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Ciclo XXVIII

Efficacy of Stanozolol on bone regeneration: *in vitro* and *in vivo* study

Efficacia dello Stanozololo nella rigenerazione ossea: studio in vitro *e* in vivo

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In primis autem constituendum est quos nos et quales esse velimus et in quo genere vitae quae deliberatio est omnium difficillima.

(Marco Tullio Cicerone)

Index

IN VIVO ----------

Chapter 3

Chapter 4

Foreword

The study of new strategies for bone regeneration represents the main goal to improve the management of demanding clinical situations in oral and maxillofacial surgery. In such a context, the combination of osteoinductive molecules with grafting biomaterials is a field of growing interest and many agents have been tested so far.

It is widely known that sex steroid hormones play a significant role in regulating morphogenesis and in maintaining homeostasis of bone throughout life. This thesis work arises from the hypothesis local androgen administration, possibly in combination with grafting materials, may favor the healing of bone defects. Following this idea, the effects of the synthetic androgen Stanozolol on bone regeneration have been evaluated in different experimental settings.

The present work is structured along a pathway which goes from *in vitro* to *in vivo* investigations; each chapter can be approached as an independent study, which corresponds to a distinct experimental phase.

Based on this idea, an overview about androgen effects on bone, is initially provided. Then, and the subsequent in vitro investigation SaOS-2 cell line is described. Lastly, an innovative analysis methodology to study bone regeneration in a rat- model is proposed and local Stanozolol administration combined to a grafting biomaterial is tested in bone defects.

Premessa

La ricerca di nuove strategie per la rigenerazione ossea rappresenta un focus di interesse centrale per migliorare la gestione di casi clinici complessi nell'ambito della chirurgia orale e maxillo-facciale. Uno degli approcci più utilizzati in tale contesto si basa sull'utilizzo di molecole con proprietà osteoinduttive e molte sostanze sono state fino ad oggi sperimentate.

E' noto in letteratura che gli androgeni svolgono un ruolo chiave nella regolazione della morfogenesi ossea e nel mantenimento della sua omeostasi durante il corso della vita. Questo lavoro di tesi nasce dall'ipotesi che la somministrazione locale di tali ormoni, eventualmente combinata a materiali da innesto, possa favorire la guarigione di difetti ossei. Stando a questa premessa, sono stati valutati gli effetti dello steroide sintetico Stanozololo sulla rigenerazione ossea in diversi settings sperimentali.

La tesi è strutturata secondo un percorso che segue le fasi della ricerca, attraverso sperimentazioni in vitro *e* in vivo*; ogni capitolo può essere approcciato come uno studio a sé stante, corrispondente ad una determinata tappa dell'iter sperimentale.*

Sulla base di questi intenti, viene fornito inizialmente un quadro d'insieme circa gli effetti degli androgeni sull'osso. A seguire, è presentata una sperimentazione in vitro *nella linea cellulare SaOS-2. Infine, è proposta un'innovativa metodologia di analisi per lo studio della rigenerazione ossea nel modello di ratto, ove viene testata la somministrazione locale di Stanozololo combinato a materiale da innesto.*

CHAPTER 1.

Androgens and bone: an overview

ABSTRACT

Androgen effects on bone morphogenesis and in the maintenance of bone homeostasis are exerted through complex and pervasive mechanisms. *In vitro* studies reported androgen influence on differentiation of pluripotent stem cells toward distinct lineages, as well as on osteoblast and osteoclast proliferation and function, providing evidence of a central role played by these hormones in maintaining a balanced bone turnover. The specific action of androgens on bone cells is mediated directly through androgen receptor (AR)-signaling pathway, but indirect effects can be achieved through androgen aromatization to estrogens and subsequent activation of estrogen receptors (ERα, ERβ)-signaling. Moreover, non-genomic molecular pathways characterized by rapid responses to androgens have been recently identified. Rodent models, and particularly rats, have been widely employed for the study of skeletal effects of androgens, due to the variety of experimental settings which can be easily achieved (i.e. models of gonadectomy, androgen depletion and/or exogenous administration, genetic knock-out of specific receptors). Many studies provided evidence of androgens positive effects on bone metabolism and homeostasis, with a prevalent action at cancellous bone sites in both sexes and at periosteal sites in males. As for androgens effect in men, they are dramatically evident during adolescence, where they contribute to the development of skeletal dimorphism between sexes. Throughout the rest of life, androgens affect bone homeostasis and function both in males and in females. These hormones were shown to stimulate periosteal bone formation and may protect men against osteoporosis via maintenance of cancellous bone mass and expansion of cortical bone. In conclusion, despite androgens play a key-role in bone physiology, further studies are needed to investigate their specific function on bone at all levels. Improvements in the comprehension of androgen effects will lead potential benefits in the prevention and treatment of a wide variety of bone disorders.

Key-words: Androgens; Testosterone; Anabolic steroids; Bone

RIASSUNTO

Gli effetti degli androgeni sulla morfogenesi scheletrica e sul mantenimento dell'omeostasi ossea si esplicano attraverso meccanismi biologici complessi e permangono durante il corso di tutta la vita. Studi in vitro rivelano l'influenza degli androgeni sul differenziamento di cellule staminali pluripotenti in lineage cellulari distinti, così come sulla proliferazione e la funzione di osteoblasti ed osteoclasti, fornendo evidenza a supporto del ruolo centrale svolto da questi ormoni nel mantenere un turnover osseo bilanciato.

L'azione specifica degli androgeni sulle cellule dell'osso è mediata direttamente dal recettore degli androgeni (AR), ma si possono ottenere effetti indiretti attraverso l'aromatizzazione degli androgeni e la susseguente attivazione dei recettori degli estrogeni (ERα, ERβ). Inoltre, pathway di signaling recettore-indipendente di tipo non genomico caratterizzati da rapida risposta agli androgeni sono stati recentemente identificati.

I modelli animali di roditore, ed in particolare il ratto, sono stati ampiamente utilizzati per lo studio degli effetti degli androgeni sull'osso, data la varietà di setting sperimentali che può essere facilmente ottenuta (es. modelli di gonadectomia, deplezione/somministrazione esogena di androgeni, knock-out genetico di recettori specifici). In questo contesto, molti studi riportano conseguenze positive dell'azione degli androgeni sul metabolismo e l'omeostasi ossea, evidenziando un'azione prevalente di questi ormoni nelle aree di osso midollare in entrambi i sessi e nelle aree periostali nei maschi.

Per quanto concerne gli effetti degli androgeni sull'uomo, essi si manifestano fisiologicamente in modo inequivocabile durante l'adolescenza, ove contribuiscono allo sviluppo del dimorfismo scheletrico nei due sessi. Nonostante solitamente si tenda a riservare più agli estrogeni un ruolo di primo piano, gli effetti degli androgeni sull'omeostasi e sulla funzione ossea permangono nel corso di tutta la vita sia nei maschi sia nelle femmine. Infatti, è stato osservato che nell'uomo questi ormoni sono in grado di stimolare l'apposizione ossea encondrale e svolgono una funzione protettiva verso lo sviluppo di osteoporosi, mantenendo la massa ossea trabecolare e promuovendo l'espansione dell'osso corticale.

In conclusione, sebbene sia evidente dalla letteratura il ruolo chiave degli androgeni nella fisiologia ossea, sono necessari ulteriori studi per investigare i loro meccanismi di azione e la loro funzione specifica a tutti i livelli.

Un maggiore sviluppo delle conoscenze sugli effetti degli androgeni sull'osso potrà portare significativi benefici in ambito clinico, nella prevenzione e nel trattamento di un'ampia varietà di patologie scheletriche.

Parole chiave: *Androgeni; Testosterone; Steroidi anabolizzanti; Osso*

1.1 Endogenous androgens and synthetic testosterone derivatives

Androgens (or androgenic hormones) can be defined as any natural or synthetic steroid that stimulates or controls the development and maintenance of primary and secondary male characteristics in vertebrates by binding to androgen receptor. Androgens are also provided with anabolic functions, which result in growth and differentiation of cells and increase in body size. Androgens can be distinguished in endogenous, which means they are physiologically produced by the organism, and synthetic, which have been artificially produced.

Endogenous androgens

Endogenous androgens are steroid hormones derived from C-19 metabolism of cholesterol, which are primarily produced in gonadal tissue, adrenal glands and placenta. The major conversion pathways which lead to androgen synthesis from cholesterol are shown in Figure 1.

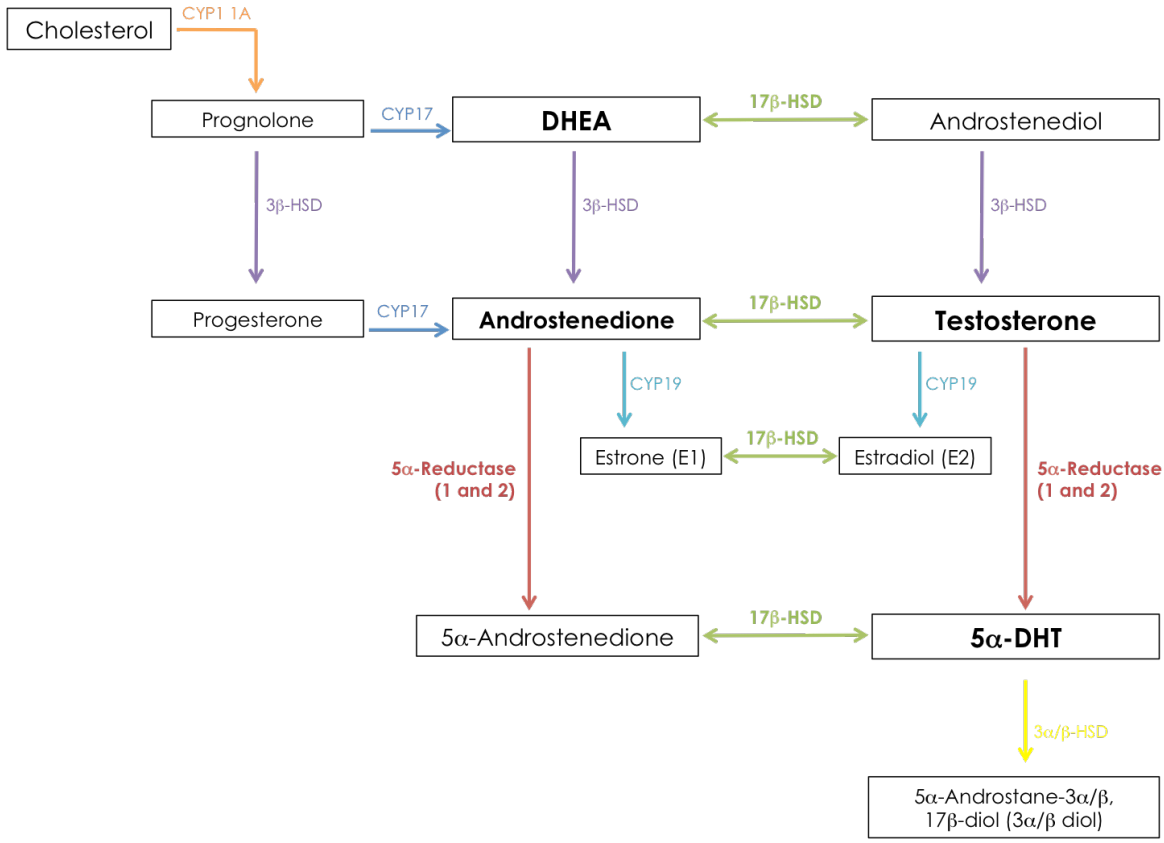

Figure 1. Principle conversion pathways and enzymes involved in w androgen synthesis and metabolism. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome

P450 17 αhydroxylase/17,20 lyase; 17β -HSD, 17 β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

T is the most abundant circulating androgen metabolite in men and is mainly is produced in Leydig cells of testes (>95%). Far smaller quantities are secreted by ovaries and placenta in women, as far as by the zona reticularis of the adrenal cortex and even skin in both sexes. T is usually transported in blood bound to a specific plasma protein, that is sex hormone-binding globulin (SHBG) (50-60%) and to less specific proteins i.e. albumin (40-50%). After reaching target tissues, T may undergo metabolic modifications through the action of specific peripheral enzymes. 5α-reductase enzyme catalyze T conversion into the more potent androgen dihydrotestosterone (DHT), while aromatase enzyme CYP19A1 is responsible for T conversion into the estrogen 17β-estradiol (E2).
Other C-19 androgens produced by the adrenal glands include Dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S), and androstenedione (AA). These androgens can also undergo metabolic conversions into T through steroid sulfatase enzymes 17β-hydroxysteroid dehydrogenase (17β-HSD) and/or 3β-HSD or into estrone through the aromatase enzyme.
It has been shown that bone is provided by aromatase, 5α-reductase, 17β-HSD, 3β-HSD enzymes. Also steroid sulfatase enzyme has been detected in bone, providing evidence for a local steroid synthesis [1][2][3][4][5][6][7][8][9][10][11][12][13][14][15][16][17][18][19][20][21].
Androgens can also be peripherally inactivated through glucuronidation, due to uridine glucuronosyl transferase enzymatic activity. Taken together, these findings support the importance of a skeletal "intracrine" metabolism of sex steroids for the regulation of local bone physiology.
The primary clinical use of testosterone and its derivatives involves a systemic administration in androgen replacement therapies, such as the treatment of male hypogonadism, induction of male puberty and gender transition [22][23][24].

Synthetic testosterone derivative: Anabolic-Androgenic Steroids (AAS)
As previously introduced, the most established physiologic functions of androgens are both androgenic and anabolic: indeed, on one side they are responsible for growth and maintenance of male primary and secondary sexual characteristics, and on the other they stimulate nitrogen fixation and increased protein synthesis. The potential therapeutic usefulness of T anabolic effect led to the synthesis of many derivatives with a low androgenic and a high anabolic action, as well as with a prolonged activity compared to endogenous androgens. These synthetic T derivatives are generally

known as anabolic-androgenic steroids (AAS). However, complete dissociation of anabolic from androgenic effects of systemically administered AAS has not been achieved.

Treatment with AAS is indicated in many other different situations, which are mainly related to the anabolic effects of these molecules. Indeed, they have been used for growth stimulation in children [25][26], preservation of lean body mass and prevention of bone loss in elderly men [27][28][29][30], treatment of chronic cachexia (i.e. patients with acquired immunodeficiency syndrome wasting and other conditions of severe catabolic burns) [31][32][33] treatment of hypoplastic forms of anemia [34] [35][36][37]. Health risks can be produced by long-term use or excessive doses of anabolic steroids; these effects include harmful changes in cholesterol levels (increased low-density lipoprotein and decreased high-density lipoprotein), liver damage, nephropathy and cardiovascular pathologies [38][39][40][41][42][43][44][45][46][47]. Conditions pertaining to hormonal imbalances such as acne, gynecomastia and testicular size reduction may also be caused by anabolic steroids [48][49][50][51]. Ergogenic use for anabolic steroids in recreational activities, racing and bodybuilding as performance-enhancing drugs is referred to as doping and banned by all major sport organizations [52].

Some of the most commonly used AAS approved by the US Food and Drug Administration there are Nandrolone decanoate, Oxandrolone, Oxymetholone and Stanozolol.

Nandrolone decanoate was firstly produced in 1950 and again in 1953 [53]. Nandrolone anabolic-to-androgenic ratio is significantly improved compared to T, which can be due to the chemical modification of a hydrogen atom in the C19 methyl group of testosterone and subsequent creation of a new asymmetric center at C10. Nandrolone metabolism is similar to that of T and this AAS can undergo aromatization in peripheral tissues [54]. Main nandrolone metabolites are 3-norandrosterone, a 5α derivative, and 2-noretiocholanolone, a 5α derivative [55].

Oxandrolone, synthesized in 1962 [56], is 17α-alkylated (methyl group) molecule with a reduced C4,5 double bond with a 5α configuration. It is characterized by the presence of an oxygen molecule at the C2 position, resulting in a lactone ring. Oxandrolone can be excreted unchanged or converted into 16β-hydroxyoxandrolone glucuronide [57].

Oxymetholone was synthesized in 1959 [58] and it is both 17α-methylated and 5α-saturated. Its chemical structure is similar to that of oxandrolone, the only difference being an oxygen instead of a hydroxymethylene group at C2 position [59].

Stanozolol is the first of a AAS series characterized by being 17α-alkylated and 5α-saturated and having a heterocyclic ring is fused to ring A of the steran skeleton. Stanozolol synthesis was firstly described in 1959 [60]. The main excreted metabolites of stanozolol include several hydroxylated byproducts, including 3'-hydroxystanozolol, 3'-hydroxy- 17 epistanozolol, 4β-hydroxystanozolol, and 16β-hydroxystanozolol. The major metabolites, including 3'-hydroxystanozolol, 4β-hydroxystanozolol, and 16β-hydroxystanozolol, are all excreted as glucuronide [61].

1.2 Androgen receptors and signaling pathways

Sex steroid classical signaling involves the interaction with specific cytoplasmic or nuclear receptors, which can be distinguished into androgen receptor (AR) and estrogen receptors (ERα, ERβ). AR and ERs are ligand-inducible transcription factors which belong to the nuclear receptor superfamily (respectively NR3C4, NR3A1, and NR3A2). They are characterized by a modular structure comprising a NH_2-terminal domain (NTD), a DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) [62] (Figure 2).

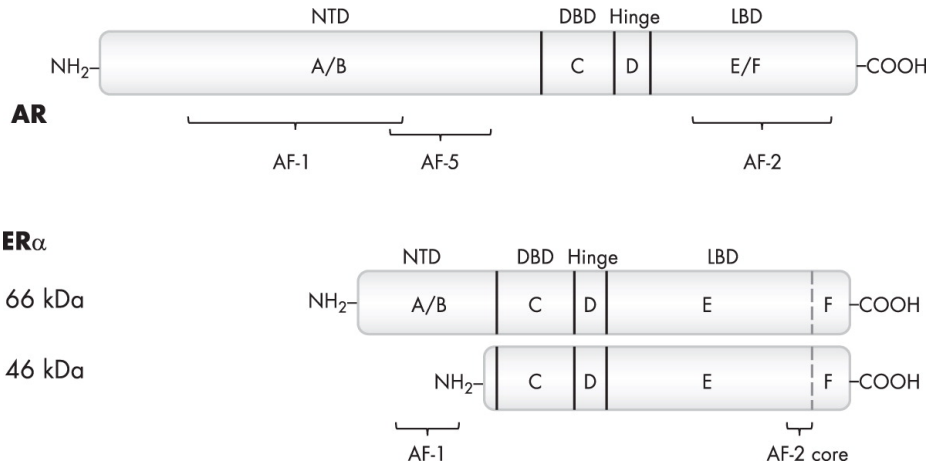

Figure 2. Scheme of the structure of AR and ERα with their different domains: the NTD (A/B domain), the C-domain containing the DBD, the D-domain containing the hinge region, and the C-terminal E- and F-domains containing the LBD. Image from Vanderschueren et al. 2015

Ligand binding to a hydrophobic pocket of the LBD induces conformational changes which reveal a nuclear translocation signal in the DBD. Once in the nucleus, sex steroid receptors bind as dimers to specific DNA sequences which have been identified as androgen response elements (AREs), interact with other modulators in order to elicit a proper transcriptional response.

AREs can be distinguished in canonical AREs, which form a clear palindromic repeat of the 5-AGAACA-3 consensus sequence, and selective AREs with a more relaxed resemblance to direct or palindromic repeats of the same hexamer [63]. A recent study in AR knock-in mice with abrogated binding to selective AREs showed that these elements have a role important in the development and maintenance of sexual dimorphism but not in the musculoskeletal system [64].

Many modulatory proteins (coactivators or corepressor) influence tissue-specific activity of sex steroid receptors on target genes and further research on this respect in bone tissue is requested. A ligand-dependent interface which is involved in N-terminal/C-terminal interactions and binding

of coregulatory proteins and is named activation function 2 (AF-2) has been identified within the LBD domain, while other surfaces for interactions with coactivators, named AF-1 and AF-5 (also called transcriptional activation unit, tau-1 and tau-5) have been identified within the NTD domain of AR [63]. The activation of sex steroid receptors may occur also through ligand-independent mechanisms, through non-classical signaling pathways: the nongenomic (or nongenotropic) pathways imply that the liganded receptor activates second messengers as phosphatases, kinases, cytoplasmatic calcium release through ion channels or nitric oxide synthesis. Nongenomic pathways usually produces immediate effects, which exert within seconds or minutes. AR has been observed to produce nongenomic effects via components of the SRC, ERK, PI3K, and AKT pathways (Figure 3). ERα isoforms may signal at the plasma membrane [65] and transmembrane receptors as G protein-coupled receptor 30 (GPR30; see Section IV.E) have been proposed as mediating nongenomic effects of ERs, although this still remains an hypothesis [66].

In addition to nongenomic pathways, another category of non-classical signaling is mediated by phosphorylation of AF-1 or other regulators which do not imply occupancy of the ligand-binding pocket. Several growth factors (IGF-1, keratinocyte growth factor, epidermal growth factor) have been shown to activate kinases responsible for AR/ER phosphorylation (MAPK, AKT, MEK1/2, and ERK1/2) as far as feedback responses from nongenomic sex steroid signaling. This mode of signaling has been distinctly demonstrated for ERα but is more debatable for AR [67] [68].

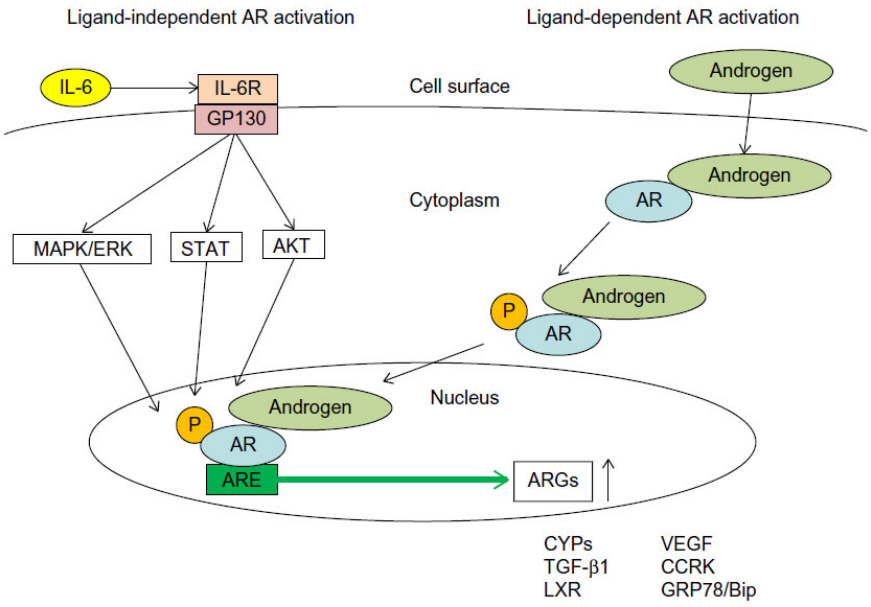

Figure 3. Scheme of AR ligand-independent and ligand-dependent activation and intracellular signaling pathways.

1.3 Androgens effects in bone cells

Proliferation of osteoblastic cells

Androgens may act directly on cells of the osteoblastic lineage and there is some evidence they are responsible for modulatory effects on the vitality, proliferation, differentiation and osteogenic activity of these elements [69].

Some authors suggested the effect of androgen on osteoblast proliferation to be biphasic in nature, with an initial enhancement of mitotic index and a further decrease at longer time-points [70].

However, too many differences between studies (i.e. as regards the cellular model, the type of androgen, the dosage, the observation period…) do not allow a univocal interpretation of data.

It was shown in osteoblast-like cell lines that in strict culture conditions T and non-aromatizable androgens (DHT and fluoxymesterone) promoted ([3H]thymidine incorporation) and cell counts [71] [72]. Stimulation of osteoblast proliferation has been observed in response to DHT also in primary human cells after 24 hours incubation [73].

Time appeared to be a key variable for the study of androgen response, as Kasperk et al. found out that prolonged DHT administration played an inhibitory effect on cell proliferation [74] and similar results were confirmed in other studies both in respose to DHT and to T [75].

A study examining the effects of DHT treatment on proliferation of an osteoblastic cell line stably expressing AR (hFOB/AR-6) observed a consistent inhibition of proliferation at any time-point [76]. Such a finding suggested the importance of AR-mediated signaling pathway, as inhibition of cell proliferation was prevented by coincubation with AR antagonist flutamide both in normal rat calvarial osteoblasts and in AR-transfected MC-3T3 cells.

From a molecular perspective, decreased viability was correlated with a reduction of mitogen-activated (MAP) kinase signaling and a subsequent inhibition of the expression of genes related to cell expansion (i.e. ELK-1) as well as of protein synthesis and extent of phosphorylation.

The investigation of molecular pathways confirmed previous results of time-dependency in cell response to androgens, as after short androgen administration it was reported a stimulation of MAP kinase signaling and AP-1 transactivation, while an inhibition of this pathway was observed after chronic androgen administration. Standing to these results, also nongenomic mechanisms have been proposed to play a role at least in the early response to the treatment [70].

As a counterpart of cell proliferation, the process of programmed cell death and its susceptibility to androgen exposure has been investigated [77].

Apoptosis provides a physiological regulation of tissue homeostasis and *in vitro* it has been shown that as an osteoblastic population progressively differentiates, a portion of cells which reach a mature bone phenotype undergoes apoptosis [78]. Prolonged DHT administration has been related to increased apoptosis both in mature mineralizing cultures and in immature proliferating osteoblasts. Indeed, androgens were responsible for an rise of Bax/Bcl-2 ratio, which was due predominantly to an inhibition of Bcl-2 dependent on functional AR. Reductions in Bcl-2 phosphorylation and protein stability were consistent with previously reported inhibition of MAP kinase pathway activation after androgen treatment. Noticeably, similar effects were observed *in vivo* in model of transgenic mice with overexpression of AR within the osteoblastic lineage [79].

In summary, the increased apoptosis which has been shown in response to androgen treatment does not conflict with increased proliferation, as a strict association between these two processes is characteristic of tissue remodeling and homeostasis [80][81].

Cell apoptosis could be functional to allow deposition and mineralization of bone extracellular matrix, which may clinically influence bone mass and density [82].

Differentiation of osteoblastic cells

Differentiation of osteoblastic cells can be monitored by specific immunohistochemical analyses or by characterization of alkaline phosphatase expression/activity and extracellular matrix components, such as type I collagen, osteocalcin, and osteonectin.

The effects of androgens on expression of these markers have not been extensively described and some controversies are still present in the literature.

Androgen treatment enhanced production of bone extracellular matrix in both primary osteoblasts and osteoblastic cell line TE-89 and increased the expression and activity of alkaline phosphatase activity, thus suggesting an overall differentiative effect [71]. Similar results and dose-dependent responses were reported by the same group in another osteoblastic cell-line (SaOS-2) and in human osteoblasts [83][74].

Androgen-mediated enhancement in type Iα-1 collagen protein and mRNA levels [75][84], and increase in osteocalcin secretion [74] was also shown. Consistently with the production of extracellular matrix elements, androgen stimulation of mineral apposition was reported and time- as well as dose-dependent responses were detected [85][86].

Nevertheless, different research groups reported that androgens either did not affect [84][86] or inhibit cell osteogenic activity [76]. Transgenic mice overexpressing AR within the osteoblast

lineage presented lowered levels of many bone markers both *in vivo* and decreased expression of genes related to bone matrix such as collagen, osterix, and osteocalcin [87].

Standing to these results, it can be concluded that androgens may influence osteoblast differentiation and play regulatory role on bone matrix apposition and mineralization.

However, androgen effects may be restricted to certain conditions (i.e. may be limited to distinct osteoblastic populations as cells of periosteal compartment [87][88].

Effects on osteoclasts

The terminal differentiation of megakariocytes into mature osteoclasts requires interactions with stromal cells of the osteoblastic lineage in the bone marrow and tight control of the receptor activator of nuclear factor κB-ligand (RANKL)/OPG system [89].

Thus, osteoclast differentiation and activity has to be considered as strictly related and dependent on that of osteoblastic stromal cells.

Surgical or chemical castration in rodents primarily lead to changes in osteoblast precursors and increase in bone marrow remodeling, which subsequently is related to increased osteoclastogenesis [90]. Studies in rodent models showed orchiectomy-induced increase of osteoclastogenesis was dependent on osteoblast/preosteoblast-derived signals [91] [92].

Increased levels of OPG were observed after T aministration in cultured osteoblasts and in both the serum and in bone derived from skeletally targeted AR3.6-transgenic male mice [93][94][87].

Additionaly, DHEA treatment enhanced the OPG/RANKL ratio in osteoblastic cells and inhibited osteoclast activity in co-cultures [95].

Whether androgens may directly affect osteoclasts and their precursors is still debated. AR has been detected in osteoclasts [92][96][97][98][99], thus indicating modulation of osteoclast action by androgen. Consistently, inhibition of osteoclast formation and activity has been reported in response to androgen treatment [100][94]. Such results which indicated and influence of androgens on osteoclasts response *in vitro* indicated this cell population as a potential androgen target *in vivo* [71]. To conclude, available evidence is suggestive for androgens effects on osteoclast to be at least in part mediated via cells of the osteoblasts lineage. Further studies are recommended to validate a direct effect of androgens on osteoclasts *in vivo*.

Effects on other cell types in bone compartment

Multiple cell types involved within bone environment have shown to be modulated by androgen exposure and are likely involved in mediating the effects of androgens on the skeleton.

Growth plate chondrocytes - Androgens regulate the proliferation and the activity of plate chondrocytes, as it is well-estabilished the role of these sex steroids in promoting longitudinal bone growth [101]. Biopsies of proximal tibial growth plate cartilage showed the expression of AR by chondrocytes [102], confirming the susceptibility of these cells to androgen classical signaling. *In vitr*o, epiphyseal chondrocytes were shown to respond to androgen exposure with increased creatine kinase and DNA synthesis [103][104]. Androgen-mediated enhancement of [35S]sulfate incorporation into newly synthesized proteoglycan and of alkaline phosphatase activity were also demonstrated [105][106].

Mesenchymal precursors -AR was found to be expressed in different cell types within the bone compartment, such as bone marrow stromal and mesenchymal precursor cells, macrophages, megakaryocytes and endothelial cells [107][108][109]. *In vitro* treatment with androgens may stimulate multipotent cells to differentiate toward the osteoblast or myoblast lineage and inhibit differentiation toward the adipocyte lineage [110]. These effects on stromal differentiation have been supported clinically by the widely observed augmentation of muscle mass in response to androgen administration [111]. The increase of bone mass which has also been reported in some cases may be at least in part a consequence of enhanced mechanical loading, suggesting a strict correlation between muscle and bone modifications in determining musculoskeletal response to androgens [112]
[113].

Others - Androgens may also play an indirect role through modulation of immune cells and hematopoiesis, although additional studies are needed to establish the importance on bone environment.

1.4 Androgens effects in rats

Rat represents one of the best described and most used animal model for the study of androgen effects on bone. Bone morphogenesis in rats is dependent on bone remodeling, which happens through both new-bone apposition and bone resorption, resulting in bone growth and shaping. Endochondral bone formation is responsible of longitudinal growth, whereas periosteal apposition results in radial bone growth. The expansion of medullary cavity is dependent on a balance between endocortical bone resorption and formation.

Young rats are usually first choice models for the study of skeleton growth. In rat skeleton, although, differently from human skeleton growth plates never fully close [114]. This fact doesn't implicate a limit-less bone growth, since it has been shown that growth plate characteristics stabilize by 12 months of age, with lack of further significant changes up to 24 months [115]. This allows aged rats to be used as a model both for bone growth and remodeling. Bone remodeling couples osteoblast and osteoclast actions to occur simultaneously and in the same place, allowing old bones to be reabsorbed and immediately replaced by new bone.

Accordingly, rodent models are an important instrument to study androgen action on skeletal structure and metabolism. Many experimental methods have been conducted to study androgen effect in male and female rats. These include surgical or chemical castration and administration of AR antagonists, ER antagonists, aromatase inhibitors, selective ER modulators (SERMs), and type II 5α-reductase inhibitors, which can be used alone or in combination with sex steroid replacement therapies.

Gonadectomy – Surgical and chemical gonadectomy in rodents represent a widely used procedure to study the effects of sex steroid depletion on bone. Chemical castration, as induced by GnRH agonists, has comparable effects on bone as surgical castration in female rats [116], but no studies have investigated its effect on skeletal in male rats. Although these procedures greatly reduce serum levels of T and E2 in male and female rats, E2 production obtained by transforming adrenal androgens into estrogens after aromatization is still maintained. Gonadectomy results in increased cancellous bone turnover and consequent bone loss, regardless of gender, age, or strain [117][118][119][120][121][122][123][124]. Histomorphometry can detect cancellous bone loss, also peripheral quantitative computed tomography (pQCT) and micr-ocomputed tomography are employed. The augmented cancellous bone turnover in early stage after gonadectomy results in a

simultaneous increase of biochemical markers of bone resorption (e.g., urinary deoxypyridinoline) and formation (e.g., serum osteocalcin) [125][118][126][127][128]. This trend in cancellous bone compartment is analogous of postmenopausal high-turnover osteoporosis [92] and improved the gonadectomized rat model consensus as animal models for osteoporosis. Comparably to postmenopausal osteoporosis in humans, the number of osteoclasts grew after castration in both females [126][129][130] and males rats [121][123][131][132]. Osteoblast hypertrophy, increased mitosis and bone matrix apposition are observed as response to augmented resorption by osteoclasts [117][133]. Results also indicate that bone resorption following orchiectomy tended to be increased than formation even al the level of single bone remodeling units [131][133].

Despite the osteoblastic reaction, resorption exceeds bone formation, resulting in net cancellous bone mass loss in each bone mineral unit. Micro-anatomically a reduction in trabecular number and thickness is observed following orchiectomy [134] [124]. Interestingly, intracortical bone loss is not early observed in gonadectomized rodents, this event may be explained by the absence of Haversian canals in rodent cortical bones. It has been reported that gonadectomized older rats have higher endocortical resorption than younger rats [119][135]. Contrarily to modifications observed in cancellous bone, different effects are observed according to sex in periosteal site and growth plate. The differences appear enhanced in younger rats. Briefly, controversial data suggest periosteal and longitudinal bone formation to be whether increased [125][136][137][138][87] or not [117][118][139] in oophorectomy, but agreement exist about decreased bone formation in the orchiectomy model [123][127][132][87]. The final results of this gonadectomy are sexually dimorphic, with a decrease in bone length and cortical bone volume in males after orchiectomy and a cortical expansion and enhanced bone length in females after oophorectomy.

Gender-dependent differences in cortical bone volume in absence of sex hormones appear to be mainly due to altered bone formation, changes in endocortical bone formation and resorption appear not to be significant [140]. This implicates male skeleton to be more influenced by serum sex hormone insufficiency, compared with female skeleton. Interestingly, cortical bone loss in gonadectomized males seemed not to be dependent on an increased resorption but mainly on a decreased formation, differently from cancellous bone.

Such differences are more evident during puberty and tend to be less important in elder rats, in which longitudinal bone growth is no more affected by orchiectomy. Viceversa, the cancellous bone compartment is significantly affected by one loss after castration, irrespective of age or gender. The intracortical bone compartment is relatively insensitive to surgical castration, whereas at periosteal sites and the growth plates responses to castration appear as gender-dependent dimorphic, mainly in

adolescent rats, with androgens stimulating and estrogens inhibiting periosteal bone expansion [122][119][120][133].

Androgen Replacement Therapy – Androgen Replacement Therapy provided effective bone-sparing results. T administration showed positive effects in preventing bone loss both in gonadectomized rats irrespective of age and gender [121][122][135][141][142]. Sex-dependent differences in response to T treatment have been highlighted within the periosteal compartment, with increases in periosteal bone formation shown in male rats and opposite effects detected in females [143].

Ovariectomized rats presented a bone-sparing response to weak aromatizable androgens, such as DHEA [144] and androstenedione [145]. Evidence that this action is mediated at least in part through the AR and not he ERs has been achieved, thus stressing the importance of androgen action also in female bone [130] [144] [145].

Not surprisingly, also the non-aromatizable androgen DHT played positive effect on bone preservation both in female and male (gonadectomized animals. Nevertheless, DHT appeared to be less effective than T on cortical bone preservation in models of old orchiectomized rats [144][146][147][121][122][143][148].

To conclude, androgens showed positive effects on bone metabolism and homeostasis, with a prevalent action at cancellous bone sites in both sexes and at periosteal sites in males.

1.5 Androgens effects in humans

Male Hypogonadism – Men suffering from hypogonadism present low serum testosterone levels due to many different causes, such as primary gonadal failure, secondary pituitary dysfunction or hypothalamic malfunction. Although there is still some controversies about the levels of T to be considered as physiological, values lower than 150 ng/dL are typically related to signs and symptoms of hypogonadism.

Independently from the primary pathology, hypogonadal men presented a significantly lower bone density than healthy controls, especially at cancellous sites [149][150]. Some studies also showed an association between hypogonadism and decreased calcium absorption and serum 1,25-dihydroxyvitamin levels [151]. An increased risk of fracture has been calculated for men suffering from hypogonadism with or without reduced bone density [152][153][154][155][156][157]. Adolescents with delayed puberty presented lower bone density than healthy controls, which tended to resolution once normal testosterone levels were reached [158][159].

Male hypogonadism also leads to various degrees of estrogen deficiency, which may be only minimally compensated by androgen aromatization [160]. Hypogonadal men presenting low E2 levels are also considered at high fracture risk compared to healthy controls [161][162].

Hypogonadism effects on bone turnover rate have not been completely clarified, with some studies suggesting an increase and others a lowering in bone remodeling activity. Such different findings may be due to the fluctuating androgen levels as well as to the high dynamism of bone tissue, which alternates phases of intense turnover to more steady stages [163][164].

Androgen Replacement Therapy in men– Androgen replacement therapy has been widely employed for the treatment hypogonadism, showing significant improvements in bone parameters.

T administration was shown to increase bone mass in hypogonadal men, as well as calcium intake in pre-adolescents [164][165]. A retrospective study demonstrated T efficacy in restoring bone density in hypogonadal, with results similar to those obtained with other antiresorptive drugs [166], and a small study showed that low-dose testosterone supplementation in young men with delayed puberty caused increased bone density [167]. However, adult men which had been suffering from hypogonadism during puberty did not took advantages from later T administration [168]. Also, the use of T replacement therapy to prevent bone loss in people suffering from Klinefelter's syndrome did not brought encouraging results [169].

A wide trial by Snyder et al. [170] showed that transdermal T therapy increased bone density over 3 years in old men with partial androgen deficiency but this increase was no greater than in the control group receiving calcium and vitamin D supplementation only. However, a significant portion of the men in this study did not suffer from severe form of hypogonadism, being in the lower part of the normal T range. Post-hoc analysis disclosed an interesting correlation between baseline T level and increases in bone mineral density after replacement therapy, revealing that men with low T levels at baseline tended to get more improvements from replacement therapy. Consistently, others showed that transdermal T administration prevented bone loss in hypogonadal men while calcium and vitamin D supplementation were not sufficient to achieve this result [171].

It has to be considered that skeletal improvements in bone density detected after androgen replacement therapy were highly variable in the literature [172][173]. Most available studies were uncontrolled, relatively short-term and involved small and various populations. As instance, research methodology may also have had a significant impact on bone outcomes, since quantitative or peripheral CT assessments generally detected greater increases in bone density than densitometric analyses [172][174].

Despite these difficulties in data interpretation, most authors agree that androgen replacement therapy in hypogonadal men led to a decrease in bone resorption markers, with some of them also reporting correspondent decreases in markers of bone formation possibly preceded by an initial increase phase [172][173][174][175].

Another aspect which deserves consideration is that androgen replacement therapies were generally based on the administration of aromatizable steroids. Thus, it is difficult to distinguish the extent of "pure" androgenic effects from results of androgen aromatization to estrogens [176].

A study investigating the effects of estrogen and/or androgen administration showed that both these sex steroids were important in maintaining bone formation [177]. Consistently, other authors observed that in younger men prolonged treatments with testosterone and estradiol independently regulate bone resorption and formation [178].

A single small short-term investigation displayed that DHT administration in men with low range T levels did not affect serum osteocalcin, a marker of bone turnover [179], but the evidence which can be driven from these observations is poor.

The use of androgen replacement to maintain musculo-skeletal integrity and function in elderly men still represents a matter of debate in the literature [134]. A direct and independent relationship between T levels and incidence of bone fractures presented difficulties to be demonstrated [162] [180][181][182]. However, a large study conducted by Meyer et al. reported that low T was

associated with low-trauma fractures in elderly men during a 5 to 8 years follow-up period [155]. Other authors revealed a similar increased risk in patients presenting low levels of both T and E2 [162][180]. Enhancements of mineral density at lumbar spine sites was reported consequently intramuscular T administration after at least 1 year of therapy, whereas the same outcome measured at hip sites showed higher variability [183][184][185][186][187].
Elderly men with high physiological T levels presented larger cortical bone areas compared with peers with low T levels, thus suggesting a role for androgen in maintaining both bone mass and density [154][188][189][190].

Supra-physiological androgen levels– Recent studies on female-to-male trans-sexuals showed no clear effect of T therapy on bone mineral density [191][192][193]. Nevertheless, a prospective trial observed an increase in cortical bone size as a consequence of T administration [191]. Decrease of bone mineral density was also response after the addition od an aromatase inhibitor to T therapy, thus reminding a combined role of estrogens and androgens in the regulation of bone homeostasis [194].

Androgen Resistance in men –Literature data reported that men suffering from androgen insensitivity syndrome have decreased peripheral bone density measured at spine and hip compared to peer controls [195]. Such a finding suggested a direct action of androgens through AR pathway in determining skeletal consequences. Nevertheless, surgical orchiectomy which is frequently performed in this population as a prevention of testes neoplasms and subsequent androgen replacement therapy may represent a confounding factor for bone evaluations in these subjects.
Moreover, androgen resistance syndrome seemed not to relate with decreased longitudinal growth in men but its effects on periosteal bone apposition remain unknown.

Selective Modulation of Androgen Receptors in men – Literature reports of some studies investigating the effects of AR and ER antagonists or of inhibitors of enzymes involved in sex steroid conversion, such as aromatase or 5α-reductase. Administration of type II 5α-reductase inhibitor finasteride in men suffering from benign prostate hyperplasia was not related to significant changes in bone density at lumbar spine site [196][197], but this finding may be explained with the predominance of type I being expressed by osteoblasts [13].

Treatment of elderly men with the aromatase inhibitor anastrazole has been shown to favor bone resorption [198], while the selective ER modulator raloxifene led benefical effects to men with low basal estradiol levels [199].

Castration – Both chemical castration through GnRH agonist therapy and surgical castration in men led to rapid bone loss and decreased lean body mass and muscle mass. Such an effect was similar to osteopenia in women after menopause or ovariectomy. Bone loss subsequent to androgen depletion was shown to affect all bone compartments, being more severe at cancellous sites, which were provided by wide resorption surfaces [200][201]. A systemic rise of biochemical markers of bone resorption was also evidenced [202].

Skeletal Effects of Androgens in Women – While male model of androgen deficiency and/or exogenous administration have been quite investigated, scarce evidence is currently available about the effects of these hormones specifically on female skeleton. Androgen levels present great variations in women, with basal T values being significantly lower than in men. The predominant androgens in females are the weaker adrenal DHEA-S and androstenedione, which are found in serum in quantities similar to those in men [203].

It has been widely documented the role played by androgens in regulating bone growth and the maintenance of bone density during puberty. Women suffering from polycystic ovary syndrome and subsequent hyper-androgenism at puberty showed a consistently increased bone density compared to peer controls [203][204]. Therapy with selective AR antagonists, GnRH agonist or both for hirsutism, acne, or menstrual irregularities could worsen bone parameters in some of these cases [205][206][207].

Nevertheless, treatment with a combination therapy consisting of GnRH agonist + spironolactone brought to stable values bone density after at least 6 months of therapy [208]. Briefly, the role played by androgen in such a complex syndrome is still not completely clarified.

The consequences of androgen depletion in women after menopause have not been extensively studies, even if this event certainly relates with a consistent decrease in circulating levels of DHEA-S and androstenedione [209]. However, a predominant effect of estrogen decrease in determining the development of osteopenia is generally accepted [210][211].

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CHAPTER 2.

Effects of Stanozolol on osteogenic activity and gene expression in SaOS-2 cells

ABSTRACT

Aim: The aim of this study was to assess the effects of the androgen Stanozolol on osteogenic activity and gene expression in SaOS-2 cellular model. **Materials and Methods:** SaOS-2 viability and proliferation in response to growing doses of Stanozolol (0-1000 nM) was evaluated through Resazurin assay up to 12 days of culture, while cell osteogenic activity was assessed through Alizarin Red S, Calcein Green and von Kossa staining techniques at 6, 12 and 24 days of culture. Expression of genes related to osteogenic differentiation (RUNX2, VDR, BMP1, BMP4, ON, SPP1) was also analyzed with RT-PCR at 12 and 24 days. Statistical analysis was performed using one-way ANOVA with Tukey's correction for multiple comparisons, considering a level of significance $p<0.05$. **Results:** Stanozolol did not significantly affect cell proliferation up to 12 days of culture, while it played a significant influence on SaOS-2 osteogenic activity: indeed, Alizarin Red S, Calcein Green and von Kossa staining showed the presence of rounded calcified nodules, which were increasing both in number and in dimensions over time and depending on the dose of administered Stanozolol. RT-PCR analysis highlighted that Stanozolol may modulate the expression of genes related to osteogenic differentiation in SaOS-2. **Conclusions:** Stanozolol promoted the osteogenic activity and the expression of genes related to osteogenic differentiation in SaOS-2 cells. Further studies are required to test Stanozolol effects on primary cells as well as to investigate molecular pathways of action of this steroid.

Key-words: SaOS-2; Cell differentiation; Osteogenesis; Bone matrix; Calcification; Real-Time Polymerase Chain Reaction; Gene expression; Androgens; Stanozolol

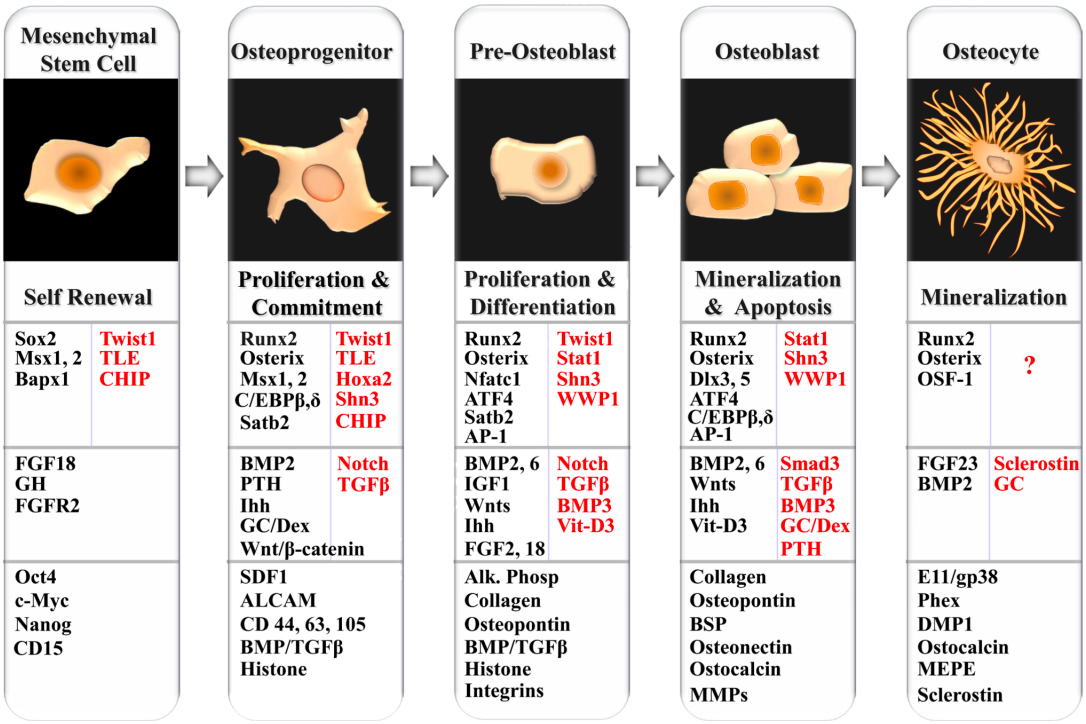
RIASSUNTO

Obiettivo: *Lo scopo di questo studio è di determinare gli effetti dell'androgeno sintetico Stanozololo sull'attività osteogenica e l'espressione genica nel modello cellulare SaOS-2.* ***Materiali e Metodi:*** *La vitalità e la proliferazione di cellule SaOS-2 in risposta a dosi crescenti (0-1000 nM) di Stanozololo sono state valutate con saggio della Resazurina fino a 12 giorni di coltura, mentre gli effetti di questo steroide sull'attività osteogenica cellulare sono stati valutati con colorazioni Alizarin Red S, Calcein Green e von Kossa a 6, 12 e 24 giorni. E' stata inoltre effettuata tramite RT-PCR una valutazione dell'espressione di geni correlati al differenziamento osteogenico (RUNX2, VDR, BMP1, BMP4, ON, SPP1) a 12 e 24 giorni. L'analisi statistica dei risultati è stata condotta con il test Anova a una via, con correzione di Tukey per i confronti multipli, considerando un livello di significatività $p<0.05$.* ***Risultati:*** *Stando ai risultati ottenuti, lo Stanozololo non influenza in modo significativo la proliferazione cellulare delle SaOS-2 in coltura fino a 12 giorni, mentre ha un notevole effetto sull'attività osteogenica delle SaOS-2: le colorazioni Alizarin Red S, Calcein Green e von Kossa mostrano infatti la presenza di noduli calcifici tondeggianti in numero e dimensione crescenti in misura correlata al time-point si osservazione e alla dose di farmaco somministrata. L'analisi dell'espressione genica con RT-PCR fa emergere gli effetti dello Stanozololo sulla modulazione di geni implicati nel differenziamento osteogenico.* ***Conclusioni:*** *Lo Stanozololo promuove l'attività di deposizione di matrice ossea e l'espressione di geni correlati al differenziamento osteogenico nelle cellule SaOS-2. Ulteriori studi sono necessari per testare gli effetti di questo steroide su colture di cellule primarie e per investigare il suo meccanismo molecolare di azione.*

Parole chiave: *SaOS-2; Differenziamento cellulare; Osteogenesi; Matrice ossea; Calcificazione; Real-Time Polymerase Chain Reaction; Espressione genica; Androgeni; Stanozololo*

2.1 Introduction

The process of osteoblast differentiation may be defined by four major stages: lineage commitment, proliferative expansion, synthesis of a peculiar ECM that undergoes mineralization by deposition of hydroxyapatite crystals. Each of these phases is characterized by sequentially expressed genes that support the progression of osteoblast differentiation through transition points [1].



Transcriptional control of osteoblastogenesis from pluripotent mesenchymal cells to terminally differentiated osteocyte. The first raw reported key transcription factors and co-regulatory proteins which promote osteoblast differentiation in black, while negative regulators are indicated in red. Physiologic mediators influencing osteoblast development, including transforming growth factor β (TGFβ), the bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs), Wnt/β-catenin signaling and hormones are also indicated. Secretory molecules, receptor and signal transducer that inhibit osteoblast maturation are highlighted in red. Last row summarize phenotypic marker genes expressed at different developmental stages of osteoblast differentiation. *(Figure drawn from Javed et al., 2010. [1])*

The first step in development of the osteoblast phenotype is the lineage commitment and formation of osteoprogenitors from pluripotent mesenchymal cells (MSC). MSC transition to cells of the osteoblastic lineage is coordinated by several transcriptional regulators, with some of theme serving as "master switches" as RUNX2, OSTERIX and ATF4 [2].

The second stage of osteoblast development is characterized by an extensive proliferation of progenitor cells, which encompasses a wide range of osteogenic cell types from the pluripotent MSC through the more committed chondro-osteoprogenitor and preosteoblast. During this phase, a

number of genes involved in extracellular matrix apposition begin to be expressed (e.g., type-I collagen, fibronectin and some growth factors such as BMP2/TGFβ) [3][4] [5].

The third stage of osteoblast differentiation corresponds to a period of osteoblasts clustering and multi-layering in cultures as well as synthesis and maturation of extracellular matrix. Early markers of the post-proliferative osteoblast phenotype and production of a collagen matrix are expressed (i.e. alkaline phosphatase, collagen-1) [6].

The final stage of osteoblastogenesis is characterized by deposition of minerals in the ECM, concomitant with peak expression of genes considered markers of mature osteoblast, which include bone sialoprotein, osteocalcin, and osteopontin [7][8].

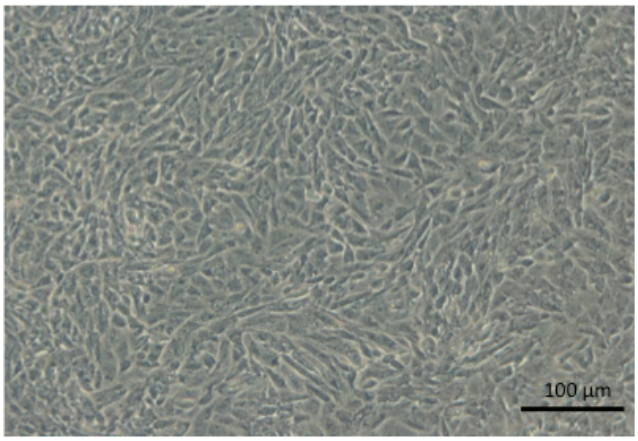
Each of these stages of osteoblast differentiation and osteogenic activity can by influenced by a variety of factors, including vitamin D3, glucocorticoids, parathyroid hormone and sexual steroids [9][10][11]. Particularly, androgen hormones play a significant influence on osteoblast commitment and subsequent deposition of extracellular matrix, as previously noticed (*see Chapter 1).*

In vitro experiments investigating the effects of androgens on different cell types showed that they enhance osteoblasts proliferation and/or inhibit osteoblastic apoptosis [12] [13] [14] [15], while the effects of these hormones on osteogenic differentiation are more controversial. However, most authors supported the notion that androgens stimulate osteoblastic differentiation and extracellular bone matrix apposition [16] [17] [18]. Also, androgens enhance osteoclast apoptosis and [19] inhibit their differentiation [20] and activity [21]. Some of the effects of androgens may be mediated by the regulation of cytokines and growth factors locally expressed in the bone. It has been shown that they can induce TGF-β production [22] [23] and reduce that of pro-inflammatory cytokines, like IL-6 [24] and PGE2 [25]. A cross-talking between androgens and IGF as also been proposed [26]. Recent *in vitro* studies showed that the pro-apoptotic effect induced by oxidative stress may be reverted with the administration of either estrogens or androgens [27] [28]. Stanozolol (ST) is a non-aromatizable synthetic steroid derived from dihydrotestosterone which combines high anabolic and low androgenic action. It was originally developed by Winthrop Laboratories in 1962, and was approved by the FDA for human use. Recent studies showed that ST may promote osteoblastic growth and activity [29], accelerate wound healing [30], increase bone mineral density [31] and improve bone mechanical properties [32].

Many different cellular models have been employed to investigate androgen influence on osteoblast commitment and activity, including both primary cells and cell-lines [33][34].

Among these, SaOS-2 (literally “Sarcoma OSteogenic”) cell line represent a validated option for the study of osteoblastic differentiation and responsiveness to exogenous stimuli [35]. These cells

have been isolated in 1973 by Fogh et al. from the primary osteosarcoma of an 11-year-old Caucasian girl [36]. Morphologically, attached SaOS-2 appear polygonal with few small cell processes and have a mean size of approximately 1/6 of that of osteoblasts. In 1987, Rodan et al. performed a study of SaOS-2 characterization and assessed that these cell line "possess several osteoblastic features and could be useful as a permanent line of human osteoblast-like cells and as a source of bone-related molecules [37]. SaOS-2 cells present the advantages of following the main molecular steps of osteoblast differentiation [38] and have the ability “to deposit a mineralization-competent extracellular matrix" [39]. Immunocytochemical assays revealed that SaOS-2 cells express osteoblast-like markers as osteocalcin (OC or BGLAP) and osteopontin (OPN or SPP1) [37], and studies of genes revealed these cells express RUNX2 and different BMPs isoforms (1 to 7) involved in osteoblast differentiation and function [40][41] [42][43]. SaOS-2 also present the advantages of low cost, worldwide availability and the possibility to obtain large amounts of cells in short time. Literature data provided evidence on SaOS-2 responsiveness to steroid stimulation; particularly, it has been shown that glucocorticoid Dexamethasone suppresses growth and promotes osteogenic differentiation (i.e. by stimulation the expression and the activity of alkaline phosphatase, a differentiation marker of the osteoblasts) [44] [45] [46] [47]. Standing to these observations, we considered Dexamethasone as a control for SaOS-2 responsiveness in our experimental setting.



Representative image of SaOS-2 cells. Optical microscopy, 10X magnification.

The aim of this study was to assess the effects of the androgen ST on osteogenic activity and gene expression in SaOS-2 cells.

2.2 Materials and Methods

The aim of this study was to investigate the effects of a differentiation medium supplemented with ST on the proliferation, osteogenic capacity and gene expression of SaOS-2 cells.
In order to check SaOS-2 responsiveness to differentiative stimuli, administration of the corticosteroid Dexamethasone (DX) was also carried out, since it is known by the literature that it may induce differentiative pathways in osteoblastic cells [48][37].

Stanozolol and Dexamethasone solutions

ST and DX powders were separately weighted and dissolved in absolute ethanol (ETOH) so that a 1000X stock solution was prepared. Sequential dilutions were further performed and final concentrations of 1 nM, 10 nM, 100 nM, 500 nM and 1000 nM of steroids were obtained. The final ETOH concentration which was used on cultured cell resulted 0.1%

Cell culture

SaOS-2 cells ranging from 8 to 12 passages were seeded with a density of 1×10^4 cells/cm^2 into 6-wells and 24-wells plates, using respectively 2 ml and 500 µl of DMEM-low glucose with 10% fetal bovine serum (FBS), penicillin (100 µg/ml), streptomycin (100 µg/ml) and L-glutammin (4 mM). After 24 hours, base culture medium was substituted with a differentiation medium consisting in DMEM-low glucose completed with ascorbic acid (50 µg/ml), L-prolin (260 µM) e β2-glicerophosphate (10 mM). ST and DX were respectively added to wells in concentrations of 0, 1, 10, 100, 1000 nM, with 0 nM concentration consisting in 0.1% ETOH and serving as a control. Culture medium was changed every three days.

Resazurin assay

After 1, 2, 4. 6, 8, 10 and 12 days of culture, Resazurin test was performed to indirectly assess cell vitality and base function. Briefly, 200 µl of Resazurin were dispensed to each well and the whole plate was incubated for 1 hour at 37°C of temperature. 120 µl of Resazurin/well were then transferred to a 96-wells reading plate and fluorescence was detected with an Enspire spectrofotometer (Perkin Elmer, Waltham, Massachusetts, USA) set on a wavelength of 570 nm.

Culture staining

After either 6, 12 or 24 days, cells lying in 24-wells plates were treated either with Alizarin Red S

staining (AR) or with Von Kossa (VK) and Calcein Green staining.

Alizarin Red S staining: Cells were washed three times with PBS and fixed by adding 200 µl paraformaldehyde /well for 30 minutes. Then, 200 µl of Alizarin Red (0,5g/50ml, pH 4,2) were added to each well and the whole plate was maintained under slight oscillation at +4°C temperature for 45 minutes in a dark environment.

Semi-quantitative analysis of AR can be performed by determining OD_{405} values of a set of known Alizarin Red concentrations and comparing these values to those obtained from unknown samples. Each well was treated with 400 µl acetic acid 10% and incubated for 30 minutes with shaking. Cells were gently scraped from the plate and transferred to a 1.5 mL microcentrifuge tube. After vortexing vigorously for 30 seconds, samples were heated to 85°C for 10 minutes. Then, they were transferred to ice for 5 minutes and centrifuged at 20000 rpm for 15 minutes. When centrifugation was finished, pH was adjusted to 4.1-4.5 by adding 10% ammonium hydroxide. After calibration and setting of a standard curve with serial dilutions of Alizarin red, samples fluorescence was detected with an Enspire microplate reader (Perkin Elmer, Waltham, Massachusetts, USA) set on a wavelength of 405 nm.

Von Kossa staining: After fixation, 200 µl of silver nitrate (AgNO3, 5% solution) were put into each well and the whole plate was maintained exposed to environmental light for 60 minutes. 200 of Sodium Tiosulphate 5% were then added and a washing with double-distilled water has been performed. Stained samples were observed with phase contrast microscopy for qualitative assessment of calcified nodules.

Calcein Green staining: 24 hours before the end of the experimental period, 2 µl of Calcein Green (10 mg/ml) were added to each well. At the end of the experimental period, samples were treated with 500 µl of acetic acid 10% dabbed with ammonium hydroxide pH 7.0. The whole plate was laid under slow oscillation for 20 minutes and was then inserted on a ultrasonic bath for 15 minutes. Each well was then washed three times with PBS. Semi-quantitative analysis of Calcein Green fluorescence was detected with an Enspire micro-plate fluorescence reader (Perkin Elmer, Waltham, Massachusetts, USA) set on a wavelength of 512 nm, as described elsewhere [49].

Gene expression analysis

At 12 and 24 days of culture, RNA was extracted from cells lying in 6-wells plates for gene evaluations with RT-PCR.

RNA extraction was performed using GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following manufacturer's instructions. 1 µg RNA/sample was retrotranscripted to cDNA

(Reverse Transcription System, Promega kit): briefly, 0.5 µl of a mixture of random hexamer oligonucleotide primers (dN6, 500 µg/µl) and 1 µl of deoxynucleotide solution (dNTP 10 mM) were added to each RNA sample. Consecutive incubations at 65°C for 5 minutes, then at 4°C for 5 minutes were carried out. After that, each sample was dispended 9.4 µl of a reaction mix containing DTT 5 mM, buffer 1X First strand buffer, Rnase OUT 40 U/µl and Superscript III RT 200 U/µl. To complete the retrotranscription, samples were heated at 25°C for 10 minutes, then at 50°C for 50 minutes and lastly at 70°C for 15 minutes.

Specific primers for target genes related to osteogenic differentiation were designed using Primer-Blast designing tool (National Center for Biotechnology Information, NCBI) (Table 1) and RT-PCR were performed (Rotor Gene TM 3000 5.0.06 version, Corbett Reaserch).

cDNA were amplified for 40 cycles in a mixture composed by 2X Platinum SYBERGreen qPCRsupermix-UDG (Invitrogen), 5 pmol specific primers and RNase-free water. Gene amplification was carried out through denaturation at 95°C for 15 seconds followed by annealing phase at 55°C to 58°C (depending on the examined gene) and an extension phase at 72°C for each cycle. Gene amplification was carried out through denaturation at 95°C for 15 seconds followed by annealing phase at 55°C to 58°C (depending on the examined gene) and an extension phase at 72°C for each cycle. Melting curve was assessed for each amplified gene and data were analyzed with relative standard curve method as described elsewhere [50]. Data normalization was carried out relatively to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was found to be homogenously expressed in all tested conditions.

Statistical analysis

Growth curves were analyzed with Boltzmann sigmoidal function and a comparison of curve fits was performed in order to verify the null hypothesis of one curve fitting all data sets and the alternative hypothesis of different curves for each culture condition. Analysis of cell differentiation and osteogenic activity was carried out with one-way ANOVA and Tukey's post-test. Linear regression analysis was performed to assess variations among different time-points. $p<0.05$ was considered as the level of statistical significance. Graphs were obtained with GraphPad Prism 6.0 software.

2.3. Results

Resazurin assay

Optical microscopy showed a typical polygonal shape of SaOS-2, which tended to become slightly elongated once they reached confluence. Resazurin assay revealed a growth pattern of cells which perfectly fitted Sigmoidal Boltzmann curve up to 10 days of culture (DMEM low: r^2=0.94, ETOH 0.1%: r^2=0.96, ST1: r^2=0.98, ST10: r^2=0.93, ST100: r^2=0.99, ST1000: r^2=0.98, DX1: r^2=0.99, DX10: r^2=0.96, DX100: r^2=0.99, DX1000: r^2=0.99), while at 12 days of culture an overall decrease in cell vitality was detected independently of the culture condition. This finding was probably due to the tendency of SaOS-2 cells to form masses once they have reached confluence and consequently to detach from the culture plate.

Comparison of curves fits did not allow to reject the null hypothesis of one curve fitting all data sets (p=0.8), thus indicating a superimposable growth pattern of SaOS-2 under all the tested conditions up to the end of the experimental period.

A graphic representation of data is reported in Figures 1 and 2.

Culture staining

Alizarin Red S staining confirmed capacity of SaOS-2 cells to produce a mineralized matrix. Cultured wells presented calcified granules with a roundly shape which were progressively increasing both in dimensions and in number depending on the concentration of the administered steroid and on the observation time-point.

Cells treated with ST revealed the presence of areas with mineralized bone matrix since the earlier observation time-point, which tended to increase with growing doses and reached a peak with the 1000 nM concentration (fold change vs control: 2.235 ± 0.559, $p < 0.05$). At 12 days, plate wells administered with ST appeared consistently more filled with calcified granules compared to controls at all the tested doses (fold change vs control ST 1nM: 1.920 ± 0.085; ST 10nM: 1.955 ± 0.092; ST 100 nM: 2.060 ± 0.113; ST 500 nM: 2.105 ± 0.163; ST 1000 nM: 2.170 ± 0.014, $p < 0.01$). A similar outcome was detected at 24 days (fold change vs control ST 1nM: 2.025 ± 0.191; ST 100 nM: 2.130 ± 0.240; ST 500 nM: 2.250 ± 0.014, ST 1000 nM: 2.200 ± 0.566, $p< 0.05$) (Figure 3, A-B).

Alizarin Red S staining of samples treated with DX confirmed the responsiveness of SaOS-2 cells to differentiative stimuli. As it was observed with ST, at 6 days only the highest dose of steroid (DX

1000 nM) could promote a significantly higher mineralization vs untreated control (fold change vs control: 2.170 ± 0.040, $p < 0.005$). At 12 days, DX doses ranging from 10 to 1000 nM revealed the presence of more Alizarin Red S staining vs controls (fold change vs control DX 10nM: 1.765 ± 0.148; DX 100 nM: 1.620 ± 0.127; DX 500 nM: 1.630 ± 0.113; DX 1000 nM: 1.900 ± 0.156, $p < 0.05$). The same range of doses was effective at 24 days (fold change vs control DX 10nM: 2.650 ± 0.099; DX 100 nM: 3.275 ± 0.304; DX 500 nM: 3.270 ± 0.311; DX 1000 nM: 3.650 ± 0.028, $p < 0.05$) and a remarkable increase of mineralization was detected at this time-point (Figure 4, A-B).

Von Kossa staining and Calcein Green labeling provided a further evidence of SaOS-2 osteogenic properties and showed a superimposable distribution pattern of mineralized areas.

Semi-quantitative analysis of Calcein Green fluorescence in ST-treated cells revealed a deposition of calcium phosphates which tended to grow with a dose-dependent trend at 6 and 12 days. At 6 days significant differences between test and control samples were not yet detectable (data not shown), while at 12 days ST doses ranging from 10 nM to 1000 nM doses produced a consistent effect over controls (fold change vs control ST 1nM: 2.001 ± 0.413, $p>0.05$; ST 10nM: 2.452 ± 0.190, $p<0.05$; ST 100 nM: 3.102 ± 0.157, $p<0.005$; ST 500 nM: 3.201 ± 0.131, $p<0.005$; ST 1000 nM: 4.103 ± 0.158, $p < 0.01$). At 24 days, all ST concentrations produced significant effects over controls (fold change vs control ST 1nM: 1.747 ± 0.098; ST 10nM: 1.793 ± 0.040; ST 100 nM: 2.074 ± 0.040; ST 500 nM: 1.674 ± 0.041; ST 1000 nM: 1.739 ± 0.041; $p<0.001$). The dose-dependent trend observed at earlier time-points was no more visible, probably due to the massive calcification which had occurred (Figure 5, A-B).

Calcein Green analysis of DX-administered cells revealed a similar outcome to that of ST, thus confirming the efficacy of steroid administration in enhancing the deposition of bone mineral. Even in this case, at 6 days a dose-dependent effect was visible although it was not sufficient to reach statistical significance over controls (data not shown). At 12 days, increasing DX doses produced correspondent significant effects over controls (fold change vs control DX 1nM: 2.254 ± 0.138, $p>0.05$; DX 10nM: 2.523 ± 0.293, $p<0.05$; DX 100 nM: 2.919 ± 0.145, $p<0.005$; DX 500 nM: 2.888 ± 0.316, $p<0.005$; DX 1000 nM: 5.434 ± 1.184, $p < 0.01$). At 24 days, all DX concentrations showed a superimposable consistent effect over controls (fold change vs control DX 1nM: 2.002 ± 0.013; DX 10nM: 1.856 ± 0.014; DX 100 nM: 1.932 ± 0.013; DX 500 nM: 1.928 ± 0.014; DX 1000 nM: 1.831 ± 0.014; $p<0.001$) (Figure 6, A-B).

Gene expression analysis

The analysis of the expression of genes related to osteogenic differentiation revealed differences depending both on the time-point (either 12 or 24 days) and on the concentration of tested steroid (Figures 7 and 8).

RUNX2: At 12 days of observation, the expression of Runx2 revealed to be increased at growing concentrations of ST, with significant differences vs controls of doses ranging from 10 to 1000 nM (fold change vs control ST 10nM: 1.701 ± 0.182, $p<0.05$; ST 100 nM: 1.847 ± 0.226, $p<0.005$; ST 500 nM: 2.061 ± 0.143, $p<0.001$; ST 1000 nM: 2.535 ± 0.295, $p < 0.001$). A similar pattern was detected at 24 days (fold change vs control ST 1nM: 2.025 ± 0.191; ST 100 nM: 2.130 ± 0.240; ST 500 nM: 2.250 ± 0.014, ST 1000 nM: 2.200 ± 0.566, $p< 0.05$).

A certain increase in Runx2 expression was also detected with DX treatment, which reached statistical significance at the doses of 1 and 1000 nM (fold change vs control DX 1nM: 1.867 ± 0.226; DX 1000nM: 2.421 ± 0.265, $p<0.001$) at 12 days.

At 24 days, Runx2 expression showed to be significantly increased vs control only at the lowest ST concentrations (1 and 10 nM) used (fold change vs control ST 1nM: 1.514 ± 0.234, $p<0.05$; ST 10 nM: 1.786 ± 0.201, $p<0.005$). A tendency to decrease at the highest ST concentrations (500, 1000 nM) was also detectable, although without any statistical significance.

A comparable pattern of Runx2 expression was evident in samples treated with DX at this time-point.

VDR: The expression of VDR showed a consistent increase vs controls with the administration of the highest ST concentrations (fold change vs control ST 10nM: 2.037 ± 0.543, $p<0.05$; ST 100 nM: 2.388 ± 0.427, $p<0.001$; ST 1000 nM: 2.255 ± 0.247, $p < 0.001$) at 12 days.

At 24, all tested ST doses were associated with significantly higher VDR expression vs controls (fold change vs control ST 1nM: 2.158 ± 0.070; ST 10 nM: 2.622 ± 0.179; ST 500 nM: 2.770 ± 0.090, ST 1000 nM: 2.901 ± 0.073, $p< 0.001$).

A superimposable pattern was observable with DX treatment, with an rise of VDR expression at the highest DX doses at 12 days (fold change vs control DX 100nM: 2.295 ± 0.125, $p<0.005$; DX 1000nM: 2.411 ± 0.350, $p<0.001$) and an overall increase of this gene at 24 days (fold change vs control DX 1nM: 3.274 ± 0.024; DX 10 nM: 3.875 ± 0.265; DX 100 nM: 3.244 ± 0.035, DX 500 nM: 2.964 ± 0.117, ST 1000 nM: 3.377 ± 0.073, $p< 0.001$).

BMP-1: BMP-1 expression was not significantly affected by ST treatment at any of the tested concentrations both at 12 and 24 days observational period ($p>0.05$).

On the other side, DX strongly decreased BMP-1 expression with similar effects after 12 and 24 days of treatment. DX-induced inhibition of BMP-1 was observable starting from the dose of 10 nM (fold change vs control at 12 days: 1.116 ± 0.074, $p<0.05$; fold change vs control at 24 days: 0.770 ± 0.021, $p<0.005$) to that of 100 nM (fold change vs control at 12 days: 0.850 ± 0.008, $p<0.005$; fold change vs control at 24 days: 0.414 ± 0.025, $p<0.001$), 500 nM (fold change vs control at 12 days: 0.803 ± 0.020, $p<0.001$; fold change vs control at 24 days: 0.243 ± 0.005, $p<0.001$) and 1000 nM (fold change vs control at 12 days: 0.869 ± 0.128, $p<0.005$; fold change vs control at 24 days: 0.308 ±0.009, $p<0.001$)

BMP-4: The highest concentration of ST (1000 nM) was related to a decrease in BMP-4 expression at 12 days observational period (fold change vs control: 0.663 ± 0.016, $p<0.005$). DX treatment at the doses of 100 nM (fold change vs control: 0.328 ± 0.049, $p<0.05$) and 500 nM (fold change vs control: 0.280 ± 0.036, $p<0.005$) was associated with a similar inhibitory effect at this time-point.

ON: The expression of ON gene was increased in response to the highest ST dose of 1000 nM (fold change vs control: 3.204 ± 0.443, $p<0.001$) at 12 days. A similar effect was observed with DX treatment at the concentrations of 10 nM (fold change vs control: 4.199 ± 0.998, $p<0.001$), 100 nM (fold change vs control: 3.461 ± 1.217, $p<0.001$) and 1000 nM (fold change vs control: 4.251 ± 1.268, $p<0.001$).

SPP1: The expression pattern of SPP1 showed variations depending on the observational period, with no significant differences in test groups over controls at 12 days ($p<0.05$) and a consistent induction observable at 24 days for all the tested concentrations of both ST (fold change vs control ST 1nM: 2.691 ± 0.145; ST 10 nM: 2.401 ± 0.416; ST 100 nM: 2.540 ± 0.197; ST 500 nM: 2.680 ± 0.166, ST 1000 nM: 2.331 ± 0.048, $p< 0.005$) and DX (fold change vs control DX 1nM: 2.215 ± 0.156; DX 10 nM: 2.228 ± 0.016; DX 100 nM: 2.302 ± 0.342; DX 500 nM: 2.654 ± 0.231, DX 1000 nM: 2.817 ± 0.210, $p< 0.05$).

2.4 Discussion

The present research investigated the effects of a differentiation medium treated with different doses of ST on proliferation, osteogenic capacity and differentiative response of SaOS-2 cells.

Growing evidence suggests that androgens act directly on bone cells playing a complex regulatory function [51]. However, to our knowledge only one old study reported ST effects on the proliferation of bone cells [52]. Here it is described that ST (10^{-8} M to 10^{-6} M) stimulated the incorporation of [3H]thymidine into DNA and increased the growth of primary bone cells up to 15 days of culture. A biphasic androgen regulation of proliferation of primary osteoblasts has later been suggested, with enhancement following short or transient treatment (up to 5 days) but significant inhibition following at longer time-points [53]. Such a biphasic pattern has been observed as a response to ST administration in primary growth plate chondrocytes, with a proliferation peak after 2 days of culture and a later decrease in cell number [54].

However, we must consider that growth behavior and androgen responsiveness of primary cells may be different from that of cancer cell lines, such as SaOS-2 cells we used in this study.

Previous authors observed the effects of androgenic steroids on cell-lines and reported positive effects of T at the doses of 10^{-10} M and 10^{-9} M on proliferation of SaOS-2 cells after 48 hours [55]. Also, DHT at concentrations ranging from 10^{-12} M to 10^{-8} M was shown to enhance the mitogenic activity of osteosarcoma cell line MG-63 [44], MC3T3-E1 [57] and osteoblast-like TE89 [58] at early time-points (2 to 4 days of culture).

Standing to our preliminary assay, ST treatment at the doses of 1 to 1000 nM did not affect the growth pattern of SaOS-2 cells up to 12 days of culture. It is interesting to observe that neither DX, which was earlier known as a regulator of growth and differentiation of SaOS-2 cells [37], gave any effects on the proliferation of our cells.

This results may be due to the specific characteristics of the cells used in our experimental setting. Indeed, various SaOS-2 subpopulations have been identified, which responded differently one from the other to proliferative and differentiative stimuli [59].

Also, the phenotypic stability of SaOS-2 may be affected by the number of passages they have undergone: it has been noticed that higher passage SaOS-2 exhibited higher proliferation rates and lower specific alkaline phosphatase activities, though mineralization was significantly more pronounced in cultures of late passage cells [38].

Such a report is consistent with our results of an overall high proliferation rate of SaOS-2 ranging from 8 to 12 passages as well as an high mineralizing activity.

It is widely documented that SaOS-2 cells are provided with osteogenic capacities and under opportune culture conditions they produce an extracellular matrix containing calcium and inorganic phosphate, which progressively accumulates to form hydroxyapatite crystals and undergoes mineralization [39].

A proper differentiation medium is necessary to allow this process and ascorbic acid, β-glycerophosphate, and dexamethasone (DX) represent some the most common compounds known to favor the expression of the osteoblastic phenotype in several bone cell systems [60] [61][62].

Although DX effects may be dependent upon the concentration, the time and duration of exposure and the specific system investigated, most studies showed DX to favor the development and the maintenance of an osteoblastic phenotype [63] [64] [65] [66].

Some authors hypothesized differential effects in short- and and long-term DX applications and suggested an initial increase in osteoblast differentiation followed by a further reduction of mineralization in primary cells [67]. Anyhow, SaOS-2 cells appeared to be favorably responsive to DX treatment for over 2 weeks of treatment [68].

Androgen treatment has also been shown to stimulate mineral accumulation in a time- and dose-dependent manner both in primary cells and in osteosarcoma cell-lines [69] [70]. However, as far as we know there is no study which analyzed the *in vitro* effects of ST on SaOS-2 cells.

In our study, we characterized the apposition of bone mineral in SaOS-2 cells with different techniques: Alizarin Red S, Calcein Green and von Kossa staining.

Alizarin Red S, an anthraquinone dye, has been widely used to evaluate calcium deposits in cell culture, as it reacts specifically with calcium cation to form a chelate. The Alizarin Red S staining is quite versatile because the dye can be extracted from the stained monolayer of cells and readily assayed [71].

Calcein Green is a fluorescent-imino-diacetic acid complex that fluoresces bright green when combined with calcium. A recent study showed that calcein bond to hydroxyapatite can be exploited for *in vitro* assessments [72] and a direct method for measuring relative changes in mineral content of cell monolayers by calcein detection with fluorescence microscopy has been suggested [49].

The von Kossa method is based on the binding of silver nitrate ions to the anions (phosphates, sulfates, or carbonates) of calcium salts and the reduction of silver salts to form dark brown or black metallic silver staining. However, a recent report observed that von Kossa staining alone may not be

considered sufficient to validate in vitro bone formation [73]. Thus, in this study we used this staining only for qualitative verifications.

Alizarin Red S allowed to detect the effect of steroid treatment on bone matrix deposition which were significantly higher vs controls. A consistent apposition of bone nodules was observed with all staining techniques especially after 12 and 24 days of culture. The addition of ST and DX to the medium notably increased the deposition of mineral bone matrix and highlighted the advantages of treating cells with steroids over the only use of a differentiation medium. It is noticeable that at 12 days observation period all tested doses showed a similar effect with Alizarin Red S quantification technique, while a dose-dependent effect different was detected with Calcein Green staining. These differences may point out a major sensitivity of Calcein Green technique vs Alizarin Red S. Nevertheless, both Alizarin Red S and Calcein Green did not reveal any differences between treatments effect at 24 days observation, when a homogeneous and abundant calcification was present in all samples.

Calcein Green provided the further advantage of not imply sample fixation or irreversible solubilization of the apposed mineral matrix and allow to perform different assays over the same sample. In the present study, we exploited this opportunity by treating the same samples both with Calcein Green and with von Kossa staining.

Von Kossa staining confirmed Calcein Green finding, as microscopic observation revealed a distribution pattern of calcified granules which was superimposable to those detected with Calcein Green.

RT-PCR analysis revealed a modulatory function played both by ST and DX on the expression of genes related to osteogenic differentiation.

RUNX2, ON and SPP1 have been widely recognized as important regulators of osteoblast differentiation and apposition of a mineralizing extracellular matrix in SaOS-2 cells [74] [75] [76] [77].

The detection of RUNX2 mRNA in control samples and confirmed previous observations which described a constitutive expression of this gene in SaOS-2 cells [40][78]. In addition, we found out that RUNX2 expression may be modulated by steroid treatment, as ST administration was related to a dose-dependent increase of RUNX2 expression and DX also played a consistent modulation on this gene, mainly at the highest concentrations.

The increase in ON ad SPP1 expression in response to DX had been previously documented in bone cell systems and was related to osteogenic differentiation [79] [80]. Our results confirmed these observations, as increases in ON and SPP1 were detected respectively at 12 and at 24 days of DX

treatment. A similar response was observed in response to ST administration, which first demonstrated the modulatory activity of this androgen on genes related to osteogenic function.

Interestingly, the expression pattern of RUNX2 and SPP1 showed to be inversely correlated, with a marked increase of SPP1 observed contemporary with a decrease of steroid effects on RUNX2 expression. This finding may indicate a genetic switching from 12 to 24 days, as it has been observed that in SaOS-2 cells RUNX2 repressed SPP1 gene expression and the induction of SPP1 expression during normal human osteoblast differentiation has been previously related to a decrease of RUNX2 [81].

Another gene whose expression was strongly enhanced by DX and ST treatment in our study was VDR. This gene encodes the nuclear hormone receptor for vitamin D3 and its expression by SaOS-2 cells has been described elsewhere [82]. Previous studies identified the activation of VDR as a central element for SaOS-2 differentiation [83] [84], while VDR inhibition was associated to a decrease of SaOS-2 osteoblastic commitment [85].

Our results also pointed out different effects of ST and DX on BMPs expression. It is known from the literature that SaOS-2 cells express BMP1 and BMP4 [43], which play a positive role on their osteogenic activity [86] [87]. In this study, DX inhibited BMP1 expression both at 12 and 24 days and BMP4 expression at 12 days, while these effects were not detected in response to ST. Thus, we may suppose that ST and DX act through at least in part different mechanisms in regulating osteogenic properties of SaOS-2 cells.

Taken together, our results indicated that both ST and DX promoted the osteogenic commitment in a SaOS-2 cell system. These steroids may exert their action through different molecular pathways, which require to be further investigated.

2.5 Conclusions

This study aimed to examine the effects of a differentiation medium treated with different doses of ST on proliferation, osteogenic capacity and differentiative response of SaOS-2 cells.

Our preliminary Resazurin assay demonstrated that neither ST nor DX treatment in a range of doses from 1 to 1000 nM produced a significant effect on the proliferation of SaOS-2 cells. This finding may be due to the specific characteristics of the cell population used in this study.

The principal investigation confirmed the osteogenic capacity of SaOS-2 cells and highlighted the advantages of treating cells with steroids over the only use of a differentiation medium. Alizarin Red S, Calcein Green and von Kossa staining showed the presence of rounded calcified nodules, which were increasing both in number and in dimensions over time and depending on the dose of steroid which was administered. Semi-quantitative analysis of dissolved Alizarin Red S and fluorescent Calcein Green validated these observations, while the use of von Kossa as a double staining with Calcein Green served to qualitatively confirm the localization of bone mineral areas.

RT-PCR analysis highlighted that both ST and DX modulate the expression of genes related to osteogenic differentiation in SaOS-2.

Taken together, our results indicated that tested steroids promoted the osteogenic commitment of SaOS-2. Further studies are required to clarify the effects of gene modulation on cell function as well as to investigate ST molecular pathways of action.

2.6 Tables and Figures

Table 1.

Gene	Abbreviation	Primer sequences (Forward and Reverse)
Runt-related transcription factor 2	RUNX2	5'-CCA GGC AGG CAC AGT CTT C-3' 5'-GTC AGA GGT GGC AGT GTC ATC-3'
Vitamin D Receptor	VDR	5'-CGC ATC ACC AAG GAC AAC C-3' 5'-CTG GCA GAA GTC GGA GTA GG-3'
Bone Morphogenetic Protein-1	BMP1	5'- TGT GAC AAG TTC GGC ATTG-3' 5'- CTC CTG AGG CTC CAT CTTC-3'
Bone Morphogenetic Protein-4	BMP4	5'- AGC CTT TCC AGC AAG TTT GTTC-3' 5'- CCA TCA GCA TTC GGT TAC CAG-3'
Alkaline Phosphatase	ALP	5'-TGA TGT GGA GTA TGA GAG TGAC-3' 5'-TGA AGT GGG AGT GCT TGT ATC-3'
Osteonectin (Secreted Protein Acidic And Rich In Cysteine)	ON (SPARC)	5'-GCA TCA AGC AGA AGG ATA-3' 5'-AAT AGT TAA GTT ACA GCT AAG AAT-3'
Osteopontin (Bone Sialoprotein I, Early T-Lymphocyte Activation 1)	SPP1 (OPN, BNSP, ETA-I)	5'-CTC CAT TGA CTC GAA CGA CTC-3' 5'-CGT CTG TAG CAT CAG GGT ACT G-3'
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	5'- TGT TCC TAC CCC CAA TGT GT-3' 5'-GGT CCT CAG TGT AGC CCA AG-3'

Figure 1.

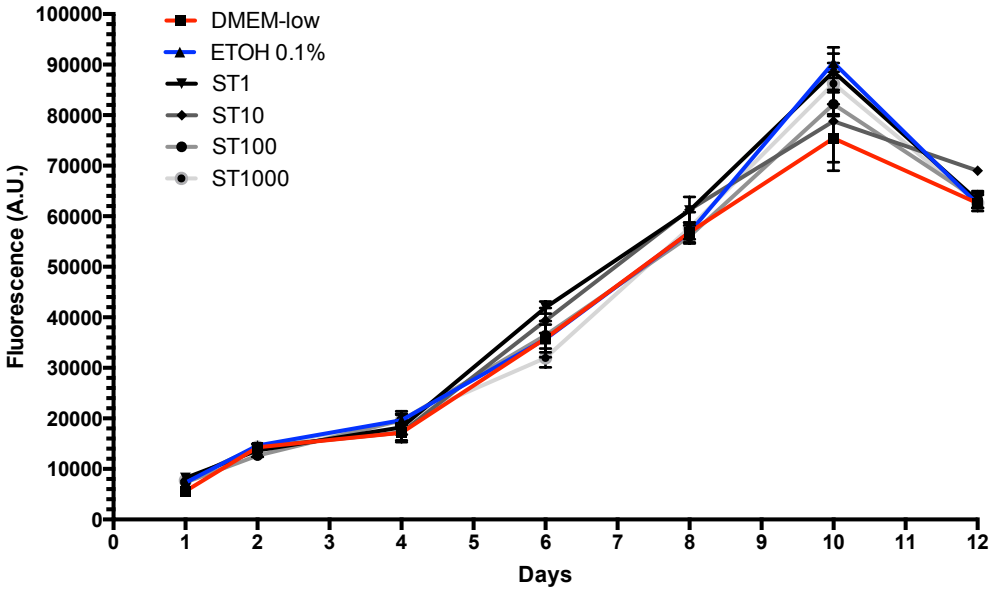


Figure 2.

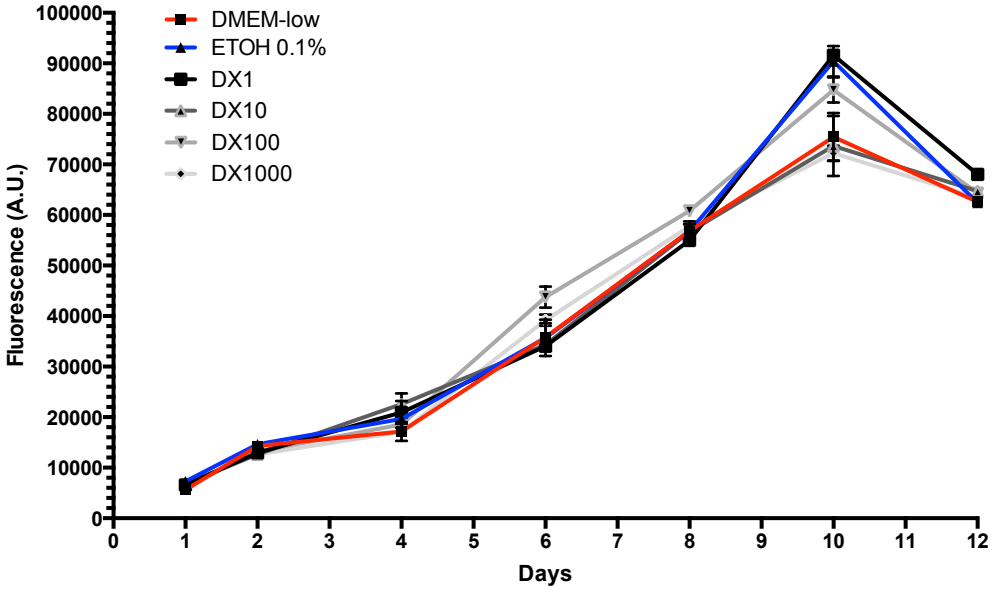
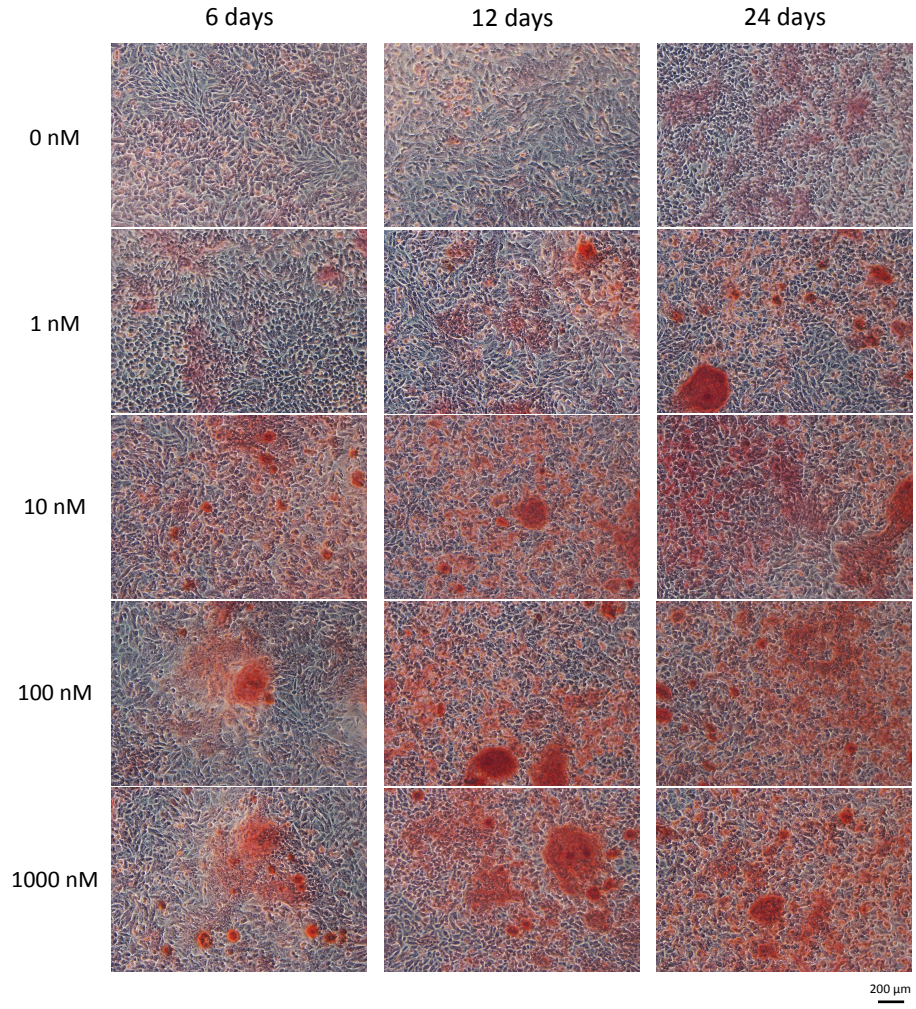


Figure 3.

A.


6 days
12 days
24 days
0 nM
1 nM
10 nM
100 nM
1000 nM
200 µm

B.

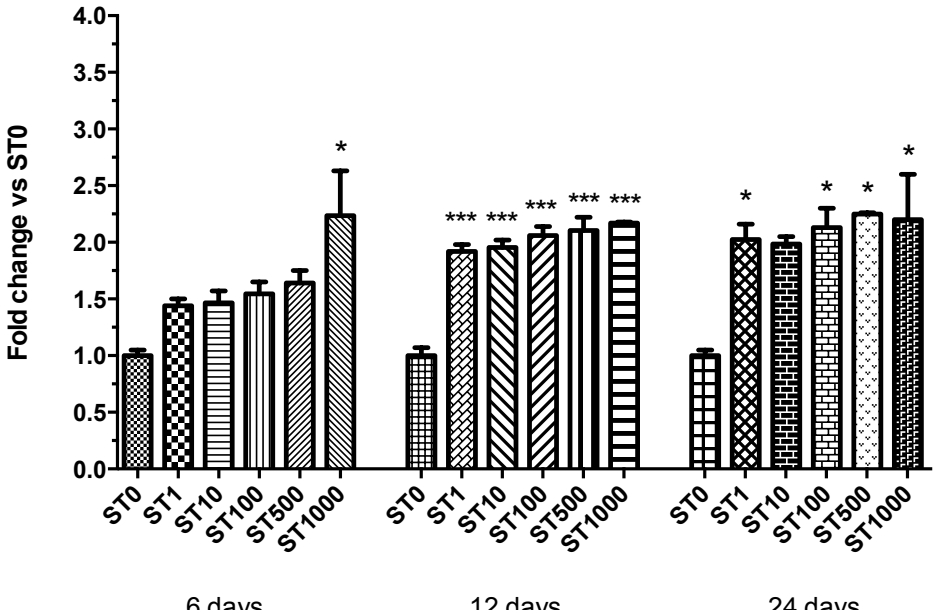
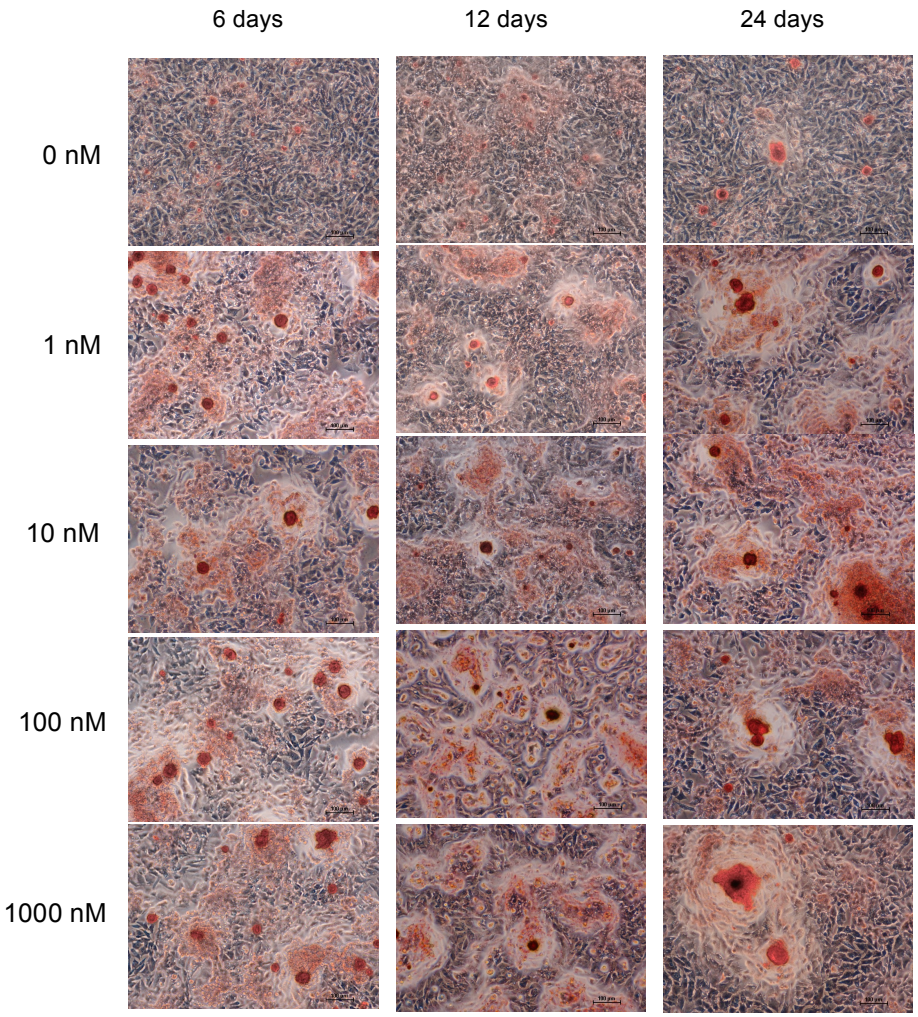

Fold change vs ST0
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ST0
ST1
ST10
ST100
ST500
ST1000
6 days
12 days
24 days

Figure 4.

A.


6 days
12 days
24 days
0 nM
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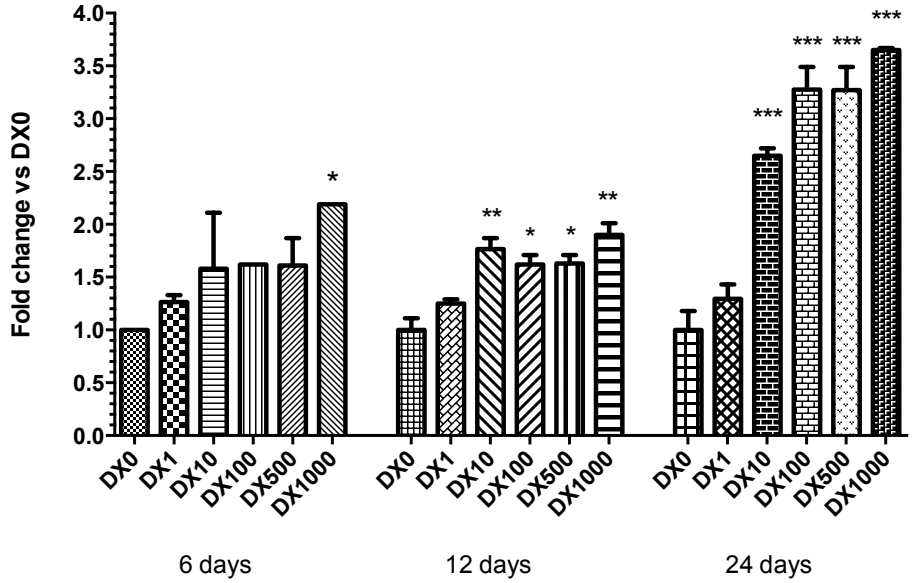
10 nM
100 nM
1000 nM

B.


Fold change vs DX0
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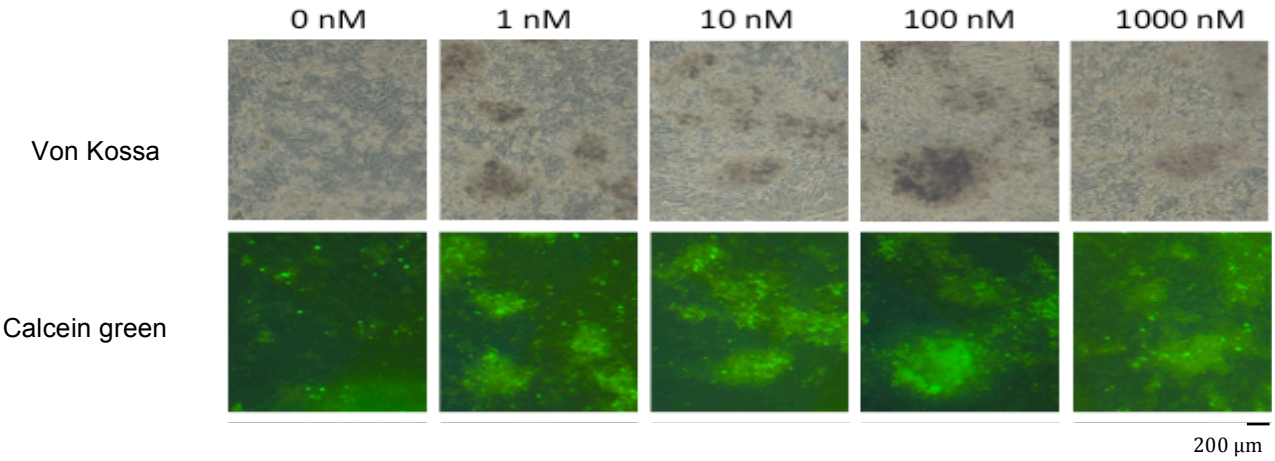
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DX0
DX1
DX10
DX100
DX500
DX1000
6 days
12 days
24 days

Figure 5.

A.


0 nM
1 nM
10 nM
100 nM
1000 nM
Von Kossa
Calcein green
200 µm

B.

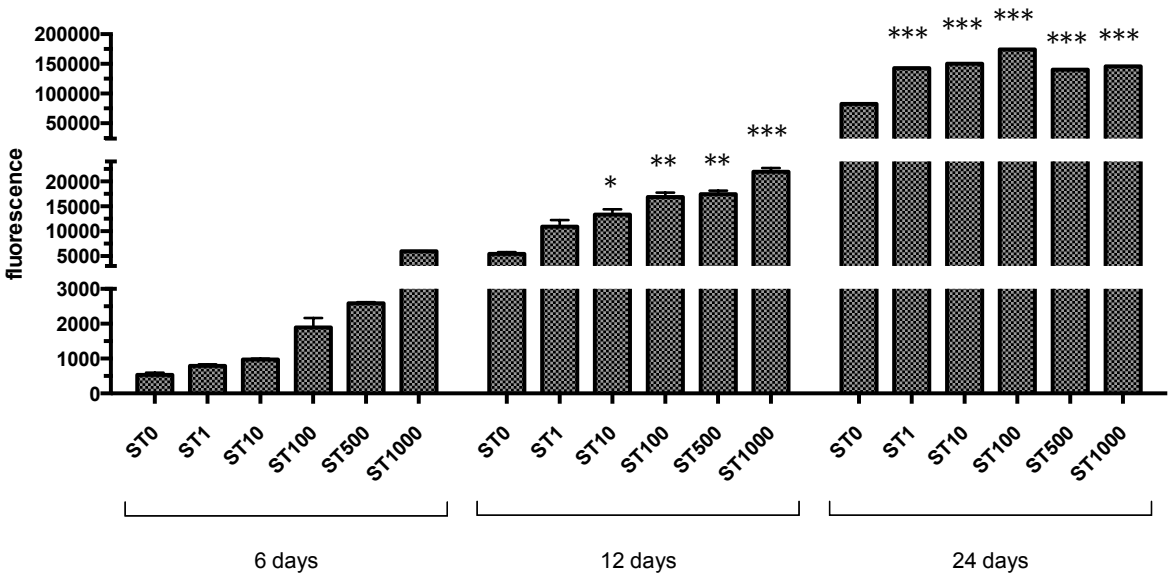
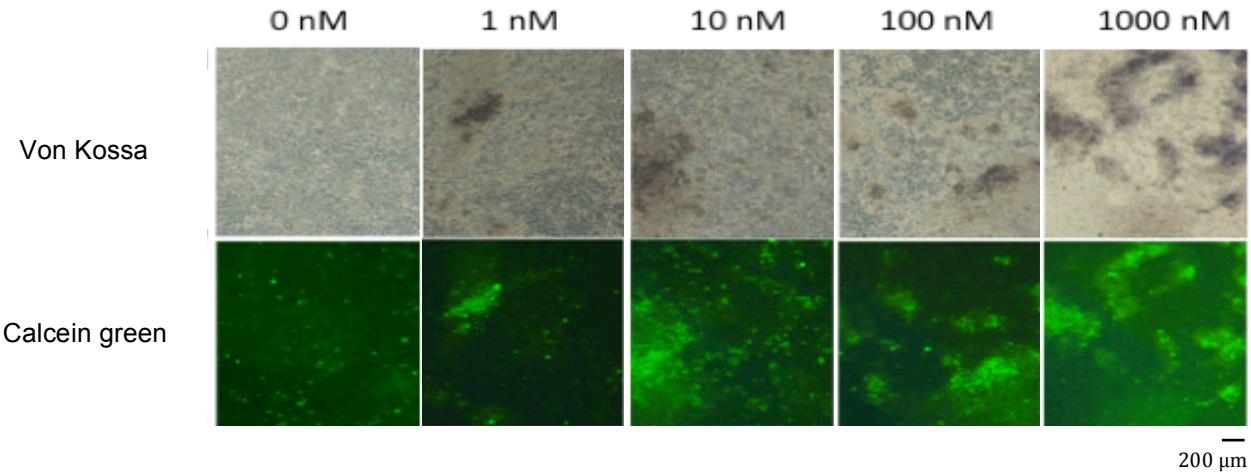
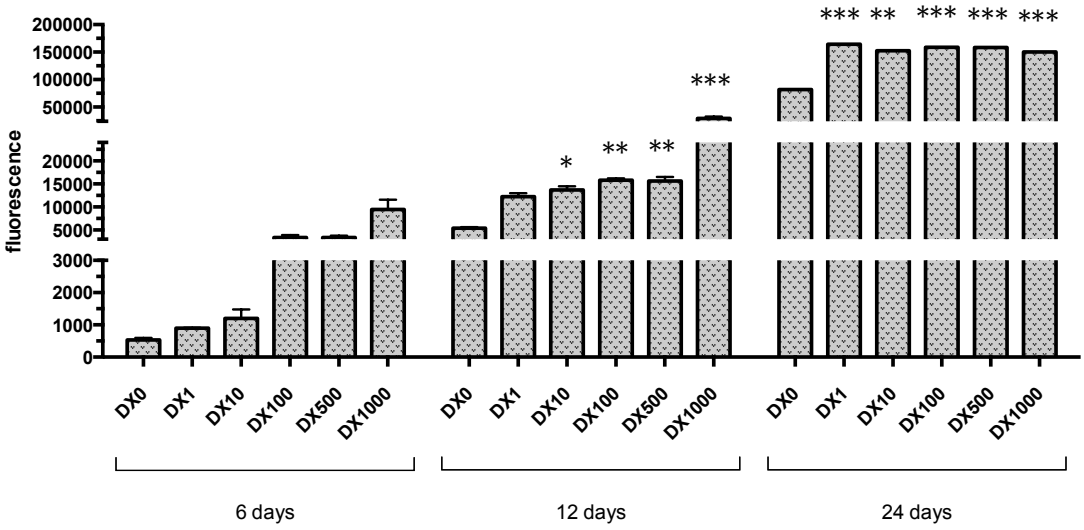

fluorescence
200000
150000
100000
50000
20000
15000
10000
5000
3000
2000
1000
0
*
**
**
***
***
***
***
***
***
ST0
ST1
ST10
ST100
ST500
ST1000
6 days
12 days
24 days

Figure 6.

A.


0 nM
1 nM
10 nM
100 nM
1000 nM
Von Kossa
Calcein green
200 µm

B.


fluorescence
200000
150000
100000
50000
20000
15000
10000
5000
3000
2000
1000
0
*
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***
***
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***
***
***
DX0
DX1
DX10
DX100
DX500
DX1000
6 days
12 days
24 days

Figure 7.

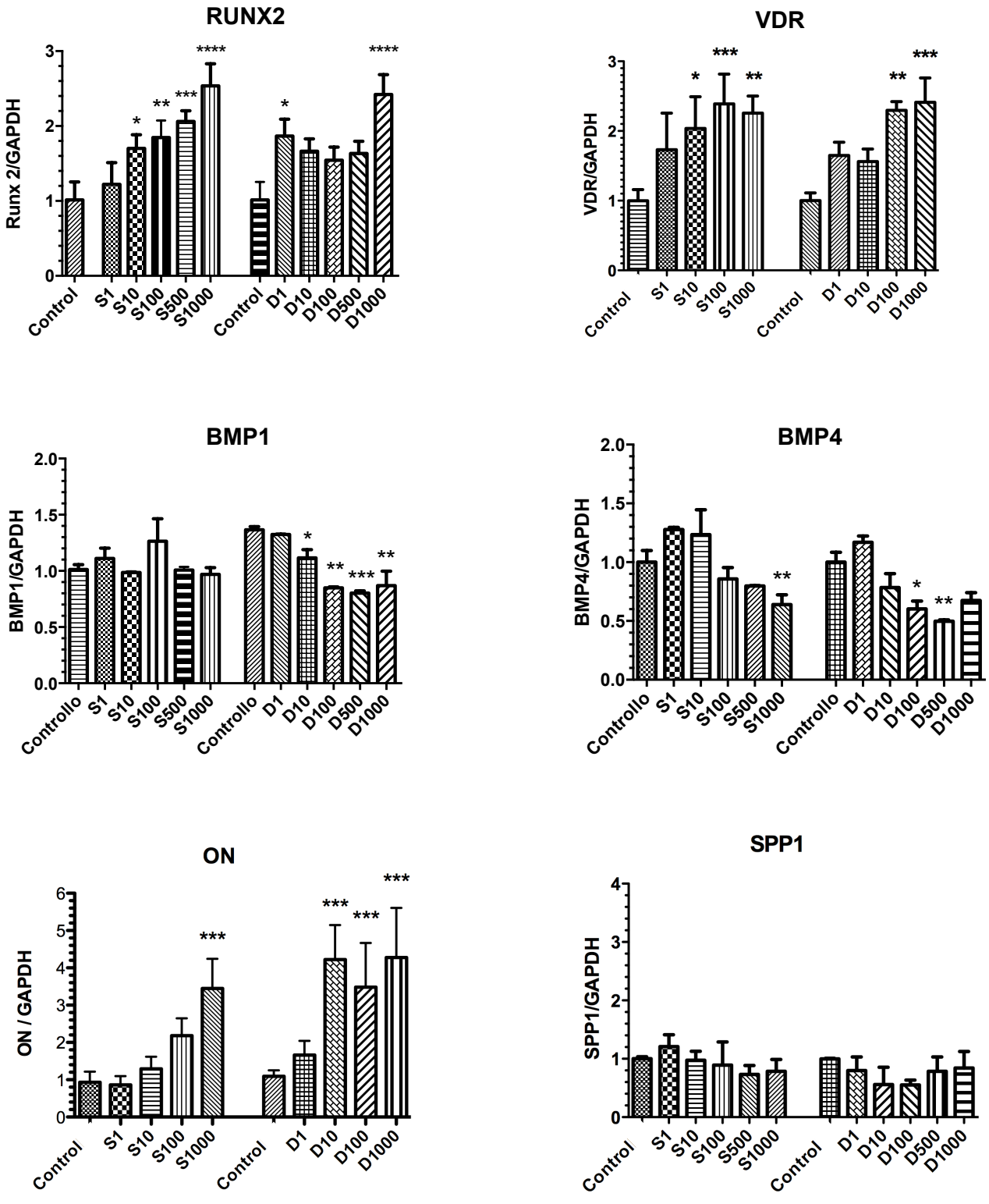
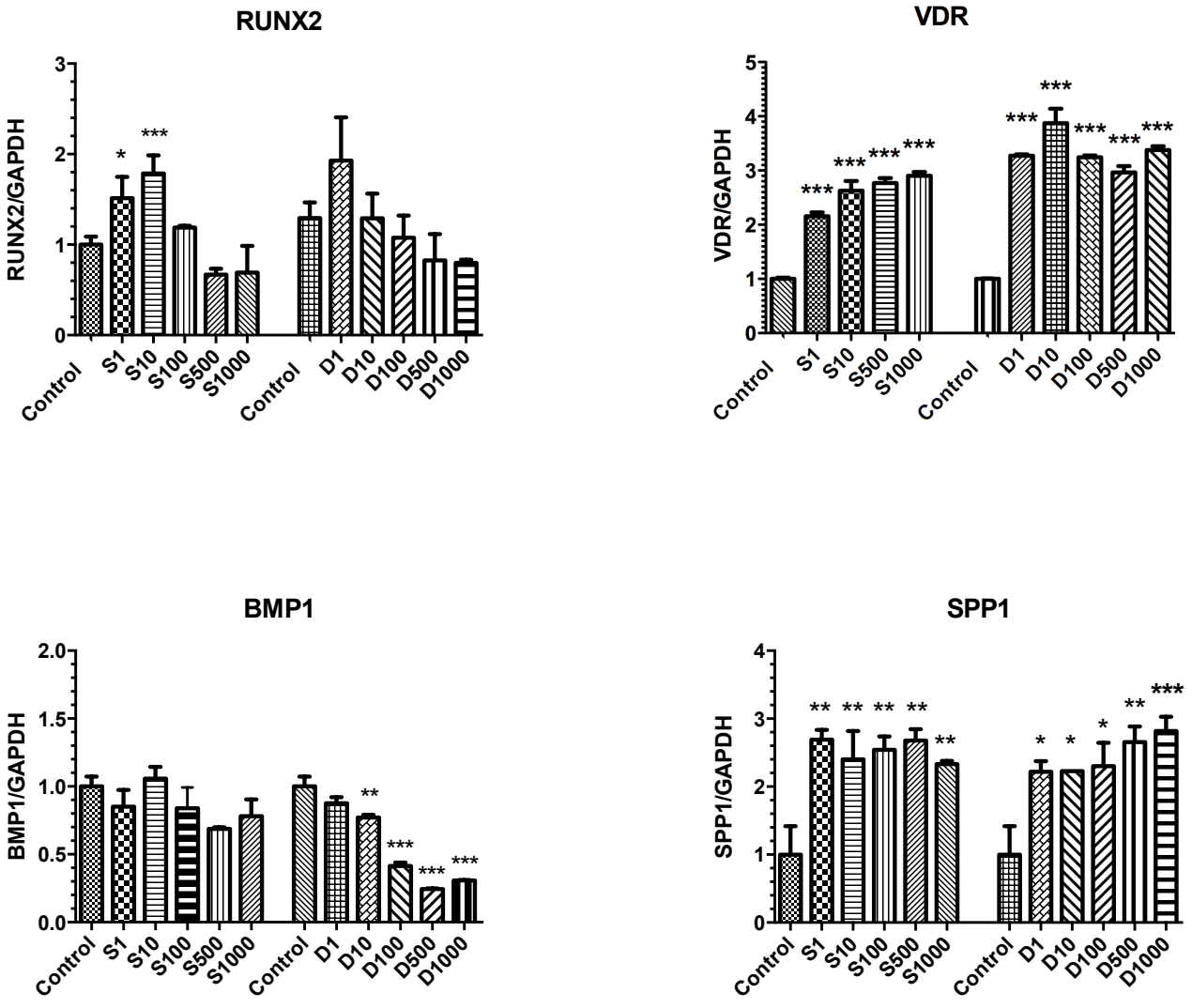

RUNX2
Runx 2/GAPDH
Control
S1
S10
S100
S500
S1000
D1
D10
D100
D500
D1000
VDR
VDR/GAPDH
BMP1
BMP1/GAPDH
Controllo
BMP4
BMP4/GAPDH
ON
ON / GAPDH
SPP1
SPP1/GAPDH

Figure 8.


RUNX2
RUNX2/GAPDH
VDR
VDR/GAPDH
BMP1
BMP1/GAPDH
SPP1
SPP1/GAPDH
Control
S1
S10
S100
S500
S1000
D1
D10
D100
D500
D1000

2.6.1 Tables and Figures Legend

Table 1. List of the genes analyzed with RT-PCR and their correspondent primer sequences.

Figure 1. Graphic representation of SaOS-2 growth under different conditions: DMEM-low (red line), ETOH 0.1% (blue line), ST 1-1000 nM (shades of grey). X axis represents the days of culture, while Y axis reports fluorescence values expressed in arbitrary units (A.U.), as they were read by spectrophotometer.

Figure 2. Graphic representation of SaOS-2 growth under different conditions: DMEM-low (red line), ETOH 0.1% (blue line), DX 1-1000 nM (shades of grey). X axis represents the days of culture, while Y axis reports fluorescence values expressed in arbitrary units (A.U.), as they were read by spectrophotometer.

Figures 3. A. Aspect of well-plates stained with Alizarin Red S staining and treated with different ST concentrations (0-1000 nM) at 6, 12 and 24 days. Optical microscopy, 10X magnification.
B. Alizarin Red S staining quantification with different ST concentrations (0-1000 nM) at 6, days and 24 days. Data are reported as fold change over controls and expressed as mean ± standard deviation. Asterisks indicate statistical significance (*: $p<0.05$ vs control; ** $p<0.005$ vs control; ***: $p<001$ vs control).

Figure 4. A. Aspect of well-plates stained with Alizarin Red S and treated with different DX concentrations (0-1000 nM) at 6, 12 and 24 days. Optical microscopy, 10X magnification.
B. Alizarin Red S staining quantification with different DX concentrations (0-1000 nM) at 6, days and 24 days. Data are reported as fold change over controls and expressed as mean ± standard deviation. Asterisks indicate statistical significance (*: $p<0.05$ vs control; ** $p<0.005$ vs control; ***: $p<001$ vs control).

Figure 5. A. Aspect of samples treated with ST (0-1000 nM) at 24 days observational period observed with phase contrast microscopy and fluorescence microscopy to reveal Von Kossa and calcein green staining (10X magnification). B. Graph illustrating fluorescence absorbance of calcein

green staining with different ST concentrations (0-1000 nM) at 6, 12 and 24 days observational period. Data are expressed as mean ± standard deviation.

Figure 6. A . Aspect of samples treated with DX (0-1000 nM) at 12 days observational period observed with phase contrast microscopy and fluorescence microscopy to reveal Von Kossa and calcein green staining (10X magnification). B. Graph illustrating fluorescence absorbance of calcein green staining with different DX concentrations (0-1000 nM) at 6, 12 and 24 days observational period. Data are expressed as mean ± standard deviation.

Figure 7. Analysis of gene expression of SaOS-2 treated with different concentrations (0-1000 nM) of either Stanozolol (S) or Dexamethasone (D) at 12 days observational period.
RUNX2: Runt-related transcription factor 2; VDR: Vitamin D Receptor; BMP1: Bone Morphogenetic Protein 1; BMP4: Bone Morphogenetic Protein 4; ON: Osteonectin; Osteopontin (SPP1). Data are reported as fold change over controls (0nM steroid) and are expressed as mean ± standard deviation. Asterisks indicate statistical significance (*: $p<0.05$ vs control; ** $p<0.005$ vs control; ***:$p<001$ vs control).

Figure 8. Analysis of gene expression of SaOS-2 treated with different concentrations (0-1000 nM) of either Stanozolol (S) or Dexamethasone (D) at 24 days observational period. RUNX2: Runt-related transcription factor 2; VDR: Vitamin D Receptor; BMP1: Bone Morphogenetic Protein 1; Osteopontin (SPP1). Data are reported as fold change over controls (0nM steroid) and are expressed as mean ± standard deviation. Asterisks indicate statistical significance (*: $p<0.05$ vs control; ** $p<0.005$ vs control; ***:$p<001$ vs control).

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CHAPTER 3.

A novel technique to study bone regeneration in rats: differential morphometric analysis of “over-inlay” block graft

ABSTRACT

Aim: The aim of this study is to describe a novel model of block graft and differential morphometric analysis to study bone regeneration in rat calvarial defects. **Materials and Method**s: Standardized critical-size defects of 5 mm diameter were surgically created into the parietal bone of 12 male Wistar rats. Deproteinized bovine bone blocks were inserted into the defects, so that a part of the block was included within calvarial bone thickness ("inlay" component of the graft) and a part exceeded calvarial height ("over" component). Half of the animals were sacrificed at 1 month and the remaining at 3 months and samples were collected for histological analyses. Differential morphometric evaluation consisted in tracing standardized regions of interest (ROIs) within each sample: periosteal area (PA), areas adjacent to native bone (BA), central areas (CA). New bone formation (NB) and fibrous tissue (FT) were quantified within each ROI. Immunohistochemical evaluation of blood vessels and co-positivity for Osterix (OSX)/Osteocalcin (OC) was carried out. Data were analyzed using 2-way Anova and Sidak's post test, considering a level of significance $p \leq 0.05$. Results are expressed as mean ± SEM. **Results**: NB was significantly higher within BA than within PA and CA, both at 1 month (BA vs PA: 42183.89±6477.27 μm^2 vs 21091.94±6477.27μm^2, $p<0.05$; BA vs CA: 42183.89±6477.27 μm^2 vs 10545.97±3238.63 μm^2, $p<0.01$) and 3 months (BA vs PA: 64131.11±7520.61 μm^2 vs 32065.55±7520.61 μm^2, $p<0.01$; BA vs CA: 64131.11±7520.61 μm^2 vs 16032.78±3760.30 μm^2, $p<0.001$) observational period. Periosteal areas presented significantly higher amounts of FT compared to BA and CA at 1 month (PA vs BA: 179192.50±29187.19 μm^2 vs 59730.83±9729.06 μm^2, $p<0.01$; PA vs CA: 179192.50±29187.19μm^2 vs 24566.72±4890.14 μm^2, $p<0.001$) and 3 months (PA vs BA: 243367.80±55447.25 μm^2 vs 81122.60±18482.42 μm^2, $p<0.001$; PA vs CA: 243367.80±55447.25 μm^2 vs 31174.89±5178.20 μm^2, $p<0.001$) observational period. Blood vessel distribution revealed a progressive migration of the angiogenic process from the interface of the graft with native bone (BA) towards the central area of the graft (CA). OSX/OC co-positivity has been mostly noticed within PA, thus suggesting periosteum to be an important source of osteoprogenitor elements. **Conclusions**: The model presented in this study allowed an investigation of distinct graft-to-host interfaces within the same histological section, thus leading to an extensive comprehension of graft biological integration. Such an advantage provides an easier achievement of complete data sets when testing biomaterials and leads to a significant reduction in the numbers of animals required for pre-clinical testing.

Key-words: Rat; Calvaria; Critical-size defect; Bone grafting; Bone regeneration

RIASSUNTO

***Obiettivo**: Lo scopo di questo studio è di descrivere un nuovo modello di innesto a blocco ed un relativo metodo di analisi morfometrica differenziale, per lo studio della rigenerazione ossea su difetti in calvaria di ratto. **Materiali e Metod**i: Difetti critici standardizzati dal diametro di 5 mm sono stati creati chirurgicamente nell'osso parietale della calvaria di 12 ratti Wistar maschi. Blocchi di osso bovino deproteinizzato sono stati inseriti all'interno dei difetti, in modo che una parte del blocco fosse inclusa entro lo spessore della calvaria (componente "inlay" dell'inesto) ed una parte eccedesse in altezza lo spessore della calvaria (componente "over" dell'innesto). Metà degli animali utilizzati sono stati sacrificati a 1 mese e metà a 3 mesi dall'intervento ed al momento del sacrificio sono stati prelevati i campioni bioptici per le valutazioni istologiche. L'analisi morfometrica differenziale è consistita nel tracciare all'interno di ogni campione regioni di interesse (ROIs) di dimensioni standardizzate, corrispondenti a diverse interfacce dell'innesto con i tessuti dell'ospite. Sono state identificate un'area periostale (PA), aree adiacenti all'osso nativo (BA), un'area centrale dell'innesto (CA). All'interno di ognuna di queste ROI è stata quantificata la formazione di tessuto osseo (NB) e fibroso (FT). E' stata inoltre effettuata una valutazione immunoistochimica dei capillari presenti in ogni area e di elementi con co-positività per (OSX)/Osteocalcin (OC). I dati ottenuti sono stati analizzati con test Anova a 2 vie e post-test di Sidak, considerando un livello di significatività $p \leq 0.05$. I risultati sono stati espressi come media ± SEM. **Risultati**: NB è risultato significativamente maggiore nella zona BA rispetto a PA e CA, sia a 1 mese (BA vs PA: 42183.89±6477.27 μm^2 vs 21091.94±6477.27μm^2, $p<0.05$; BA vs CA: 42183.89±6477.27 μm^2 vs 10545.97±3238.63 μm^2, $p<0.01$) sia a 3 mesi (BA vs PA: 64131.11±7520.61 μm^2 vs 32065.55±7520.61 μm^2, $p<0.01$; BA vs CA: 64131.11±7520.61 μm^2 vs 16032.78±3760.30 μm^2, $p<0.001$). La zona PA si è rivelata invece ricca di FT in confronto alle altre ROI analizzate sia a 1 mese (PA vs BA: 179192.50±29187.19 μm^2 vs 59730.83±9729.06 μm^2, $p<0.01$; PA vs CA: 179192.50±29187.19μm^2 vs 24566.72±4890.14 μm^2, $p<0.001$) sia a 3 mesi (PA vs BA: 243367.80±55447.25 μm^2 vs 81122.60±18482.42 μm^2, $p<0.001$; PA vs CA: 243367.80±55447.25 μm^2 vs 31174.89±5178.20 μm^2, $p<0.001$).La distribuzione dei capillari ha mostrato una progressiva migrazione del processo angiogenico dalle zone di interfaccia tra innesto ed osso nativo BA verso le zone centrali dell'innesto CA. Elementi cellulari con co-positività per OSX/OC sono stati in prevalenza osservati a livello della zona PA, suggerendo che il periostio possa costituire una fonte importante di cellule osteoprogenitrici. **Conclusioni**: Il modello presentato in questo studio ha consentito uno studio di distinte interfacce tra innesto e sito*

ricevente nella stessa sezione istologica, consentendo una comprensione più completa del processo biologico di osteointegrazione. Grazie ai vantaggi portati da questo modello, data sets completi nel testing di nuovi materiali da innesto potranno più facilmente essere ottenuti, portando ad una significativa riduzione del numero di animali richiesti per sperimentazioni pre-cliniche.

Parole chiave: *Ratto; Calvaria; Difetto critico; Innesti ossei; Rigenerazione ossea*

3.1 Introduction

Bone grafting procedures are widely used for the management of alveolar bone deficiencies in oral and maxillofacial surgery. The clinical success of bone grafts depends on their osseointegration, that is the clinical and histological incorporation of the grafting material into the recipient site. The achievement of this goal is depending on complex biological interactions occurring at the graft-host interfaces, which finally lead to new bone formation and graft remodeling [1].

In the early healing phase, inflammatory cells (macrophages, monocytes, lymphocytes, and polymorphonucleated cells) infiltrate the grafted area following chemotactic stimuli; blood vessels developing from adjacent areas progressively grow in a network and mesenchymal precursors initiate their migration to the grafted area. Before the inflammatory phase subsides, a reparative phase initiates and results in the development of a reparative callus tissue in grafted area, which will eventually be replaced by bone [2] [3] [4].

Native bone marrow and periosteum play an essential role in this process, by providing the grafted area with osteogenic cells, vascular supply and osteoinductive factors [5]. New bone formation within the callus may occur through intramembranous ossification, with direct differentiation of precursor cells into osteoblasts and no intermediate phase of cartilage apposition. Many authors showed that bone marrow contains a population of pluripotent mesenchymal stem cells, able to commit into bone forming elements [6] [7] [8] and histological evidence of this process has been achieved at fracture sites [9]. Also, periosteal contribution to bone healing may lead to endochondral ossification: mesenchymal or undifferentiated cells from the inner layer of periosteum migrate to the grafted area, become larger, start to take on the appearance of chondrocytes and synthesize a basophilic collagen matrix, which is progressively vascularized and substituted with new bone [10] [11] [12]. It is known from clinical experience that preservation of the periosteum or use of a periosteal graft significantly improves bone graft incorporation and remodeling [13].

The composition and rate of repair tissue may differ depending on factors such as the morphology of the defect, graft type and mechanical stability of the wound. Particularly, the grafting techniques may substantially influence the graft-host interface and thus the biological process of osseointegration.

In vivo research in rodents has been extensively employed to improve graft osseointegration to test surgical procedures and a variety of biomaterials. Particularly, rat calvarial bone provides a well

described experimental setting, combining the advantages of a relatively low cost of animals and a favorable anatomic access and surgical handling [14].

Among grafting materials, deproteinized bovine bone (DBBM) represents is one of the most investigated materials and it is widely employed as a scaffold in regenerative procedures, both in animal models and in clinical practice [15] [16] [17] [18].

As regards the grafting technique, a distinction can be traced between *inlay* grafts, which are placed in retentive recipient sites, as the cavity between two surgical bone sections, and *onlay* grafts, which are apposed onto the surface of the recipient bone.

Both inlay and onlay bone grafting techniques have been employed in rat calvarial bone [19].

Inlay grafts are mainly tested within "critical–size" defects, defined as "the smallest intrabony wound which does not undergo spontaneous healing during the lifetime of the animal" [20]. Defects are surgically created by trephine burs, i.e. a circular full-thickness defect involving the parietal and/or occipital bone [21]. Inlay grafts represent a favorable environment for angiogenesis and osteogenic cell mobilization, as they lie in direct contact with native bone marrow and periosteum of the recipient site; however, by definition they can not be performed in non-containing defects.

Onlay grafts are usually fixed over the top of the cranium and provide the achievement of vertical bone regeneration, which is the most challenging clinical condition [22] [23]. Nevertheless, they present the operative difficulty to be opportunely fixed and stabilized to the recipient site and the biological hindrance to be vascularized and colonized by osteogenic cells [24] [25] [26]. Some authors described the use of domes or barrier membranes [27] [28] [29] for the stabilization of particulated onlay grafts. However, these methods of graft covering impede blood vessels and cells from periosteum to participate in bone graft incorporation and remodeling with negative biological implications [30]. Studies where onlay grafts were covered with native periosteum reported the finding of sub-periosteal bone formation [31] [32], thereby showing a prolific periosteal osteogenic and angiogenic response. Also, the role of host bone marrow has been emphasized as a key factor to improve graft vascularization and colonization by osteogenic elements: onlay grafts showed better results in terms of early osseointegration when the cortical bone of the recipient site was grinded or perforated and cancellous bone was in direct contact with the grafted area [33][34]. Previous authors who used block grafts for inlay procedures in graft calvarial critical-size defects reported new bone at the graft borders adjacent to host bone marrow [35] and underneath periosteum [36]. However, a clear distinction between periosteal and medullary contribution in bone apposition and in graft incorporation has not been clearly documented [37].

The aim of this study was to describe a block graft technique sharing some inlay and onlay characteristics and a differential morphometric analysis of graft-to host interfaces within the same surgical site of rat calvaria.

3.2 Materials and Methods

Surgical protocol

The study protocol was in accordance to EU Directive 2010/63/EU and was approved by the local Ethics Committee for Animal Testing of Parma. Twelve male, 4 months old Wistar rats were included in the study. During the whole experimental period rats were in a monitored environment (21°C; 12:12 light cycle) and received a solid diet and water *ad libitum*.

Fifteen minutes prior the surgical procedure, all animals received a single intra-muscular inoculation of enrofloxacin (10 mg/kg Baytril, Bayer*, Germany)*. Anaesthesia was obtained with intraperitoneal injection of tilethamine hydrochloride zolazepam hydrochloride (30 mg/kg Zoletil 100, Virbac, France). The surgical site in the parietal region of the calvaria was shaved off and disinfected with povidone-iodine 10%.

A midline incision from the bipupillar line to the occipital process was performed: after skin elevation, the subcutaneous fascia was incised and the calvarial bone surface is exposed by a blunt dissection through the periosteum. Particular attention was paid to maintain the integrity of periosteum. Standardized full-thickness critical-size defect were created into parietal bone by means of a trephine burr of 5.0 mm external diameter (Biomet 3i, USA), under abundant irrigation with sterile saline (Figure 1, A-B).

Discs of deproteinized bovine bone measuring 5 mm-diameter and 1.5 mm-height were placed into the defects so that the upper part of the disc exceeded the external cortical bone. A straight line was traced between the cortical margins of the defect and the vertical extension of the exceeding part of the discs was measured towards the axis of this segment and was found corresponding to 43.73 % ± 2.96 of disc height.

The part of the graft included within calvarial bone thickness represents the “inlay” component, while the exceeding part represents the “over” component (Figure 2, A-B).

A first layer suture was carried out in order to fix the periosteum, then a secondary layer of subcutaneous suture was performed out to allow primary intention wound healing (Vycril 5-0, Ethicon, Johnson & Johnson, Netherlands; Prolene 3-0, Ethicon, Johnson & Johnson, Netherlands). Attention was paid not to put the two layers of suture over the surgical defects, in order to avoid scar tissue formation within the grafted sites. All surgeries were performed by the same trained operator.

Half animals were sacrificed at 1 month and the remaining at 3 months. Euthanasia was obtained with an intraperitoneal injection of pentobarbital 150 mg/kg.

A mid-thickness incision was performed at the surgical site, paying attention not to remove periosteum. A rectangular panel was containing the original surgical defect area and the surrounding tissues was harvested.

Histomorphometry

Specimens were fixed in 10% buffered formalin for 48 hours, washed in running water, decalcified in a 0.5 M ethylene-diamine-tetra-acetic acid (EDTA) solution pH 7.4 and paraffin embedded. For each sample, serial longitudinal 5μm-thin serial sections were cut with a rotative microtome in a plane parallel to the sagittal suture. Central sections were stained with haematoxylin-eosin for histomorphometric evaluation. Microphotographs of tissue from different regions of interest (ROI) were captured by an optical microscope (Nikon Eclipse 90i, Nikon, Japan) connected to a digital camera.

The ROIs to be analyzed were respectively defined as: periosteal area (PA), identified as a rectangular area corresponding to the "over" component of the graft / delimited by the periosteum and a straight line traced between the external cortical bone at the margins of the defect; lateral areas adjacent to native bone (BA), described as rectangular areas extending from native bone towards the center of the defect; central area of the defect (CA), defined as a rectangular area traced at the remaining central portion of the defect. Areas of PA, BA and CA were standardized in order to allow multiple comparisons (Figure 3, A-B).

Morphometric evaluation of new bone formation (NB) and fibrous tissue (FT) was carried out as described elsewhere [38] by image analysis software (Image Pro-Plus 4.0, Media Cybernetics, USA) within each ROI.

Immunohistochemistry

The immunohistochemical analysis was conducted on optical and fluorescence microscopy.

Capillary and venule density were assessed in PA, BA and CA following immunohistochemical (IHC) staining. Samples were incubated with anti-von Willebrandt Factor antibody (vWF, rabbit polyclonal, Dako, Denmark; 1:30, o.n), stained with streptavidin-conjugated peroxidase (using DAB as chromogen) and finally counterstained with hematoxylin. Sections were examined at 1000X magnification taking advantage of an ocular reticle (9604 mm^2 area). A total of 20 fields within each ROI were analyzed in order to compute the capillary or venule numerical density/mm^2 of tissue.

Osteoblasts were evaluated analyzing samples processed for IHC staining with an anti-Osterix (OSX) antibody (rabbit polyclonal, Santa Cruz, CA, USA; 1:50 o.n), revealed by anti-rabbit secondary antibody FITC-conjugated (1:20 60' 37°C, Jackson Laboratories, PA, USA) and IHC staining with anti-Osteocalcin (OC) antibody (rabbit polyclonal, Santa Cruz, CA, USA) revealed by anti-rabbit secondary antibody TRITC-conjugated (1:20 60' 37°C, Jackson Laboratories, PA, USA). The nuclear counterstaining was performed with DAPI (4',6-diamidine-2-phenyndole, Sigma St. Louis, MO, 5mM, 18' RT). Slides were mounted with fluorescence mounting medium, Vectashield (VECTOR, USA).

OSX positive cells were computed at 1000X magnification by using fluorescent microscopy. The analysis was performed separately within each ROI.

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA) and differences between groups were assessed using the Bonferroni post-test for multiple comparisons. A p-value ≤ 0.05 was considered statistically significant. Results are reported as mean ± SEM. Graphs were obtained with GraphPad Prism 6.0 software.

3.3 Results

Surgical aspects

Intraoperative mortality was less than 10% and healing carried on uneventfully. This finding indirectly revealed that the block graft did not exert a significant compression over the underlying tissues, thus supporting the safeness of the procedure.

The suturing technique was simple and the wound was closed by primary intention. The limited height of the "onlay" graft component allowed to carry out a suture without any tension, thus preventing the risk of dehiscences

Histomorphometry

Grafts showed a good integration in the recipient sites and no signs of abnormal inflammation nor immune response were observed at any time. Analysis of tissue composition revealed no significant differences in NB and FT at 1 vs 3 months within each analyzed area (BA, PA, CA; $p>0.05$). However, the differential morphometric analysis applied in this study allow to recognize the implication of different host-to-graft interfaces in the process of bone healing.

BA appeared to give a substantial contribution in new bone formation: NB was significantly higher within BA than within PA and CA, both at 1 month (BA vs PA: 42183.89±6477.27 μm^2 vs 21091.94±6477.27μm^2, $p<0.05$; BA vs CA: 42183.89±6477.27 μm^2 vs 10545.97±3238.63 μm^2, $p<0.01$) and 3 months (BA vs PA: 64131.11±7520.61 μm^2 vs 32065.55±7520.61 μm^2, $p<0.01$; BA vs CA: 64131.11±7520.61 μm^2 vs 16032.78±3760.30 μm^2, $p<0.001$) observational period.

Periosteal areas presented significantly higher amounts of FT compared to BA and CA at 1 month (PA vs BA: 179192.50±29187.19 μm^2 vs 59730.83±9729.06 μm^2, $p<0.01$; PA vs CA: 179192.50±29187.19μm^2 vs 24566.72±4890.14 μm^2, $p<0.001$) and 3 months (PA vs BA: 243367.80±55447.25 μm^2 vs 81122.60±18482.42 μm^2, $p<0.001$; PA vs CA: 243367.80±55447.25 μm^2 vs 31174.89±5178.20 μm^2, $p<0.001$) observational period.

It has to be noted that NB and FT were overall poor within CA, enlightening that it is difficult for the central portion of the graft to be colonized by cells migrating from adjacent host tissues. Results are shown in Table 1, A-B and in Figure 4.

Immunohistochemistry

Presence of a rich network of new vessels was detected at the host-graft interfaces and capillary density showed tendencies to be different between the analyzed areas. At 1 month, BA was the

region with the highest capillary density (13.89 ± 2.58 n/mm^2) compared to PA (11.21 ± 1.32 n/mm^2, p>0.05) and CA (8.64 ± 2.43 n/mm^2, p>0.05): such a finding suggested an important participation of this region in the process of early graft vascularization, as new vessels developing from native bone initiate to colonize the grafted site. At 3 months observational period, an inverted trend was visible, with CA being provided with a consistent capillary invasion (12.16 ± 0.42 n/mm^2) compared to other areas (PA: 10.44 ± 2.49 n/mm^2, p>0.05; BA: 6.93 ± 1.63 n/mm^2, p>0.05). Taken together, these data may indicate a progressive migration of the angiogenic process from lateral to central areas of the graft, which is consistent with the biological process of graft integration (Table 2; Figure 5).

Analysis of OSX/OC distribution revealed a more abundant presence of OSX/OC-positive cells in PA zone when compared with other ROIs at 1 month observational period (PA: 4.71 ± 0.40 n/mm^2 vs BA: 1.95 ± 0.40 n/mm^2, p<0.01; PA: 4.71 ± 0.40 n/mm^2 vs CA: 2.60 ± 0.25 n/mm^2, p<0.01). This finding enlightened the presence of cells with osteoblastic features within the sub-periosteal areas and suggested that the inner layer of periosteum to be an important source of osteoprogenitor elements. An overall decrease of cellularity was visible at 3 months, as it is compatible with a more mature tissue pattern, with no significant differences observed between groups (BA: 0.65 ± 0.13 n/mm^2; PA: 0.89 ± 0.31 n/mm^2; CA: 1.50 ± 0.24 n/mm^2) (Table 3; Figure 6).

3.4 Discussion

The study of biological mechanism leading to bone regeneration and repair often entails the use of *in vivo* models. Rat calvaria is one of the most commonly used pre-clinical setting for the screening of bone graft biomaterials, combining the advantages of a relatively low cost of animals and a favorable anatomic access and surgical handling. Both inlay and onlay grafting techniques have been tested in this model [19].

We proposed a modified block graft technique that combines aspects of both inlay and onlay procedures ("over-inlay" model) and a differential morphometric analysis of graft-to host interfaces which provides extensive biological insights about graft interaction with host tissues.

The diameter of surgical wounds was set at 5 mm, which can be considered as a "critical-size" [39] [40] in rat calvaria of male rats ranging between 3 and 6 months of age up to 12 months observational period, standing to a recent review of the literature [41]. In our model, we chose this defect dimension, so that a complete healing was not expected and distinct tissue apposition fronts could be defined within each region of interest. Moreover, a 5 mm diameter is also compatible with the creation of bilateral defects on each parietal bone. This will give the chance to set paired study designs and to compare the healing of defects receiving experimental treatment with non-treated defects within the same animal.

As for grafting materials, autogenous bone is still looked at as the "gold standard" in oral and maxillofacial surgery, even thus the high morbidity related to harvesting procedures and the limited amount availability let the room for many alternative biomaterials. Among these, deproteinized bovine bone matrix (DBBM) represents one of the most commonly employed, due to its of low morbidity versus autogenous grafts and its property of osteoconductivity. Many case series reported good clinical outcomes of DBBM blocks used in onlay [42][43][44][45] and inlay procedures [46][47].

Onlay DBBM blocks presented difficulties to be stabilized on the recipient site, as they tended to break under the pressure of fixation screws [48]. In order to overcome this issue, alternative surgical techniques (i.e. tunnel technique instead of traditional flap) have been proposed by studies in dogs [49] and in humans [50]. However, these procedures often increased the technical difficulty of the surgery, thus raising the risk of an operator-related outcome variability. The use of barrier membranes for graft fixation has also been proposed, basing on the biological concept of isolating the grafted area from the invasion of surrounding epithelial cells [27] [28] [29]. Nevertheless, the potential advantages given by the use of membranes combined with onlay DBBM blocks have not

been univocally documented [51][52][53][54] and the formation of a thick layer of dense connective tissue has been associated with the use of resorbable barriers [55]. On the other hand, some authors stressed the importance of a direct contact between the grafted area and native periosteum, which may act as a source of osteoprogenitor elements and contribute in graft osseointegration [56].Some authors reported the difficulty of DBBM blocks to be invaded with newly formed bone, which was primarily found in proximity of native medullary bone or periosteum, both in onlay [57] [53][54][58][59] and inlay [60] grafts. Others reported problems of onlay DBBM block grafts are complications during the healing phase, as dehiscences due to an excessive tension over the flap [61].

In our model, we adopted a surgical technique which shared both inlay and onlay grafting characteristics: the creation of a surgical defect within the host bone provided a recipient site which could be filled with an inlay graft with proper lateral stability. This inlay component overcome the need of further fixation means and permitted to examine the interface between the graft and the medullary spaces of native bone.

The differential morphometric analysis performed in our study allowed to study distinct contributions of different graft-to-host interfaces in graft integration. Histological results showed the fundamental role of host bone in supporting this process: indeed, analyzed BA are actively involved in new vessels as well as bone formation within the grafted area. Previous authors who used block grafts for inlay procedures in graft calvarial critical-size defects reported new bone at the graft borders adjacent to host bone [35] and underneath periosteum [36].

The onlay component of our graft model provided an easier histomorphometric definition of sub-periosteal area (PA), which was delimited by the periosteum and a straight line traced between the external cortical bone at the margins of the defect. The limited height of the “onlay” component granted the wound to be sutured without any tension and the block graft to be fully covered with native periosteum. Studies where onlay grafts were covered with native periosteum reported periosteal bone formation [31] [32], showing a significant periosteal angiogenic and osteogenic response. The finding of blood vessels and OSX/OC-positive cells at periosteal sites is consistent with these data, suggesting that the inner layer of periosteum may act as a source of osteoprogenitor elements. In addition, the evidence of increased FT in PA regions that we observed may be indicative of a regenerative process initiating within this area. Further characterization of sub-periosteal tissues are required to support this speculation.

As it was expected, tissue remodeling within the central areas of the grafts (CA) was limited. However, an increase in the number of blood vessels was detectable between 1 and 3 months observational period, showing the progressive development of a vascular network within the graft.

3.5 Conclusions

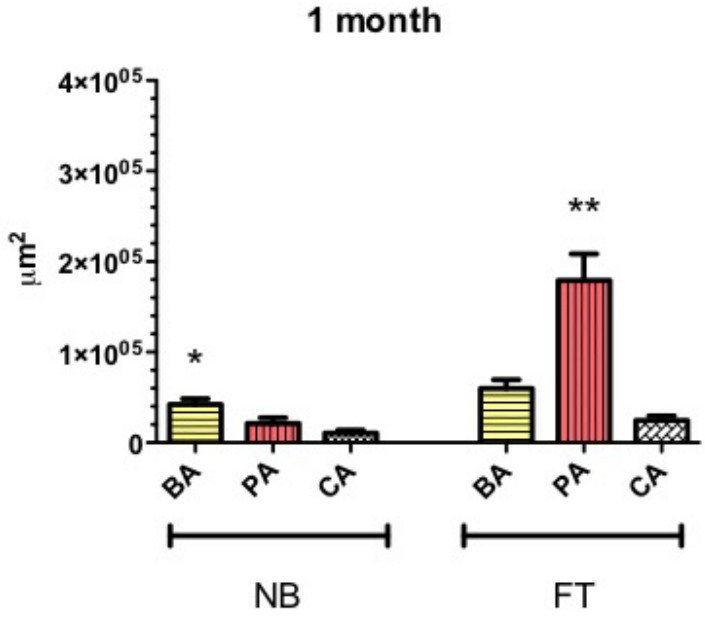
The “over-inlay” block graft technique offered the possibility to investigate distinct graft-to-host interfaces within the same histological section. The method of differential histomorphometric analysis allowed to define different regions of interests (sub-periosteal area, graft-to-native bone interface, central graft area) and their relative contributions in graft osseointegration.

On the whole, the novel approach presented in this study provided an extensive comprehension of graft biological behavior at different sites, which entails an easier achievement of complete data sets when testing new biomaterials and leads to a significant reduction in the numbers of animals required for pre-clinical testing.

3.6 Tables and Figures

Table 1.

A.



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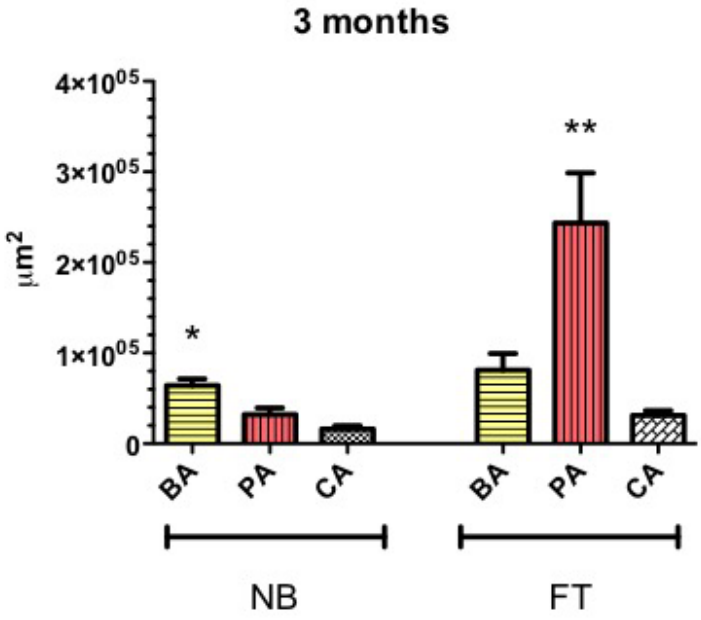

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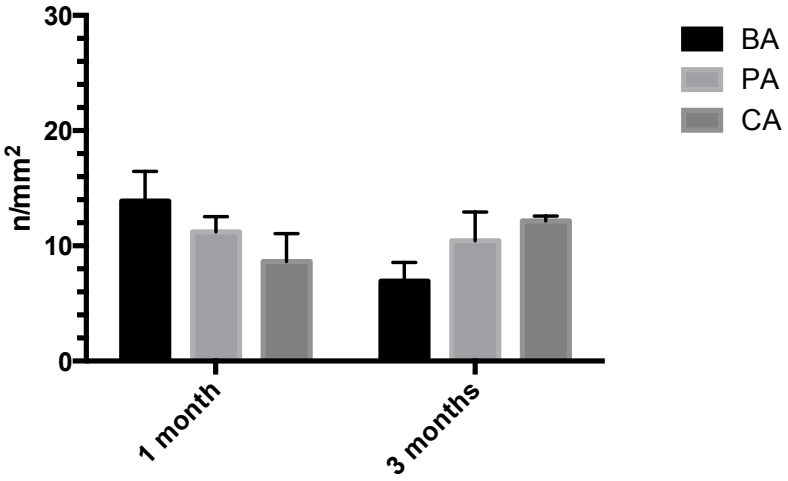

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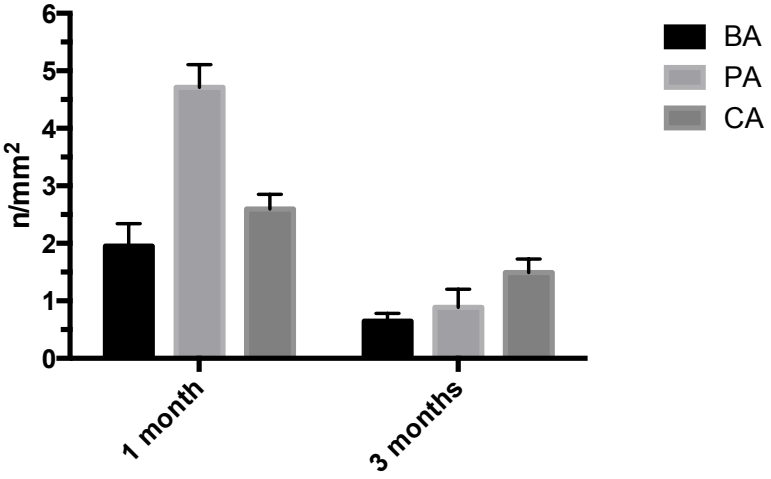
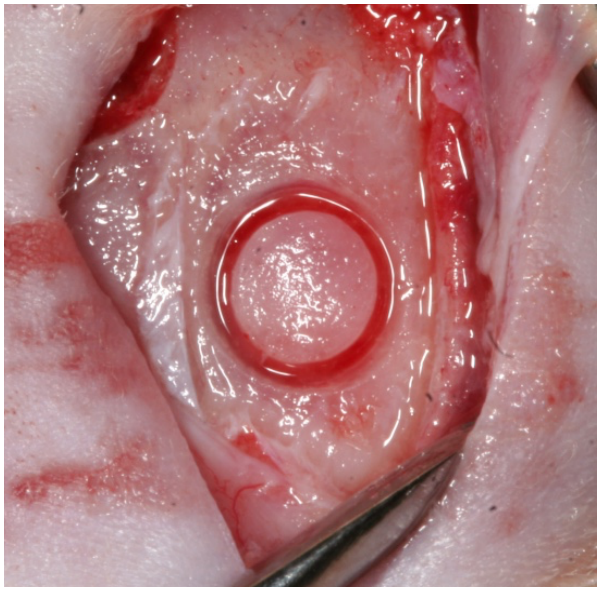


Figure 1.

A.



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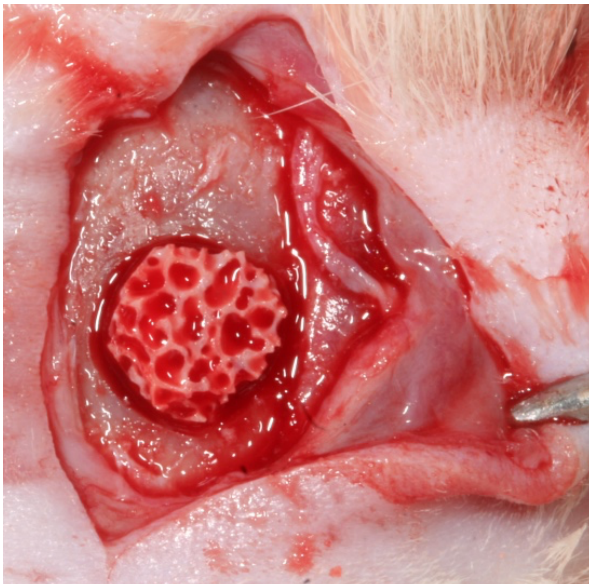

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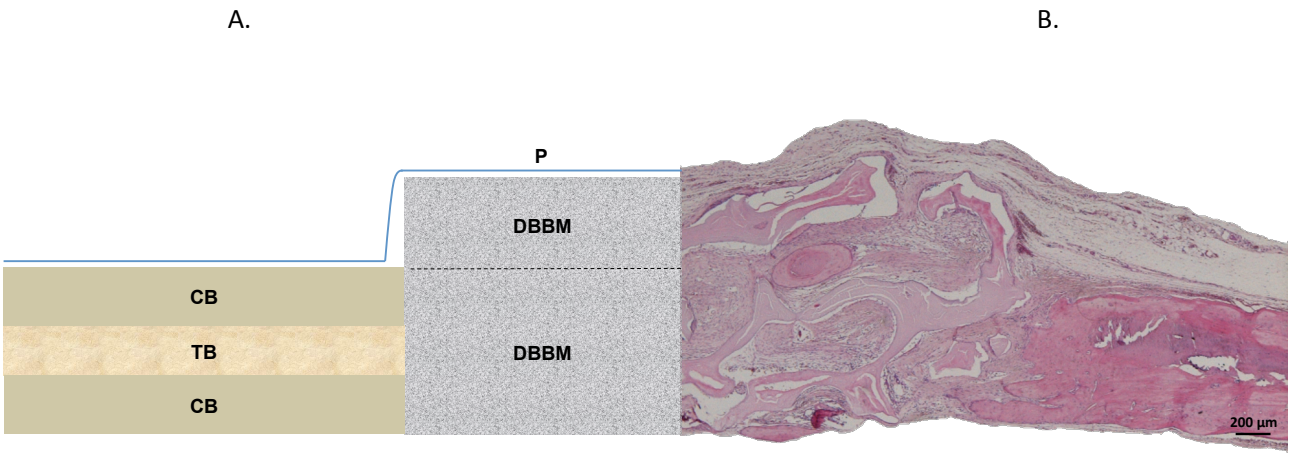

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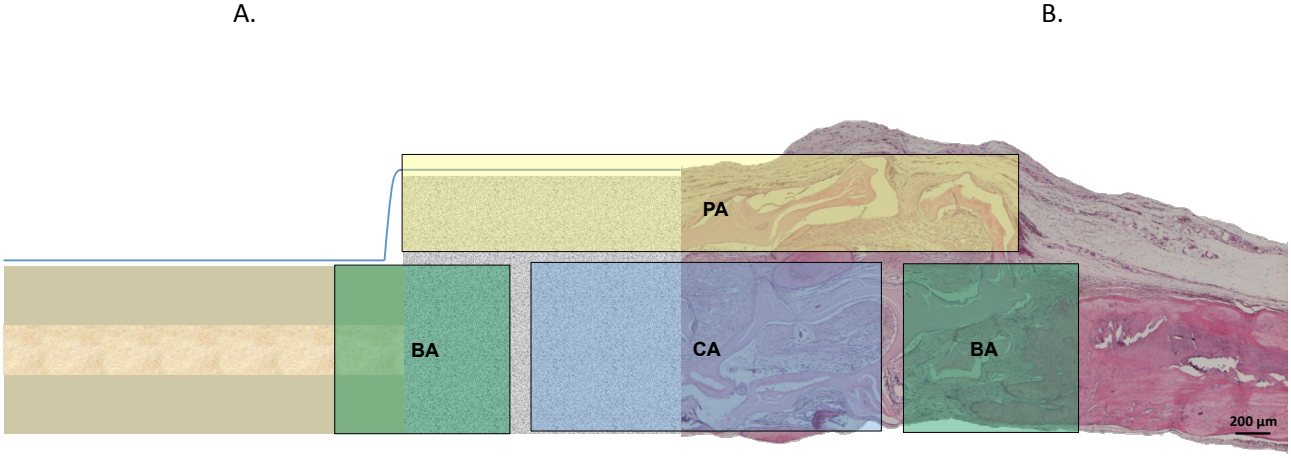

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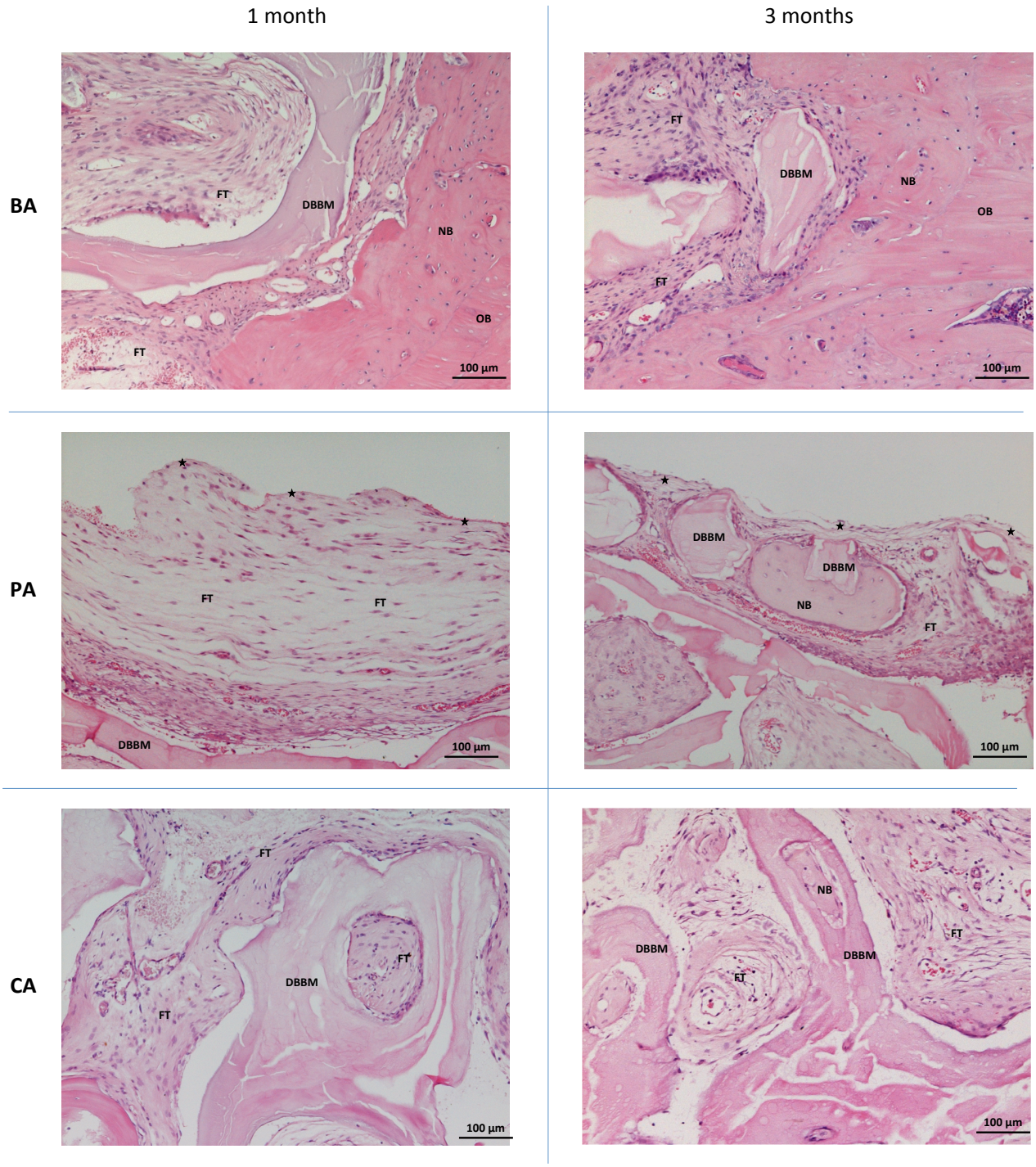

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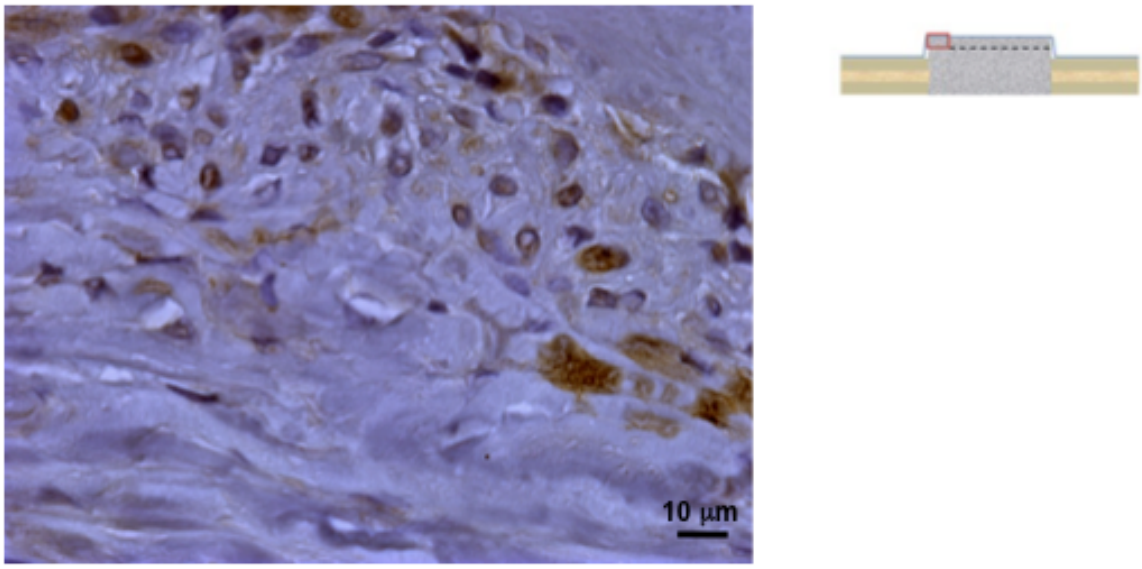
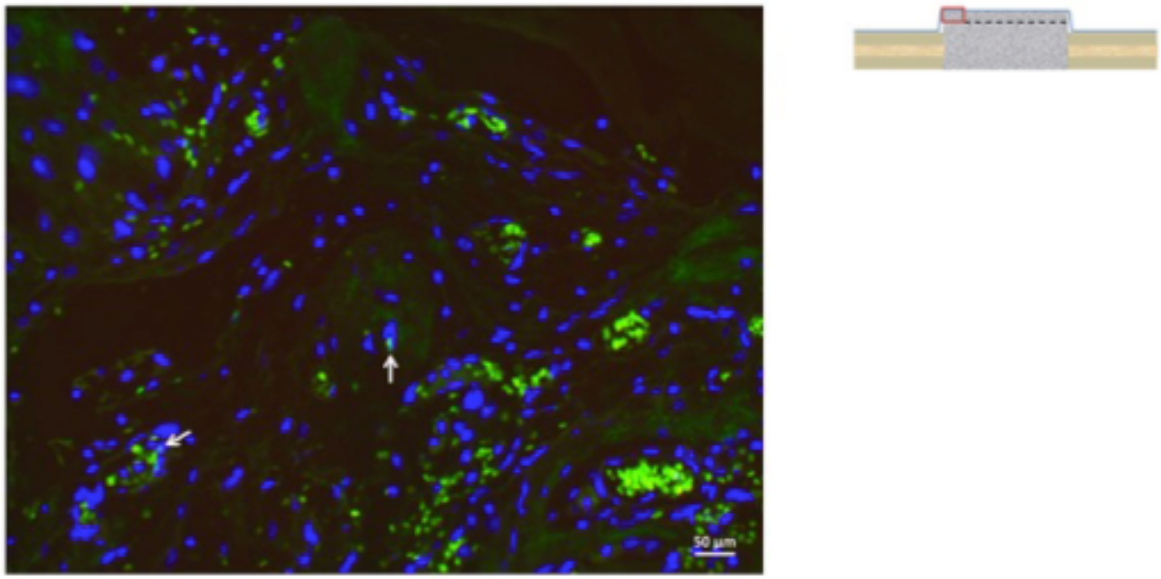


Figure 6.



3.6.1 Tables and Figures Legend

Tables Legend

Table 1. A. New bone formation (NB) within lateral areas adjacent to native host bone (BA), periosteal areas (PA) and central area of the defect (CA) at 1 and 3 months of healing. Results are expressed as μm^2, mean ± SEM.

B. Fibrous tissue formation (FT) within lateral areas adjacent to native host bone (BA), periosteal areas (PA), and central area of the defect (CA) at 1 and 3 months of healing. Results are expressed as μm^2, mean ± SEM. Asterisks indicate statistical significance ($p< 0.05$).

Table 2. Blood capillaries density within lateral areas adjacent to native host bone (BA), periosteal areas (PA) and central area of the defect (CA) at 1 and 3 months of healing.

Results are reported as number of capillaries (n) over mm^2, mean ± SEM. Asterisks indicate statistical significance ($p< 0.05$).

Table 3. Density of OSX/OC co-positive cells within lateral areas adjacent to native host bone (BA), periosteal areas (PA) and central area of the defect (CA) at 1 and 3 months of healing.

Results are reported as number of OSX/OC co-positive cells (n) over mm^2, mean ± SEM.

Figures Legend

Figure 1. Surgical procedure of "over-inlay" grafting: (A) osteotomy on parietal bone created by means of a trephine bur with a 5 mm external diameter (B) filling of the defects with a cylindrical block graft which partially exceeded calvarial height. Optical camera (Nikon D3x, 24.5 Megapixels).

Figure 2. A. Scheme of "over-inlay" graft and surrounding host tissues in a sagittal section: grafting material (DBBM), host trabecular bone (TB), host periosteum (P), host cortical bone (CB). Dotted line: distinction between "over" and "inlay" component of the graft.

B. Histological specimen stained with hematoxylin-eosin, showing the typical aspect of "over-inlay" graft. Optical microscopy, original magnification 2X.

Figure 3. A. Scheme of ROIs considered for histomorphometric evaluation: periosteal area (PA), lateral areas adjacent to native host bone (BA) and central area of the defect (CA).
B. Histological specimen stained with hematoxylin-eosin, showing PA, BA and CA ROIs considered for histomorphometric evaluation. Optical Microscopy, original magnification 2X.

Figure 4. Representative histological details of lateral areas adjacent to native host bone (BA), periosteal areas (PA) and central areas of the defect (CA) at 1 and 3 months of healing. Areas of new bone formation (NB), fibrous tissue (FT), grafting material (DBBM) and native bone (OB) are marked with letters. Black stars outline periosteum level.
Lateral margins of the defect (BA) show consistent areas of newly formed woven bone adjacent to native lamellar bone both at 1 and 3 months. Numerous, big and roundish osteocytes are visible within this area, as well as abundant blood vessels. Projections of newly formed bone towards the grafted area are clearly identifiable in the test sample.
Periosteal area (PA) presents thick layer of fibrous tissue with abundance of cells and blood vessels at 1 month. At 3 months, nodules of newly-formed bone are also visible in strict contact with residual grafting material.
Central area (CA) is mainly composed by fibrous tissue and residual biomaterial, with poor new bone formation at both observational periods.
Residual biomaterial showed a good integration within the host tissues and no signs of inflammatory reaction were detectable at any of the analyzed ROIs.
Hematoxylin-eosin staining. Optical Microscopy, original magnification 10X.

Figure 5. Microscopic image showing blood capillaries, recognized by brown of FVIII IHC-staining revealed by DAB. The region where the image was captured is marked in red in the scheme. Optical Microscopy, scale bar: 20 μm.

Figure 6. OSX positive cells visualized with Green Fluorescence. The blue fluorescence of DAPI recognize nuclei. The region where the image was captured is marked in red in the scheme. Fluorescence Microscopy, scale bar 50 μm.

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CHAPTER 4.

Local Stanozolol administration and bone healing of rat calvarial critical-size defects

ABSTRACT

Aim: The aim of this *in vivo* study was to assess the effects of Stanozolol (ST) combined to a demineralized bovine bone matrix (DBBM) scaffold on bone regeneration in rat calvarial critical-size defects at 1 and 3 months of healing. **Materials and Methods:** *Biomaterial preparation* - DBBM blocks (Geistlich Bio-Oss® Block 1x1x2, Geistlich Biomaterials) were cut into discs of 5 mm diameter and 1.5 mm of width under sterile conditions with a wheel diamond dental bur. Discs were weighed with a precision balance and a matching between disc pairs was performed. After that, one disc of each pair was treated with 150 µl of 0.1% ETOH + 100nM ST (DBBM+ST, test group), while the other was treated with 150 µl of 0.1% ETOH (DBBM, control group). *Surgical procedures* – Twenty-six male Wistar rats aged 4 months were included in the study. Standardized critical size defects were created bilaterally on each parietal bone by means of a trephine burr of 5.0 mm external diameter. The defect on one side was filled with DBBM+ST (test) and the controlateral with DBBM scaffold alone (control). All surgeries were performed by the same operator. Fourteen animals were allocated to the 1 month healing period, while 12 animals were allocated to the 3 month healing time. In order to evaluate mineral apposition rate (MAR), at days 23 and 24 rats were treated with a daily administration of 10% Xylenol Orange at the dosage of 30 mg/kg. From day 80 to 84, animals allocated to the healing period of 3 months were administered with Calcein Green 20 mg/kg once a day. At the end of the experimental period, rats were euthanized and samples were harvested. *Histology* - Samples were embedded in paraffin and serial slices were obtained with a rotative microtome in a plane parallel to the sagittal suture. Central sections were stained with haematoxylin-eosin for histomorphometric evaluation. The region of interest (ROI) to be analyzed was determined by identifying the lateral margins of the intrabony defect and the upper and lower superiosteal adjacent areas. The amount of new bone formation (NB), fibrous tissue (FT) and residual biomaterial (DBBM) were calculated and expressed as a percentage within the ROI. A secondary morphometric evaluation of NB and FT within sub-ROIs BA, PA and CA was carried out, as described in *Chapter 3*. *Immunohistochemistry* - The number and distribution of blood capillaries, the co-positivity for OSX/OC, ALP expression and COL1A1 expression were evaluated and quantified with immunohistochemistry. *Gene expression evaluation* - Gene expression of GAPDH, RUNX2, OSX, COL1A1, VDR, COL1A2, POSTN, TGFb, FN, ALP and BGLAP was analyzed through RT-PCR. *Statistical analysis*- Gaussian distribution of data was verified with D'Agostino-Aearson omnibus normality test. DBBM discs homogeneity and the efficiency of the matching between test and control samples was evaluated with using a paired t-

test. As for primary morphometrical, immunohistochemical and gene expression data, differences between test and control group were assessed using paired Student's t test with Welch's correction. Secondary morphometric outcomes were analyzed with two-way Anova and Bonferroni's post-test for multiple comparisons. $p<0.05$ was considered as the level of statistical significance. Results are reported as mean values ± SEM. **Results:** Qualitative histology showed a good integration of grafts in the surrounding tissue both in test and control samples. Direct deposition of bone was found on the surface of biomaterial and fibrous connective tissue was visible within the defect, with abundance of blood vessels. Histomorphometry revealed a significantly higher NB in DBBM+ST compared to DBBM specimens at 1 month of observation (24.41% ± 4.14% vs 15.01% ± 2.43%, $p<0.05$), while this difference was no more detectable at 3 months (27.36% ± 1.76% vs 27.38% ± 2.921%, $p>0.05$). FT presented a tendency to be lower in test vs control group both at 1 month (45.53% ± 4.95 vs 54.21% ± 4.83, $p>0.05$) and 3 months (46.19% ± 2.995% vs 47.62% ± 3.44%, $p>0.05$). Residual DBBM was not significantly different between test vs control group neither at 1 month (29.16% ± 4.14% vs 30.78% ± 4.15%, $p>0.05$) nor at 3 months (24.99 % ± 2.51% vs 23.28% ± 6.78, $p>0.05$). Secondary morphometry analysis revealed a superimposable pattern of NB and FT deposition within test and control sub-ROIs. MAR evaluation revealed a noticeable bone remodeling activity at days 23-24 in both groups, with a significantly higher apposition rate in test vs control group (9.20 μm/day ± 0.37 μm/day vs 6.50 μm/day ± 1.09 μm/day, $p<0.05$). At 3 months, MAR was very slowand no differences between groups were detectable (7.07 μm/day ± 1.24 μm/day vs 7.92 μm/day ± 1.51 μm/day, $p>0.05$). IHC analysis revealed that 1 month, the number of capillaries detected in test samples did not significantly differ from that of controls (11.43 n/mm^2 ± 2.01 n/mm^2 vs 11.25 n/mm^2 ± 2.30 n/mm^2, $p>0.05$), while at 3 months test samples appeard to be significantly more colonized with capillaries compared to control samples (28.26 n/mm^2 ± 5.62 n/mm^2 vs 9.84 n/mm^2 ± 2.01 n/mm^2, $p<0.05$). BA appeared to give a substantial contribution to graft vascularization and at 3 months a significantly higher capillary density was found in this sub-ROI compared to others in test samples (BA: 28.26 n/mm^2 ± 2.17 n/mm^2 vs PA: 10.70 n/mm^2 ± 2.18 n/mm^2, $p<0.05$ and vs CA:17.81 n/mm^2 ± 3.63 n/mm^2, $p>0.05$). As for osteoblast identification, a significantly lower OSX/OC co-positivity in test vs control group at 1 month (2.00 n/mm^2 ± 0.21 n/mm^2 vs 3.9 n/mm^2 ± 0.32 n/mm^2, $p<0.05$), while an opposite pattern was detected at 3 months (2.05 n/mm^2 ± 0.27 n/mm^2 vs 1.01 n/mm^2 ± 0.20 n/mm^2, $p<0.005$), principally due to a decrease in control group over time. A tendency of ALP to be more

expressed in control group samples was visible at 1 month and an opposite trend was visible at 3 months, although these differences were not statistically significant.
RT-PCR revealed a consistent activation of genes related to osteogenic differentiation and apposition of bone matrix in both test and control samples at both time-points. **Conclusions:** Standing to our results, ST fastens new bone formation in the early healing phase of rat calvarial critical-size defects. Also, the model used in this study provides evidence about the advantages of local administration approach, which allows to use low steroid concentrations and get a focused anabolic effect. Further studies are required to investigate ST effects on bone regeneration and its potential application in a clinical environment.

Key-words: Rat; Critical-size defect; Bone grafting; Bone regeneration; Androgens; Stanozolol

RIASSUNTO

Obiettivo: *Lo scopo di questo studio* in vivo *consiste nella valutazione degli effetti dello Stanozololo (ST) combinato ad un scaffold di osso bovino deproteinizzato (DBBM) sulla rigenerazione ossea in difetti critici di calvaria di ratto a 1 e 3 mesi.*

Materiali e Metodi: Preparazione del biomateriale – *Blocchi di DBBM (Geistlich Bio-Oss® Block 1x1x2, Geistlich Biomaterials) sono stati tagliati in modo da ricavarne dischi di 5 mm di diametro e 1.5 mm di spessore. I dischi sono stati pesati con una bilancia di precisione ed è stato effettuato un matching tra coppie di dischi di dimensioni omogenee. In seguito, un disco di ogni coppia è stato trattato con 150 μl di 0.1% ETOH + 100nM ST (DBBM+ST, gruppo test), mentre l'altro è stato trattato con 150 μl di 0.1% ETOH (DBBM, gruppo controllo).* Procedure chirurgiche –
Ventisei ratti Wistar maschi dall'età di 4 mesi sono stai inclusi nello studio. Difetti critici standardizzati sono stati create bilateralmente sull'osso parietale dei ratti,per mezzo di una fresa trephine dal diametro esterno di 5 mm. I difetti chirurgici sono stati riempiti su un lato con DBBM+ST (lato test) e sull'altro con il solo scaffold DBBM (lato controllo). Tutti gli interventi sono stati effettuati dallo stesso operatore. Quattordici animali sono stati allocati al periodo di guarigione 1 mese e 12 al periodo di guarigione 3 mesi. Allo scopo di valutare il tasso di apposizione minerale (MAR), nei giorni 23 e 24 i ratti sono stati trattati con somminstrazione giornaliera di 10% Xylenol Orange alla dose di 30 mg/kg. Dal giorno 80 al giorno 84, gli animali allocati al periodo di guarigione di 3 mesi sono stati trattati con Calcein Green 20 mg/kg una volta al giorno. Alla fine del periodo sperimentale, i ratti sono stati sacrificati e sono stati raccolti i relativi campioni biologici. Istologia – *I campioni sono stati inclusi in paraffina e tagliati in sezioni parallele alla sutura sagittale. Le sezioni centrali sono state colorate con ematossilina-eosina per valutazioni morfometriche. La regione di interesse (ROI) da analizzare è stata determinata identificanto i margini laterali del difetto infraosseo ed i limiti del periostio. La quantità di osso neoformato (NB), tessuto fibroso (FT) e biomateriale residuo (DBBM) sono state calcolate ed espresse come percentuale rispetto alla ROI. Una valutazione morfometrica secondaria di NB e FT è stata effettuata all'interno di sub-ROIs (BA, PA, CA), secondo il metodo descritto al* Capitolo 3. Immunoistochimica – *Il numero e la distribuzione di capillari sanguigni, gli elementi con co-positività per OSX/OC, l'espressione di ALP e di COL1A1 sono stati valutati e quantificati con immunoistochimica.* Valutazione dell'espressione genica – *L'espressione dei geni GAPDH, RUNX2, OSX, COL1A1, VDR, COL1A2, POSTN, TGFb, FN, ALP e BGLAP è stata analizzata con RT-PCR.* Analisi statistica – *La distribuzione gaussiana dei dati è stata verificata con il test di*

normalità D'Agostino-Aearson omnibus. L'omogeneità dei pesi dei dischi di DBBM tagliati e l'efficienza del matching è stata valutata con t-test per dati appaiati. Per quanti riguarda l'analisi degli outcomes primari morfometrici, immunoistochimici e di espressione genica, le differenze tra gruppo test e gruppo controllo sono state valutate con test t di Student e correzione di Welch. Per l'analisi degli outcome secondari si è utilizzato il test Anova a due vie e il post-test di Bonferroni. $p<0.05$ è stato considerato il livello di significatività statistica. I risultati sono riportati come medie ± SEM.

***Risultati**: L'analisi qualitativa dei campioni istologici rivela una buona integrazione dell'innesto nei tessuti circostanti, sia nel gruppo test sia nel gruppo controllo. Sono stati osservati osso neodeposto e tessuto firboso a diretto contatto con la superficie del DBBM , con abbondanti vasi neoformati ed assenza di reazione immuno-infiammatoria. L'istomorfometria ha rivelato una quantità di osso neodeposto NB significativamente maggiore nel gruppo test rispetto al gruppo controllo ad 1 mese (24.41% ± 4.14% vs 15.01% ± 2.43%, $p<0.05$), mentre a 3 mesi tale differenza non è stata più riscontrata (27.36% ± 1.76% vs 27.38% ± 2.921%, $p>0.05$). Il tessuto fibroso FT presentava una tendenza ad essere meno rappresentato nel gruppo test vs controllo ad 1 mese (45.53% ± 4.95 vs 54.21% ± 4.83, $p>0.05$) e 3 mesi (46.19% ± 2.995% vs 47.62% ± 3.44%, $p>0.05$). Il DBBM residuo non si è mostrato differire quantitativamente tra gruppo test e controllo né a 1 mese (29.16% ± 4.14% vs 30.78% ± 4.15%, $p>0.05$) né a 3 mesi (24.99 % ± 2.51% vs 23.28% ± 6.78, $p>0.05$). L'analisi morfometrica secondaria ha rivelato un pattern sovrapponibile di distribuzione di NB e FT all'interno delle diverse sub-ROIs analizzate. La valutazione del MAR ha evidenziato una notevole attività di rimaneggiamento osseo ai giorni 23 e 24 in entrambi i gruppi, con un tasso di apposizione minerale significativamente più alto nel gruppo test vs controllo in questo frame temporale (9.20 μm/day ± 0.37 μm/day vs 6.50 μm/day ± 1.09 μm/day, $p<0.05$). A 3 mesi, si è osservata una consistente riduzione del MAR in entrambi i gruppi, senza differenze rilevanti tra i due (7.07 μm/day ± 1.24 μm/day vs 7.92 μm/day ± 1.51 μm/day, $p>0.05$). L'analisi immunoistochimica ha rivelato che a 1 mese il numero di capillari sanguigni del gruppo test non differiva significativamente dai controlli (11.43 n/mm^2 ± 2.01 n/mm^2 vs 11.25 n/mm^2 ± 2.30 n/mm^2, $p>0.05$), mentre a 3 mesi i campioni del gruppo test apparivano più colonizzati da capillari rispetto ai controlli (28.26 n/mm^2 ± 5.62 n/mm^2 vs 9.84 n/mm^2 ± 2.01 n/mm^2, $p<0.05$). E' emerso che il maggiore contributo alla vascolarizzazione dell'innesto venisse fornito dalla regione BA, ed infatti a 3 mesi una densità di capillari significativamente maggiore è stata misurata in questa sub-ROI rispetto alle altre nei campioni del gruppo test (BA: 28.26 n/mm^2 ± 2.17 n/mm^2 vs*

PA: 10.70 n/mm^2 ± 2.18 n/mm^2, p<0.05 and vs CA:17.81 n/mm^2 ± 3.63 n/mm^2, p>0.05). Elementi con co-positività per OSX/OC sono stati riscontrati in numero significativamente minore nel gruppo test vs controllo a 1 mese (2.00 n/mm^2 ± 0.21 n/mm^2 vs 3.9 n/mm^2 ± 0.32 n/mm^2, p<0.05), mentre un trend opposto è stato rilevato a 3 mesi (2.05 n/mm^2 ± 0.27 n/mm^2 vs 1.01 n/mm^2 ± 0.20 n/mm^2, p<0.005), dato principalmente legato ad una diminuzione delle positività nel gruppo controllo. Allo stesso modo, si è riscontrata una tendenza di ALP ad essere maggiormente espresso nel gruppo controllo a 1 mese, mentre un trend opposto si è evidenziato a 3 mesi. La RT-PCR ha rivelato una consistente e variegata modulazione di geni correlati al differenziamento osteogenico e all'apposizione di matrice minerale in campioni del gruppo test e del gruppo controllo a 1 e 3 mesi.

***Conclusioni**: Stando ai risultati ottenuti, la somministrazione locale di ST accelera la neoformazione ossea nella fase precoce di integrazione di innesti in difetti critici in calvaria di ratto. Inoltre, il modello animale usato in questo studio ha fornito evidenza circa i vantaggi di un approccio di somministrazione locale di steroidi, che consente di utilizzare basse concentrazioni di farmaco e di ottenere un effetto anabolico focalizzato. Ulteriori studi sono necessari per testare gli effetti dello ST sulla rigenerazione ossea e le sue potenziali applicazioni in ambito clinico.*

***Parole chiave:** Ratto; Difetto critico; Innesti ossei; Rigenerazione ossea; Androgeni; Stanozololo*

4.1 Introduction

Alveolar bone deficiency is a common finding of clinical practice and may represent a central concern in planning an adequate oral rehabilitation, especially in case of large defects. Autologous bone is still considered as the "gold-standard" grafting material, but the high morbidity of the sampling procedure and the limited availability of this material boosted the search to find efficient alternatives. Combining osteoconductive scaffolds with bioactive molecules known to promote bone regeneration represents a field of growing interest in current research and many agents have been tested so far [1] [2] [3].

Androgen hormones play a significant role in regulating skeletal morphogenesis and in maintaining bone homeostasis during the whole life [4] [5].

In vitro experiments investigating the effects of androgens on different cell types showed that they enhance osteoblast proliferation and/or inhibit osteoblastic apoptosis [6] [7] [8] [9], while the effects of these hormones on osteogenic differentiation are more controversial. Most authors support the notion that androgens stimulate osteoblastic differentiation and extracellular bone matrix apposition [10] [11] [12]. Inhibitory effects on osteoclast differentiation [13] and activity [14], as well as enhancing of osteoclast apoptosis [15] have been reported as well.

Animal models of gonadectomized rats showed an early increase of cancellous bone turnover and osteoclast activity, resulting in a later decrease in bone mass, irrespective of gender and age [16] [17] [18] [19]. In these models, bone loss could be prevented by exogenous androgen administration [20] [21] [22] [23] [24] [25]. Probably this outcome was in part due to androgens aromatization to estrogens, whose protective effects on bone mass maintenance are well known [26]. However, treatment of orchiectomized rats with non-aromatizable androgens yielded beneficial effects on bone, thus allowing to perceive a distinct androgen action [27], [28]. In female animals, blockade of specific androgen receptor led to osteopenia, while androgen administration improved bone mineral density [29][30].

Pathological conditions reproducing a lack of androgens in men (i.e. hypogonadotropic hypogonadism, Klinefelter syndrome, delayed puberty, androgen insensitivity syndrome, androgen deprivation therapy in patients suffering from prostate cancer) are commonly associated to a decrease of bone mineral density and total bone mass [31] [32] [33][34]. In such cases of severe androgen depletion, exogenous administration of an androgenic supportive therapy helped to preserve the total bone mass of the body, particularly at cancellous sites. Bone density tended to

stabilize over about 2 years, similar to what is seen during therapy with other anti-resorptive drugs used in the treatment of osteoporosis. [35] [36] [37]. Nevertheless, the overall long-term beneficial effects of androgen administration for the treatment of osteoporosis still remains controversial [38].

Stanozolol (ST) is a non-aromatizable synthetic steroid derived from dihydrotestosterone which combines high anabolic and low androgenic action. Available studies revealed that ST may promote osteoblastic growth and activity *in vitro* [39], while it increased bone mineral density and improved mechanical properties *in vivo* as well as bone mass in osteoporotic conditions [40] [41]. A recent study demonstrated beneficial effects of local ST administration in the treatment of osteoarthritis [42].

Despite ST showed a positive impact on bone metabolism, no literature is currently available regarding the effects of local ST administration on bone healing.

The aim of this *in vivo* study was to assess the effects of ST combined to a deproteinized bovine bone block (DBBM) on bone healing in rat calvarial critical-size defects.

4.2 Materials and Methods

Biomaterial preparation

Fifty-two DBBM blocks (Geistlich Bio-Oss® Block 1x1x2, Geistlich Biomaterials) were cut into discs of 5 mm diameter and 1.5 mm of width under sterile conditions with a wheel diamond dental bur. Discs were weighed with a precision balance (Mettler Toledo Analytical Balance AE240, Marshall Scientific) and a matching between disc pairs was performed.

After that, one disc of each pair was treated with 150 µl of 0.1% ETOH + 100nM ST (DBBM+ST, test group), while the other was treated with 150 µl of 0.1% ETOH (DBBM, control group). Discs were left for 20 minutes at a room temperature to consent ST adsorption on DBBM surface, as observed previously (data not shown) and were incubated at 50°C for 4 hours to allow ETOH volatilization. Then, discs were singularly packed, stocked in pairs and randomly marked with an alphanumeric code.

Surgical procedures

The study protocol was approved by the Ethics Committee for Animal Testing of Parma. Twenty-six male, Wistar rats aged 4 months were included in the study.

Fifteen minutes prior the surgical procedure, all animals received a single intra-muscular administration of 10 mg/kg enrofloxacin (Baytril, Bayer, Germany). Anesthesia was obtained with intraperitoneal injection of 30 mg/kg tilethamine hydrochloride zolazepam hydrochloride (Zoletil 100, Virbac, France). The surgical site in the parietal region of the calvaria was shaved off and disinfected with povidone-iodine 10% (Betadine), then a midline incision from the bipupillar line to the occipital process was performed through skin and periosteum at a bone level. Surgical flaps were gently raised. Standardized critical size defects were created bilaterally on each parietal by means of a trephine bur of 5.0 mm external diameter (Biomet 3i, USA) attached to a ATR 3000 Plus motor (Simit, Italia) 800 rpm, under abundant irrigation with sterile saline. The defect on one side was filled with DBBM+ST (test) and the contralateral with DBBM scaffold alone (control). A 2-layers suture (Vycril 5-0, Ethicon, Johnson & Johnson, Netherlands; Prolene 3-0, Ethicon, Johnson & Johnson, Netherlands) was carried out to allow primary intention wound healing, paying attention to preserve periosteum (Figure 1, A-D).

All surgeries were performed by the same trained operator. After surgery, each rat was randomly allocated to an healing period of 1 or 3 months and was kept in a separate cage.

In order to evaluate mineral apposition rate (MAR), at days 23 and 24 rats were treated with a daily administration of 10% Xylenol Orange (Sigma Aldrich, USA) at the dosage of 30 mg/kg. From day 80 to 84, animals allocated to the 3 month healing period were administered with Calcein Green 20 mg/kg (Sigma Aldrich, USA) once a day. During the whole duration of the study, rats were in a monitored environment (21°C; 12:12 light cycle) and received a solid diet and water ad libitum.
At the end of the experimental period, each rat was euthanized with an intraperitoneal injection of pentobarbital 150 mg/kg. A mid-thickness incision was performed at the surgical site and two rectangulars specimens resepectively containing the test and the control defect area with its surrounding tissues were removed.

Histomorphometry

Samples from 18 rats were fixed in 10% buffered formalin for 48 hours, washed in running water and decalcified in a 0.5 M ethylenediaminetetraacetic acid (EDTA) solution pH 7.4. They were dehydrated in ascending grades of alcohol from 70% to 100% (Carlo Erba reagents, Italy), clarified in a xylene solution (Carlo Erba reagents, Italy) and embedded in paraffin (Lab-O-Wax, Histoline) using an automated embedder (ATP 700 Histo-Line). For each sample, serial longitudinal 5µm-thin serial sections were obtained with a rotative microtome (RM -2155, Leika) in a plane parallel to the sagittal suture.
Central sections were stained with haematoxylin-eosin for histomorphometric evaluation, while adjacent sections were used for immunohistochemical evaluations.
The region of interest (ROI) to be analyzed was determined by identifying the lateral margins of the intrabony defect and the upper and lower periosteal adjacent areas. After verification of ROI homogeneity, the amount of new bone formation (NB), fibrous tissue (FT) and residual biomaterial (DBBM) were computed as primary outcomes.
Additional standardized sub-regions of interest were traced at periosteal areas (PA), lateral areas adjacent to native bone (BA) and central area of the defect (CA), as previously described (*see Chapter 3*). A secondary morphometric evaluation of NB and FT within each of these additional ROIs was then conducted in 12 rats (6 sacrificed at 1 month and 6 at 3 months).
Images were captured with a binocular optical microscope (Nikon Eclipse 90i, Tokyo, Japan) connected with digital camera (Nikon model DS-5M) with an original magnification of 1X, 2X, 4X, 10X. Data were analyzed with the aid of NIS-Elements AR 2.1 (Nikon) software.
Morphometric data were computed by image analysis software (Image Pro-Plus 4.0, Media Cybernetics, USA).

Immunohistochemistry

Immunohistochemical analysis (IHC) was conducted on optical and fluorescence microscopy.

Sections were deparafinized and rehydrated through xylene and serial dilutions of ethanol to distilled water. They were incubated in an antigen retrieval solution composed by sodium citrate buffer 10 mM (pH 6.0) and EDTA 1 mM (pH 8.0) at 95°C for 15 min. Sections were washed in PBS (pH 7.4), with 0.02% Triton X-100 (PBS) twice for 3 min each. All incubations were performed in a humidity chamber at room temperature and followed by two, 3-min washes in PBS.

For the evaluation of capillaries, samples were incubated with anti-von Willebrandt Factor antibody (vWF, rabbit polyclonal, Dako, Denmark; 1:30, o.n), stained with streptavidin-conjugated peroxidase (using DAB as chromogen) and finally counterstained with hematoxylin. Sections were examined at 1000X magnification taking advantage of an ocular reticle (9604 mm^2 area).

As regard protein expression investigation, Alkaline Phospatase expression was assessed using an anti-ALP antibody (mouse monoclonal, Santa Cruz, CA, USA) revealed by anti-mouse secondary antibody FITC-conjugated (1:20 60' 37°C, Jackson Laboratories, PA, USA). Osteoblasts were evaluated analyzing samples processed for IHC staining with an anti-Osterix (OSX) antibody (rabbit polyclonal, Santa Cruz, CA, USA; 1:50 o.n), revealed by anti-rabbit secondary antibody FITC-conjugated (1:20 60' 37°C, Jackson Laboratories, PA, USA) and IHC staining with anti-Osteocalcin (OC) antibody (rabbit polyclonal, Santa Cruz, CA, USA) revealed by anti-rabbit secondary antibody TRITC-conjugated (1:20 60' 37°C, Jackson Laboratories, PA, USA). Cells with co-positivity for OSX/OC were computed at 1000X magnification by using fluorescent microscopy.

Collagen1 (COL1) apposition was assessed with an anti-Col1 antibody (mouse polyclonal, Santa Cruz, CA, USA; 1:50 o.n), revealed by anti-mouse secondary antibody FITC-conjugated (1:20 60' 37°C, Jackson Laboratories, PA, USA). The nuclear counterstaining was performed with DAPI (4',6-diamidine-2-phenyndole, Sigma St. Louis, MO, 5mM, 18' RT). COL1 semi-quantitative evaluation was carried out with the following arbitrary units scoring: no detectable expression (score: 0), 1 to 3 positivity/mm^2 (score: 1), 3 to 6 positivity/mm^2 (score: 2),

6 to 9 positivity/mm^2 (score: 3), 9 to 12 positivity/mm^2 (score: 3), 12 to 15 positivity/mm^2 (score: 4), > 15 positivity/mm^2 (score: 5). Slides were mounted with fluorescence mounting medium, Vectashield (VECTOR, USA).

All IHC parameters were primarily within the total defect area (ROI) and secondarly within additional sub-ROIs BA, PA and CA.

Gene expression evaluation

Samples from 6 rats were collected in liquid nitrogen for gene expression analysis. RNA isolation was perfomed using TRIzol Reagent® (Life Technologies, California, US), following manufacturer's instruction. Concisely, samples were crushed with a pestle in a mortar filled with liquid nitrogen to a fine powder and 1ml Trizol / 0.1g bone powder was added each sample. After homogenization and short stand at room temperature, samples were incubated with 0.2 ml chloroform/ml TRIzol. Then, sequential washing and spinning with 0.5ml isopropanol/mL Trizol and 1ml 75% ethanol/mL Trizol were performed and finally samples were redissolved in RNAse-free water.

Samples were retrotranscripted to cDNA (Reverse Transcription System, Promega kit) following manufacturer's instructions. Briefly, 0.5 µl of a mixture of random hexamer oligonucleotide primers (dN6, 500 µg/µl) and 1 µl of deoxynucleotide solution (dNTP 10 mM) were added to each µg of RNA sample. Consecutive incubations at 65°C for 5 minutes, then at 4°C for 5 minutes were carried out. After that, each sample was dispended 9.4 µl of a reaction mix containing DTT 5 mM, buffer 1X First strand buffer, Rnase OUT 40 U/µl and Superscript III RT 200 U/µl. To complete the retrotranscription, samples were heated at 25°C for 10 minutes, then at 50°C for 50 minutes and lastly at 70°C for 15 minutes.

Specific primers for target genes related to osteogenic differentiation were designed using Primer-Blast designing tool (National Center for Biotechnology Information, NCBI) and RT-PCR were performed (Rotor Gene TM 3000 5.0.06 version, Corbett Reaserch). A list of the examined genes and their corresponding primers sequences is reported in Table 7.

cDNA were amplified for 40 cycles in a mixture composed by 2X Platinum SYBERGreen qPCRsupermix-UDG (Invitrogen), 5 pmol specific primers and RNase-free water. Gene amplification was carried out through denaturation at 95°C for 15 seconds followed by annealing phase at 57°C to 59°C (depending on the examined gene) and an extension phase at 72°C for each cycle. Gene amplification was carried out through denaturation at 95°C for 15 seconds followed by annealing phase at 57°C to 59°C (depending on the examined gene) and an extension phase at 72°C for each cycle. Melting curve was assessed for each amplified gene and data were analyzed with relative standard curve method as described elsewhere [43].

Data normalization was carried out relatively to the amount of cDNA in each sample, measured by means of Oligreen Assay Kit (OliGreen ssDNA Quantitation Reagent and OliGreen ssDNA Quantitation Kit, Molecular Probes).

Statistical analysis

Gaussian distribution of data was verified with D'Agostino-Aearson omnibus normality test. DBBM discs homogeneity and the efficiency of the matching between test and control samples was as evaluated with using a paired t-test.

As for primary morphometrical, immunohistochemical and gene expression data, differences between test and control group were assessed using paired Student's t test with Welch's correction.

Secondary morphometric outcomes were analyzed with two-way Anova and Bonferroni's post-test for multiple comparisons.

$p<0.05$ was considered as the level of statistical significance. Results are reported as mean values ± SEM.

4.3 Results

Biomaterial preparation

Mean weight of DDBM discs used for test group (14.00 ± 0.64) was not significantly different from that of control group (13.79 ± 0.59, $p>0.05$). The pairing between test and control samples showed to be significantly effective ($p<0.0001$), with correlation coefficient r of 0.92 (Table 1, A-B).

Surgical procedures

Intraoperative mortality was less than 10%. 14 animals were allocated to the 1 month healing period, while 12 animals were allocated to the 3 months healing period.

After surgery, one rat died due to wound infection while all the others animals showed good healthy conditions and neither changes to their normal behavior nor graft exposures or other complications were reported throughout the experimental period.

Histomorphometry

Qualitative histology showed a good integration of grafts in the surrounding tissue. Neither signs of inflammation nor immune reaction were detected. Direct deposition of bone was found on the surface of biomaterial and fibrous connective tissue was visible within the defect, with abundance of blood vessels in periosteal areas. None of the samples showed complete bone regeneration within the defect area, at the times tested, thus confirming the "critical" dimensions of surgical wounds (Figures 2, A-D and 3, A-D).

NB was significantly higher in test vs control group at 1 month of observation (24.41% ± 4.14% vs 15.01% ± 2.43%, $p<0.05$), while this difference was no more detectable at 3 months (27.36% ± 1.76% vs 27.38% ± 2.921%, $p>0.05$). FT presented a tendency to be lower in test vs control group both at 1 month (45.53% ± 4.95 vs 54.21% ± 4.83, $p>0.05$) and 3 months (46.19% ± 2.995% vs 47.62% ± 3.44%, $p>0.05$). Residual DBBM was not significantly different between test vs control group neither at 1 month (29.16% ± 4.14% vs 30.78% ± 4.15%, $p>0.05$) nor at 3 months (24.99 % ± 2.51% vs 23.28% ± 6.78, $p>0.05$) (Tables 2, A and 3, A).

A further differential morphometric analysis which was carried out allowed to assess the role of distinct areas (BA, PA and CA) in graft integration (*see Chapter 3*). NB and FT presented a similar distribution within BA, PA and CA in test vs control group, thus suggesting a similar healing pattern. Consistently with previous observations (*see Chapter 3*), BA was the region which mostly

contributed in new bone formation. At 1 month, both in test ad in control group NB in this area was significantly higher than in PA (test group: BA: 16.0% ± 0.27% vs PA: 8.0% ± 0.66%, $p<0.05$. control group: BA: 8.49% ± 0.52% vs PA: 4.25% ± 0.39% μm^2, $p<0.05$.) and CA (test group: BA: 16.0% ± 0.27% vs CA: 3.08% ± 0.33% μm^2, $p<0.05$. control group: BA: 8.49% ± 0.52% vs CA: 2.12% ± 0.21% μm^2, $p<0.0001$) (Table 2, B).

A superimposable pattern was observed at 3 months, with BA being the area containing more NB compared to PA (test group: BA: 16.06% ± 0.12% vs PA: 7.92% ± 0.48%, $p<0.05$. control group: BA: 16.28% ± 0.45% vs PA: 8.15% ± 0.45%, $p<0.05$.) and CA (test group: BA: 16.06% ± 0.12% vs CA: 2.97% ± 0.21% μm^2, $p<0.05$. control group: BA: 16.28% ± 0.45% vs CA: 4.06% 0.45± %, $p<0.05$) both in test and control group (Table 3, B).

It has to be noticed that an overall increase in NB was observed over time in all examined areas of control group, with a major contribution within BA ($p<0.05$). On the other side, NB values did not undergo significant variations in any of the examined areas of test group, thus indicating that the main part of newly formed bone had already been deposed at 1 month ($p>0.05$).

As for FT, the sub-periosteal area PA exhibited significantly greater amounts of this component compared to BA and CA ($p<0.05$) in both test and control group at 1 and 3 months (1 month, test group: PA: 31.36% ± 1.56%; BA: 10.25% ± 0.51%; CA: 3.91% ± 0.81%. 1 month, control group: PA: 36.06% ± 1.80%; BA: 12.02% ± 0.60%; CA: 4.94% ± 1.41%. 3 months, test group: PA: 35.001% ± 0.87% ; BA: 11.65% ± 0.30 ; CA: 2.94% ± 1.08%. 3 months, control group: PA: 31.92% ± 1.17% ; BA: 10.64% ± 0.39% ; CA: 4.08% ± 0.72%). No significant variations in FT were observed over time in any of the analyzed areas, although an overall decrease of this parameters in ROIs of control group was detectable (Tables 2, C and 3, C).

MAR analysis showed consistent bone remodeling activity at days 23-24 in both groups, with a significantly higher apposition rate in test vs control group (9.20 μm/day ± 0.37 μm/day vs 6.50 μm/day ± 1.09 μm/day, $p<0.05$). At 3 months, MAR was very slowand no differences between groups were detectable (7.07 μm/day ± 1.24 μm/day vs 7.92 μm/day ± 1.51 μm/day, $p>0.05$) (Table 4, A-B; Figure 4, A-H).

Immunohistochemistry

IHC analysis revealed a rich vascular network was detected in all specimens, as a sign of the physiological process of bone healing and graft integration within the host tissue. At 1 month, the number of capillaries detected in test samples did not significantly differ from that of controls

(11.43 n/mm^2 ± 2.01 n/mm^2 vs 11.25 n/mm^2 ± 2.30 n/mm^2, p>0.05), while at 3 months test samples appeard to be significantly more colonized with capillaries compared to control samples (28.26 n/mm^2 ± 5.62 n/mm^2 vs 9.84 n/mm^2 ± 2.01 n/mm^2, p<0.05) (Table 5; Figure 5).

The secondary analysis within sub-ROIs provided further insights on capillary distribution, revealing a homogenoues capillary distribution within distinct sub-ROIs of both test and control group at 1 month (test group: BA: 10.50 n/mm^2 ± 2.46 n/mm^2; PA: 11.86 n/mm^2 ± 2.09 n/mm^2; CA 11.92 n/mm^2 ± 2.25 n/mm^2, p>0.05. control group: BA: 13.89 n/mm^2 ± 2.58 n/mm^2; PA: 11.21 n/mm^2 ± 1.32 n/mm^2; CA: 8.64 n/mm^2 ± 2.43 n/mm^2, p>0.05).

A noticeable raise in capillary density was observed within BA in test group (p<0.01) from 1 to 3 months observation, thus revealing a consistent capillary invasion which was mainly coming from adjacent native bone. Consistently, at 3 months, control samples presented again a quite homogeneous capillary distribution within different sub-ROIs (BA: 6.93 n/mm^2 ± 1.63 n/mm^2; PA: 10.44 n/mm^2 ± 2.50 n/mm^2; CA: 12.16 n/mm^2 ± 0.42 n/mm^2, p>0.05), while in test samples a higher number of capillaries was observed within BA (28.26 n/mm^2 ± 2.17 n/mm^2) compared to PA (10.70 n/mm^2 ± 2.18 n/mm^2, p<0.05) and CA (17.81 n/mm^2 ± 3.63 n/mm^2, p>0.05).

As for osteoblast identification, a significantly lower OSX/OC co-positivity in test vs control group at 1 month (2.00 n/mm^2 ± 0.21 n/mm^2 vs 3.9 n/mm^2 ± 0.32 n/mm^2, p<0.05), while an opposite pattern was detected at 3 months (2.05 n/mm^2 ± 0.27 n/mm^2 vs 1.01 n/mm^2 ± 0.20 n/mm^2, p<0.005) (Table 6, A; Figure 6, A), principally due to a decrease in control group over time. It is interesting to notice that osteoblasts were particularly abundant within the subperiostal area, thus suggesting a contribution of this tissue compartment to graft integration.

Indeed, the secondary evaluation of sub-ROIs highlighted that at 1 month both in test and control samples the number of cells with co-positivity for OSX/OC was significantly higher within PA (test group: 4.76 n/mm^2 ± 0.49 n/mm^2; control group: 4.71 n/mm^2 ± 0.40 n/mm^2) compared to BA (test group: 0.39 n/mm^2 ± 0.11 n/mm^2; control group: 1.95 n/mm^2 ± 0.39 n/mm^2, p<0.01) and CA (test group: 0.86 n/mm^2 ± 0.15 n/mm^2; control group: 2.60 n/mm^2 ± 0.25 n/mm^2, p<0.01).

OSX/OC co-positivity in test samples did not undergo significant changes over time within any sub-ROI analyzed (BA: 0.79 n/mm^2 ± 0.17 n/mm^2; PA: 3.97 n/mm^2 ± 0.46 n/mm^2 ; CA: 1.39 n/mm^2 ± 0.28 n/mm^2, p>0.05), while an overall decrease was observed at 3 months in control samples, which reached statistical significance within PA (BA: 0.65 n/mm^2 ± 0.13 n/mm^2, p>0.05; PA: 0.89 n/mm^2 ± 0.31 n/mm^2 , p<0.05; CA: 1.49 n/mm^2 ± 0.24 n/mm^2, p>0.05).

Statistically unsignificant differences in ALP detection were observed between test and control group neither at 1 month (8.83 n/mm^2 ± 3.80 n/mm^2 vs 12.14 n/mm^2 ± 6.29 n/mm^2, p>0.05), nor at 3 months (7.62 n/mm^2 ± 3.19 n/mm^2 vs 6.29 n/mm^2 ± 2.73 n/mm^2, p>0.05). However, a tendency of ALP to be more expressed in control group samples was visible at 1 month and an opposite trend was visible at 3 months. Similarly to OSX/OC, samples of control group presented the greatest variations in ALP over time, with a noticeable decrease at 3 months (Table 6, B; Figure 6, B).

Analysis of sub-ROIs revelaed PA to be the area which the highest expression of ALP within both test (BA: 3.37 n/mm^2 ± 0.78 n/mm^2; PA: 14.29 n/mm^2 ± 2.09 n/mm^2 ; CA: 8.84 n/mm^2 ± 1.78 n/mm^2. PA vs BA, p<0.05; PA vs CA, p<0.05) and control group samples (BA: 11.31 n/mm^2 ± 2.21 n/mm^2; PA: 14.68 n/mm^2 ± 2.00 n/mm^2 ; CA: 10.42 n/mm^2 ± 1.13 n/mm^2. PA vs BA, p<0.05; PA vs CA, p<0.05) at 1 month observation. Such a finding was still evident at 3 months in test samples (BA: 5.71 n/mm^2 ± 1.46 n/mm^2; PA: 12.14 n/mm^2 ± 1.86 n/mm^2 ; CA: 5.00 n/mm^2 ± 0.96 n/mm^2. PA vs BA, p<0.05; PA vs CA, p<0.05), while control samples presented an overall lowered and homogeneous ALP expression in all sub-ROIs (BA: 6.12 n/mm^2 ± 1.05 n/mm^2; PA: 6.12 n/mm^2 ± 1.05 n/mm^2 ; CA: 6.63 n/mm^2 ± 0. 99 n/mm^2).

Semi-quantitative evaluation of COL1A1 expression revealed a higher positivity in test vs control samples both at 1 month and 3 months, consistently with RT-PCR results (Table 6, C; Figure 6,C).

Gene expression evaluation

Gene expression analysis allowed to detect variations in genes related to cellular metabolism, osteogenic differentiation as well as of bone and connective tissue apposition.

The expression of GAPDH, an important enzyme for energy metabolism and the production of ATP and pyruvate, was significativly higher in test vs control group at 1 month (fold change test/control: 5.86 ± 3.52, p<0.05), while no differences were detectable at 3 months (fold change test/control: 1.143 ± 0.62, p>0.05). Such a finding may indicate an increase in cellular metabolism related to the anabolic effect of ST, which remained evident at 1 month observation period, while disappeared at a longer time-point.

The significantly higher increase in RUNX2 expression in test vs control group at 1 month (fold change test/control: 4.78 ± 2.36, p<0.05) suggested that some cellular activity may be oriented to an osteogenic differentiation, as this gene is well known to increase its expression since the early to the late stages of osteoblast differentiation. As for GAPDH, at 3 months no more differences in

RUNX2 expression were reported between test and control group (fold change test/control: 0.81 ± 0.36, $p>0.05$).

The expression pattern of SP7, a gene related with osteoblast commitment, was consistent with that of RUNX2, with higher values reported for test vs control samples at 1 month (fold change test/control: 4.20 ± 3.23, $p>0.05$) and no differences between groups at 3 months (fold change test/control: 0.68 ± 0.29, $p>0.05$).

It was interesting to notice that the expression of COL2A1, a gene which is found primarily in cartilage, was significantly higher in test vs control group at 1 month1 month (fold change test/control: 5.52 ± 2.49, $p<0.05$): such a finding led to speculate an induction of enchondral bone formation, although no morphologic evidence of chondrocytes was reported in hematoxylin-heosin stained samples. At 3 months no more differences were detectable, with COL2A1 test values being at the level of the essentially unvariated controls (fold change test/control: 0.75 ± 0.27, $p>0.05$).

The expression of genes related to osteoblast differentiation and deposition of mineral bone matrix has been detected also in control samples, as a sign of the physiological process of bone healing and graft integration. VDR gene, which encodes nuclear hormone receptor for vitamin D3 and is principally involved in mineral metabolism, was significantly more expressed in control vs test samples at 1 month (fold change test/control: 0.56 ± 0.10, $p<0.05$), thus revealing the activation of canonical mineralzying pathways. Other genes related to mineral matrix apposition were abundantly expressed by control samples: FN gene, which encodes a protein involved in osteoblast compaction and in the regulation of type I collagen deposition, was significantly more expressed in control vs test samples at 1 month (fold change test/control: 0.43 ± 0.64, $p>0.05$). Consistently, also COL1A2 (fold change test/control: 0.01 ± 0.002, $p<0.05$), as well as ALP, a gene linked with matrix mineralization (fold change test/control: 0.003 ± 0.001, $p>0.05$) and BGLAP, a gene encoding one of the most abundant non-collagenous proteins in the mineralized matrix of bone, presented a similar behavior. None of these genes presented different expression between test and control samples at 3 months (VDR fold change test/control: 1.00 ± 0.18, $p>0.05$; FN fold change test/control: 0.64 ± 0.55, $p>0.05$; COL1A2 fold change test/control: 1.52 ± 1.29, $p>0.05$; ALP fold change test/control: 1.11 ± 0.43, $p>0.05$; BGLAP fold change test/control: 0.98 ± 0.96, $p>0.05$), as it is compatible with a more advanced and stabilized healing phase. Nevertheless, BGLAP presented a tendency to be higher in test vs control group at this time-point (fold change test/control: 4.14 ± 1.86, $p>0.05$).

Genes identified as critical regulators of fibrosis presented a different expression between test and control samples. Particularly, both TGFb and POSTN were significantly less expressed in test vs control samples at 1 month (TGFb fold change test/control: 0.13 ± 0.06, $p<0.05$; POSTN: fold change test/control: 0.02 ± 0.01, $p<0.05$), while at 3 months similar levels of expression were detected (TGFb fold change test/control: 0.84 ± 0.49, $p>0.05$; POSTN fold change test/control: 11.88 ± 11.57, $p>0.05$). A general prospect of gene expression analysis is reported in Tables 7 and 8, A-K.

4.4 Discussion

Androgen hormones are strictly implicated in skeletal morphogenesis as well in maintaining bone homeostasis throughout life. It has been demonstrated that androgens affect growth plate maturation and closure contributing to regulate longitudinal bone growth during development, mediate bone mass control and prevent bone loss in adults [44] [45].

Despite plenty of data are available about androgen action on bone, the local application of anabolic steroid to improve bone healing has not been adequately investigated so far. This study allowed to use low doses of ST compared to parenteral administrations and consequently to reduce the risk for unfavorable side effects: *in vivo* toxicity tests performed in rats reported adverse events (hepatic toxicity, hypertension, cardiac hypertrophy, aggressive behavior) in response to ST doses that were thousand times higher than those used in this study [46] [47] [48] [49] [50]. Consistently, neither behavioral changes nor signs of pain were noticed during the whole experimental period.

Qualitative histological evaluation did not reveal inflammation/immune reaction neither in test nor in control specimens, while newly formed tissues and blood vessels showed a close proximity to residual biomaterial in all samples. These findings confirm literature data about DBBM, which is one of the most employed xenogenic materials for clinical regenerative procedures and it is known to provide a safe osteoconductive environment [51][52]. DBBM quantity showed only small variations at 1 and 3 months of observation in both test and control samples, consistently with earlier reports which described DBBM persistence at grafted sites through long time periods [53].

The presence of higher amounts of newly formed bone (NB) in test vs control samples is suggestive of a positive action of ST in enhancing bone formation at 1 month healing period. Previous literature data reported that ST-mediated positive regulation of bone metabolism, by accelerating bone formation rate, increasing bone apposition and reducing urinary calcium excretion [54]**.** Also, the consistent mineral apposition rate (MAR) observed in test samples revealed the persistence of an apposing and remodeling activity of bone extracellular matrix at this time-point [55].

RT-PCR analysis of GAPDH expression supported the hypothesis of an increase in cellular activity as an effect of ST in test samples. Despite GAPDH represents a widely used housekeeping gene, recent studies showed that it not so suitable to this purpose neither in primary mesenchymal cells [56], nor in fibroblasts [57], nor in bone cells [58] [59]. Moreover, androgen administration was found to modulate GAPDH expression [60] and the transcirpional activity of androgen receptor was upregulated by GAPDH in prostate cancer cells [61]. Taken together, these observations may

suggest an regulation played by androgens on GAPDH expression and function, although to our knowledge no studies with bone cells are currently available on this regard.

The high expression of RUNX2 and SP7 in test samples indicate an ongoing process of osteoblast commitment, as these genes are essential transcription factors in the cell-fate decision process by which mesenchymal cells become chondrocytes and osteoblasts [62] [63]. Studies involving controlled bone injuries followed by healing in animals have demonstrated that RUNX2 and SP7 are expressed intensely during bone healing at fracture sites [64]. The relatively abundant expression of COL2A1 gene in test samples at 1 month lead to hypothesize the presence of a cell population with chondroblastic features, as this gene encodes for a fibrillar collagen typical of cartilage [65] [66]. Considering that the overexpression of RUNX2 and VDR we found in test group has been previously associated with the development of hypertrophic chondrocytes during enchondral bone formation [67] [68] [69] and also GAPDH raise has been correlated with an hypoxia-induced metabolic shift during periosteal chondrogenesis and with endochondral bone formation [70], we may not exclude that in our model osteogenesis occurred at least in part through this mechanism. Nevertheless, no histological evidence was provided to support this speculation.

Control group samples showed evidence of a delayed graft integration and bone healing at 1 month, with a significantly lower MAR than in test group and a strong expression of genes which mark early phases of osteoblast differentiation (i.e. BGLAP). Genes which play a master role in matrix deposition of collagen-rich connective tissues (i.e. (i.e. FN, POSTN, TGFb, COL1A1) were also remarkably expressed in control samples [71][72][73][74] [75]: such a result can be associated to the histological findings of both new bone and fibrous tissue formation.

At 3 months, morphometrical differences between test and control group were no more detectable in any of the analyzed histomorphometrical parameters (NB, FT, DBBM). We cannot rule out that ST had been completely metabolized by 3 months and accordingly removed from the defect area, so that the physiological healing process occurring at control group sites leaded new bone formation towards levels comparable to test group. Additional studies analyzing ST pharmacokinetics after local administration are therefore recommended. However, some qualitative differences in the composition of newly formed tissues was still detectable at this time-point: test samples presented an increase in blood capillaries density throughout time providing evidence of a vital remodeling tissue. On the other hand, control samples presented an almost unvaried capillary density throughout time, thus suggesting the formation of a stable tissue with a prevalence of fibrotic component. Similarly, a decrease of OSX/OC as well and ALP IHC-positive elements from 1 to 3

months were also observed in control samples, and such a finding may indicate a progressive reduction of osteogenesis together with wound stabilization.

4.5 Conclusions

In conclusion, local ST administration enhanced new bone formation in the early healing phase of rat calvarial critical-size defects. Standing to our results, it can also be speculated that ST promoted a delayed improvement in graft osteointegration at a later time point, by maintaining an active remodeling tissue and thus reducing scar formation.

The osteoinductive activity of ST was consistent with a previous *in vitro* study (see *Chapter 2*), which showed a significant increase in the expression of genes related to osteogenic differentiation after ST treatment.

With the awareness of the positive effects of androgens on bone homeostasis, further investigations are required to bring their local administration to the clinical practice.

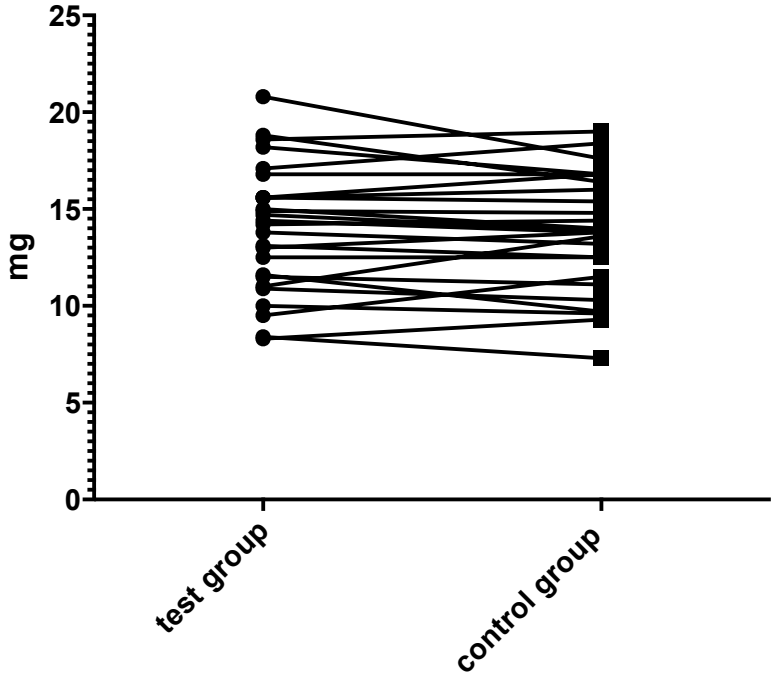
4.6 Tables and Figures

Table 1.

A.

Number of values	26	26
Minimum	8,300	7,300
25% Percentile	11,38	11,40
Median	14,30	13,85
75% Percentile	15,90	16,50
Maximum	20,80	19,00
Mean	14,00	13,79
Std. Deviation	3,290	3,038
Std. Error of Mean	0,6451	0,5958
Lower 95% CI of mean	12,67	12,56
Upper 95% CI of mean	15,32	15,02

B.



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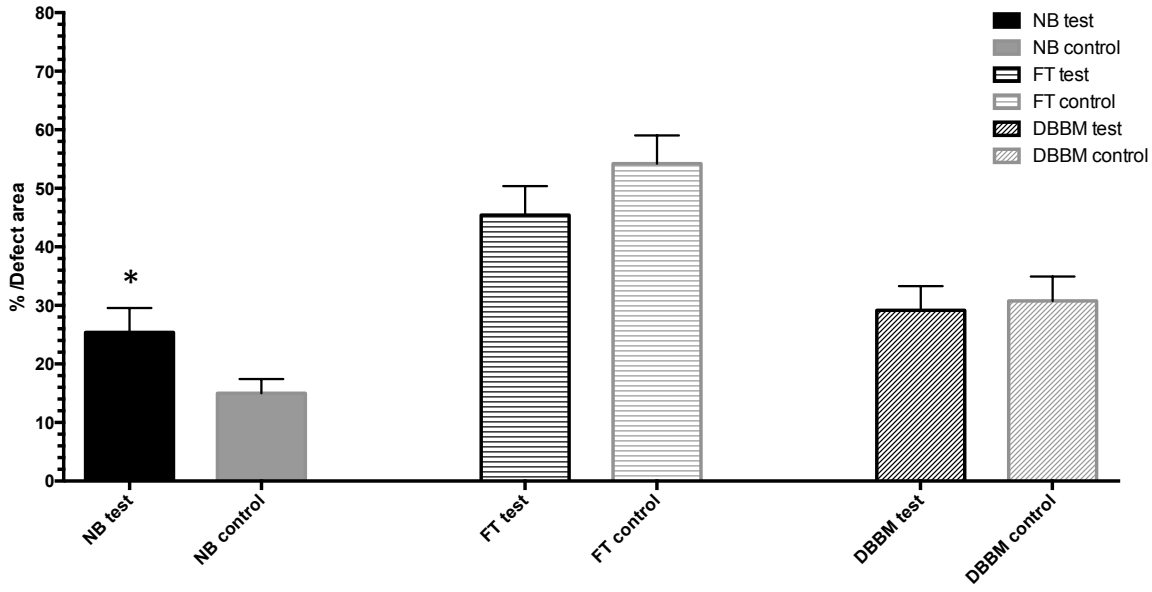
NB test
NB control
FT test
FT control
DBBM test
DBBM control
% /Defect area
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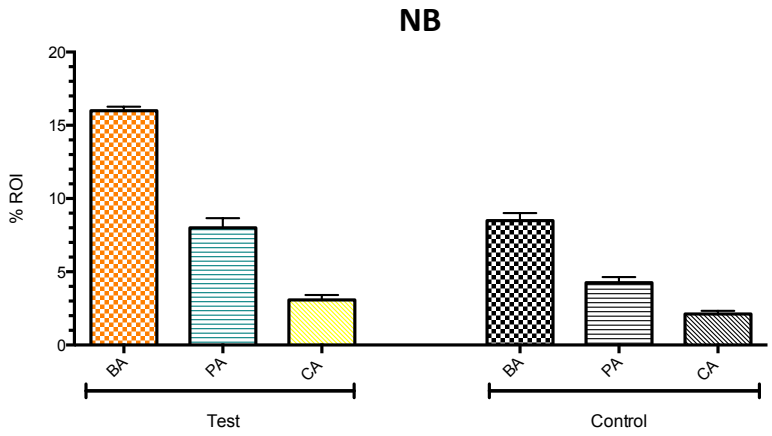
70
60
50
40
30
20
10
0
*
NB test
NB control
FT test
FT control
DBBM test
DBBM control

B.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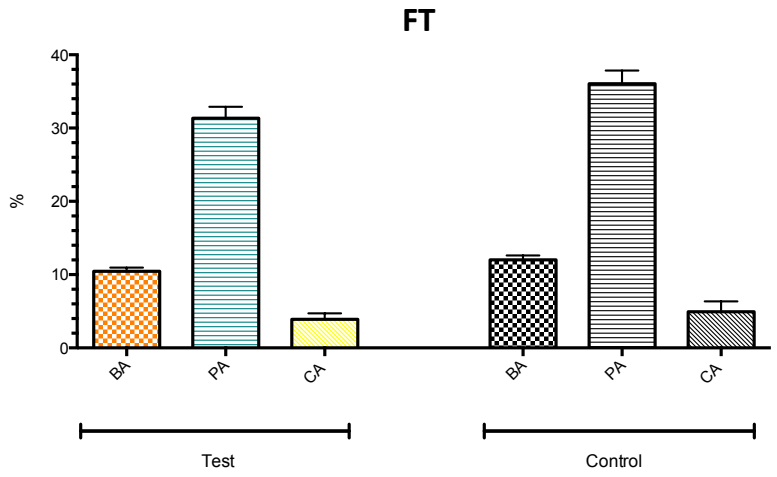
% ROI
20
15
10
5
0
BA
PA
CA
BA
PA
CA
Test
Control

C.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%
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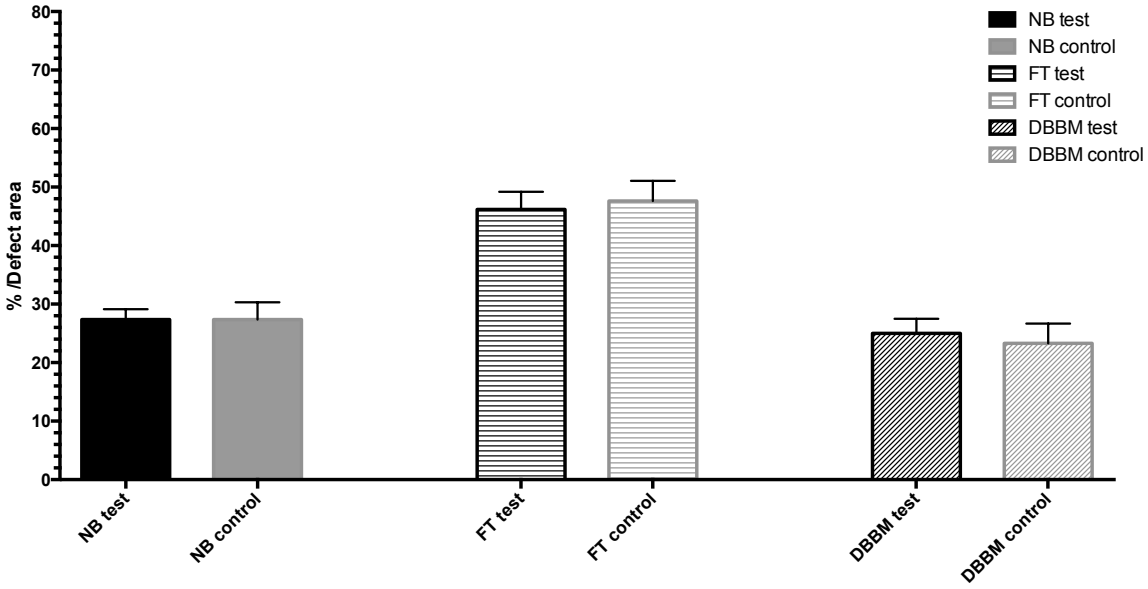
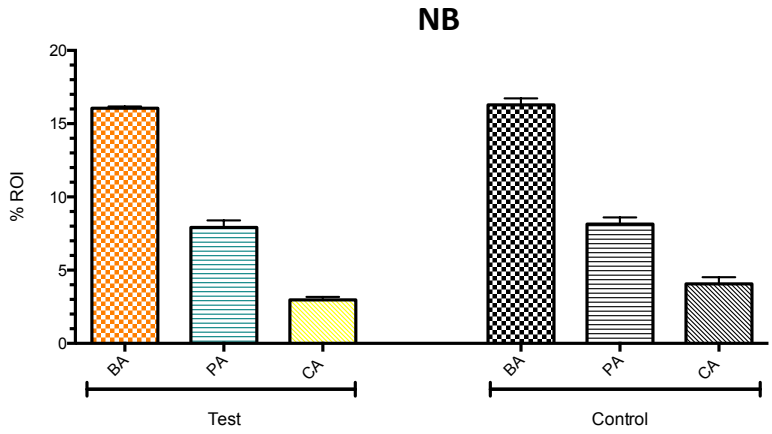
30
20
10
0
BA
PA
CA
BA
PA
CA
Test
Control

Table 3.

A.


NB test
NB control
FT test
FT control
DBBM test
DBBM control
% /Defect area
80
70
60
50
40
30
20
10
0
NB test
NB control
FT test
FT control
DBBM test
DBBM control

B.


NB
% ROI
20
15
10
5
0
BA
PA
CA
BA
PA
CA
Test
Control

C.

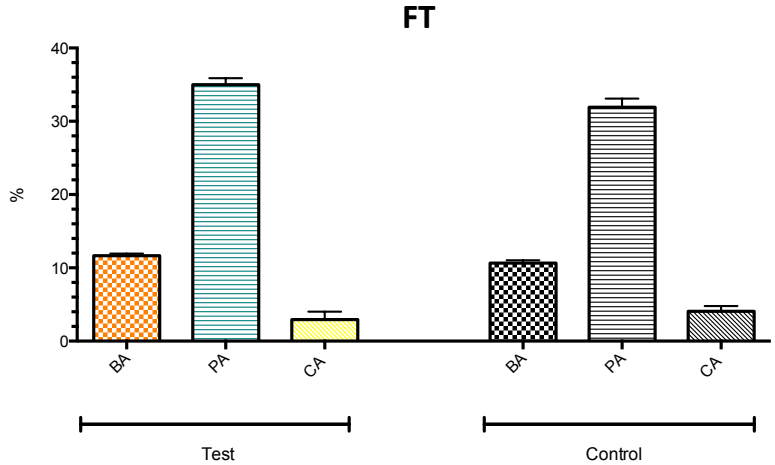
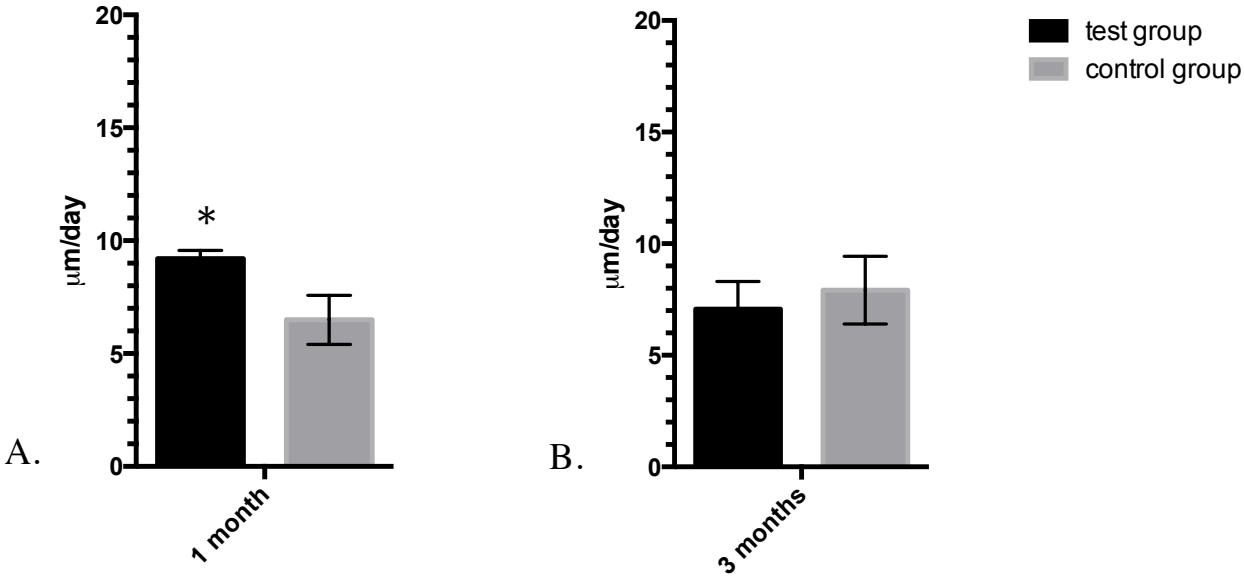

FT
%
40
30
20
10
0
BA
PA
CA
BA
PA
CA
Test
Control

Table 4.



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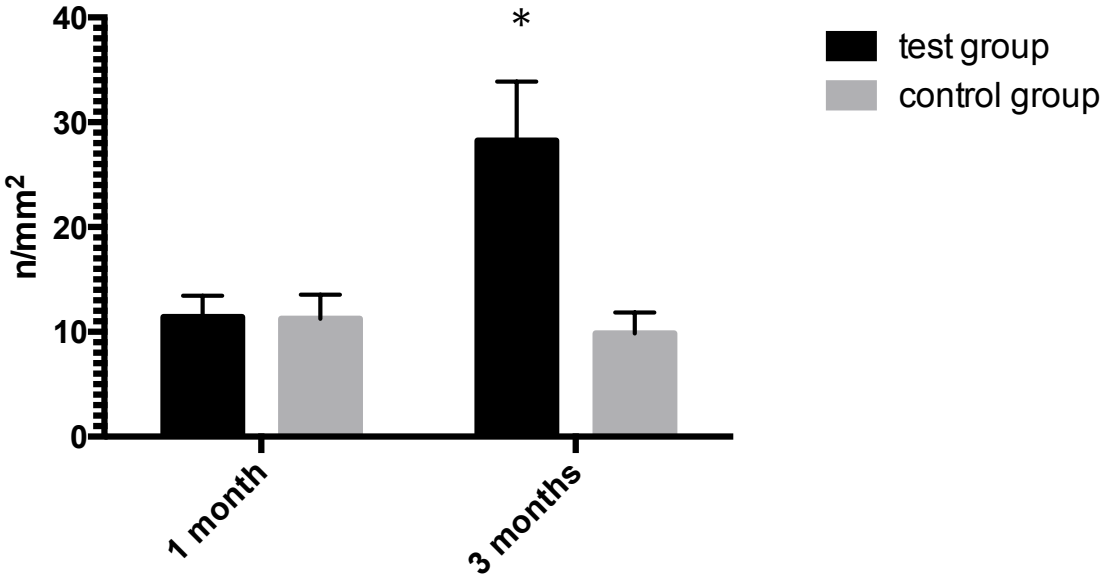
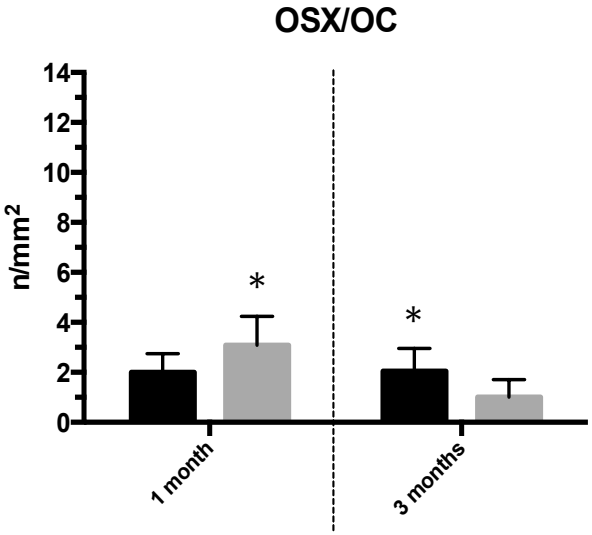
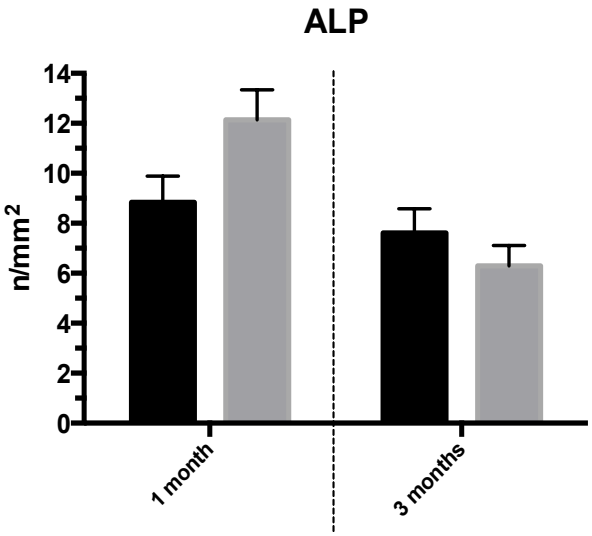


Table 6.

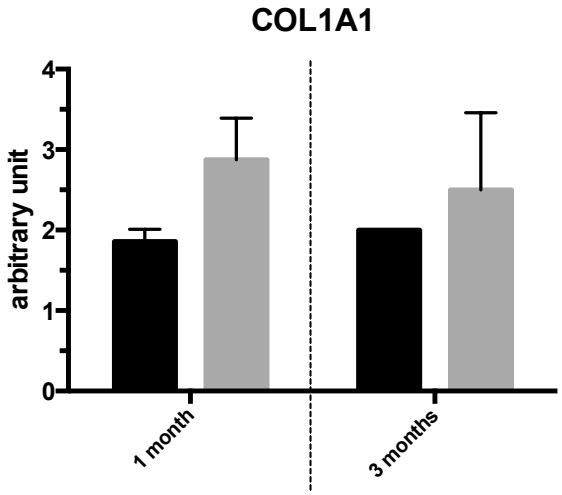
A.


OSX/OC
n/mm2
14
12
10
8
6
4
2
0
*
*
1 month
3 months

B.


ALP
n/mm2
14
12
10
8
6
4
2
0
1 month
3 months

C.


COL1A1
arbitrary unit
4
3
2
1
0
1 month
3 months

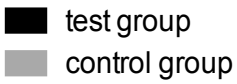
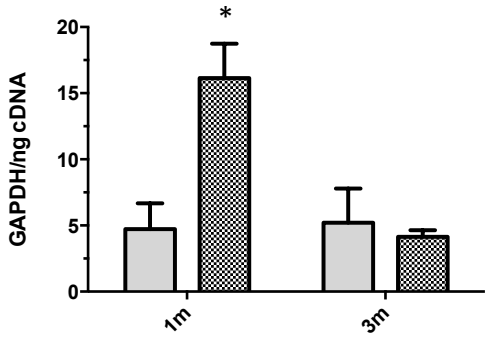

test group
control group

Table 7.

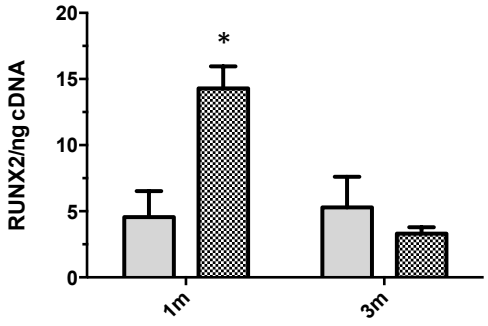
Gene	Abbreviation(s)	Primer sequences (Forward and Reverse)
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	5’-AGGTTGTCTCCTGTGACTTCA-3’ 5’-TGTTGCTGTAGCCATATTCATTGT-3’
Runt-Related Transcription Factor 2	RUNX2	5’-ACTTCGTCAGCGTCCTATCAG-3’ 5’-CATCAGCGTCAACACCATCATT-3’
Osterix	SP7 (OSX)	5’-TTCACCTGTCTGCTCTGCTC-3’ 5’-GCGGCTGATTGGCTTCTTC-3’
Collagen, Type II, Alpha 1	COL2A1	5’-AGCAGCAAGAGCAAGGAGAA-3’ 5’-AGTGGACAGTAGACGGAGGAA-3’
Vitamin D Receptor	VDR	5’-CCACAGGCTTCCACTTCAATG-3’ 5’-CGGTTGTCCTTGGTGATGC-3’
Collagen, Type I, Alpha 2	COL1A2	5’-GCAACAGCAGATTCACCTACAC-3’ 5’-AAGGAATGGCAGGCGAGAT-3’
Periostin	POSTN (OSF2)	5’-ACAATGCGTTCTCTGATGACACT-3’ 5’-CGATACACGAAGACACGGAGTT-3’
Transforming Growth Factor beta	TGFb	5’-AGCAACAATTCCTGGCGTTAC-3’ 5’-CTGTATTCCGTCTCCTTGGTTCA-3’
Fibronectin	FN	5’-CTGTGAAGAACGAGGAGGATGT-3’ 5’-CACGCTGGAGACACTGACTAA-3’
Alkaline Phoshatase	ALP	5’-GCAAGGACATCGCCTATCAG-3’ 5’-TCCAGTTCATATTCCACATCAGTT-3’
Bone Gamma-Carboxyglutamate (Gla) Protein	BGLAP	5’-GCAGACCTAGCAGACACCAT-3’ 5’-GGCTCCAAGTCCATTGTTGAG-3’

Table 8.

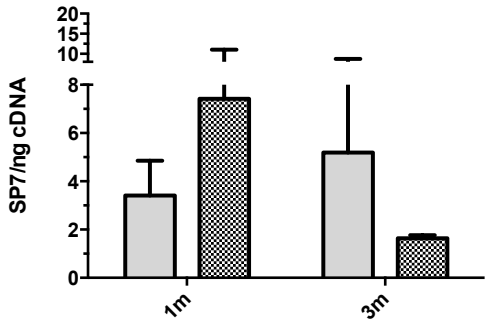
A.



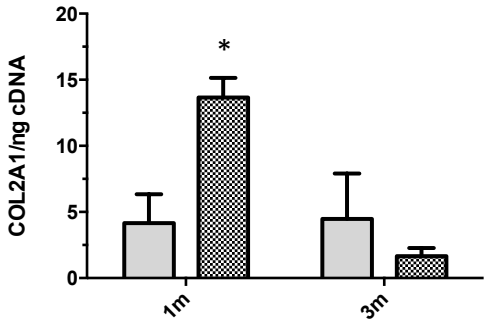
B.



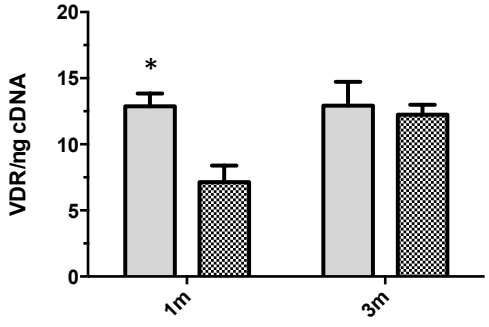
C.



D.



E.



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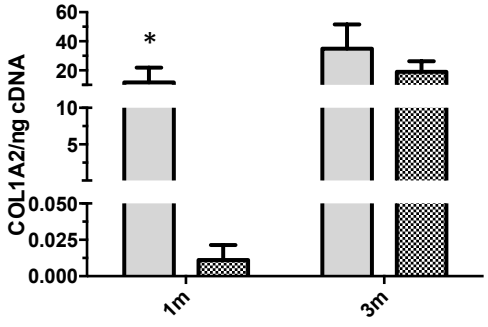
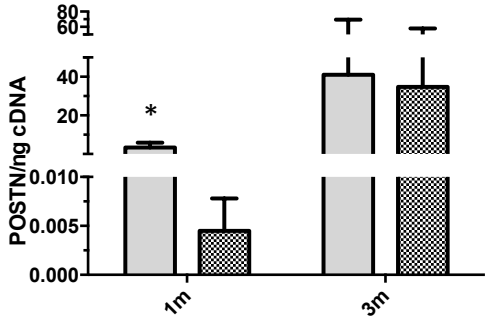


G.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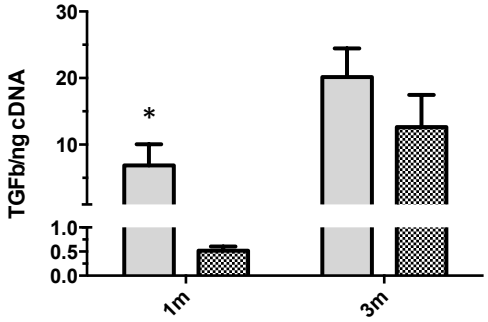
80
60
40
20
0.010
0.005
0.000
*
1m
3m

H.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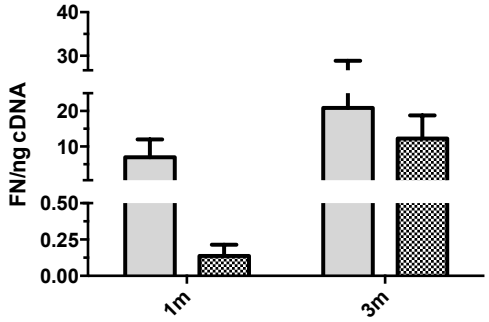
0.5
0.0
*
1m
3m

I.


FN/ng cDNA
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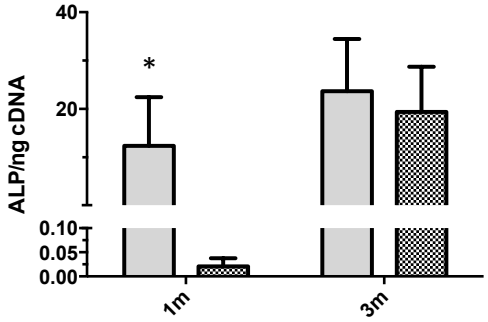
0.25
0.00
1m
3m

J.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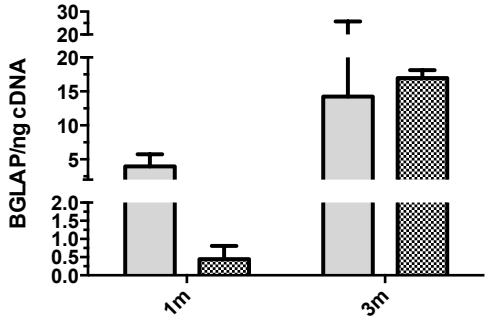
0.00
*
1m
3m

K.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1.0
0.5
0.0
1m
3m

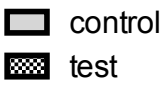

control
test

Figure 1.

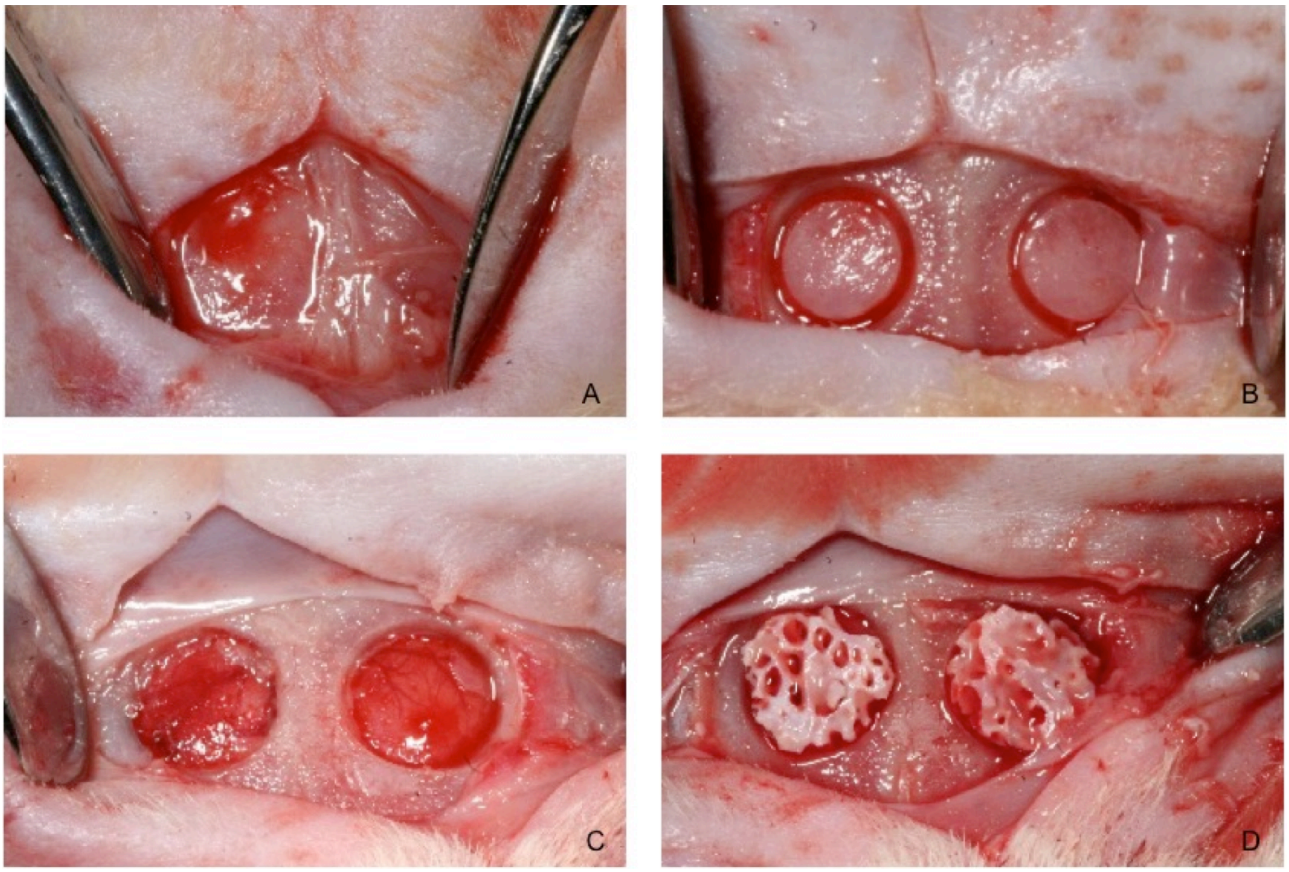


Figure 2.

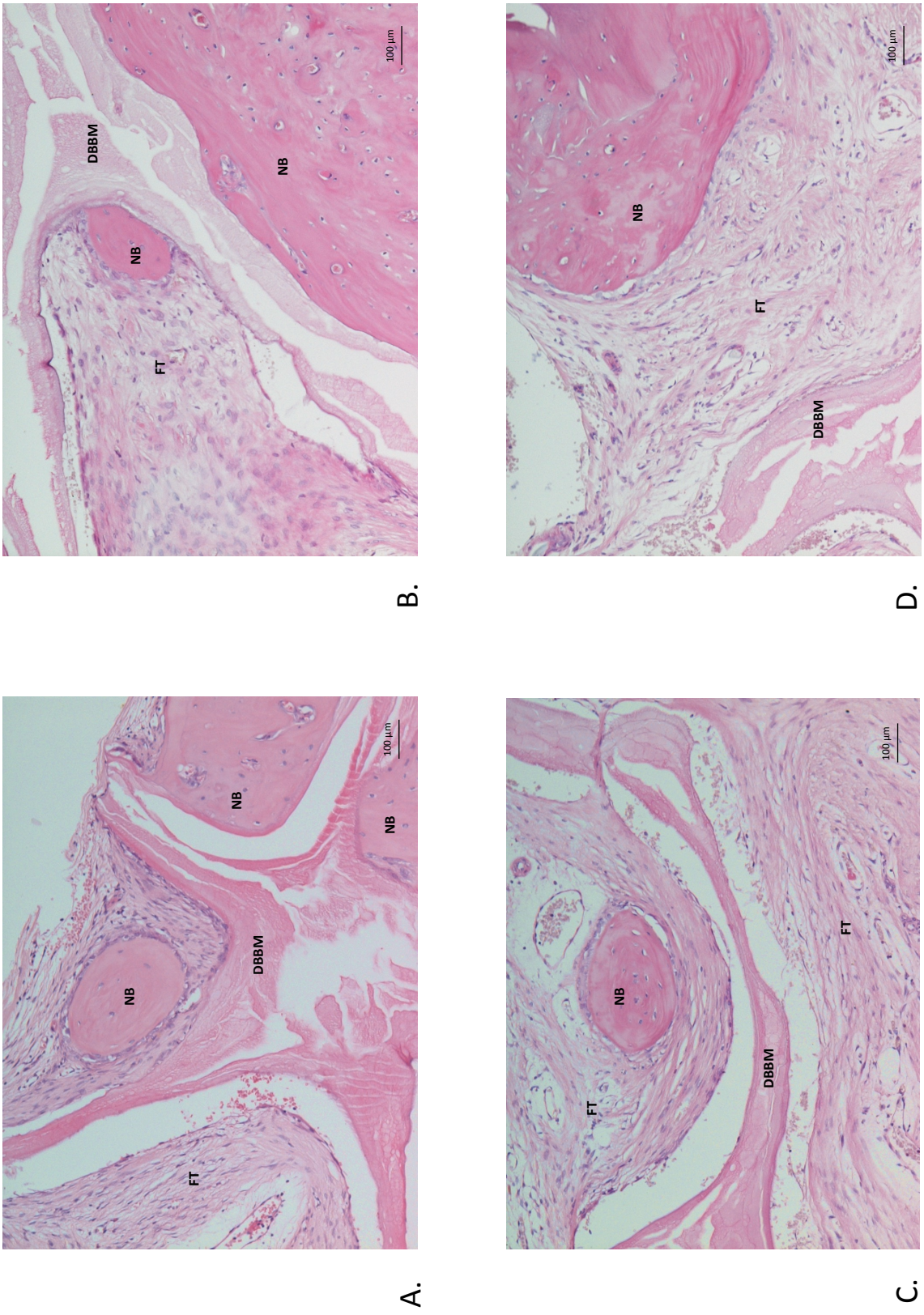
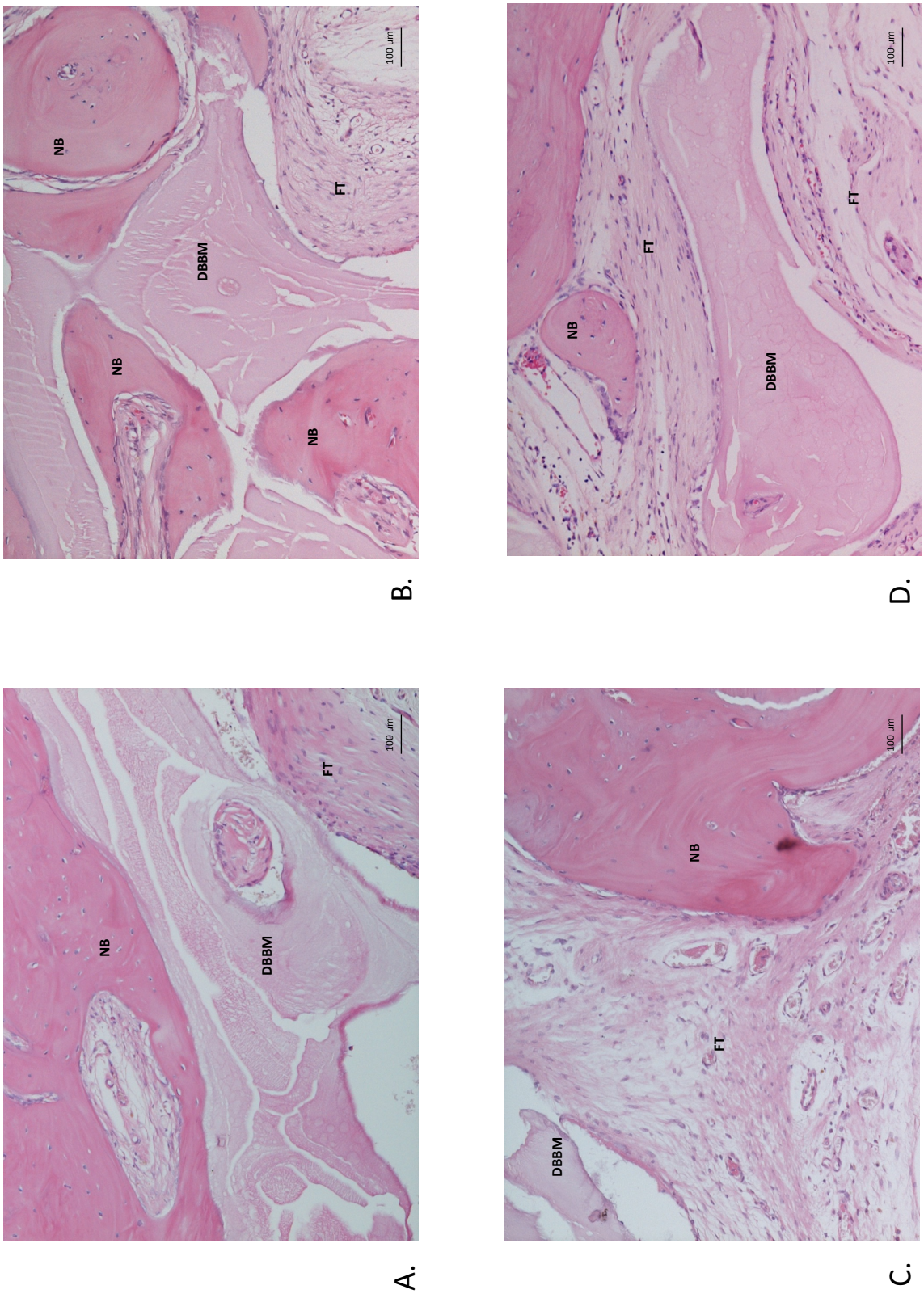

B.
DBBM
NB
NB
FT
100 µm
D.
NB
FT
DBBM
100 µm
A.
NB
NB
NB
DBBM
FT
100 µm
C.
NB
FT
DBBM
FT
100 µm

Figure 3.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NB
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NB
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100 µm
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A.
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C.
NB
FT
DBBM
100 µm

Figure 4.

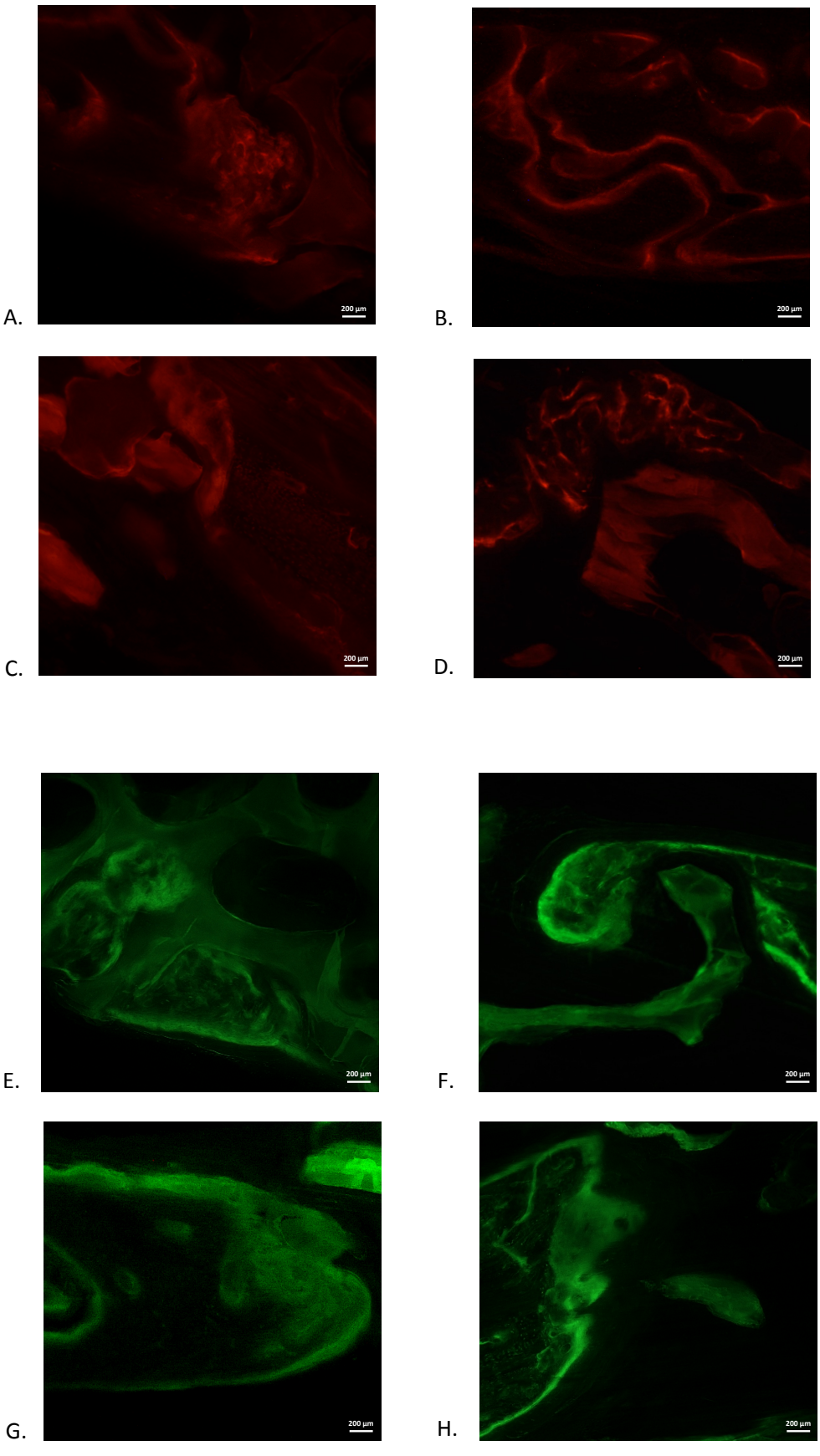


Figure 5.

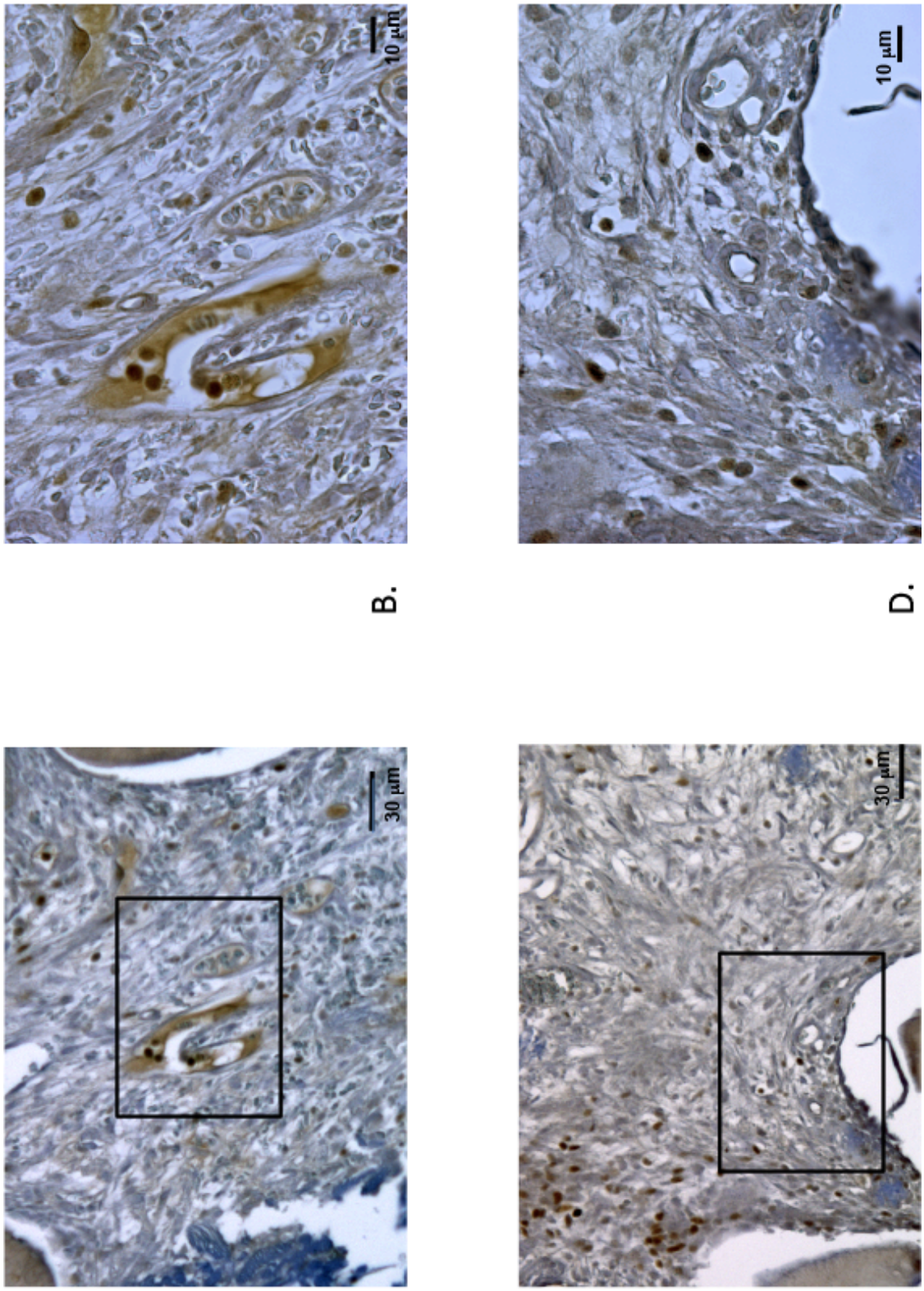
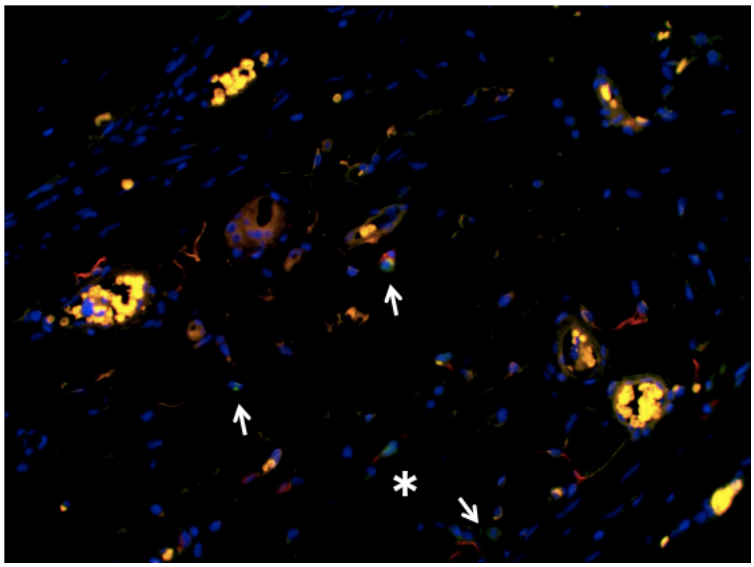


Figure 6.

A.



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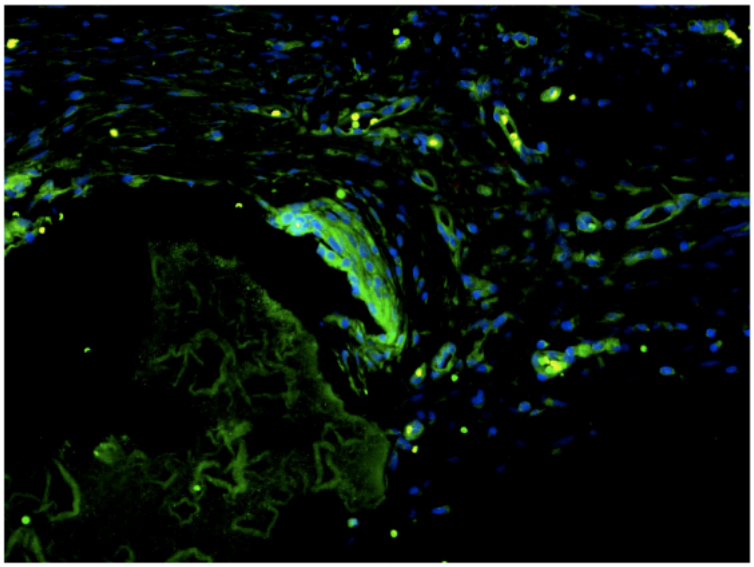
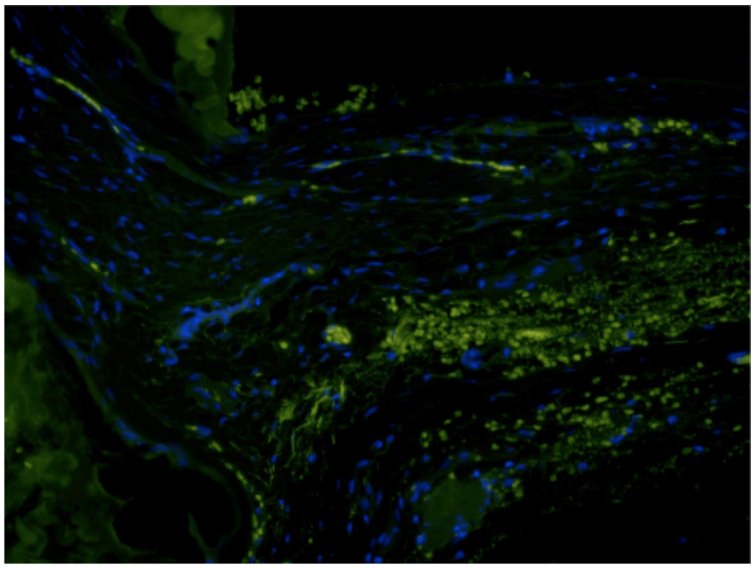


C.



4.6.1 Tables and Figures Legend

Tables Legend

Table 1. A. Table resuming the main characteristics of DBBM discs, in terms of number (n), weight (mg) and distribution. B. Graphical represantiation of the pairing of DBBM discs. Data as reported as mg values.

Table 2. A. New bone (NB), fibrous tissue (FT) and residual biomaterial (DBBM) within test and control samples at 1 month observation period. B. New bone (NB) within distinct sub-ROIs BA, PA and CA at 1 month. C. Fibrous tissue (FT) within distinct sub-ROIs BA, PA and CA at 1 month. Data are reported as mean percentages over the total defect area ± SEM. Statistical significance ($p<0.05$) is marked with an asterisk.

Table 3. New bone (NB), fibrous tissue (FT) and residual biomaterial (DBBM) within test and control samples at 3 months observation period. B. New bone (NB) within distinct sub-ROIs BA, PA and CA at 3 months. C. Fibrous tissue (FT) within distinct sub-ROIs BA, PA and CA at 3 months. Data are reported as mean percentages over the total defect area ± SEM. Statistical significance ($p<0.05$) is marked with an asterisk.

Table 4. Mineral apposition rate (MAR) in test and control samples evaluated A. at days 23-24 with Xylenol Orange labeling B. at days 80-84 with Calcein Green labeling. Data are reported as mean values (μg/day) ± SEM. Statistical significance ($p<0.05$) is marked with an asterisk.

Table 5. Capillary density in test and control samples at 1 and 3 months observation period. Data are reported as mean values (n/mm^2) ± SEM. Statistical significance ($p<0.05$) is marked with an asterisk.

Table 6. Immunohistochemical quantification of A. OSX/OC co-positive elements, B. ALP positive elements, C. COL1A1 positivity. Data are reported as mean values ± SEM. Statistical significance ($p<0.05$) is marked with an asterisk.

Table 7. List of primers used for RT-PCR and their corresponding forward and reverse nucleotide sequences.

Table 8. RT-PCR evaluation of GAPDH (A), RUNX2 (B), SP7 (C), COL2A1 (D), VDR (E), COL1A2 (F), POSTN (G), TGFb (H), FN (I), ALP (J) and BGLAP (K) genes in test and control samples at 1 and 3 months observation period. Data are normalized to the relative amount of cDNA (ng) in each sample and are reported as mean ± SEM. Statistical significance ($p<0.05$) is marked with an asterisk.

Figures Legend

Figure 1. Surgical procedure of bone grafting. A. Raising of the flap along the median sagittal suture; B. Bilateral osteotomy on parietal bone created by means of a trephine bur with 5 mm external diameter; C. Aspect of bilateral critical-size defects; D. Filling of the defects with block grafts loaded with (test side) or without ST (control side). Optical camera (Nikon D3x, 24.5 Megapixels).

Figure 2. Histological aspect of test (A-B) and control (C-D) samples at 1 month observation period. Abundant fibrous tissue (FT) rich in blood vessels is visible both in test and in control samples, as well as calcified nodules of newly formed bone (NB) surrounded by numerous osteoblasts. Residual biomaterial (DBBM) is well integrated in the recipient site and no signs of inflammatory reaction are detectable. Hematoxylin-eosin staining. Optical Microscopy, original magnification 10X, scale bar 100 μm.

Figure 3. Histological aspect of test (A-B) and control (C-D) samples at 3 months observation period. Newly formed bone (NB), fibrous tissue (FT) and residual biomaterial (DBBM) are observable both in test and in control samples show a good integration one with each other. Hematoxylin-eosin staining. Optical Microscopy, original magnification 10X, scale bar 100 μm.

Figure 4. A-D. Xylenol Orange labeling showing consistent areas of newly formed calcified tissues in days 23-24 from surgery, both in test (A-B) and in control (C-D) samples. Fluorescence Microscopy, original magnification 4X, scale bar 200 μm.

E-H. Calcein Green labeling showing areas where calcium precipitated from day 80 to 84 from surgery. Wide sites of mineralization at the margins and in the central portion of the defect are visible both in test (E-F) and in control (G-H) samples. Fluorescence Microscopy, original magnification 4X, scale bar 200 μm.

Figure 5. A-D. Microscopic image showing blood capillaries, recognized by brown of FVIII IHC-staining revealed by DAB in test (A-B) and control (C-D) samples at 3 months. Optical Microscopy, original magnification 20X (A;C), 40X (B;D).

Figure 5. A-C. Microscopic images of IHC in test samples at 3 months: OSX positivity (A), ALP positivity (B) and COL1A1 positivity visualized with Green Fluorescence. OC positivity (A) visualized with Red Fluorescence. The Blue Fluorescence of DAPI recognize nuclei (A-C). White arrows recognize OSX/OC co-positive cells (A). Fluorescence Microscopy, original magnification 20X.

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