spatial disorientation	Inability to operate space craft or other complex machinery Risk of falls due to postural and loco motor instability after landing	Antiemetics	Tactor vests, visual displays to provide orientation cues

6.2 Effects of Bed Rest on the Skeletal System

The integrity of the skeletal system is based on dynamic, mechanical loading which is opposing the gravitational vector and further, the contraction of muscles. On earth bones are under the constant influence of a gravitational field of 1 g (9,81m/s$^{2)}$, this gravitational force is only reduced by about < 5% in typical space missions. Though, spacemen seem to be weightless, this fact does not arise from a lacking gravitational field but from two opposite directed forces: the centrifugal (vanquishing earth gravitation) and centripetal force (gravitation), which compensate each other and lead to a condition of free falling around the earth. Thus, it seems to be reasonable that the net force generated by gravity, to be called weight, accounts more to bone quality and quantity than the magnitude of the gravitational field (78).

Weight is acting upon the skeleton by impairing a mechanical load in an upright position and is one of the main factors for bone maintenance. Moreover, muscles play a vital role in keeping up bone´s strength. Muscle contraction implies a large force on bones, mainly because of the quite short levers they are working against (79). Consequentially, these two factors are representing the strongest excitation for bone loss in weight bearing structures during bed rest. Thus, bed rest seems to be an appropriate model to investigate the influence of microgravity on bone.

The mechanostat hypothesis (see p. 24) obviously explains the loss of bone during bed rest very well (80–82). Bone metabolism is connected tightly to the homeostasis of calcium. It was first believed that the elevated levels of measured calcium excretion during bed rest reflect a form of primarily endocrine dysfunction. Neither calcium nor vitamin D supplementation did show an effect in preventing bone loss (83). During immobilization bone loss shows different patterns due to a large inter – individual variability (84,85), moreover there is a large fluctuation considering the same bone at various sites within different subjects (84). Which in turn would suggest that it is not the mechanostat alone affecting bone loss during immobilization. On the contrary, patients after a spinal cord injury show unique patterns of bone loss over the course of years (86).

Table 4 Key bone changes measured in microgravity (87)

Observed Changes in Bone Tissue Due to Microgravity
• Reduction of 1 % areal BMD/month (spine) • Reduction of 1 – 1,6 % areal BMD/month (hip)
• Reduction of 0,4 – 0,5 % cortical BMD/month (hip) • Reduction of 2,2 – 2,7 % trabecular BMD/month (hip)
• Reduction of 2,6% bone fracture strength/month (hip)

Accordingly to the loss of bone mineral content the rate of calcium excretion starts to exceed. Levels of plasma calcium will start to rise about the third day of immobilization and losses can be measured in the urine. If the calcium level exceeds the kidney´s excretion capacity the risk for urolithiasis increases, which could also lead to heterotopic calcification and myositis ossificans in some cases. Secondary hypercalcaemia will set in, affecting smooth muscles and neurones, nausea, vomiting and anorexia may occur. After about 7 weeks of immobilization 93 mg of phosphorus and 61 mg of calcium will be excreted (88).

Connective tissue, such as ligaments, articular cartilage and tendons are also affected by bed rest. Changes become apparent on the 4^{th} – 6^{th} day, due to structural transformations in collagen fibers. A immobilization period of 20 days leads to stiffness and increased viscosity in tendons (89) which affects the ability to apply dynamic force upon bones. Ligaments lose about 31% in stiffness and 39% of load bearing, even after one year decreases will not be diminished (90). Obviously, contractures also contribute to the negative effects of bed rest and will disable physiological motion.

7 OMICS ANALYSIS

"It has been said: The whole is more than the sum of its parts. It is more correct to say that the whole is something else than the sum of its parts, because summing up is a meaningless procedure, whereas the whole-part relationship is meaningful." Kurt Koffka (91).

The sequencing of the whole human genome in 2001 represents a milestone in human biology. Though, the complete information about all genes and most of their related biomolecules was available at that time, a lot of questions remain unanswered up to day. The idea or hope to develop new therapy concepts or illuminate disease mechanisms from scratch, based on this knowledge, has not been fulfilled for the most part up to now and gave birth to a new era in biology: the "omics-era".

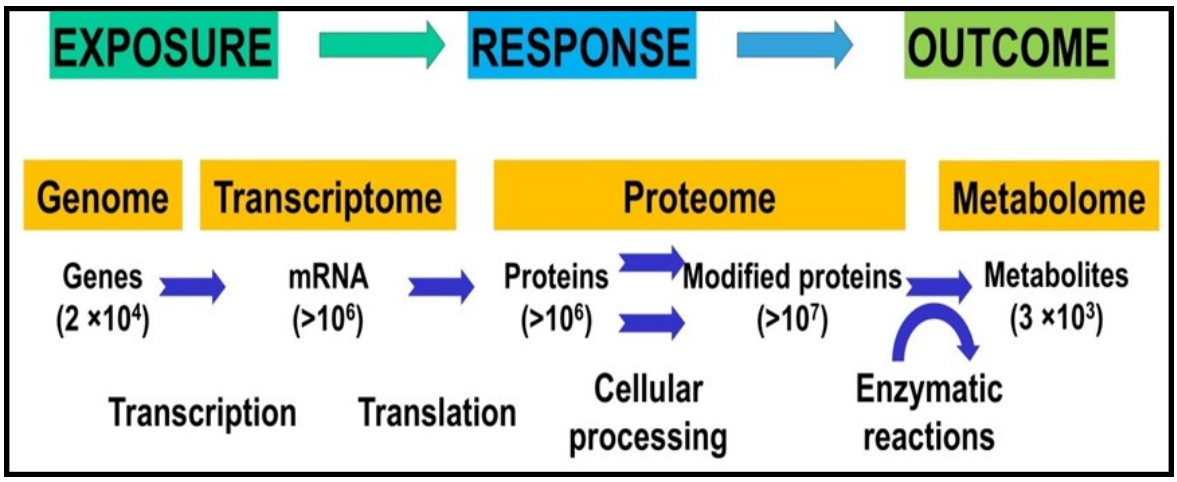

Figure 19 The molecular biological dogma: DNA transcribed to mRNA, translated to proteins (provided by Dr. Goswami Nandu)

The suffix "Ome" has its origin in the Greek language and stands for "every", "complete" or "whole". An omics is a neologism referring to a large study field in biology, for example genomics, epigenomics, transcriptomics, proteomics or lipidomics. In essence, it is based on combining or summing up information on a given biological system, instead of splitting up and reductionism. The new approach in system biology is to obtain systematic insight into biological processes on multiple levels (RNA, proteins, metabolites) and to combine this information to gain an integrated, more kinetic, real-time based understanding of biology (92). The rapidly evolving technical progress made it possible to investigate hundreds or thousands of genes simultaneously in one experimental setup, which is often related to the term

"high-throughput technology". Instead of focusing on single targets or values of interest this technology might help to unravel the interaction of cause and effect in living systems.

Due to high-throughput measurements system biology and researcher face huge amounts of data material, whose quality relies on measurements which have to be conducted in a very short time under highest accuracy to obtain reasonable results. These results undergo numerous analytical algorithms and pattern recognition methods and are analysed by comparing results with, for the most part, public data bases or linked to other published data available. This retrieved information about a particular gene or gene set and its biological function is further used to generate pathway analysis with appropriate software tools. Thus, making it a new, broad interdisciplinary field involving mathematicians, informaticians and medical staff.

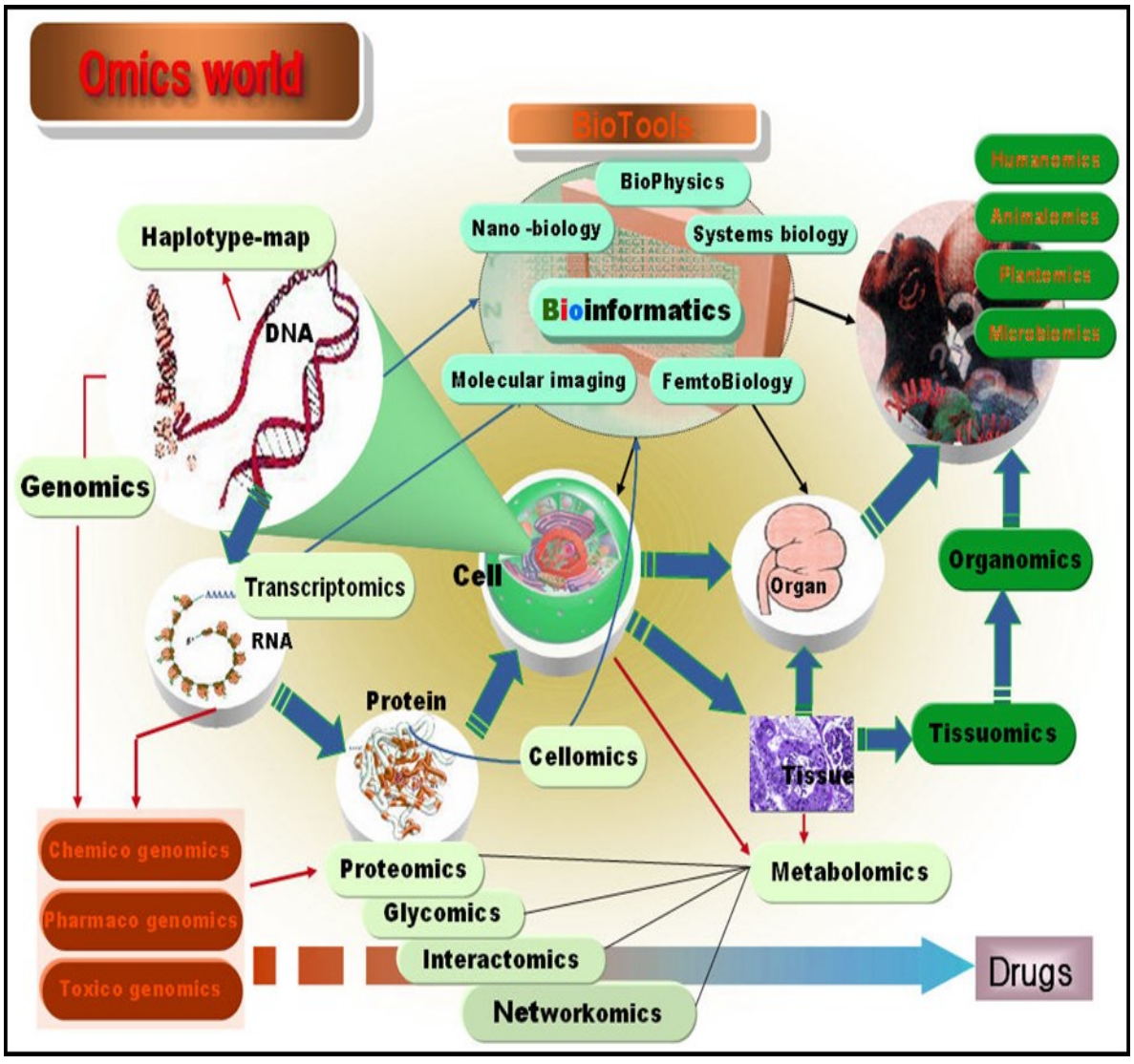

Figure 20 Overview of omics technologies and fields of interest (93)

In principal, the most common used high throughput measurements are as follows:

- Genomics
- Transcriptomics
- Proteomics
- Metabolomics

Every one of these disciplines is unique and provides a different view on the mechanisms underlying disease onset and progression, likewise on ways of preventing, treating or predicting disease.

7.1 Genomics

Genomics is used to identify the physiological role of genes and their relation to the susceptibility of certain diseases. The human genome project (HGP) elucidated the number of genes and their location in the human genome (94). By sequencing, assembling and analysing the combined effect of variability in multiple genes the development of complex diseases can be studied. Although there are several types of genetic variations (eg, copy number variations (CNVs), deletions and insertions of nucleotide base pairs), the most frequently investigated are single nucleotide polymorphisms (SNPs) because of their high abundance (95). SNP genotyping measures a couple of hundred thousand SNPs spread over the whole genome. Genomics has to be considered as a statical tool because the genotype remains constant over lifetime (excluding individual mutations of cells). This makes the measurement of SNPs very useful in terms of predicting the risk for certain diseases.

7.2 Transcriptomics

Transcriptomics deals on the assessment of the quantity of specific mRNA transcripts and evaluation of their composition variability. Focus of interest is the whole transcriptome. By measuring the amount of mRNA transcripts a reflection of corresponding genes and their expression levels are obtained. The transcriptome is a fast responder to any environmental changes and very fluctuating over time. Thus, making it a valuable method for gene expression profiling to elucidate differences in individuals, who have a similar phenotype (e.g.: diseases, age and sex) (95).

By collecting whole genome gene expression data, which can be extracted from different kinds of organic samples, a systemic snapshot of the organism is created. The transcriptome captures early microbiological changes and provides insight into transcriptome pattern changes, which might be influenced by environmental factors, physiological stimuli, diseases or a process of development. These specific changes integrated on a systemic level may help to understand the complex molecular organisation and shade some light on the dynamics of biological pathways by establishing an understanding of intra- and intercellular pathways and also control mechanisms of intracellular processes. Functional analyses are widely applicable in drug-, biomarker- and cancer research, translational or personalized medicine and to discover disease mechanisms.

7.3 Proteomics

Proteomics focuses on the assessment of all proteins which are present in a specific tissue or cell to a given time point. By identifying the quantity of proteins in the intercellular and intracellular compartments inferences to functions might be drawn. Though all proteins derive from mRNA precursors, gene expression analysis reclusively cannot predict the abundance of proteins due to post translational modifications and environmental interactions. Because of its high responsiveness and fluctuation over time the proteome is a good indicator for external and internal influences on biological systems. Further, expression levels provide an insight into the abundance of a certain protein, showing the balance of degradation and translation in cells and their related function (92). Therefore, the proteome is a useful tool to detect diseases in very early stages.

7.4 Metabolomics

Metabolomics is the aggregation measurement of all small molecules (metabolites) in a specific cellular compartment, a cell or body fluid to a given point of time. Metabolites represent end- or by-products of all physiological or pathological processes. The metabolome reacts very fast upon all internal and external influences, such as nutrition, lifestyle, environment or diseases. Therefore, conclusions to related enzymes and metabolic pathways can be drawn (95). The biggest challenge in metabolomics is the

huge number of involved pathways for one molecule and the large concentration shifts (96).

7.5 The Peripheral Blood Transcriptome

Blood is a special connective tissue in fluid form; cells are suspended in a liquid matrix to functionally connect the entire human body at a physiological level. Blood as a tissue pervades the whole human body and is associated with manifold tasks: heat regulation, first line of immune response, coagulation and transport medium for gases, nutrients, metabolites, cytokines, hormones and antibodies.

Based on the demand of personalized medicine future concepts and difficulties of organ tissue sampling, several studies have been performed to explore the potential of peripheral blood as a biopsy tissue surrogate in combination with microarray technology. Therefore the term bloodomics was introduced.

It has been shown that blood cells are able to differentially react upon physiological, pathological or environmental impact and cover about 80% of the transcriptome in several tissues (60). More importantly, organ specific genes have been detected (e.g.: ß – myosin heavy chain) and genes that react on physiological stimuli, which were thought to be only tissue, related (e.g.: insulin) (61).

Due to the high overlap in gene expression compared to specific tissues and the good responsiveness to environmental or physiological processes, the peripheral blood transcriptome can be considered as a potential biopsy surrogate. Moreover it has contact to every cell in the human body and a very high turnover rate. Finally, it can be accessed very easily in large amounts using minimal invasive techniques people are very familiar with and it is readily available.

7.6 DNA Microarray Technology

The simultaneous measurement of whole genome RNA concentrations requires special “high-throughput technologies”. A DNA microarray (also Biochip or DNA-chip) experiment is the state of the art technology to explore the molecular-genetical situation of a cell to a certain time point, under certain conditions.

DNA–chips are usually very small glass- or plastic plates, which represent a classified arrangement of small DNA-molecules with known sequences. Each molecule

corresponds to an exactly defined spot on the chip with a diameter of approximately 200 um, called probes. Using up to date technology it is possible to apply 250.000 to 1.000.000 oligonucleotides (probes) on a surface of 1 cm2. These probes are used to hybridize cRNA or cDNA samples, also called targets. Fluorescent colour is applied to label targets before hybridization. Laser scanners detect relative signal changes due to concentration ratios and data is analysed by specific software arrangements (97).

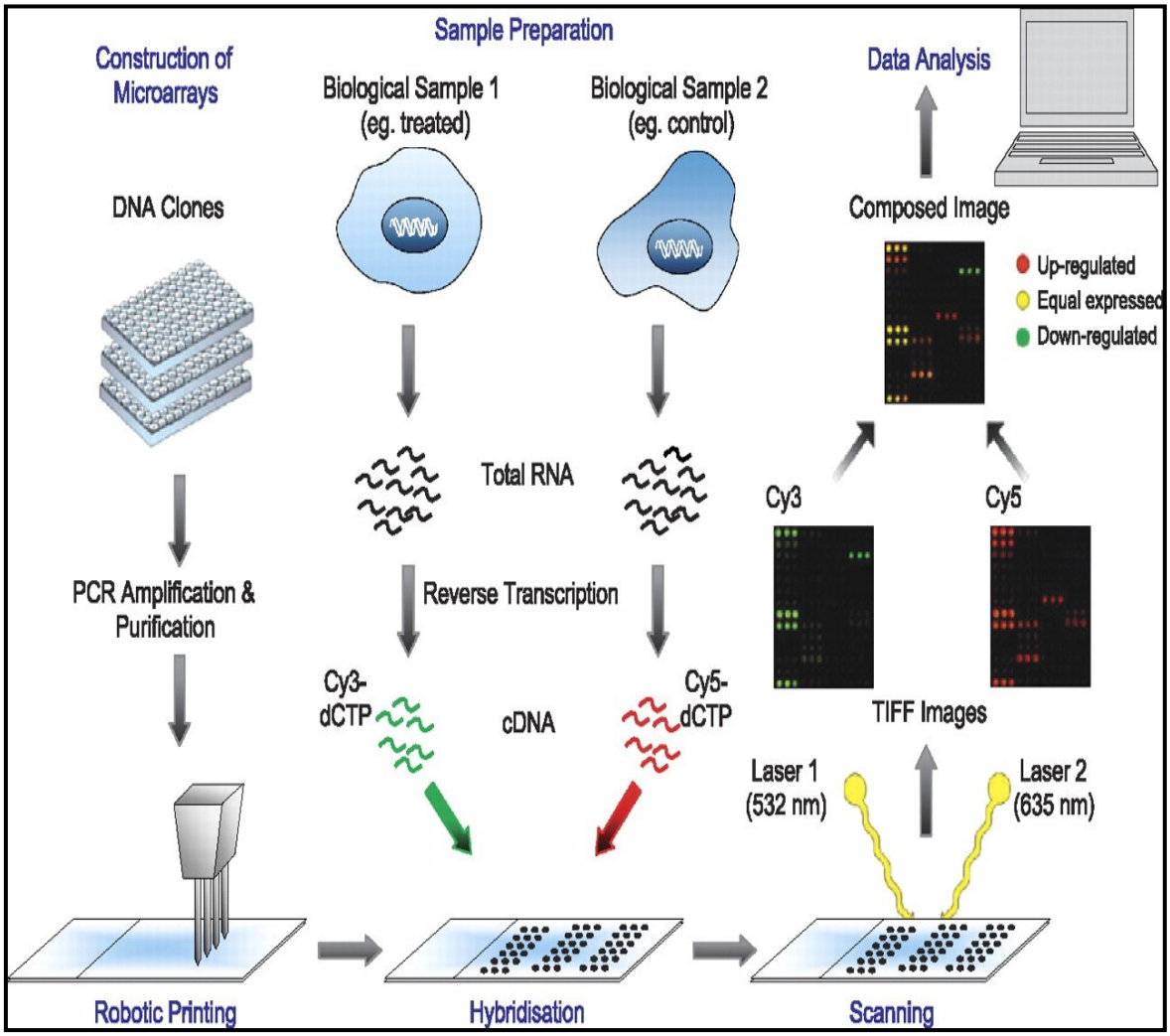

Figure 21 Schematic process of a microarray experiment (98)

II AIMS AND OBJECTIVES

Bone tissue is known to react very fast in terms of deconditioning with a loss of bone mass and reduction of bone mineral density. Well-established countermeasures against bone loss are physical exercise and an adapted diet. In this study, we will investigate the effects of immobilization and the two mentioned countermeasures on molecular mechanisms of bone metabolism. Our hypotheses:

- **Blood gene expression levels are affected during 21 days of bed rest**
- **Differences in the transcriptome can be observed depending on the type of intervention (BR, BR + EX, BR + EX + Diet)**
- **Microarray analysis is an appropriate technique to capture molecular biological changes on bone metabolism due to bed rest**
- **Blood is an appropriate surrogate to investigate molecular biological changes on bone metabolism due to bed rest**

12 healthy male subjects were put in a 6° head-down-tilt position to simulate bed rest and gravitational unloading. Further, the influence of mechanical stimulation and a special diet were investigated as important countermeasures to prevent negative effects of microgravity.

This diploma thesis explores the physiological basis of bone function and how it is affected by immobilization with special focus on molecular changes. Therefore, a search of the existing literature and data that were recently collected during bed rest campaigns will be performed to provide up to date knowledge, considering proteins that are known to be affected during bed rest. Moreover, data from a microarray experiment, with respect to the total of mRNA (transcriptome) abundant in the peripheral blood will be investigated. However, this thesis only examines proteins specific to bone function and bone metabolism, which will be systematically checked for known proteins. In addition, any other new or previously unknown protein important for bone function and metabolism will be identified on the basis of the database.

III MATERIALS AND METHODS

1 MNX BED–REST STUDY

The MNX Bed–Rest Study (**M**edium duration **N**utrition and vibration e**X**ercise) was performed under the leadership of the French and European space agencies, from November 2012 till November 2013, at the Institute for Space Medicine and Physiology (MEDES) in Toulouse, France. Aim of the study was to evaluate the effects of immobilization and microgravity on human physiology and the effectiveness of two countermeasures to attenuate the effects of microgravity to which study objects were subjected:

- Exercising the lower limbs ("squats") under resistance, on a vibrating platform absorbing the up and down motion. This exercise was conducted twice a week (Figure 23).
- The same physical exercise programme combined with protein and potassium bicarbonate supplementation in the daily diet (99).

Figure 22 Study subject in 6° head-down-tilt position (100)

1.1 Experimental Set-Up

12 healthy male study subjects took part in this randomized, cross-over study. They were exposed to bed rest with a 6° head-down-tilt position (simulation of spaceflight induced fluid shifts) and divided into 3 groups, respectively: bed rest (BR), bed rest plus exercise (BR+EX) and bed rest plus exercise plus diet (BR+EX+DIET). Each of the 12 volunteers acted as their own control, and changed group after each campaign Lots were drawn to determine the order in which they changed between the three groups. The study was divided into three campaigns, each campaign or hospitalisation period was followed by a break of three months.

Each hospitalisation period of 35 days was broken down into three phases:

- An ambulatory control period of 7 days before bed rest
- Anti-orthostatic bed rest (inclined at -6°) for a period of 21 days (Figure 22)
- An ambulatory recovery period of 7 days after bed rest

Figure 23 Physical exercise under resistance on a vibrating platform (101)

Following each hospitalisation period, the volunteers returned for follow-up visits, 14 days and 28 days after the end of each bed-rest campaign.

Peripheral blood, withdrawn from an upper arm vein, was used as a material of investigation. Blood collection was performed every day between 7.00 am and 8.30 am on seven predefined time points by medical stuff persons of MEDES. In addition, blood pressure, heart rate and body weight were checked every morning before experiments started.

Table 5 MNX - Study design

	Campaign I 21 days	4 months	Campaign II 21 days	4 months	Campaign III 21 days
Group 1 n = 4	BR	WASHOUT	BR + EX	WASHOUT	BR + EX + Diet
Group 2 n= 4	BR + EX		BR + EX + Diet		BR
Group 3 n = 4	BR + EX + Diet		BR		BR + EX

1.2 Subject Selection

Subject recruiting was done throughout French media and via the homepage of ESA (European Space Agency) and MEDES. A preliminary selection was made considering lifestyle, profession and education. Secondly, a form on individual and family anamnesis was taken into account. Test protocols were explained in detail to the study participants and given informed consent in written form. Every study participant was granted to quit the study at all times independent of reason. Each proband underwent a comprehensive medical examination prior to study participation. Test persons were instructed to avoid exhaustive physical exercise one week before the beginning of the study. The consumption of stimulants such as coffee, two days prior the start of the trial was also interdicted.

Inclusion criteria:

- Healthy male subject (according to the performed medical tests plus laboratory analysis)

- Age: 20 - 45
- BMI (weight Kg/height m^2): 20 - 26
- Height: 1,58 – 1.90m
- No family nor personal past record of acute or chronic diseases
- No psychological abnormalities
- Fitness assessment:
    - < 35 years: 35ml/min/kg < VO2 max < 60ml/min/kg
    - > 35 years: 30ml/min/kg < VO2 max < 60ml/min/kg
- Mobile and active (no orthopaedic musculoskeletal nor cardiovascular disorders
- No tobacco, drug, or alcohol dependence
- Not under medical attendance
- Information consent: fully understood and signed
- No engagement during hospitalization periods

Non-inclusion criteria:

- No orthostatic intolerance
- Cardiac rhythm abnormalities
- Back pain
- Reported hiatus hernia, thyroid dysfunction, gastro-oesophageal reflux, diabetes, renal stones, migraines
- Record of thrombophlebitis (personal and family history: thrombosis)
- Claustrophobia
- Reported genetic muscle or bone diseases
- BMD: T-score ≤ -1,5
- Metallic implants
- Reported knee problems or joint surgery
- Intolerance to blood collecting

- Blood collections 8 weeks or less prior the study (more than 8ml/kg)
- Special diet (vegan, vegetarian)
- Reported lactose intolerance
- Hepatitis A, B, C
- Anti - HIV_{1+2} antibodies
- Inappropriate thoracic acoustic window
- Participation in another clinical research
- Declined permission to contact personal general practitioner
- Cancer
- Likeliness of noncompliance throughout the study
- Not able to cooperate due to language barriers
- Having gained more than 4500 Euros in the last 12 months from research projects
- Guardian- or trusteeship

1.3 Sample Collection

Blood sampling was performed using butterfly catheters and Tempus Blood RNA tubes (*Life Technology*, Halle Belgium). Each study subject donated a probe of 6ml per time point. After collection the tubes were mixed 10 times and frozen within 20 minutes in a -80° C freezer. RNA sample analyses were done in Belgium by the Flemish Institute for Technological Research under the supervision of Prof. Dr. Patrick De Boever.

1.4 Sample Collection Time Points

Test subjects had to stay in hospital 5 days prior the start of the study. First blood samples were taken at this time point (BDC-5). Further blood collections were performed within the following 3 weeks of bed rest and after bed rest (R = recovery period). Namely on day 2, 7, 14 and 21 of head down tilt (HDT) -6° position. Further blood samples were taken at the beginning of recovery before getting up (R 0) and the second day of recovery (R 2).

Table 6 Sample collection time points

Sample collection time points							
	Pre – bed rest	**Bed rest (6° HDT)**				**Post – bed rest (standing up)**	
Day	**- 5**	**2**	**7**	**14**	**21**	**0**	**+2**
Abbreviation	**BDC -5**	**HDT 2**	**HDT 7**	**HDT 14**	**HDT 21**	**R = 0**	**R = +2**

According to the World Medical Association (WMA) Declaration of Helsinki, the MNX bed rest study protocol was approved by the Institute for Space Medicine and Physiology Ethics Board in France.

2 MICROARRAY ANALYSIS PROTOCOL

Microarray analysis was performed under the supervision of Prof. Dr. Ir. Patrick De Boever in Belgium, at the Flemish Institute for Technological Research.

With reference to the MNX bed-rest study design, only 3 individuals (out of 4) per condition (BR, BR+EX, BR+EX+Diet) from the first campaign were chosen randomly (Table 7).

Table 7 Study design for microarray analysis

	Group 1 n = 3	Group 2 n = 3	Group 3 n = 3
Campaign 1 21 days	BR	BR + EX	BR + EX + Diet

Due to cost issues only blood samples from two defined time points were analysed: 5 days prior to bed rest (baseline data collection: BDC - 5) and at the end of the study, just before subjects were allowed to stand up (R 0) (Table 8).

Table 8 Blood collection timeline for microarray analysis

Sample collection time points							
	Pre – bed rest	Bed rest (6° HDT)				Post – bed rest (standing up)	
Day	- 5	2	7	14	21	0	+2
Abbreviation	BDC -5	HDT 2	HDT 7	HDT 14	HDT 21	R = 0	R = +2

Each individual donated a 6-mL non-fasting blood sample between 7 am and 8.30 am by using butterfly catheters and Tempus Blood RNA tubes (*Applied Biosystems*, Halle, Belgium). After phlebotomy the tubes were mixed gently for ten times and frozen at -80 °C within 20 minutes after collection.

2.1 Workflow

All procedures mentioned below were exactly performed according to the instructions of the manufactures.

2.1.1 RNA processing and quantification

- Extraction of total RNA from blood samples by use of the *Tempus Spin RNA Isolation kit* (Applied Biosystems)
- Measurement of RNA yields by use of the *Nano Drop Spectrophotometer* (Isogen Life Science, PW De Meern, the Netherlands)
- Depletion of globin mRNA from total RNA-preparations by use of the *Ambion Globinclear kit* (Applied Biosystems)
- Integrity determination of remaining globin-depleted RNA by use of the *Agilent 2100 Bioanalyzer* using *RNA 6000 Chips* (Agilent Technologies, Diegem, Belgium)
- Storage of all samples at -80 °C

2.1.2 RNA amplification and labeling

- Amplification and labeling of the globin depleted RNA in order to generate complementary RNA (cRNA) by use of the *Low Input Quick Amp Labeling (one color) kit* (Agilent Technologies)
- Reverse transcription of 100 - 200 ng RNA into complementary DNA (cDNA) by the use of *T7-promotor primer* and *MMLV reverse transcriptase*
- Transcription of cDNA into cRNA with an incorporation of cyanine 3-CTP (pre bed rest samples) and cyanine 5-CTP (post – bed rest samples)
- Purification of single-stranded, labeled cRNA by use of *Qiagen's RNeasy mini spin columns* (Qiagen, KJ Venlo, Netherlands)
- Determination of specific activity and yield by use of the *Nano Drop spectrophotometer*

2.1.3 Microarray analysis and data preprocessing

- Hybridization of 1.65ug cRNA on *4x44K Agilent Whole Human Genome microarray* slides (design 014850) for the duration of 17 hours by the use of automated *HS4800TM pro hybridization station* (Tecan, Männedorf, Switzerland)
- Array scan on an *Agilent DNA microarray scanner* (G2565BA)
- Processing by the use of *Agilent Feature Extraction Software* (Version 10.7)
- Conversion of the scanned tiff-images into text files by the use of *Agilent protocol GE1-10.7-SEP09*
- Analysis per probe of the g processed signal applying a quantile normalization and log2-transformation by the use of *GeneSpring 12.1* (Agilent Technologies).
- Replicates were reported as a median signal
- Removing of control probes
- Result: data files with the expression signals of 41000 unique probes were obtained
- Filtering by an expression intensity filter an mapping of the probes to HUGO gene symbols

2.1.4 Statistical analysis

- For each probe a paired Student t – test was considered
- Adjusting all p–values for multiplicity by the use of the Benjamini-Hochberg false discovery rate (BH-FDR)

Probe significance:

- False discovery rate p-value < 0.05
- Absolute fold change (FC) > 1.5

2.1.5 Biological interpretation

The software "*GeneSpring*" served as entry point for biological interpretation. Analysed probes were matched to their according gene symbol. Further, we performed a Gene Ontology calculation with a Benjamini-Hochberg false discovery rate (BH-FDR) set at 0.1.

Using Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 313398M) a list of genes was constructed that are related to *"Skeletal and muscular system development and function"*. These genes were mapped back to their corresponding probes and fold change (FC) results were calculated per experimental conditions. All expression values with absolute values ≤ -1.5 or ≥ 1.5 were considered as debatable for biological interpretation. $FC > 1.5$ means an up regulation after bed rest; $FC < -1.5$ means a down regulation after bed rest (R=0), which is to say a 50% increase/decrease compared to baseline data values (BDC – 5).

IV RESULTS

12 healthy male study subjects completed this randomized, cross – over, pilot study. Due to cost issues, only 3 subjects from each group of the first campaign (n=4) were chosen randomly for investigation of the molecular mechanisms associated with bed rest, with and without exercise and nutrition interventions (Table 7). Blood samples of only two different time points were considered for statistical analysis: BDC -5 and R 0 (Table 8).

Anthropometric data of the study participants:

- Age: 20 - 45 (mean: 34,3 years, +/- 8,3)
- Weight: 58 - 83 kg (mean: 69,8 kg, +/- 8,0)
- Height: 1,58 - 1,90 m (mean: 1,76 m, +/- 0,06)
- BMI: 20 - 26 kg/m^2 (mean: 22,4 kg/m^2 / +/- 1,7)

Whole blood samples were processed using standard workflows and samples were hybridized on Agilent microarrays using a 2-color design. Post bed rest samples were hybridized using Cy5 dye and pre bed rest samples were hybridized using Cy3 dye. Raw data files were processed using standard Agilent workflows (including normalization and filtering steps). Data plots of expression values of 41.093 probes were generated. On the base of Gene Ontology these results were mapped to known HUGO gene symbols. Thus, 30,953 results were considered for further analysis. Results were assessed by using different cut–offs for p–values and fold changes.

Figure 24 shows the unfiltered results for the whole blood transcriptome for all 9 subjects in all 3 groups, respectively: bed rest (BR), bed rest plus exercise (BR +EX) as countermeasure and bed rest plus exercise plus diet (BR + EX + Diet) as countermeasure.

The y – axis shows the normalized fold change expression values of all genes present in the whole blood transcriptome. A value above zero implies an up – regulation of that particular gene, whereas a value below zero means the expression of this gene was down – regulated after bed rest (R = 0), compared to the baseline expression (BDC -5)

before bed rest. Intensities of the same genes in different participants were linked with lines. A flat line describes similar gene intensities in different study participants

The x – axis refers to the 9 study subjects separated into the 3 different groups.

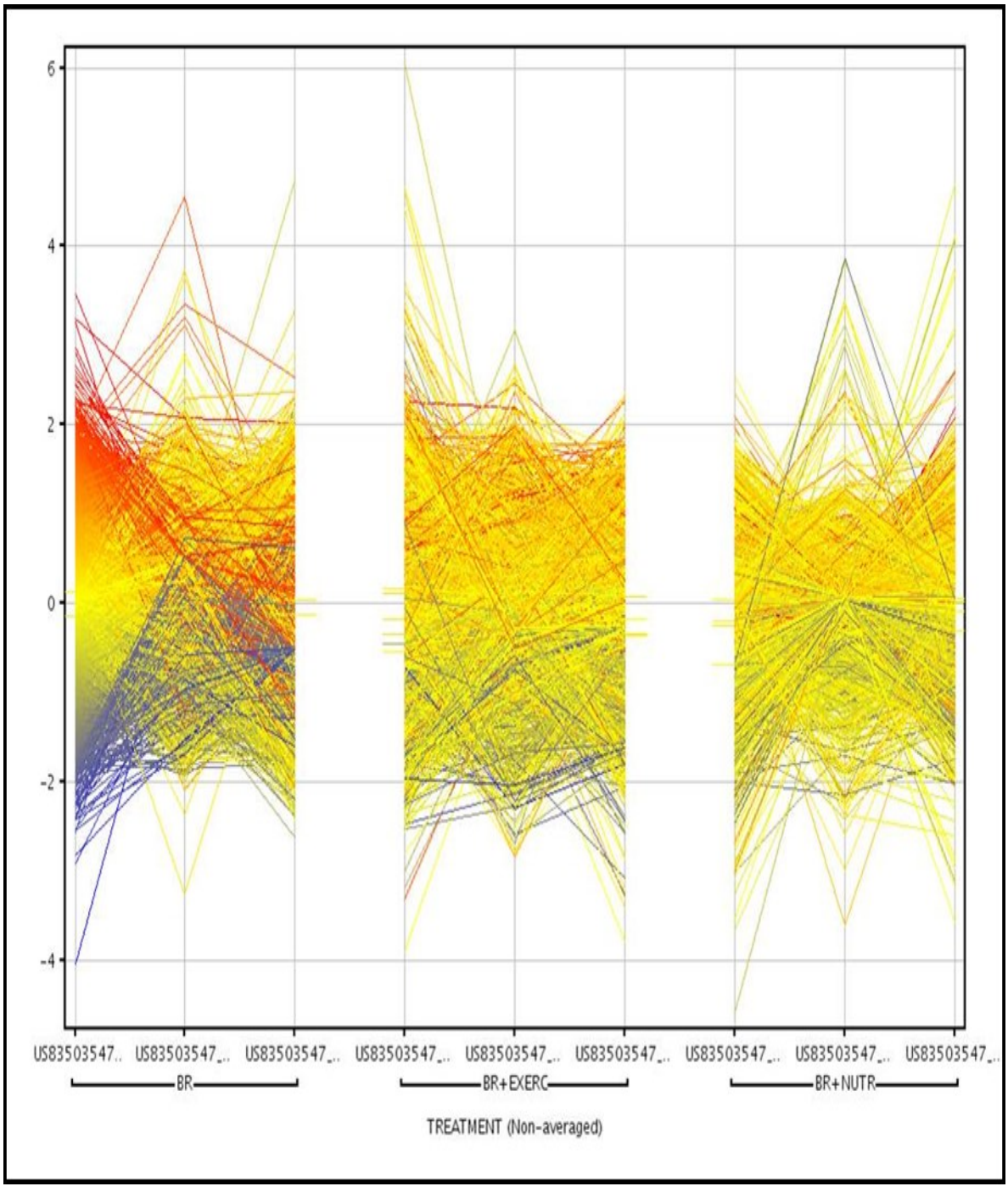

Figure 24 Unfiltered microarray results after the first campaign (provided by Dr. Patrick de Boever)

Statistical analysis was performed using independent t-test and Benjamini-Hochberg correction ($p < 0,05$). No significant genes were identified (no genes passed the

threshold of 0.05), which might be due to the low number of samples in combination with the high inter-individual variability (Figure 25 -27).

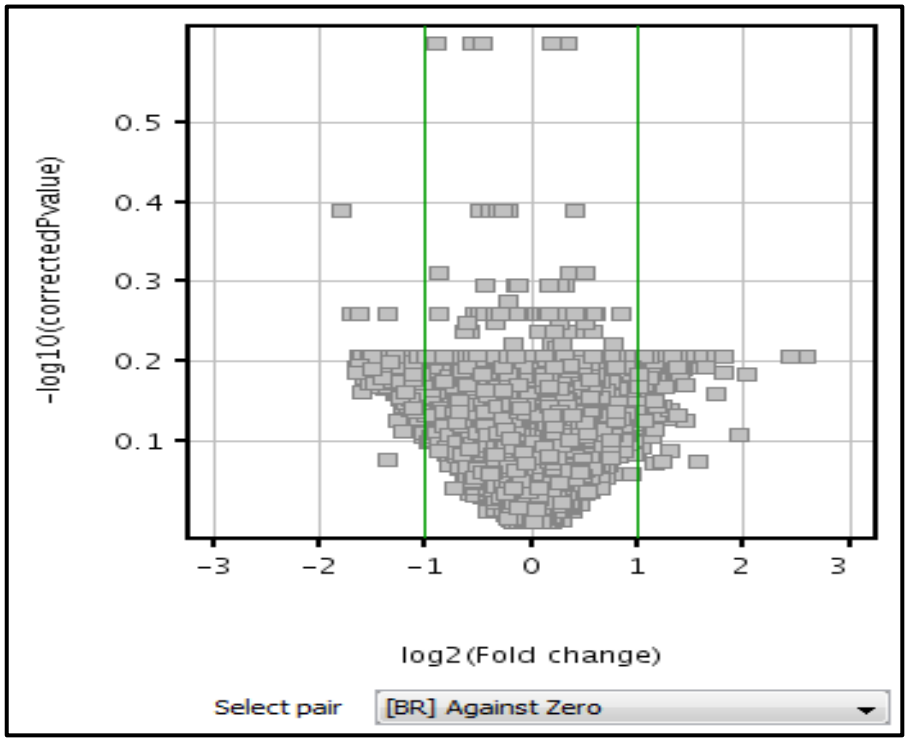

Figure 25 Volcano plot (p ≤ 0,05): BR

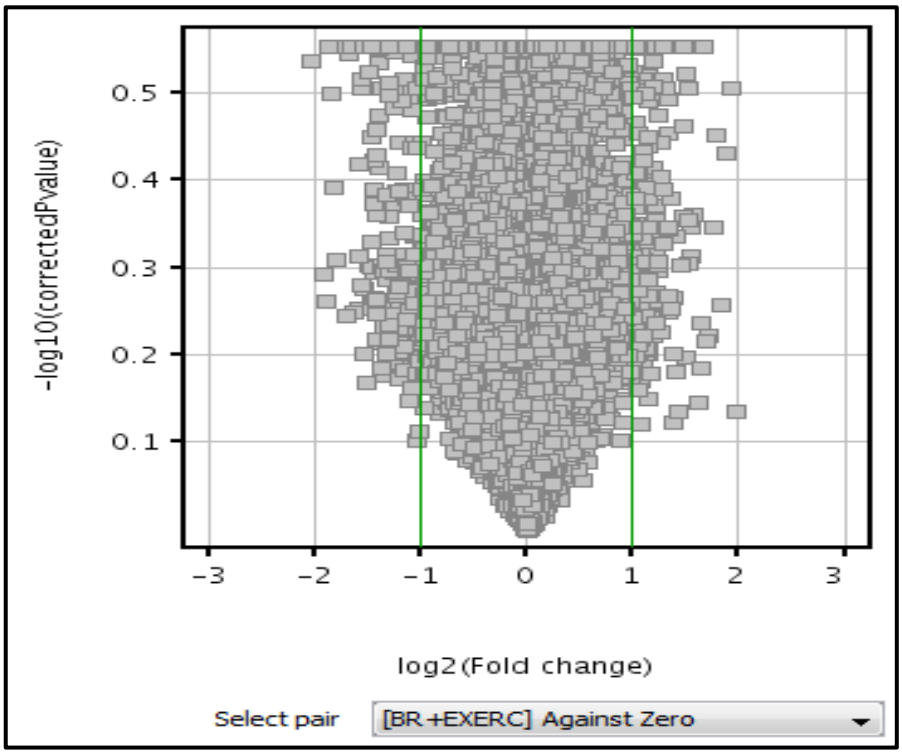

Figure 26 Volcano plot (p ≤ 0,05): BR + EX

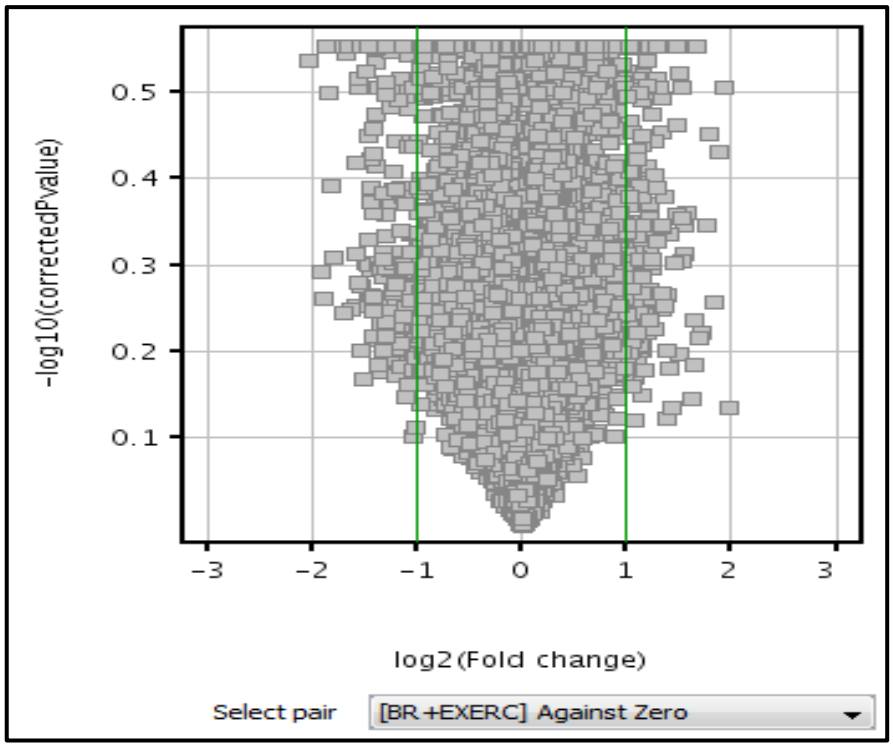

Figure 27 Volcano plot (p ≤ 0,05): BR + EX + Diet

Due to the fact that no distinction was made between treatments, for purposes of analysis only bed rest was considered as the main factor (n=9). Then an independent t-test identified 350 probes, which were differentially expressed (Figure 28).

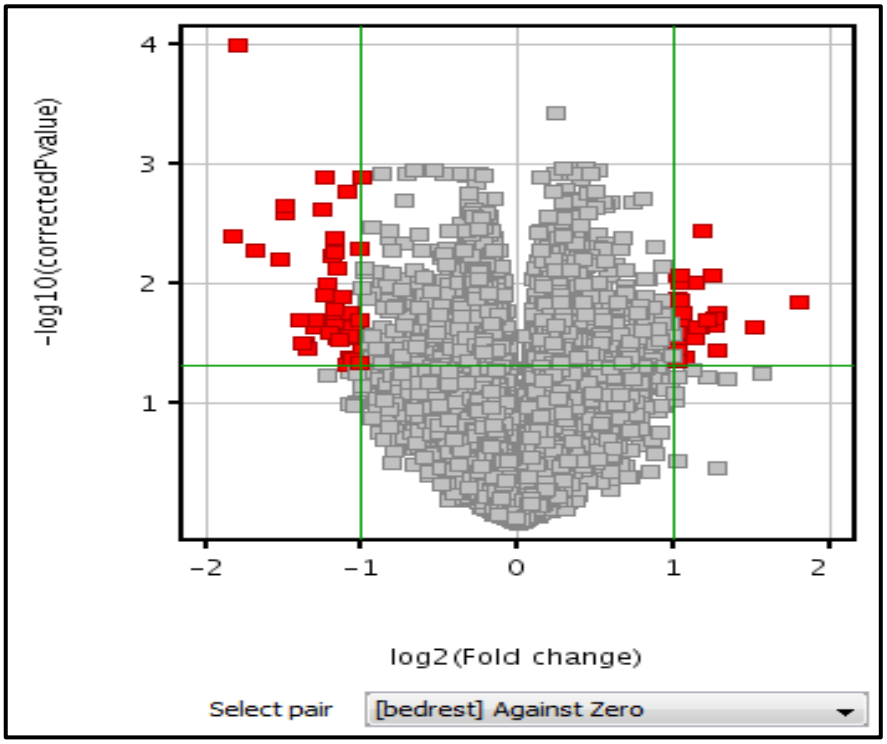

Figure 28 Volcano plot (p ≤ 0,05): BR as single condition for all 9 subjects

As a next step, a specific physiological system was focused because of the need to balance between discovery mode (with issues of multiple testing corrections) and biological interpretation of data. Using Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 313398M) a list with 233 genes was constructed that are related to *"Skeletal and muscular system development and function"*. These genes map back to 409 probes and the fold change results are given in Figure 26. Fold changes (FC) of these probes were calculated per experimental conditions. FC > 1.5 means up regulation after bed rest; FC < -1.5 means down regulation after bed rest (R=0), which means a 50% increase/decrease compared to baseline data values (BDC – 5). All expression values with absolute values ≤ 1.5 or ≥ 1.5 are considered as debatable for biological interpretation.

After applying a threshold of FC = 1.5, 15 genes of the BR – group, 33 genes of BR + EX and 16 genes of the BR + EX + Diet group passed the cut – off criteria, which are listed in Figure 29 with their corresponding expression values.

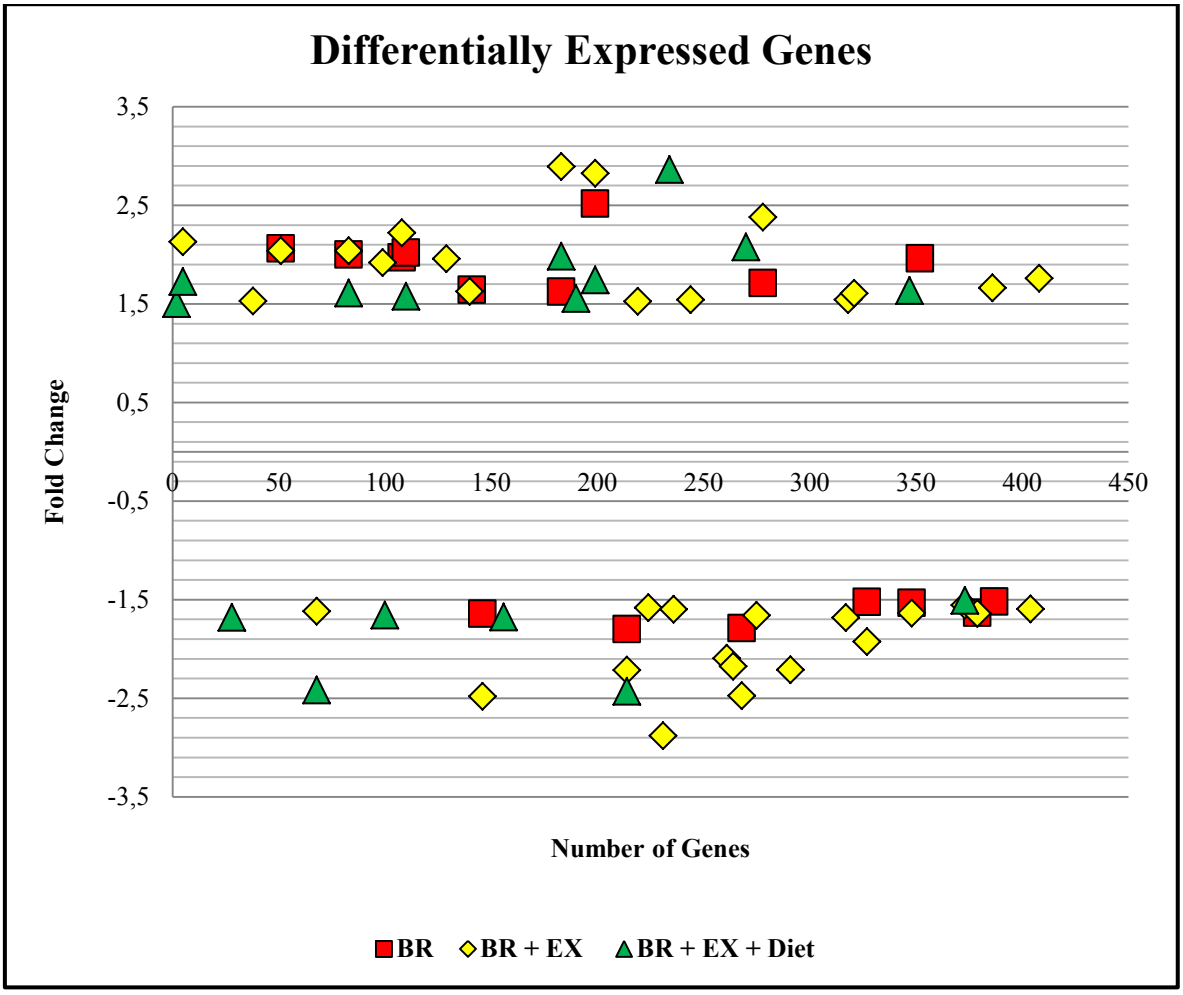

Figure 29 FC values of all differentially expressed genes in all 3 groups

However, we only focused on the effects of immobilization on bone metabolism. Hence, Figure 30 lists all 15 differentially expressed genes of the BR – group with their corresponding expression values.

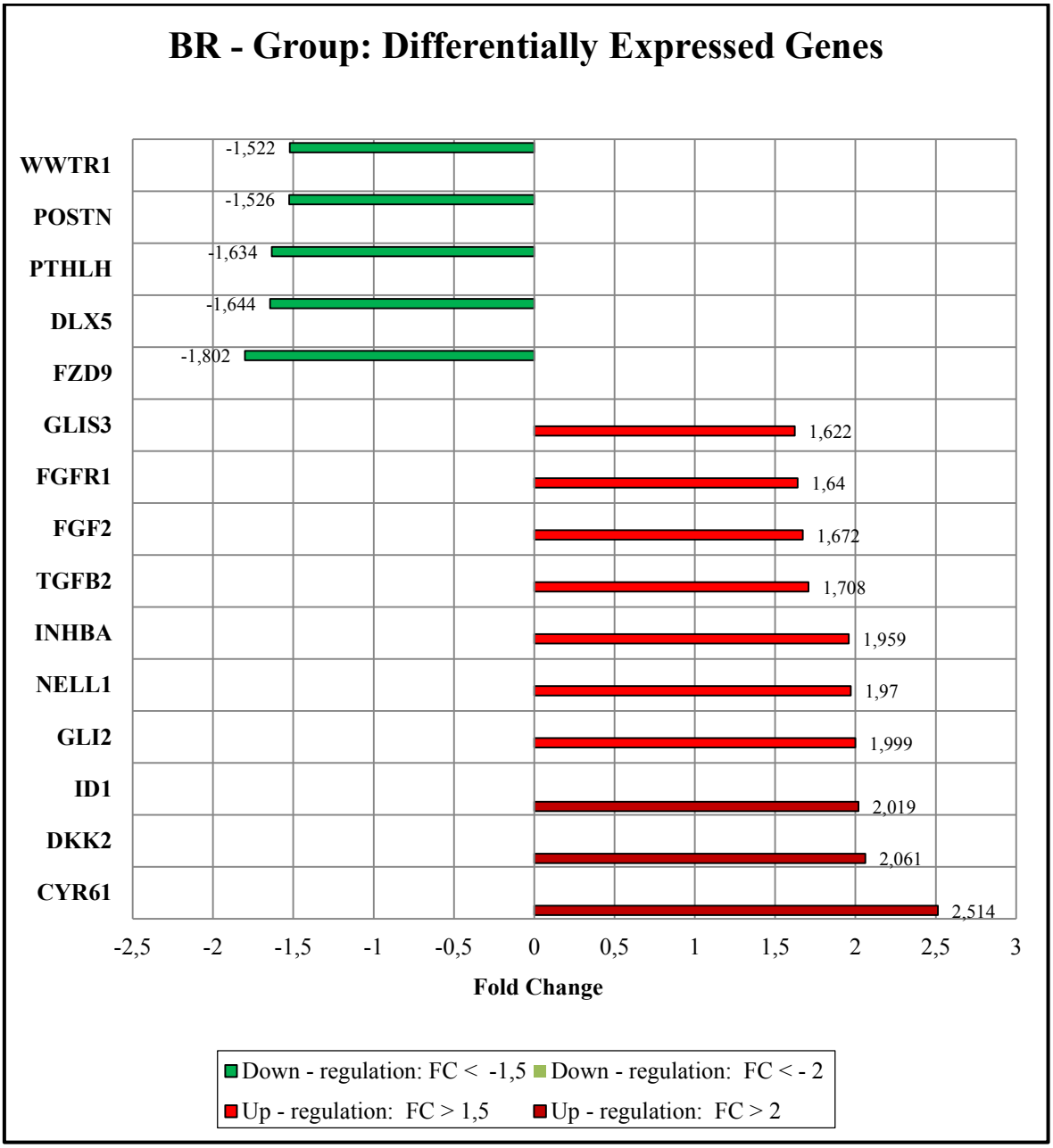

Figure 30 Differentially expressed genes in the BR - group

These 15 genes will be elaborated in detail under discussion. For a more comprehensive biological interpretation and understanding, we will compare these genes to those of the treatment groups. Figure 31 gives a schematic overview of the gene distribution in all 3 groups.

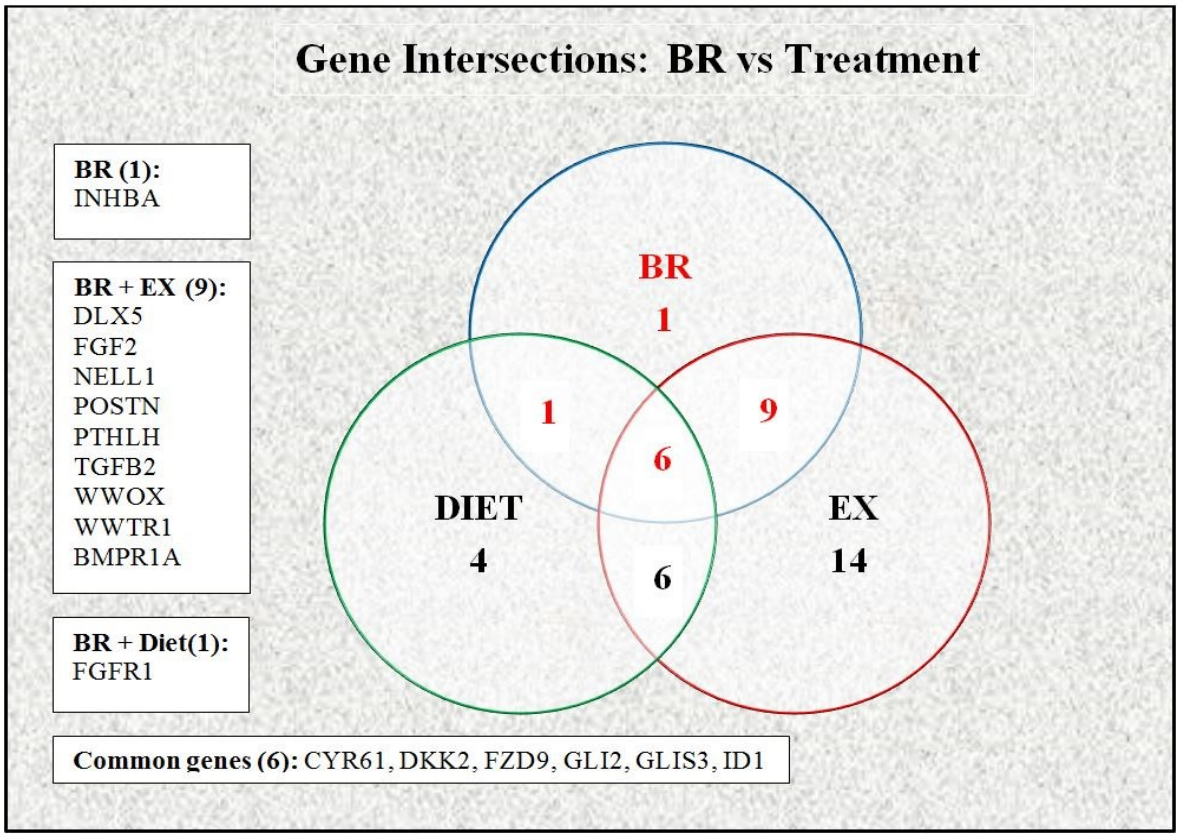

Figure 31: Gene distributions and overlapping of all 3 groups

We primarily placed special emphasis on those genes that were differentially expressed in each group. Secondarily, we categorized them into similar directional regulation patterns (all up - regulated or down – regulated) as shown in Figure 32.

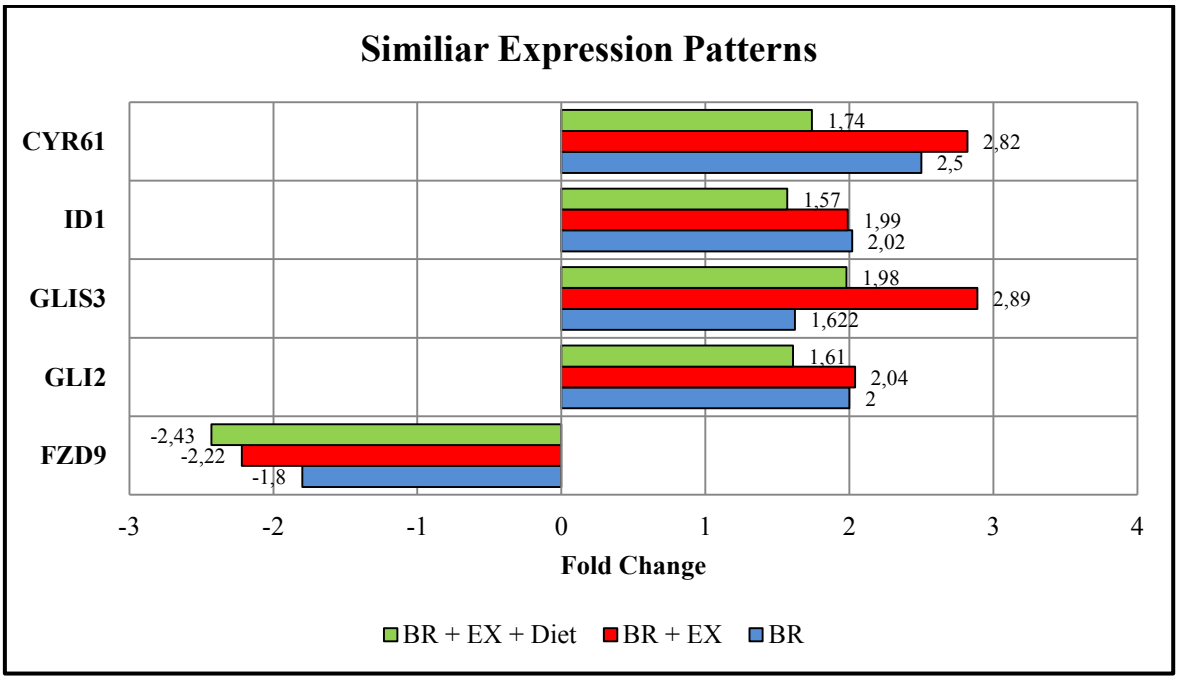

Figure 32 Common genes of all 3 groups with a similar regulation polarity

Furthermore, we focused on genes that were differentially expressed in the BR – group and were significantly counter regulated by treatments (EX, EX +Diet) as shown in Figure 33. This was important to identify the key factors involved in the control of mechanical skeletal adaption.

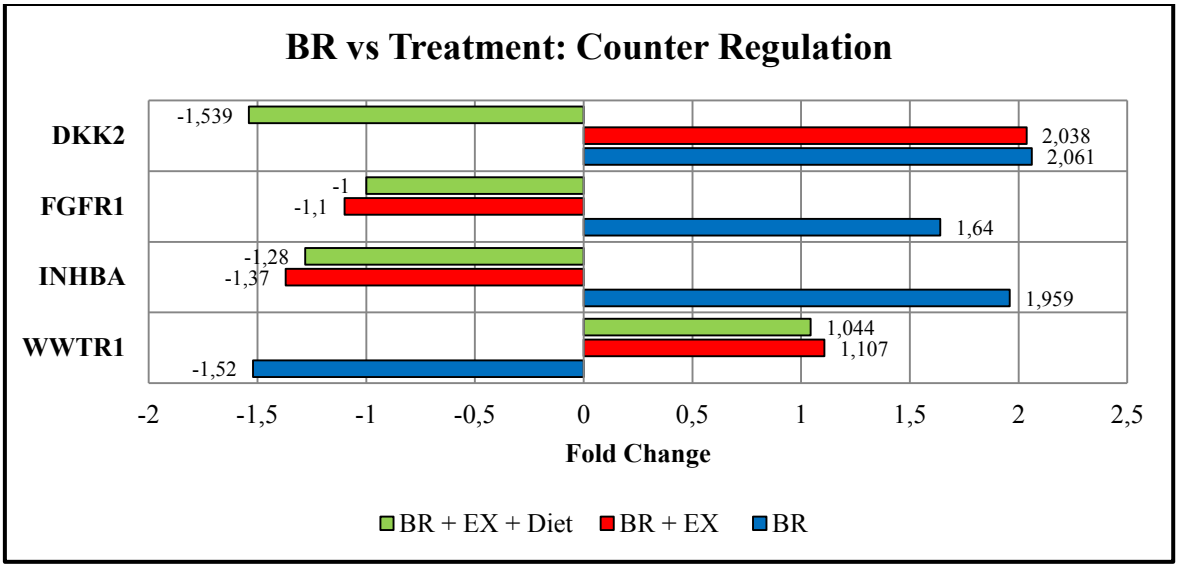

Figure 33 Counter regulated genes: BR compared to treatment

Figure 34 shows all genes that were differentially expressed in the BR and BR + EX group but have no significance in the BR + EX + Diet group (FC = 1,5).

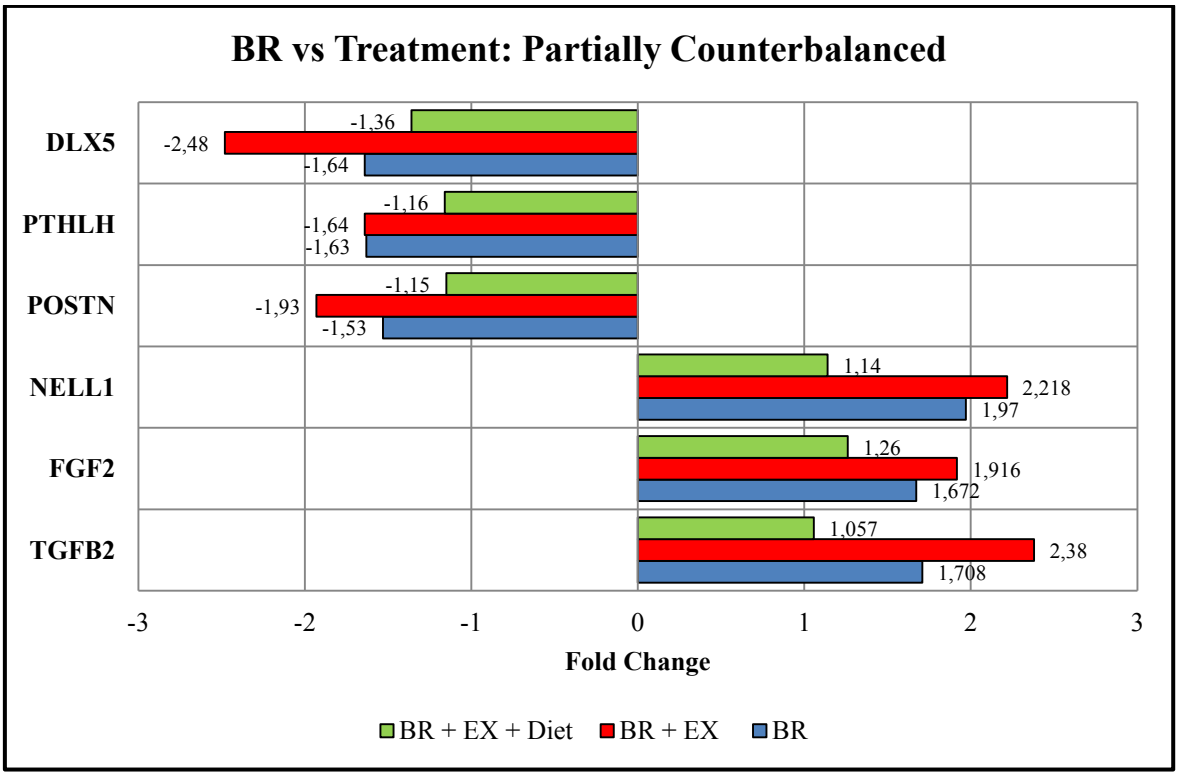

Figure 34 Partially counter regulated genes: BR compared to treatment

Figure 35 shows a biological interpretation of the 15 differentially expressed genes from the BR – group and their bone related functions ((http://www.ingenuity.com, application build 313398M).

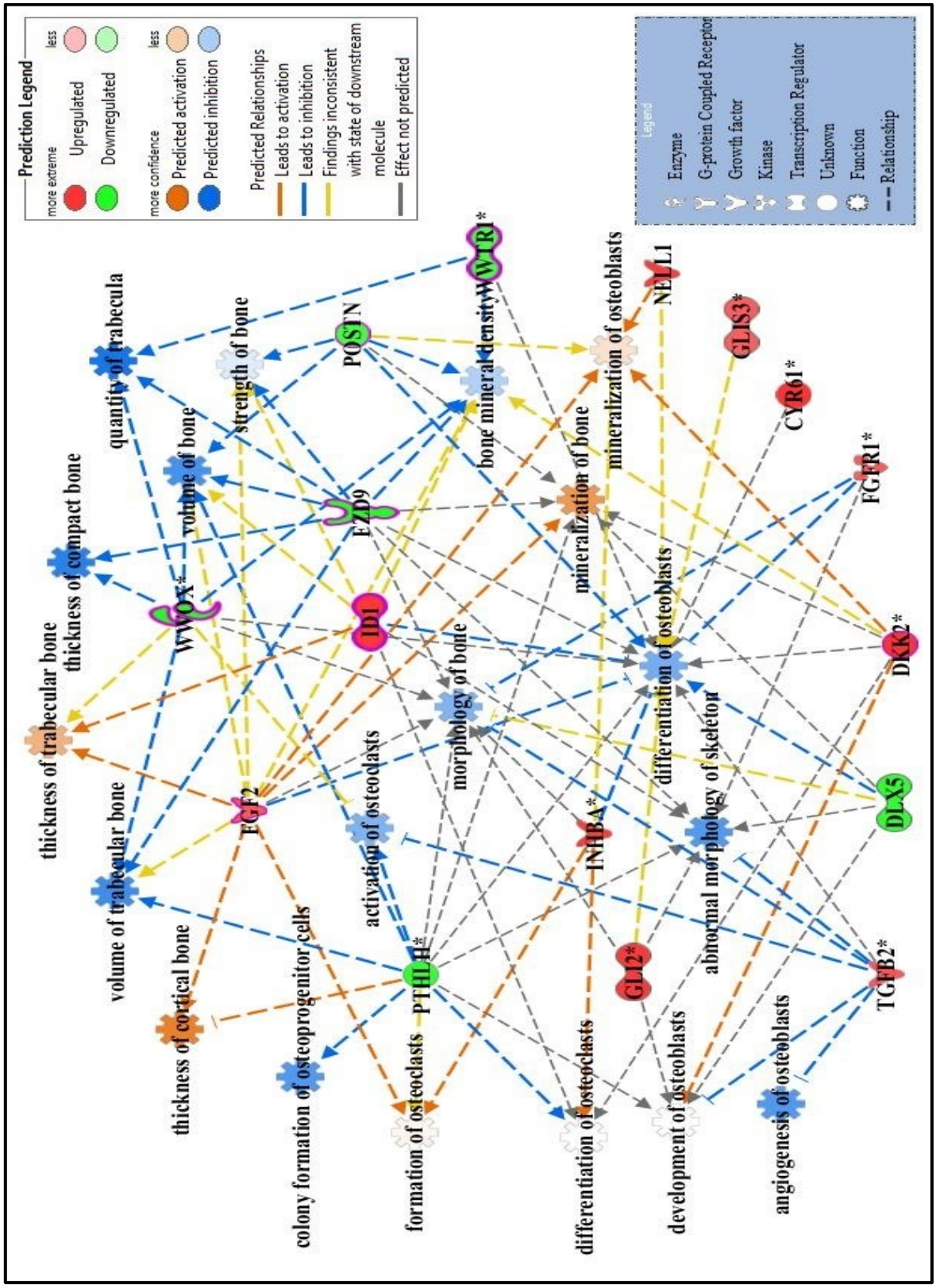

Figure 35 Pathway analysis: bone related diseases and functions (BR)

The *Upstream Analysis* option in Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 313398M) identified FGF2 as an activated upstream regulator of CYR61, DKK2, FGFR1, INHBA and TGFß2 in the BR group. Figure 36 shows direct downstream targets of FGF2 and the most significant related canonical pathways.

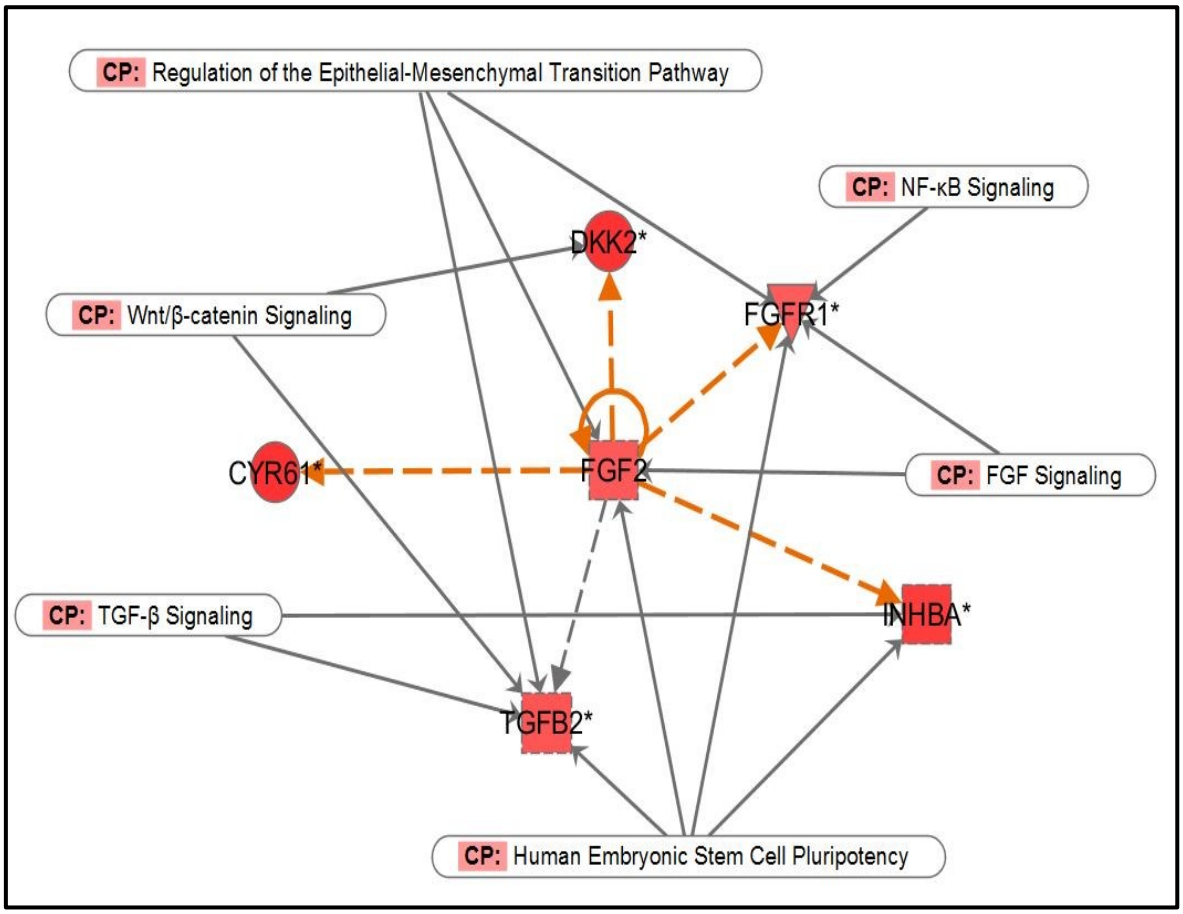

Figure 36 FGF2 as upstream regulator and associated canonical pathways (BR)

Using the *Networks analysis* option in Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 313398M) a biological network related to "Skeletal and Muscular System Development and Function", "Tissue Development" and "Cellular Development" was revealed (Figure 37).

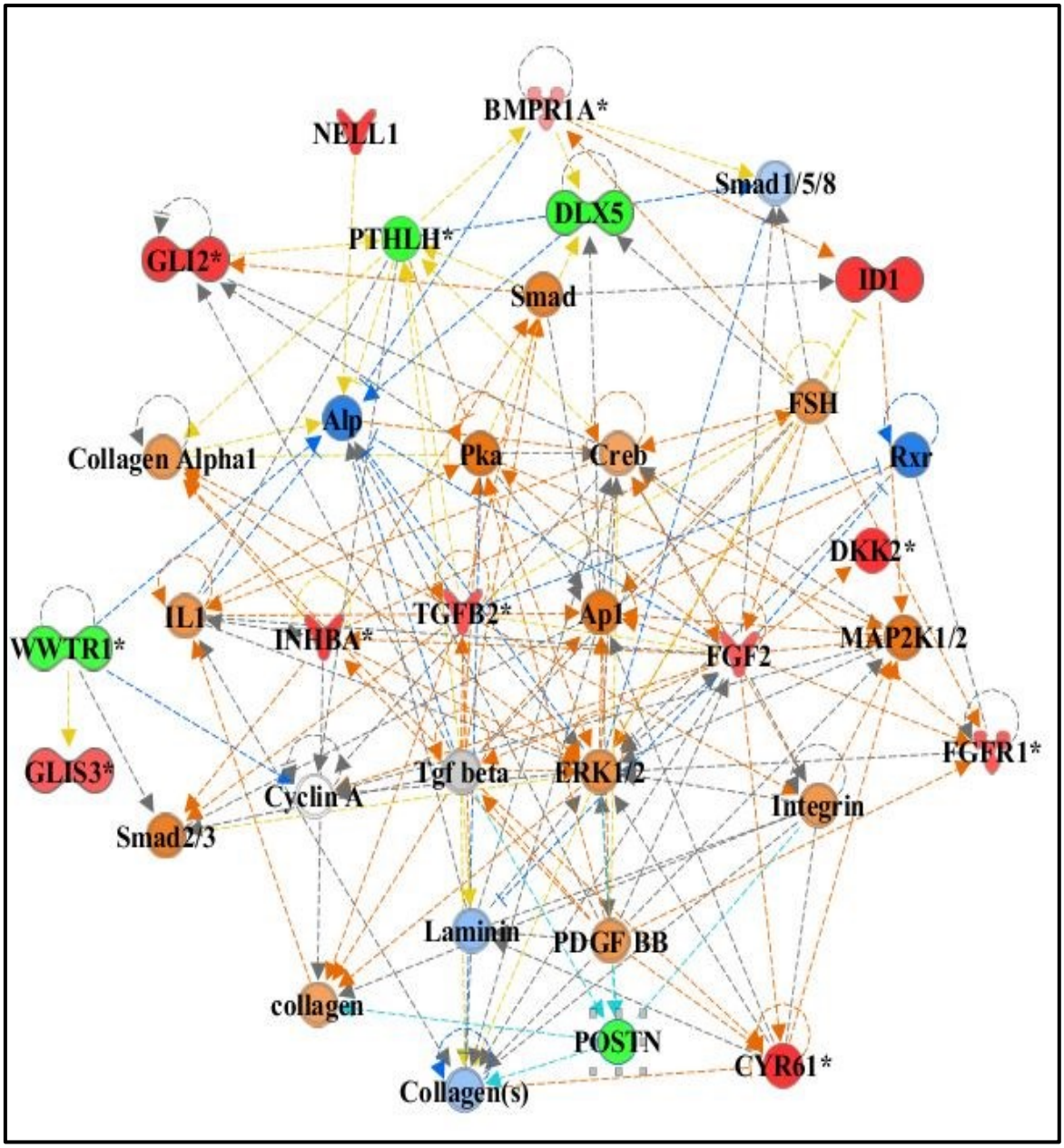

Figure 37 Biological network related to the differentially expressed genes

V DISCUSSION

Statistical analysis revealed 15 genes that were differentially expressed in the BR – group (Figure 30). For a better biological understanding and interpretation these genes will be discussed in detail based on their expression values and polarity compared to those of the treatment groups (BR + EX, BR + EX + Diet).

What we have to keep in mind is the fact that we are looking at expression values derived from two different time points: BDC -5 and R = 0 (Table 8). This data represents a snapshot of complex, molecular biological processes that have been initiated 21 days earlier and are highly dynamic and varying over time. This is a crucial point, as bone remodelling is tightly regulated in space as well as over time (see p. 14).

Moreover, the effect of a single gene is not only dependent on its expression level but also on its microenvironment. Thus, one gene could have inverse effects based on its concentration levels and time of expression.

1 DIFFERENTIALLY EXPRESSED GENES

1.1 Counter - Regulated Genes

4 genes in the BR - group were recognized to be counter regulated in at least one of the treatment groups. These genes include **FGFR1**, **DKK2**, **INHBA**, and **WWTR1** (Figure 33). Discussed below is what each of these genes represent as well as what it does.

1.1.1 FGFR1

Gene name: fibroblast growth factor receptor 1, transcript variant 1

Accession: mRNA [NM_023110]

"*The protein encoded by this gene is a member of the fibroblast growth factor receptor (FGFR) family… The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. It is involved in limb induction. Mutations in this gene have been associated with Pfeiffer syndrome, Jackson-Weiss syndrome,*

Antley-Bixler syndrome, osteoglophonic dysplasia, and autosomal dominant Kallmann syndrome" (102).

FGFR1 is up regulated in the BR – group, whereas it shows negative regulation pattern in both treatment groups. FGFR1 knockout mice have an increased bone mass and increased osteoblast proliferation, whereas osteoblast differentiation and mineralization are decreased. FGFR1 enhances the differentiation from mesenchymal progenitors to preosteoblasts, whereas the proliferation of mesenchymal progenitor cells is down regulated, as well as the maturation and mineralization of osteoblasts. Furthermore, FGFR1 is essential for a sufficient osteoclast formation and function (103).

The findings mentioned above are coherent with IPA analysis: prediction of reduced osteoblast differentiation, mineralization and low bone mass. Though, there is only a direct linkage to osteoblast differentiation in IPA analysis the predicted results are in confirmation with what has been reported from the literature (103). Further, FGFR1 leads to enhanced osteoclast activity which is also consistent with IPA results. Due to skeletal unloading, proliferation of mesenchymal progenitor cells is reduced; these results are consistent with what I obtained using the IPA.....

1.1.2 INHBA

Gene name: inhibin, beta A

Accession: mRNA [NM_002192]

"*The inhibin beta A subunit joins the alpha subunit to form a pituitary FSH secretion inhibitor…. Because expression in gonadal and various extragonadal tissues may vary several folds in a tissue-specific fashion, it is proposed that inhibin may be both a growth/differentiation factor and a hormone. Furthermore, the beta A subunit forms a homodimer, activin A, and also joins with a beta B subunit to form a heterodimer, activin AB, both of which stimulate FSH secretion"* (104).

INHBA is significantly up regulated in the BR group and down regulated in both treatment groups but showed no significance. INHBA is known to have a bimodal effect on bone metabolism. A constant exposure in vivo showed an anabolic and protective effect in mice (concerning BMD, bone volume and improved biomechanical properties). Whereas a cycling exposure of inhibin decreases bone turnover by suppressing osteoblast and osteoclast development plus decreasing the commitment to

the osteoblastic linage and later differentiation of osteoblasts (105). Moreover, the local stimulatory effects of BMPs, Activins and TGFßs are dominantly antagonized by INHBA. Since INHBA is a gonadal derived reproductive hormone it's significant up regulation suggests bone as a reproductive endocrine target organ.

Therefore, an up regulation in the BR group could indicate a protective mechanism by antagonizing local factors to prevent excessive bone loss due to a high bone turnover rate or an endocrine anabolic stimulus as a counter regulation against low mechanical stimuli. The latter one seems to make more sense because osteogenic factors (e.g. BMPs, Activin, TGFß) are known to be down regulated in lack of mechanical stimulation.

1.1.3 WWTR1

Gene name: WW domain containing transcription regulator 1, transcript variant 1

Accession: mRNA [NM_015472]

"*This gene encodes a protein that is expressed at high levels in cardiac and skeletal muscle. Mutations in this gene have been associated with a number of clinical disorders including Barth syndrome, dilated cardiomyopathy (DCM), hypertrophic DCM, endocardial fibroelastosis, and left ventricular noncompaction (LVNC)….***"** (106).

TAZ (WWTR1) is down regulated in the BR – group and up regulated in both treatment groups but showing no significance. TAZ has been shown to play an important role in mechantransduction and osteogenic differentiation in mesenchymal stem cells. Upon mechanical stimulation osteogenic differentiation markers are expressed (e.g. RUNX2, DLX 5, MSX2). Further, TAZ is involved in the commitment of MSCs to the osteogenic linage by stimulating the activities of RUNX2 and suppressing the activities of PPARγ (107). Therefore, a down regulation of TAZ results in a decreased osteoblast differentiation and enhances the commitment of mesenchymal stem cells to the adipogenic linage. Moreover, TAZ has been shown to be involved in WNT signaling, which underlines its importance as an osteogenic factor. These results are consistent with IPA findings and the down regulation of TAZ in the BR group could be explained because of the lack of mechanical loading. Moreover, DLX 5 as a downstream target of TAZ is also down regulated.

1.1.4 DKK 2

Gene Name: Dickkopf 2

Accession: mRNA [NM_014421]

"*This gene encodes a protein that is a member of the dickkopf family. The secreted protein contains two cysteine rich regions and is involved in embryonic development through its interactions with the Wnt signaling pathway. It can act as either an agonist or antagonist of Wnt/beta-catenin signaling, depending on the cellular context and the presence of the co-factor kremen 2. Activity of this protein is also modulated by binding to the Wnt co-receptor LDL-receptor related protein 6 (LRP6)* **"** (108).

DKK2 is up regulated in the BR and BR + EX group, whereas it shows a negative expression level in the BR + EX + Diet group. DKK2 is a WNT inhibitor by binding to LRP5/6 and Kremen. Unexpectedly, DKK2 knock out mice show osteopenic bones and exhibit defective mineralization, increased osteoid and reduced expression of osteoblastic genes. Furthermore, the lack of DKK2 results in up regulation of RANKL and increases osteoclastogenesis. These results might implicate a role of DKK2 in later stages of osteoblast differentiation, by enhancing osteoblast maturation and therefore bone mineralization, possibly favouring new laid matrix (109). Since WNT signaling is also involved in mechanical sensing and transduction, an up regulation of DKK2 in the BR group could be the result of skeletal unloading, impairing osteoblast differentiation and enhancing osteoclast differentiation plus activity. As bed rest leads to a negative bone net balance these findings seem to be reasonable at least for the BR group.

1.2 Common Genes with a similar Expression Polarity

We will now discuss those genes that are common in all 3 treatment groups and show a similar regulation pattern, namely: **CYR 61**, **GLI 2**, **GLIS 3**, **ID 1** and **FZD 9** (Figure 32).

1.2.1 CYR 61

Gene Name: Cysteine-rich, angiogenic inducer

Accession: mRNA [NM_001554]

"*The secreted protein encoded by this gene is growth factor-inducible and promotes the adhesion of endothelial cells. The encoded protein interacts with several integrins and with heparan sulfate proteoglycan. This protein also plays a role in cell proliferation, differentiation, angiogenesis, apoptosis, and extracellular matrix formation***"** (110).

CYR 61 is significantly up regulated in all 3 groups. CYR61 is associated with increased bone formation and also participates in bone repair (111). Additionally, human mesenchymal stem cells express CYR61 protein and mRNA during an early proliferative stage (112). CYR61 is significantly up regulated by FGF2 and modulates numerous signaling pathways; therefore it is a potential candidate in coordinating tissue repair and in bone regeneration. Moreover, it has been suggested that CYR61 expression plays a vital role in osteoblast differentiation by co - regulating Wnt3a signaling pathway (113), which might be established by Sclerostin via inhibiting CRY61 mediated fibroblast attachment and accordingly increasing osteoblast differentiation. At an early stage of Wnt3A stimulation Cyr61 is up regulated and gets back to basal levels at day 7 (114). CYR61 was also proven to be significantly up regulated due to mechanical strain (115) but in most cases it is co induced by FGF2, TGF-β, VEGF, GFG, PG, ATII and bioactive lipids (116).

Since CYR61 is highly up regulated upon mechanical stimulation, this option should be out of question. We can assume that in our case osteoblast differentiation is initiated. That would make sense since bone remodelling is probably in the reversal phase, presuming the induction of remodelling in the first days of supine position due in all 3 groups by the lack of gravitational loading.

1.2.2 GLI2

Gene name: GLI family zinc finger 2

Accession: mRNA [NM_005270]

"*This gene encodes a protein which belongs to the C2H2-type zinc finger protein subclass of the Gli family. Members of this subclass are characterized as transcription factors which bind DNA through zinc finger motifs…. GLI family zinc finger proteins are mediators of Sonic hedgehog (Shh) signaling…. The protein encoded by this gene localizes to the cytoplasm and activates patched Drosophila homolog (PTCH) gene*

expression.... The encoded protein is associated with several phenotypes- Greig cephalopolysyndactyly syndrome, Pallister-Hall syndrome, preaxial polydactyly type IV, postaxial polydactyly types A1 and B" (117).

The GLI 2 transcription factor is important for the activation of Indian hedgehog (Ihh) dependent osteoblast differentiation, therefore an upregulation of GLI2 should increase osteoblast differentiation (118). Ihh is a member of the Hedgehog family (Hh) which is involved in numerous developmental processes. The lack of GLI2 results in an elevated proliferation of chondrocytes and reduced resorption of mineralised cartilage and bone formation (119). New studies have shown that Ihh signaling has an inverse effect on MSCs: antiadipogenic and proosteogenic (120). As mechanical unloading inhibits the commitment of MSCs to the osteogenic lineage, upregulation of GLI2 could be a late respond to maintain a sufficient number of osteoblasts to keep up bone integrity.

1.2.3 GLIS3

Gene name: GLIS family zinc finger 3, transcript variant 1, mRNA

Accession: mRNA [NM_001042413]

"*This gene is a member of the GLI-similar zinc finger protein family and encodes a nuclear protein with five C2H2-type zinc finger domains. This protein functions as both a repressor and activator of transcription....*" (121).

Glis3 has been approved to induce osteoblast differentiation in multipotent mesenchymal stem cells (MSC) and is up regulated by FGF18, which is a potent activator of osteogenesis. However, there is also evidence that FGF18 is a direct target of GLIS3 by binding GliBS and therefore act as a possible regulator of osteogenesis. Moreover, it was recognized to act synergistically with bone morphogenetic protein 2 (BMP2) and Sonic Hedgehog signaling in osteoblast differentiation (122). It has also been shown the patients with Glis3 down regulation develop osteopenia (123).

GLIS3 is significantly up regulated in all 3 groups, which implies an enhancement of osteoblast differentiation from multipotent mesenchymal stem cells and supresses the adipogenetic linage of MSCs. Though, FGF18 shows only an expression value of 1,472 (cut off = 1,5) in the BR – group, FGF18 passes the cut off criteria in the treatment groups: BR + EX: 1,83 and BR + EX + Diet: 1,55. Therefore, osteoblast differentiation from multipotent mesenchymal stem (MSCs) cells should be induced in all 3 groups.

Again, this would make sense since the lack of mechanical stimulation leads to a decreased stimulation of the osteoblast lineage. It has also been shown the patients with Glis3 down regulation develop osteopenia (123).

1.2.4 ID1

Gene name: Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein, transcript variant 1,

Accession: mRNA [NM_002165]

"*The protein encoded by this gene is a helix-loop-helix (HLH) protein that can form heterodimers with members of the basic HLH family of transcription factors. The encoded protein has no DNA binding activity and therefore can inhibit the DNA binding and transcriptional activation ability of basic HLH proteins with which it interacts. This protein may play a role in cell growth, senescence, and differentiation….***"** (124).

ID1 is expressed in osteoblast progenitor cells and helps to maintain the pool of progenitor population. Further, genes which enhance differentiation of mature osteoblasts are inhibited. ID1 also contributes in the co – regulation of osteoclast related genes. Thus, ID1 knockout mice show an osteoporotic phenotype (125). In generally, ID proteins are considered as positive regulators of proliferation and as negative regulators of differentiation. This characteristic might function as a switch for lineage specification and makes sense concerning our data. Because of the negative effects of bed rest on proliferation and mobilization of the proosteogenic lineage, ID1 could help to maintain the pool of osteoprogenitors. Moreover, ID1 expression is induced by TGF – ß which is also up regulated in the BR group.

1.2.5 FZD9

Gene name: Frizzled family receptor 9

Accession: mRNA [NM_003508]

"*Members of the 'frizzled' gene family encode 7-transmembrane domain proteins that are receptors for Wnt signaling proteins. The FZD9 gene is located within the Williams syndrome common deletion region of chromosome 7 and heterozygous deletion of the*

FZD9 gene may contribute to the Williams syndrome phenotype. FZD9 is expressed predominantly in brain, testis, eye, skeletal muscle, and kidney" (126).

The frizzled family is one of the most important activator of the canonical WNT-signaling pathway. FZD9 knockout (KO) mice show a decreased matrix mineralization and low bone mass due to impaired bone formation, although the WNT canonical pathway was not affected. Further, FZD9 is known to be up regulated upon early stages of osteoblast differentiation and remains high for another 25 days. Osteoclast functions were not affected by FZD9 KO mice, which implicates an autonomous osteoblast defect. Moreover, the lack or down regulation of FZD9 seems to block the WNT - non – canonical pathway (127). FZD9 is the only gene that is down regulated in all 3 groups. According to its functions mentioned above, these findings are matching those of IPA analysis: impaired bone formation and therefore insufficient matrix mineralization, low bone mass and mineral density.

1.3 Partially Counter – Regulated Genes

6 genes show a differential expression pattern in the BR and BR + EX group but have no significance in the BR + EX + Diet group (FC = 1,5): **DLX 5**, **PTHLH**, **POSTN**, **NELL1**, **FGF2** and **TGFB2** (Figure 34).

1.3.1 DLX5

Gene name: Distal-less homeobox 5

Accession: mRNA [NM_005221]

"*This gene encodes a member of a homeobox transcription factor gene family similar to the Drosophila distal-less gene. The encoded protein may play a role in bone development and fracture healing. Mutation in this gene, which is located in a tail-to-tail configuration with another member of the family on the long arm of chromosome 7, may be associated with split-hand/split-foot malformation*" (128).

DLX5 is significantly down regulated in the BR and BR + EX group but shows no significance in the BR + EX + Diet group. DLX5 is a direct downstream target of RUNX2 and an osteogenic differentiation marker. Studies have shown an involvement of DLX5 in bone formation and development. Further, it seems to play a role in the commitment of mesenchymal stem cells to the osteogenic linage and differentiation of

mature osteoblast and therefore enhancing bone mineralization. Since DLX5 is down regulated in the BR – group the commitment of mesenchymal stem cells to the osteogenic cell lineage should be inhibited, thereby resulting in impaired osteoblast development, differentiation and consequently decreased mineralization.

1.3.2 PTHLH

Gene name: Parathyroid hormone-like hormone, transcript variant 1

Accession: mRNA [NM_198965]

"*The protein encoded by this gene is a member of the parathyroid hormone family. This hormone, via its receptor, PTHR1, regulates endochondral bone development and epithelial-mesenchymal interactions during the formation of the mammary glands and teeth. It is responsible for most cases of humoral hypercalcaemia of malignancy, and mutations in this gene are associated with brachydactyly type E2 (BDE2)….* **"** (129).

PTHLH is down regulated in all 3 groups but shows no significance in the BR + EX + Diet group. In contrast to PTH, PTHLH is ubiquitously produced and both proteins derive from two different genes. PTHLH is expressed in osteoblasts and osteocytes and contributes to bone specific functions. Besides that, PTHLH also controls the levels of ionized calcium in the blood circulation and extracellular fluids. Due to low levels of calcium PTHLH is secreted and upon binding to its corresponding receptor (PTH1-R) bone resorption is initiated releasing Ca^{++}, which in turn decreases PTHLH secretion in a direct negative feedback loop. A permanently increase of PTHLH causes high bone turnover with a loss of bone net balance, whereas intermittent levels have an anabolic effect. Since prolonged bed rest is known to increase calcium levels it seems to be reasonable that PTHLH expression levels are kept low to protect bone tissue from excessive resorption (18) due to immobilization.

1.3.3 POSTN

Gene name: Periostin, osteoblast specific factor, transcript variant 1

Accession: mRNA [NM_006475]

"*Enhances incorporation of BMP1 in the fibronectin matrix of connective tissues, and subsequent proteolytic activation of lysyl oxidase LOX. Induces cell attachment and*

spreading and plays a role in cell adhesion. May play a role in extracellular matrix mineralization" (130).

Periostin is down regulated in all 3 groups but only BR and BR + EX show significant expression values. Periostin is known to be involved in bone formation and mechanotransduction by enhancing osteoblast function and simultaneously inhibiting the expression of Sclerostin. Furthermore, it has been shown that physical loading leads to an increased expression of POSTN. KO mice show an impaired bone formation and mechanical loading is not sufficient to improve bone quality or quantity. In addition, Periostin plays an important role during collagen fibre synthesis and participates in the arrangement of the extracellular matrix due to mechanical stimuli (131). These findings are consistent with IPA results and make sense because of the lack of mechanical stimulation, at least for the BR group.

1.3.4 TGFB2

Gene name: Transforming growth factor, beta 2, transcript variant 2

Accession: mRNA [NM_003238]

"*This gene encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types by transducing their signal through combinations of transmembrane type I and type II receptors (TGFBR1 and TGFBR2) and their downstream effectors, the SMAD proteins....* " (132).

TGFß2 is significantly up regulated in the BR and BR + EX group but shows no significant expression levels in the BR + EX + Diet group. It is known to regulate the differentiation and function of both osteoblasts and osteoclasts from lineage recruitment to mature differentiation. TGFß2 plays an important role in bone formation by a coordinated activity of its downstream targets RUNX2 and SMADs. However, these genes show no significant expression values. Paracrine and autocrine stimulation of TGFß2 is essential for the maintenance, recruitment and proliferation of mesenchymal stem cells and their commitment to the osteoblastic cell lineage. Furthermore, TGFß2 is involved in matrix production and differentiation of osteoblasts while inhibiting the secretion of RANKL and thereby osteoclast formation (133),

whereas this function is inhibited in late differentiation stages. There is also a complex interplay between TGFß, WNT, PTH, FGF and BMP signaling. Which makes a reasonable appointment difficult based on the underlying data. A possible interpretation would be the increased commitment of MSCs towards the osteoblastic cell lineage, due to skeletal unloading to provide a large enough cell population because of a negative bone net balance and simultaneously inhibiting mineralization. Further, it is a direct downstream target of FGF2 which was recognized by IPA to be an activated upstream regulator.

1.3.5 FGF2

Gene name: Fibroblast growth factor 2 (basic)

Accession: mRNA [NM_002006]

"*The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members bind heparin and possess broad mitogenic and angiogenic activities. This protein has been implicated in diverse biological processes, such as limb and nervous system development, wound healing, and tumor growth....* **"** (134).

FGF2 is significantly up regulated in the BR and BR + EX group but shows no significance in the BR + EX + Diet group. Upon binding to their FGFR family of tyrosine kinase receptors (FGFR 1-3) FGF2 exerts its function. The effect on bone cells is attained indirectly by up regulating local factors such as TGF–ß, IGF–1 and VEGF. Mice with overexpressed FGF2 show a dwarfism phenotype. Down regulation leads to low bone mass, impaired formation and poorly mineralized bone matrix. FGF2 enhances differentiation of bone marrow stromal cells into osteoblasts and extends their survival (103). The literature findings mentioned above do match IPA findings, since osteoblast differentiation is down regulated and mobilization of mesenchymal stem cells up regulated. Together with TGFB2 this could be a late response due to mechanical unloading to balance the early effects of increased resorption and maintain bone mass by providing a large enough progenitor pool to maintain bone integrity.

1.3.6 NELL 1

Gene name: NEL-like 1, transcript variant 1

Accession: mRNA [NM_006157]

"*This gene encodes a cytoplasmic protein that contains epidermal growth factor (EGF)-like repeats. The encoded heterotrimeric protein may be involved in cell growth regulation and differentiation. A similar protein in rodents is involved in craniosynostosis…***"** (135).

NELL1 is significantly up regulated in the BR and BR + EX group but shows no significance in the BR + EX + Diet group. Nell1 has osteoinductive properties: overexpression promotes osteoblast differentiation and mineralization, whereas down regulation results in impaired osteoblastogenesis and low mineral density. It is a direct downstream target of RUNX2 and is inhibited by Osterix. Studies have shown that NELL1 is a potential and promising candidate in bone regeneration (136). Integrinß1 was revealed to be the first cell surface receptor of NELL1. Together with MAPK, HH and WNT signaling osteogenesis is induced, thereby enhancing preosteoblasts mineralization, attachment and antiosteoclastic effect. Moreover, NELL1 has an antiadipogenic effect which was associated with HH signaling (120). By decreasing the commitment of MSCs to the adipogenic cell lineage, more cells can be mobilized towards the osteogenic cell lineage. Mechanical unloading leads to an increased commitment of MSCs towards the adipogenic cell line. With regards to our data the findings mentioned above make sense. By antagonising the adipogenic linage more MSCs can be mobilized towards the osteogenic cell line. Thereby providing a large enough number of osteoprogenitors to maintain bone net balance as a reparation response to skeletal unloading.

2 LIMITATIONS

Since independent t – tests ($p \leq 0,05$) showed no statistical significance for any gene in none of the three study groups (BR, BR + EX, BR + EX + Diet) we focused on more specific biological interpretation using Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 313398M). Thereupon, 233 genes (mapped to 409 probes) that are related to *"Skeletal and muscular system development and function"* were investigated on the basis of fold change cut offs only ($FC \leq -1,5$ / $FC \geq 1,5$).

Based on these results some limitations of the study could be:

- Only one expression value for each gene per group, representing the averaged expression values of 3 individuals per gene and group
- Low number of study subjects
- Only 2 probes for each study subject
- Large inter-individual variability

As a consequence of the mentioned limitations and the restricted statistical power the results obtained and discussed in this thesis have to be considered as exploratory only. However, our approach has to be regarded as a novel one since no literature exists up to now investigating this subject. For a more detailed examination it would be necessary to compare data derived from various time points, especially at the beginning of the bed rest, when bone resorption is expected to set in. Moreover, it would be beneficial to compare data derived from each individual after completing the entire study (BR, BR + EX, BR + EX + Diet) acting as their own controls because of the high inter-individual variability.

3 CONCLUSIONS

It is well known that prolonged bed rest or skeletal unloading causes negative impacts on bone quality and quantity. However, it still remains challenging to capture early response changes in bone health. In this thesis we used microarray DNA analysis to investigate blood gene expression changes based on the blood transcriptome during 21 days of bed rest. Study subjects acted as their own control and results where compared prior (BDC -5) bed rest and just before standing up (R=0). We were able to observe differences in gene expression changes based on the type of intervention. Differentially expressed genes in the BR group have been reported to play an important role in bone metabolism, regeneration and also in mechanotransduction. Hence, we conclude that microarray analysis is an appropriate technique to investigate gene expression changes in the blood, due to physiological and pathophysiological alterations on a systemic, molecular level in very early stages.

Overall, we observed a trend towards decreased osteoblast differentiation and mineralization, whereas the differentiation, proliferation and maintenance of mesenchymal stem cells towards the osteogenic cell lineage was increased. This underlines the importance of mechanical loading to maintain and recruit a large enough number of mesenchymal stem cells to the osteogenic linage and simultaneously, decrease the number of stem cells available for the adipogenic linage to keep up bone integrity.

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