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EFFECTS OF MICRONUTRIENTS, BORON, SELENIUM AND VITAMIN D, ON OSTEOBLAST CELL DIFFERENTIATION AND MINERALISATION

EFFETTI DEI MICRONUTRIENTI BORO, SELENIO E VITAMINA D SUL DIFFERENZIAMENTO E MINERALIZZAZIONE DELLE CELLULE OSTEOBLASTICHE

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RIASSUNTO

Nutrienti, minerali e vitamine svolgono un ruolo vitale nel rimodellamento osseo. Il **boro** è un micronutriente essenziale degli organismi e svolge un ruolo importante nell'osteogenesi e nel mantenimento dell'osso; è benefico per la crescita e il mantenimento delle ossa attraverso il suo effetto sulla presenza o l'attività di osteoblasti e/o osteoclasti, oltre che attraverso il suo effetto sulla concentrazione di calcio osseo. Anche il calcitriolo (vitamina D_3) è in grado di ridurre rischi legati a malattie ossee, come l'osteoporosi. Le colture *in vitro* di osteoblasti umani hanno mostrato che il **calcitriolo** può migliorare il differenziamento e la mineralizzazione delle cellule. Gli effetti diretti del calcitriolo sugli osteoblasti comprendono il controllo della produzione di proteine della matrice extracellulare (osteocalcina, collagene di tipo I, osteopontina) e gli effetti sull'attività dell'enzima fosfatasi alcalina responsabile della deposizione di minerali. È stato inoltre dimostrato che un inadeguato apporto di **selenio** può modificare il metabolismo osseo e ritardare la crescita, aumentando il rischio di malattie ossee e diminuendo la densità minerale ossea (BMD).

A partire da queste evidenze, l'obiettivo di questo studio è stato quello di verificare come il boro possa svolgere un ruolo importante nella biomineralizzazione degli osteoblasti e il selenio possa regolare il turnover osseo. Per il nostro studio è stata utilizzata la linea cellulare di osteoblasti umani hFOB1.19, che rappresenta è un modello *in vitro* consolidato per lo studio del metabolismo osseo. È stato investigato come differenti concentrazioni dei due micronutrienti selezionati, boro e selenio, e la loro combinazione insieme alla vitamina D, influenzino la differenziazione e la mineralizzazione della matrice extracellulare e l'espressione dei geni coinvolti nelle varie fasi di sviluppo degli osteoblasti, dopo aver sottoposto le cellule ai diversi trattamenti per 7 giorni. Le cellule sono state trattate con le seguenti concentrazioni

di micronutrienti: B 10 ng / ml, B 100 ng / ml, SE 10 ng / ml, VD 100 nM da soli e in combinazione. Le prime analisi sono state volte a verificare gli effetti citotossici e la vitalità delle cellule dopo 24 e 48 ore di incubazione, utilizzando il test XTT. Successivamente sono state valutate le proprietà osteoinduttive attraverso colorazioni citochimiche (Alizarin Red and ALP staining) per la determinazione della deposizione di sali di calcio e dei livelli di fosfatasi alcalina.Mediante analisi in Real time PCR è stata valutata l'espressione di alcuni geni coinvolti negli stadi di proliferazione (*RUNX2*: fattore di trascrizione specifico per gli osteoblasti; *ALP*: fosfatasi alcalina; *TGFB1*: fattore di crescita trasformante B1), maturazione (*ITGB1*: integrina beta 1; *COL3A1*: collagene di tipo III; *SPP1*: osteopontina; *SPARC*: osteonectina) e mineralizzazione della matrice extracellulare *(BGN*: bioglicano; *DCN:* decorina). Complessivamente, i dati raccolti hanno mostrato come il boro e il selenio alle concentrazioni testate non siano tossici per le cellule. Il gruppo che ha ricevuto la vitamina D e il boro alla concentrazione più elevata ha mostrato un decremento significativo della vitalità cellulare che dopo 48 ore è diminuito ristabilendo un livello di proliferazione sempre inferiore alle cellule di controllo ma non statisticamente significativo. La sinergia tra boro, selenio e vitamina D è risultata evidente grazie alla maggiore capacità di stimolare il differenziamento in senso osteogenico rispetto all'azione esercitata dal singolo micronutriente, portando ad un aumento significativo nella capacità di deposizione dei noduli di calcio e a livelli maggiori della fosfatasi alcalina. Per ciò che riguarda i risultati dell'espressione genica, è emerso che il boro, rispetto al selenio è in grado di favorire la proliferazione e mineralizzazione in maniera più efficace se somministrato con il selenio e la vitamina D. Al contrario il selenio da solo sembra essere più efficace rispetto al boro.

INTRODUCTION

Bone tissue

Bone tissue is the main structural and supporting connective tissue of the body. The most important function of bone tissue is to be strong and to transmit forces to the bones without causing any breakage. As reported in the study conducted by Seeman and Delmas, the optimal characteristics of the bone tissue are determined by a good level of hardness, flexibility, and lightness [1]. To support and transmit loads efficiently, the bone must be rigid and capable of resisting deformation. The bone tissue, similar to all connectives, originates from the mesenchyme and is characterized by the presence of a mineralized matrix, which organic phase consists mainly of collagen (type I is 97% and the remaining 3% is type V collagen) and non-collagenous proteins. Focusing on the organic phase, among non-collagenous proteins, Biglycan (BGN) and Decorin (DCN) are the predominant proteoglycans expressed in the bone. BGN is expressed during cell proliferation and mineralization, whereas DCN is expressed continuously beginning with bone matrix deposition [2]. Osteocalcin (OCN) is expressed specifically by bone-forming osteoblasts and binds calcium and modulates its metabolism by mediating its association with hydroxyapatite (HA). Its serum concentration can be used as a biochemical marker of bone formation [3]. Noteworthy, among of glycoproteins in the bone matrix, is the “secreted protein acidic and rich in cysteine” SPARC, commonly known as Osteonectin. It is found in mineralized tissues and is highly expressed in bone osteoblasts. Osteonectin is an important calcium release regulator because it binds collagen and HA crystals, influencing collagen

mineralization during bone formation [4]. In addition, small Integrin-Binding Ligand N-Linked Glycoproteins (SIBLINGs) are a family of glycophosphoproteins that includes Bone Sialoprotein (BSP) and Osteopontin (OPN). These proteins are predominantly found in mature, mineralized tissues, such as dentin and bone [5]. BSP is a highly glycosylated non-collagenous phosphoprotein that is expressed at the beginning of hard connective tissue mineralization and plays an important role in the regulation of osteoblast differentiation and matrix mineralization in bone tissue [6]. OPN, similarly to BSP, is a key regulator of bone formation and mineralization, particularly bone turnover. It is abundant in osteoblasts, odontoblasts, and osteocytes. OPN regulates osteoclastogenesis and osteoclast activity in bone remodeling, thus contributing to bone formation and resorption [7].

The inorganic phase is instead formed by calcium and phosphate salts deposited in the form of hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$, calcium carbonate, $CaCO_3$ and other salts in smaller quantities. Furthermore, bone tissue represents the primary source of calcium ion for the metabolic needs of the whole organism. The cellular component of bone tissue consists of cell types, mainly osteoblasts, osteocytes and osteoclasts. These precisely represent the group of cells that synergistically guide the continuous process of remodeling, regeneration and repair of bone.

Osteoblast

Several cell lines originate from multipotent mesenchymal stem cells (MSC): osteoblasts, adipocytes and chondrocytes and they also have similar characteristics that suggest the presence of common precursors. The enrollment and differentiation of MSCs towards adipocytes or osteogenic cells depends on the activation of various signaling pathways and transcription factors. In particular, the existence of an inverse

correlation between adipogenesis and osteogenesis emerged with the induction of one cell line that occurs at the expense of the other (**Fig. 1**) [8].

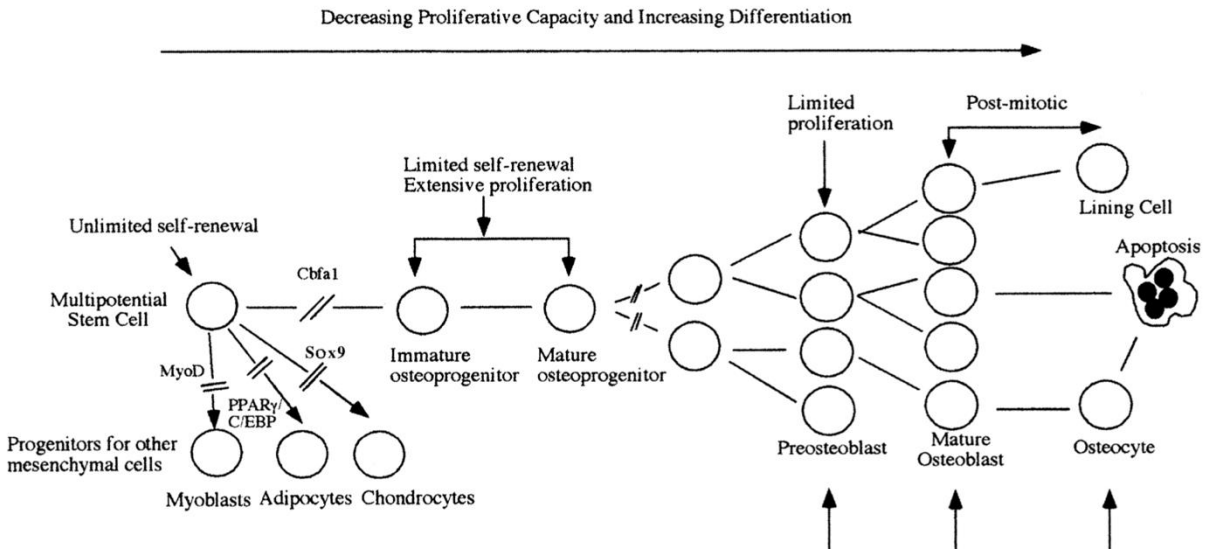

Fig.1 : Hypothesized stages of proliferation and differentiation along the osteoblastic line, proven by in vitro and in vivo studies[8]

The environment in where the cells are kept in culture, can condition the genetic pathway involved in the differentiation of this type of precursors[8]. The use of supplements, such as glucocorticoids and vitamin D in colonies that show markers typical of both cell lines, pivot the differentiation into osteoblasts, as well as the regression of mature adipocytes first into more proliferative precursors presenting fibroblastic phenotype, and later in cells with osteoblastic phenotype.

The main transcription factors able to orient MSCs towards adipocytes or osteoblast precursors are peroxisome proliferator receptor-γ (PPARγ) and RUNT-related-transcription factor2 (RUNX2). Heterozygous *Runx2* knockout mice had impaired calvarian osteogenesis, whereas homozygous knockout mice had no mineralization, leaving the entire skeleton cartilaginous. Because their ribcage could not produce air traction, the homozygous knockout animals died at birth. Osteoblast differentiation

appears to have been halted at an early stage, as osteocalcin was completely absent and osteopontin and alkaline phosphatase were barely detectable[9]. Further research revealed that *Runx2* plays an important role at two distinct times in the cell cycle: during the exit of pre-osteoblasts from the cell cycle (end of growth – start of matrix maturation) and during the late maturation stages of osteoblasts [10].

Osteoblast differentiation

It is becoming increasingly clear that osteoblast differentiation is a multistep series of events influenced by an integrated cascade of gene expression that promotes proliferation as well as the sequential expression of genes involved in the biosynthesis, organization and mineralization of the bone extracellular matrix. The osteoblast from primary cultures showed bone nodules with properties of bone tissue during histochemical and ultrastructural studies. Thus, a foundation was laid for studies that have mapped the transient expression of cell growth and tissue-specific genes during the progressive establishment of the osteoblast phenotype[11]. Over the last few years, studies using bone marrow-derived cells *in vivo* and *in vitro* have increased our understanding of the process by which an undifferentiated mesenchymal stem cell undergoes differentiation. These progenitor cells progress through the osteoblast lineage in a series of intermediate stages. Osteoblast differentiation occurs in 3 stages: in Stage 1, the cells continue to proliferate and express fibronectin, collagen, TGFβ receptor 1 and osteopontin. In Stage 2, they exit the cell cycle and start differentiating, while maturating the extracellular matrix with ALP and collagen. Matrix mineralization occurs in Stage 3 when the organic scaffold is enriched with osteocalcin, which promotes mineral substance deposition. In fact,

osteocalcin is the second most abundant protein in bone, after collagen. The osteoblast takes on its characteristic cuboidal shape at this stage [12].

The isolation of osteoblasts from transgenic animals allows for a comparison of transcriptional regulatory mechanisms at work during the development of bone tissue-like organization in culture with those at work in developing bone tissue *in vivo* [13].

Gene expression sequence during osteoblast development

The sequential expression of cell growth and tissue-specific genes has been mapped during the progressive development of the bone cell phenotype (**Fig. 2**) [14,15].

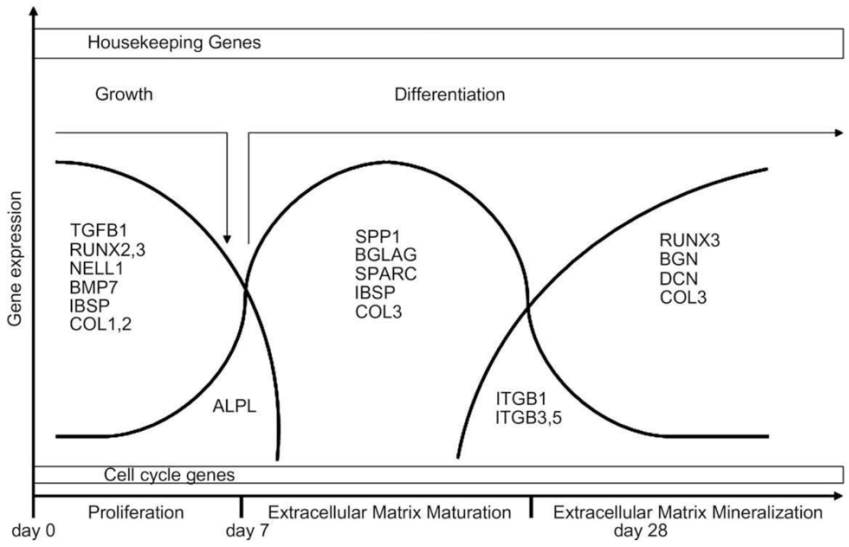

Fig 2: Osteoblast lineage cell growth and differentiation [15]

This temporal sequence of gene expression defines four major stages of development. Proliferation initially promotes the expansion of the osteoblast cell population and the biosynthesis of type I collagen bone extracellular matrix. At this time, genes required for proliferation activation are expressed alongside genes encoding cell adhesion proteins (fibronectin) and others involved in the regulation of

extracellular matrix biosynthesis (*TGF-β*, type I collagen) and its interaction with the cytoskeleton (integrins). Following the initial proliferation period, the expression of genes involved in the maturation and organization of the bone extracellular matrix is increased, contributing to the extracellular matrix's readiness for mineralization (alkaline phosphatase). The third developmental period is characterized by gene expression associated with the ordered deposition of HA. Osteopontin and osteocalcin exhibit maximal expression. When bone tissue-like organization is maturing, a fourth developmental period occurs in mature cultures, when collagenase and type I collagen gene expression are elevated, apoptotic activity occurs, and compensatory proliferative activity is visible [16]. Although the gene expression pattern of the fourth developmental stage has not yet been formally recognized, it mainly acts on extracellular matrix to maintain the tissue's structural and functional properties. During the first developmental period, all cells equivalently engage in pre-confluent proliferation and express genes that support cell cycle progression and extracellular matrix biosynthesis. But after the completion of proliferation phase, the genes which are expressed during maturation and mineralization are only observed in the developing bone tissue like nodules. However, post-proliferatively, expression of genes that support maturation, organization, and mineralization of the bone extracellular matrix are confined to the developing bone tissue-like nodules[11]. *In situ* hybridization analysis revealed that post-proliferative gene expression is restricted to osteoblasts within nodules at the single-cell level. There are specific requirements for proliferation to support the development of bone tissue-like organization throughout the sequential stages of osteoblast differentiation. Pre-confluent proliferation occurs alongside osteoblast extracellular matrix biosynthesis during the initial developmental period, resulting in a monolayer of cells associated with type I collagen. Post-confluent proliferation occurs at multiple foci and promotes cell multilayering in developing bone nodules. A third period of proliferation occurs

alongside collagenase-mediated apoptotic restructuring of the bone nodule [17]. There are developmental stage-specific regulatory requirements for each stage proliferation control. These are accompanied by developmental changes in the expression of early response genes [14,17], competency factors, and growth regulatory factors all of which contribute to cell cycle progression.

Development transition-restriction points mediate Proliferation-Differentiation interrelationships

There are a reciprocal and functionally coupled relationship between proliferation and differentiation as a sequential and stringently regulated expression of genes that defines periods of osteoblast phenotype development (**Fig. 3**)*:* Extracellular matrix formation suppresses expression of genes associated with proliferation, and extracellular matrix mineralization suppresses extracellular matrix maturation related genes. The presence of Fos-Jun and/or related proteins in *AP-1* sites in osteocalcin (*OC*) and alkaline phosphatase (*AP*) gene promoters is thought to suppress both basal and vitamin D-enhanced transcription of phenotypic genes in proliferating osteoblasts. Apoptosis occurs in mature osteoblasts and, along with increased collagenase activity, promotes remodeling of the developing bone extracellular matrix, which is required for the formation of bone tissue-like organization[18].

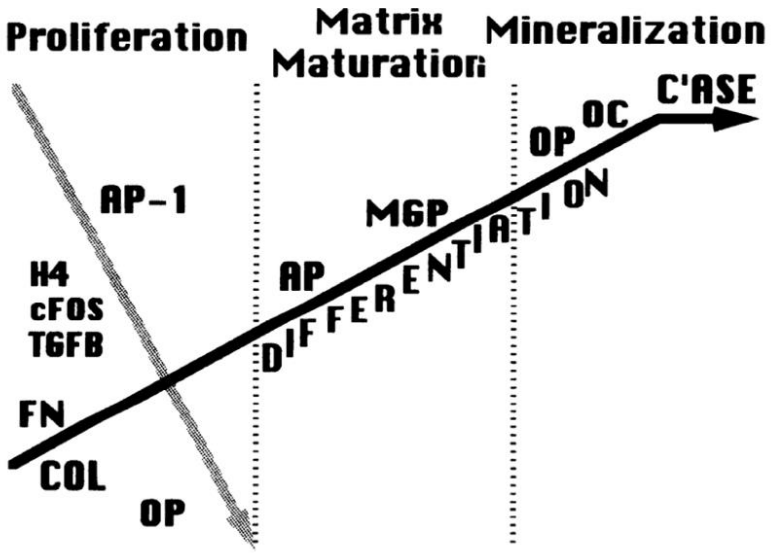

Fig. 3: Reciprocal and functionally coupled relationship between cell growth and differentiation-related gene expression in osteoblasts[11].

Two major points are critical components of this temporal expression of genes that promotes cell growth and differentiation. The first transition point occurs at the end of the proliferation period, when genes involved in cell cycle and cell growth control are downregulated and expression of genes encoding proteins involved in extracellular matrix maturation and organization begins. The second occurs when extracellular matrix mineralization begins. At all the transition points, the gene expression can proceed to the next phase only with additional cellular signaling [14].

The genes involved in extracellular matrix biosynthesis, maturation, or mineralization that are prematurely upregulated or delayed in expression initiation are then identified [14]. The growth-differentiation relationship can be explored further by conducting studies in which the onset and progression of differentiation are systematically altered, and the resulting changes in gene expression during proliferation as well as the influence of proliferative activity are observed[19–21]. Biochemical changes show that inhibiting proliferation promotes the expression of genes that are only expressed during the progression of the osteoblast developmental

sequence up to the stage where mineralization is initiated (e.g., alkaline phosphatase and osteopontin but not osteocalcin). There is also evidence that the expression of at least a second set of genes is not directly linked to the downregulation of proliferation, but rather to the development of the more differentiated osteoblast in a mineralized extracellular matrix. Without mineralization, osteopontin and osteocalcin are not induced to high levels, and alkaline phosphatase does not decline [14]. Bone cell phenotypic genes expression such as collagen, matrix GLA protein, and osteonectin are not affected [22].

Extracellular matrix mediated control of osteoblast proliferation and differentiation

The temporal pattern of expression suggests a working model for the relationship between growth and proliferation, in which genes involved in extracellular matrix production and deposition must be expressed during the proliferative period for differentiation to progress (**Fig. 4**).

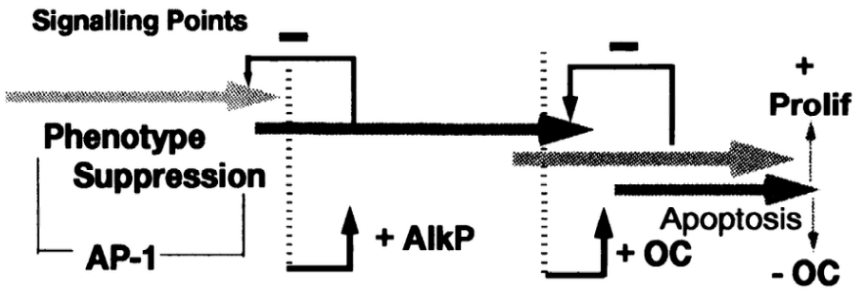

Fig. 4: A series of signaling mechanisms is schematically illustrated whereby proliferation period supports biosynthesis of a type I collagen extracellular matrix, which continues to mature and mineralize[11].

Proliferation may be functionally related to the synthesis of a bone-specific extracellular matrix. The maturation and organization of the extracellular matrix

contribute to the shutdown of proliferation, which then promotes the expression of genes that make the matrix capable of mineralization, a final process required for full expression of the mature osteoblast phenotype. The onset of extracellular matrix mineralization and/ or events during the mineralization period may be responsible for the downregulation of genes expressed during extracellular matrix maturation and organization. In this model, the development of an extracellular matrix is integrally related to the differentiation stages. This relationship is evident from a series of studies in which cells were cultured at various concentrations of ascorbic acid[19,23,24]. Proliferation stops at lower cell density due to increased collagen synthesis in extracellular matrix accumulation. Alkaline phosphatase mRNA levels and enzyme activity per cell are both increasing. Thus, extracellular matrix signals promote progressive differentiation in primary cultures of normal diploid rat osteoblasts, as has been demonstrated for other bone cell systems [24,25]. Vitamin D also was found to modulate the biosynthesis of collagen in a similar signaling mechanisms as that of the extra cellular matrix mediated ones [26]. Culturing primary diploid osteoblasts in type 1 collagen film or collagen gels is a complementary experimental approach for demonstrating the support and/or inductive effects of the collagen extracellular matrix in osteoblast differentiation [23,25,27].

Mature osteoblast

Mature osteoblasts have a size of around 20-30 µm, a rounded euchromatic nucleus with a large nucleolus, an abundant and basophilic cytoplasm and specific markers [28]. *In vivo*, however, the markers expressed by all osteoblasts are only two: alkaline phosphatase (*ALP*) and parathyroid hormone (*PTH)*. The other markers depend on the

state of bone maturation, the type of bone, the age of the osteoblasts and the microenvironment in which they reside;

Parathyroid hormone (PTH): is a physiological regulator of bone mineralization. When administered intermittently, it induces an increase in bone mass, if administered by continuous infusion it inhibits differentiation along the osteoblastic line. Despite this, further studies seem to show how PTH increases the life of osteoblasts by decreasing their apoptosis[8].

Alkaline phosphatase (*ALP*): is a metalloenzyme belonging to a class of cell surface proteins covalently linked to phosphatidyl inositol, phospholipid of cell membranes. It as a marker expressed by pre-osteoblasts in an early stage of differentiation, much earlier than mineralization and expression of non-collagenic matrix proteins [29]. The osteoblast, once the matrix has been synthesized, can face three possible fates: undergo cell death by apoptosis, differentiate into an osteocyte or become a bone lining cell [30] (**Fig. 5).**

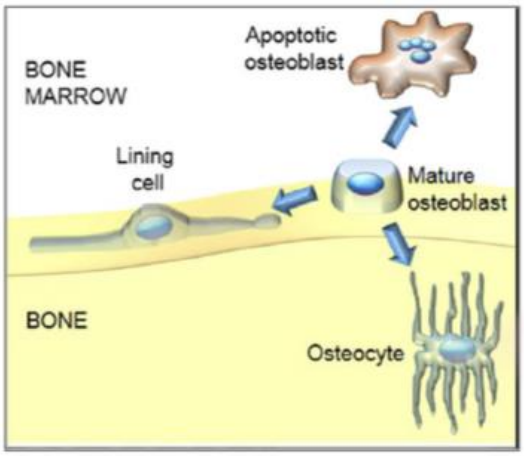

Fig. 5: Possible fate of mature osteoblasts [30].

Osteocytes

Osteocytes represent the most abundant cells in bone tissue (representing 90-95% of the total) are located within the bone matrix and derive from mesenchymal stem cells through the differentiation of osteoblasts [31]. About 15% of osteoblasts remain trapped in the bone matrix and become osteocytes, entering a state of quiescence. These cells are characterized by a flattened body from which up to fifty branched cytoplasmic extensions can form, lodged in the bone canaliculi within the mineralized matrix [32].

Osteocytes in in bone formation and resorption

Mature osteocytes present in the matrix secrete sclerostin, the product of the *SOST* gene. Sclerostin represents a potent inhibitor of bone formation. Genetic studies in mice have confirmed that this glycoprotein inhibits bone formation [33–35] by interacting directly with the *LRP5* / 6 co-receptor of *Wnt*, inhibiting the canonical pathway of *Wnt* [36,37] The loss of the expression of the *SOST* gene both in humans leads to an increase in bone mass, while the over-expression of *SOST* determines a reduction [34,38]. *SOST,* produced by osteocytes, represents the main negative regulator of osteoblastic differentiation and function. Osteocytes regulate the recruitment of osteoclast precursors and stimulate osteoclastogenesis. The programmed death of osteocytes determines an increase in *RANKL* secretion both by the osteocytes themselves and by the stromal cells and osteoblasts [31]. Osteocytes can also secrete osteoprotegerin (*OPG*), which by binding to *RANKL* inhibits osteoclastogenesis. *OPG* secretion is regulated by *Wnt* / β-catenin; mice with β-catenin deficiency in osteocytes have an osteoporotic picture due to an increase in the number of osteoclasts and resorption [39].

Osteoclasts

Osteoclasts are giant multinucleated cells, which are formed by the fusion of mononuclear precursors of the monocyte / macrophage family [40,41]. Macrophage colony stimulating factor (M-CSF) and activating receptor ligand NFkB (RANKL) are two necessary and sufficient factors to promote osteoclastogenesis. The activating receptor NFkB (RANK) ligand (RANKL) is essential for the formation of osteoclasts. It is expressed by osteoblasts, T lymphocytes and endothelial cells. The interaction of RANKL with its receptor amplifies the signal activated by M-CSF in the precursors, stimulating their differentiation towards the osteoclastic phenotype [42]. Osteoclasts are cells responsible for resorption of the bone matrix during the remodeling process [40].

Bone remodeling

Bone is a dynamic tissue constantly subjected to turnover thanks to the bone remodeling process. This process ensures the continuous replacement of aged bone with more mechanically competent newly formed bone. It is a multicellular event involving osteoclasts, osteoblasts, osteocytes and lining cells [43]. The coordination of these cells allows to maintain the bone shape and structure substantially unchanged throughout life, despite the repeated events of resorption and new formation to which the bone is subjected [44,45]. It has long been recognized that this coordination is

made possible by the organization of local bone remodeling teams, called “basic multicellular units” (BMUs) which are activated in a desynchronized manner at the bone surface [46]. There are three distinct and sequential phases of this process: activation, transition, and termination (**Fig. 6**).

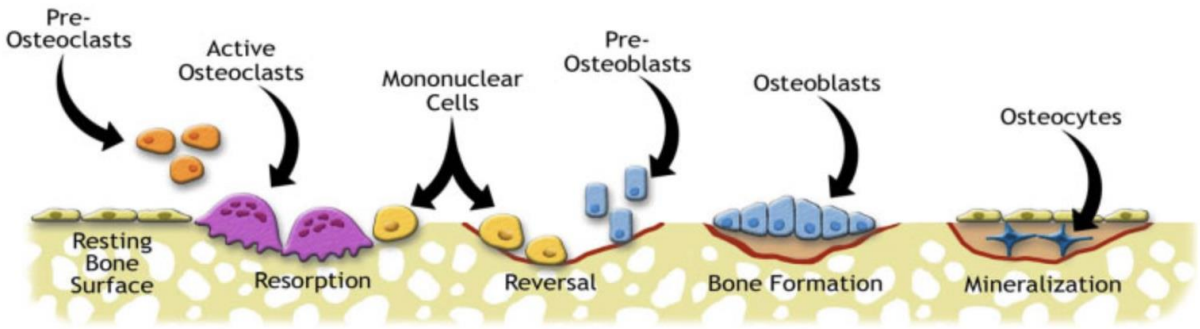

***Fig. 6: Process of bone remodeling*[47]**

Activation phase

In the first phase, monocytes, the precursors of osteoclasts, are attracted to the resorption site by various chemokines. Fundamental for the differentiation of monocytes in osteoclasts is the interaction between the RANK receptor present on the surface of monocytes and its RANKL ligand exposed on the cell membrane of osteoblasts. The interaction triggers the cascade activation of various transcription factors which stimulate the expression of osteoclastic differentiation genes. Osteoclasts degrading bone cause the release from the bone matrix of growth factors such as *TGF-β* (Transforming Growth Factor β), *BMP* (Bone Morphogenetic Protein) and *IGF-II* (Insulin-like Growth Factor II) [43].

Transition and termination phase

In this phase, we are witnessing at the cellular level the transition from resorption to bone formation, that is coupling. The factors released from the matrix, such as TGF-β, BMPs and IGF-II, stimulating the recruitment of pre-osteoblasts and promoting osteogenic differentiation by regulating the expression of important transcriptional factors such as RUNX-2, OSX, Dlx5. Osteoclasts produce clastokines to amplify the process and these stimulate the migration of the osteoblastic line towards the Howship lacuna and the proliferation of osteoblast precursors [48]. Another important factor in the transition between resorption and bone formation is the direct contact between osteoclasts and osteoblasts mediated by ephrins which have the ability to transduce the signal in a bidirectional way. Bidirectional signaling suppresses osteoclastic resorption and stimulates bone formation, facilitating the transition between these two states [49]. Bone formation involves the restoration of resorbed bone with a new osteoid matrix which is subsequently mineralized [50]. Finished bone formation, the osteocytes produce sclerostin (*SOST*) blocking the action of the osteoblasts that become bone lining cells [51]; [52].

Nutrients and bone tissue

Nutrients, minerals and vitamins play a vital role in remodel bone remodeling and are essential for maintaining bone strength. Numerous studies have demonstrated the effects of micronutrients on bone health (**Fig. 7**) and it is not difficult to understand why a dietary intake, alterations in absorption or excessive loss can negatively affect bone mass [53].

Micronutrient	Effect on bone health
Sodium	Excessive intake is a risk factor for osteoporosis
Phosphorus	Chronic, greater than calcium intake potentially linked with bone loss
Copper	Deficiency linked with cartilage and bone disorders
Zinc	Incompetence during growth reduces peak bone density
Magnesium	Improves bone quality
Manganese	Involved in bone metabolism
Vitamin K	Low levels associated with reduced bone density and increased risk of fractures
Vitamin C	Increased intake linked with increased bone density
Vitamin A	Hypervitaminosis A causes bone resorption and decrease in bone formation
Vitamin B12	Deficiency associated with reduced bone development and maintenance

Fig. 7: Micronutrients and their effect on bone health [53]

Few studies have focused previously on how micronutrients including boron, selenium and their combination with vitamin D can positively affect the activity of bone cells.

Focus on Boron

Boron (B) is an essential micronutrient of organisms and plays an important role in osteogenesis and the maintenance of bone. Many researchers investigated the biological impacts of boron including: transportation methods, cellular membrane roles [54] functions in cell-wall creation [55] osteogenesis and the maintenance of bone prevention of calcium loss and bone demineralization [56] . Deficiency of boron in cell cultures was found to induce the mitogen-activated protein kinase (MAPK) pathway [57] and this pathway plays a major role in skeletal development. Boron is not found alone, so it is found in conjunction with sodium or oxygen-containing organoboron complexes, which are the physiologically essential type of boron in organisms [58]. The majority of boron is found in the form of boric acid B $(OH)_3$; however, a small percentage of boron is found in the form of borate anion $B(OH)_4$ [59]. Experiments in rats revealed that boron deficiency increased plasma homocysteine and decreased S-adenosylmethionine (SAM-e), supporting the hypothesis that boron biological

capability is mediated by an effect on SAM-e formation [60]. Furthermore, a decrease in SAM-e has been observed in syndromes such as arthritis, osteoporosis, urolithiasis, and diabetes that are influenced by the nutritional utilization of boron [61]. Considering all these evidences from literature, it can be hypothesized that boron's bioactivity is expressed by fastening cyclic NAD+ and ADP ribose and suppressing calcium ion liberation, which is important in a variety of processes such as brain activity, bone formation, immune response, embryonic development, liver function, and preventing heavy metal influences (**Fig. 8).**

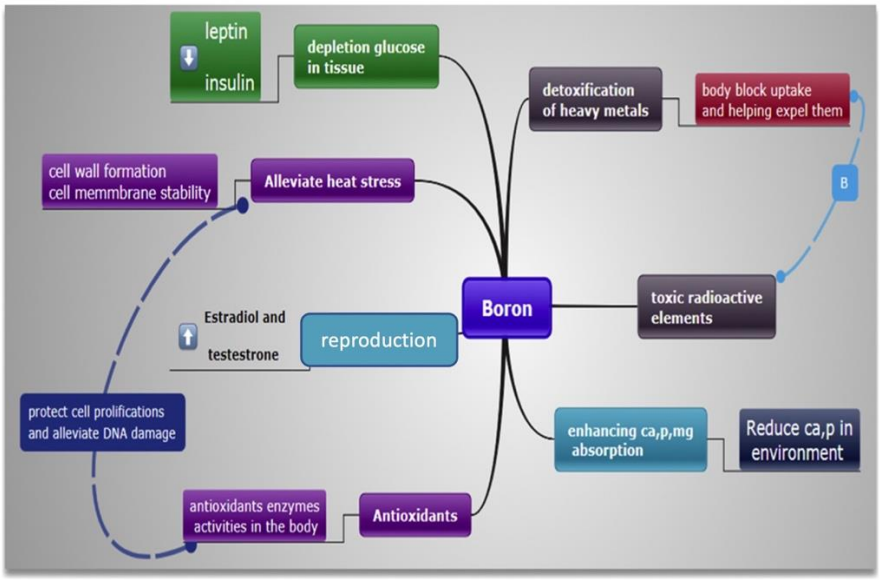

Fig 8: Functions and mode of action of boron in animals[62]

Results of many scientific reports support the positive benefits of boron in bone metabolism of animals. Boron plays an important function in the development [63], mineralization and proliferation of bones [56]. It is proposed that boron has an impassive impact on a variety of metabolic actions in bones due to its biological importance on metabolism and absorption of some other minerals. In recent years, the effect of boron on osteogenesis and bone maintenance has been investigated in vivo [64,65]. In animals, boron deficiency causes stunted growth and abnormal bone

development [66]. The mineral changes in bone, combined with the fact that its deprivation reduces the alveolar bone surface and activity of osteoblasts in mice, suggest that boron is beneficial to bone growth and maintenance via its effect on the presence or activity of osteoblasts and/or osteoclasts, rather than through its effect on bone calcium concentration [67]. Aside from its interactions with other nutrients, supplemental boron in the form of boric acid has been shown to improve bone structure and strength in rats [67].

A study demonstrated that boron is able to regulate turnover of the extracellular matrix and increases *TNF*-alpha release [68]. They studied the direct effect of boron on specific enzymes (trypsin-like enzymes, elastase, alkaline phosphatase and collagenase) implicated in extracellular matrix turnover and they discovered that boron may modulate these enzymes in fibroblasts, *in vitro* [69]. It was also found that boric acid solution improved wound healing through action on the extracellular matrix*, in vitro* [63]. Boron also interacts with other minerals and vitamins, such as calcium, magnesium, and vitamin D, as well as hormones that are important for bone formation, to play a role in bone metabolism. Bone morpho-genetic proteins (BMPs) are multi-functional growth factors belonging to the transforming growth factor b (TGFb) superfamily and promote new bone formation of both cartilage and bone. Vitamin D deficiency and boron supplementation to rats' feed improve calcium (Ca) and phosphorus (P) retention and absorption, as well as femur magnesium (Mg) levels [70]. In ostrich chicks, Cheng et al. added boron at various concentrations to the drinking water, to study the effects of those levels on the tibia [71]. Most variables increased significantly, and 0.2 g/L was found to be an effective level for improving bone strength. The improvement in bone strength after boron supplementation may be attributed to boron's effect on leptin and steroid hormone levels, which are important for bone metabolism. In pigs, boron supplementation showed beneficial effects on bone characteristics [72]. It is widely accepted that boron improves bone strength and

increases bone ash content in chickens [73]. The discovery that boron can partially correct leg abnormalities in Vitamin D-deficient chicks has increased the study of Boron essentiality in animal and human nutrition. Boron supplementation has also been shown to improve variables related to bone strength and microstructure of cortical and trabecular bone in diabetic animals [62]. Boron may also reduce bone abnormalities caused by diabetes, and administration may be beneficial to bone health even in non-disease situations [74]. Similarly, boron addition into cell culture at 1.0 ng/mL levels demonstrated positive influences. A boron deficiency can lead to osteoporosis; however, a proper level of boron leads to an increase in bone morphogenetic proteins [56]. Boron seems to be a very crucial partner in calcium metabolism and accordingly, it plays a fundamental function in the incidence of osteoporosis [75]. Based on what has been said so far, it can be considered that boron play an important role in the regulation of development of the skeleton, the preservation of bone health throughout life, and the metabolism of minerals such as calcium, magnesium, and vitamin D.

Focus on Vitamin D

Vitamin D is found in two forms: ergocalciferol or vitamin D_2, which is obtained through irradiation of plants, plant materials, or foods and vitamin D_3 or cholecalciferol, which is formed in the skin after exposure to sunlight or ultraviolet light. Calcitriol (1,25-dihydroxyvitamin D_3 ($(1,25(OH)_2D)$) is the active metabolite form of vitamin D produced by the two-step subsequent hydroxylation of cholecalciferol (vitamin D_3) in the liver and kidney. Calcitriol is responsible for calcium absorption from the gut and it enters the cell and binds to the vitamin D receptor [76]. This complex, together with the retinoid receptor, forms a heterodimer and binds to a vitamin D

responsive element on a responsive gene, such as osteocalcin, calcium binding protein, or 24-hydroxylase. This is followed by transcription and translation, which results in the formation of proteins such as the calcium binding protein or osteocalcin [76]. In the intestinal cell, the classic effect of calcitriol on active calcium transport occurs. Calcium enters cells via membrane proteins. When calcitriol binds to the vitamin D receptor in the intestinal cell, the calcium binding protein is synthesized, which regulates active transport through the cell. The calcium is transported to the extracellular fluid by an ATP- dependent mechanism [76]. *In vitro* cultures of human osteoblasts revealed that calcitriol has direct effects on bone forming cells (osteoblasts) to enhance differentiation and mineralization [77–79]. Osteoblast expect the Vitamin D receptor to enable this direct effect. Direct effects of calcitriol on osteoblast include the control of the production of extracellular matrix proteins (osteocalcin, collagen type I, osteopontin) and effects on the activity of the alkaline phosphatase enzyme to provide phosphate for the deposition of mineral [80]. Beneficial effects of vitamin D on mineralization and differentiation have been demonstrated *in vitro* studies using human mesenchymal stem cells, human osteoblasts and even induced pluripotent stem cells stimulated into the osteogenic lineage [77,78,81–84]. Mineralization defects in humans may be the result of vitamin D deficiency, leading to osteomalacia and an increased risk of fractures in the long run. Vitamin D supplementation increases bone mineral density, decreases bone turnover, and reduces fracture incidence. As reviewed by Lips and van Schoor, a relevant decrease in the incidence of fractures was proved by several randomized placebo- controlled trials using vitamin D, first with calcium and then without calcium [85].

The biphasic formation and deposition of HA crystals in the extracellular matrix is balanced by mineralization promoters and inhibitors. [86–88]. It was discovered that calcitriol upregulated *Enpp1* gene expression (encoding for NPP1 which is an inhibitor of mineralization) in mature murine MLO-A5 osteoblasts, resulting in mineral

incorporation suppression[89,90]. A previous study using human osteoblasts showed an increase in alkaline phosphatase in the extracellular vesicles following treatment with calcitriol, which resulted in improved mineralization by more production and deposition of HA crystals [79].(**Fig**. **9**)

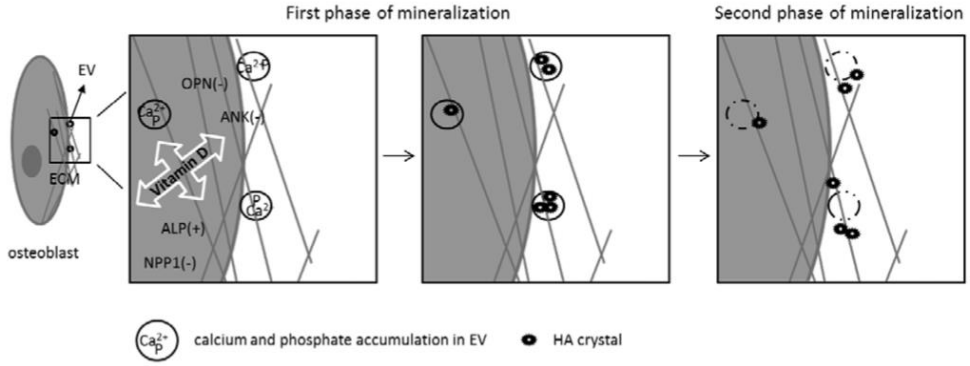

Fig. 9: Process of mineralization with promotors and inhibitors that are regulated by vitamin D [91].

This direct effect of vitamin D occurred in the period before mineralization and also caused an accelerated extra cellular matrix maturation [79]. After the start of mineralization, vitamin D treatment had no effect. Direct effects of calcitriol on human osteoblast activity depend on phase of differentiation of osteoblasts, as is also demonstrated in rat osteoblasts [92]. Direct effects of vitamin D on osteoblasts are caused by binding to the nuclear vitamin D receptor (VDR) [82,93]. Differences in VDR protein expression during osteoblast development could explain the osteoblast differentiation dependent effects of calcitriol. In mouse bone was found that the VDR is highly expressed in immature osteoblasts, instead in matured osteoblasts there is low or no expression, including lining cells and osteocytes [94]. VDR expression was also found in hypertrophic chondrocytes, and not in bone marrow stromal cells, chondroclasts and osteoclasts. Upon calcitriol binding, the nuclear VDR heterodimerizes with the Retinoid X Receptor (RXR). The dimeric VDR complex binds to genomic DNA through the regulatory regions of primary vitamin D target genes

[93,95]. The interaction of the VDR with *RUNX2*, a key transcription factor of osteoblast development, is critical for the effect of vitamin D on osteoblast differentiation and mineralization. In rat osteoblasts, this interaction has been shown to regulate the expression of the non-collagenous proteins osteocalcin and osteopontin [96,97]. Moreover, the calcitriol-activated VDR interacts with the *Wnt* signaling cascade to regulate osteoblast differentiation [98].

Focus on Selenium

The physiological functions of the essential micronutrient selenium (Se) are primarily mediated by a class of proteins containing selenocysteine (Sec) known as selenoproteins. The 25 selenoproteins found in humans are thioredoxin reductases (TrxR1, 2, 3), glutathione peroxidases (GPx1, 2, 3, 4, 6), iodothyronine deiodinases (Dio1, 2, 3), selenophosphate synthetase 2, 15 kDa selenoprotein, and other selenoproteins. Many of them act as a redox gatekeeper and play a fundamental role in maintaining cellular antioxidant homeostasis [99–101]. The co-translational incorporation of Sec, the 21st amino acid, into the growing polypeptide is a critical component of the selenoprotein biosynthesis machinery (**Fig. 10**).

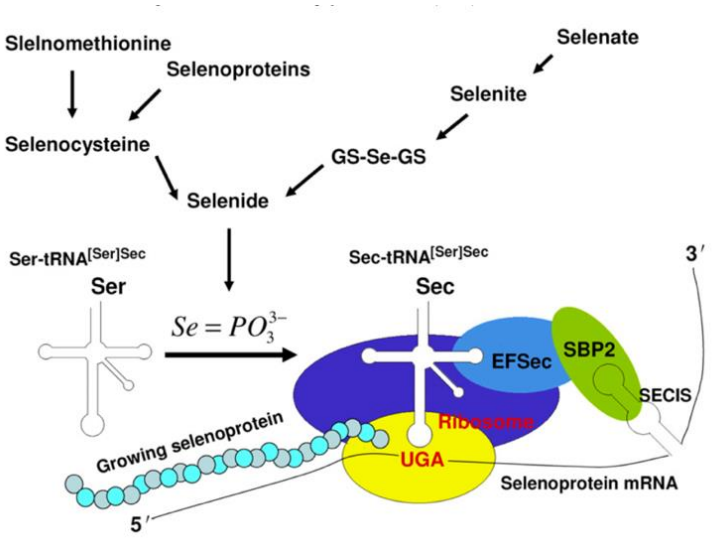

Fig. 10: Co-translational incorporation of Sec into selenoprotein occurs at the UGA codon [102].

Oxidative stress caused by an increased level of reactive oxygen species (ROS) in the bone system is detrimental to normal bone physiology because excessive amounts of ROS suppress osteoblastic differentiation and promote osteoclastic differentiation independently of NFB activation [103,104]. ROS stimulates RANKL expression in osteoblasts [105] and acts as a fundamental intracellular signal mediator for RANKL-stimulated osteoclastic differentiation [106,107]. During the extracellular signal-regulated kinase signaling in rabbit BMSCs and MC3T3-E1 pre-osteoblastic cells, ERK1/2 is known to mediate the inhibitory effect of H_2O_2 on osteoblastic differentiation [108]. The majority of known selenoprotein genes, as well as some important factors required for selenoprotein biosynthesis, have been discovered in osteoblasts and osteoclasts [109–111]. There are only a few studies of effects of Selenium on in bone cells, but in standard cell cultures, with a condition of selenium deficiency [112], supplementation of it in human fetal osteoblast cells (hFOB) restored GPx and TrxR activities [113]. Similarly, Selenium supplementation increased GPx and TrxR activities in primary bone marrow stromal cells (BMSCs) capable of differentiating into mesenchymal cells such as osteoblasts (**Fig. 11**), reducing intracellular ROS levels and DNA damage [110] and counteracting hydrogen peroxide-suppressed osteoblastic differentiation [114].

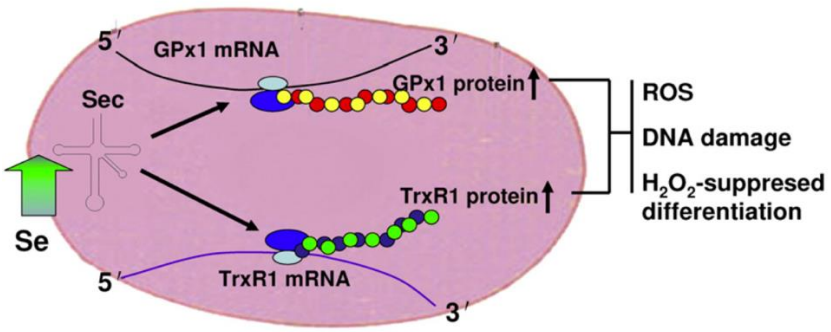

Fig. 11: Se supplementation increases GPx and TrxR activities, consequently reducing intracellular ROS levels and DNA damage and counteracting hydrogen peroxide-suppressed osteoblastic differentiation [102]

Thus it has been reported that Selenium inadequacy can change bone metabolism and retard growth [115]. The concentration of Selenium has been shown to be inversely related to the bone turnover rate correlated in a positive way with the prevalence of low bone mineral density (BMD) in humans [33]. Low Selenium intakes have been correlated with increased risk to bone disease [116]. Genetic data demonstrated that a single nucleotide polymorphism (SNP) at codon 198 of *GPX1*, is correlated with low bone mineral density (BMD), increased bone turnover markers [117] and Kaschin-Beck chondrodystrophy [118]. Animal studies have linked selenium deficiency to lower levels of pituitary growth hormone, plasma insulin-like growth factor I, and calcium, as well as higher levels of parathyroid hormone, 1,25-dihydroxyvitamin D_3, and urinary calcium concentrations; the fact that these changes were linked to lower BMD, bone volume, and impaired bone microarchitecture suggests that they were linked to increased bone resorption [116,119]. Downey *et al* demonstrated that the selenoproteins activity preservation in osteochondroprogenitors is needful to skeletogenesis and the maintenance of cartilage viability [120]. The study conducted by Zheng and collaborators showed that human umbilical cord mesenchymal stem cells (HUMSCs) showed increased mineralization following treatment with Selenium nanoparticles, which appear to be able to promote the deposition of calcium nodules [121]. Recent research has shown that treatment with nanomolar amounts of Selenium increases the expression of type I collagen, alkaline phosphatase, and calcium deposition in rat marrow stromal cells (MSCs), implying that selenium is capable of enhancing osteoblastic differentiation at cellular level [114].

In-vitro models to study bone formation

Cell culture has become an indispensable tool to help uncover fundamental biophysical and biomolecular mechanisms that govern how cells assemble into tissues and organs, how these tissues function, and how that function becomes disrupted in disease.

In vitro cell cultures are frequently used to advance understanding of the mechanisms that underlie cell behavior in vivo. Cell differentiation, migration, growth, and mechanics are all influenced by their biochemical and biomechanical microenvironment [122]. Deciphering the mechanisms behind these behaviors is vital to understanding in vivo processes that result in formation and function of tissues and organs. Two-dimensional (2D) cell cultures have been used as in vitro models to study cellular responses to stimulations from biophysical and biochemical cues for over a century. Although these approaches are widely accepted and have significantly advanced our understanding of cell behavior, growing evidence suggests that, in some cases, the 2D systems can result in cell bioactivities that deviate significantly the in vivo response.

Traditional 2D cell culture relies on adherence to a flat surface, typically a petri dish of glass or polystyrene, to provide mechanical support for the cells. Cell growth in 2D monolayers allows for access to a the same amount of nutrients and growth factors present in the medium, resulting in homogenous growth and proliferation [123]. This characteristic makes 2D platforms attractive to biologists and clinical users due to simplicity and efficiency. Most of these 2D methods, however, do not allow for control of cell shape, which determines biophysical cues affecting cell bioactivities in vivo. For example, Hepatocytes in the liver are surrounded by a complex ECM, and several researchers have shown that conventional 2D culture methods, flasks with or without

ECM protein coatings, do not establish well enough the complex microenvironment that hepatocytes need to function [124,125].

Several attempts have been made in recent years to develop model systems that represent bone metabolism and vasculature. It goes without saying that these processes cannot be demonstrated in traditional 2D monocultures. The organic (mostly collagen) and inorganic (mostly hydroxyapatite) matrix characteristics for bone are missing in conventional 2D cultures on cell culture plastic. However, this bone matrix primarily serves as a regulator of bone cell function and differentiation [126,127]. The *in vitro* models purpose must be clearly defined. Bone forming osteogenic cells and bone resorbing osteoclastic cells are essential when studying the biocompatibility of implant materials or bone metabolism. Endothelial cells and even hematopoietic cells have been shown to influence bone metabolism [128,129]. However, to obtain a sufficient number of osteoblasts and/or osteocytes for experiments, the cells must be cultured for several weeks to months, or the use of osteoprogenitor cells, such as MSCs derived from bone marrow or fat tissue, may speed up the expansion time but may lengthen the period of differentiation [130]. It should be noted that the cell line must be carefully chosen in order to best represent the primary counterpart, and even then, cell lines cannot represent the inter-individual differences that are characteristic of humans. It should also be noted that the bone is not an isolated organ, so a more straightforward approach would be to test the effect of not only native but also activated or metabolized substances on bone cultures. For example, the microenvironment of cells can be affected by the stiffness of the substrate that cells adhere to, which affects bioactivities ranging from migration to differentiation[131]. Engler et al. demonstrated that the differentiation of mesenchymal stem cells (MSCs), which possess the ability to develop into osteoblasts, can be regulated by substrate stiffness. MSCs were cultured on collagen I-coated, inert polyacrylamide gels, with the elasticity of the matrix determined by the degree to

which the gels were cross-linked and cells cultured on the stiffest matrix expressed fourfold higher osteogenic factor messages[132]. The majority of mechanistic studies use cell lines, which has significant limitations. The most commonly used cell line is MC3T3-E1. [56,133], which is a preosteoblast but is frequently used to represent osteoblasts and even osteocytes. Primary cells are also used, but they are much more difficult to obtain. Rat and mouse bone marrow stromal cells [134] and primary calvarian osteoblasts COB [135]are isolated. Human material is also used, ranging from fetal osteoblasts hFOB [136] to mature osteoblasts [137], both of which are commercially available. However, new culture methods are continuously appearing, and older methods are constantly evolving to address the current challenges in the research of cancer [138,139], stem cells [140,141], and many organs (such as liver, kidney, bone, etc.) [142,143].

hFOB1.19 cells

The availability of good model cell lines for studies of human osteoblast function, differentiation, hormone regulation, and mechanical stress, among other things, has been limited [144]. Human fetal osteoblastic cells (hFOB 1.19) have been conditionally immortalized with a gene encoding a temperature-sensitive mutant (tsA58) of the simian virus 40 (SV40) large T antigen. The temperature-sensitive gene is expressed, and the cells proliferates rapidly at permissive temperatures (37°C), whereas at restrictive temperatures (39.5°C), the gene is not expressed, and the cells proliferate less rapidly while expressing increased alkaline phosphatase activity and osteocalcin levels in comparison to cells cultured at permissive temperatures. Thus, temperature can control hFOB 1.19 proliferation and differentiation *in vitro* to some extent, providing a useful model for studying osteoblastic differentiation and extracellular

matrix mineralization [145]. According to one study, hFOB cells can generate bone-like tissue *in vivo*, secrete an ECM in vitro with ultrastructural elements similar to those deposited by primary osteoblasts *in vitro*, and do not transform over multiple passages *in vitro* or *in vivo* in mice, making them excellent laboratory models [144]. Recent studies have been conducted on the osteogenic differentiation process on human osteoblasts and on cells of other organisms. O'Gorman and co-workers evaluated the effect of Aquamin on osteoblast behaviour and mineralisation in the mouse calvarial pre-osteoblastic MC3T3-E1 cell line, proving that it directly influences the bone-forming cells [146]. Another research compared the quality of minerals in vesicles produced by two distinct human cell lines: fetal osteoblastic hFOB 1.19 and osteosarcoma Saos-2 to see if the origin of vesicles and their properties predetermine the onset of mineralization at the cellular level [147,148].

AIM

To date, further research is needed to clarify the exact process by which micronutrients and trace elements act on bone cells and mineralized matrix. In this regard, this project aims to establish whether treatment with different micronutrients can have a positive effect on osteogenesis in human osteoblast cells. Boron is known to play an important role in osteogenesis and bone maintenance, preventing calcium loss and inducing mineralization by regulating the expression of related genes in osteoblasts [62]. It has been reported that Selenium inadequacy can retard growth and change bone metabolism [119]. Vitamin D is also known to play a crucial role in promoting calcium absorption and preventing bone loss by reducing the risk of fractures and by stimulating the differentiation of progenitor cells [149]. Therefore, we aim to establish whether different treatments, namely boron, vitamin D and selenium, alone or in combination with each other, can affect the ossification and mineralization process of human osteoblasts. For this purpose, fetal human osteoblast cell line hFOB1.19 was selected among commercially available cell lines and a trial consisting of 9 treatment groups was set up, to verify the effects of treatments on mineralization. The Alizarin Red staining and Alkaline Phosphatase staining will provide macroscopic insights regarding the formation of calcium nodules and osteogenic differentiation respectively, while the expression of the genes involved in the proliferation, mineralization and maturation of the extra cellular matrix will clarify differences induced by the different treatments suggesting their role in osteogenesis process.

MATERIALS AND METHODS

Cell culture

The experiments were conducted using hFOB1.19 (ATCC CRL-11372) (**Fig. 12**). This cell line was cultured in 1:1 mixture of HAM'S F12 Medium Dulbecco's Modified Eagle's Medium (DMEM) with 2.5 mM L-glutamine (w/o phenol red) supplemented with 0.3 mg/ml geneticin (G418) and fetal serum bovine (FBS) to a final concentration of 10%. For proliferation cells were cultured in 75 cm^2 flasks at 34°C in a suitable incubator with humidified atmosphere of 5% CO_2. Once 90% confluence is reached, the cells were washed with 3ml of PBS (Phosphate Buffered Saline w/o calcium or magnesium), trypsinated with 3ml of Trypsin-EDTA solution to disperse the cell layer for 3 minutes and added 9ml of complete growth medium to inactivate the action of trypsin. Subsequently the cells were counted using a Neubauer cell counter before seeding in plates or to prepare flask subcultures (**Fig 12**). For the experiments the cells were seeded in complete medium (DMEM) at a density of $1x10^5$ cell/well in 12-well plates with 3 replicates of every treatment and incubated at 34°C in CO_2 at 5%. On the second day the medium was removed from each well and the different compounds of treatment (1ml / well) were added to the monolayer and the media with respective treatments were changed on the fifth day. The cells were kept in the different conditions for seven days and then sampled for performing various biochemical assays and the RNA was extracted in order to analyze the various gene expression profiles.

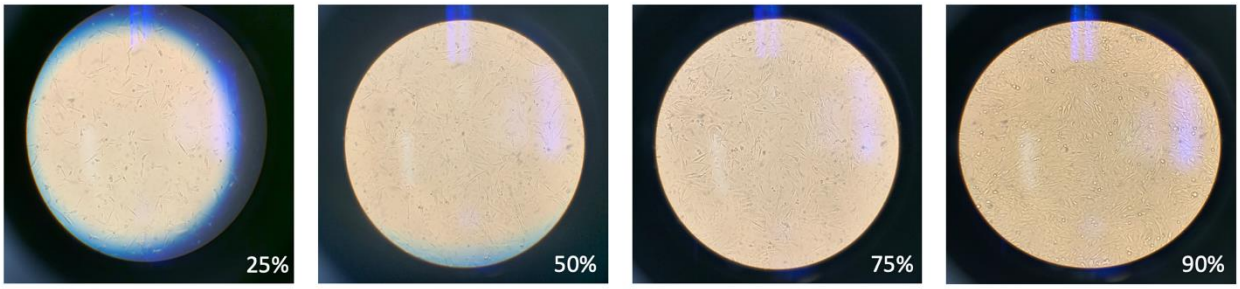

Fig. 12. hFOB1.19 cells at different stages of confluence, 25%, 50%, 75% and 90%.

Treatments

To verify the effects on cell mineralization and differentiation, Boron (B) in the form of boric acid, Selenium (Se) as sodium selenite, calcitriol or vitamin D_3 (VD) were tested in various concentrations and combinations. Boric acid, $B(OH)_3$ and sodium selenite, NA_2SO_3 were used to obtain Boron and Selenium concentration. They were prepared as filtered stock solution of 10 ug/ml to obtain the final concentrations to test, 50 ug / ml of vitamin D3 powder (Sigma, Milan, Italy) was dissolved in 4ml of ethanol. Compounds were tested on cells using the following concentrations: B 10 ng / mL, B 100 ng / mL, Se 10 ng / mL, VD 100 nM alone and in combination with each other for a total of 9 treatment groups, including vitamin D (**Table 1**). A Mineralization Mix (MM) (ascorbic acid 50 ug / ml + 7.5 mM β glycerophosphate) was added to all groups to enhance mineralization. All compounds of treatment have been prepared in 50 ml the basal medium of DMEM supplemented with 10% FBS and 0.3 mg / ml G418 (previously aliquoted to maintain sterility conditions) and stored in the fridge at 4 ° C. Before each use, all the groups were warmed in a water bath to 34 ° C

***Table 1**: Compositions of groups of treatment.*

NAME	CONCENTRATION
CONTROL	DMEM + FBS 10 % + 0.03 ug/ml G418
VD	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + 100 nM VD
B10	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + B10ng/ml
B100	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + B100ng/ml
B10VD	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + B10ng/ml + 100 nM VD
B100VD	DMEM + FBS 10 % + 0.03 ug/ml G418+ MM + B100ng/ml + 100 nM VD
Se	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + Se10ng/ml
SeB	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + Se10ng/ml + B10ng/ml
SeVD	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + Se10ng/ml + 100 nM VD
SeBVD	DMEM + FBS 10 % + 0.03 ug/ml G418 + MM + Se10ng/ml + B10ng/ml + 100 nM VD

Alizarin red staining

The ability to deposit calcium nodules was evaluated using Alizarin Red Staining. It is an anthraquinone dye capable of chelating calcium which allows both the microscopic visualization of the mineral deposits and their estimation. The protocol provides for the treatment of the cultures with the dye, the removal of its excess, the subsequent extraction of the complexed alizarin and its quantification by determining the absorbance at 550 nm. Cells were cultured in 12-well plates under the different processing conditions as previously described in the section “cell culture”. After 7 days, the medium was removed and the cells were washed 3 times with PBS and fixed in 4% (v / v) PFA for 30 minutes at 4 ° C (1 ml / well). The PFA was discarded, and the cells were washed 3 times with milliQ water. The cells thus fixed were stained with 40 mM alizarin red (pH 4.2) for 30 minutes in the dark at room temperature. pH of the

alizarin red was adjusted with 1% NH_4OH. Finally, monolayers were washed with milliQ water 4 times and were observed under Lionheart XF Automated Microscope (Biotek). Calcium nodules appeared in red. For the spectroscopic quantification of calcium deposits, the distilled water was removed and the cells dissolved in 1 ml of 10% cetylpyridinium chloride (CPC) for 30 minutes at room temperature. Finally, 250 ul of cells were taken from each well and transferred to a 96-well plate to measure absorbance at 550 nm. The recorded values were converted into dye concentration using a standard curve obtained by determining the absorbance of alizarin samples in CPC at known concentrations, starting from a 2 mM solution. Serial dilutions of Alizarin red in 10% CPC was made to obtain the following concentrations: 2, 1, 0.5, 0.25, 0.125 and 0.

Alkaline phosphatase staining

For the alkaline phosphatase (ALP) staining the cells were cultured in 12-well plates under the different processing conditions as previously described in the section “cell culture”. After 7 days, the medium was removed and the cells were washed 2 times with PBS (1 ml / well) to eliminate any non-adherent cells, and fixed in 4% PFA (v / v) (1 ml / well) for 30 minutes at 40 ° C. Subsequently the PFA was removed and the cells were washed 3 times with milliQ water (1 ml / well). The thus fixed cells were incubated with a staining mixture for ALP provided by the BCIP / NBT kit (Sigma Aldrich) for 45 minutes at 37 ° C in dark conditions following the kit protocol. After the established time, the dye was removed and mono layers washed twice and covered with milliQ water (1 ml / well) and observed under a microscope to verify the staining. Images were taken using Lionheart XF Automated Microscope (Biotek).

Image analysis using ImageJ

ImageJ software was used for the image analysis for both Alizarin Red staining and Alkaline Phosphatase staining; the scale was set at the beginning and maintained for all the pictures of each staining. RGB stack was selected to split the image into red, green and blue channels and green channel was chosen to analyze further. Then threshold was adjusted to not to have non-specific pixels stained and maintained the same for all the images analysed in the experiment. Intensity was measured for the thresholded range, and the data was further analysed statistically using R software.

XTT assay

The cell viability of hFOB1.19 under different treatments for two time points- 24 hours and 48 hours, was determined using the XTT assay kit (Abcam) following the protocol provided by the manufacturer. The assay is based on the extracellular reduction of XTT by NADH produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator. Reduction of XTT produces a water-soluble formazan which dissolves directly into the culture medium, eliminating the need for an additional solubilization step. For reagent preparation, equal volumes (600 ul) of XTT developer reagent and electron mediator solution were combined to make enough XTT mixture for the number of wells in the experiment (10 ul/well) and mixed well and stored until use (**Table 2**).

***Table 2**: Reagents and storage for XTT assay*

ITEM	QUANTITY
XTT Developer Reagent	600 ul
Electron Mediator Solution	600 ul

Cells were seeded in two 96 well plates at a density of 10^4 cells/well in 100 ul of culture medium with all 12 group of compounds to be test, and incubated in a CO_2 incubator set at 5% and 37°C for 24h and 48h. After 24h, 10 ul of the prepared XTT mixture were added to each well of the first plate and this one was incubated for 2 hours at 37°C and 5% of CO_2. Before measuring the absorbance using a microplate reader at 450 nm, the plate was mixed gently on an orbital shaker for one minute to ensure homogenous distribution of color. The same protocol was repeated for the second plate after 48h. The data obtained from the spectrophotometer was then statistically analysed using Graphpad Prism version 8.2.1 (279).

RNA extraction

For RNA extraction from the cells, RNAzol (Sigma Aldrich) was used which is a mixture of guanidine thiocynate and phenol in a monophase solution for use in the single-step isolation of total and small RNA from DNA, protein, polysaccharides and other molecules. Cells were cultured in 12-well plates as previously described in M&M. After 7 days of culture, the cells were detached and lysed directly into plates by removing the media and adding 100 µl of RNAzol to each well (vigorously pipetting to detach all adherent cells) and the contents of 3 wells for each treatment group were pooled in a 1.5 ml eppendorfs and stored at -20°C. Four replicates per group were sampled for extraction. 120 ul of RNase-free water was added to each sample, shaken

vigorously for 15 seconds, and allowed to stand for 10 minutes at RT. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 4°C (centrifugation separates the mixture in a semisolid pellet containing DNA protein and polysaccharides and an upper supernatant containing RNA). 300 ul of supernatant were transferred to new tubes (leaving a layer of the it above the pellet). Then 300 ul of 100 % isopropanol were added to precipitate RNA (mixing vigorously with the pipette to dissolve the RNA), and allowed to stand 10 minutes at RT. Subsequently samples were centrifuged at 12000 g for 10 minutes at 15°C. The RNA was observed as a pellet on the bottom of the tubes. Supernatant was discarded and RNA pellet was washed thrice with 150 ul of EtOH at 75% and centrifuged at 8000 g for 3 minutes at 15°C. Ethanol was completely removed with a syringe and dried RNA pellet was solubilized in 20 ul of RNase free water and stored at -80°C.

RNA quality and quantity measurement

A nanophotometer (Implen,Germany) was used to determine the purity and concentration of the RNA (**Fig. 13**).

Fig. 13: Spectrophotometer NanoPhotometer@ P-Class

The blank of the measurement was obtained by measuring at the spectrophotometer 1 ul of the same RNase-free water with which the RNA was eluted. The measurement of the samples was carried out using 1 ul of nucleic acid sample which was diluted in RNase-free water during the extraction step. The instrument allows to measure the absorbance (A) at 260 nm, the absorption wavelength characteristic of the aromatic heterocyclic rings of nucleic acids (**Fig. 14**). In addition, the absorbance is also measured at the following wavelengths: 230 nm for the absorption wavelength of carbohydrates and phenols and 280 nm for the wavelength of protein absorption. The concentration (ng / µL) of RNA calculated by the instrument and the various absorbance ratio estimates the degree of purity of RNA. Good RNA preparations must have the following ratios: A260 / A280 between 1.8 -2 and A260 / A230 between 1.8-2.2. Significantly different purity ratios may indicate the presence of DNA contamination, proteins, phenols or other strongly absorbing contaminants. Purity ratios that are significantly lower than expected values may indicate that the nucleic acid extraction and purification technique used was unsuccessful.

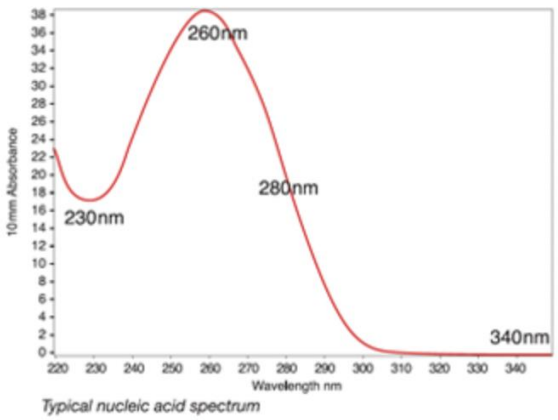

Fig.14: Typical spectrum of nucleic acids.

Gel Electrophoresis

The agarose gel was prepared with TAE 50X (tris-acetate-EDTA buffer) with the addition of GelRed, a non-carcinogenic intercalating agent alternative to ethidium bromide, which overlaps between the nitrogenous bases and is detectable by an ultraviolet lamp (UV). For each sample, mixes of 1 ul of RNA diluted in RNase free water, 2 ul of TAE blue and 9 ul of RNase free water were prepared and run at 400 A for 20 minutes. In this way it was possible to view the integrity of the 28s and 18s RNA bands on gel which further indicates the RNA quality in addition to the results observed in the spectrophotometer (**Fig 15**).

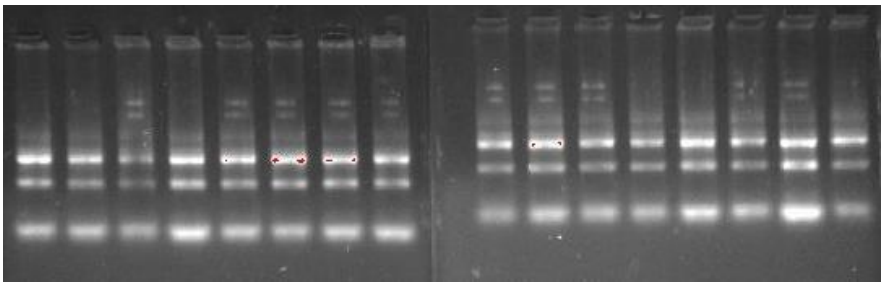

Fig. 15: Results of electrophoresis run of treatment groups.

Reverse transcription

The reverse transcription consists of the synthesis of a molecule of complementary DNA, called cDNA from the RNA. Total RNA (also containing ribosomal RNA) is incubated with:

- An RNA-dependent DNA polymerase enzyme, commonly called reverse transcriptase or retro-transcriptase.
- Deoxyribonucleotides triphosphate (dNTPs).
- A buffer solution.
- Divalent Mg^{2+} ions, which act as cofactors for the polymerase.

- Primer sequences which act as a primer by pairing in a complementary way to the RNA strand.

The most commonly used primers are oligos (dT), which are thymidine oligonucleotide sequences designed to pair with the polyadenylated tail of RNAs. If, on the other hand, you want to amplify a sample of RNA without the polyadenosine tail, the most suitable primers are the "random hexamers" random sequences of 6 or 9 bases which, being just random, can potentially act as a primer for any RNA template sequence. The primer provides a free 3'-OH that can be used by the reverse transcriptase to generate a DNA strand complementary to the transcript. Once the first strand is synthesized, before proceeding with the amplification of the cDNA, the RNAse enzyme is added to the reaction which degrades the original RNA strand which was used as a template by

reverse transcriptase (**Fig. 16**). When reverse transcription is complete, the generated cDNA is amplified by a standard PCR method.

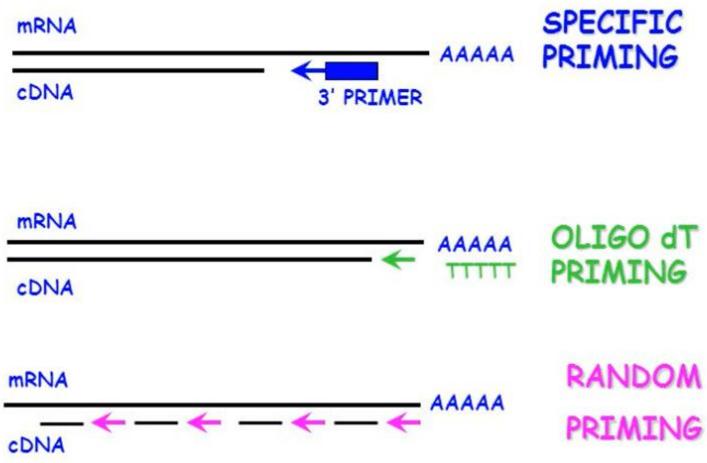

Fig. 16: Reverse transcription reaction leading to the formation of a cDNA-RNA hybrid compound.

In this study, the reverse transcription was carried out using the Iscript cDNA synthesis kit (Bio-Rad), in compliance with the experimental conditions recommended by the manufacturer. Therefore 1 ug of total RNA was back-transcribed as per protocol. The master mix was prepared using all the components of the kit (**Table 3**).

***Table 3**: Components details of Iscript cDNA synthesis kit.*

Component	Volume per Reaction, µl
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA template (100 fg–1 µg total RNA)*	Variable
Total volume	**20**

The kit chosen provides a final volume of 20 ul where the quantity of 5 ul is fixed and consists of iScript reaction mix (4 ul) and iScript reverse transcriptase (1 ul), while the remaining 15 ul are composed of nuclease-free water and RNA, calculating the respective volumes based on the concentrations of each individual RNA sample. Below is the protocol used by the thermal cycler characterized by 4 different steps (**Table 4**). Once the reverse transcription was completed, the cDNA samples diluted 1:10 were prepared and stored at -20 ° until they were used in RT-PCR.

***Table 4**: Four step protocol used in the thermal cycler*

Reaction Protocol

Incubate the complete reaction mix in a thermal cycler using the following protocol:

Priming	5 min at 25ºC
Reverse transcription	20 min at 46ºC
RT inactivation	1 min at 95ºC
Optional step	Hold at 4ºC

Real time PCR

PCR (Polymerase Chain Reaction) was used to produce many copies of target DNA fragments by repeating cycles consisting of three distinct phases:

- Thermal denaturation of the mold
- Pairing of oligonucleotides
- Extension of oligonucleotides with synthesis of a new DNA chain complementary to the template by means of Taq polymerase.

Real-Time PCR allows to quantify the synthesis of the PCR product at each amplification cycle in real time. This allows for a relative quantitative analysis of the initial template DNA concentration. The signal that is quantified is represented by the fluorescence emitted by a fluorophore, the SYBR @ Green, which is intercalated in the double strand of the DNA. The DNA-SYBR @ Green complex absorbs blue light at a wavelength λmax = 488 nm and emits green light at a wavelength λmax = 522 nm, as can be seen from the figure below (**Fig. 17**).

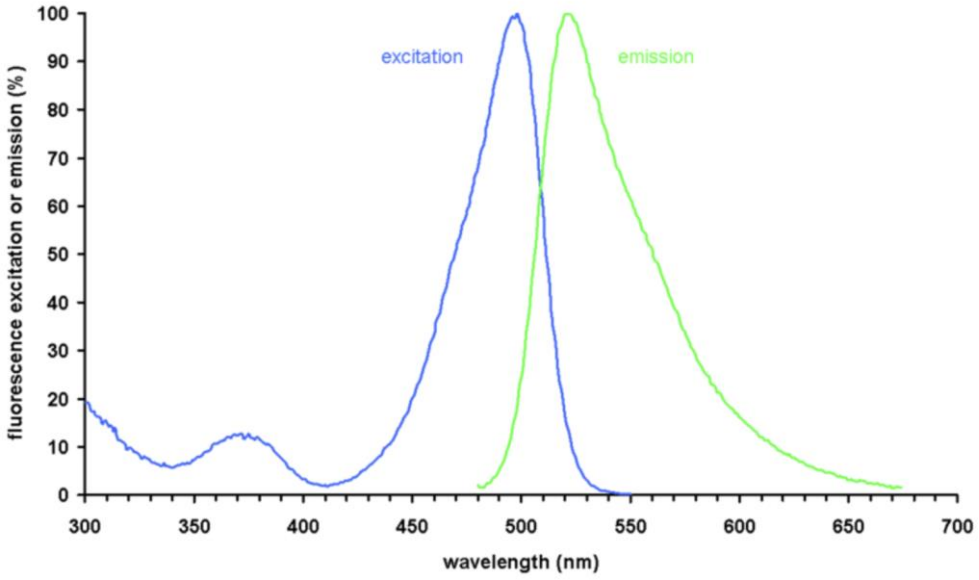

Fig. 17: SYBR Green excitation and emission spectrum.

The real-time PCR instrument used in this study is the CFX96 Real-Time PCR system (Bio-Rad), with has an analysis capacity of 96 wells and a sample reaction volume between 1 and 50ul. In the present study, duplicates were made for each sample under examination. The reaction was performed by preparing a Master Mix for each target gene, obtained by adding 5.0 ul of SYBR Green Mix, 3.8 ul of H2O, 0.2 ul of Primers (Forward + Reverse) for each sample (2*n samples + 5 extra-samples + 2 blanks= x) (**Table 5**). A 1:10 dilution was prepared for each pair of primers.

***Table 5**: Master Mix and primer quantity for each sample*

FILTERED-AUTOCLAVED H2O	3.8 μl * X
SYBER GREEN	5 μl * X
PRIMERS	0.2 μl * X
TOTAL	9 μl * X

9 ul of this master mix was pipetted into each well of 8 strips, and finally added 1 ul of cDNA diluted 1: 10 (or 1 ul of H2O for blanks). The amount of PCR product doubles at each amplification cycle: as an effect, the fluorescence, which is proportional to the number of cDNA copies present, shows a sigmoid trend. The melting curve obtained at the completion of the RT-PCR reaction provides an index on the purity of the reaction product. Gene expression analysis was carried out using the CFX manager (Bio-Rad) software. First of all, the integrity control of the cDNAs and their normalization were verified through the amplification of the 2 housekeeper genes *ACTB* (actin beta) and *RPLP0* (ribosomal protein lateral stalk P0); then the expression of the genes of interest was evaluated using the primers shown in the Table 6. The following genes were analyzed: *RUNX2*: osteoblast-specific transcription factor 2;

ALP: alkaline phosphatase; *TGFB1*: transforming growth factor B1 about proliferation; *ITGB1*: integrin beta 1; *COL3A1*: collagen type III Alpha 1 Chain; *SPP1*: secreted phosphoprotein 1 or osteopontin; *SPARC*: secreted protein acidic and cysteine or osteonectin about extracellular matrix maturation; *BGN*: biglycan; *DCN:* decorin about extracellular mineralization.

***Table 6**: List of primers used in gene expression analysis by Real-Time PCR.*

Gene	Forward primer (5'-3')	Reverse Primer (3'-5')
ACTB	ATTGGCAATGAGCGGTTC	GGATGCCACAGGACTCCAT
RPLP0	CTGGAAAACAACCCAGCTCT	GAGGTCCTCCTTGGTGAACA
RUNX2v1	GTGCCTAGGCGCATTTCA	GCTCTTCTTACTGAGAGTGGAAGG
ALP	CCATCCTGTATGGCAATGG	CGCCTGGTAGTTGTTGTGAG
TGFB1	GCAGCACGTGGAGCTGTA	CAGCCGGTTGCTGAGGTA
ITGB1	TCCAAAGTCAGCAGAGACCTT	ATTTCCAGGGCTTGGGATA
COL3A1	CTGGACCCCAGGGTCTTC	CATCTGATCCAGGGTTTCCA
SPP1	TTGCAGCCTTCTCAGCCAA	CAAAAGCAAATCACTGCAATTCTC
SPARC	GTACATCGCCCTGGATGAGT	CGAAGGGGAGGGTTAAAGAG
BGN	CAGCCCGCCAACTAGTCA	GGCCAGCAGAGACACGAG
DCN	GGAGACTTTAAGAACCTGAAGAACC	CGTTCCAACTTCACCAAAGG

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc tests were applied to determine the gene expression differences between groups. All the tests were performed using R version 4.0.2 and plots were generated using ggplot2 within R. The results of XTT assay were investigated using Two-way ANOVA followed by Bonferroni's test using GraphPad Prism (version 8.2.1). The results are reported in

terms of mean and standard deviation and the statistical significance was set to $p < 0.05$.

RESULTS

XTT assay

Cytotoxic effects and cell viability of all the treatment groups were verified using the XTT assay after two time points of incubation- 24 hours and 48 hours. After 24 hours the groups with boron and selenium alone (B10, Se) and the synergistic groups SeVD and SeBVD led to a significant increase in cell viability compared to the control and the other treatment groups. The synergy group B100VD instead led to a significant decrease in cell proliferation compared to control cells and other groups.

The remaining groups (VD, B100, B10VD, SeB) showed an increase in absorbance compared to the control although not statistically significant.

After 48 hours none of the groups induced a significant change of cell viability respect to control cell. Groups receiving VD, B100, B100VD, SeB, SeBVD presented a slight, not statistically significant decrease of viability, while B10 and Se groups induced a slight not significant increase.

Comparing 24 and 48 hours results, only control, B10 and B100VD cells showed a significant increase of cell viability (Fig 18).

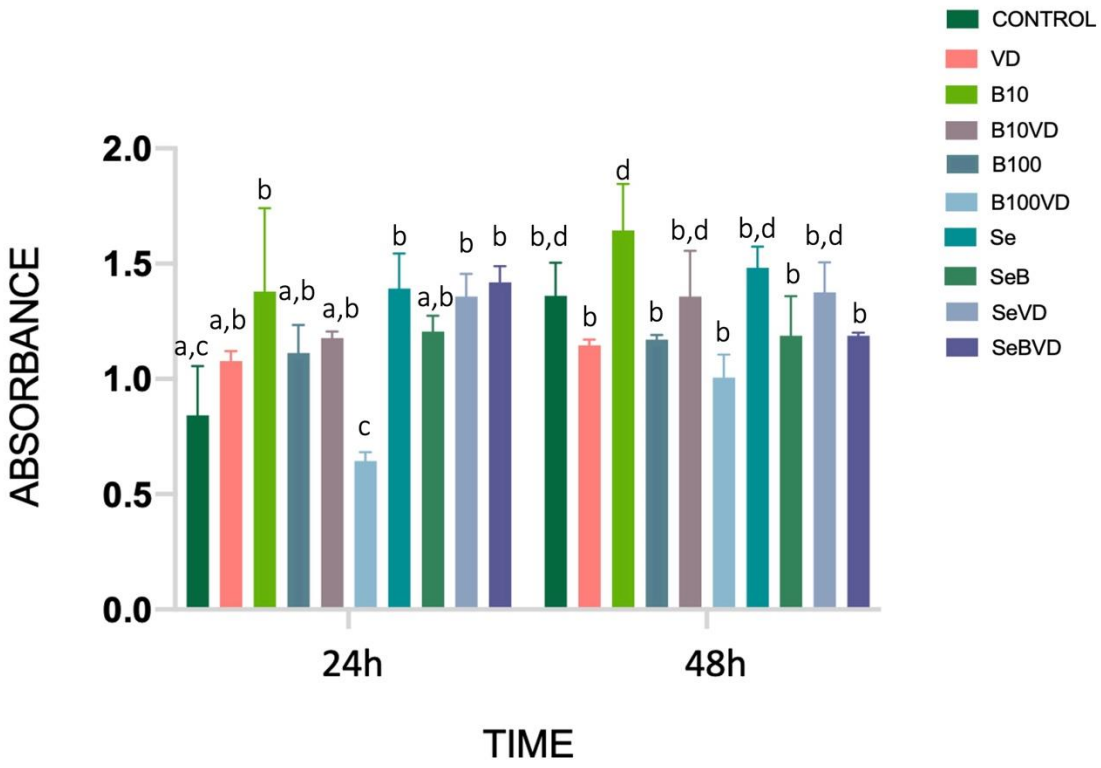

Fig 18. Cell viability of human fetal osteoblast (hFOB1.19) cell line in the presence of different treatments was performed using the XTT assay. The graphs show the mean and standard deviation as error bars. The different letters above each graph indicate statistically significant differences in the different groups. Statistical significance was set at p <0.05.

Alizarin Red staining and quantitative analysis

The ability to deposit calcium nodules was detected by Alizarin Red staining.

Already at a qualitative level it can be noted that there is a difference between the control and the VD group which shows a more evident red color. Treatments with boron and selenium alone and the combination group SeB present a similar mineralization to control. The appearance of appreciable calcium nodules, demonstrated by a strong intensification of the red coloring, can be observed in groups B10VD, B100VD, SeVD, SeBVD. (**Fig 19**).

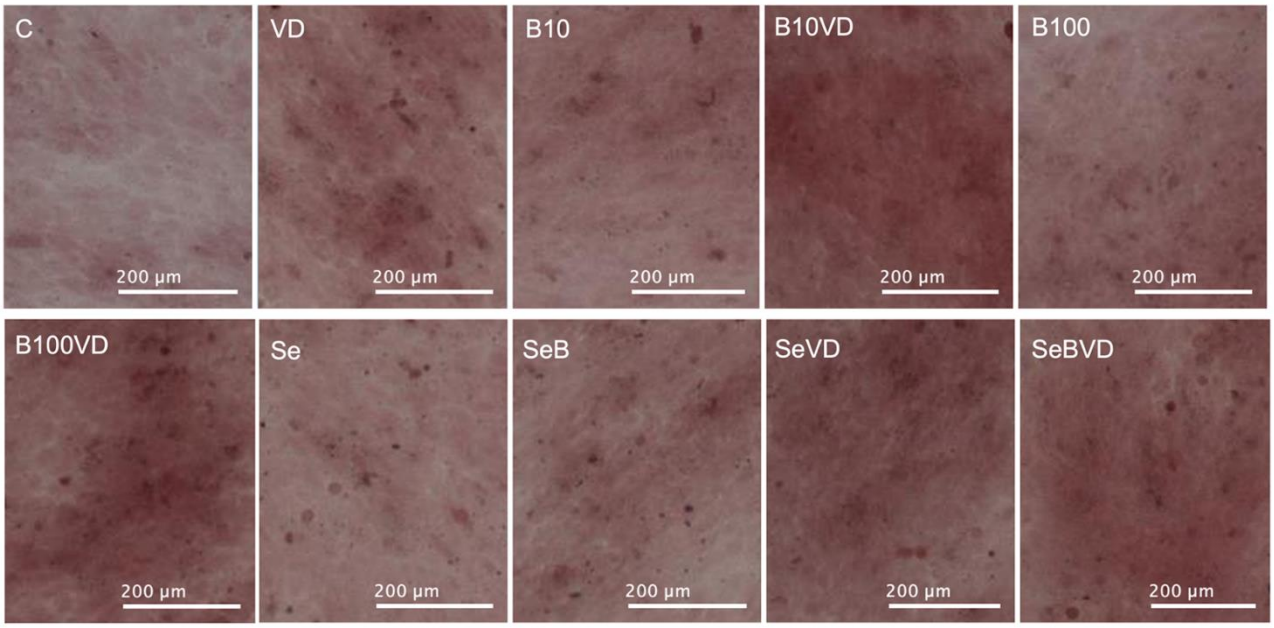

Fig 19. Alizarin Red staining of the hFOB1.19 cells cultured on the different treatments. Each image is representative of the different treatments applied to the cells for 7 days in mineralization inducing media. Scale bar: 200 µm

A quantitative spectroscopy analysis of the mineralization of the extracellular matrix was subsequently carried out. The elaborated data about the intensity of fluorescence measured, relating to the deposition of calcium nodules, are shown in the graph (**Fig 20**). The synergy groups (B10VD, B100VD, SeVD, SeBVD) showed the significantly strongest stimulation capacity in mineralization compared to other groups, including control. Groups with boron and selenium alone (B10, B100, Se) and the combination group (SeB) did not show significant effects, with a fluorescence intensity slightly lower or similar to control cells. VD alone also highlighted an increase of nodules calcium compared to control although not statistically significant.

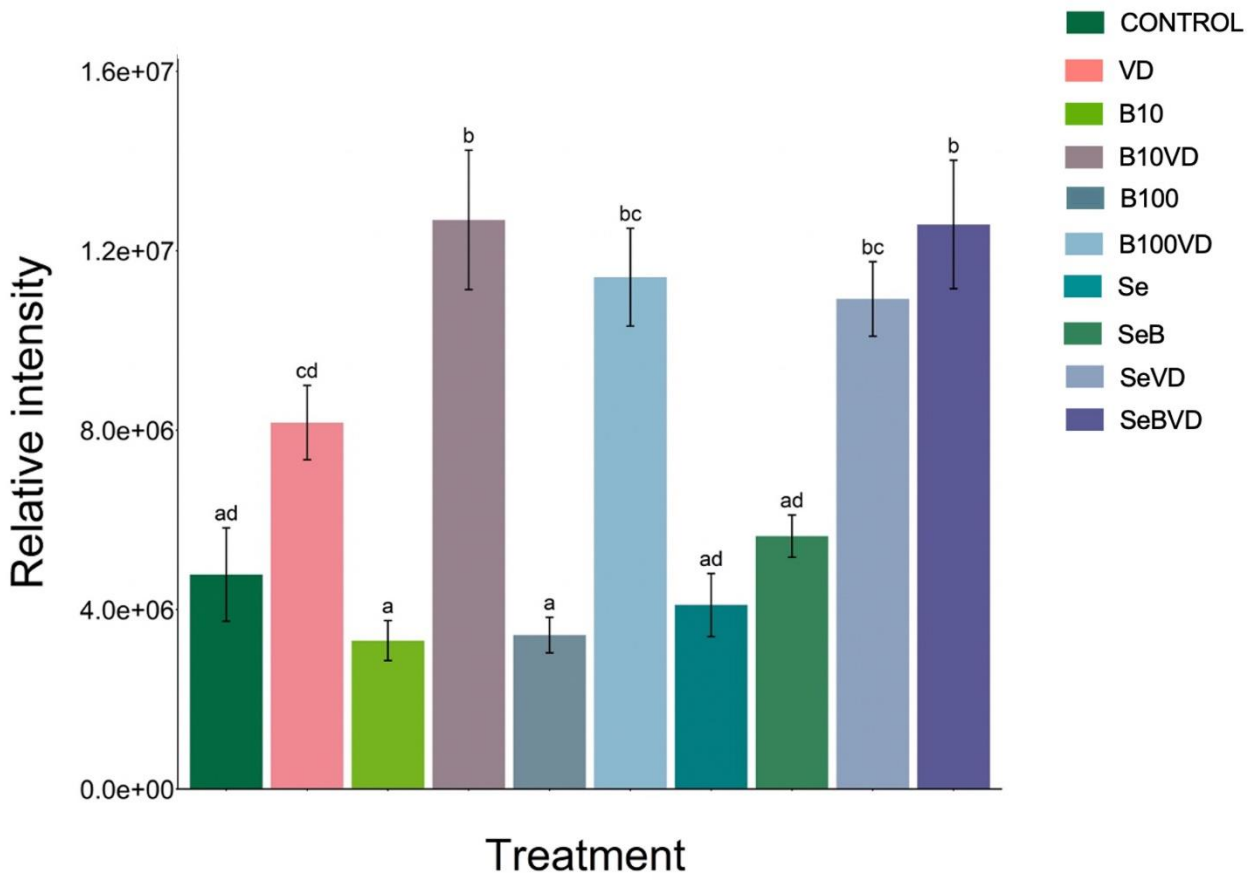

Fig 20. Quantitative analysis of the extracellular matrix mineralization following the different treatments. The graphs show the mean and standard deviation as error bars. The different letters above each graph indicate statistically significant differences in the different groups. Statistical significance was set at p <0.05.

Alkaline phosphatase staining and quantitative analysis

The level of osteogenic differentiation of the hFOB1.19 cell population was qualitatively assessed by cytochemical staining of Alkaline Phosphatase. All treatment groups show an increase in the activity of ALP compared to cells cultivated in a medium base (C). VD, B10VD, B100VD, SeB, SeVD, SeBVD groups show the superior intensity of blue-violet color compared to control demonstrating a superior effect in

increasing ALP activity. B10 and Se showed a lower ALP activity, respect to combination groups (**Fig 21**).

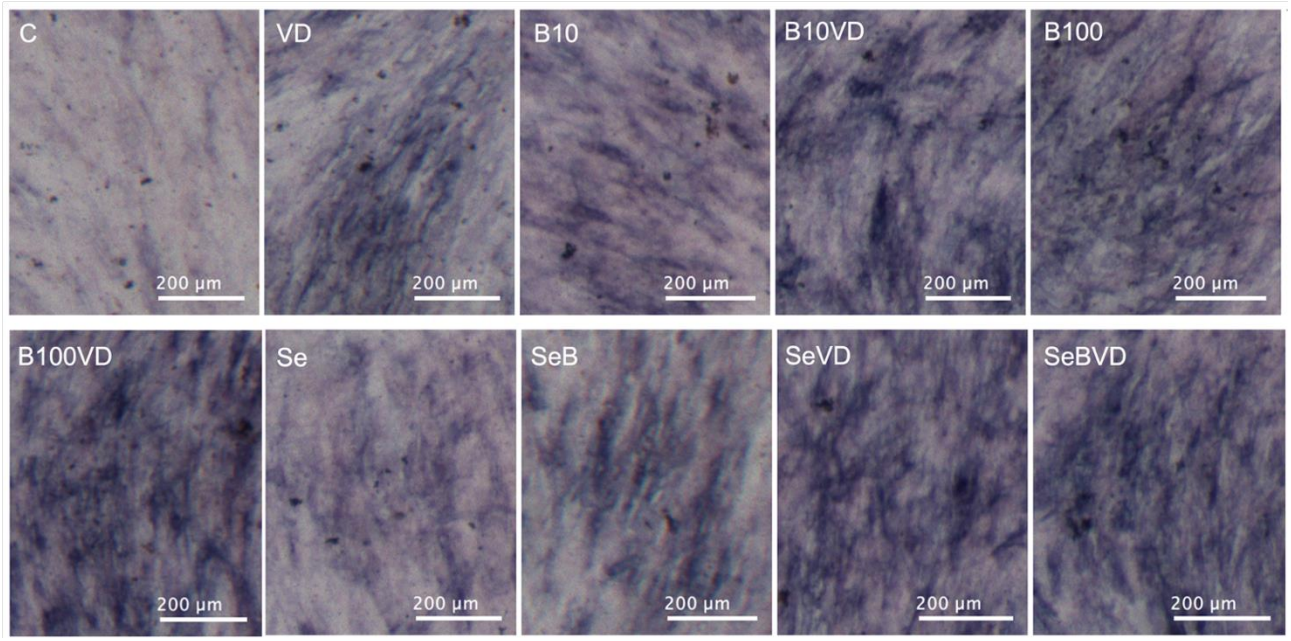

Fig 21. Alkaline phosphatase staining of the hFOB1.19 cells cultured on the different treatments. Each image is representative of the different selenium treatments applied to the cells for 7 days in mineralization inducing media. Scale bar: 200 µm

A quantitative spectroscopy analysis of the Alkaline Phosphatase was subsequently conducted. The processed data about the intensity of fluorescence measured, relating to ALP levels, are shown in the graph (**Fig 22**). B10 and Se groups, induced the lowest fluorescence intensity levels, with no significant difference respect to control group. VD group and those with boron and selenium in combination with vitamin D (B10VD, B100VD, SeVD) as well as SeBVD group, show the significantly highest increase in ALP levels if compared to control and groups alone. VD, B100 and SeB groups induced a similar ALP activity.

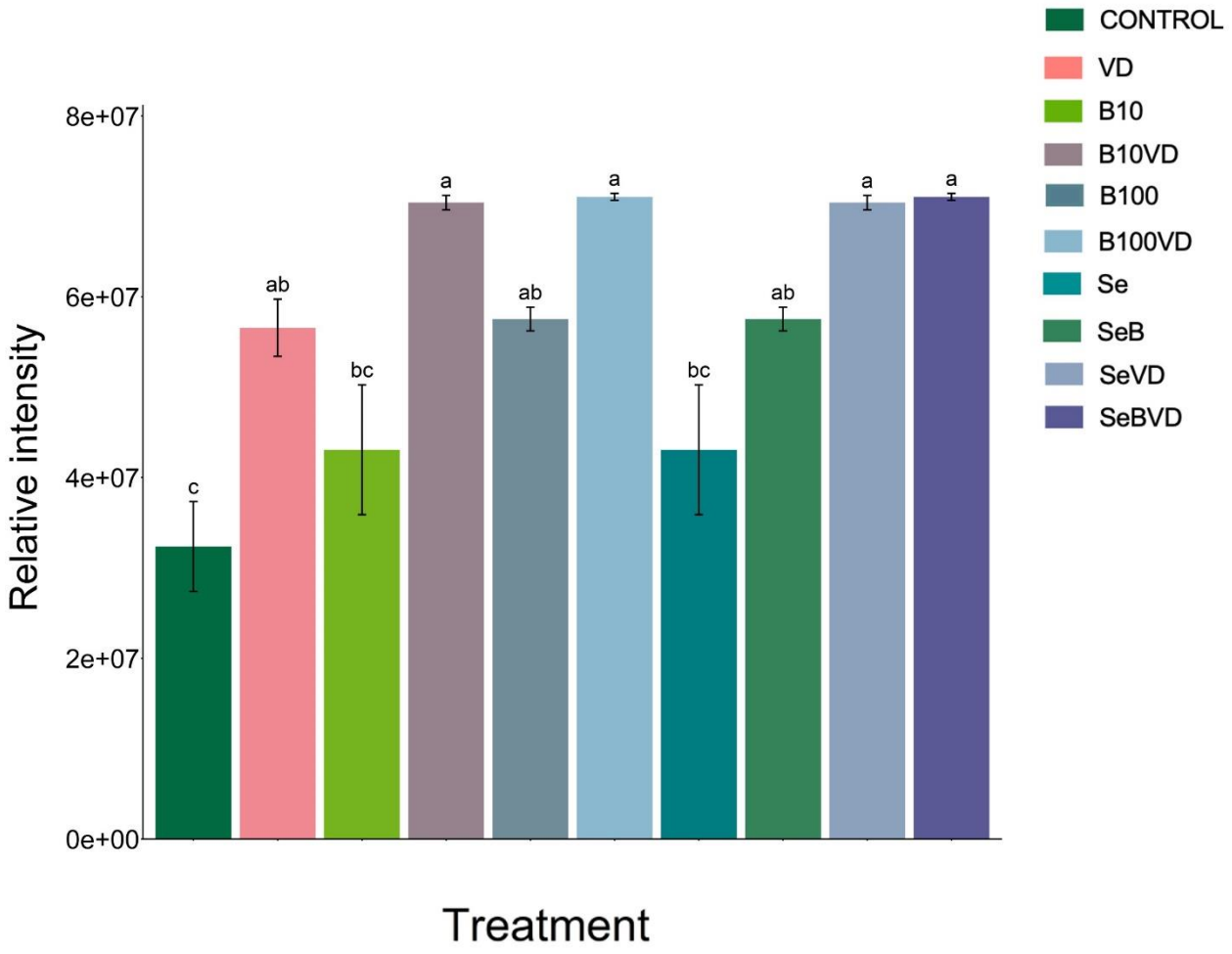

Fig 22. Quantitative analysis of Alkaline Phosphatase levels following the different treatments. The graphs show the mean and standard deviation as error bars. The different letters above each graph indicate statistically significant differences in the different groups. Statistical significance was set at p <0.05.

Gene expression

Gene expression was evaluated following treatments with boron, selenium and vitamin D alone and in combination with each other (**Fig 23**). Regarding the genes involved in "proliferation stage", for *ALP*, the simple treatment with both Boron concentrations and selenium alone did not induce significant changes in mRNA levels respect to control. Similarly, VD treatment alone induced an increase, although not significant of gene expression, but when co-administered, in B10VD, SeVD and SeBVD, mRNA levels reached the highest expression respect to control.

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For *RUNX2* mRNA, all groups exhibit greater expression than the control. The highest increase was observed in B10VD, SeB and SeVD groups. In VD and SeBVD groups, a lower induction if compared to the above mentioned groups, but still significantly higher than the control, was measured. Groups with boron at the highest concentration alone and in combination with vitamin D (B100, B100VD) induced a lower stimulation respect to VD alone although not significant. Groups with boron alone (B10 and B100) displayed the lowest effects. Concerning TGFβ mRNA levels, all changes induced by the different micronutrient exposures did not induce significant changes. More precisely, *TGFB* mRNA exhibits the greatest stimulation following treatment with B10VD compared to the control and other treatment groups, significantly higher than B100 treatment and groups with selenium combined with boron and vitamin D (SeB, SeVD). B10, B100VD, and Se groups displayed a similar expression to control, while there is a decrease in mRNA levels following treatment with VD, B100, SeB, SeVD and SeBVD with no significant difference.

Regarding the genes involved in "maturation of the extracellular matrix stage", *ITGB mRNA* show the significantly highest stimulation following synergy groups SeB and SeVD. Group with selenium alone (Se), also exhibit a significant increase in gene

expression compared to control and the groups VD, B100, B10VD, B100VD. B10 and SeBVD lead to a similar expression pattern greater than in control and VD group, although without significant effect. For the *COL3A*, the significantly strongest increase in stimulation was observed in synergy groups SeVD and SeBVD. Lack of changes were induced by VD, B10, B100, B100VD, Se and SeB, respect to control group. Among synergy groups, B10VD also leads to a significant increase in gene expression compared to control and B100, Se and SeB groups. Concerning *SPARC*, synergy groups SeVD and SeB, lead to the significantly highest mRNA stimulation compared to control and other groups. All the others micronutrients did not induce any changes in mRNA expression. For *SPP1*, VD group shows the greatest increase in gene stimulation, significant compared to control and other groups, except for B10VD. Synergy groups with boron and vitamin D (B10VD, B100VD) are the groups which expressed significantly higher *SPP1* mRNA than groups with selenium alone and combined with boron and vitamin D (Se, SeB, SeBVD), which exhibited lower, but not significant, levels of expression than control cells.

Regarding the genes involved in "mineralization of the extracellular matrix stage", *DCN* mRNA showed the significantly highest levels of expression in all groups presenting selenium alone or in combination with other micronutrients (Se, SeB, SeVD and SeBVD), compared to control. VD and boron treatments alone and combined (B10, B10VD, B100, B100VD), did not induced any mRNA level changes. For *BGN*, all groups lead to a strong gene stimulation than the control, except for Se. The best significant effects compared to control cells were obtained following vitamin D alone or in synergy (B10VD, B100VD, SeVD, SeBVD). Groups with boron alone or combined with selenium (B10, B100 and SeB) showed the same expression pattern, higher than control although not statistically significant, while selenium alone (Se) displayed the same level of control.

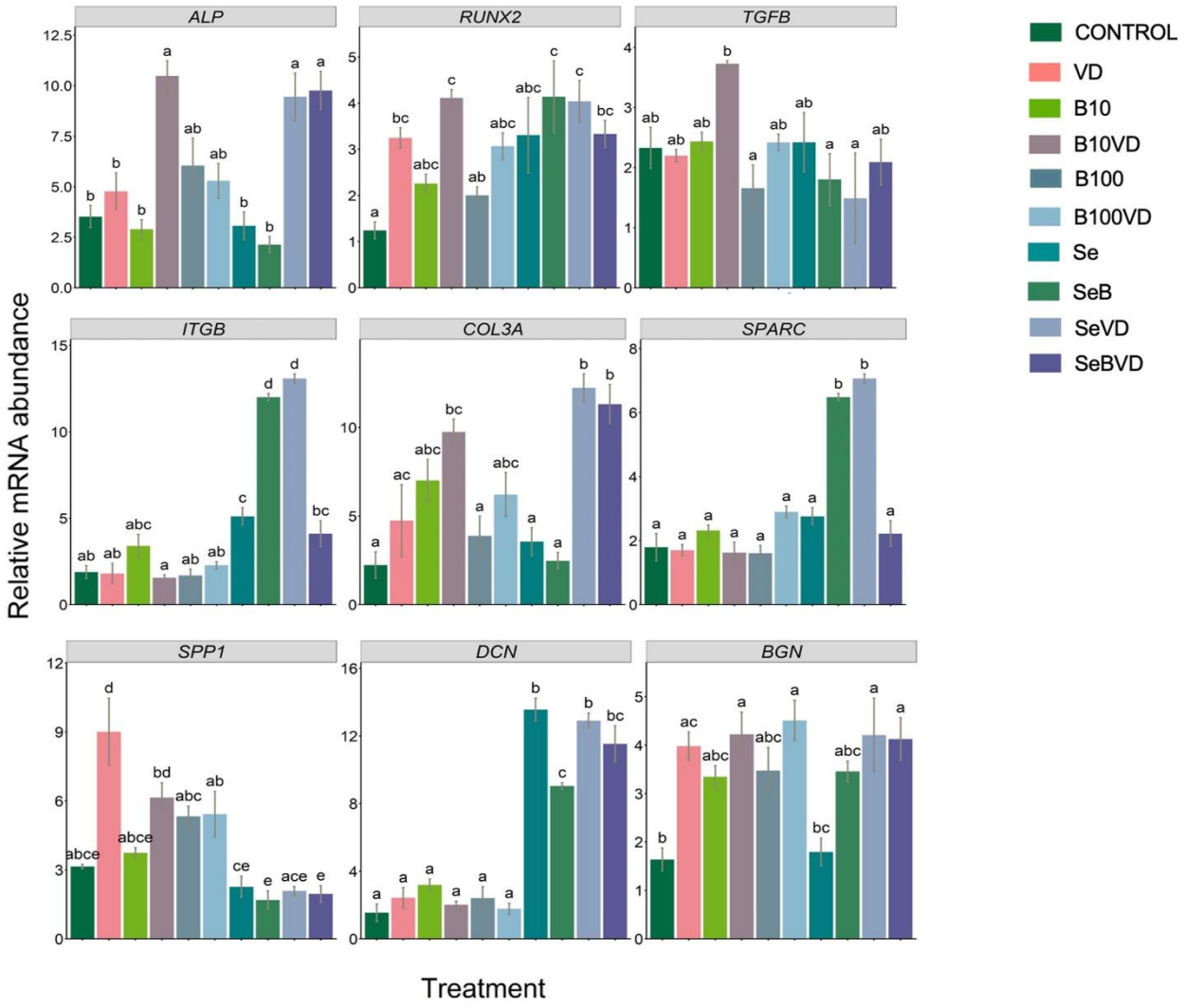

Fig 23. Expression of genes involved in the proliferation (ALP, RUNX2, TGFB), maturation (ITGB, COL3A, SPARC, SPP1) and mineralization (DCN, BGN) of the extracellular matrix after treatments. The graphs show the mean and standard deviation as error bars relating to gene expression in cells cultured with the different treatment groups. The different letters above each graph indicate statistically significant differences in the different groups. Statistical significance was set at p <0.05.

DISCUSSION

To clarify the process of osteogenic differentiation and determining which micronutrients are involved in differentiation and mineralization of osteoblastic cells several micronutrients were analyzed. It was decided to base the study on the use of the cell line hFOB1.19, which is an established *in vitro* model to study bone formation, capable of generating an ECM *in vitro* with ultrastructural elements similar to those deposited by primary osteoblasts *in vitro* [144]. In this regard we investigated, *in vitro*, how different micronutrients, boron at two different concentrations, selenium and vitamin D and their combinations influenced the differentiation and mineralization of the ECM. The effects of the different micronutrient administration were further analyzed considering the expression of the genes involved in the various developmental phases of osteoblasts. We based our hypothesis on several previous results in mice and rats [150,151], which gave evidence that boron can play an important role in biomineralization of osteoblasts, and selenium may also have a link with bone turnover. Therefore, it was decided to determine whether the evidence found in the literature for these elements in animals could be also valid for the human osteoblastic line. In addition, the possible beneficial effects of administering combination of micronutrients were further evaluated.

Cell viability was analyzed using XTT assay for all the treatment groups. Boron and selenium show a certain level of toxicity when administered at high concentrations. Concentration of micronutrients were selected on the bases of previous published studies. The study by Hakki and collaborators showed that boron at 1,000 ng/ml will decrease the mouse MC3T3-E1 cell survival in the short term of study[56]. In addition, the study conducted by Liu and collaborators on bone marrow stromal cells (MSCs) showed that selenium was able to inhibit the adverse effect of H_2O_2 on osteoblastic differentiation. However, these authors found that when MSCs were treated with 1

μM selenium for more than 10 days, cell viability was significantly decreased. With higher dose of selenium (10 μM), cell viability was significantly decreased with prolongation of the treatment time[114]. Therefore, to avoid toxicity problem, for our experiments we decided to test concentration corresponding to 100 and 10 ng/ml and to use a concentration of 10 ng / ml of selenium. We found that after 24 hours, when hFOBs were treated with 10 ng / ml boron and selenium (B10, SE), cell viability increased and reached a maximum which was maintained even after 48 hours.

In our study the group B100VD after 24 hours instead showed a significant decrease in cell viability compared to the control cells and the other groups, while after 48 hours this decrease was clearly reduced, without negative effects compared to cells grown in the base medium. Following others treatment groups (……), cell viability increased after 24 hours and then decreased to the control level or slightly below. Therefore, we can conclude that boron and selenium are generally nontoxic if they are not used in high doses and cells are safe under the experimental conditions selected in the present study. Considering the different results obtained between this study and a previous one with MC3T3-E1, which is an osteoblast precursor cell line derived from *Mus musculus* (mouse) calvaria[56] and bone marrow stromal cells (MSCs)[114], we can hypothesize that cell tolerance depends on cell lines which may have a different sensitivity to boron and selenium. In our study also vitamin D did not affected cell viability in agreement with the previous reports which also showed that vitamin D has no toxic effects on cell viability [152].

In our study, ALP has been used as a biomarker of osteoblast cell differentiation. The highest induction was observed mainly in groups presenting VD, suggesting that the increase could depend on how the synergistic action of the compounds is able to increase the effects of the already positive individual micronutrients in a beneficial way. In our results, the fact that vitamin D when combined with boron has shown superior beneficial effects respect to when singularly administered, is corroborated

by a study in which boron manifests additional integrative effects on bone metabolism in its actions relating to vitamin D, by acting as a helper, backup agent, and/or facilitators to maintain bone integrity[153]. A previous study also demonstrated that boron modulates turnover of the ECM and regulates the formation of calcium nodules modulating the activity of enzymes such as ALP in fibroblasts [68]. The result that selenium in combination with boron and vitamin D led to a significant increase in ALP levels is in line with the study of Liu and co-workers conducted on primary rat MSCs that showed that selenium is able to significantly enhance ALP activity after 14 days of treatment.[154]

The appearance of calcium nodules was also analyzed to verify the mineralization profile. Cells cultured in base medium produced small, weakly stained minerals. The number of granules in their cytoplasm was smaller compared to that of stimulated cells which instead had more markable and bigger mineral deposits mainly in co-administerd groups. This is in line with what was obtained for the ALP. Here the combination of boron and selenium with each other and with vitamin D have highlighted an appreciable and significant deposition of calcium nodules compared to the individual administration, including vitamin D, confirming that the co-administerd groups are those that produce a greater beneficial effect in the differentiation and mineralization of osteoblastic cells. The study conducted by Ying and collaborators on MSC cells showed that calcium depositions were increased in 1 and 10 ng/ml boric acid treatment groups, and they were reduced at the concentration of 100 ng/ml treatment group [155]. This is in line with our results, and the fact that even the concentration of 100 ng/ml boron, when combined with vitamin D, led to an increase in calcium nodule deposition compared to control cells may lie in the different sensitivity with which hFOB1.19 cells have responded to treatment. In fact, the study conducted by Strzelecka-Kiliszek and collaborators showed that osteosarcoma Saos-2 cells are able to lay 2.6 time more calcium nodules than hFOB1.19 osteoblasts when

they are stimulated for mineralization[156] and this might be due to the more mature osteoblast phenotype of Saos-2 cells19 in comparison to hFOB1.19 cells as demonstrated by the study by Yen and collaborators [157] and Bozycki and co-workers [147]. The study conducted by Zheng and collaborators showed that human umbilical cord mesenchymal stem cells (HUMSCs) showed increased mineralization following treatment with selenium nanoparticles, which appear to be able to promote the deposition of calcium nodules [121]. In the results obtained in our research, all the co-administerd groups with selenium led to an increase in mineralization and osteogenic differentiation. Our results , obtained after 7 days exposure are validated by the study by Liu and co-workers in which primary rat MSC cells show an increase in calcium deposition following selenium treatment after 14 days[114]. Liu et collaborators work demonstrated that matrix mineralization in MSCs, as measured by calcium deposition in the matrix, was undetectable in early stage of culture, low in middle stage of culture, and reached a maximum in late stage of culture (at day 21 of culture) [158]. On this regard, it could be interesting to investigate on the effects that treatments could have on mineralization in human osteoblasts after longer time exposition.

In our study, to elucidate morphological fine tuning, we have analyzed also the expression of gene involved in the proliferation, maturation and mineralization stage of the extracellular matrix. In our results co-administered groups showed better effects on gene expression than treatments alone and this is in line with what was obtained at physiological and biochemical levels by calcium nodules and alkaline phosphatase levels analysis. However transcriptional level this was not evident for all the tested combinations; in fact, in some cases, also the treatment alone led to a significant increase of expression (i.e. VD, SE groups). When boron is in combination with vitamin D (B10VD, B100VD) it is able to lead to a significant increase in gene expression respect to when used alone, and this is in agreement with what is reported in the literature by Devirian and Volpe study [159]. For genes involved in osteoblast

proliferation stage (*RUNX2*, *ALP* and *TGFB)* co-administered group with boron at lower concentration and vitamin D (B10VD) exhibited a significant effect on expression and this is in line with the study conducted by Ying and collaborators in which boron showed an increase in gene expression on bone marrow stromal cells (BMSCs) [155]. For other genes like *DCN*, *SPARC* and *ITGB* the greatest stimulation was observed following groups with selenium alone and in combination with boron and vitamin D (Se, SeB, SeVD, SeBVD), while boron alone and in combination with vitamin D had no effects. In the studies conducted by Hakki and collaborators and by Ying and collaborators, a greater gene expression is obtained in all the genes analyzed on osteoblast precursor cell line derived from *Mus musculus* (mouse) calvaria (MC3T3-E1) and on bone marrow stromal cells (BMSCs), respectively[56,160]. An explanation for this different trend in our research, regarding boron, could lie in the time at which the analyzes were carried out, which was always 7 days after the induction, and in the type of cell line used; MC3T3-E1 cells are a partially differentiated cell line and they can respond differently than human osteoblasts. Also another study conducted by Wiper-Bergeron and collaborators showed opposite effects of vitamin A on *RUNX2* expression in mesenchymal stem cells and committed osteoblasts [161]. On the other hand, the study conducted by Armstrong and co-workers, reported that boron apparently does not markedly affect bone calcification because it did not affect plasma alkaline phosphatase activity [162]. In our results selenium showed a great ability in gene stimulation, especially when in combination with boron and vitamin D, except for *SPP1*. These results are in line with the results obtained in the study conducted by Zheng and collaborators on human umbilical cord mesenchymal stem cells (HUMSCs), which shows how selenium nanoparticles are able to enhance osteoblastic differentiation *in vitro*. They found a higher expression of osteoblast specific genes and a greatest mineralization following the interaction of cells with selenium nanoparticles [121]. The result that for some genes, treatments with selenium have led

to an increase in expression compared to boron, could depend on the fact that selenium has a positive effect in protecting against oxidative stress and in the proliferation and regulation of apoptosis in mouse osteoblasts as previously described on osteogenic differentiation by Ren and collaborators [163]. Also the study conducted by Liu and collaborators suggested that selenium alone was capable of enhancing the expression of genes like osteocalcin and type III collagen and osteoblastic differentiation of MSCs by reducing basal oxidative stress [114].

In our results, in some cases the synergy between the compounds seems to have modified some pathways acting on gene expression and this led to a better effect than the treatments alone. Similarly, selenium shows the ability to enhance the effect of vitamin D in thioredoxin reductase gene expression on human osteoblasts [164]. A study in which lithocholic acid (LCA) shows the ability to modify the effect of vitamin D in gene expression on human osteoblasts interacting with VDR receptor[152] could confirm this hypothesis. Therefore, the mode of action of boron and selenium on the osteogenic differentiation of human fetal osteoblasts has not yet been deeply clarified. The results here obtained show a general positive effects both on the mineralization well supported by the expression of specific genes typically expressed by osteoblasts, although the results need further validation.

CONCLUSION

In our study, it was found that boron should be considered as a regulator in the osteogenic ability of human fetal osteoblasts *in vitro*. The highest effects are observed in cases where the boron is in association with vitamin D and results are in agreement with what is reported in the literature by Devirian and Volpe study [159]. Future work needs to be focused on how and whereby these osteogenic differentiation-related marker genes are modulated by boron to clarify its osteogenic role. The same was observed for selenium, but the data in literature concerning the impact of selenium in combination with vitamin D and boron on bone, are unclear and require further research. However, its effects may contribute to the increased bone formation and human osteoblast differentiation is in agreement with what is reported in the literature by Zheng and co-workers and Liu and collaborators study on other human and rat type of cells [121,154]. Further investigation could be carried out and verify whether prolonged treatments with boron and selenium alone and combined, are able to maintain the increased effects on the osteogenic differentiation process and gene expression pattern and analyze, through the action of promotors and inhibitors, how the origin and properties of vesicles released by osteoblasts can determine the onset of mineralization at the cellular level.

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