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# Gallium, a promising candidate for bone pathologies treatment

Ivana Strazic

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UNIVERSITE NICE SOPHIA ANTIPOLIS, UFR SCIENCES  
Ecole Doctorale des Sciences de la Vie et de la Santé (ED85)

THESE DE DOCTORAT

# **Gallium, un candidat prometteur pour le traitement des pathologies osseuses**

En anglais :

# **Gallium, a promising candidate for bone pathologies treatment**

Pour obtenir le grade de  
Docteur en Sciences de l'Université Nice Sophia Antipolis  
Spécialité : Interactions moléculaires et cellulaires

Par

Ivana Strazic

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Présentée et soutenue publiquement le 9 octobre 2015 devant le jury composé de :

|                        |                                  |
|------------------------|----------------------------------|
| Dr Valérie GEOFFROY    | Rapporteur et Présidente du jury |
| Pr Giovanna MAZZOLENI  | Rapporteur                       |
| Dr Nicolas AMORETTI    | Examineur                        |
| Pr Jean-Michel BOULER  | Membre invité                    |
| Dr Jean-Claude SCIMECA | Directeur de thèse               |
| Dr Elise VERRON        | Co-directrice de thèse           |

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## ABSTRACT

In bone reconstructive surgery, biomaterials commonly replace the missing tissue, and can also serve as vectors for local drug delivery. Due to its inhibitory action on bone-resorbing cells (osteoclasts), a semi-metallic element gallium (Ga) is used for the treatment of disorders associated with accelerated bone resorption. Since Ga can be incorporated into the structure of bone substitutes, we embarked on characterising the biological properties of novel Ga-loaded biomaterials. *In vitro*, in the presence of Ga-loaded biomaterial, we observed a decrease in osteoclast differentiation and the upregulated expression of several osteoblastic markers (bone-forming cells). *In vivo*, using a rat bone defect model, we showed an increase in newly formed bone tissue in implants filled with Ga-loaded biomaterial. Taken together, our data indicate that Ga-loaded biomaterial improved bone reconstruction by modifying the balance construction/degradation. Taking into account known antitumoral effects of Ga, we also investigated its impact on a bone metastatic model. Using an aggressive human cancer cell line selected for its ability to invade bone tissue, we showed that Ga could reduce cancer cell proliferation and viability, and reverse excessive osteoclastogenesis in bone metastatic environment. Moreover, we demonstrated that Ga modulated the expression of several marker genes hindering the tumour-propagating potential of cancer cells. Thus, due to its inhibitory action on cancer cells, Ga could represent an attractive additive to biomaterials used for bone tissue reconstruction after tumour resection.

Keywords: gallium, biomaterials, osteoporosis, bone metastases, second-harmonic generation



## **RESUME DETAILLE DE LA THESE**

Ce projet de thèse s'inscrit dans la problématique générale du comblement des pertes de substance osseuse résultant de traumatismes, de pathologies ostéo-articulaires ou de la résection de tumeurs osseuses.

La structure et la physiologie du tissu osseux sont présentées dans le premier chapitre de l'introduction. La différenciation et l'activité des éléments cellulaires clés de l'os sont détaillées en commençant par les ostéoblastes, les cellules formatrices de l'os, puis les ostéocytes et enfin les ostéoclastes, les cellules qui résorbent le tissu osseux. Le mécanisme et les molécules impliquées dans la régulation du remodelage osseux sont aussi décrites.

Le deuxième chapitre qui porte sur la physiopathologie osseuse est plus particulièrement concentré sur l'ostéoporose, le trouble le plus récurrent du remodelage osseux avec 440 000 nouveaux patients par an dans les pays de l'Union Européenne. Cette maladie est caractérisée par une activité excessive des ostéoclastes qui résulte en une diminution de la densité osseuse et se manifeste cliniquement par des fractures ostéoporotiques. Le deuxième accent fort de ce chapitre est mis sur les métastases osseuses - la complication récurrente des nombreux cancers solides, comme le cancer de la prostate et le cancer du sein. Il a été montré que les métastases osseuses sont capables de moduler le microenvironnement osseux en favorisant l'activité ostéoclastique et donc la formation de lésions ostéolytiques, mauvais pronostic pour les patients.

Le troisième chapitre introduit les biomatériaux synthétiques à base de phosphate de calcium (CaP) qui sont couramment utilisés comme substituts en chirurgie reconstructive de l'os. Ces matériaux biocompatibles remplacent le tissu au sein de défauts osseux permettant de rétablir partiellement ses propriétés mécaniques. En même temps, ils servent de matrice pour la reconstruction de l'os et en fonction de la structure chimique, ils sont résorbés au cours du temps et remplacés par le tissu osseux du patient. Dans le cadre de pathologies telles que l'ostéoporose ou les tumeurs osseuses, ces biomatériaux pourraient également assurer un délivrage local de molécules actives.

Dans le traitement de plusieurs pathologies osseuses, de nombreuses données issues de la recherche et de la pratique clinique mettent en évidence les effets bénéfiques de l'élément semi-métallique, le gallium (Ga).

Il a été montré que le Ga exerce des effets inhibiteurs sur les cellules cancéreuses et également sur les ostéoclastes, les cellules résorbantes de l'os. Dû à cet effet, le gallium est utilisé dans le traitement de différentes pathologies liées à la résorption ostéoclastique accélérée du tissu osseux, telle que l'hypercalcémie maligne.

En partenariat avec la société Graftys et dans le but de développer de nouveaux biomatériaux de comblement pour les pertes de substance osseuse, qui seront également pharmacologiquement actifs, ce travail de thèse a évalué les propriétés biologiques d'un ciment phosphocalcique (CPC) incorporant du gallium dans sa structure (CPC/Ga). Ce biomatériau composé pourrait être utilisé dans le cadre de la prévention de fractures ostéoporotiques ou bien pour le comblement de cavités après la résection de tumeurs osseuses. Considérant les effets majeurs que le Ga exerce sur l'ensemble du tissu osseux, il est important d'étudier de façon approfondie son impact sur le recrutement et la différenciation des cellules osseuses, ainsi que sur la formation du tissu osseux lui-même.

*In vitro*, la culture de cellules osseuses sur les pastilles de CPC/Ga a mis en évidence une diminution de la prolifération des ostéoclastes et une sous-expression des marqueurs tardifs de la différenciation ostéoclastique. D'autre part, nous avons montré une sur-expression de plusieurs marqueurs caractérisant les ostéoblastes sur le CPC/Ga par rapport au CPC contrôle. Les propriétés reconstructives de biomatériaux chargés en Ga ont aussi été étudiées *in vivo* dans un modèle murin de comblement du défaut osseux. Pour tous les biomatériaux implantés, nous avons pu observer une bonne ostéointégration, accompagnée d'une reconstruction avec colonisation par la moelle osseuse. De plus, pour un des biomatériaux galliés, l'analyse quantitative en 3D par micro-CT a révélé une augmentation du remplissage total du défaut osseux par rapport au contrôle.

Néanmoins, la technique de micro-CT ne permet pas de distinguer clairement la phase minérale des biomatériaux phosphocalciques et la phase minérale du tissu osseux dû à la grande ressemblance des leurs structures chimiques. Au cours de

cette thèse, et en collaboration avec Sébastien Schaub de la plateforme PRISM de l'IBV, nous avons pu développer un outil de quantification en 3D du tissu osseux au sein des implants par la microscopie SHG (*angl.* second- harmonic generation). Le constituant organique majeur du tissu osseux, les fibres de collagène, ont la propriété d'émission de « second harmonique » grâce à leur structure physico-chimique. C'est pourquoi, dans l'analyse de nos échantillons, le signal SHG est spécifiquement lié au tissu osseux. Dans notre modèle du défaut osseux, la quantification en 3D du signal SHG au sein des implants galliés a montré une augmentation du tissu osseux par rapport aux implants contrôles. En effet, ce résultat a confirmé l'effet observé précédemment en analyse quantitative par micro-CT suggérant que la microscopie SHG ouvre une nouvelle piste non-destructive pour la quantification spécifique du tissu osseux dans le domaine de l'ingénierie tissulaire de l'os.

D'autre part, étant donné que des effets anti-tumoraux du Ga sont largement rapportés dans la littérature, nous nous sommes également intéressés aux effets cellulaires que le Ga pourrait exercer sur les cellules tumorales dans l'os. En utilisant une lignée cellulaire métastatique agressive, choisie pour son affinité pour le tissu osseux, nous avons montré que le Ga réduit la prolifération des cellules cancéreuses et également la différenciation ostéoclastique induite par les cellules cancéreuses. De plus, nous avons mis en évidence que le Ga module l'expression de gènes susceptibles de réduire le potentiel tumoral des cellules métastatiques.

L'ensemble de ces données indique que les biomatériaux chargés en Ga sont des substrats non-cytotoxiques et compatibles avec la survie et la prolifération des cellules osseuses *in vitro* et *in vivo*. De plus, en agissant à la fois sur les ostéoclastes et les ostéoblastes, le Ga améliore la repousse osseuse intervenant au sein des biomatériaux phosphocalciques implantés. Cette propriété peut être un avantage dans le contexte ostéoporotique où le remodelage et la reconstruction osseuse sont fortement impactés. Il serait donc intéressant d'évaluer le potentiel ostéogénique des biomatériaux galliés dans un modèle ostéoporotique. Enfin, dû au fait de ses effets inhibiteurs sur les cellules cancéreuses, le Ga constitue un candidat intéressant pour le couplage avec des substituts phosphocalciques destinés à la reconstruction osseuse après la résection tumorale.



Mots-clés: gallium, biomatériaux, ostéoporose, métastases osseuses, génération de second harmonique

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*"My utmost respect and admiration to all the engineers, especially the greatest of them all: God."*

Thomas A. Edison (1847 – 1931)

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period is over so let's celebrate now all the wonderful days in front of us! My joy, my star, my blessing! Kog' ja volim?!



# THESIS GENERAL STRUCTURE

## I INTRODUCTION

## II THESIS GENERAL OBJECTIVES

## III RESULTS

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*In vitro* and *in vivo* effects of gallium-loaded calcium phosphate biomaterials on bone cells and bone reconstruction

1. Context and objectives of the study
2. Article I : Incorporation of gallium enhances reconstructive properties of a calcium phosphate bone substitute

### **PART ONE B:**

Development of quantitative second-harmonic generation microscopy for bone tissue engineering applications

1. Context and objectives of the study
2. Methods
3. Results and discussion
4. Conclusions

### **PART TWO:**

Effects of gallium in a bone metastatic model

1. Context and objectives of the study
2. Article II : Gallium, a promising candidate to disrupt the vicious cycle driving osteolytic metastases

## IV GENERAL THESIS DISCUSSION AND PERSPECTIVES





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## LIST OF PUBLICATIONS AND COMMUNICATIONS

### Published articles:

Verron E, Loubat A, Carle GF, Vignes-Colombeix C, Strazic I, Guicheux J, Rochet N, Bouler JM, Scimeca JC (2012) Molecular effects of gallium on osteoclastic differentiation of mouse and human monocytes. *Biochem Pharmacol* 83(5); 671-679

### Articles in preparation:

Strazic I., Melis N., Boukhechba F., Schaub S., Mellier C., Janvier C., Laugier JP., Rochet N., Bouler JM., Verron E., Scimeca JC (2015) Incorporation of gallium enhances reconstructive properties of a calcium phosphate bone substitute.

Strazic I., Guberovic I., Didak B., Schmid-Antomarchi H., Schmid-Allianai A., Bouler JM., Scimeca JC., Verron E. (2015) Gallium, a promising candidate to disrupt the vicious cycle driving osteolytic metastases.

### Communications:

Strazic I., Melis N., Boukhechba F., Lesoeur J., Mellier C., Rochet N., Bouler JM., Verron E., Scimeca JC. (2014) Biological and reconstructive properties of gallium-doped calcium phosphate bone substitutes.

*24th Interdisciplinary Research Conference on Injectable Osteoarticular Biomaterials and Bone Augmentation Procedures (GRIBOI)*, May 5th - 7th, 2014, Nantes, France. (Abstract is published in *European Cells and Materials Journal*, Vol. 28, Suppl. 5, 2014, page 14)

Strazic I., Schaub S., Rochet N., Boukhechba F., Rekima S., Dutilleul M., Lesoeur J., Verron E., Scimeca JC. (2015) Second-harmonic generation microscopy: a deep look into bone tissue engineered constructs.

*Third Workshop of Physics of living matter series*, September 22nd, 2015, Nice, France.



## LIST OF ABBREVIATIONS

|        |   |
|--------|---|
| 2PEF   | two-photon excited fluorescence           |
| ABC    | acrylic bone cement                       |
| ALP    | alkaline phosphatase                      |
| Ang    | angiopoetin                               |
| BCP    | biphasic calcium phosphate                |
| BMD    | bone mineral density                      |
| BMP    | bone morphogenetic protein                |
| BMU    | basic multicellular unit                  |
| BP     | bisphosphonate                            |
| BSP    | bone sialoprotein                         |
| Ca     | calcium                                   |
| CA     | carbonated hydroxyapatite                 |
| CAII   | carbonic anhydrase II                     |
| CALCR  | calcitonin receptor                       |
| CaMKs  | calmodulin-dependent kinases              |
| CaP    | calcium phosphate                         |
| CCR3   | chemokine receptor 3                      |
| CDA    | calcium-deficient apatite                 |
| CDA/Ga | gallium-loaded calcium- deficient apatite |
| COL1A1 | type I Collagen                           |
| CPC    | calcium phosphate cement                  |
| CPC/Ga | gallium-loaded calcium phosphate cement   |
| CREB   | cAMP response element-binding protein     |
| CSC    | calcium sulphate cement                   |
| CTR    | calcitonin receptor                       |
| CTSK   | cathepsin K                               |
| CXCL   | C-X-C motif chemokine                     |
| CXCL10 | C-X-C motif chemokine 10                  |
| CXCR   | C-X-C chemokine receptor                  |
| CXCR4  | C-X-C chemokine receptor type 4           |
| DCPA   | anhydrous dicalcium phosphate             |



|         |  |
|---------|--|
| DCPD    | dicalcium phosphate dihydrate                    |
| DDS     | drug delivery system                             |
| DDS     | drug delivery systems                            |
| Dkk1    | dickkopf homologue 1                             |
| DO      | distraction osteogenesis                         |
| DXA     | dual- energy x-ray absorptiometry                |
| ECM     | extracellular matrix                             |
| EGF     | epidermal growth factor                          |
| EMT     | epithelial to mesenchymal transition             |
| ERK     | extracellular-signal-regulated kinases           |
| ET-1    | endothelin-1                                     |
| FDA     | Food and Drug Administration                     |
| FGF     | fibroblast growth factor                         |
| Ga      | gallium  |
| Ga-CaP  | gallium- loaded calcium phosphate biomaterials   |
| GAS6    | growth arrest-specific 6                         |
| GDF     | growth differentiation factor                    |
| GM-CSF  | granulocyte/macrophage-colony stimulating factor |
| H&E     | hematoxylin and eosin                            |
| HA      | hydroxyapatite                                   |
| HFG     | halofuginone                                     |
| HIF     | hypoxia-inducible factors                        |
| HPMC    | hydroxypropyl methyl cellulose                   |
| HSC     | hematopoietic stem cell                          |
| ICP- MS | inductively coupled plasma–mass spectrometry     |
| IGF     | insulin-like growth factor                       |
| IGFBP   | IGF-binding proteins                             |
| IGF-IR  | insulin-like growth factor receptor              |
| ITGB3   | integrin, beta 3                                 |
| IκB     | inhibitor of κB                                  |
| JUN-D   | jun D proto-oncogene                             |
| KP      | kyphoplasty                                      |
| LPA     | lysophosphatidic acid                            |
| αVβ3    | alpha 5, beta 3 integrin                         |

|                |  |
|----------------|--|
| MAPK           | mitogen-activated protein kinase   |
| MCP-1          | monocyte chemotactic protein-1   |
| M-CSF          | macrophage colony stimulating factor   |
| MDSC           | myeloid-derived suppressor cell  |
| Micro-CT       | micro-computed tomography  |
| MIP-2          | macrophage inflammatory protein-2  |
| MMP            | matrix metalloproteinase   |
| MSC            | mesenchymal stem cell  |
| NFATC1         | nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent<br>1 |
| NF- $\kappa$ B | nuclear factor kappa-light-chain-enhancer of activated B cells               |
| NK             | natural killer cells   |
| NLOM           | non-linear optical microscopy  |
| NMR            | nuclear magnetic resonance   |
| NOF            | National Osteoporosis Foundation   |
| OCN            | osteocalcin  |
| OCP            | octacalcium phosphate  |
| OI             | osteogenesis imperfecta  |
| ONJ            | osteonecrosis of the jaw   |
| OPG            | osteoprotegerin  |
| OPN            | osteopontin  |
| Osx            | osterix  |
| PBMC           | peripheral blood mononuclear cell  |
| p-CREB         | phosphorylated CREB  |
| PDGF           | platelet- derived growth factor  |
| PGs            | prostaglandins   |
| PMMA           | polymethylmethacrylate   |
| PTH            | parathyroid hormone  |
| PTHrH          | parathyroid hormone-like hormone   |
| RANKLr         | receptor activator of nuclear factor kappa-B ligand                          |
| ROS            | rat osteogenic sarcoma   |
| ROS            | reactive oxygen species  |
| RUNX2          | runt-related transcription factor 2  |
| SDF-1          | stromal-derived factor-1   |

|               |   |
|---------------|---|
| SEM           | scanning electron microscopy                                      |
| SHG           | second-harmonic generation  |
| TCP           | tricalcium phosphate  |
| TF            | transferrin   |
| TGF- $\beta$  | transforming growth factor, Beta 1                                |
| THG           | third-harmonic generation   |
| TNFR2         | tumour necrosis factor receptor 2                                 |
| IL            | interleukin   |
| TNF- $\alpha$ | tumour necrosis factor alpha                                      |
| TRAP          | tartrate-resistant acid phosphatase                               |
| TRF           | transferrin receptor  |
| TRPV1         | transient receptor potential vanilloid type 1                     |
| TRPV-5A       | transient receptor potential cation channel subfamily V member 5A |
| TSP1          | thrombospondin 1  |
| VCAM1         | vascular cell adhesion molecule 1                                 |
| VEGF          | vascular endothelial growth factor                                |
| VEGFR         | VEGF receptor   |
| VP            | vertebroplasty  |
| WHO           | World Health Organisation   |
| WNT           | wingless/integrated   |
| XRD           | X-ray diffraction powder patterns                                 |
| ZEB-1         | zinc finger e-box binding homeobox 1                              |
| $\beta$ -TCP  | $\beta$ -tricalciumphosphate                                      |

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# I INTRODUCTION





# 1. Bone structure and physiology

## 1.1. Bone systemic functions

Bone is composed of several tissues, predominantly a highly specialized connective tissue that enables locomotion and provides mechanical protection for the vital organs. It serves as an environment for blood forming and fat storage, and acts as a homeostatic reservoir of growth factors, cytokines, and minerals important for the body.

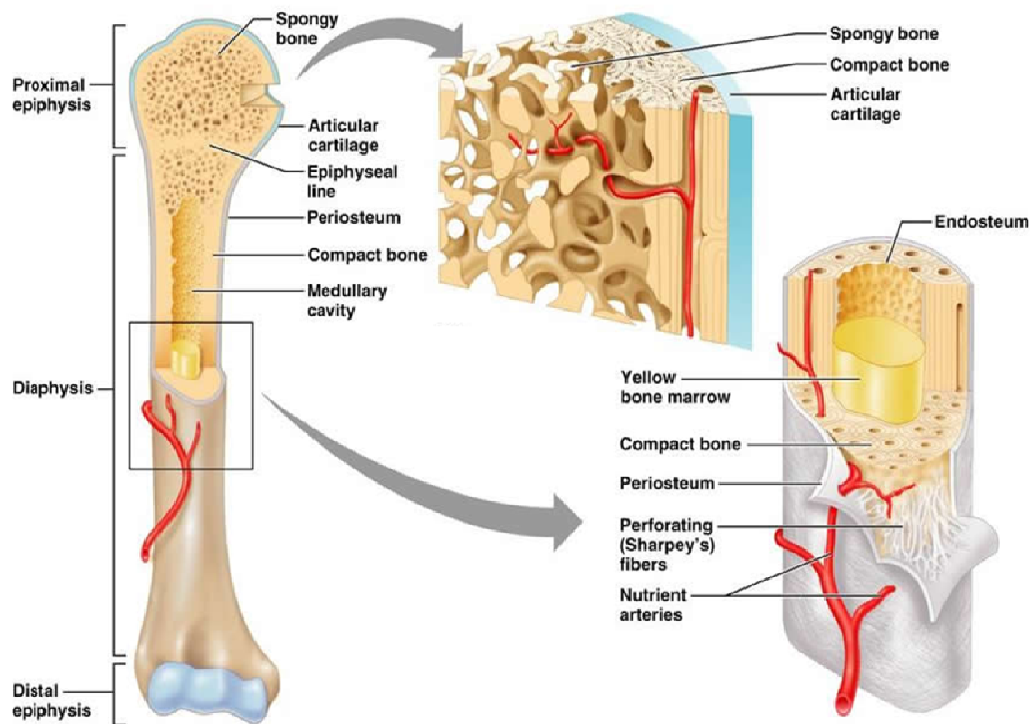
Amongst different minerals deposited in the bone, the most important ones are calcium with 95% and phosphorus with 85% of total body content contained in the bone tissue. The minerals can be mobilized and replaced in bone to meet systemic homeostatic needs regardless of reduction of skeletal structural integrity. Hence, this particular metabolic function of bone tissue outweighs its structural function (Kini & Nandeesh, 2012).

Mineralized bone matrix serves also as a depository for a great number of prominent cytokines and growth factors, such as insulin-like growth factors (IGFs), transforming growth factor- $\beta$  (TGF- $\beta$ ) and bone morphogenetic proteins (BMPs) that can be released upon bone resorption and act locally or systemically. Regarding the endocrine function, one of the notable hormones released by bone is osteocalcin, known to increase bone density by helping to funnel calcium and other minerals into the bone matrix. Osteocalcin also contributes to the regulation of blood glucose metabolism and fat deposition (Kini & Nandeesh, 2012). The yellow bone marrow acts as storehouse of adipocytes that can secrete leptin, a hormone largely involved in energy balance that also participates in bone mass control (Ducy *et al.*, 2000). Residing in red bone marrow, self-renewing haematopoietic stem cells (HSCs) are progenitors to all of the different mature blood cell types (Alenzi *et al.*, 2009). And by its capacity to store heavy metals and other extraneous elements and to remove them from the circulation, bone also participates in body detoxification (Kini & Nandeesh, 2012).

## 1.2. Bone tissue organisation

### 1.2.1. Cortical and cancellous bone

Based on the porosity and density, bone tissue can be divided into dense solid cortical (compact) bone surrounding the bone marrow space, and the cancellous (trabecular, spongy) bone, that is composed of a honeycomb-like network of plates and rods. The outer cortical surface of bone, except at joints, is protected by periosteum, a fibrous connective tissue sheath containing blood vessels, nerve fibers and certain bone cell types. It plays an important role in appositional bone growth and fracture repair. A membranous structure called endosteum covers the inner surface of cortical and cancellous bone and the blood vessel canals (Volkmann's canals) present in bone (Kini & Nandeesh, 2012).



**Figure 1. The structure of a long bone.**

Typical honeycomb-like network of cancellous (spongy) bone and dense cortical (compact) bone (taken from *Human Anatomy and Physiology 8th edition, 2010.*).

Numerous cell types are found in bone, with the main ones being (i) mesenchymal stem cells that can differentiate in (ii) osteoblasts, the bone forming cells that endure maturation becoming (iii) osteocytes, the cells orchestrating bone remodelling. The fourth main cell type, (iv) osteoclast, is derived from hematopoietic cell lineage and is responsible for bone tissue resorption. These cell types are entrapped in osteoid matrix built up of collagen and noncollagenous proteins with deposits of inorganic mineral salt (Kini & Nandeesh, 2012).

Based on the spatial pattern of collagen forming the osteoid, two types of bone are distinguished: the woven bone, which is characterized by a haphazard organization of collagen fibers, and mechanically stronger lamellar bone, characterized by a regular parallel alignment of collagen into sheets (lamellae). A rapid osteoid production results in woven bone and this occurs initially in all foetal bones and in fracture healing. The woven bone is replaced in a process called remodelling by the deposition of more resilient lamellar bone and virtually all the bone in the healthy mature adult is lamellar bone (Eriksen *et al.* 1994).

### **1.2.2. Mesenchymal stem cells**

Mesenchymal stem cells (MSCs), or bone stromal cells, are progenitor cells present in adult bone marrow, adipose tissue, muscles, synovial and placental tissue, and teeth. They participate in synthesis of extracellular matrix, immune tolerance, development, inflammation and fibrosis. MSCs are currently being studied for tissue engineering applications, including bone and cartilage repair, because of their potential to differentiate into different lineages such as chondrocytes, osteoblasts or adipocytes. Recently, these cells have also been shown to have immunosuppressive and healing capacities, to improve angiogenesis and to prevent fibrosis (Pers & Jorgensen, 2013).

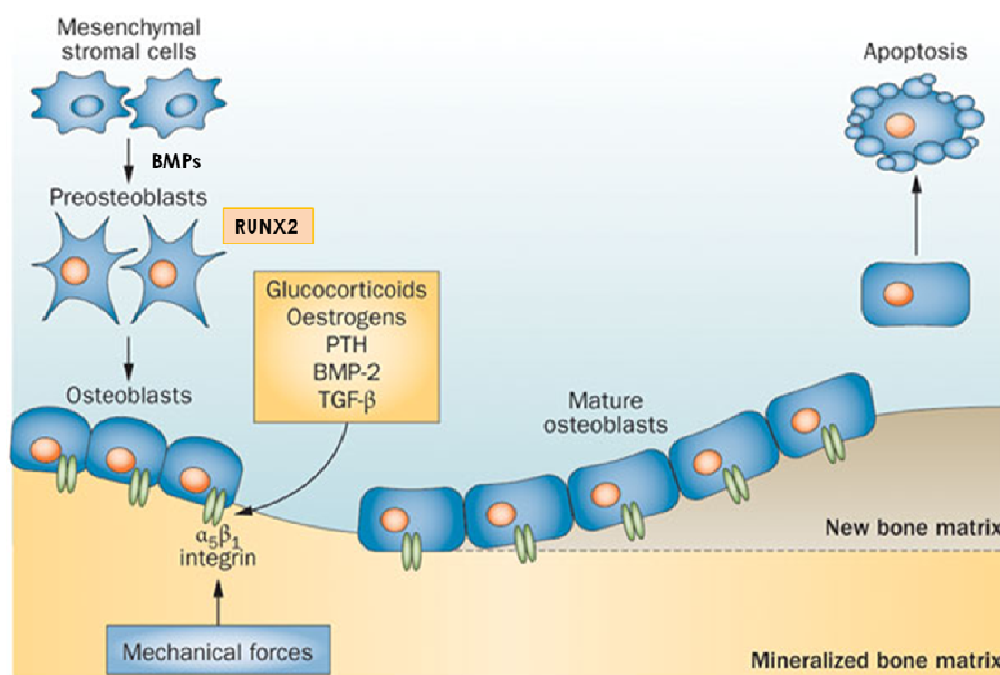
### **1.2.3. Osteoblasts and bone lining cells**

Osteoblasts are bone-forming cells developed from the mesenchymal stem cell lineage. They secrete organic matrix at the bone resorption sites and further they

induce its mineralization. Eventually, flat mature osteoblasts lying on quiescent bone surface differentiate into bone lining cells. Mature osteoblasts that remain entrapped inside the new bone tissue become osteocytes.

Runx-related transcription factor 2 (RUNX2) is an essential transcription factor in the regulation of mesenchymal cell differentiation into the osteoblast lineage and is needed for proper bone development. Osteoblastogenesis is tightly regulated by canonical WNT and BMP signalling pathways. Activation of canonical WNT/ $\beta$ -catenin signalling pathway is essential for normal osteogenesis, and its suppression causes bone-related pathologies in humans (Lee *et al.*, 2012). Preosteoblasts are usually considered to express RUNX2 and osterix (OSX) and mature osteoblasts are often characterized by the expression of type I collagen (COL1A1), osteocalcin (OCN) and alkaline phosphatase (ALP) (Huang *et al.*, 2015).

In the end of bone formation phase, elongated mature osteoblasts give rise to bone lining cells covering all quiescent bone surfaces. These cells are connected to the osteocytes network via canaliculi within the bone matrix (Kini & Nandeesh, 2012).



**Figure 2. The major steps in osteoblastogenesis.**

Mesenchymal stem cells begin to differentiate into preosteoblasts. The activation of transcription factor RUNX2 is the major trigger for further differentiation into fully functional osteoblasts (adapted from Marie, 2013).

#### 1.2.4. Extracellular matrix composition and mineralization

Bone matrix is constituted of water (5%), inorganic mineral component (69%), predominantly hydroxyapatite (99 %), and organic component (22%) constituted mostly of collagen type I (90-95%) and noncollagenous structural proteins including proteoglycans, sialoproteins, osteocalcin, alpha-2-HS-glycoprotein and gla-containing proteins (Kini & Nandeesh, 2012). Osteoblasts synthesize and lay down precursors of collagen 1, which are deposited in parallel or concentric layers to produce mature (lamellar) bone. When bone is rapidly formed, as in the fetus or certain pathological conditions (e.g., fracture callus, fibrous dysplasia, hyperparathyroidism), the collagen is not deposited in a parallel array but in a basket-like weave resulting in immature woven bone. Osteoblasts also secrete noncollagenous proteins, such as osteocalcin, the most abundant noncollagenous protein of bone matrix, glycosylated proteins such as alkaline phosphatase, and g-carboxylated (gla) proteins (Kini & Nandeesh, 2012).

The hardness and rigidity of bone is due to the presence of mineral salt in the osteoid matrix, which is a crystalline complex of calcium and phosphate. Amorphous calcium phosphate matures through several intermediate stages to form crystalline hydroxyapatite  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ , which is progressively substituted with carbonate to increase its solubility. Finally, the mineral component of the bone is deposited along the collagen fibrils forming a particular mineral-collagen network that ensures the structural integrity of the bone tissue and supports its mechanical function (Kini & Nandeesh, 2012).

#### 1.2.5. Osteocytes

The most abundant cells in bone are the osteocytes. These terminally differentiated osteoblasts embedded in bone matrix change their morphology by extending cellular processes for connection and intercommunication with other cell types present in bone. They are considered to be the mechanosensors of the bone, instructing osteoblasts and osteoclasts to start bone remodelling in response to mechanical stimuli (You *et al.*, 2008). Osteocytes are interlinked metabolically and electrically through gap junctions composed primarily of connexin (You *et al.*, 2008).

In aging bone, the presence of empty lacunae suggests that osteocytes may have undergone apoptosis, probably caused by disruption of their intercellular gap junctions or cell-matrix interactions. It is also known that estrogen deficiency or glucocorticoid treatment is harmful to bone structure because it induces osteocyte apoptosis. As a consequence of this, estrogen and bisphosphonate therapy accompanied by physiologic loading of bone may help in prevention of osteoblast and osteocyte apoptosis (Plotkin, Aguirre, Kousteni, Manolagas, & Bellido, 2005).

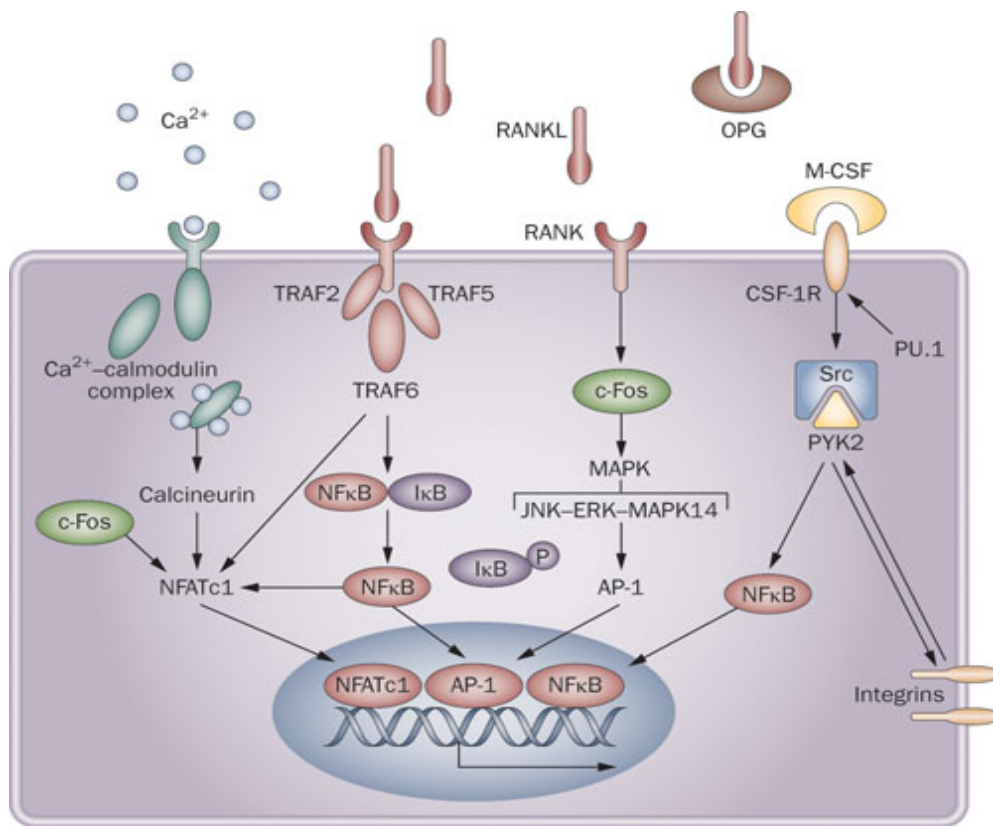
### **1.2.6. Osteoclasts**

Osteoclasts, the bone resorbing cells, originate from monocytes/macrophage lineage and their differentiation requires direct contact with the surrounding osteoblasts, stromal cells, and immune cells (Weiss, Iqbal, Zaidi, & Mechanick, 2010).

Mature polarized osteoclasts adhere to the bone surface through  $\alpha\text{v}\beta3$  integrin, form an actin ring, and acidify the sealed zone by secreting hydrogen ions. Proteases (i.e. cathepsin K) that degrade extracellular matrix proteins such as type I collagen (Kini & Nandeesh, 2012) are also secreted in the sealing zone.

Osteoblasts and marrow stromal cells secrete macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor  $\text{NF}\kappa\text{B}$  ligand (RANKL) that direct certain hematopoietic precursor cells to become mature multi-nucleated osteoclasts (M. Nakamura, Hentunen, Salonen, Nagai, & Yamashita, 2013). As shown in Figure 3, the binding of RANKL to its cognate membrane receptor activates wide downstream pathways which include nuclear factor kappa-light-chain-enhancer of activated B cells ( $\text{NF}\kappa\text{B}$ ), c-Fos and mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinases (ERK) or p38. Afterwards,  $\text{NF}\kappa\text{B}$ , MAPK and c-Fos are implied in the regulation of "nuclear factor of activated T-cells, cytoplasmic 1" (NFATc1) expression that is the master regulator of osteoclast differentiation. It is responsible for the induction of many osteoclast-typical markers such as (ACP5/TRAP) tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK),  $\beta3$ -integrin (ITGB3), calcitonin receptor (CTR), and matrix metalloproteinase 9 (MMP9) (Takayanagi *et al.*, 2002). Although many different pathways target NFATc1 expression,  $\text{NF}\kappa\text{B}$  is the main inducer of the early phase of its transcription. RANKL has been shown to induce a specific set of about 70 genes, including chemokines and

growth factors [RANTES, platelet-derived growth factor (PDGF $\alpha$ ) or IGF-1], which are indispensable for osteoclastic differentiation (Borovecki *et al.*, 2007).



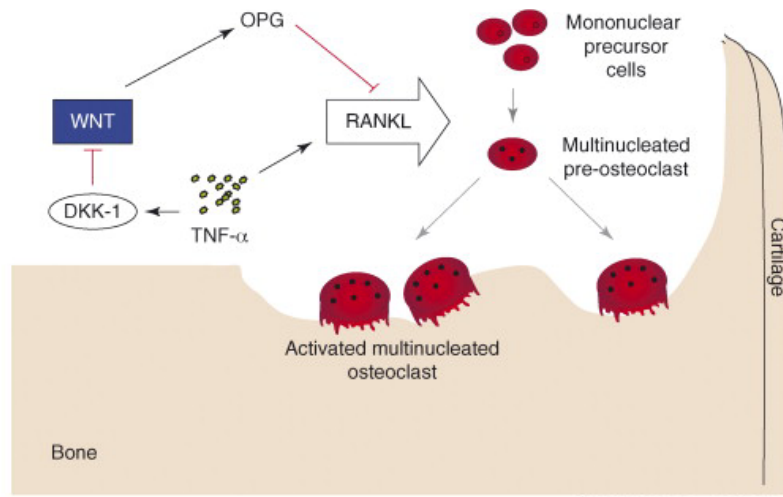
**Figure 3. Central signalling pathways in osteoclastogenesis.**

Activation of RANK by RANKL represents a well-accepted signalling mechanism that initiates osteoclast differentiation and leads to the activation of NFATc1, the principal transcription factor in osteoclastogenesis (taken from Edwards & Mundy, 2011).

Osteoprotegerin (OPG) is an endogenous decoy receptor of RANKL and thus inhibits the recruitment, proliferation, and activation of osteoclasts. *In vivo* models show that the overexpression of OPG, or the deletion of RANK or RANKL cause severe osteopetrosis, this demonstrating the critical role of this pathway in osteoclastogenesis (Weilbaecher, Guise, & McCauley, 2011). Indeed, abnormalities in the balance of RANK/RANKL/OPG can lead to an increased bone resorption that underlies for example the bone damages observed upon postmenopausal osteoporosis, Paget's disease, bone loss in metastatic cancers or rheumatoid arthritis. Further supporting the importance RANK/RANKL/OPG balance, the canonical Wnt/ $\beta$ -catenin signalling pathway was shown to inhibit osteoclastogenesis via the



modulation of RANKL expression in osteoblasts (Spencer, Utting, Etheridge, Arnett, & Genever, 2006).



**Figure 4. The Wnt signalling pathway with its ability to inhibit osteoclastogenesis.**

Wnt-signaling proteins are able to block osteoclastogenesis via induction of OPG expression. Under physiological circumstances, OPG binds to RANKL and provides a balance between bone resorption and bone formation. Through increased induction of DKK1, the Wnt/ $\beta$  catenin signaling is inhibited, which consequently leads to a decrease in production of OPG and more active osteoclastogenesis (taken from *Herman, Krönke, & Schett, 2008*).

In addition to RANK/RANKL/OPG system, calcium functions as an important second messenger in the regulation of skeletal cells. Indeed, calcium signalling pathway is pivotal for osteoclastic maturation due to, for example, its involvement in the late phase of NFATc1 nuclear translocation. Cytoplasmic calcium can originate either from intracellular calcium stores or extracellular calcium uptake. Oscillations in intracellular calcium level activate the major calcium-binding protein calmodulin, which can notably activate multifunctional CaMKs (calmodulin-dependent kinases). Binding of  $\text{Ca}^{2+}$ / calmodulin to CaMKs induces a conformational change that leads to an active form of these kinases. In the case of CaMKIV, maintaining maximal levels of activity requires either autophosphorylation or phosphorylation by another calmodulin-dependant upstream kinase. Further, p-CaMKIV induces CREB-mediated transcription through its phosphorylation (CREB, C-AMP Response Element-binding protein). Then, phosphorylated CREB (p-CREB) cooperates with the master regulator NFATc1 to induce the transcription of osteoclast-typical markers mentioned above.

Furthermore, p-CREB is also involved in activation of c-Fos transcription factor (Sato *et al.*, 2006).

### 1.3. Bone dynamics

Bone is a highly metabolically active tissue that undergoes continuous remodelling by two counteracting processes: bone formation by osteoblasts and bone resorption involving osteoclasts. Under normal physiological conditions, bone resorption and formation are tightly coupled to each other, so that the amount of bone removed is always equal to the amount of newly formed bone at each remodelling site. This critical balance preserves the skeletal integrity and is mediated by the activation of mechanosensing osteocytes (Seibel, 2005). In addition, bone remodelling is influenced by numerous paracrine and endocrine factors, including osteoblast- and osteoclast-specific signalling proteins and transcription factors, as well as hormones and growth factors (Borovecki *et al.*, 2007). These processes also control the reshaping or replacement of bone during growth (bone modeling) and following injuries like fractures.

#### 1.3.1. Bone modeling

Bone modeling (reshaping) can be found both in cancellous and cortical bone of evolutionary higher mammals. In a growing mammalian skeleton, cancellous bone turnover is dominated by modeling, whereas remodelling is the major turnover activity in a mature skeleton (Erben, 2015). Modeling is the process by which bones change their overall shape (longitudinal and radial growth) responding to physiological or biomechanical stimuli in order to gradually adjust to the forces that skeleton encounters. Longitudinal growth occurs at the growth plates, where cartilage proliferates in the epiphyseal and metaphyseal areas of long bones, before subsequently undergoing mineralization to form primary new bone (Clarke, 2008). Bone widening occurs with aging as a result of periosteal new bone apposition and old bone removal on the endosteal surface. Additionally, modeling may be modified in hypoparathyroidism (Ubara *et al.*, 2005), renal osteodystrophy (Ubara *et al.*, 2003), or as a consequence of treatment with anabolic agents (Lindsay *et al.*, 2006).

### 1.3.2. Bone remodelling

Following the changes in biomechanical forces, and with a goal to replace old micro-damaged bone with new and mechanically stronger tissue, the skeletal system is subjected to lifelong remodelling. Both trabecular and cortical bone are continuously renewed by removal of discrete packets of old bone, replacement of these packets with newly synthesized proteinaceous matrix, and subsequent mineralization of the matrix (Fernández-Tresguerres-Hernández-Gil, Alobera-Gracia, del-Canto-Pingarrón, & Blanco-Jerez, 2006). Although cortical bone makes up 75 % of the total bone volume, the turnover rate is ten times higher in trabecular bone, since the surface area-to-volume ratio is much higher in the later. Therefore, trabecular bone turnover is more important in mineral homeostasis. Approximately 5–10 % of total bone turnover per year is adequate to maintain biomechanical strength of bone, with 25 % of trabecular and 2-3 % of cortical bone tissue being remodeled annually (Kini & Nandeesh, 2012).

Intracortical bone remodelling results in typical microanatomical structures, named Haversian canals or osteons. In this type of bone remodelling, osteoclasts and osteoblasts are organized in a complex structure called basic multicellular unit (BMU). Each BMU comprises a cutting cone of osteoclasts, followed by a closing cone lined by osteoblasts, together with connective tissue, blood vessels, and nerves. The average lifespan of a typical human BMU is 2–8 months, with the greater part of this time being used for bone formation. In cancellous bone, and at least in post-menopausal osteoporosis, it is not entirely established whether BMUs exist as distinct entities because the time lapse between the end of resorption and the beginning of new bone formation at one remodelling site appears to be quite variable (Andersen *et al.*, 2013).

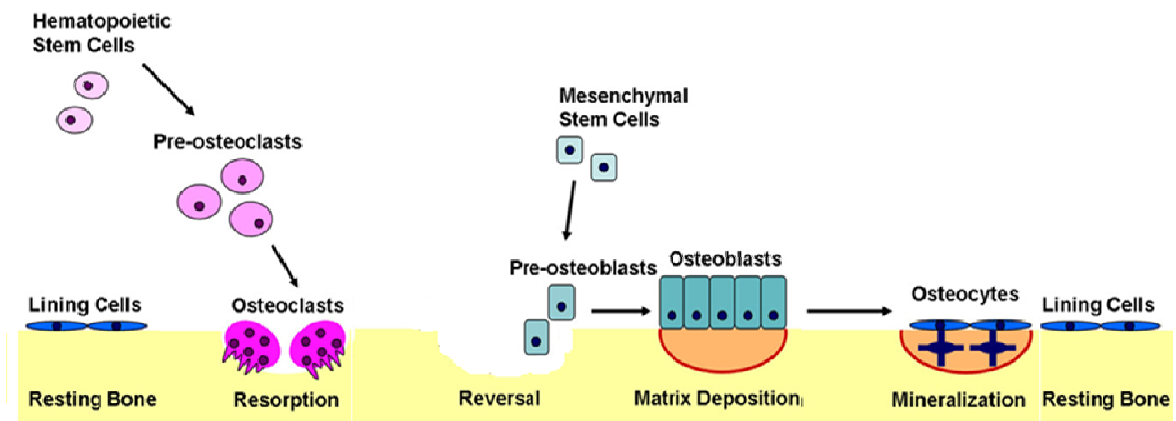
It is currently believed that remodelling can be initiated by either hormone-driven stochastic mechanism, or microdamage-driven targeted mechanism. Stochastic remodelling is considered to be under endocrine control, with sex steroids and parathyroid hormone being the most salient endocrine determinants of bone metabolism (Khosla, Oursler, & Monroe, 2012). On the other hand, the purpose of targeted remodelling is to remove microdamage that occurs during normal physical activities. However, the distinction between stochastic and targeted remodelling

may be arbitrary, because there is currently no sufficient data supporting the fact that these two mechanisms operate really independently (Erben, 2015).

In the adult skeleton bone remodelling is a balanced process, with the amount of resorbed bone proportional to the newly formed tissue. In the third decade of life, bone mass peak is attained and maintained with small variations until the age of 50. Periosteal bone balance is mildly positive, whereas endosteal and trabecular bone balances are mildly negative, this resulting in cortical and trabecular thinning with aging. These relative changes occur with endosteal resorption outstripping periosteal formation. Bone turnover actually increases in perimenopausal and early postmenopausal women, but the balance is shifted towards resorption resulting in a decrease of bone mass. With further aging, turnover rate slows down but remains higher than in premenopausal women (Kini & Nandeesh, 2012).

At least in humans, the initial event in bone remodelling is most likely the detachment of bone lining cells from the quiescent bone surface. Following this, blood-borne osteoclastic precursors can attach to the bone surface and start bone resorption (Sims & Martin, 2014). Normal bone remodelling cycle takes place in a highly coordinated fashion with a complex time-space coupling between bone resorption and bone formation. Osteocytes appear to be the main cell type orchestrating the bone remodelling process, due to their unique ability to detect microdamages in old bone and to secrete RANKL, a crucial cytokine for osteoclastic differentiation (Xiong *et al.*, 2011).

Bone remodelling can be divided into six phases namely, quiescent, activation, resorption, reversal, formation, and mineralization (Fernández-Tresguerres-Hernández-Gil *et al.*, 2006).



**Figure 5. Bone remodelling process.**

Osteoclasts are recruited to the bone site to resorb the organic and the mineral component of the bone matrix. Further on, osteoblasts arrive on the resorbed bone surface and lay down the new organic bone matrix, which is subsequently mineralized. Osteoblasts then differentiate to their final maturation stages (osteocytes or bone lining cells (adapted from Kapinas & Delany, 2011)).

- 1. Quiescent phase:** the state of the bone when at rest.
- 2. Activation phase:** retraction of the bone lining cells and digestion of the endosteal membrane by collagenase action. In this stage, mononuclear monocyte-macrophage osteoclast precursors are recruited from the blood circulation to the bone site enabling their interaction with other bone cell types. This leads to the differentiation, fusion and activation of large multinucleated osteoclasts that attach to the mineralized bone surface.
- 3. Resorption phase:** osteoclasts secrete hydrogen ions lowering the pH and dissolving the bone mineral. They also produce lysosomal enzymes, particularly cathepsin K, which can degrade the organic components of bone matrix such as the collagen. This process is completed by the macrophages and permits the release of numerous hormones, cytokines and growth factors contained within the bone matrix, with the most important ones being transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet-derived growth factor (PDGF), and insulin-like growth factors I and II (IGF-I and II). Osteoclast-mediated bone resorption takes approximately 2–4 weeks in each remodelling cycle, and produces irregular cavities on the trabecular bone surface.

These cavities are called Howship's lacunae in cancellous bone or cylindrical Haversian canals in cortical bone.

**4. Reversal phase:** when a resorption cavity is created, osteoclasts detach from the bone surface and are replaced by cells of the osteoblast lineage that initiate bone formation. The preosteoblasts are attracted by the growth factors liberated from the matrix. These factors act as chemoattractants and in addition, they stimulate preosteoblasts proliferation. The biochemical signals linking the end of bone resorption and the beginning of bone formation are not yet firmly established, but some of the candidates are bone matrix-derived factors such as TGF- $\beta$ , IGF-1, IGF-2, bone morphogenetic proteins (BMPs), PDGF, or fibroblast growth factor (FGF) (Bonewald & Mundy, 1990; Locklin, Oreffo, & Triffitt, 1999).

**5. Formation phase:** the preosteoblasts now present in the resorption cavity synthesize a cementing substance upon which the new tissue will be attached. A few days later, some of the osteoblasts fill the resorption cavity by secreting the osteoid matrix (Lind *et al.*, 1995). The remaining osteoblasts continue to synthesize bone until they evolve into quiescent lining cells that completely cover the newly formed bone surface and connect with the osteocytes embedded in the bone matrix.

**6. Mineralization phase:** 30 days after osteoid secretion, minerals are deposited in the bone matrix and this process lasts up to 90 days in the trabecular and up to 130 days in the cortical bone. When the cycle is completed at one remodelling site, with the amount of bone formed equal to the amount of bone resorbed, the quiescent phase is reached.

### 1.3.2.1. Bone resorption

Bone development and turnover, as well as systemic mineral homeostasis, are essentially influenced by osteoclasts activated to resorb calcified cartilage, enamel, dentin, and mineralized bone. Mature osteoclasts display different levels of actin and vinculin distribution and organisation (Väänänen & Horton, 1995). The podosome belt found in mature osteoclasts is thought to evolve into thick circular band of actin,

referred to as a sealing zone in actively resorbing osteoclasts. This zone enables tight osteoclastic attachment to the bone surface and seals the resorption cavity where protons and proteases are released (Lakkakorpi & Väänänen, 1991). Compact osteoclasts have greater bone substrate resorption efficacy than spread osteoclasts (Arkett, Dixon, & Sims, 1992). The morphology of the actin ring depends on surface roughness, crystallinity, energy, and solubility (Nakamura *et al.*, 2013). Osteoclasts recognize bone surface through the detection of bone matrix proteins (type I collagen, osteopontin) through  $\beta 1$  integrins and vitronectin receptor  $\alpha v\beta 3$  (Helfrich *et al.*, 1996; Nakamura *et al.*, 2007). Osteoclastic recognition of the substrate determines whether they behave as resorbing or non-resorbing osteoclasts. Osteoclasts cultured on bone slices have been shown to have thick actin rings and TRAP localized in their upper levels and around the nuclei. By contrast, osteoclasts cultured on glass coverslips have been shown to have thin actin rings and TRAP evenly distributed in the cytoplasm (Nakamura *et al.*, 2013). The involvement of integrins in the recognition by osteoclasts of synthesized inorganic substrates is not yet completely elucidated. The differences in actin ring structure between osteoclasts cultured on hydroxyapatite (HA) and carbonate-substituted hydroxyapatite (CA) substrates indicate that inorganic components have also an important role in the recognition mechanism. For example, actin ring morphology is similar between osteoclasts cultured on bone slices and CA, while the ring quality is inferior when osteoclasts are cultured on HA and tricalcium phosphate (TCP) (Nakamura *et al.*, 2013). One of the indicators for a resorbing osteoclast is also the morphology and localization of vinculin. Indeed, noticeable amounts of cytoskeletal structures co-localize with actin and are found in cytoplasmic sites close to the resorption lacuna (Lakkakorpi & Väänänen, 1991).

Polarized and activated multinuclear osteoclasts exhibit high levels of expression of genes associated with the dissolution of bone mineral or involved in the degradation of bone organic matrix. Carbonic anhydrase II (CAII) is a good genetic marker for activated osteoclast. Actually, its function to supply protons for the vacuolar-type proton-ATPase is essential for the dissolution of bone mineral (Riihonen *et al.*, 2007). Another good marker for functional osteoclasts is CTSK, which is essential for the degradation of bone organic matrix (Troen, 2006). Protons and CTSK are transported from the ruffled border areas of osteoclasts into resorption lacuna, where the actual resorption takes place in low pH conditions. The carbonate-substituted HA

of the bone is more soluble than the stoichiometric HA, which increases the advantages of decalcification and the release of calcium ions. In turn, calcium ions contribute to the activation of non-resorbing osteoclasts (Helfrich *et al.*, 1996).

### 1.3.2.2. Bone formation

Ossification (or osteogenesis) is the process of formation of new bone by cells called osteoblasts. A rapid formation of bone mass occurs in the foetus and infant, while it slows somewhat during childhood until the age of 11 for females and 12 for males. Adolescence is characterized by tremendous bone formation and most of adult bone mass is acquired by the age of 18, with only a small amount added during the next ten years (Kini & Nandeesh, 2012). Two important processes contribute to normal healthy bone synthesis:

**1. Intramembranous ossification** characterized by laying down of bone into the primitive connective tissue (mesenchyme), this resulting in the formation of bones (e.g. skull, clavicle, mandible). This process also occurs in the healing of compound fractures treated by open reduction and stabilization by metal plate and screws.

**2. Endochondral ossification** where a cartilage model acts as a precursor for bone formation (e.g. femur, tibia, humerus, radius). This is the most important process occurring during fracture healing when treated by cast immobilization.

Both types of ossification include synthesis of extracellular organic matrix (osteoid) and its subsequent mineralization yielding the newly formed bone. The boundary between the old and new bone is called a cement line or reversal line, and it can be distinguished on an hematoxylin and eosin (H&E) histological sections as a blue (basophilic) line. If the process of bone tissue formation occurs at an extraskeletal location, it is termed as heterotopic ossification (Kini & Nandeesh, 2012).



### 1.3.2.3. Intramembranous (mesenchymal) ossification

During foetal development of the mammalian skeletal system, bone tissue is formed from connective tissue such as mesenchyme. It mainly occurs during formation of the flat bones of the skull, but also the mandible, maxilla, and clavicles. It is also an essential process during the natural healing of bone fractures or during the rudimentary formation of bones of the head. The steps in intramembranous ossification are:

1. Formation of ossification center
2. Calcification
3. Formation of trabeculae
4. Development of periosteum.

Located within human mesenchyme or in the medullary cavity at the site of a bone fracture, mesenchymal stem cells (osteoprogenitor cells) initiate the process of intramembranous ossification. These unspecialized cells undergo characteristic changes as they develop into an osteoblast. The process of membranous ossification is essentially the direct mineralization of a highly vascular connective tissue, and it starts at certain constant points known as centers of ossification. At such a center, the mesenchymal cells proliferate and condense around a profuse capillary network. The space between the cells and around the vessels is filled by the amorphous ground substance with a fine meshwork of collagen fibers. The MSCs then differentiate into osteoblasts, which secrete an osteoid matrix in the center of the aggregate. Consequently, they become entrapped in bone matrix and develop into osteocytes. At this point, the mineralization of osteoid begins, forming a nidus structure that contains osteocytes and that is lined by active osteoblasts. The process of entrapping of osteoblasts proceeds, the trabeculae gradually thicken, and the intervening vascular spaces (spongy layer) become progressively narrowed. The nidus that began as a diffuse collection of MSCs has now become rudimentary bone tissue. Where the bone persists as cancellous bone, the process slows down and the spaces later become occupied by hemopoietic tissue. As changes in the ossification center proceed, the surrounding mesenchyme condenses as a fibrovascular periosteum around its edges and surfaces. Then, the periosteum is formed and bone growth continues at the surface of trabeculae. The increasing growth of trabeculae

results in interconnection and this network is called woven bone. Eventually, woven bone is replaced by lamellar bone (Kini & Nandeesh, 2012).

#### **1.3.2.4. Intracartilaginous (endochondral) ossification**

In endochondral ossification, bone tissue grows from hyaline cartilage. It occurs during the growth of the length of long bones, most of the other bones in the body, as well as in natural fracture healing (Kini & Nandeesh, 2012). The basic steps include:

1. Development of cartilage model
2. Growth of cartilage model
3. Development of the primary ossification centre
4. Development of the secondary ossification centre
5. Formation of articular cartilage and epiphyseal plate.

The process begins at points within the cartilage called “primary ossification centres.” They mostly appear during fetal development with the exception of a few short bones that begin their primary ossification after birth. They are responsible for the formation of the diaphysis of long and short bones, and certain parts of irregular bones. After birth, secondary ossification forms the epiphyses of long bones and the extremities of irregular and flat bones. A growing zone of cartilage (the epiphyseal plate) separates the diaphysis and epiphyses of a long bone. When the child reaches skeletal maturity at age 18–25, all of the cartilage is replaced by bone tissue that fuse the diaphysis and both epiphyses together in a process called epiphyseal closure (Kini & Nandeesh, 2012).

#### **1.3.2. Models of bone remodelling**

Numerous attempts have been made to explain how, in bone remodelling cycle, the information about the amount of bone resorbed by osteoclasts is transmitted to osteoblasts. It is currently thought that coupling between bone resorption and formation occurs (i) through growth factors stored in the bone matrix and released during resorption, (ii) through soluble cytokines secreted by osteoclasts,

and (iii) through molecules expressed in the cell membrane of osteoclasts (Sims & Martin, 2014). Most of our current understanding of the mechanisms involved in coupling comes from experiments in gene-targeted mice. However, mice and rats lack true intracortical Haversian remodelling. Therefore, it is uncertain whether there are differences in the coupling mechanisms between intracortical and cancellous bone remodelling (Erben, 2015).

The current explanatory models of the coupling mechanism are associated with a number of problems. First, none of these models can precisely explain why the amount of bone formed during the formation phase matches the amount of bone resorbed during the resorption phase. Second, and perhaps more critical, is the fact that in human cancellous bone remodelling, the time span between the end of osteoclastic resorption and the initiation of bone formation is in the range of several weeks (Delaisse, 2014). Any biochemical signal linking bone resorption to bone formation will have dissipated during this long period of time. Therefore, it is unclear how information is actually transmitted from osteoclasts to osteoblasts. Moreover, a diligently conducted histomorphometric study in human iliac biopsies of patients with postmenopausal osteoporosis revealed a large percentage (~30%) of remodelling cycles that were arrested in the reversal phase (Andersen *et al.*, 2013), suggesting that in humans and in cancellous bone remodelling, formation is not always tightly coupled to resorption.

These problems led certain authors to hypothesize that coupling in cancellous bone remodelling may rather be a mechanically controlled process within the newly formed bone package. An extensive review of cell-based and biomechanical bone remodelling theories is presented in Hambli *et al.* (Hambli, 2014). Using finite element models, Ruimerman *et al.* (Ruimerman *et al.*, 2005) and Smit and Burger (Smit & Burger, 2000) provide mathematical descriptions of cancellous bone remodelling and of the potential strain distributions around a resorption cavity in cortical bone. They suggest that strains sensed by osteocytes within resorption cavities could account for subsequent activation of osteoclasts and osteoblasts. Additionally, based on the microanatomy of newly formed bone packages during cancellous bone remodelling, Erben (Erben, 2015) proposes that self-regulating mechanisms are involved in the refilling control of the resorption cavity. In this model, the resorption cavity created by osteoclasts results in mechanical weakening of the structural element and thus, in increased strain around the resorption cavity under constant

loading conditions. The increased strain is detected by osteocytes entrapped in the underlying bone matrix. When the strain exceeds a certain threshold, the osteocytes initiate bone formation by secreting osteogenic signals through the canalicular network opened by osteoclastic bone resorption. After osteoblastic bone formation has started, because the underlying osteocyte canaliculi system is sealed by the new cement line, it is the newly built osteocyte–osteoblast network that detects strain. In addition, newly formed bone is less mineralized and has, therefore, different material properties compared with the higher mineralized surrounding old bone. It is likely that the differences in material properties between old and new bone affect strain energy distribution within the newly formed bone package and thus, mechanosensing of matrix-embedded osteocytes. Once the mechanical strain within the newly built bone structural unit falls below a certain threshold, bone formation stops (Erben, 2015).

These provocative biomechanical strain-driven models challenge the notion of hemisteonal remodelling with predetermined BMUs acting during cancellous bone remodelling. Indeed, they suggest that osteoclastic bone resorption and osteoblastic bone formation are not directly associated but rather indirectly linked through mechanosensing osteocytes.

### **1.3.3. Regulation of bone remodelling**

The sensitive balance between bone resorption and bone formation rates is maintained through the action of various interrelated factors such as genetic, mechanical, vascular, nutritional, hormonal and local determinants.

Genetic factors determine between 60 and 80 % of the bone mass. In humans, black population has greater bone mass than Caucasians, who in turn have higher mass than Asians. Also, daughters of mothers with osteoporosis are more predisposed to developing this condition themselves (Grant & Ralston, 1997). Bone tissue adapts its structure in response to the mechanical loading, one of the major driving forces in remodelling. Physical activity is essential for the correct development of the bone. It is considered that muscular action transmits tension to the bone, which is detected by osteocyte network, and that new trabeculae tend to align with maximum stresses in many bones. Loading improves bone strength by influencing collagen alignment as new bone is being formed. In cortical bone tissue, regions

subjected to predominantly tensile stresses have a higher percentage of collagen fibers aligned along the bone long axis. In regions of predominant compressive stresses, fibers are more likely to be aligned transverse to the long axis. On the other hand, the absence of muscular activity has an adverse effect on bone by accelerating its resorption.

Vascularization is a prerequisite in the first phase of ossification since it supplies blood cells, oxygen, minerals, ions, glucose, hormones, and growth factors. In endochondral ossification, blood vessels invade the cartilage, allowing later resorption via the osteoclasts originating from the nearby vessels. In the same way, angiogenesis is one of the first events in the repair of fractures and bone regeneration.

Lastly, nutritional factors such as caffeine, alcohol, excess salt and smoking constitute risk factors for lowering bone mineral density. On the other hand, calcium uptake accompanied by sufficient vitamin D levels contributes to systemic calcium homeostasis, this impacting the mobilization of calcium stores from the skeleton.

**1.3.3.1. Systemic regulators of bone remodelling**

Normal development of the skeleton is conditioned by the correct activity of the endocrine system. The main hormones that regulate bone metabolism are summarized in Table 1., with PTH, calcitonin, and vitamin D being the most prominent ones that regulate the bone minerals (calcium and phosphorus).

**Table 1. Hormones involved in the regulation of bone remodelling.**

|                                 |  |
|---------------------------------|--|
| <b>Increase bone formation</b>  | <b>growth hormone, vitamin D metabolites, androgens, insulin, low-dose PTH/PTHrP, progesterone</b> |
| <b>Decrease bone formation</b>  | glucocorticoids  |
| <b>Increase bone resorption</b> | PTH/PTHrP, glucocorticoids, thyroid hormones, high-dose vitamin D                                  |
| <b>Decrease bone resorption</b> | calcitonin, estrogens  |

Acting on both bone resorption and formation, **thyroid hormones** are critical for the maintenance of normal bone remodelling. Firstly, favoring the synthesis of IGF-I, they stimulate the synthesis of the osteoid matrix and its mineralization. Secondly, increasing the number and activity of osteoclasts, a contrary effect is produced. The clinical manifestation of this effect is the bone loss occurring in hyperthyroidism (Kini & Nandeesh, 2012).

**Parathyroid hormone** (PTH) controls the homeostasis of calcium and phosphorous by direct action on the bone and the kidneys, and indirectly on the intestine. It is produced by the parathyroid glands in response to hypocalcaemia. Its skeletal effects are mediated through the osteoblast, which are the major expressers of PTH receptors in bone. PTH is a potent stimulator of bone resorption and exerts dual effects on bone formation. High concentrations of PTH result in an inhibition of collagen synthesis. Plasma PTH tends to increase with age, and this may produce an increase in bone turnover and a loss of bone mass, particularly of cortical bone. Conversely, a prolonged intermittent administration of PTH stimulates bone formation, and this is clinically exploited in the treatment of osteoporosis and hypothyroidism (Dempster, Cosman, Parisien, Shen, & Lindsay, 1993).

**Calcitonin** is a hormone produced by parafollicular C cells of the thyroid and other neuroendocrine cells. It is an inhibitor of bone resorption through the reduction of the number and activity of osteoclasts. However, this action is rather transitory since osteoclasts seem to become insensitive to calcitonin within a few days (Kini & Nandeesh, 2012).

**Vitamin D** is a steroid hormone that favours bone mineralization by stimulating the intestinal absorption of calcium and phosphorous. It is necessary for normal growth of the skeleton (Raisz, 1993). It comes from both an endogenous (vitamin D<sub>3</sub>/cholecalciferol) and an exogenous (vitamin D<sub>2</sub>/ergocalciferol) supply. The endogenous form of vitamin D, (cholecalciferol) is synthesized in the skin from the cholesterol metabolite 7-dehydrocholesterol under the influence of ultraviolet radiation. The exogenous form of vitamin D (ergocalciferol) is produced by ultraviolet irradiation of the plant sterol ergosterol and is available through the diet. Both forms of vitamin D require further metabolism to be activated. Vitamin D regulates the expression of several bone proteins, notably osteocalcin by promoting its gene transcription. It has also bidirectional effects on the transcription of type I collagen and alkaline phosphatase.

Concerning sex hormones, **androgens** have an anabolic effect on bone through the stimulation of the osteoblasts. Likewise, they act as mediators of the growth hormone in puberty. Androgens increase cortical bone size via stimulation of both longitudinal and radial growth. While androgen deficiency is associated with lower bone density, administration of testosterone in young people before the closure of the epiphyses increases bone mass. In the same way, women with an excess of androgens present higher bone densities. Androgens present a dual effect on endochondral bone formation. Indeed, at the start of puberty, they stimulate endochondral bone formation, whereas they induce epiphyseal closure at the end of puberty. This effect of androgens may be important because bone strength in males seems to be determined by relatively higher periosteal bone formation and therefore, greater bone dimensions relative to muscle mass at older age. **Estrogen** receptors have been described in human osteoblasts, osteocytes, and osteoclasts. They are essential for the closure of the growth plates and have an important role in the development of the skeleton. Estrogens can affect bone formation by increasing the number and function of osteoblasts. On the other, they can reduce resorption. Several studies have found that estrogens can increase the levels of OPG, an inhibitor of osteoclastic differentiation. By doing so, they may play an important role in the regulation of osteoclastogenesis (Hofbauer *et al.*, 1999). Alternatively, estrogens may enhance local factors involved in bone formation and inhibit those that result in its impairment. For this reason, estrogen deficiency during menopause constitutes the most important pathogenic factor in bone loss associated with osteoporosis. Lastly, loss of estrogens or androgens increases the rate of bone remodelling by removing restraining effects on osteoclastogenesis. This also causes a focal imbalance between resorption and formation by prolonging the lifespan of osteoclasts and shortening the lifespan of osteoblasts (Kini & Nandeesh, 2012).

Called "the pregnancy hormone", **progesterone** has an anabolic effect on bone, either directly through the osteoblasts that possess progesterone receptors, or indirectly through a competition for the binding of glucocorticoids to their osteoblastic receptors.

Indeed, **glucocorticoids** are indispensable for bone cell differentiation during development, but their greatest postnatal effect is to inhibit bone formation (at high doses, they have a catabolic effect on bone), since they inhibit the synthesis of IGF-I by the osteoblasts and directly suppress BMP-2, critical factors in osteoblastogenesis.

This is the major mechanism in glucocorticoid-induced osteoporosis. Indirect effects of glucocorticoids on calcium absorption and steroid hormone production may, however, increase bone resorption (Manolagas, 2000).

The **growth hormone** acts directly on the osteoblasts. It stimulates their activity, this leading to an increased synthesis of collagen, osteocalcin, and alkaline phosphatase. An indirect action is produced through the upregulation of IGF-I and II synthesis by the osteoblasts. In turn, these two factors further stimulate the proliferation and differentiation of premature osteoblasts (Hull & Harvey, 1998; Rosen & Donahue, 1998).

The adipocyte-derived hormone **leptin** binds to its receptors in hypothalamic region and, via the sympathetic nervous system, activates  $\beta$ 2-adrenergic receptors situated on osteoblast surface. The activation of these receptors is adverse to osteoblastic proliferation and reduces the rate of bone formation (Fu *et al.*, 2005). On the opposite, they switch on the activating transcription factor 4 (ATF4) and stimulate RANKL expression, this favouring the bone resorption (Takeda *et al.*, 2008).

Lastly, **Insulin** stimulates bone matrix synthesis both directly and indirectly by increasing the hepatic synthesis of IGF-I.

### 1.3.3.2. Local regulators of bone remodelling

Growth factors, cytokines, and bone matrix proteins are implicated as modulators in local bone metabolism (Fernández-Tresguerres-Hernández-Gil *et al.*, 2006).

#### *Growth factors*

Bone contains a large number of growth factors. These are polypeptides produced by bone cells themselves or coming from extraosseous tissue, and which act as modulators of cellular functions including proliferation, and differentiation. Among the most abundant are the IGFs (Fraher, 1993; Hakeda *et al.*, 1996; Rosen & Donahue, 1998) , TGF- $\beta$ , and the related family of BMPs that have important functions not only in remodelling but also in skeletal development. Other growth factors, such as PDGF, PTHLH, and FGF may play an important role in physiologic remodelling, and an even more important role in skeletal repair.



**IGF-I and IGF-II** are synthesized by the liver and osteoblasts, and these factors are found in high concentrations in the osteoid matrix (Cohick and Clemmons 1993). They stimulate collagen synthesis by increasing the number and function of the osteoblasts. They circulate linked to IGF-binding proteins (IGFBP), which in turn can exercise stimulatory or inhibitory effects on bone (Conover 2008). IGFs synthesis is upregulated by growth hormone, estrogens, and progesterone, while the glucocorticoids inhibit it. They also mediate osteoblast-osteoclast interactions and actively participate in bone remodelling (Hill *et al.* 1995). IGF-II is the most abundant factor in the bone matrix and it plays an important role during embryogenesis (Canalis *et al.* 1989; Mohan and Baylink 1991).

**TGF- $\beta$**  is the second most represented superfamily in bone tissue. These proteins are latently present in the matrix, and are activated during osteoclastic resorption. TGF- $\beta$  is a potent stimulator of bone formation, promoting osteoblastic differentiation and the synthesis of the osteoid matrix. Conversely, it inhibits the synthesis of proteases, especially the matrix metalloproteinases (MMPs) that degrade bone organic matrix (Bonewald and Dallas 1994). TGF- $\beta$  inhibits bone resorption by reducing the formation and the differentiation and the activity of osteoclasts, as well as stimulating their apoptosis (Baylink *et al.* 1993).

**BMPs** are comprised in the TGF- $\beta$  family as a group of 15 proteins able to achieve the transformation of connective tissue into bone tissue. Thus, they are considered as osteoinductive factors (Sakou 1998). They are highly abundant in bone tissue and during embryogenesis; they participate in the formation of bone and cartilage. They stimulate the differentiation of the mesenchymal stem cells toward different cell lines (adipose tissue, cartilage, bone) (Yamaguchi *et al.* 2000). They strongly promote osteoblastic differentiation and they are believed to inhibit osteoclastogenesis (Canalis *et al.* 2003).

**PDGF**, on one side stimulates protein synthesis by osteoblasts and, on the other, favors bone resorption. Its other effects favour scarring since it stimulate fibroblast and smooth muscle cells proliferation, neovascularization, as well as collagen synthesis (Nash *et al.* 1994).

**FGF** has an anabolic effect on bone, as it is a mitogen for osteoblasts, vascular endothelial cells, and fibroblasts. For example, it is known that mutations in FGF receptors produce alterations in the craniofacial skeleton such as achondroplasia, Apert's syndrome, and Crouzon's syndrome (Marie 2003).

Epidermal growth factor (**EGF**) is a powerful mitogen for cells of mesodermic or ectodermic origin. It has a dual formative and destructive action, although the latter is the most well known.

**VEGF** induces angiogenesis and vascular endothelial proliferation. It produces vasodilation and an increase in vascular permeability. It is produced upon hypoxia and is currently considered as one of the key factors in the first phases of fracture repair, for bone regeneration, and in tumor growth (Kini & Nandeesh, 2012).

Granulocyte / macrophage - colony stimulating factor (**GM-CSF**) is important in osteoclastogenesis and may play a role in the pathogenesis of osteopetrosis.

Osteoblasts and medullar stromal cells produce **M-CSF**, an essential factor that is required for osteoclastogenesis initiation and the formation of giant multinucleated cells. However, it has no effect on osteoclastic activity.

Concerning **TNF**, *in vitro* it stimulates resorption activity and consequently, it has been related with bone loss in arthritis and in periodontal disease.

### *Cytokines*

Through both autocrine and paracrine effects, polypeptides synthesized in lymphocytic and monocytic cells play an important role in immunological response, inflammation, and hematopoiesis. The following are the most important in bone.

(a) Interleukin 1 (**IL-1**) directly increases osteoclastic resorption through the stimulation of preosteoclastic cells proliferation and differentiation. It also enhances osteoclasts activity, while inhibiting their apoptosis (Compston 2001). More specifically, three different related molecules (IL-1 a, IL-1 b, and their inhibitor IL-1 receptor antagonist) act both directly and indirectly on resorption through the synthesis of prostaglandins.

(b) Interleukin 6 (**IL-6**) stimulates bone resorption and appears to be implicated in the development of Paget's disease (Roodman 1999). It is believed to play an important role during the initial stages of osteoclastogenesis and it is produced in response to PTH, IL-1, and vitamin D.

(c) Interleukin 11 (**IL-11**) is produced in bone marrow and also induces osteoclastogenesis.

(d) Prostaglandins (**PGs**), particularly prostaglandin E2 (PGE2), stimulate bone resorption (Kawaguchi *et al.* 1995). This cytokine could also be important in inflammatory bone loss. The first step in PGE2 synthesis is mediated by an enzyme

called cyclooxygenase 2 (COX2), and inhibitors of this enzyme can prevent bone formation in response to mechanical stress in animals. PGE2 may be required for exercise-induced bone formation.

(e) **Leukotrienes** are another set of lipid molecules that appear to regulate bone remodelling.

#### *Matrix proteins*

Since matrix proteins are found in concentrations about 1,000 times higher than growth factors, they could play a more pivotal role in the regulation of the different bone cells functions, and thus in bone remodelling (Horowitz 2003). For example, collagen I is one of the earliest markers that regulate osteoprogenitor cells. Additionally, alkaline phosphatase (ALP) is a surface protein that could participate in the regulation of the proliferation, migration, and differentiation of osteoblastic cells. Lastly, osteonectin, fibronectin, and osteocalcin promote the migration, the attachment, and the activation of bone cells.

### **1.3.3. Markers of bone metabolism**

Biochemical markers provide useful dynamic informations about the turnover of bone tissue, and they are used in diagnosis and management of different metabolic bone diseases. They are usually classified according to the metabolic process they are considered to reflect. Most biochemical indices of bone resorption are collagen breakdown products such as hydroxyproline or the various collagen cross-links and telopeptides. Other markers of bone resorption include non-collagenous matrix proteins such as bone sialoprotein (BSP), or osteoclast-specific enzymes like TRAP or CTSK. In contrast, markers of bone formation are either by-products of collagen neosynthesis (e.g. N- and C-terminal propeptides), or osteoblast-related proteins such as osteocalcin (OCN) and alkaline phosphatase (ALP).

However, this distinction is not as sharp as it may appear since certain marker components reflect both bone formation and bone resorption (e.g. hydroxyproline, certain OC fragments). Furthermore, most of the molecules used as markers of bone turnover are also present in tissues other than bone, and non-skeletal processes may therefore influence their circulating or urinary levels. Finally, changes in markers of

bone turnover are not disease specific, but reflect alterations in the metabolism of the entire skeleton independently of the underlying cause (Seibel, 2005).

## 2. Bone tissue physiopatology

### 2.1. Bone strength and disease

Bone strength depends on several structural properties. The bone mass accounts for 50–70% of bone strength (Pocock *et al.* 1987) and peak bone mass is determined largely by genetic factors, with contributions from nutrition, endocrine factors, physical activity, and health during growth. Bone geometry and composition are important as well. The strength of bone increases by the radius of the involved bone raised to the fourth power. Consequently, larger bones are stronger than smaller bones with the equivalent bone mineral density (BMD). On the other hand, at a given skeletal site, the amount and proportion of trabecular and cortical bone independently affect bone strength.

Bone strength can be compromised by abnormalities in bone metabolism/remodelling. Subsequently, a variety of skeletal disorders appear such as fractures, osteomalacia/rickets, vitamin D deficiency, Paget's disease, or osteoporosis. Bone tissue can be weakened by mutations occurring in certain bone-typical proteins (e.g., collagen defects in osteogenesis imperfecta, impaired  $\gamma$ -carboxylation of gla proteins), but also by fluoride therapy, or hypermineralization states (Kini & Nandeesh, 2012). Regarding bone metabolism, cytokines such as IL-1, IL-6, and TNF, as well as growth factors, have been implicated in pathologic responses, particularly in osteoporosis associated with oestrogen deficiency, hyperparathyroidism, and Paget's disease (Lorenzo, 1991; Mills & Frausto, 1997; Papanicolaou *et al.*, 1998; Raisz, 1999).

### 2.2. Fracture healing

One of the notable characteristics of bone tissue is its potential for repair in response to injury or surgical treatment. Because of their great importance in orthopaedic, maxillofacial, and periodontal treatment, a great effort is taken in understanding the basis of these regeneration mechanisms.

### 2.2.1. Fracture repair

Fracture repair is a well-orchestrated multistage process conducted by numerous cell types, growth factors, and the extracellular matrix. In fracture healing, the pathway of normal embryonic development of bone is recapitulated through the coordinated and precisely timed activation of cells originating from the cortical bone, periosteum, bone marrow and surrounding soft tissue (Ferguson *et al.*, 1999; Gerstenfeld *et al.*, 2003). Most fractures heal by the combination of both intramembranous and endochondral ossification, with the later one being the predominant form (Ai-Aql, 2008). Endochondral bone formation usually occurs externally to the periosteum in regions that are mechanically less stable and immediately adjacent to the fracture site. On the other hand, intramembranous ossification that forms hard callus occurs internal to the periosteum at the proximal and distal edges of the callus (Dimitriou *et al.*, 2005) The eventual bridging of hard callus areas across the central fracture gap, which helps to regain bone biomechanical functions, is the first step in defect stabilization (Gerstenfeld *et al.*, 2006). Fracture repair can be divided into four overlapping phases presented in Table 2. (Ai-Aql, 2008). Firstly, an immediate inflammatory response appears at the injury site. Inflammatory cytokines are believed to initiate the repair cascade following an injury. Secondly, MSCs are recruited to the site where they differentiate into osteoblasts and chondrocytes, the later ones producing the cartilage. Thirdly, the cartilage matrix is mineralized and subsequently resorbed. This process is followed by primary bone formation performed by osteoblasts. At last, the process of bone remodelling begins, in which the initial bony callus is reshaped by resorption and secondary bone formation, which restores the anatomical structures that can support greater mechanical loads (Gerstenfeld *et al.*, 2003).

Signalling molecules that can be distinguished into three main groups tightly regulate the underlying biological processes in each of these stages: pro-inflammatory cytokines, TGF- $\beta$  superfamily members, and angiogenic factors (Peng *et al.*, 2005).

**Table 2. Summary of the multiple stages of fracture healing and the accompanying expression of signalling molecules.** (Based on published results from Kon *et al.*, 2001; Cho *et al.*, 2002; Gerstenfeld *et al.*, 2003 ; Dimitriou *et al.*, 2005 and taken from Ai-Aql, 2008).

| Stage of Fracture Repair                               | Biological Processes  | Expression of Signalling Molecules and their Proposed Functions  |
|--|---|--|
| <b>Inflammation</b>                                    | Hematoma  | IL-1, IL-6, and INF- $\alpha$ play a role in initiating the repair cascade.  |
|  | Inflammation  | TGF- $\beta$ , PDGF, and BMP-2 expression increases to initiate callus formation.  |
|  | Recruitment of mesenchymal stem cells   | GDF-8 is restricted to day 1, suggesting its role in controlling cellular proliferation.   |
| <b>Cartilage Formation and Periosteal Response</b>     | Chondrogenesis and endochondral ossification begins   | TGF- $\beta$ 2, $\beta$ 3, and GDF-5 peak due to their involvement in chondrogenesis and endochondral bone formation.  |
|  | Cell proliferation in intramembranous ossification  | BMP-5 and -6 rise.   |
|  | Vascular in-growth  | Angiopoietins and VEGFs are induced to stimulate vascular in growth from vessels in the periosteum.  |
|  | Neo-angiogenesis  |  |
| <b>Cartilage Resorption and Primary Bone Formation</b> | Phase of most active osteogenesis   | TNF- $\alpha$ rises in association with mineralized cartilage resorption.  |
|  | Bone cell recruitment and woven bone formation  | This promotes the recruitment of mesenchymal stem cells and induces apoptosis of hypertrophic chondrocytes. RANKL and MCSF rise in association with mineralized cartilage resorption.          |
|  | Chondrocyte apoptosis and matrix proteolysis<br>Osteoclast recruitment and cartilage resorption | BSIP-3, -4, -7, and -8 rise in association with the resorption of calcified cartilage. They promote recruitment of cells in the osteoblastic lineage.  |
|  | Neo-angiogenesis  | BMP-5 and -6 remain high during this stage, suggesting a regulatory effect on both intramembranous and endochondral ossification.<br><br>VEGFs are up-regulated to stimulate neo-angiogenesis. |
| <b>Secondary Bone Formation and Remodelling</b>        | Bone remodelling coupled with osteoblast activity   | IL-1 and IL-6 rise again in association with bone remodelling, whereas RANKL and MCSF display diminished levels  |
|  | Establishment of marrow   | Diminished expression of members of the TGF- $\beta$ superfamily.  |

### 2.2.1.1. Pro-inflammatory cytokines in fracture healing

Macrophages, inflammatory cells, and cells of mesenchymal origin secrete pro-inflammatory cytokines that are active for a limited time period following an injury. Later on, at a mid-stage in healing, some of the inflammatory cytokines are still upregulated and stimulate the osteoclastogenesis to remove mineralized cartilage. Next, other cytokines are induced at the final stage of healing when bone remodelling is triggered (Ai-Aql, 2008).

Interleukins-1 and 6 (IL-1 and IL-6) and TNF- $\alpha$  have been shown to play a role in initiating the repair cascade. They recruit inflammatory cells, enhance extracellular matrix synthesis, and also stimulate angiogenesis (Kon *et al.*, 2001). Their expression peaks within the first 24 hours following the injury, and then declines rapidly to nearly undetectable levels by day 3 (Cho *et al.*, 2002; Kon *et al.*, 2001). IL-1 and IL-6 are again upregulated, in association with bone remodelling during the stage of secondary bone formation (Gerstenfeld *et al.*, 2003).

After the day 3 decline, the expression of TNF- $\alpha$  rises again in the later stages, in association with mineralized cartilage resorption. During this stage of fracture healing, TNF- $\alpha$  stimulates osteoclast function, promotes the recruitment of MSCs and induces the apoptosis of hypertrophic chondrocytes. Its absence delays the resorption of mineralized cartilage and consequently, prevents the primary formation of new bone. In pathological situations where TNF- $\alpha$  is over-expressed, such as diabetic healing, there is a premature cartilage removal that results in a deficient bone formation and healing (Kayal *et al.*, 2007).

In addition, the expression of RANKL, OPG, and MCSF is upregulated after the initial injury and during the resorption of mineralized cartilage. Afterwards, during the phase of secondary bone formation and bone remodelling, the expression of RANKL, OPG, and MCSF is downregulated (Gerstenfeld *et al.*, 2003).

### 2.2.1.2. Transforming growth factor beta superfamily in fracture repair

During fracture healing, specific members of this family, such as BMPs (2-8), growth differentiation factors (GDFs) (1, 5, 8, and 10), and TGF- $\beta$  isoforms promote various stages of intramembranous and endochondral bone ossification (Cho *et al.*,



2002). It has been suggested that BMP-2, 6, and 9 may be the most prominent inducers of MSCs differentiation to osteoblasts, while the remaining BMPs promote the maturation of already committed osteoblasts (Cheng *et al.*, 2003).

Mesenchymal cells, osteoblasts, and chondrocytes produce BMPs that trigger a cascade of events promoting the formation of cartilage and bone. At the injury site, BMPs are largely involved in cellular processes like chemotaxis, mesenchymal cell proliferation and differentiation, angiogenesis, and the synthesis of extracellular matrix. Suggesting a role in the initiation of the repair cascade, studies of fracture healing in mice show maximal levels of BMP-2 expression within 24 hours following the injury. Accordingly, Tsuji *et al.* and Xiong *et al.* showed that BMP-2 expression is genetically associated with the maintenance of normal bone mass, and that it is a prerequisite for post-natal bone repair. Interestingly, BMP-2 does not appear to be necessary for normal embryological bone formation. Other *in vitro* studies examining bone marrow MSCs have shown that BMP-2 controls the expression of several other BMPs, while blocking its activity results in MSCs fail to differentiate into osteoblasts (Ai-Aql, 2008).

BMP- 3, 4, 7, and 8 show a restricted period of expression during fracture healing (days 14 to 21), when the resorption of mineralized cartilage and osteoblastic recruitment are the most active. By contrast, implying that they have a regulatory effect on both intramembranous and endochondral ossification, BMP-5 and 6 are constitutively expressed from day 3 in fracture healing (Cho *et al.*, 2002).

BMP antagonists also play an important role in fracture healing. It has been reported that the expression of noggin, a blocking agent for BMP-2, 4, and 7, is modulated during fracture healing (Yoshimura *et al.*, 2001). The pattern of noggin expression is similar to that of BMP-4, suggesting that the noggin/BMP-4 balance could be an important factor in the regulation of callus formation during fracture healing. On the other hand, in the absence of noggin, an excess of bone and cartilage formation occur during the development, indicating that noggin plays an important role in limiting the formation of new tissue (Ai-Aql, 2008).

TGF- $\beta$ , which induces the expression of extracellular matrix proteins, is thought to play an important role in chondrogenesis and endochondral bone formation (Barnes, Kostenuik, Gerstenfeld, & Einhorn, 1999). All three isoforms of TGF- $\beta$  are involved in fracture repair. They are initially produced by degranulated platelets after the injury, which suggests their involvement in the initiation of callus formation.

At later stages, they are also produced by osteoblasts and chondrocytes, enhancing the proliferation of these cells (autocrine stimulation), as well as MSCs and pre-osteoblasts multiplication (Lieberman, Daluiski, & Einhorn, 2002). In a mouse model, the expression of TGF- $\beta$ 1 remains constant throughout the fracture healing process, whereas the expression of TGF- $\beta$ 2 and TGF- $\beta$ 3 peaks on day 7 post-fracture. This suggests that TGF- $\beta$ 2 and TGF- $\beta$ 3 may play a more important role during fracture healing, since their expression peaks during the critical phase of cartilage formation (Cho *et al.*, 2002).

### **2.2.1.3. The role of angiogenic factors in fracture repair**

Optimal bone healing is highly dependent on adequate vascularization and therefore, the development of new blood vessels is an imperative in fracture repair. In endochondral healing, the transition from a cartilaginous tissue to new bone formation is tightly linked to angiogenesis. This transition includes chondrocyte apoptosis, cartilaginous matrix degradation, vascularization of the repair site, osteogenic cell recruitment and differentiation, and bone matrix production. Disruption of any one of these steps leads to delayed or impaired healing. For example, disruption of cartilaginous matrix degradation through the loss of MMP-9 expression leads to a massive expansion of the hypertrophic zone (Vu *et al.*, 1998). Moreover, in *mmp-9*<sup>-/-</sup> mice, the delay observed in fracture repair can be rescued by the addition of exogenous VEGF, suggesting that angiogenesis is linked to the apoptosis of chondrocytes (Colnot *et al.*, 2003).

Embryonic angiogenesis is regulated by two pathways, a vascular endothelial growth factor-dependent pathway and an angiopoietin-dependent pathway (Suri *et al.*, 1996). Both pathways are believed to be activated during fracture repair.

It has been demonstrated that VEGFs signalling plays a central role in neo-angiogenesis and in endochondral bone formation (Street *et al.*, 2002). After an injury, existent vessels are first dissociated into a pool of endothelial cells that are kept in growth arrest by the action of angiopoietin-2 and vascular endothelial growth inhibitor. In the stage of cartilage resorption and primary bone remodelling, VEGFs levels rise. This increase stimulates the proliferation of endothelial cells progenitor pool and promotes the participation of these cells in neo-angiogenesis (Ai-Aql, 2008).

Osteoblasts are known to express considerable amounts of VEGFs and therefore, they have been implicated as primary regulators of angiogenesis in fracture healing. Moreover, several studies have shown that BMPs stimulate the expression of VEGFs and their receptors in different cell types, suggesting a BMPs/VEGF interplay that promotes the formation of new bone (Deckers et al., 2002). The VEGF gene family members detectable during fracture healing are VEGF-D, VEGF-A, and VEGF-C. The VEGF gene family members are expressed throughout the chondrogenic phase of healing, reaching maximal levels of expression during the late phases of calcification of the cartilage tissues, at the time when resorption is initiated (Ai-Aql, 2008).

A second pathway involves vascular morphogenetic proteins angiopoietin-1 and 2 that are associated with the formation of larger vessels and the development of collateral branches from existing vessels. The expression of angiopoietin-1 is induced during the initial stages of fracture repair suggesting that, in the periosteum, initial vascular in-growth from vessels plays an important role in the repair process. Nevertheless, the role of angiopoietins in fracture repair is not yet understood as well as for the VEGF family members (Ai-Aql, 2008).

Platelet-derived growth factor (PDGF) is a third, and more distantly related member of the angiogenic signalling molecules family. PDGFs are secreted from the alpha granules of platelets, endothelial cells, vascular smooth-muscle cells, and macrophages. Because PDGFs enhance osteoblast migration and proliferation, and are secreted from osteoclasts, they have been proposed as key factors in bone remodelling ((Kubota, Sakikawa, Katsumata, Nakamura, & Wakabayashi, 2002) Accordingly, *in vitro*, PDGF enhances the formation of a mineralizing matrix. *In vivo*, it enhances bone formation in periodontal regeneration. Systemic administration of PDGF in ovariectomized rats results in increased bone density and strength. PDGF can be detected in the callus tissue obtained from healing fractures during bone formation, and exogenous PDGF enhances callus density and bone formation associated with healing osteotomies. In contrast, along with TGF- $\beta$ , fibroblast growth factor- $\beta$ , BMP-2, and BMP-14, PDGF is lacking in fractures that do not heal properly, suggesting that PDGF is an essential component of normal fracture healing (Ai-Aql, 2008).

The relationship between the expression of some angiogenic factors and pro-inflammatory cytokines has also been studied. In mice lacking TNF receptors, the expression of angiopoietins, metalloproteinases, and vascular endothelial growth

inhibitor is diminished during fracture healing. However, the expression of VEGF family members that directly promote new vessel formation is not inhibited (Ai-Aql, 2008).

### **2.2.2. Distraction osteogenesis**

Distraction osteogenesis (DO) is a surgical model used: (i) to bridge gap defects such as non-healing fractures; (ii) to treat disease conditions where there has been a net destruction of the bone tissues (osteomyelitis); (iii) to augment the alveolar bone around lost teeth; (iv) to correct congenital skeletal deformities where there is a deficiency in the original structure of the skeleton (Ai-Aql, 2008). With a view to creating new bone, it is a controlled procedure that initiates a regenerative process and uses mechanical strain to enhance biological responses of the injured tissue. Compared to fracture healing, the main differences are three modes of ossification that take place during DO. Although endochondral ossification occurs during early stages of DO, intramembranous bone formation is the predominant mechanism of ossification, particularly in later stages. A third form of ossification is called 'transchondroid bone formation', where chondroid bone is formed directly by chondrocyte-like cells, with a gradual transition from fibrous tissue to bone (Yasui *et al.*, 1997; Choi, Chung, Cho, & Yoo, 2002). Cartilage that forms during DO is usually observed at the level of the periosteum, but not between the ends of the cortices within the distraction gap.

As in the case of fracture healing, biological mechanisms involved in distraction osteogenesis are governed by complex molecular interactions that involve local and systemic factors (pro-inflammatory cytokines, TGF- $\beta$  superfamily, angiogenic factors), and cells that are recruited to the surgical site from the surrounding tissues and circulation. Angiogenesis was shown to contribute significantly to new bone formation in DO. Indeed, results from a study conducted on a rat model revealed at the surgical site an increased expression of numerous angiogenic factors including angiopoietin (*Ang*) 1 and 2, *Tie* 1 and 2 receptors, VEGF-A and D, VEGF receptor 2 and neuropilin 1 (Ai-Aql, 2008).

## 2.3. Osteoporosis

### 2.3.1. Medical and economical toll

Osteoporosis is the most common bone disease in humans, representing a major public health problem as outlined in “A Report of the Surgeon General (2004)” by Bone Health and Osteoporosis (Cosman, Lindsay, LeBoff, Jan de Beur, & Tanner, 2014). It is a silent disease until it is complicated by fractures that occur following minimal trauma or, in some cases, with no trauma. For example, osteoporosis is responsible for weakening of the structural strength of bone to the extent that normal daily activities can create stress that exceeds the vertebra's strength, resulting in osteoporosis-induced vertebral compression fractures. The incidence and associated socio-economic costs of these fractures are very high, with about 440,000 new cases per year in the European Union countries and 700,000 in the United State (EPOS Group, 2002). Annually in the U.S., two million fractures are attributed to osteoporosis, causing more than 432,000 hospital admissions, almost 2.5 million medical office visits, and about 180,000 nursing home admissions (Cosman *et al.*, 2014). Estimated direct annual costs reach US\$440 million and US\$750 million in the EU countries and in the United States, respectively (Johnell, 2003; Melton, 1997) Moreover, increasing life expectancy will probably lead to a severe rise in the human and financial costs associated with osteoporosis. Osteoporotic fractures often lead to high degree of morbidity, a decrease in both general and health-related quality of life, an increase in various psychological disorders, and an increase in mortality rate as well (Lewis, 2006). Osteoporosis can be prevented, diagnosed and treated before fractures occur. Importantly, even after the first fracture has occurred, there are treatments to decrease the risk of further fractures. Usual recommendations for the management of osteoporosis apply to postmenopausal women and men age 50 and older (Cosman *et al.*, 2014). After 50 years old, 40% of women are osteoporotic and one in three women older than 50 years will develop at least one osteoporotic fracture. Therefore, it is rightly regarded as a major health problem.

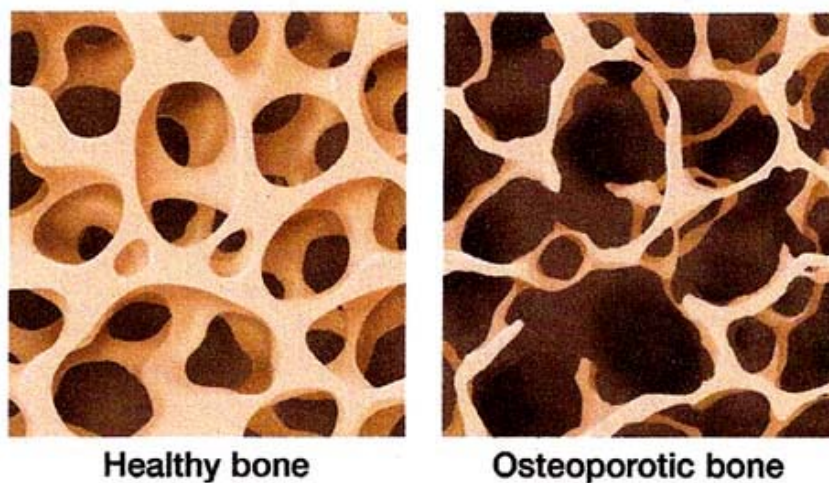
### 2.3.2. Pathogenesis of osteoporosis

Osteoporosis is characterized by low bone mass, disruption of bone microarchitecture, and compromised bone strength, these factors increasing the risk of fractures. According to the World Health Organisation (WHO) diagnostic classification, and measured at the hip or lumbar spine, osteoporosis is defined by a bone mineral density (BMD) that is less than or equal to 2.5 standard deviations below the mean BMD of a young-adult reference population.

The most common fractures are those of the vertebrae (spine), proximal femur (hip) and distal forearm (wrist). Some notable exceptions are those of the fingers, toes, face and skull, which are primarily related to trauma rather than underlying weakened bone tissue. Fractures may be followed by full recovery or by chronic pain, disability and death. Hip fractures are associated with an 8.4 to 36% excess mortality within one year, with a higher mortality in men than in women. Additionally, hip fractures are accompanied with a 2.5-fold increased risk of future fractures. Approximately 20% of hip fracture patients require long-term nursing home care, and only 40% fully regain their pre-fracture level of independence (Cosman *et al.*, 2014). Although the majority of vertebral fractures are initially clinically silent, these fractures are often associated with symptoms like pain, disability, deformity and mortality. Postural changes associated with kyphosis (height-reduction of vertebrae) may limit simple physical activities like bending and reaching. Wrist fractures are less disabling but can interfere with some activities of daily living as much as hip or vertebral fractures. Pelvic fractures and humerus fractures are also common and contribute to increased morbidity and mortality. Fractures can also cause psychosocial symptoms, most notably depression and loss of self-esteem, as patients grapple with pain, physical limitations and lifestyle changes.

As mentioned before, the process of bone remodelling that maintains a healthy skeleton depends on a delicate regulation of osteoblast-osteoclast activity and crosstalk. The well-accepted pathophysiological mechanisms for low bone mass include prolongation of the life span of osteoclasts and early apoptosis of osteoblasts and osteocytes. With aging and menopause, osteoblastic activity decreases. The impairment in osteoblast function can be the consequence of cellular senescence, but also may be the result of a decreased synthesis or activity of systemic and local growth factors. On the other hand, the lack of sex hormones leads to enhanced

osteoclastic resorptive activity. In addition, the amount and variety of noncollagenous proteins in bone is altered. Taken together, these events result in bone loss and disordered skeletal microarchitecture that increase the appearance of bone fractures with a risk of death outcome. With the onset of menopause, the rate of bone remodelling increases, magnifying the impact of the remodelling imbalance. Figure 6 shows the changes within cancellous bone as a consequence of osteoporosis; individual trabecular plates are lost, leaving an architecturally weakened structure with a significantly reduced mass (Cosman *et al.*, 2014).



**Figure 6. Illustration of healthy bone vs. osteoporotic weakened bone structure.**

In vertebrae, the thinning of trabecular bone structure comprises bone strength, with increased risk of fracture (taken from <http://www.drwolgin.com/Pages/osteoporosis.aspx>).

Other aging-associated factors contribute to an increased risk of osteoporosis-related fractures. This includes general factors like sex steroid deficiency, as well as specific risk factors such as the use of glucocorticoid treatment that causes decreased bone formation and bone loss. Since the majority of osteoporotic fractures result from falls, it is also important to evaluate risk factors for falling, such as personal history of falling, muscle weakness and gait, selected medications, balance and visual deficits, and dehydration (American Geriatrics Society, British Geriatrics Society and American Academy of Orthopaedic Surgeons Panel on Falls Prevention, 2001).

Depending on the cause, two types of osteoporosis are distinguished: primary (postmenopausal/senile) and secondary osteoporosis (nutrition, endocrine, drug,

malignancy, chronic disease, idiopathic) (Cosman *et al.*, 2014). The loss of bone mass and strength can be attributed to: (i) the failure to reach an optimal peak bone mass as a young adult; (ii) an excessive resorption of bone after peak mass has been attained; (iii) an impaired bone formation during remodelling process.

Several studies have assessed the mechanisms that regulate osteoporosis onset from a genomic point of view. A study by Klein *et al.* identified the lipoxygenase gene *Alox15* as a major negative regulator of peak bone mineral density in mice. In a more recent report, Liu *et al.* used the approach of expression profiling of circulating monocytes (progenitors of osteoclasts) isolated from patients with low BMD. They observed a strong correlation between low BMD scores and the upregulation of chemokine receptor 3 (CCR3), histidine decarboxylase and glucocorticoid receptor expression. Furthermore, Orlic *et al.* identified significantly enriched pathways in bones of OVX rats. Among bone-specific genes, they observed the upregulation of interleukin 7 (IL-7), IL-7 receptor and MMP9 expression, while genes such as TGF- $\beta$ 3, procollagen type I and procollagen type VI exhibited a significant decrease in their expression. The control animals exhibited marked enrichment of IGF-1-related pathways, including IGF-1 receptor pathway, insulin signalling pathway, glycogen metabolism, as well as glycolysis and gluconeogenesis. Indicating and increased cytokine production in OVX animals, this group showed an enrichment of fatty acid metabolism, IL-12 and TNFR2 pathways. In addition, OVX animal displayed an enrichment of the caspase pathway that might indicate an increased rate of apoptosis. Lastly, pointing to an enhanced protein degradation, the proteasome pathway was also significantly enriched following ovariectomy (Borovecki *et al.*, 2007; Cosman *et al.*, 2014).

## **2.3.2. Osteoporosis management**

### **2.3.2.1. Recommendations for the prevention of osteoporosis**

With the objective of strengthening bone, health professionals point out several considerations. These include an adequate intake of dietary calcium and vitamin D, lifelong participation in regular weight-bearing and muscle-strengthening exercise, avoidance of tobacco and excessive alcohol use, and treatment of risk factors for falling. National Osteoporosis foundation (NOF) supports



recommendations that men age 50-70 consume 1,000 mg per day of calcium and that women age 51 and older and men age 71 and older consume 1,200 mg per day of calcium (Cosman *et al.*, 2014). Clinical trials have demonstrated that the combination of supplemental calcium and vitamin D is a safe and inexpensive way to reduce the risk of fracture. There is no evidence that excessive calcium intake confers additional bone strength (Larsen, Mosekilde, & Foldspang, 2004).

**2.3.2.2. Diagnostic tools**

BMD is an excellent predictor of future fracture risk since it correlates with bone strength. Dual-energy x-ray absorptiometry (DXA) measurement of BMD of the hip and spine is the method used to diagnose osteoporosis, to predict future fracture risk, and to monitor patients. The BMD diagnosis of low bone mass (osteopenia), osteoporosis, and severe/established osteoporosis is based on the WHO diagnostic classification (Table 3.). Instead of a specific threshold, fracture risk increases exponentially as BMD decreases (Cosman *et al.*, 2014). BMD is expressed in terms of grams of mineral per square centimetre scanned (g/cm<sup>2</sup>) and as a relationship to two norms: (i) compared to the BMD of an age-, sex- and ethnicity-matched reference population (Z-score) (ii) compared to a young-adult reference population of the same sex (T-score). An individual's BMD is presented as the standard deviation above or below the mean BMD of the reference population.

**Table 3. WHO diagnostic classification of decreased bone mineral density.**

| <b>WHO Definition of Osteoporosis Based on BMD</b> |  |   |
|--|--|---|
| <b>Classification</b>                              | <b>BMD</b>   | <b>T-score</b>                                      |
| Normal   | Within 1 SD of the mean level for a young-adult reference population                       | T-score at -1.0 and above                           |
| Low Bone Mass (Osteopenia)                         | Between 1.0 and 2.5 SD below that of the mean level for a young-adult reference population | T-score between -1.0 and -2.5                       |
| Osteoporosis                                       | 2.5 SD or more below that of the mean level for a young-adult reference population         | T-score at or below -2.5                            |
| Severe or Established Osteoporosis                 | 2.5 SD or more below that of the mean level for a young-adult reference population         | T-score at or below -2.5 with one or more fractures |

Independently of BMD measurements, the age, and other clinical risk factors, radiographically confirmed vertebral fractures (even asymptomatic), are a strong

sign of impaired bone quality and strength, and a good predictor of new vertebral and other fractures. The presence of a single vertebral fracture increases the risk of subsequent vertebral fractures 5-fold, and the risk of hip and other fractures 2- to 3-fold (Cosman *et al.*, 2014).

Beside the parameters listed above, different biochemical assays have been extensively used as predictive tools in relation to rates of bone loss (total ALP and OCN in plasma, hydroxyproline and calcium in fasting urine). They are also useful in monitoring the effects of specific anti-osteoporotic treatments (e.g., oestrogens, calcitonin and bisphosphonates, or bone-forming agents) in individual patients (Kini & Nandeesh, 2012).

All postmenopausal women and men age 50 and older should be evaluated for osteoporosis risk in order to determine the need for BMD testing and/or vertebral imaging. Osteoporosis is preventable and treatable during the early phase of the disease, but since there are no warning signs prior to a fracture, many patients are not being diagnosed in time to receive an effective therapy (Cosman *et al.*, 2014).

Even though osteoporosis affects a significant number of men, it often remains undetected and untreated (Cosman *et al.*, 2014).

### **2.3.2.3. Current treatments and perspectives**

Current FDA-approved pharmacological options for the prevention and/or treatment of postmenopausal osteoporosis include: bisphosphonates (alendronate, alendronate plus vitamin D, ibandronate, risedronate, zoledronic acid), calcitonin, oestrogen, oestrogen agonist/antagonist (raloxifene), tissue-selective estrogen complex (conjugated estrogens/bazedoxifene), parathyroid hormone (PTH[1-34], teriparatide), and the RANKL inhibitor denosumab (Cosman *et al.*, 2014). None of the pharmacological options is considered indefinite in duration, and a comprehensive risk assessment should be performed in different stages of disease.

Due to their pronounced anti-osteoclastic effect, bisphosphonates (BPs) are the preferred anti-resorptive agents largely prescribed for osteoporosis prevention and treatment. Despite their clinical efficiency, treatment with BPs may be associated with complications that are partially due to their mode of administration. The major drawbacks resulting from oral administration are: (i) a poor bioavailability; (ii) the development of gastrointestinal disorders; (iii) the considerable constraints

related to administration modalities that can lead to poor patient compliance. However, in patients receiving high doses of BPs by intravenous administration, complications such as osteonecrosis of the jaw and low-energy fractures have been recently reported (E. Verron & Bouler, 2014). Taking into account the clinical benefit of BPs for patients, it is not conceivable to remove them from the anti-osteoporosis medication list. Thus, a possible strategy would be to optimize their use or to implement alternative modes of administration that are more specific, such as bone drug delivery systems. Releasing a controlled dose of BPs directly at the bone site that is weakened by osteoporosis, such as the femoral neck or vertebrae, could help to circumvent forenamed limitations related to systemic administration. BPs would most probably remain localized at the delivery bone site due to their strong affinity for hydroxyapatite (E. Verron & Bouler, 2014). Indeed, measurements of released BPs locally or at bone sites distant from implants support this hypothesis (Stadelmann *et al.*, 2008).

Although primarily known as a major bone resorption hormone, PTH has been approved as a first bone anabolic therapy for treatment of osteoporosis. Despite the fact that sustained PTH stimulation results in increased bone resorption, an intermittent administration of PTH leads to overall anabolic effect and large increases in BMD (Borovecki *et al.*, 2007). In a study using RNA microarrays, Reppe *et al.* analysed frozen bone biopsies of patients with primary hyperparathyroidism. Most of the differentially expressed genes represented structural and adhesion proteins, as well as proteases and protease regulators indicating an increased bone resorption. Remarkably, collagen type I and OCN were significantly increased, pointing to possible PTH anabolic action (Reppe *et al.*, 2006).

Beside pharmacological options, vertebroplasty (VP) and kyphoplasty (KP) are two procedures that have been introduced to surgically manage vertebral compression fractures. They aim to augment the weakened vertebral body, to stabilize it, and to restore as much as possible its normal height while relieving the pain. To achieve these goals, and using minimally invasive procedures, a paste of adaptable bone cement is carefully injected either directly into the fractured vertebral body (VP) or into a cavity created in the fractured vertebral body by the inflation of a balloon tamp (KP) (Lewis, 2006).

## 2.4. Other disorders of bone remodelling process

In syndromes like **osteopetrosis** or **osteosclerosis**, bone resorption is defective because of osteoclast impaired formation and/or function. In these disorders, bone remodelling is modified and the architecture of the skeleton can be quite abnormal, increasing the risk of fractures (Cosman *et al.*, 2014).

Another well-known disorder of bone remodelling is **Paget's disease**. In this disorder, osteoclasts are excessively resorbing and osteoblasts in turn respond by an intense irregular new bone formation, often in the form of woven bone. This leads to increased bone density, but because of the irregular architecture, bone strength is compromised and pathologic fractures are commonly occurring in patients with Paget's disease. It is reported that IL-6, which is implicated in the early phases of osteoclastogenesis, is linked to Paget's disease development (Kini & Nandeesh, 2012).

**Osteomalacia** is a disorder characterized by impaired mineralization of the osteoid, resulting in soft or fragile bones. It typically occurs as a consequence of vitamin D diet deficiency, or more commonly, when the body is unable to properly absorb and metabolize vitamin D, this resulting in an impaired absorption of calcium. In children, it is called rickets and is characterized by obvious skeletal deformities, whereas in adults the disease has more subtle manifestations, hence being difficult to be diagnosed and distinguished from osteoporosis (Kini & Nandeesh, 2012).

**Osteogenesis imperfecta** (OI), also known as Lobstein syndrome, is a congenital bone disorder characterized by brittle bones that are prone to fracture. Patients affected by OI are born with defective connective tissue, or without the ability to make it, usually due to the deficiency of type I collagen. Indeed, most types of OI are caused by mutations in the *COL1A1* and *COL1A2* genes (Rauch & Glorieux, 2004).

Both parathyroid hormones and thyroid hormones can stimulate bone formation as well as resorption. Although not a direct orthopaedic disorders, **hyperparathyroidism** and **hyperthyroidism** result in increased bone turnover that is sometimes accompanied by a decrease in bone mass. Increased levels of IL-6, a stimulating interleukin in bone resorption, have been reported in hyperparathyroidism (Kini & Nandeesh, 2012).

## 2.5. Bone metastases

### 2.5.1. Generalities

Bone metastases are a common complication of different cancers and occur in 65–80% of patients with metastatic breast and prostate cancers (Coleman et al., 2008). The incidence of bone metastases is also increasing in other cancers, probably owing to improved tumour control at primary disease sites. Once they have metastasized to bone, tumours are generally considered incurable. After lung and liver, bone tissue is the third most common site for metastatic disease and it is more prevalent in adult patients (>40 years of age) (Davila, Antoniou, & Chaudhry, 2015). More than 600 000 cases of cancer bone metastases are diagnosed every year in the USA (Randall, 2014). Some of the notorious complications provoked by bone metastases are: fractures, hypercalcaemia, spinal cord compression, immobility, and ultimately mortality (Quinn, 2013).

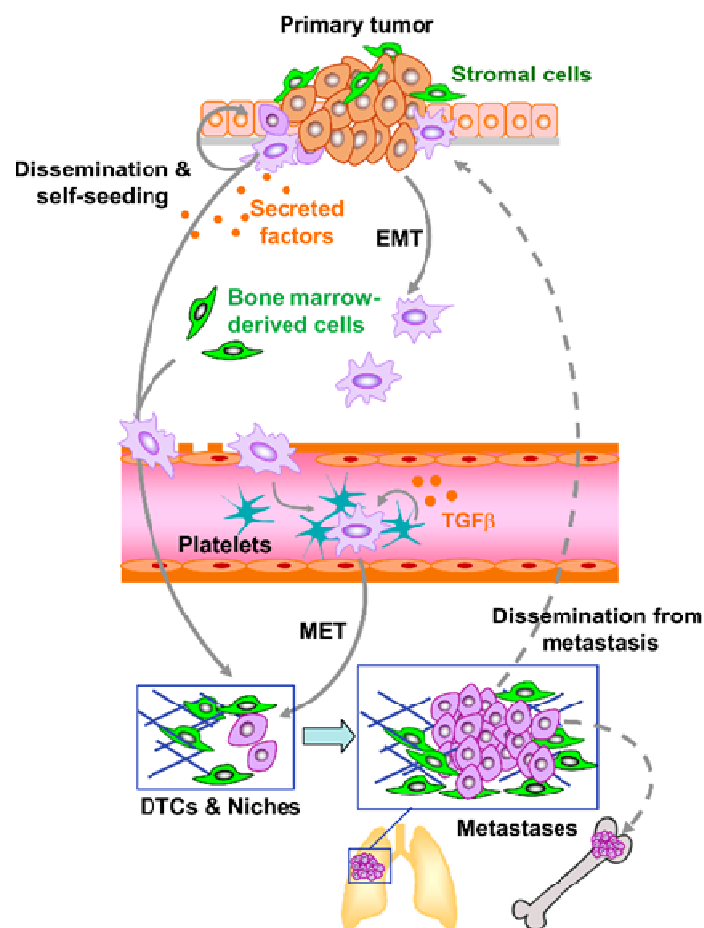
The special milieu of bone microenvironment provides a fertile soil for many cancer types. Most solid tumours metastasize to bone and induce both destructive osteolytic and/or bone-forming osteoblastic lesions. Although there is considerable bone deposition in osteoblastic lesions, these abnormal structures are weak and are subjected to fractures. The distinction between these two types of lesions is not absolute, and an individual metastatic lesion can contain both osteolytic and osteoblastic components (Krzyszinski & Wan, 2015).

Tumour invasion into the bone is tightly associated with osteoclast recruitment, resulting in the release of growth factors and calcium from mineralized bone matrix. This liberation further enhances tumour growth, forming the 'vicious cycle' of bone metastases (Weilbaecher *et al.*, 2011). Indeed, when anti-resorptive bisphosphonate therapy is applied in patients with bone osteolytic metastases, an improved survival rate is observed due to the suppression of bone resorption. In addition to the effects on osteoclasts, tumours in the bone microenvironment induce the formation of new blood vessels, while recruiting and modulating the function of osteoblasts, platelets, myeloid cells, immune and nerve cells. The bone marrow compartment also serves as a reservoir for dormant tumour cells that can resist chemotherapy, and later emerge again as metastases in bone or other organs (Weilbaecher *et al.*, 2011).

## 2.5.2. Bone metastatic cascade

In the complex process of bone metastatic cascade, several crucial steps are identified (Figure 7):

- (a) epithelial–mesenchymal transition;
- (b) degradation of the extracellular matrix;
- (c) escape from the primary tumour and invasion into lymphatic and/or blood vessels;
- (d) survival in the circulation;
- (e) homing to the bone niche;
- (f) adhesion to bone tissue and establishment of metastasis



**Figure 7. Bone metastatic cascade.**

Under the influence of stromal cells in the primary tumor microenvironment, tumor cells undergo EMT or use other means of invasion to escape from the primary tumor. Platelets protect tumour cells in circulation and further stimulate EMT by TGF- $\beta$  and NF $\kappa$ B signalling. Tumour-derived factors and exosomes mobilize bone marrow-derived cells to form pre-metastatic niche to promote the homing of tumour cells to bone (taken from *Kang & Pantel, 2013*).

### 2.5.2.1. Epithelial to mesenchymal transition (EMT)

Although still under debate, increasing evidence suggest that the EMT process is essential for carcinoma cells to undergo fundamental cytoskeleton reorganization, which results in the loss of cell cohesiveness and enables certain cancer cells to escape from primary disease site (Lamouille, Xu, & Derynck, 2014).

It is proposed that EMT activators drive tumour cells specifically to the bone. Thus, a pro-EMT factor Snail is believed to direct prostate cancer cells to bone and stimulate tumour-bone vicious cycle through calcium and RANKL signalling. By examining the immunohistochemical expression of EMT markers (i.e., E-cadherin, vimentin, NF- $\kappa$ B, Notch-1, ZEB1, and PDGF-D), Sethi *et al.* identified Notch-1 as a signature gene in the EMT involved in prostate cancer bone metastases (Sethi, Macoska, Chen, & Sarkar, 2010). In addition, showing that it up regulates the expression of the proinvasive factor miR-10b, Croset *et al.* proposed TWIST1, a well-known EMT-inducing transcription factor, as an important contributor to breast cancer bone metastases (Croset *et al.*, 2014).

### 2.5.2.2. Degradation of the extracellular matrix (ECM)

The invasion of carcinoma cells requires degradation of ECM, which forms the structural framework of most tissues. Several genes related to the ECM degradation have elevated expression in highly metastatic tumours (Eckhardt *et al.*, 2005). Various types of proteinases are implicated in ECM degradation, with the most prominent ones being the Zn<sup>2+</sup>-endopeptidases MMPs that cleave the constituents of the ECM. MMP-2 and 9 are the predominant MMPs responsible for ECM protein degradation, and thus play key roles in tumour development, growth, and metastasis (Coussens, Fingleton, & Matrisian, 2002). In addition, besides its strong implication in synthesis of ECM, TGF- $\beta$  participates also in ECM degradation (Hubmacher & Apte, 2013).

### 2.5.2.3. Angiogenesis

After ECM degradation, tumour cells produce angiogenic stimuli that endothelial cells into the perivascular space to form new blood vessels (Papetti &

Herman, 2002). As other steps in metastatic cascade, this is also a highly regulated process that involves essential signalling factors such as VEGF, VEGF receptors (VEGFRs), anti-angiogenic (e.g., thrombospondin-1) and pro-angiogenic factors (e.g., HIFs), Notch, and several ECM proteins (Dimova, Popivanov, & Djonov, 2014). Endothelial cells that constitute tumour blood vessels have generally weak cell-to-cell junctions. This renders them leaky and facilitates cancer cell entrance into new-formed vessels (intravasation). As an illustration of this, circulating factors found in patients with breast cancer bone metastases may promote angiogenesis, intravasation and survival of circulating tumour cells (Krzyszinski & Wan, 2015). On the other hand, the extravasation of tumour cells at a specific bone site is linked to *CXCR4*, *IL-11*, *OPN*, and *MMP1* expression. Indeed, in a bone-metastatic model of human breast cancer cell line MDA-MB-231, it has been demonstrated that combined over-expression of these markers in the parental cell line enhances the extravasation of circulating tumour cells into the bone niche (Y Kang et al., 2003). In the same line, it was shown that targeting stromal and endothelial cells present the bone marrow with the tyrosine kinase inhibitor sunitinib resulted in bone marrow vessel leakage, stromal cells apoptosis. By doing so, sunitinib disrupts bone colonization by lung cancer cells, as well as the subsequent osteolysis (Weilbaecher et al., 2011).

### **2.5.2.3. Survival in the circulation**

After intravasation, a small percentage of tumour cells is able to survive in the circulation, mostly through the expression of various genes, the cooperation with other cell types, and the escape from immune system recognition (Krzyszinski & Wan, 2015). Cooperation with platelets and surface shielding from natural killer (NK) cells are thought to be the major approaches that circulating tumour cells use to survive and successfully develop into metastases (Palumbo et al., 2007). It has also been shown that platelet-derived TGF $\beta$ , and direct platelet-tumour cell contacts, synergistically activate TGF $\beta$ /Smad and NF- $\kappa$ B pathways in cancer cells, this enhancing the metastatic potential of tumour cells (Labelle, Begum, & Hynes, 2011).



#### 2.5.2.4. Homing to the bone

Ample evidences strongly suggest that bone homing of tumour cells depends on both tumour-intrinsic traits and various factors supplied by the bone niche itself (Krzyszinski & Wan, 2015). Cells at the tumour primary site may produce circulating factors that modulate the microenvironment at distant organ sites, thus generating pre-metastatic niches conducive to secondary tumours localization and colonization. In preclinical melanoma and lung cancer models for example, **VEGFR1**-positive bone marrow-derived haematopoietic cells home to the sites of future metastasis and form cellular clusters that precede tumour cell arrival and increase fibronectin production in tumour target sites (Kaplan *et al.*, 2005). On the other hand, endocrine-like actions further support the development of a pre-metastatic niche in the bone microenvironment. Examples include: (i) **heparanase** produced by breast cancer cells, and which increases bone resorption (Kelly *et al.*, 2005); (ii) **OPN** secreted by tumour cells and/or senescent fibroblasts, and which promotes bone marrow cells recruitment or tumour formation (Anborgh *et al.*, 2010; McAllister *et al.*, 2008; Pazolli *et al.*, 2009); (iii) **MMPs** produced by osteoclasts, and which support prostate cancer skeletal metastasis (Lynch *et al.*, 2005).

Among factors implied in organ-specific cancer metastasis, chemokines and their receptors are believed to play a key role (Krzyszinski & Wan, 2015). The best-studied example is C-X-C chemokine receptor type 4 (**CXCR4**) and its ligand the stromal-derived factor-1 (**SDF-1**). Multiple bone-metastatic tumours including breast, prostate, and multiple myeloma express CXCR4. This chemokine receptor controls the metastatic destination of cancer cells by directing them to organs where SDF-1 is abundant, such as the bone marrow. Therefore, the disruption of the CXCR4/SDF-1 axis is a promising therapeutic strategy to prevent bone colonization by tumour cells (Hirbe *et al.* 2010). In mice, an intracardiac injection of CTCE-9908, a peptide analogue of SDF-1 and a competitive inhibitor of CXCR4, reduces the size of metastatic lesions derived from MDA-MB-231 cancer cell line (Richert *et al.*, 2009). Plerixafor/AMD3100, a small-molecule antagonist of CXCR4, also delays metastatic outgrowth of orthotopically transplanted 4T1 mouse breast cancer cells (Smith, 2004). However, the effects of the CXCR4/SDF-1 axis on tumour cell growth within the bone are not yet fully defined. Indeed, some studies point out that within the bone microenvironment, SDF-1 and CXCR4 could have conflicting roles. Genetic disruption

of *Cxcr4* in hematopoietic cells seems to enhance osteoclast activity and thereby stimulates tumour cell growth in bone (Hirbe *et al.*, 2007). Even more, there is a concern that the blockade of this chemokines/receptor axis could impair many important physiological processes that include stem cell homing, angiogenesis and immune cell trafficking (Lapidot & Kollet, 2002). Therefore, it is desirable to design therapeutics that would specifically target CXCR4/SDF in tumour cells (Krzyszinski & Wan, 2015).

Besides CXCR4, a second SDF-1 receptor, **CXCR7**, has been involved in tumour development (Burns *et al.*, 2006). Azab *et al.* found that the inhibition of CXCR7 delayed tumour progression in multiple myeloma, by specifically abrogating the trafficking of angiogenic mononuclear cells. These results highlight a novel role of CXCR7 in the recruitment of angiogenic mononuclear cells to areas of multiple myeloma in the bone marrow niche (Azab *et al.*, 2009).

Similarly, C-X-C motif chemokine 10 (**CXCL10**) has been shown to facilitate the trafficking of CXCR3-expressing cancer cells to bone, which augments its own production, enhances tumour growth, and promotes osteoclastic differentiation. Thus, CXCL10/CXCR3 axis may be another attractive therapeutic target for osteolytic metastases prevention (Lee *et al.*, 2012).

Several cytokine products of breast cancers, such as **PTH1H**, **IL-11**, and **IL-8**, have been shown to modify the bone microenvironment and promote bone resorption and colonization by tumour cells (Krzyszinski & Wan, 2015). More recently, an over-expression of **adrenomedullin**, a small peptide secreted by breast cancer cells and increased in hypoxia conditions, was shown to accelerate osteolytic bone metastasis development and to increase osteoclastic activity. On the contrary, a small-molecule antagonist of adrenomedullin dramatically suppressed both tumour burden in bone and osteoclastic activity (Siclari *et al.*, 2014).

**TGF- $\beta$**  is one of the most abundant cytokines released upon bone tissue resorption. It stimulates tumour cell expression of **PTH1H**, which activates osteoclasts (Johnson *et al.*, 2011). More bone resorption results in additional release of TGF- $\beta$  that again promotes tumour cell proliferation and survival. Consequently, the inhibition of TGF- $\beta$  with small molecules or neutralizing antibodies can reduce osteolytic lesion and tumour burden in mouse models for breast cancer and melanoma (Bandyopadhyay *et al.*, 2006; Mohammad *et al.*, 2011).

High serum levels of **IGFs** are linked to high risk for breast, prostate, and colorectal cancer. As for bone metastases, neutralizing antibody against IGF-1 receptor (IGF-IR) blocks breast cancer bone anchorage. This supports an important role of bone-derived IGF-1 in the crosstalk between the bone microenvironment and the cancer cells (Hiraga *et al.*, 2012).

The bone marrow microenvironment where HSCs are thought to reside comprises of several cell types such as vascular endothelium and endosteal osteoblasts (Kiel *et al.*, 2005; Xie *et al.*, 2009). **Components of the HSC niche** constitute fertile ground and it has been speculated that bone metastatic cells disseminate in the same manner as HSCs home to bone marrow, that is to say, via the CXCR4/SDF1 axis (Müller *et al.*, 2001; Taichman *et al.*, 2002). In addition, HSC-derived BMPs, such as BMP-2 and 6, can promote osteoblast differentiation (Jung *et al.*, 2008). Osteoblasts in turn maintain the HSC niche, creating a positive feedback loop with HSCs. In line with this, it has been shown that prostate cancer cells could interfere with BMP signalling pathway and alter the regulation of normal bone formation. Lastly, BMPs are involved in HSC quiescence and tumour cell dormancy, therefore representing promising candidates for therapeutic targeting of bone metastases (Grassinger *et al.*, 2007; Kobayashi *et al.*, 2011).

#### 2.5.2.6. Adhesion of tumour cells to bone

Adhesion between tumour cells and bone marrow stromal cells is crucial for cancer colonization of bone tissue.

Integrins are a family of transmembrane receptors that function as the bridges for cell-to-cell and cell-to-ECM interactions. Various *in vivo* and *in vitro* experiments suggest that  $\alpha v\beta 3$  integrin increases the potential of human and murine breast cancer cells to form bone metastases (Felding-Habermann *et al.*, 2001; Sloan *et al.*, 2006; Zhao *et al.*, 2007). ATN-161, a peptide that interferes with  $\alpha v\beta 1$  and  $\alpha v\beta 3$  binding, has successfully passed Phase I and II clinical trials (Cianfrocca *et al.*, 2006). And in a preclinical animal model using intracardiac injection of MDA-MB- 231 human breast cancer cells into nude mice, ATN-161 treatment results in a significant reduction in the number of bone metastases (Khalili *et al.*, 2006). This further supports a crucial role of integrins in cancer metastasis development.

### 2.5.3. Osteoclasts and the vicious cycle

In osteolytic bone metastases, tumour cells enhance osteoclast formation by continually secreting pro-osteoclastogenic factors including PTHLH, RANKL, interleukins, prostaglandin E, TNF, and MCSF. On the other hand, osteoclastic bone resorption releases growth factors, such as TGF- $\beta$ , IGFs, PDGFs, and BMPs that further enhance tumour growth. This tumour-osteoclast cooperation is the basic foundation of the 'vicious cycle' hypothesis (Guise, 2002).

TGF- $\beta$  is undoubtedly a major cytokine of bone metastases vicious cycle. Over-production of active TGF- $\beta$  by osteoclastic bone resorption in the skeletal metastases, not only increases tumour cell invasiveness and angiogenesis but also induces immunosuppression. Thus, blocking the TGF- $\beta$  signalling pathway to interrupt this vicious cycle offers a promising therapeutic approach (Massagué, 2008). Thus, in preclinical studies as well as in clinical trials, TGF- $\beta$  neutralizing antibodies (1D11, Fresolimumab), tyrosine kinase inhibitors (LY2109761, LY2157299), and soluble decoy receptor proteins have been developed and tested for their efficacy in inhibiting bone metastasis (Buijs, Stayrook, & Guise, 2011; Connolly, Freimuth, & Akhurst, 2012).

### 2.5.4. Osteoblast role in bone metastasis

The role of osteoblasts in skeletal metastasis is less well defined. Whether they exert pro-metastatic or anti-metastatic properties may depend on multiple factors including, the type of primary cancer, and whether the metastatic lesion is more osteolytic or osteoblastic (Krzyszinski & Wan, 2015).

Activated osteoblasts produce factors including TGF- $\beta$ , IGFs, IL-6, monocyte chemotactic protein-1 (MCP-1), VEGF, and macrophage inflammatory protein-2 (MIP-2), which in turn favour cancer cell colonization and amplification upon arrival in the bone niche (Krzyszinski & Wan, 2015).

On the other hand, tumour cells are able to produce osteoblast-stimulating factors such as BMPs, FGFs and PDGF (Weilbaeher *et al.*, 2011). It is likely that canonical WNT signalling is the central point of osteoblast-cancer cell crosstalk. WNT signalling results in high levels of RUNX2, a major transcription factor in osteoblastogenesis. The WNT signalling inhibitor, dickkopf homologue 1 (DKK1) has a crucial role in osteolytic skeletal metastasis in which osteoblast formation is

suppressed and osteoclast activity is enhanced. Serum DKK1 levels are increased in patients with multiple myeloma and in women with breast cancer skeletal metastasis. And in experimental models of prostate cancer, *Dkk1* expression is high during early carcinogenesis and decreases with skeletal progression, suggesting a molecular switch between osteolytic and osteoblastic disease (Weilbaecher *et al.*, 2011).

In osteoblastic bone lesions, there is also a vicious cycle between tumour cells and osteoblasts. It has been shown that tumour cells can secrete endothelin-1 (ET-1), which downregulates *Dkk1* expression (Clines *et al.*, 2007) and therefore stimulates excessive osteoblast formation responsible for the deposition of poor quality and immature bone.

## **2.5.5. Other cell types involved in bone metastasis**

### **2.5.5.1. Bone marrow MSCs**

It has been argued that bone marrow MSCs are also implicated in tumour growth and metastasis through their ability to promote and protect cancer stem cells. Interestingly, the potential of MSCs to interact with tumour cells has prompted investigations into whether MSCs can be used for the targeted delivery of antitumor agents to cancer cells (Weilbaecher *et al.*, 2011).

### **2.5.5.2. Adipocytes**

The number of bone marrow adipocytes increases greatly with aging and obesity. Marrow adipocytes secrete adipokines including hormones, cytokines, and fatty acids that may have profound effects on hematopoietic niche, neighbouring bone cells, and inflammation, hence modifying the local milieu (Krzyszinski & Wan, 2015). In the same line, MSCs-derived adipocyte stem cells were shown to promote prostate tumour growth (Weilbaecher *et al.*, 2011).

It has been suggested that tumour cells can be attracted to marrow adipocytes and are affected by adipokines. Yet, little is known about how exactly they modulate tumour colonization of the skeleton. The interaction between these

two cell types may create a chronic pro-tumourigenic inflammatory environment that promotes the translocation of adipocyte-stored lipids to the metastatic tumour cells, increasing their motility. Marrow adipocytes may promote tumour growth in bone in a FABP4-dependent manner. FABP4 is a cytosolic lipid chaperone that is predominantly expressed in adipocytes, macrophages, and endothelial cells. Herroon *et al.* propose that FABP4 inhibition can block lipid trafficking between marrow adipocytes and cancer cells, thus reducing the formation of tumour metastases in bone (Krzyszinski & Wan, 2015).

### 2.5.5.3. Myeloid and immune cells

Bone marrow-derived myeloid cells include macrophages, monocytes, myeloid-derived suppressor cells (MDSCs), myeloid dendritic cells, and osteoclasts. In addition, lymphocytes are recruited to bone metastases sites. Both myeloid cells and lymphocytes have a dual role in the regulation of tumour growth. In a recent report, mice with osteoclast and immune cell defects were protected from tumour-associated bone loss but did not have decreased tumour burden in bone (Vaira *et al.*, 2008). Increasing evidence support myeloid cell participation in angiogenesis, but the effective measurement of the vascular supply to a tumour in bone is challenging (Stockmann *et al.*, 2008).

Since many animal models of bone metastasis are immune-compromised mice that lack T cells, their role in regulating tumour growth within the bone microenvironment remains largely unstudied. Notably, pro-inflammatory CD4<sup>+</sup>Th17 T cells secrete RANKL, TNF and TGF- $\beta$ . All of these factors activate osteoclasts and promote bone resorption (Sato *et al.*, 2006; Takayanagi, 2009), and thus may enhance tumour growth in bone by promoting the vicious cycle. It is anticipated that future studies will probably set an important role for T cell subpopulations in regulating bone metastasis (Weilbaecher *et al.*, 2011).

For what it concerns the macrophage contribution to tumour growth and metastasis, an intense investigation has been conducted in the past few years. However, no direct evidence that clearly demonstrates a suppressive effect of macrophages on bone metastasis was found. This is mainly due to the fact that most strategies for depletion and/or inhibition of macrophages, as well as for manipulating macrophage intrinsic factors, also target osteoclasts at the same time (Krzyszinski &

Wan, 2015). Regarding their regulation of inflammation, macrophages are classified into two main subsets: (i) M1 macrophages that generally promote inflammation; (ii) M2 macrophages that suppress inflammation and assist tissue repair (Lawrence & Natoli, 2011). M1 macrophages have been proposed to be anti-tumourigenic by releasing reactive oxygen species (ROS), nitrogen intermediates, and inflammatory cytokines that eliminate cancer cells. By contrast, M2 macrophages are generally regarded as pro-tumourigenic, promoting tumour growth and invasion by releasing a variety of growth factors such as FGFs and VEGF (Zhang *et al.*, 2014). Consistently with this, it was reported that the small GTPase Rac2, which facilitates macrophage M1 to M2 polarization, promoted tumour metastasis (Joshi *et al.*, 2014).

#### **2.5.5.4. Platelets and megakaryocytes**

Platelets and their megakaryocytic precursors interact with cancer cells before, during and after metastasis to bone (Weillbaecher *et al.*, 2011). Activated platelets are an important source of pro-angiogenic (VEGF) and anti-angiogenic (thrombospondin 1/TSP1) factors whose aggregation at tumour sites affects tumour growth (Gay & Felding-Habermann, 2011). Tumour cells that are able to respond to platelet-derived lysophosphatidic acid (LPA) have enhanced bone metastatic potential in mice, partly through LPA-mediated induction of osteolytic factors such as IL-6 and IL-8 (Boucharaba *et al.*, 2004). Likewise, it has been demonstrated that genetic and pharmacological inhibition of platelet activation or platelet-specific integrins ( $\alpha\text{IIb}\beta\text{3}$ ) decreases osteolytic bone metastases in mice (Bakewell *et al.*, 2003; Uluçkan *et al.*, 2008). Platelet progenitors (mature megakaryocytes) are located at vascular sinusoids, and are among the first cells to encounter cancer cells that enter the bone marrow environment. Conversely to the major platelet effect on bone metastasis, bone marrow megakaryocytes inhibit prostate cancer tumour growth in bone (Li *et al.*, 2011). Interestingly, bisphosphonates increase megakaryocyte proliferation and increase platelet concentration of the anti-angiogenic integrin ligand TSP1 (Bernasconi *et al.*, 1995; Zaslavsky *et al.*, 2010), which suggests that bisphosphonates have additional non-osteoclast mechanisms that decrease tumour growth in bone.

### **2.5.6. Tumour cell dormancy in bone**

Paradoxically, only 0.001–0.02% of cancer cells that are experimentally introduced into the circulation actually forms metastases (Fidler, 1970; Luzzi *et al.*, 1998; Schneider *et al.*, 2005). Preclinical evidence suggests that metastatic tumour cells can home to the HSC niche of the bone, and survive there in a dormant state for long periods being often resistant to cancer therapies (Weilbaecher *et al.*, 2011). For example, myeloma cells interact with bone marrow stroma, partly through integrin  $\alpha 4$  and vascular cell adhesion molecule 1 (VCAM1). This facilitates quiescence and protection from apoptosis, providing resistance to chemotherapy (Damiano *et al.* 1999).

It has been speculated that tumour cells have the ability to mimic HSC cells, and the research on factors that induce HSC dormancy could explain tumour cell dormancy in bone marrow niche. Prostate cancer cells bind to osteoblast annexin II, which in turn induces the expression of growth arrest-specific 6 (GAS6) receptors. GAS6 receptors are well-known inducers of dormancy in HSCs and were found to reduce cell cycle progression in prostate cancer cells as well (Shiozawa *et al.*, 2010). Due to a possibility of inducing long-term remission and overcoming resistance to anti-cancer treatments, understanding tumour cell dormancy mechanism within the bone marrow is one of the priorities in tumour investigation (Weilbaecher *et al.*, 2011).

### **2.5.7. Bone metastasis current prevention and treatment**

When cancer metastasizes to bone, it causes considerable pain and deregulation of bone remodelling, which greatly reduces the possibility of cure. Tumour cells are able to modify the bone microenvironment to be receptive to tumour cell attachment and tumour growth. Important advances in understanding the basic biology of tumour–bone interactions, bone remodelling, and haematopoiesis, have uncovered new therapeutic possibilities for the prevention and treatment of bone metastasis (Weilbaecher *et al.*, 2011). Improvements have been made in the medical management of bone metastases including surgery approaches and radiation therapy, as well as targeted pharmacological therapy. Nevertheless, to date, these strategies remain only palliative and do not significantly improve overall patient survival (Taubenberger, 2014). Identifying more effective and specific molecularly targeted therapy to prevent and cure bone metastases as well



as enhancing the quality of life of the patients remain one of the major challenges of future biomedical research.

### *Targeting osteoclast resorption*

Clinically, anti-resorptive drugs have been effective in slowing down the process of cancer bone metastasis (Krzyszinski & Wan, 2015).

Bisphosphonates are a group of pyrophosphate analogues that preferentially bind to calcium, which is abundant in bone tissue. Upon bone resorption, bound bisphosphonates are ingested by osteoclasts and induce their apoptosis (Li, Wong, & Pavlakis, 2014). Moreover, bisphosphonates can affect directly tumour cells, as well as other cell types that promote bone metastases, including  $\gamma\delta$ -T cells, monocytes, MDSCs and endothelial cells. Clinical data suggest that bisphosphonates can limit the progression of breast cancer metastasis in bone and in other tissues (Aft *et al.*, 2010; Gnant *et al.*, 2009); Misso *et al.*, 2012; Santini *et al.*, 2002, 2003, 2007; Vincenzi, 2003). Thus, bisphosphonate therapy is currently a gold standard in treatment of patients with established bone metastases of many tumour types (prostate, breast, lung, and multiple myeloma) (Wu, Dahut, & Gulley, 2015). Bone matrix-targeted bisphosphonates, such as pamidronate, zoledronic acid (zoledronate) and ibandronate, are amino-bisphosphonates that block farnesyl pyrophosphate synthase and that disrupt protein prenylation (Rogers, 2003). In one study, zoledronate treatment of women suffering from localized breast cancer modulated the presence of disseminated tumour cells in the bone marrow (Weilbaecher *et al.*, 2011).

Human monoclonal antibodies against RANKL, the main inducer of osteoclastogenesis, are also under extensive investigation as potential inhibitors of malignant bone resorption. One such antibody, Denosumab, has been approved for the treatment of skeletal complications in patients with bone metastases secondary to breast or prostate cancer, as well for the treatment of osteoporotic patients (Drooger, van der Padt, Sleijfer, & Jager, 2013). In addition, denosumab may be effective in directly targeting subtypes of breast and prostate cancers that express RANKL (Gonzalez-Suarez *et al.*, 2010).

Other osteoclast-targeted therapies under clinical evaluation include CTSK inhibitors, SRC inhibitors and  $\alpha\text{v}\beta\text{3}$  inhibitors (Desgrosellier & Cheresch, 2010; Jensen *et*

*al.*, 2010; Rucci, Susa, & Teti, 2008). These therapies can directly affect tumour cells and/or stromal components that participate in bone metastases (Weilbaecher *et al.*, 2011).

Despite the ability of these osteoclast inhibitor drugs to control and reduce bone metastases, significant challenges lie ahead. Firstly, ineligible side effects of currently approved bisphosphonates are yet to be tackled. This includes osteonecrosis of the jaw (ONJ), hypocalcaemia, and renal toxicity (Brown & Coleman, 2012; Coleman, 2012; Khosla *et al.*, 2007). Secondly, none of these drugs can completely eradicate bone metastases, nor increase patient long-term survival. Unfortunately, 30–50% of patients on this therapy still develops new bone metastases, deteriorating skeletal-related events and disease progression (Weilbaecher *et al.*, 2011). Hence, with a goal to provide new improved therapeutics, investigation of tumour influence on osteoclasts incessantly continues (Krzyszinski & Wan, 2015).

#### *Targeting osteoblasts*

An alternative therapeutic strategic to osteoclast inactivation, is to delay bone loss by stimulating osteoblast function. Proteasome inhibitors are reported to promote bone formation by stimulating MSCs proliferation and osteoblast differentiation. The first FDA-approved therapeutic proteasome inhibitor, bortezomib, has shown efficacy in the treatment of multiple myeloma and lymphomas in humans. Interestingly, in preclinical models bortezomib has also been shown to inhibit breast cancer osteolytic metastasis (Krzyszinski & Wan, 2015).

On the other hand, treatments for osteoblastic bone lesions are also being developed. Endothelin 1 (ET-1) is a vasoconstrictive agent that stimulates osteoblast proliferation and differentiation (Nelson *et al.*, 1995; Tang *et al.*, 2009). ET-1 promotes cancer cell proliferation and invasion via upregulating MMPs, inhibiting tumour cells apoptosis, and enhancing VEGF-induced angiogenesis (Carducci & Jimeno, 2006). Inhibition of ET-1 is reported to have dual suppressive effects on both tumour cells and osteoblasts. Both ET-1 neutralizing antibodies and ET-1 receptor antagonists are under clinical evaluation (Chiao *et al.*, 2000; Pinto *et al.*, 2012). Atrasentan was the first ET-1 receptor subtype A antagonist to be investigated in patients with prostate cancer, while zibotentan is a more selective and promising inhibitor that is currently in clinical trials (Shepard & Dreicer, 2015).

### *Targeting TGF- $\beta$ signalling pathway*

Several classes of TGF- $\beta$  inhibitors have been tested in preclinical models and clinical trials in the treatment of bone metastases: (i) neutralizing TGF- $\beta$  antibodies that prevent TGF- $\beta$  ligand-receptor interactions; (ii) small molecules that inhibit T-bRI (and T-bRI II) kinase activity; (iii) halofuginone (HFG) that inhibits TGF- $\beta$ , although the exact mechanism of action remains to be investigated (Weilbaecher *et al.*, 2011). In patients with advanced metastatic melanoma, pheochromocytoma, and colon, prostate and breast cancer, dose escalation studies demonstrated that the small molecule TGF- $\beta$  inhibitor LY2157299, was well tolerated with minimal toxicity.

### *Targeting bone metastases angiogenesis*

Due to its indisputable role in cancer metastasis, angiogenesis has been studied extensively as a potential target of anti-cancer therapy. FDA-approved bevacizumab, a monoclonal antibody against VEGF-A, was the first commercially available angiogenesis inhibitor that has been clinically used to treat metastatic cancers of colorectal, lung, breast, and renal origin. Cabozantinib, a dual inhibitor of VEGFR2 and receptor tyrosine kinase MET, has exhibited beneficial effects on radiographically evident bone metastases. Other inhibitors of VEGFRs include sunitinib, sorafenib, cediranib, and aflibercept, which is a small recombinant protein decoy receptor for VEGFs. However, none of these drugs has been proven to decrease the mortality rate of the treated patients. In order to achieve significant clinical benefit, it is proposed that angiogenesis inhibitors may require concomitant administration of cytotoxic chemotherapeutic agents (Krzyszinski & Wan, 2015).

### *Targeting bone metastases dormancy*

Due to their cell cycle arrest, cancer cells homed in the bone marrow are often resistant to chemotherapy. An exciting new approach takes advantage of the fact that cancer cells use CXCR4 and VLA4 to home to and engraft in the bone marrow. In mice, HSC mobilizing agents such as AMD3100 and anti-VLA4-targeted molecules were shown to mobilize leukaemia and myeloma cells into the blood, this release into bloodstream making cancer cells more susceptible to chemotherapy.

This approach is currently being tested in clinical trials in the treatment of acute myeloid leukaemia and multiple myeloma (Weilbaecher *et al.*, 2011).

### *Bone pain treatment*

Bone tissue is densely innervated by primary sensory afferent and sympathetic neurons located in the periosteum and the intramedullary bone (Goblirsch, Zwolak, & Clohisy, 2006). That is why bone metastases result in considerable pain, which is extremely difficult to treat and that is often rather resistant to opioids (Meuser *et al.*, 2001). A study by Honore *et al.* found that the acidic environment, which is produced by resorbing osteoclasts, activates the transient receptor potential vanilloid type 1 (TRPV1) expressed by pain neurons (Honore *et al.*, 2000). Consequently, bone resorption inhibitors substantially decrease the pain caused by the growth of bone metastases (Coleman *et al.*, 2008). Another pain-relieving tactic in patients with bone metastases has been the use of radiopharmaceuticals, such as strontium, samarium and radium that exhibit strong avidity towards bone calcified matrix. These agents can exert only a small and localized anti-tumour effect through radiation. However, due to their substantial effects on bone pain, they are primarily indicated to provide bone metastases-related pain relief (McEwan, 2000).

### **2.5.8. Future directions in bone metastasis research**

Most current therapeutic strategies only attenuate the progression of existing bone metastases, and do not significantly increase long-term survival. Thus, strategies interfering with early stages of metastatic cascade are highly desirable. In order to achieve this goal, the major aims for future bone metastasis investigation are the following: (i) identification of early bio-markers, ideally circulating factors, for the prediction and diagnosis of metastatic disease; (ii) cancer stem cells eradication; (iii) inhibition of epithelial-to-mesenchymal transition (EMT); (iv) halt of dissemination of circulating tumour cells; (v) better general characterization of the mechanisms by which cells of various cancers cooperate with bone cells; (vi) identification of genetic or humoral factors that allow cancer cell dormancy reversal; (vii) development of therapeutic approaches that can simultaneously target multiple steps of the bone metastatic cascade; (viii) the design of pharmacological agents

that specifically target tumour cell functions, without considerably interfering with normal physiological functions, thus resulting in less side-effects; (ix) improvements in relief of skeletal metastasis-related pain (Nakanishi, 2010); (x) development of powerful imaging technologies to visualize different stromal cell elements that are involved in bone metastasis in live animals; (xi) development new improved *in vitro* and animal models that are more representative of real physiological bone metastasis process such as patient-derived cancer cell xenograft models that can predict patient-specific predisposition for bone metastasis as well as patient-specific efficacy of envisioned treatments (personalized medicine) (Krzyszinski & Wan, 2015; Villasante, Marturano-Kruik, & Vunjak-Novakovic, 2014; Weilbaecher *et al.*, 2011).

Hopefully, the bone metastasis investigation will yield so called theranostic agents whose application is at the same time diagnostic and therapeutic. Eventually, by using safer agents for treatment and diagnostic, these innovations will allow for even more efficient attenuation of bone metastatic disease. Ultimately, improving the overall clinical outcome for cancer patients, they will result in effective prevention of cancer bone metastasis (Krzyszinski & Wan, 2015).

## 3. Biomaterials in bone tissue engineering

### 3.1. Applications and properties

Despite the tremendous potential of bone tissue for repair and regeneration in response to injury or surgical treatment, different osteoarticular pathologies can result in significant loss of bone tissue. Spontaneous bone repair is valid only for small-sized, 'non-critical' defects. In order to replace the missing or damaged bone tissue and to restore its mechanical function, a surgical autograft technique is employed as a gold standard (Verron *et al.*, 2010c). A secondary site is chosen, most often the patient's iliac crest, where certain amount of healthy bone tissue is harvested and implanted at the initial site where bone loss occurred. The main advantages of this technique are the absence of immunological reaction and adverse compatibility events, and its clinical efficiency due to the living cells and growth factors present in the harvested bone. Despite good clinical efficiency in replacing the damaged and/or missing bone tissue, this method presents notable limitations in terms of quantity of replacement bone tissue that can be harvested, but also in increased morbidity (pain, infections) at the secondary operation site.

Alternatively, allograft, in the form of donor bone from a regular bone bank, might partly overcome the drawbacks of bone supply. However, in this technique, the sterilization treatment results in degradation of biological factors and impaired bone strength. Furthermore, there are concerns regarding immunological reaction between the patient and the donor bone, as well as disease transmission (Zhang *et al.*, 2014).

In the past decades, autograft and allograft limitations have prompted the development of synthetic materials that serve as bone substitutes, which aim to imitate autograft properties. When implanted at the injury site, these materials provide mechanical stability of the damaged area and support bone reconstruction. Nevertheless, autograft method is more efficient and remains the reference treatment for bone loss indications (Mellier *et al.*, 2015) .

The development of bone substitutes and their clinical implementation are commonly named bone tissue engineering. An ideal bone substitute has at least two characteristics:

- (i) it is well-tolerated by surrounding host tissue = **biocompatibility**;
- (ii) it is capable to recruit bone cells to form new bone on its surface = **osteoconductivity**

The first generation of bone substitutes was comprised of a wide range of synthetic materials, such as metals, polymers and porous ceramics. The second generation introduced injectable calcium phosphate cements (CPCs) that are currently under intense investigation. Indeed, their primary mechanical properties are close to those of cancellous bone. Moreover, their injectability makes them suitable for implantation under minimally invasive surgery, while their adaptability allows them to fit with any shape of defect. Further developments of a third bone biomaterials generation aim at expanding potential benefits of injectable substitutes by combining them with patients own cells, osteogenic factors or active molecules to enhance bone regeneration in normal healing and in pathological situations. Thus, bone biomaterials would have another valuable role as drug delivery systems (DDS).

Since calcium phosphate is one of the major mineral components of the bone tissue, CaP synthetic substitutes are well tolerated by the surrounding host tissue. In this regard, calcium phosphate (CaP) biomaterials, mainly ceramics, cements, composites and thin coatings, are considered excellent materials for bone tissue engineering (Verron *et al.*, 2010c).

### 3.2. Calcium phosphate ceramics

CaP ceramics are characterized by three types of porosity: macro, meso and microporosity. **Microporosity** comprises of intercrystal pores with a diameter smaller than 10  $\mu\text{m}$ , and is formed by high sintering temperatures during biomaterial synthesis. This porosity grade enables ionic exchange in biomaterial contact with fluids. **Mesoporosity** refers to pores between 10-100  $\mu\text{m}$ . **Macroporosity** corresponds to pores with a diameter superior to 100  $\mu\text{m}$ . These pores are created by porogens, such as naphthalene or sugar, used in ceramic synthesis. High temperature during the sintering step results in complete porogen evaporation and pore formation. The ratio of these three types of porosity influences biological performance of CaP materials. On the other hand, atomic Ca/P ratio is generally inversely proportional to dissolution rate and therefore, to ceramics resorption behaviour *in vivo*.

Only two CaP species are resistant to thermal treatment  $\geq 1000^{\circ}\text{C}$  during ceramic synthesis, namely hydroxyapatite (HA) and  $\beta$ -tricalciumphosphate ( $\beta$ -TCP). HA and  $\beta$ -TCP possess properties required for bone substitute materials and are frequently used for implantation in orthopaedic and dental fields (Nakamura *et al.*, 2013).

Hydroxyapatite, with a chemical formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , is the naturally occurring mineral that crystallizes in hexagonal system and closely resembles in structure to human bone and tooth enamel. Stoichiometric HA has a Ca/P atomic ratio equal to 1.67, while the biological apatite present in bone has a Ca/P atomic ratio between 1.5-1.67. It has the same crystal structure as stoichiometric HA but their chemical composition differs. Calcium can be partly substituted with other ion species, forming so-called calcium deficient apatite (CDA), which is more soluble than stoichiometric one. Calcium deficiency is commonly attributed to the low driving force available for growth under biomineralization conditions. Since Calcium deficiency affects mechanical and biological properties of HA, more profound understanding of the defect chemistry is essential for the design of novel improved synthetic biomaterials based on CDA (Bhat, Waghmare, & Ramamurty, 2014).

In biological apatite, both phosphate and hydroxyl ions can be partially substituted with carbonate ions (2-8%) while calcium ions can be substituted by small amounts of sodium, magnesium and potassium (Bhat *et al.*, 2014). This interesting characteristic of biological apatite has incited numerous investigations of synthetic carbonated hydroxyapatite (CA) as an ideal clinical biomaterial. Indeed, it is empirically hypothesized that artificial implants should most closely resemble the natural mineral phase of the bone tissue (Nakamura *et al.*, 2013).

Biomaterials in which HA and  $\beta$ -TCP phases are combined are called biphasic calcium phosphates (BCPs).  $\beta$ -tricalciumphosphate ( $\beta$ -TCP), has a chemical formula  $\text{Ca}_3(\text{PO}_4)_2$  and crystallizes in rhomboedric system. With its atomic Ca/P ratio equal to 1.5, this ceramic is more soluble than HA, and thus, more readily biodegradable. Thus, the bioactivity and the resorbability of BCPs are proportional to  $\beta$ -TCP percentage.



### 3.3. Injectable bone cements

Bone cements are prepared by mixing a solid powder with a liquid phase, which results in the formation of an injectable paste. *In vitro* properties and *in vitro* behaviour of injectable cements depend highly on: additives to cement paste; powder particle size; powder-to-liquid ratio; the method used to mix the powder with the liquid phase (Lewis, 2006).

Regarding their chemical composition, injectable bone cements can be classified into three categories: (i) acrylic bone cements (e.g. PMMA); (ii) calcium sulphates, (iii) calcium phosphates (Lewis, 2006). In this chapter, main features of each group will be presented, and calcium phosphate cements will be discussed more in detail in chapter 3.4.1.

**Polymethylmethacrylate (PMMA)**, commonly described as orthopaedic “cement”, is an organic polymer resin with good mechanical properties, widely used in orthopaedic applications such as joint replacement, vertebroplasty, arthroplasty, and defect repair. The main drawback of PMMA cement is its biological inertness. Hence, no biological interaction occurs at the cement-bone interface and no material resorption takes place. For this reason, after implantation, aseptic loosening and shifting of the prosthesis are a common cause of surgical failure (52-55% of surgical revisions) (Li *et al.*, 2015). In total joint replacements, the second important inconvenience of PMMA is the thermal necrosis of the periprosthetic soft tissue during the cement exothermic setting reaction. However, this risk seems to be lower in the case of vertebroplasty and kyphoplasty procedures (Lewis, 2006). Conversely, due to the heat-induced necrosis of residual tumoral tissue, exothermic setting reaction of PMMA can be beneficial when filling bone cavities after tumour resection.

**Calcium sulphate cements (CSCs)**,  $\text{CaSO}_4$ , known as plaster of Paris or gypsum, have a long clinical history as bone graft substitutes in various skeletal sites. Upon mixing with a diluent, the cement powder is converted to calcium sulphate dihydrate, producing a paste or putty with a solid or partially solid structure. When injected to surgical site, CSCs inhibit fibrous tissue ingrowth and slightly acidify the environment, which favours osteogenesis and angiogenesis. CSCs are degradable cements and take about 2 months to be resorbed *in vivo*, depending on the implantation site. Although they are eventually replaced by new bone tissue, there is

a major concern about their rapid resorption rate that is usually not accompanied by immediate bone formation. Hence, the mechanical properties of the implantation site are decreased. Consequently, they are employed to a lesser extent in today's clinical practice.

### 3.3.1. Calcium phosphate cements (CPCs)

Since their initial formulation in the 1980s, injectable bone substitutes based on calcium phosphate cements (CPCs) have been extensively developed and numerous formulations are commercially available for various orthopaedic applications (Zhang *et al.*, 2014). The concept of CPCs was first introduced by LeGeros *et al.* in 1982, while Brown and Chow further broadened clinical applications of these biomaterials by adding a range of possible indications in orthopaedic surgery (i.e. kyphoplasty, vertebroplasty, prosthesis fixation, defect filling after tumoral resection), as well as in dental and maxillofacial surgery (i.e. alveolar ridge defect filling, sinus lift, implants fixation, bone regeneration after periodontitis, pulp covering and protection). *In vivo*, CPCs are resorbable and exhibit evidenced osteoconductive properties. Thus, they are efficient in most non-load bearing clinical situations in orthopaedics, dental, ear, nose and throat surgeries.

As bone void fillers, CPCs present many advantages compared to ceramics:

- (i) **injectability**, which allows for minimal invasive surgery approach, thus limiting surgical risks and post-operative complications, as well as reducing patient's hospitalization and recovery time;
- (ii) **adaptability** to complex irregular defects due to the moldability of the cement paste;
- (iii) generally, **greater mechanical support** compared to ceramic bone substitutes.

When compared to acrylic cement, important improvements due to CPCs are:

- (i) **negligible exothermic setting reaction**;
- (ii) **intrinsic microporosity** useful for biological fluids impregnation;
- (iii) **resorption rate** *in vivo* that is neither too fast nor too slow, and follows bone remodelling kinetics;

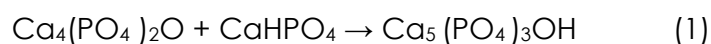
(iv) **osteoconductivity** that leads to new bone formation (this phenomenon is discussed in details in chapter 3.5.2.).

The synthesis of the cement solid phase (CaP powder) begins by sintering (typically, at 1350°C for 1 h), followed by quenching to room temperature in a desiccator, and grinding in a planetary ball or attrition mill (typically, at 600 rpm for 12 h), in ambient air. Regarding powder preparation of commercially available CPCs, there are great variations mainly depending on: the sintering temperature and time; the atmosphere in which the powder grinding takes place; the planetary ball milling time; the rotation rate, mass, and surface area of the balls; the pressure used to compact the mixture (Lewis, 2006).

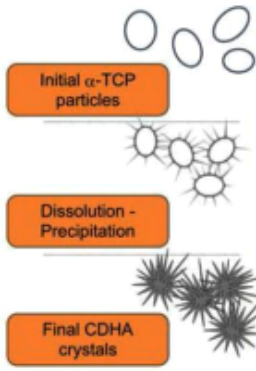
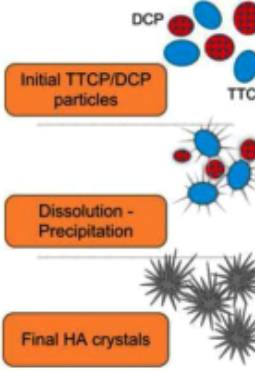
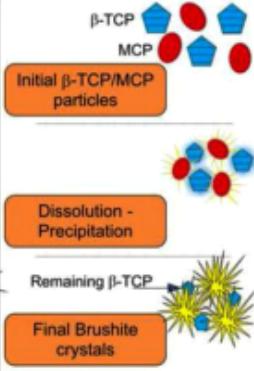

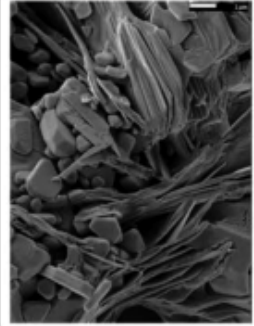
Thorough mixing of solid powder phase and liquid phase of the cement, in defined ratio, results in a viscous and mouldable paste. Solid phase is constituted of one or several phosphate- or calcium-based compounds, whereas the liquid phase may be water, or solution of calcium or phosphate that can contain alginate, chitosan, hyaluronate, gelatine, chondroitin sulphate, succinate or citric acid (Zhang *et al.*, 2014). Liquid phase insures the dissolution of initial reactants till solution oversaturation is achieved and crystal precipitation begins. The hardening of the cement progresses with crystal entanglement while cement mechanical properties gradually augment till reaching the final solidified state. Increase in the powder-to-liquid ratio markedly reduces both initial and final setting time of the cement (Lewis, 2006). In addition, solid phase initial granulometry and the presence of crystals also determine the cement solidifying time. It was demonstrated that CPCs harden through a low exothermic reaction (without reaching high curing temperatures), that does not result in cement shrinking or thermal necrosis of adjacent bone tissue (Lewis, 2006).

Two types of setting reactions are possible when a CPC solid phase is mixed with an aqueous solution (Bohner, 2000; Chow & Takagi, 2011) (Figure 8.).

The first is an acid-base reaction where a relatively acidic CaP phase reacts with a relatively basic CaP to produce a relatively neutral CaP. An example is:



The final product is brushite cement (dicalcium phosphate dihydrate, DCPD) that is 1-2 orders of magnitude more soluble than apatite cement at physiological pH (Zhang *et al.*, 2014), and which is more quickly resorbed *in vivo*. However, if bone ingrowth does not follow the brushite resorption rate, it results in a decrease in bone strength at the implantation site (Lewis, 2006). As brushite is a metastable phase, *in vivo* transformation to apatite may occur (Zhang *et al.*, 2014).

|  | Apatite cements   |  | Brushite cements   |
|--|---|--|--|
|  | Single Component  | Multiple Components  |  |
| Reactives                                | $\alpha$ -TCP   | TTCP + DCPA/DCPD   | $\beta$ -TCP + MCPM/MCPA   |
| Reaction                                 | $3\alpha\text{-Ca}_3(\text{PO}_4)_2 + \text{H}_2\text{O} \rightarrow \text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5(\text{OH})$ | $2\text{Ca}_4(\text{PO}_4)_2\text{O} + 2\text{CaHPO}_4 \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$   | $\beta\text{-Ca}_3(\text{PO}_4)_2 + \text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} + 7\text{H}_2\text{O} \rightarrow 4\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ |
| Type of Reaction                         | Hydrolysis  | Acid-Base  | Acid-Base  |
| Setting mechanism and crystal morphology |   |    |   |
| SEM                                      |                                        | <div style="display: flex; align-items: center; justify-content: center;"> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">APATITE</div> <div style="writing-mode: vertical-rl; transform: rotate(180deg);">BRUSHITE</div> </div> |    |

**Figure 8. Synthesis of brushite and apatite cement.**

Two types of calcium phosphate cement synthesis and their final products. Scanning electron micrographs of set apatite and brushite cements (taken from Dorozhkin, 2013).

The second type of setting reaction is hydrolysis, where the initial and final CaPs have the same Ca/P molar ratio, an example being:



This chemical reaction results in the formation of different kinds of apatite cements, such as stoichiometric hydroxyapatite, calcium-deficient hydroxyapatite (CDA) or carbonated apatite (dahllite) that differ in solubility rates.

Even though they possess superior mechanical properties compared to ceramics, CPCs mechanical resistance (in terms of strength, toughness, brittleness and reliability) remains rather poor in comparison to healthy bone or autograft (Table 4.). Thus, for the moment, their use is limited to moderate- or non-load-bearing clinical situations (Verron *et al.*, 2010c). Nevertheless, it is not often realized by researchers and clinicians that poor mechanical performances are not a critical issue for bone repair applications. Indeed, CPCs with compressive strengths comparable to those of cortical bones can be produced through densification and/or homogenization of the cement matrix. Actually, the real mechanical limitation for CPCs appears to be their low fracture toughness and poor mechanical reliability (Weibull modulus), which have been so far only rarely studied (Zhang *et al.*, 2014).

The second main drawback resulting from CPCs use is the absence of macroporosity. These results *in vivo* in poor cellular colonization of the implant centre, leading to the absence of new bone formation inside the CPC. Hence, the material resorption is mostly limited to the implant surface and slowly continues layer by layer to the interior (Lewis, 2006). Therefore, in current CPC research, there is a focus on developing macroporous substitutes by incorporation of soluble crystals during synthesis or by adding sodium hydrogencarbonate (NaHCO<sub>3</sub>) in initial powder composition.

**Table 4. Typical mechanical resistance of healthy human bone and calcium phosphate cement.**

(summarized from Charron, 2008).

|                                 |             |
|---------------------------------|-------------|
| <b>cortical bone</b>            | 150-200 MPa |
| <b>cancellous bone</b>          | 8-12 MPa    |
| <b>calcium phosphate cement</b> | 20-35 MPa   |

## 3.4. Calcium phosphate biomaterial behaviour *in vivo*

### 3.4.1. Resorption mechanisms

*In vivo*, CaP biomaterials are resorbed from implantation site and partially or completely replaced by new bone tissue. It is crucial that no foreign material remains permanently in the implantation site and that the load-bearing capability of the bone is not affected over the long-term process of implant replacement. Solubility rates of different biomaterials stem from their physicochemical composition (Nakamura *et al.*, 2013).

After CPC implantation, biological fluids set off the physico-chemical dissolution of the material, followed by surface precipitation of carbonated apatite. As mentioned before, this kind of apatite is the closest in chemical composition to biological apatite. Consequently, bone cells that create a tight interface between biomaterial and bone tissue readily colonized the implant, and are responsible for CPCs good osseointegration into the surrounding intact host bone.

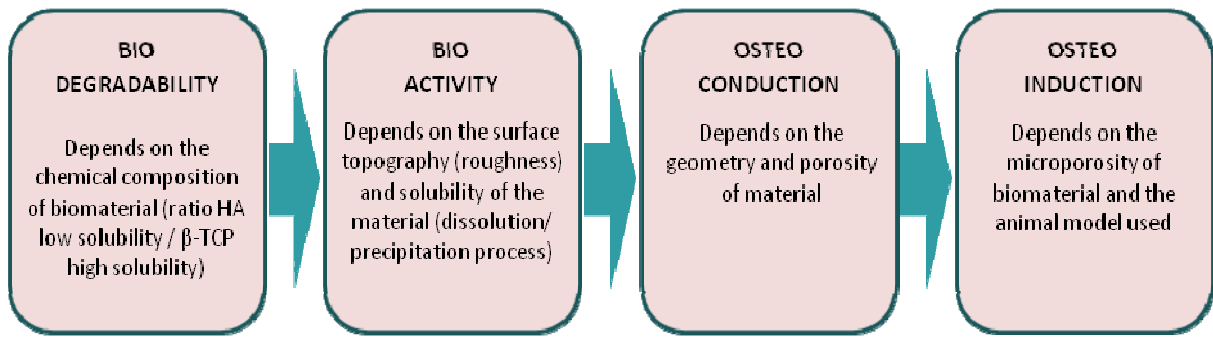
The second phase of resorption is performed by joint action of macrophages and osteoclasts present at the material-bone interface, and dissolved materials are incorporated material dissolution into remodelling process. The cellular resorption rate depends on the implantation site and ideally, to assure implant stability, it should be close to the rate of new bone formation. In the case where a too-rapid material resorption is not accompanied by sufficient new bone ingrowth, issues occur regarding the mechanical stability of the implant area.

Numerous studies on different inorganic biomaterials have been conducted to evaluate osteoclast survival and activity *in vitro*, and to assess bone substitute resorption *in vivo*. *In vitro*, osteoclast resorption is affected by: the incorporation of ions such as carbonate or silicon into the structure of inorganic material; the surface energy; the physicochemical dissolution characteristics; the surface roughness; the surface crystallinity. For example, the incorporation of carbonate ions into the HA lattice has been shown to increase osteoclastogenesis. This is in accordance with the fact that main bone mineral is carbonated apatite (Nakamura *et al.*, 2013). On the other hand, osteoclast mononuclear precursors fusion is influenced by the material surface topography. It has been reported the roughened HA surfaces increased the

number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells derived from rat bone marrow, as well as the differentiation rate of human peripheral blood mononuclear cells (PBMCs) cultured with M-CSF and RANKL. Interestingly, regardless of the similar numbers of TRAP-positive multinucleated cells and the similar viability of osteoclasts, there were differences in the resorption pits created by human and rat osteoclasts, depending on the type of inorganic material. On HA, human osteoclasts formed stronger actin rings and thereby deeper resorption pits compared to TCP. Conversely, Yamada *et al.* reported that rabbit osteoclasts resorbed TCP but not HA. These differences that result in alternation of biological properties are due to cell origin as well as cement characteristics such as sintering temperature and time, porosity, surface roughness, and grain size (Nakamura *et al.*, 2013).

### **3.4.2. Bone neoformation**

Bioactivity is the property of a material to directly bond new forming bone without formation of unmineralized fibrous tissue at the biomaterial-bone interface. This property depends on the surface topography (roughness) and also on the solubility of the material, responsible for a dissolution/precipitation process. As explained above, the dissolution of CaP biomaterials leads to an oversaturation of the biological fluid with calcium and phosphate ions. Further precipitation of biological carbonated apatite on the material surface permits the new bone formation, thus restoring mechanical, metabolic and haematopoietic functions at the implantation site. Three fundamental phenomena participate in bone tissue neoformation: (i) osteogenesis/osteoinduction; (ii) osteoconduction; (iii) osseointegration.



**Figure 9. Fundamental phenomena in degradable biomaterial-induced bone formation.**

Physico-chemical properties of calcium phosphate biomaterials determine the rate of its *in vivo* resorption and subsequent new bone formation (taken from Graftys internal brochure *Boukhechba, 2011*).

### *Osteogenesis/Osteoinduction*

Besides mature cells, undifferentiated cells are found in bone and adjacent tissues. At the time of the injury, local biochemical and biophysical stimuli are released and help surviving cells to respond in a proper manner. Some of these messengers guide the differentiation and organisation of cells, while others function as mitogens. In the injury response, both differentiated and osteoprogenitor cells are involved for adequate bone repair. Even more, progenitor cells are of utmost importance for proper bone healing. The majority of newly formed bone depends on mesenchymal stem cells (MSCs) transition to bone-forming osteoblasts. This process starts immediately after the injury, and it is very active during the first week, even though the action of the newly recruited preosteoblasts is not obvious until several weeks later, during the callus stage (Albrektsson & Johansson, 2001).

At ectopic site, a phenomenon that resembles to osteogenesis, is called osteoinduction. The classical papers describing osteoinduction at various non-skeletal sites were published a long time ago. To demonstrate ectopic bone formation, the authors used gall bladder epithelium, alcohol extracts of bone, demineralised bone and transplants to muscles or the anterior chamber of the eye. Today, it is considered that the best way to demonstrate the osteoinductive properties of a biomaterial is to inject it into the subcutaneous or muscle tissue, where bone formation does not occur under normal physiological conditions, and to observe the formation of ectopic bone tissue. Inductive agents naturally function in



bone surroundings too, but it is difficult to differentiate between bone induction and bone conduction in an orthotopic site. That is why it is hard to discuss whether or not a particular orthotopically implanted biomaterial acts as a real osteoinductor (Albrektsson & Johansson, 2001).

The assessment of biomaterial osteoinductive properties in ectopic sites has its purpose as a fast, easy and discriminating way to test new biomaterials in preclinical studies. Meanwhile, it has little importance in actual clinical practice, since the injury in itself is sufficient to recruit a great number of osteoprogenitor cells.

Calcium phosphate materials are generally known to be osteoconductive but not osteoinductive. However, several CaP biomaterials have been reported to have the ability to form bone in non-bony sites in different animal models without addition of exogenous osteogenic factors. These include hydroxyapatite,  $\beta$ -tricalcium phosphate, biphasic calcium phosphates with varying HA/ $\beta$ -TCP ratio, as well as calcium phosphate cements. Some authors compared different CaP ceramics implanted in muscles or in femoral cortical bone in dogs and goats, and concluded that the osteoinductive capacities of the biomaterial tested in non-osseous sites in large animals is correlated to a better performance in clinically relevant orthotopic sites. However, in all these studies, bone formation was much lower in ectopic sites than in orthotopic sites.

To date, the exact mechanism of osteoinduction by biomaterials is still unknown, while some authors hypothesize that BMPs could concentrate at the material surface after implantation, thus stimulating ectopic bone formation. On the other hand, the osteoinductivity of CaP materials could be modulated by their intrinsic physicochemical characteristics (geometry, topography, macropore size and percent of microporosity).

### *Osteoconduction*

Osteoconduction refers to bone growth from bone defect edges towards the the surface or down into the pores of a biomaterial, which serves as a scaffold or template to guide the formation of the new bone tissue (Albrektsson & Johansson, 2001). This phenomenon is regularly seen upon the implantation of bone substitutes and is considered dependent both on biological factors for bone repair, but also on the intrinsic properties (geometry, porosity) of the implanted biomaterial. Materials of

low biocompatibility, e.g. copper, silver and acrylic cement, show little or no osteoconduction (Nakamura *et al.*, 2013). Surprisingly, bone conduction is observed with biomaterials such as stainless steel, which is not regarded as ideal from the point of view of biocompatibility (Albrektsson & Johansson, 2001).

Bone growth at the surface of an implant involves differentiated bone cells at the injury site and progresses in six stages: (1) serum adsorption; (2) the recruitment of various cell types; (3) the attachment, migration and proliferation of recruited cells; (4) osteoblast differentiation and osteoid production; (5) osteoid matrix mineralization; (6) bone remodelling. In the final stage of osteoconduction, osteoclasts have an important role in resorbing the osteoid and implanted biomaterial, as well as in interacting with osteoblasts that are responsible for the bone formation (Nakamura *et al.*, 2013).

Importantly, bone growth, including bone conduction, does not occur without a proper vascularisation, and the principal action of many growth factors implicated in bone repair is both mitogenic and angiogenic.

The osteoconductive response to a particular material is rather short lived, and maintaining the successful bone anchorage over a long period of time is attributed to osseointegration phenomenon (Albrektsson & Johansson, 2001).

### *Osseointegration*

Osseointegration is the stable anchorage of an implant achieved by direct bone-to-biomaterial contact and maintained over time (Albrektsson & Johansson, 2001). The same as osteoconduction, it is determined not only by biological factors, but also by the response to a foreign material. It was first described by Branemark as a direct contact, observed at light microscope magnification levels, between living bone and implant. In histological terminology, osseointegration is defined as the direct anchorage of an implant by the formation of bony tissue around the implant without the growth of fibrous tissue at the bone-implant interface. Another more biomechanically oriented definition of osseointegration has also been suggested: "A process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved, and maintained, in bone during functional loading". It is not an isolated phenomenon, but instead depends on previous osteogenesis/osteoiduction and

osteoconduction. Thus materials that are too toxic to allow osteoconduction will not be osseointegrated (Albrektsson & Johansson, 2001).

In oral and craniofacial implantology, osseointegrated implants have been a real breakthrough, yielding excellent functional results, in contrast to alternatively anchored implants, which have generally shown very poor success rates. Functional osseointegrated implants demonstrate interfacial bone density similar to that of the bone area in which the biomaterial was implanted. Screw-type implants inserted using a minimally traumatising technique have been convincingly osseointegrated, e.g. in hip arthroplasties and interphalangeal implants (Albrektsson & Johansson, 2001).

Osseointegration refers to general implant anchorage in the bone, so one- or two-point bone-biomaterial contact cannot be characterized as a real osseointegration. Even a slight implant displacement can compromise osseointegration, and it is shown that interfacial implant movement of more than 150  $\mu\text{m}$  inevitably lead to soft tissue formation instead of bone (Albrektsson & Johansson, 2001).

### **3.5. Calcium phosphates as drug delivery systems**

In the design of new generation of bone substitutes, there is a growing interest in coupling bone biomaterials with pharmacological products. These combined devices provide mechanical stability, which stems from the structural properties of the material, and they also display enhanced biological activity due to the pharmacological action of the loaded drug. Thus, efficient pharmacological effects of the drug are confined to specific target site. This strategy overcomes several serious limitations of systemic drug administration including: (i) the need for high drug doses due to poor bioavailability at the disease site; (ii) low efficiency; (iii) drug side-effects; (iv) toxicity issues; (v) poor patient compliance (Mellier *et al.*, 2011). The focus is on the development of biomaterials capable of precisely timed drug release with predictable and reproducible pharmacokinetics (Verron *et al.*, 2012). Special attention is paid to the prevention of uncontrolled and excessive drug release that could result in local tissue toxicity.

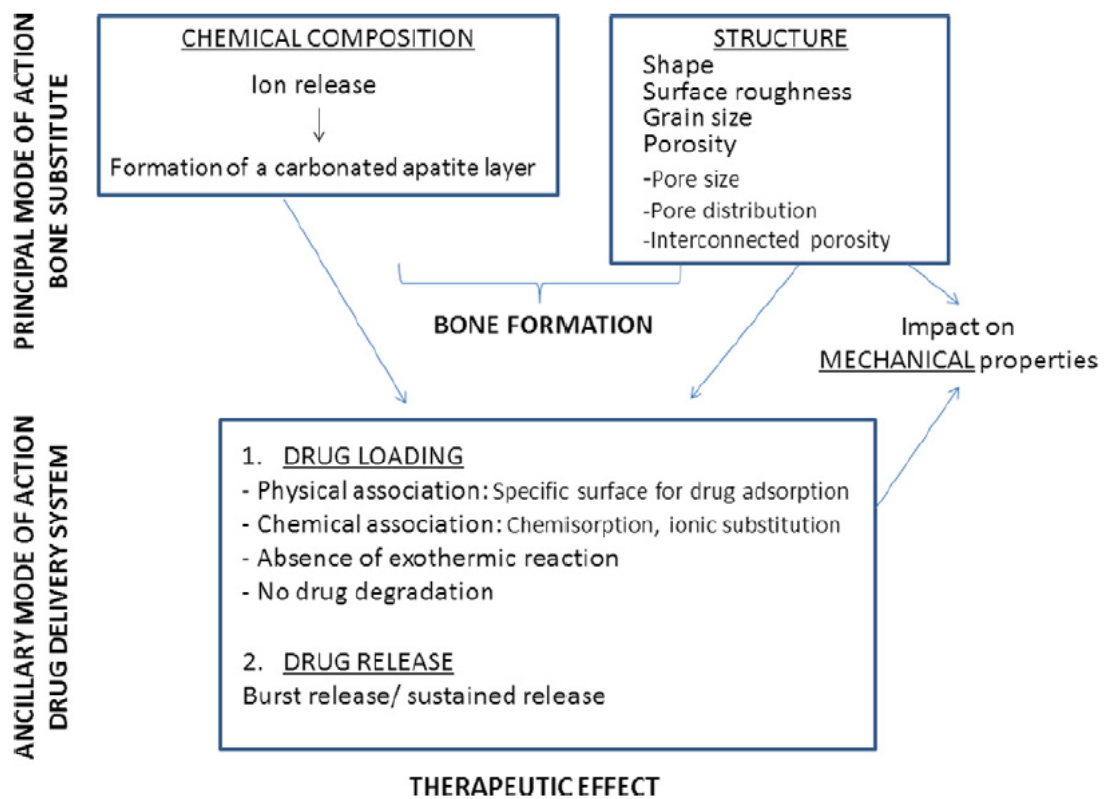
Although the use of implants as drug delivery systems (DDSs) is well developed for cardiovascular applications (drug-eluting stents) and increasing for diabetic management (insulin pumps), this concept is still in infancy for bone applications.

Regulatory aspects are important obstacles that have to be tackled by orthopaedic industry. Indeed, such bone DDS are considered as combination devices by the FDA and, correspondingly, the registration of these new systems follows a longer procedure than that of traditional orthopaedic implants (Verron *et al.*, 2010c).

Nowadays, efforts are focused on developing mixed systems that combine calcium phosphate bone substitutes with active molecules. Current research on these bone drug delivery systems aims to improve the osteogenic potential of CaP in healthy bone sites and to provide a bone response in pathological ones.

Great number of commercially available CaP-based bone substitutes has the capacity to integrate molecules of different chemical composition. Excellent biocompatibility, complete resorbability, very low exothermic behaviour that does not affect loaded drug, as well as clinical efficacy, have prompted calcium phosphate cements as attractive candidates for combining with therapeutic agents. Physicochemical structure of CPCs (specific surface area, porous size distribution and connectivity, roughness, grain size, etc.) (Verron *et al.*, 2012) permits pharmacological compounds incorporation either by ionic substitution (chemical association) or by surface adsorption (physical association). When implanted, CPCs combined with drugs may act, as their primary function, as mechanically resistant sacrificial calcium phosphate source for bone reconstruction, but as well as vectors for active compounds that address bone-related disorders or infections. Therefore, CPCs have been proposed as good carriers for local and controlled supply of antibiotics, anti-inflammatory, anti-resorptive or anti-cancer agents (Mellier *et al.*, 2015).

As mentioned before, the structure of a biomaterial (macro- and microporosity) affects its resorption by dissolution or/and cellular processes, and therefore greatly influences the drug-release profile. The degradation of the biomaterial carrier and previous sterilization step should not produce toxic metabolites or degrade the loaded drug. Figure 11 summarizes general requirements of a biomaterial-based DDS to be used in bone tissue engineering (Verron *et al.*, 2012).



**Figure 10. General requirements of a bone drug delivery system.**

The implantation of bone substitute reinforces mechanical properties of damaged bone site and if resorbed, can induce new bone formation. In pathological situations, bone substitutes can have an auxiliary beneficial effect if loaded with active molecules (taken from Verron *et al.*, 2012).

### *Combined devices in bone reconstructive surgery*

Combining different osteogenic and angiogenic factors with calcium phosphate cements has the potential to improve bone reconstruction at the implantation site.

Numerous studies have analyzed reconstructive properties of cement-**bone morphogenetic protein** combined devices. For example, Haddad *et al.* (Verron *et al.*, 2010c) evaluated bone repair in a critical-size calvarial defect in rabbits, after implantation of apatitic cement loaded BMP-2. After 12 weeks of implantation, they observed a 45.8% increase of bone formation in cement/BMP-2 group compared to unloaded control cement. Moreover, some clinical studies focused on BMP-2, have

begun to confirm the encouraging results of preclinical studies (Verron, Bouler, & Guicheux, 2012). In a clinical study, Dimar *et al.* used a vertebral fusion model to compare the efficiency of iliac crest autograft versus CaP biomaterial combined with BMP-2. The implantation of combined device gave a clear improvement compared to the iliac crest bone graft in terms of vertebral fusion rate (88% vs 73%), duration of the intervention (2.4 h vs 2.9 h), haemorrhages (273 cm<sup>3</sup> vs 465 cm<sup>3</sup>), and morbidity (leg and back pain) (Verron, Khairoun, Guicheux, & Bouler, 2010c). In addition, several studies have shown a beneficial effect of associating growth factors with CaP during the filling of bone defects (Verron, Khairoun, Guicheux, & Bouler, 2010c).

Due to its osteogenic properties, **transforming growth factor-beta** also constitutes an attractive candidate for a bone DDS. Indeed, Sumner *et al.* shown that the *in situ* release of TGF- $\beta$ 2 from a CaP-based substitute, increased twofold the amount of newly formed after a four week implantation period in the dog proximal humerus (Verron, Khairoun, Guicheux, & Bouler, 2010).

Another preliminary study demonstrated an increased proliferation of MG-63 cells and the phosphatase alkaline activity of SaOS-2 cell line in the presence of **insulin growth factor** (IGF) released *in vitro* from a biomaterial composite (Verron, Khairoun, Guicheux, & Bouler, 2010c).

Since **VEGF** contributes significantly to angiogenesis during bone healing (E. Verron, Bouler, & Guicheux, 2012), it has been proposed that a CaP substitute loaded with VEGF could enhance new bone formation. A short-term release of VEGF is supposed to promote angiogenesis, while a sustained release of VEGF should improve bone regeneration thanks to its stimulatory effects on both angiogenesis and osteoblast differentiation. However, most studied delivery systems induced the release of high amounts of VEGF during the initial phase of biomaterial resorption, and high VEGF concentrations are known to provoke an increase in vascular permeability with formation of non-functional blood vessels (Verron, Bouler, & Guicheux, 2012).

#### *Combined devices in bone pathological situations*

Any pathological situation (e.g. infection, irradiation, tumour) unfavourably affects bone implant performances in terms of substitution and/or resorption process.

The local administration of active agents has numerous advantages compared with systemic treatments in terms of therapeutic efficiency and tolerance, mainly due to specific tissue response and optimal bioavailability (Verron, Khairoun, Guicheux, & Bouler, 2010c).

One major focus is the combinations of CaP materials with **antiresorptive drugs**, such as bisphosphonates (BPs) or strontium. Seshima *et al.* envisaged apatite as a potential vector for alendronate and have studied the influence of certain material characteristics (crystallinity, specific surface, solubility) on the drug release profile. In another study, various zoledronate concentrations were grafted on titanium substitute coated with HA, and implanted in either healthy or osteoporotic female rat condyles (Peter *et al.*, 2005; Peter *et al.*, 2006). In both healthy and osteoporotic group, a significant increase in the peri-implant bone volume fraction was observed, reinforcing the idea of using a local bisphosphonate delivery from CaPs. Moreover, injection of BP-loaded calcium-deficient apatite (CDA) within proximal femurs of osteoporotic ewes led to a significant increase in relative bone content and an improvement of the bone microarchitecture (Verron, Bouler, & Guicheux, 2012). Considering strontium, it was recently introduced for the prevention and the treatment of osteoporosis. The implantation of strontium-doped CaP in the foreleg radius of rabbits showed increased bone formation in Sr-CaP group when compared to control group. Interestingly, no difference was observed in terms of degradability between both groups 16 weeks after implantation (Tian *et al.*, 2009).

Although rare, infections associated with the implantation or the revision of orthopaedic devices are known to generate additional morbidity (prolonged hospitalization, poor functional outcome, risk of septicemia, etc.). In extreme cases, acute or chronic osteomyelitis can compromise the vital prognosis of the patient. Therefore, bone DDSs releasing **antibiotics** present an interesting clinical approach for postoperative infection prevention. A meta-analysis of 19 studies covering 35 659 patients confirmed the usefulness of loaded cements in treating bone infections. They reduced the bone infection rate by half in primary prevention, and by approximately 40% in secondary prevention. However, resistance of some pathological strains has appeared, suggesting that the application of these loaded cements should be restricted only to multi-resistant strains (Elise Verron, Khairoun, Guicheux, & Bouler, 2010c).

Concerning the field of bone tumours, 80% of osteosarcomas are treated with pre- and postoperative chemotherapy coupled to surgery, with a healing rate between 60% and 70%. To reduce the risk of developing metastases in other organ sites, wide excision of the tumour and curing of the surrounding tissues are systematically associated with chemotherapy. Since biomaterials can be used to fill the bone cavities after tumour excision, several teams looked into the development of combined systems with the goal of providing high **anti-cancer** drug release at bone sites. Considering the major side-effects of chemotherapy (e.g. digestive complications, haematotoxicity, nephrotoxicity, hepatotoxicity) that cause the interruption of usual systemic treatments, it is anticipated that the local release of high chemotherapeutic doses will be more efficient and better tolerated by the patient (Elise Verron, Khairoun, Guicheux, & Bouler, 2010c). As an illustration, Itokazu *et al.* characterized the release of anti-cancer compound methotrexate from two different CaPs. In the first days, approximately 1 mg/mL of methotrexate was released and this reduced progressively to reach, on the 12th day, a local concentration of approximately 0.1–1 mg/mL, which remained efficient. In a another study, it was reported that the use of combined biomaterial loaded with chemotherapeutic agent paclitaxel slowed by 140% the appearance of paralysis linked to bone metastases in rat vertebral metastasis model, and increased the survival rate by 150% (Elise Verron, Khairoun, Guicheux, & Bouler, 2010c). Lastly, acute postoperative pain following bone surgery is a severe complication that considerably disturbs patients' convalescence by reducing mobility, functional recovery and increasing hospital stay. Moreover, if this postoperative pain persists, it generally turns to chronic pain that is more difficult to cure]. So, to facilitate patients' recovery, it is critical to manage bone postoperative pain by the administration of conventional **analgesics**, such as morphine-derived products or anti-inflammatory drugs. In line with this, several preclinical developments have been conducted, associating biomaterials with the non-steroidal anti-inflammatory drug indomethacin, aspirin, or bupivacaine, one of the most commonly used local anaesthetics (Verron, Bouler, & Guicheux, 2012).



## 4. Gallium

### 4.1. Physicochemical properties

Gallium (Ga) was discovered by Lecoq de Boisbaudran in 1875 as a semi-metallic element with atomic number 31, and position in group IIIa of the periodic table of elements. Its main physicochemical properties are summed up in Table 5. In most of its compounds, gallium comes in the form of a trivalent ion,  $\text{Ga}^{3+}$ . Charged  $\text{Ga}^{3+}$  is almost completely hydrolyzed over a wide pH range, thus, forming hydroxide species, particularly gallate ( $\text{Ga}(\text{OH})_4^-$ ) (Hancock & Marsicano, 1980). In aqueous solutions, under physiological conditions, essentially all  $\text{Ga}^{3+}$  ions (98.4%) are incorporated in gallate (Harris & Pecoraro, 1983). Ga hydrolysis is accompanied by formation of hydronium ion,  $\text{H}_3\text{O}^+$ , which lowers the pH of the aqueous solution. Conversely, in conditions of elevated pH, the precipitation of highly insoluble amorphous  $\text{Ga}(\text{OH})_3$  occurs (Benézéth *et al.*, 1997).



**Figure 11. Gallium typical silver-white appearance.**

(taken from <https://www.flickr.com/photos/argentoratum/3305472652>).

The chemical behaviour of  $\text{Ga}^{3+}$  resembles to that of  $\text{Fe}^{3+}$ , in terms of electric charge, ion diameter, coordination number and electronic configuration (Collery, Keppler, Madoulet, & Desoize, 2002). Due to this similarity, in biological systems Ga can compete with iron for binding to  $\text{Fe}^{3+}$ -binding proteins.

**Table 5. Main physicochemical properties of gallium.**

(adapted from <http://www.lenntech.com/periodic/elements/ga.htm>)

|                   |              |
|-------------------|--------------|
| atomic number     | 31           |
| atomic weight     | 69.72        |
| melting point     | 29.78 °C     |
| boiling point     | 2400 °C      |
| crystal structure | orthorhombic |
| appearance        | silver-white |

## 4.2. History of clinical application of gallium compounds

The first mentioning of Ga as a possible therapeutic agent appeared in 1931 in the work of Levaditi who tested Ga tartrate effects in syphilis treatment in a rabbit model and for the eradication of *Trypanosoma evansi* in a mouse model. In 1940s, the focus was shifted to investigating Ga radioisotopes tissue distribution (Bernstein, 1998). It was found that Ga presented a strong affinity for growing and remodelling tissues like tumours and bone (Dudley & Maddox, 1949). <sup>67</sup>Ga and <sup>68</sup>Ga radioisotopes are readily absorbed by cancer deposits in bone and have been extensively used as imaging agents in diagnosis and study of neoplastic diseases (Collery *et al.*, 2002; Cutler *et al.*, 2013).

In bone pathologies, Ga efficiently suppresses accelerated bone resorption and bone pain associated with multiple myeloma and more generally, it decreases bone malignancy-induced hypercalcaemia (Bernstein, 1998). Citrated Ga(NO<sub>3</sub>)<sub>3</sub> for intravenous injection (Ganite®) is a FDA-approved drug for the treatment of hypercalcaemia of malignancy (Mellier *et al.*, 2011). In addition, Ga was found to be also effective in the treatment of Paget's disease of bone (R. S. Bockman & Bosco, 1994), and due to its antiresorptive properties, it was suggested as a possible treatment for osteoporosis as well (Verron, Bouler, & Scimeca, 2012b).

Considering that some similar elements, like platinum, exhibit antitumor properties, there was an extensive investigation of Ga potential antitumor effects. In clinical trials with patients suffering from relapsed malignant lymphomas, 43% of the

subjects responded to a Ga nitrate treatment. On the other hand, Ga efficacy was controversial in few cases of treatment of melanoma and epithelial ovarian cancer (Verron, Bouler, & Scimeca, 2012b).

Besides its avid bounding to tumour or bone tissue, Ga also accumulates at the sites of inflammation and infection, and can have anti-inflammatory and immunosuppressive action. Indeed, subcutaneously administrated Ga nitrate suppressed the development of adjuvant arthritis in rats (Verron, Bouler, & Scimeca, 2012b).

Despite obvious clinical efficiency, the exact mechanism of Ga action on bone and cancer cells remained unknown for a long time. Recent *in vitro* and *in vivo* studies, which will be discussed later, elucidated important cellular and molecular targets of Ga action.

### 4.3. Gallium interactions with plasma membrane proteins

By the virtue of similarity to  $\text{Fe}^{3+}$ ,  $\text{Ga}^{3+}$  can occupy ferric sites in several proteins and chelate complexes. Hence,  $\text{Ga}^{3+}$  can compete with  $\text{Fe}^{3+}$  for binding to transferrin (TF), the major iron transport protein (Kubal, Mason, Patel, Sadler, & Woodworth, 1993). On the other hand,  $\text{Ga}^{3+}$  ion is irreducible under physiological conditions so that it could not compete with  $\text{Fe}^{2+}$  in binding to haemoglobin, which would otherwise result in major haematological toxicity (Verron, Bouler, & Scimeca, 2012b). Transferrin binds to two different receptors, TF receptors 1 and 2 (TFR1 and TRF2). All nucleated cells of the body seem to express either one of these receptors, but their concentration varies considerably. TFR1 is highly expressed by rapidly proliferating cells, such as cancer cells, that need large amounts of iron. In the course of osteoclast differentiation, mitochondrial biogenesis requires an increased iron uptake, which results in the upregulation of TFR1 on its plasma membrane. Since Ga is capable of binding to TFR1 ligand, transferrin, it is hypothesized that TF/TFR1-mediated uptake is the mechanism of Ga cellular internalization (Ishii *et al.*, 2009). In line with this, Ga concentration was found to strongly correlate with TRF expression in malignant tissue. And it has been shown that tumours overexpressing TFR internalised Ga more avidly than tumours lacking a functional TFR (Luttrupp *et al.*, 1998). Accordingly, in lung carcinoma tumour cells, as well as for several types of

lymphomas, it was found a high correlation between TFR expression and  $^{67}\text{Ga}$  binding capacity (Bernstein, 1998).

TFR-mediated Ga uptake does not exclude the possibility of Ga interaction with other membrane transporters. Sohn *et al.* proposed a TF-independent Ga uptake by bone cells, based on the fact that Ga concentration in bone was not affected in hypotransferrinemic mice lacking TF protein (Sohn *et al.*, 1993). Lastly, and beside Ga transport into cells, calcium channels such as TRPV-5A were also proposed as potential partners involved in Ga action on extracellular Ca entry into cells (Verron *et al.*, 2010d).

#### 4.4. Gallium and bone tissue

In bone, Ga tends to bind in metabolically active regions, such as metaphyseal growth plate, growth cartilage and fracture sites. To a lesser extent, Ga also accumulates on the endosteal and periosteal surfaces of diaphyseal bone (Bernstein, 1998). Concerning the mechanism of Ga accumulation within bone tissue, it is hypothesized that Ga could attach to the mineral matrix by substitution with calcium (Ca) ions (Bockman *et al.*, 1993). This was confirmed later by Korbas *et al.* who found that Ga uptake by bone depends on the maturation stage of the mineral phase (Korbas, Rokita, Meyer-Klaucke, & Ryczek, 2004). Indeed, in the initial phase of mineralization, Ga substituted Ca and modified the apatite configuration, blocking its growth. Thus, further maturation of the mineral phase included only apatite that did not contain Ga. In contrast, when added in late mineralization stage, Ga could not incorporate into the already formed apatite. Nevertheless, Ga can be adsorbed on the surface of already formed apatite crystals, interfering with their solubility.

##### 4.4.1. *In vitro* studies of gallium effect on bone cells

###### *Ga action on osteoclast*

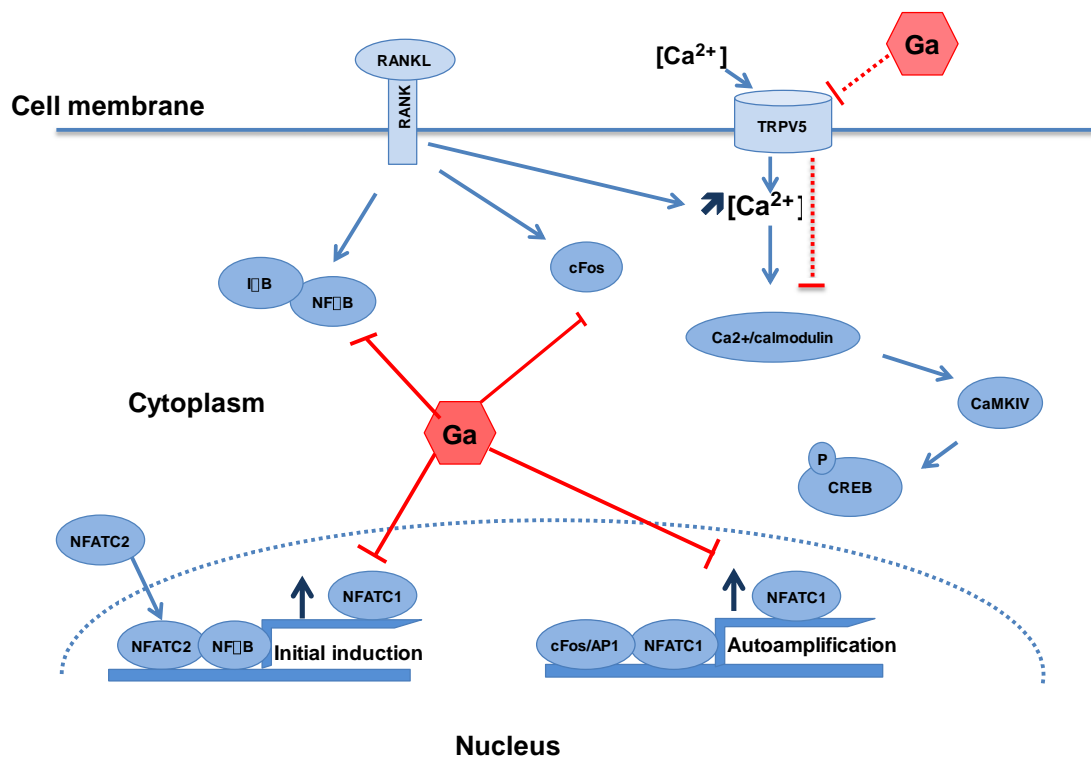
Several studies have established the inhibitory effect of gallium on osteoclast-mediated bone resorption (Blair, Teitelbaum, Tan, & Schlesinger, 1992; Bockman, 2003; Hall & Chambers, 1990; Lakatos, Mong, & Stern, 1991; Liewehr *et al.*, 1995;

Shaini, Shelton, Marquis, & Shortall, 2000). Blair *et al.* described *in vitro* Ga interactions with bone slices, bone cells and cell culture medium (Blair *et al.*, 1992). They observed a 50% reduction in osteoclastic resorption when bone slices were pre-treated with Ga. Even more, at high Ga doses, osteoclast activity was totally abolished. Similarly, Hall *et al.* (Hall & Chambers, 1990) found that Ga, in solution at 391  $\mu\text{M}$ , reduced by 64% the osteoclast-mediated resorption of cortical bone slices. However, when bone slices were treated to pre-adsorb Ga, the resorption inhibition observed was approximately twice lower (34%). and Ga did not affect cell morphology nor altered osteoclast number. By contrast, in another study, it was reported that Ga displayed cytotoxicity towards osteoclast for concentrations starting from 50  $\mu\text{M}$  (Blair *et al.*, 1992).

Concerning, the molecular mechanisms underlying Ga anti-osteoclastic actions, an early study by Mills *et al.* (Milles *et al.* 1988) showed that  $^{67}\text{Ga}$  localized almost exclusively in the osteoclast nuclei, suggesting that Ga could interfere with gene expression. More recently, molecular pathways impacted by Ga treatment were more precisely documented by Verron *et al.* (Verron *et al.*, 2012c; Verron *et al.*, 2010d). The authors found that 100  $\mu\text{M}$  Ga dramatically reduced *in vitro* osteoclast formation from unfractionated primary rabbit bone cells, and also that Ga dose-dependently inhibited the resorption activity of differentiated rabbit osteoclasts. Using another model, the mouse monocyte-macrophage cell line RAW 264.7, they showed that Ga downregulated the expression of several osteoclastic marker genes (*Nfatc1*, *Trap*, *Calcr*, *Ctsk*, *Rank*, *Oc-stamp*) in a dose-dependent manner (0-100  $\mu\text{M}$ ), without any cytotoxic effects.

A general scheme is proposed in Figure 13, which illustrates Ga action on two main signalling pathways involved in RANKL-induced osteoclastogenesis, namely NF- $\kappa\text{B}$  and calcium pathways. NF- $\kappa\text{B}$  is constitutively present in cytoplasm, coupled to its inhibitor I $\kappa\text{B}$ . I $\kappa\text{B}$  phosphorylation is a marker for ubiquitination and subsequent proteasome-mediated degradation, which unmasks the nuclear localization signal motif of NF- $\kappa\text{B}$ . Upon its nuclear translocation, NF- $\kappa\text{B}$  stimulates the initial induction of *Nfatc1* gene, the master regulator of osteoclastogenesis. In line with this, gallium inhibitory action on I $\kappa\text{B}$  degradation leads ultimately to a down-regulation of *Nfatc1* gene expression. Concerning calcium signalling, this pathway is of particular importance during osteoclastogenesis. In response to RANKL, the calcium channel TRPV-5A would participate in calcium entry into the cell. Indeed, in response to

gadolinium, an agonist of TRPV-5A, intracellular  $[Ca^{2+}]$  oscillations needed for proper osteoclastogenesis are observed. Importantly, Ga completely abolishes gadolinium-induced intracellular  $[Ca^{2+}]$  oscillations. These results strongly suggest that an alternative mode of Ga action on osteoclast differentiation may be the blocking of specific calcium channels in the plasma membrane of undifferentiated osteoclasts. Lastly, Ga completely reversed calcium-induced CREB phosphorylation, thus blocking its cooperation with NFATc1 for the induction of osteoclast marker genes expression program (Verron *et al.*, 2012c).



**Figure 12. Gallium molecular targets in osteoclast differentiation.**

Gallium can interfere at different levels in RANK-L-induced osteoclast differentiation (taken from Verron *et al.*, 2012c).

#### *Ga action on osteoblast - osteocytes*

Only a few studies addressed Ga effects on bone-forming cells osteoblasts. In early works using rat osteogenic sarcoma (ROS) osteoblast-like cells treated with Ga, several authors reported an increase in type I collagen gene expression, but also a decrease in osteocalcin mRNA and protein levels (Bockman *et al.*, 1993; Jenis,

Waud, Stein, Lian, & Baran, 1993). Using rat primary osteoblasts, Bockman *et al.* revealed that Ga stimulated the production of fibronectin and type I collagen, but this effect was only transient (Bockman *et al.*, 1993). Lakatos *et al.* observed that Ga inhibited PTH-stimulated calcium transport in UMR-106 osteoblast model (ROS cell line), and they speculated that by reducing osteoblastic response to PTH, it is possible to decrease PTH-stimulated bone resorption (Lakatos *et al.*, 1991).

Later, it was demonstrated that Ga did not affect the viability, the proliferation rate nor the alkaline phosphatase activity in MC3T3-E1 cell line or in primary murine osteoblasts (Verron *et al.*, 2010d). In the same study, Ga treatment stimulated the expression of *Alp*, *Osx*, and *Runx2* markers in MC3T3-E1 cells.

As explained previously, at the end of a bone remodelling cycle mature osteoblasts can differentiate in osteocytes. Although they make up 90% of bone cells, at the best of my knowledge, so far no studies addressed potential Ga effects on osteocytes. This is due to the difficulty to set up a relevant *in vitro* model to study this particular bone cell type.

#### **4.4.2. *In vivo* studies of gallium effect on bone**

Most insights into *in vivo* Ga properties were obtained from normal or osteoporotic rat models. For example, studies by Ma *et al.* (Ma & Fu, 2009, 2010) showed positive Ga effects on ovariectomized osteopenic female rats, which received orally 120 µg/kg/day of Ga during eight weeks. Compared to the non-treated group, Ga-treated rats exhibited an increase in trabecular bone volume (37%), trabecular thickness (48.4%), and cortical thickness (32.2%). Reflecting an inhibition of bone resorption, a decrease in calcium and phosphate plasma levels was also observed. At the same time, the bone mineral content of the vertebrae and femur was elevated and femoral neck strength was significantly higher in Ga group (110 N) compared to control (80 N). Similarly, Stern *et al.* demonstrated that Ga administration stimulates osteoblasts activity in ovariectomy-induced osteopenia associated with low-Ca diet (Stern *et al.*, 1994). Nevertheless, this stimulation was not sufficient to prevent osteopenia bone loss in rats. In another study using organic yeast-derived Ga, it was shown that Ga can promote callus bone formation in a fracture healing model in ovariectomized rats (Pei & Fu, 2011).

On the contrary, a study by Wakley *et al.*, in normal rats, evidenced an inhibition of bone formation and apposition (32% and 36% respectively) without any significant effect on bone resorption, when 900 µg/kg of Ga was injected intraperitoneally during 21 days (Wakley *et al.*, 1992). However, at higher doses (1.8-3.5 mg/kg), Ga lowered calcium and vitamin D serum levels, and inhibited bone resorption, but also the bone formation. In another study in normal rats, Jenis *et al.*, found that a subcutaneous 3-week Ga treatment (500 µg/kg/day) decreased calcium serum levels without any significant effect on bone mineral density (Jenis, Waud, Stein, Lian, & Baran, 1993).

The discrepancy in Ga effects on bone in abovementioned studies could be due to the differences in Ga treatment duration, doses and administration mode, as well as to the fact that some studies used osteoporotic models while others were conducted in normal rats. Thus, although Ga antiresorptive activity *in vivo* is fairly evidenced in the literature, its anabolic effects on bone tissue are still controversial.

#### **4.4.3. Clinical studies of gallium therapeutic potential in bone disorders**

Ga clinical efficiency as an antiresorptive agent was first demonstrated in a pilot study by Warrell *et al.* (Warrell, Bockman, Coonley, Isaacs, & Staszewski, 1984). Patients suffering from cancer-related hypercalcaemia were treated with gallium nitrate by continuous infusion, and all subjects responded by a reduction of total serum calcium to normal or subnormal concentrations, indicating that Ga suppressed excessive bone resorption related to malignancy. The same group showed that a short-term treatment with gallium nitrate could reduce biochemical parameters of disease activity in patients with advanced Paget disease of bone (R P Warrell *et al.*, 1990). Several other clinical trials confirmed that a daily dose of 100-300 mg/kg of Ga was effective against cancer-induced hypercalcaemia resulting from an excessive bone resorption (Collery *et al.*, 2002). Matkovic *et al.* (Matkovic *et al.* 1991) conducted a study on postmenopausal women and found that Ga nitrate treatment significantly elevated serum level of alkaline phosphatase, a classical marker for bone formation. More recently, a small clinical trial, involving myeloma patients receiving chemotherapy, showed that Ga could attenuate the rate of bone loss (Niesvizky, 2003).



On the whole, *in vitro* and *in vivo* studies, as well as clinical trials, are in favour of using Ga compounds for the regulation of excessive osteoclastic activity seen in osteoporosis or in other osteolytic bone diseases. Although not yet firmly established, Ga seems to potentiate also bone formation by acting on osteoblasts.

## 4.5. Gallium and tumours

### 4.5.1. *In vitro* studies of gallium antitumor effects

Several molecules and cellular processes are identified as possible Ga targets in tumour cells. It was demonstrated that Ga could: (i) bind to phosphates and nucleic bases of DNA molecule, thus destabilizing the DNA helix; (ii) compete with magnesium for DNA binding (Manfait & Collery, 1984); (iii) induce chromatin condensation, an early apoptosis step (Haq, Wereley, & Chitambar, 1995).

When cell growth-inhibiting concentrations of Ga are applied, most of the cell biosynthesis is reduced, including the protein synthesis (Collery *et al.*, 2002). Ga was reported to decrease osteocalcin, transferrin receptor and haemoglobin levels. Conversely, it increases fibronectin and type I procollagen (Bockman *et al.*, 1993; Chitambar & Zivkovic, 1987; Guidon, Salvatori, & Bockman, 1993). Ga is also capable of interfering with different enzymes' activity, such as ATPases, DNA polymerases, ribonucleotide reductase, and tyrosin-specific protein phosphatases. In the form of chloride, Ga destabilizes microtubules and prevents their assembly (Perchellet, Ladesich, Collery, & Perchellet, 1999). Due to its chemical similarity with ferric ion, Ga can bind to several Fe<sup>3+</sup>-binding proteins and thus disrupt important intracellular pathways in which iron is implicated. Mitochondrial function can also be altered by Ga treatment. It was shown that Ga induced calcium efflux from mitochondria, a process that is recognized as a preliminary step in apoptosis (Collery *et al.*, 2002).

Some authors reported also that Ga disturbed cell cycle of human T lymphoblastic cells in a way that it provoked an accumulation of cells in S phase of the cycle (Hedley, Tripp, Slowiaczek, & Mann, 1988).

Lastly, in different tumour cell lines, a combined treatment consisting of Ga and one other well-established chemotherapeutic drug showed that Ga could circumvent drug resistance of tumour cells. These results are of particular interest for the treatment of multidrug resistant cancer cells (Collery *et al.*, 2002).

#### **4.5.2. *In vivo* studies of gallium antitumor effects**

In animal models, gallium anticancer properties were first described in a study by Hart *et al.* (Hart & Adamson, 1971), where Ga efficiently inhibited solid tumour growth in rodents. In mice, intraperitoneal administration of Ga during 10 days inhibited by 90% the growth of subcutaneously transplanted solid tumours. Ga was also effective against 256 Walker sarcoma by increasing the survival time by 138%, compared to control. In the same line, intraperitoneal Ga injection suppressed tumour growth and the formation of metastases after Lewis lung carcinoma transplantation (Capel *et al.*, 1981). On the other hand, Ga was not shown to be effective against different leukaemia types and Ehrlich carcinoma (Collery *et al.*, 2002). Carpentier *et al.* assessed the efficacy of Ga treatment in different tumour stages in mouse adenocarcinoma CA755 (Carpentier *et al.* 1987). They observed a rapid decrease in tumour volume when Ga was administered during the exponential phase of tumour growth, while the treatment during the plateau phase was less efficient.

Studies on Ga intracellular distribution in tumour cells yielded variable results. Some authors found Ga bound to ferritin and mostly localized in the nuclei, while others showed high Ga concentration in lysosomes or lysosome-like bodies. Additionally, it was reported that Ga also concentrated in lysosomes of tumour-associated macrophages (Bernstein, 1998).

#### **4.5.3. Clinical studies of gallium antitumor potential**

As a result of its antiproliferative and antimitotic properties, gallium is the second metal (after platinum) to be used in cancer treatment (Collery *et al.*, 2002). Some earlier clinical trials proved Ga efficacy against bladder and urothelial carcinomas (Bernstein, 1998). One other study of Ga influence on prostate cancer in elder patients proved only modest clinical effects (Senderowicz *et al.*, 1999), and although exhibiting no major toxicity, oral administration of Ga yielded only partial responses in ovarian cancer treatment (Collery *et al.*, 2002).

Certain studies failed to show any anti-cancer effect of gallium compounds (Collery *et al.*, 2002). Nevertheless, due to administration modalities, Ga concentration at tumours sites was probably low. Thus, poor bioavailability of Ga in tumour microenvironment probably explains the absence of Ga effect in these studies (Hall *et al.*, 1979).

It was proposed that Ga could be used to potentiate the action of chemotherapeutic agents and consequently, in phase I or II clinical studies, Ga efficacy has been tested in patients that are refractory to conventional chemotherapeutics. Indeed, its combined administration with cisplatin/vinblastine/ifosfamide indicated that Ga can potentiate their anticancer effects (Collery *et al.*, 2012, 2002). However, in some of these studies, a high degree of toxicity was observed (e.g. renal dysfunction, anaemia, granulocytopenia, thrombocytopenia, temporary blindness), probably due to the cumulative effects of administered drugs.

#### 4.6. Other potential therapeutic applications of gallium

In addition to bone and tumour sites preference, it is known that Ga also concentrates at sites of inflammation or infection, and several animal studies describe Ga immunomodulating properties.

Some authors demonstrated Ga efficacy in asthma (Apsehoff *et al.*, 1996), type I diabetes (Flynn *et al.*, 1992) in murine models. After being the first studied biological effect of gallium, the antibacterial activity of Ga compounds against several important pathogens, such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* has been demonstrated both *in vitro* and *in vivo* (Rangel-Vega, Bernstein, Mandujano-Tinoco, Garc-a-Contreras, & Garc-a-Contreras, 2015). For example, a study on murine sepsis model showed that Ga could efficiently suppress the pathogen-induced production of nitric oxide (Krecic-Shepard *et al.*, 1999). And, with a view to inhibit bacterial infections after bone substitute implantation, it was recently proposed to graft gallium and silver ions on titanium bone implants to counteract the biofilm formation (Cochis *et al.*, 2014).

Concerning immunomodulation, it was reported that, in the absence of any general immunosuppressive action, Ga suppressed adjuvant-induced arthritis

(Matkovic, 1991), experimental encephalomyelitis (Whitacre *et al.*, 1992), experimental autoimmune uveitis (Lobanoff *et al.*, 1997), and allograft rejection (Orosz *et al.*, 1996). In *in vitro* studies, Ga also inhibited T cell and macrophage activation, without inducing their cytotoxicity (Huang *et al.*, 1994); (Makkonen *et al.*, 1995); Drobyski, Ul-Haq, Majewski, & Chitambar, 1996). Lastly, in the context of organ transplantation, Ga was found to suppress acute tissue rejection of cardiac allografts and severe graft versus host disease in mice (Bernstein, 1998).

#### **4.7. Calcium phosphate biomaterials as vectors for local delivery of gallium**

Oral administration of Ga salts, such as nitrate or chloride, results in the formation of an acidic solution due to the  $\text{Ga}^{3+}$  hydrolysis and possibly induces tissue damage extremely inconvenient for drug administration. Such acidified solutions facilitate the precipitation of poorly soluble  $\text{Ga}(\text{OH})_3$ , affecting the pharmacokinetics of Ga compounds. The observed low Ga absorption upon oral administration is most probably a consequence of formation of poorly soluble Ga species in the gastrointestinal tract (Verron, Bouler, & Scimeca, 2012b). Some studies reported that the systemic administration (oral or parenteral) of gallium compounds resulted in elevated concentration of gallium in kidneys and liver, possibly generating toxicity issues together with a poor delivery of Ga to the aimed tissue. These important drawbacks related to systemic administration of Ga could be circumvented by a local delivery strategy.

Taking into account Ga anti-resorptive effects on bone tissue, as well as its potential use as an antiosteoporotic drug, calcium phosphate bone substitutes are suggested as attractive vectors for local delivery of Ga to bone. Even though osteoporosis is a systemic bone disease, most osteoporotic fractures occur in three common sites: wrist, hip and spine. This preference for particular bone sites justifies the idea of Ga delivery only to these specific sites that present high fracture risk. These sites often present low bone density or microfractures and so could benefit of: (i) new bone ingrowth induced by CaP/Ga composite; (ii) secondly, Ga antiosteoclastic properties on a local site usually displaying an excessive bone resorption (Verron, Bouler, & Scimeca, 2012b). Thus, delivering Ga locally through

CaP biomaterials is proposed as a clinical strategy to reinforce osteoporotic bone. Although preclinical research provided strong evidence on Ga antiresorptive potential, no clinical trials were conducted to assess Ga effects in the context of osteoporosis prevention or treatment.

In addition, the implantation of CaP/Ga composites could also be beneficial after bone tumour or metastases excision, due to the well-documented anticancer effects of Ga. In this context, Ga could inhibit the proliferation of eventual remaining tumours cells after the excision and thus, prevent cancer local recurrence and spreading.

More generally, a local delivery strategy would provide optimal Ga bioavailability at bone site, and help to reduce toxicity issues related to systemic administration. A study by Donnelly *et al.* analysed the properties of Ga-loaded bone particles implanted subcutaneously in rats. Particles incorporating Ga were less readily resorbed, suggesting that Ga treatment inhibited local mineral resorption activity (Donnelly, Bockman, Doty, & Boskey, 1991). The observed effect was not associated with a reduction of multinucleated osteoclast-like cells, suggesting that Ga did not interfere with their recruitment or their differentiation *in vivo*. In a recent study by Mellier *et al.* (Mellier, *et al.*, 2015) two different Ga-loaded calcium phosphate cements were implanted in rabbit femora for 4 weeks. No significant difference was observed in biological performance of these 2 cements compared to control, but the amount of resorbed biomaterial was rather low, and consequently, the quantity of released gallium was small. For the moment, these are the only two *in vivo* studies that reported on Ga local delivery.

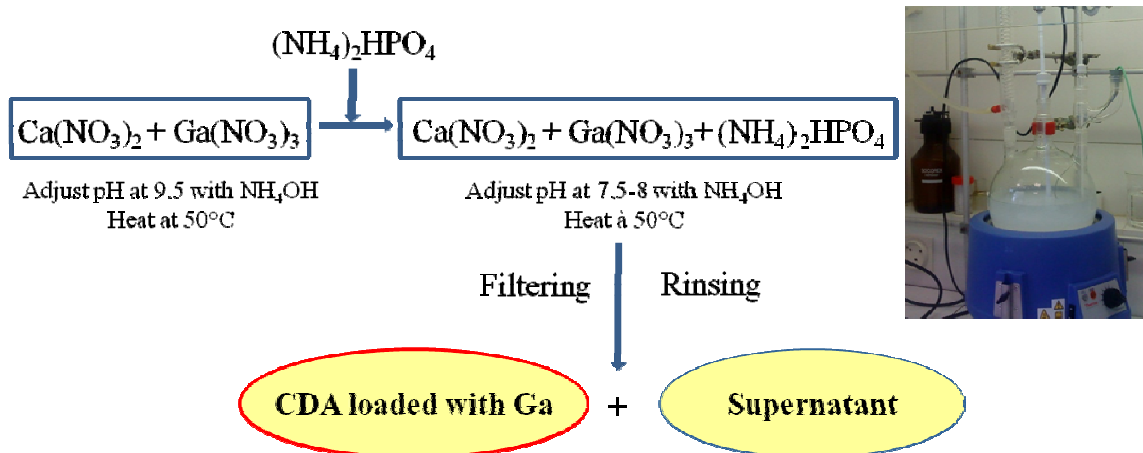
#### **4.7.1 Incorporation of gallium in calcium phosphate biomaterials**

As mentioned before, Ga can be integrated in the structure of calcium phosphate materials by partial substitution with calcium. It can be either chemisorbed onto the surface of calcium-deficient apatite or inserted in the structure of  $\beta$ -tricalcium phosphate. In terms of injectability and setting time, the addition of these gallium-doped components into a CaP cement final formulation did not significantly affect the main properties of the cement (Mellier, *et al.*, 2015). The suitable protocols for combining Ga and calcium phosphate cements, while

preserving the textural, mechanical and setting properties, have been developed recently by Mellier *et al.* (Mellier *et al.*, 2011; Mellier *et al.*, 2015). Generally, the introduction of gallium(III) ions into an injectable apatitic cement includes: (i) dissolution of a gallium salt in the liquid phase; (ii) mixing a gallium salt with the ground solid phase; (iii) chemical combination of gallium ions with one of the calcium phosphate components of the solid phase (Mellier, *et al.*, 2015).

#### **4.7.1.1 Incorporation of gallium in calcium-deficient apatite (CDA)**

Composites of gallium and calcium-deficient apatite (CDA) were synthesized by co-precipitation of a mixture of gallium nitrate hydrate and calcium nitrate tetrahydrate with diammonium hydrogen phosphate. The reaction was performed in the 9–9.5 pH range with (Ca + Ga)/P molar ratio = 1.515. Electron and X-ray diffraction (XRD) analysis of the nanostructure of precipitate confirmed that the obtained material is CDA (Charlotte Mellier, Fayon, Boukhechba, Verron, LeFerrec, *et al.*, 2015). The nanocrystalline CDA obtained by precipitation is known to possess a peculiar structure, which consists of a nearly stoichiometric apatitic crystalline core and a disordered hydrated surface layer. This hydrated surface layer, which involves labile ionic species with non-apatitic chemical environments, confers specific physicochemical properties to CDA such as capability to reversibly exchange ions and adsorb organic molecules and proteins. To investigate the nature of the gallium species in the obtained CDA, a detailed structural characterization by solid-state nuclear magnetic resonance (NMR) was performed (Charlotte Mellier, Fayon, Boukhechba, Verron, LeFerrec, *et al.*, 2015).



**Figure 13. Preparation of calcium-deficient apatite (CDA) loaded with gallium by co-precipitation.**

(obtained by courtesy of C. Mellier).

#### 4.7.1.2. Incorporation of gallium in calcium phosphate cement (CPC)

A commercially available apatitic-type calcium phosphate cement (CPC), Quickset (Graftys SARL), is used in clinical practice as a bone substitute in non-load bearing orthopaedic indications. Its liquid phase is 0.5 wt %  $\text{Na}_2\text{HPO}_4$ , and its solid phase is mainly composed of:

- 78 wt.%  $\alpha$ -TCP ( $\alpha$ -tricalcium phosphate,  $(\text{Ca}_3(\text{PO}_4)_2)$ );
- 10% DCPA (anhydrous dicalcium phosphate,  $\text{CaHPO}_4$ );
- 10 wt.% CDA (calcium-deficient apatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ );
- 2 wt.% HPMC (hydroxypropyl methyl cellulose).

Upon mixing of solid and liquid cement phases, the basic driving force of the setting reaction is the transformation of  $\alpha$ -TCP into CDA (Mellier, *et al.*, 2015). Due to the fact that Quickset cement is already successfully used as a bone substitute in clinical practice, it was chosen as a substrate for Ga incorporation. In the preparation of Ga-loaded cement, the replacement of CDA by Ga-loaded CDA (CDA/Ga) extended the setting time of the cement proportionally to the quantity of incorporated Ga. This observation suggested that the release of some Ga species might alter the setting

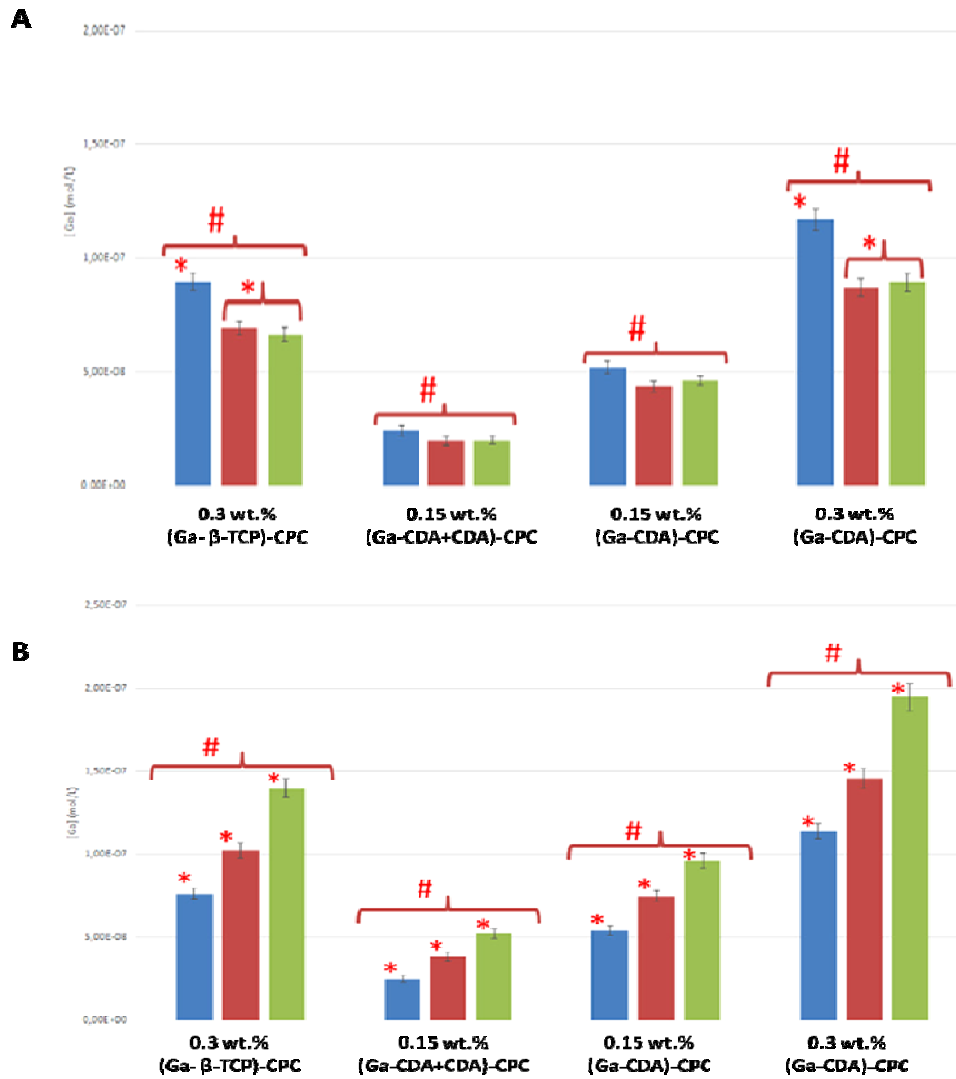
reaction. Interestingly, in comparison to standard preparation protocol, slightly higher compressive strengths of the cement were obtained when CDA/Ga was used as a solid phase in cement preparation (Charlotte Mellier, Fayon, Boukhechba, Verron, LeFerrec, *et al.*, 2015). Indeed, comparative scanning electron microscopy (SEM) observations of hardened cement samples showed that, during the cement setting process, the presence of CDA/Ga in the cement composition led to the formation of significantly larger CDA crystals. This might result from the slower rate of the setting reaction observed for these samples, together with a texture of higher fluidity, compared to the standard cement without Ga (Mellier, *et al.*, 2015).

#### **4.7.2 *In vitro* release of gallium from calcium phosphate cement (Quickset)**

*In vitro*, chemical dissolution of Ga-loaded calcium phosphate cement (CPC/Ga) in the cell culture medium was shown to release Ga species at relatively low concentration. Using ICP-MS (inductively coupled plasma–mass spectrometry) analysis, this release was estimated to correspond to less than 0.001% of the total amount of gallium initially loaded in the cement. This is not surprising since the limited porosity of the material probably confined the process only to the cement surface. In addition, it cannot be excluded that, once released from the CPC, part of the gallium species precipitate. More generally, Ga release was found to increase in direct relation with the amount of Ga initially loaded in the cement (Mellier, *et al.*, 2015). Ga release by active cellular resorption from CPC/Ga was also studied using murine RAW 264.7 cells seeded on the surface of the cement and stimulated with 20 nM RANKL. In sharp contrast to the situation where no cells were seeded on cement surface, an increase of Ga release was observed in the presence of RAW 264.7 cells (Figure 14). This gradual release over a period of 7 days is very likely due to the osteoclastic resorption activity that increased with the maturation of RAW 264.7 cells.

As a whole, these results strongly suggest that Ga release from CPC might occur from both passive chemical dissolution and active cellular resorption processes (Mellier, *et al.*, 2015).





**Figure 14. *In vitro* release from Ga-loaded calcium phosphate cement.**

Measurements of gallium ions in the cell culture media show that gallium can be successfully released from gallium-loaded calcium phosphates cement pellets (different chemical components of cement ( $\beta$ -TCP or CDA) are loaded with different amounts gallium (0.3 or 0.15 wt.%)). A) Without cells; B) with RAW 264.7. Blue histograms represent measurements at day 2, red at day 4, green at day 7. (Taken from Mellier *et al.*, 2015).

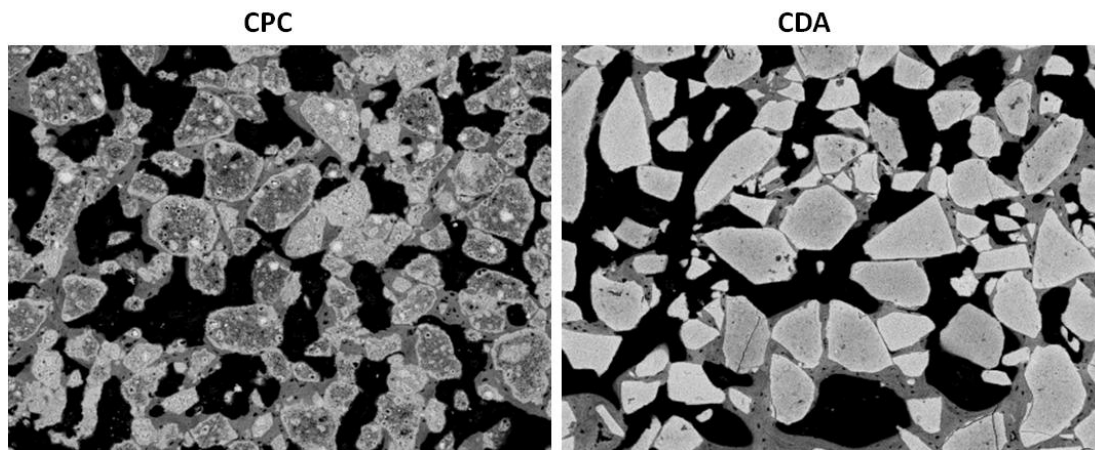
## 5. Second-harmonic generation microscopy (SHG)

### 5.1. Current quantitative assessment methods in bone tissue engineering and their limitations

**Two-dimensional histomorphometry** of tissue slides remains the gold standard in bone tissue analysis, despite the fact that this time-consuming and destructive method is limited to a very small percentage of the sample that can be taken for quantitative analysis.

**Micro-computed tomography (micro-CT)** is a non-destructive technique, based on X-ray absorption, which is widely used for analysis of three-dimensional bone structure. Compared to classical 2D histomorphometric analysis, it is far more appropriate for assessment of structural parameters of bone, e.g. plates and rods dimensions, connectivity, and thickness (Peyrin, 2011). Micro-CT scans can be performed at high resolutions, such as few  $\mu\text{m}$ , allowing the detailed study of tissue microarchitecture in healthy and pathological bone. The 3D quantitative micro-CT analysis enables the characterization of disease processes and therapeutic agent effects in bone. It has been useful for the assessment of bone reconstructive properties of different kinds of biomaterials in the field of bone tissue engineering. In the particular case of calcium phosphate-based (CaP) biomaterials, their high chemical composition similarity with biological apatite results in the same X-ray attenuation coefficients and therefore, to very similar micro-CT images. This arises difficulties in manual thresholding between biomaterial and bone mineral phase (Peyrin, 2011). Even though some groups reported on automated thresholding of micro-CT images (Polak *et al.*, 2012; Tuan & Hutmacher, 2005), to date this approach remains limited to a small number of scaffolds, and is not widely applicable for CaP biomaterial-bone tissue segmentation.

In contrast, **scanning electron microscopy (SEM)** is a commonly used technique that provides unambiguous distinction between CaP biomaterials and bone tissue (Figure 16). SEM can achieve very high resolution of only few nm. However, this approach requires sample surface coating with a heavy metal or carbon, which is expensive and also restricts the imaging to the surface, thus allowing only for 2D analysis as in histomorphometry (Ambekar *et al.*, 2012).



**Figure 16. Scanning electron microscopy images of CaP and CDA 80-200  $\mu\text{m}$  microparticles in a cavitory femoral defect in rat.**

At 2 months post-operatively, microparticles are surrounded by zones of newly formed bone (our unpublished data).

## 5.2. The principle and simplicity of SHG

Non-linear multiphoton optical microscopy has gained a lot of interest in recent years, due to its unique ability of in-depth imaging of thick biological samples. Thus, it permits the observation of complex structures with a micrometric 3D resolution (Beaurepaire *et al.*, 2007). These techniques include two-photon excited fluorescence (2PEF), as well as second- and third-harmonic generation (SHG and THG respectively) (Beaurepaire *et al.*, 2007).

Physical features of certain molecules and molecule assemblies determine if they can produce a second-harmonic generation phenomenon, and the first requirement for SHG is a lack centro-symetry. In addition, these molecules/assemblies have to fulfil the phase-matching condition, which means that the incident and the resulting second harmonic waves travel at the same velocity. Only materials with a high degree of organization and orientation meet this requirement, i.e. anisotropic crystals or certain structural protein arrays that are called harmonofors. Thus, SHG is a highly specific phenomenon, which is produced by a limited number of biological structures including type I collagen fibers, myosin II, and tubulin (Vielreicher *et al.*, 2013). So far, the most important use of SHG was for collagen organisation detection in diseases such as sclerodermia and epithelial cancer types. Imaging type I collagen by SHG microscopy requires excitation laser wavelengths from 720-960 nm,

while the generated SHG signal will be exactly half of the excitation wavelength. This signal originates from intrinsic properties of the molecule and no additional staining or labelling is needed. Other convenient characteristics of SHG are that this technique is non-destructive, and that no special sample preparation protocol is required (Vielreicher *et al.*, 2013).

### 5.3. SHG application in bone tissue imaging

Type I collagen is the major organic constituent of bone matrix. Collagen molecules (tropocollagen), mineralized with hydroxyapatite crystals, represent mineralized collagen fibrils that are the basic structural units of bone. These mineralized fibrils are bundled into fibers of 2–3  $\mu\text{m}$  diameters that form woven or lamellar bone structures. Woven bone, found in the developing embryo and very young bone, is less organized and contains randomly distributed fibers. During bone maturation, woven bone is eventually resorbed and replaced by lamellar bone, which is made up of preferentially oriented collagen fibers, organized in 3–7- $\mu\text{m}$  thick lamellae. Alterations to collagen's chemical composition and structural organization have serious implications on bone strength. This is the case for osteoporosis, osteogenesis imperfecta, and osteosarcoma. The ability to carry out both a qualitative and quantitative assessment of the composition and structural organization of collagen fibers in bone is critical for understanding its health and pathogenesis, and it could be used in early diagnosis of bone disorders (Ambekar *et al.*, 2012).

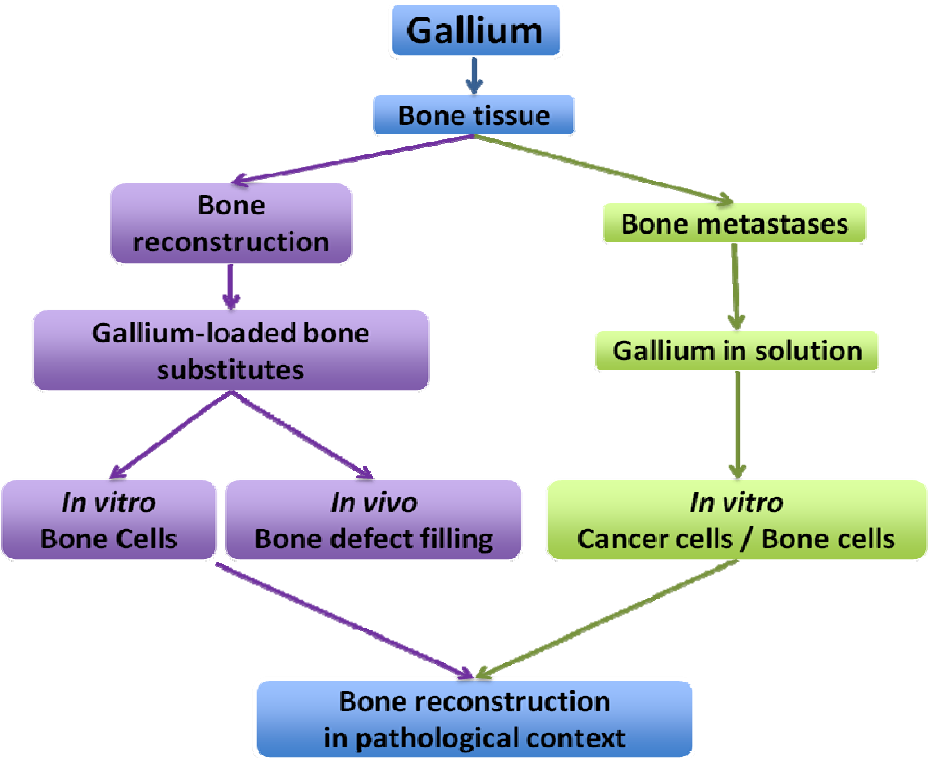
SHG microscopy's ability to image predominantly collagen fibers has been experimentally validated by numerous studies using techniques such as immunofluorescence-stained light microscopy and polarized light microscopy (Ambekar *et al.*, 2012). To my knowledge, its potential has not yet been fully explored in bone imaging and the study of age-related structural alterations, with the exception of the vertebral endplate in the human lumbar spine (Paietta, Burger, & Ferguson, 2013).



## **II THESIS GENERAL OBJECTIVES**



Considering data available in the literature, as well as previous work performed in the laboratory, it is well-established that gallium (Ga) displays several potentially beneficial properties for bone remodelling in pathological context. In the present thesis, this general idea of using Ga in bone pathologies treatment was addressed from two different angles: (i) investigation of antiresorptive properties of Ga in the context of bone tissue reconstruction, and (ii) investigation of Ga anti-neoplastic properties in bone metastases model.



**Figure 15. Gallium biological properties were assessed in the view of bone reconstruction and bone metastases.**

Ga antiresorptive properties were studied by using calcium phosphate-based bone substitutes that were loaded with Ga. We sought to evaluate a possible Ga impact on human primary bone cells *in vitro* and these experiments were extended to *in vivo* implantations of Ga-loaded substitutes to document potential Ga action on bone tissue regeneration within a critical-sized femoral defect in rat.

Experiments assessing Ga anti-neoplastic features were performed using human breast carcinoma cells and their bone-seeking clone. These cancer cells were cultured alone or in combination with osteoclast precursor cells, in order to



determine if Ga can affect cancer cells directly and also, if it can interfere with cancer cells-induced osteoclastogenesis. Consistently with the first part of the thesis, future developments will address the concept of using Ga-loaded bone substitutes to fill a defect generated by a bone tumour resection.

The context and particular objectives of each of these approaches are briefly summarized before the two publications in preparation, and, in a more detailed manner, in the Introduction part of publication manuscripts.

## **III RESULTS**



## PART ONE A:

# *In vitro* and *in vivo* effects of gallium-loaded calcium phosphate biomaterials on bone cells and bone reconstruction

### 1. Context and objectives of the study

In osteoporosis, one of the most common bone disorders, bone remodelling balance is shifted towards osteoclastic bone resorption, resulting in the weakening of bone structure and an increased risk of fracture. In osteoporotic fractures treatment, bisphosphonates are the most widely used therapeutic agents. Despite a proved clinical efficiency, important side-effects inducing also a poor patient compliance have prompted the investigation of alternative antiresorptive agents.

Due to its inhibitory effect on osteoclastic bone resorption, the semi-metallic element gallium (Ga) has been used in systemic treatment of malignancy-induced hypercalcemia. In their vast majority (80%), osteoporotic fractures occur at specific skeletal sites (femoral neck, spine and wrist), this opening the possibility for their local treatment and prevention. Mellier *et al.* have shown that Ga readily incorporates in the structure of calcium phosphate biomaterials (CaP), allowing the implantation of these bone substitutes within fragilized osteoporotic sites. By doing so, it is expected that intrinsic properties of CaP would restore mechanical stability at the implantation site, while loaded Ga could locally reduce osteoclastic resorptive activity. Thus, investigating CaP-Ga biological properties is crucial in order to propose Ga as an attractive adjuvant candidate for the management of osteoporotic fracture.

In the present study, we wanted to document *in vitro* the interactions of CaP-released Ga with bone cells, as well as its performances *in vitro* in terms of bone defect reconstruction. Specific objectives were to determine whether Ga affected: (i) the viability, morphology and differentiation of human primary bone cells; (ii) bone reconstruction induced by CaP biomaterials in a critical-sized femoral defect in rats.

## **2. Methods and results**

Article I (in preparation): Incorporation of gallium enhances reconstructive properties of a calcium phosphate bone substitute

# Incorporation of gallium enhances reconstructive properties of a calcium phosphate bone substitute

Abbreviated title: **Gallium stimulates bone reconstruction**

Ivana Strazic <sup>a, b, c, d</sup>, Nicolas Melis <sup>a, b, c, §</sup>, Florian Boukhechba <sup>a, b, c, d</sup>, Sébastien Schaub <sup>a, b, c</sup>, Charlotte Mellier <sup>d</sup>, Pascal Janvier <sup>e</sup>, Jean-Pierre Laugier <sup>f</sup>, Nathalie Rochet <sup>a, b, c</sup>, Jean-Michel Bouler <sup>g</sup>, Elise Verron <sup>e, ¶</sup>, Jean-Claude Scimeca <sup>a, b, c, ¶, \*</sup>

<sup>a</sup> Université Nice Sophia Antipolis, iBV, UMR7277, 06100 Nice, France

<sup>b</sup> CNRS, iBV, UMR7277, 06100 Nice, France

<sup>c</sup> Inserm, iBV, U1091, 06100 Nice, France

<sup>d</sup> GRAFTYS SA, 13854 Aix en Provence, cedex 3, France.

<sup>e</sup> LIOAD, Université de Nantes, Inserm UMR791, BP84215, 44042 Nantes, France.

<sup>f</sup> CCMA, Université Nice Sophia Antipolis, BP71, 06108, Nice, France.

<sup>g</sup> CNRS, CEISAM, UMR6230, 44322 Nantes, France.

**§ Present address:** Université Nice Sophia Antipolis, CNRS, LP2M, UMR7370, 06107 Nice cedex 2, France

**¶:** EV and JCS contributed equally to the supervision of the study

**\* Correspondence:** Jean-Claude Scimeca; Université Nice Sophia Antipolis, institut de Biologie Valrose, iBV, UFR Médecine, 28 av de Valombrose, 06107 Nice cedex 2, France. Email: scimeca@unice.fr; Phone: +33 493-377-724; Fax: +334 9337 7750

## **ABSTRACT**

Calcium phosphate-based (CaP) biomaterials are commonly used in bone reconstructive surgery while, due to its inhibitory action on osteoclasts, the semi-metallic element gallium (Ga) is used for the treatment of disorders associated with accelerated bone resorption. As it was demonstrated that Ga could be incorporated in the structure of CaP biomaterials, we investigated in the present work the biological properties of novel Ga-substituted CaP biomaterials. Culturing bone cells on Ga-CaP, we observed a decrease in osteoclast number and a downregulation of late osteoclastic markers expression, while Ga-CaP upregulated the expression of several osteoblastic marker genes. Using a murine bone defect-healing model, we next investigated *in vivo* bone reconstructive properties of Ga-CaP. All implanted biomaterials showed a good osseointegration into the surrounding host tissue, accompanied by a successful bone ingrowth and bone marrow reconstruction. Lastly, quantitative analysis of implants revealed that Ga enhanced total defect filling (3D micro-CT), as well as mature organised collagen synthesis (SHG). Taken together, our data indicate that Ga-CaP provides biocompatible and non-cytotoxic substrates for bone reconstruction. Moreover, acting both on osteoclasts and osteoblasts, Ga enhances new bone formation triggered by CaP bone substitutes.

### ***Key Words***

Bone reconstruction

Calcium phosphate cement

Gallium

Osteoclast/osteoblast crosstalk

Second Harmonic Generation (SHG) analysis

## **1. Introduction**

In the field of bone tissue engineering, major efforts are currently focused on the development of novel biomaterials usable for bone reconstruction. CaP biomaterials are considered to be the best alternative to bone grafting because of their chemical properties, which are suitable for bone remodelling [1-7]. In fact, CaP is the main component of bone and is a biocompatible and biodegradable biomaterial. Various forms of CaP biomaterials have been developed (granules, injectable suspensions, cements) that are suitable for different clinical applications including orthopaedics, and dental, ear, nose and throat surgeries. Among CaP biomaterials, CaP cements (CPCs), based on studies performed originally by LeGeros in 1982 [8] and Brown and Chow in 1986 [9], has considerably revolutionized the world of bone tissue engineering. To restore bone mechanical properties, an ideal biomaterial should be slowly resorbed while concomitantly replaced by natural bone. The concept of CPCs [10] as possible restorative materials might fulfil this requirement due to their bioactivity and ability to be resorbed. Optimized formulations of injectable bioresorbable CPCs have been developed for dental and orthopaedic indications, and these products are commercialized in both EU and US markets since 2009.

Apatite cements are constituted of entangled microcrystals that are closer to biological apatites in terms of size and composition than hydroxyapatite ceramic particles [11-13]. One of the major advantages of CPCs is their moldability. CPCs can fit perfectly into the implant site, thus optimizing bone-biomaterial contact, even in geometrically complex bone defects. Moreover, due to their properties of injectability, CPCs are considered to be promising materials for minimally invasive surgery. After implantation into the bone defect, CPCs are able to self-set [14] and harden without any significant exothermic reaction that could be prejudicial to the bone tissue and/or loaded drug. CPCs rapidly integrate into the bone structure and are then progressively resorbed, mainly by cellular processes. Thus, CPC combined devices loaded with active molecules represent good candidates for local drug delivery [15].

In the early 1970s, it was demonstrated that Ga had several therapeutic effects (for review see [16]) including: (i) the decrease of accelerated bone mineral resorption, which occurs during osteolytic



bone diseases, and the subsequent lowering of elevated plasma calcium levels associated with these pathologies; (ii) the inhibition of neoplastic proliferation; (iii) the treatment of some intracellular pathogens such as species of *Mycobacterium* [17]. Studies on Ga, which were mainly focused on its antitumor activities [18], have shown that Ga caused a mild hypocalcaemia [19]. In this context, Ga has been used to treat hypercalcemia resulting from malignant tumours such as parathyroid carcinoma and Paget's disease of bone [20-22]. This antihypercalcemic effect of Ga results from an inhibition of bone resorption [20, 23]. Indeed, due to its chemical characteristics, Ga presents an affinity for biological apatite that explains the presence of Ga deposits in bone tissue, and preferentially at sites of rapid bone remodelling such as active metaphyseal growth plate and healing fractures [24-26].

Few studies have explored the effect of Ga on bone cells. Hall et al. demonstrated that Ga inhibited bone resorption in a dose-dependent manner, and for example, at 100 µg/mL, Ga reduced the osteoclastic resorption activity by 64% without inducing modifications to the morphology or number of osteoclasts [27]. In contrast to these findings, Blair et al. showed a cytotoxic effect of Ga on osteoclasts [28]. Considering this discrepancy, we previously investigated the biological effect of Ga on bone cells. We reported that Ga used in solution inhibited both the differentiation and the resorbing activity of osteoclasts in a dose-dependent manner (0-100 µM) [29, 30]. Furthermore, Ga did not affect the viability or proliferation of osteoblasts [29].

In addition to the medical applications reported above, a potential application of gallium compounds as anti-inflammatory agents has been described. Transferrin-gallium and gallium nitrate were shown to inhibit the mixed lymphocyte reaction and increase the survival of mice with severe graft-versus-host disease in a murine bone marrow transplant model [31]. Interestingly, gallium nitrate has been shown to prevent adjuvant inflammatory arthritis through suppression of macrophage function and T-cells in rat models [32]. Other studies showed that gallium nitrate suppressed lupus in MRL/lpr mice, a murine model of systemic lupus erythematosus (SLE) [33-35]. At last, and more recently, the elimination of arthritis pain and inflammation for over 2 years with a single 90 min, topical 14% gallium nitrate treatment was reported [36].

As a whole, these data strongly support the therapeutic potential of Ga compounds [37]. With a view to evaluate the impact of Ga addition on bone tissue engineering, we used apatitic matrices loaded with Ga [38] as a new model to document Ga action on bone cells *in vitro* and on bone tissue reconstruction *in vivo*.

## 2. Materials and methods

### 2.1 Biomaterial synthesis and preparation

Graftys SARL (Aix-en-Provence, France) provided all biomaterials used in this study. Calcium phosphate cement (CPC) consisting of 80%  $\alpha$ -tricalcium phosphate ( $\alpha$ -TCP), 10% dicalcium phosphate anhydrous (DCPA) and 10% calcium-deficient apatite (CDA) was loaded with gallium (Ga) by chemical substitution with  $\text{Ca}^{2+}$  ions so that the final Ga content was 0.3%. The same composition of CPC without Ga was used as a control. Calcium-deficient apatite loaded with gallium (CDA/Ga) was synthesized as previously described in Mellier *et al.* [38]. Briefly, CDA/Ga was synthesized from a mixture of gallium nitrate hydrate, calcium nitrate tetrahydrate and diammonium hydrogen phosphate with  $(\text{Ca}+\text{Ga})/\text{P}$  molar ratio at 1.51 and  $\text{Ga}/\text{Ca}$  molar ratio in the 0-0.08 range. X-ray powder diffraction (XRD) patterns were recorded to verify the level of gallium in the obtained powder (Philips PW 1830 (Cu K $\alpha$ , 40 kV, and 30 mA) equipped with a vertical goniometer PW 1050 ( $\Theta/2\Theta$ ) and PW 1711 Xe detector). CDA without gallium was used as a control. For *in vitro* experiments, the powder phase of CPC or CPC/Ga was mixed with 5% aqueous solution of  $\text{Na}_2\text{HPO}_4$  (Merck, Germany) in ratio 0.45, according to manufacturer's instructions. The obtained paste was placed in silicon moulds and set at 37°C in physiological saline for 72h. Solidified pellets with surface of approximately 0.5 cm<sup>2</sup> were polished and cleaned from dust three times for 15 sec in ultrasound bath. Pellets were steam-sterilized at 121 °C for 20 min and incubated 48 hours prior to cell seeding in 1 ml of  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) (Sigma-Aldrich, USA) supplemented with 10% Hyclone FCS (GE Healthcare, USA), 100 U/ml of penicillin (Lonza, Switzerland), 100 U/ml of streptomycin (Lonza, Switzerland) and 2% of ultraglutamine 1 (Lonza, Switzerland). Medium was replaced three times to neutralize the pH and ion exchange. For *in vivo* experiments, the powder phase of CPC, CPC/Ga, CDA and CDA/Ga was compacted, grinded

and water-sieved to retrieve microparticles of 80-200 µm diameters. Microparticles were further heat-sterilized at 180°C for 3 h and placed in 1-ml syringes (Therumo Europe N.V., Belgium) before implantation.

## *2.2 Cell culture*

Both cell types used in this study were cultured in α-MEM medium supplemented with 10% Hyclone FCS, 100 U/ml of penicillin, 100 U/ml of streptomycin and 2% of ultraglutamine 1 with the medium replacement every third day. Human primary osteoblasts (#CC-2538, Lonza, Switzerland) were expanded to P<sub>7</sub> at 6 000 cells/cm<sup>2</sup>. For experiments, 100 000 cells/pellet were seeded. Human primary monocytes were isolated from whole blood samples of healthy individuals (provided by Etablissement Français du Sang) using Uni-SepMaxi tubes (NOVAMED Ltd., Israel) and Easy Sep Human Monocyte Enrichment Kit (STEMCELLS Technologies, Canada) according to manufacturer's instructions. For monoculture experiments, 200 000 cells were seeded on plastic or biomaterial pellets. Osteoclast differentiation was triggered by adding 66 ng/ml of receptor activator of NFκB ligand (RANK-L) (Peprotech, France) and 33 ng/ml of macrophage-colony stimulating factor (M-CSF) (Peprotech, France) in culture medium. For co-culture experiments osteoblasts were seeded at 100 000 cells/pellet and four hours later, monocytes were added at 200 000 cells/pellet.

## *2.3 Cell viability assays*

At days 3, 6 or 14 of culture, cells were rinsed two times in phosphate-buffered saline (PBS) (Lonza, Switzerland) and incubated in 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) for 1h in cell incubator. MTT was aspirated delicately and 0.3 ml of lysis buffer (SDS 10%, HCl 0.01 N) was added/well and left overnight at room temperature, in dark. Next morning, supernatants were centrifuged 2 min at 20 000 g and absorbance was measured in 100 µl at 562 nm. In parallel experiments, using increasing amounts of cells cultured on plastic or pellets, we verified the relationship between cell number and MTT assay result (data not shown).

## *2.4 Tartrate-resistant acid phosphatase (ACP5) staining*

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After 7 or 14 days of culture, cells were rinsed two times in PBS and fixed in citrate/acetone solution for 30 s. ACP5<sup>+</sup> cells were revealed using the Acid Phosphatase, Leukocyte kit following manufacturer's instructions (# 387A; Sigma-Aldrich, USA).

#### *2.5 Gene expression analysis*

At day 7 and 14, cells were rinsed 2 times in PBS and incubated in 0.25 % trypsin/EDTA (Lonza, Switzerland) at 37 °C for 5 min. Total RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel, Germany) following manufacturer's instructions and RNA concentration was measured on Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). 150 ng of RNA was used as a template for cDNA synthesis using random primers and SuperScript II reverse transcriptase (Invitrogen, USA) in Flexcycler (Analytik Jena, Germany). Total cDNAs were diluted ten times and 5 µl of template was used for quantitative real-time PCR assay (StepOne Plus, Life Technologies, USA). Reactions were performed in a 20-µl final volume with qPCR mastermix GoTaq (Promega, USA). Amplification conditions were as following: 2 min at 50 °C, 10 min at 95 °C, 40 cycles (15 s at 95 °C, 1 min at 60 °C). The expression of genes of interest was normalized to ribosomal protein large P0 (*RPLP0*) gene expression, quantified by comparative  $2^{-\Delta Ct}$  method and expressed as a fold change compared to control condition without Ga. Primers used in qPCR are listed in Table 1. Gene symbols (abbreviations) are as follows: *ACP5*, Acid Phosphatase 5 Tartrate Resistant; *ALPL*, Alkaline Phosphatase; *BGLAP*, Bone Gamma-Carboxyglutamate (Gla) Protein (Osteocalcin); *COL1A1*, Collagen Type I, Alpha 1; *CTSK*, Cathepsin K; *FBJ*, c-Fos; *IBSP*, Integrin-Binding Sialoprotein; *ITGB3*, Integrin, Beta 3; *JDP2*, Jun Dimerization Protein 2; *MCSF*, Macrophage Colony-Stimulating Factor 1; *MMP9*, Matrix Metalloproteinase 9; *NFATC1*, Nuclear Factor Of Activated T-Cells Cytoplasmic 1; *ON*, Osteonectin; *OPG*, Osteoprotegerin; *OPN*, Osteopontin; *POSTN*, Periostin Osteoblast Specific Factor; *RANKL*, Receptor Activator Of Nuclear Factor Kappa-B Ligand; *RPLP0*, Ribosomal Protein Large P0; *RUNX2*, Runt-Related Transcription Factor 2.

#### *2.6 Field-emission scanning electron microscopy analysis (MEB)*

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After an overnight fixation at 4°C in a buffered glutaraldehyde solution, samples were rinsed, dehydrated in a graded ethanol series, immersed in hexamethyldisilazane (Sigma-Aldrich) for 5 min, and dried at room temperature. The samples were then mounted on aluminium stubs and sputter coated with gold-palladium (Cressington, 308R, UK). Examination was performed using a field-emission scanning electron microscope (FESEM JEOL 6700F, Tokyo, Japan).

#### *2.7 Orthotopic implantation in rat*

All animal procedures obtained the approval of local animal health care committee (CIEPAL NCE/2012-69) and were conducted in accordance to European Community guidelines for the care and use of laboratory animals (Directive 2010/63/UE). Bilateral femoral implantations were performed on 17 male Lewis rats weighing approximately 300 g (Janvier Labs, France), in aseptic conditions under general anaesthesia induced by inhalation of isoflurane 5% and maintained by intramuscular injections of xylazine 10 mg/kg and ketamine 50 mg/kg. 60 mg of CPC, CPC/Ga, CDA or CDA/Ga microparticles were extemporaneously coagulated with 100 µl of previously prepared rat platelet-poor plasma, 10 µl of 2 % CaCl<sub>2</sub>, 2 H<sub>2</sub>O (Merck, Germany) and 1 U of thrombin (Sigma-Aldrich, USA). After at least 15 min, coagulated microparticles were implanted in a 3 x 2.7 mm cylindrical critical-sized defect at the distal epiphyso-metaphysal junction in rat femora. At the end of the experiment animals were sacrificed by inhalation of CO<sub>2</sub> and the bone specimens were retrieved immediately and stored in 70 % ethanol.

#### *2.8 Three dimensional (3D) microtomography analysis*

Physiological saline-immersed rat femora were scanned with Skyscan 1172 microtomograph (Bruker, USA) equipped with 12-bit digital CCD camera. Samples were placed with the axis of the femur perpendicular to the plane of scanning and cross-section images are acquired under 90 kV voltage and 88 µA current with 1-mm aluminum filter. Rotation step was set at 0.7° and exposure time per step was 776 ms. 3D reconstruction of 6 µm-resolution cross-section images was done in NRecon software (Bruker, USA) using a modified Feldkamp cone-beam algorithm, as provided by manufacturer. The parameters were set as following: smoothing at 3, ring artifact correction at 10,

beam hardening correction at 50%, rotation angle 180 ° and image conversion values in 0.003-0.03 ranges. To quantify bone regeneration and biomaterial degradation in the defects, the region of interest (ROI) was set to whole implant area excluding the cortical part. Regenerated bone was discriminated from biomaterial microparticles by manual grey scale thresholding in CTAn (Bruker, USA) performed by three independent operators.

### *2.9 Histological preparation and analysis*

CPC and CPC/Ga samples were fixed for 24 h in neutral formalin solution (pH 7.2), and then dehydrated in baths with ethanol concentration increasing from 70% up to 100 % for (3 days/bath). Bone specimens were then infiltrated and embedded in methyl methacrylate (MMA) as previously described [39]. CDA and CDA/Ga samples were fixed for 24 h in neutral formalin solution (pH 7.2), and then decalcified in 10% EDTA (pH=8) for four weeks. For CPC and CPC/Ga implants, undecalcified serial 5- $\mu$ m sections were cut using a hard tissue microtome (Supercut 2050, Reichert-Jung, Austria) equipped with a D profile tungsten carbide knife. From these sections, haematoxylin/eosin (HE) and Movat's pentachrome staining were assessed to visualize mature bone, osteoid tissue, bone marrow and residues of biomaterial microparticles. Femurs with CDA and CDA/Ga implants were embedded in paraffin and 5- $\mu$ m thick sections were prepared and stained with HES. All histological sections were imaged by Nikon Eclipse 80i for qualitative observations.

### *2.10 SHG preparation and analysis*

Second harmonic generation (SHG) microscopy has intrinsic specificity for collagen fibres, which results in high contrast images, with no additional staining needed [40]. Thus, tissue sections prepared for histological study were also used for SHG imaging (Zeiss LSM 710 microscope, Germany). Mai Tai laser excitation wavelength was set to 880 nm and the SHG signal was detected in transmission and reflection mode. For CPC and CPC/Ga, 5- $\mu$ m sections were qualitatively analysed and for CDA and CDA/Ga, 100- $\mu$ m sections were prepared in order to do quantification as well. Quantitative comparison of collagen signal between Ga and control implants was performed on whole implant

area using an in-house quantification tool developed in Matlab programming language by S. Schaub from iBV PRISM platform (Platform of Resources in Imaging and Scientific Microscopy).

### 2.11 Statistical analysis

*In vitro* results are expressed as a mean  $\pm$  standard deviation of three independent experiments each performed in triplicate. Differences between gallium and the control group were evaluated by bi-directional Mann and Whitney U test with the risk threshold set at 5 %. For microtomography experiments, significant differences in total defect filling between Ga and control groups were determined by bi-directional Kruskal-Wallis one-way analysis of variance with the risk threshold set at 5 %. Fisher's combined probability test (confidence interval set at 95%) was used in assessment of the differences in biomaterial particles and newly formed tissue percentages between Ga and control group. For SHG quantitative analysis, bi-directional Mann and Whitney U test with the risk threshold set at 5 % was used to assess significant differences between Ga and control biomaterials.

## 3. Results

### 3.1 Human primary bone cells culture on apatitic matrices loaded with Ga

Human primary monocytes were seeded on CPC or CPC/Ga pellets, and cultured for 3 or 6 days before performing an MTT assay. In parallel experiments (not shown), we verified that MTT assay results correlated with the number of viable cells seeded on apatitic matrices. As shown in Fig. 1A, and compared to CPC control condition, seeding monocytes on CPC/Ga resulted for both time in a slightly decreased cell viability/number. We also performed cell structure examination using MEB analysis. As depicted in Fig. 1B, we observed monocytes on CPC and CPC/Ga (white arrowheads), as well as cell processes interacting with the cement surface. In the presence of the osteoclastogenic agent RANKL (Receptor activator of nuclear factor kappa-B ligand), some cellular interactions foreshadowing osteoclastic differentiation were also observed. However, these events remained rare for both cements. Lastly, we performed RT-qPCR experiments to quantify the expression of early and late osteoclastogenesis marker genes (gene symbols are defined in Materials and Methods). As shown in Fig. 1C, RANKL induced the differentiation of monocytes seeded on plastic, as attested the

expression of early (*NFATC1*, *JDP2*) and late (*ACP5*, *MMP9*, *CTSK*) marker genes. By contrast, RANKL failed to induce any osteoclastic differentiation of monocytes seeded either on CPC or CPC/Ga. We also observed that, whatever the condition (control or RANKL), the expression of the transcription factors *JDP2* and *FBJ* was diminished on CPC/Ga, while the late marker genes *MMP9* and *CTSK* were induced in the presence of Ga. As a whole, this first set of experiments indicated that human primary monocytes did not efficiently differentiate into osteoclasts when seeded alone on CPC or CPC/Ga.

We next seeded human primary osteoblasts on CPC or CPC/Ga and we quantified cells viability/number. As shown in Fig. 2A, after 3 days of culture, the presence of Ga induced a 40% inhibition of cell viability/number. At day 7, cellular proliferation was observed on both cements, while the inhibition observed in the presence of Ga tended to decline (30% instead of 40%). Cells structure was also examined using MEB analysis. Whatever the cement used, and as shown in Fig. 2B, osteoblasts proliferated and interacted with the surface of the biomaterial. Lastly, we used RT-qPCR experiments to quantify the expression of osteoblast marker genes (gene symbols are defined in Materials and Methods), and three kinds of situation were observed (Fig. 2C). Firstly, *RUNX2*, *ALPL* and *OPN* were expressed at similar levels at both times. Considering the cement, the expression of these three genes was inhibited on CPC/Ga at day 3, and this inhibition tended to decrease at day 7. Secondly, *BGLAP* expression increased between day 3 and day 7, and no difference was observed comparing CPC and CPC/Ga. Thirdly, *COL1A1*, *ISBP*, *ON* and *POSTN* expression was identical on CPC or CPC/Ga at day 3. At day 7, the expression level of the four genes decreased on both cements but remained at higher levels on CPC/Ga compared to CPC (or stayed identical for *POSTN*). As a whole, these data indicated that following 7 days of primary osteoblasts culture on cements, the expression level was slightly diminished on CPC/Ga for *RUNX2* and *OPN*, was identical for *ALPL* and *BGLAP*, and was enhanced on CPC/Ga for *COL1A1*, *ISBP*, *ON* and *POSTN*.

To document monocyte/osteoblast interactions, human primary osteoblasts and human primary monocytes were then co-cultured up to 14 days on both cements, in the absence of any additional effectors such as MCSF and RANKL. As depicted in Fig. 3A, cell viability/number decreased between day 3 and day 7, and in a lesser extent between day 7 and day 14. Except for day 3, no difference was observed between the two cements. Regarding cellular morphology (Fig. 3B), we observed



osteoblasts on both surfaces (white arrowheads), as well as monocytes differentiating into osteoclasts (red arrowheads). We next performed histochemical demonstration of ACP5 activity. As shown in Fig. 3C (left panels), osteoblasts were stained in yellow/brown (white arrowheads) and no difference was observed on CPC/Ga compared to CPC. Regarding osteoblasts/monocytes co-culture, a purple staining was observed within osteoclasts (right panels, red arrowheads) on both cements, but the number of differentiated cells was decreased on CPC/Ga compared to CPC. To further document this inhibition at the molecular level, we eventually performed RT-qPCR experiments (gene symbols are defined in Materials and Methods) using RNAs prepared from osteoblasts alone or from osteoblasts co-cultured with monocytes (Fig. 3D). Concerning the transcription factors involved in the early steps of osteoclastogenesis, we observed that *NFATC1*, *JDP2* and *FBJ* were expressed in both conditions (osteoblasts alone versus co-culture), with no significant differences between CPC and CPC/Ga. The same situation was observed for the late marker genes of osteoclastic differentiation *ITGB3* and *CTSK*. By contrast, the late marker genes *ACP5* and *MMP9* were detected only in co-culture conditions and not in osteoblasts alone condition, suggesting an expression restricted to monocytes differentiated into osteoclasts. In this co-culture situation, *ACP5* and *MMP9* expression was strongly inhibited on CPC/Ga compared to CPC. Lastly, we also tested the expression of *RANKL*, *MCSF* and *OPG*, the triad of osteoblastic genes regulating osteoclastic differentiation. Considering the co-culture condition, we shown that no difference could be observed between CPC and CPC/Ga. In conclusion, we observed that ACP5 activity histochemical staining revealed an inhibition of osteoclastic differentiation on CPC/Ga compared to CPC. Moreover, this inhibition could also be quantified by RT-qPCR experiments measuring the expression of marker genes restricted to differentiated osteoclasts, i. e *ACP5* and *MMP9*.

### 3.2 Orthotopic *in vivo* implantation of apatitic matrices loaded with Ga

We next performed *in vivo* implantation experiments to determine Ga impact on the reconstructive properties of the CPC under study. As illustrated in Fig. 4A, cements were first processed into 80-200 µm diameter microparticles, and then implanted within cylinder defects generated in distal position of rat femurs. After 2 months, implants were retrieved and processed to perform HES staining and SHG

analysis on tissue sections, as well as micro-CT quantification of the whole implant. Both HES (Fig. 4B) and SHG (Fig. 4C) methods demonstrated for CPC and CPC/Ga a successful bone reconstruction including newly formed tissue up to the central part of implants and a complete cortical bone restoration. Based on image acquisitions illustrated in Fig. 4D, micro-CT was used to quantify total defect filling. Results presented in Fig. 4E did not show any significant impact of Ga on bone reconstruction.

With a view to increase Ga content of biomaterial used for bone defects filling, we shifted from CPC to calcium deficient apatite (CDA) loaded or not with Ga. Indeed, as detailed in Materials and Methods, Ga was brought to CPC through the CDA part (10%) of the cement formulation. Thus, using pure CDA allowed increasing Ga content by a factor of ten. As illustrated by HES staining and SHG analysis (Fig. 5A-5B), and similarly to CPCs, CDA and CDA/Ga implantations led after 2 months to a good osseointegration into the surrounding host tissue, accompanied by a successful bone ingrowth and bone marrow reconstruction. Taking advantage of the power of SHG thresholding, we performed 100- $\mu$ m sections in order to do quantification on larger implant areas. As shown in Fig.5B (right panel), we measured a 39% increased SHG signal mean intensity with CDA/Ga. These data indicated that higher amounts of fibrillar collagen were synthesized when the reconstruction occurred in the presence of Ga. Lastly, 3D micro-CT was used to quantify total defect filling (Fig.5C). We found that Ga induced a significant 23 % increase of defect filling (53.51% versus 76.89%). As a whole, our results indicated that the Ga presence within CDA used for bone defect filling led to a significant enhanced bone reconstruction.

#### 4. Discussion

*In vitro*, using CPC and CPC/Ga as growing surfaces, primary human monocytes do not differentiate into osteoclasts when cultured alone in the presence of MCSF/RANKL, while successful differentiation was achieved on plastic surface. In a previous publication characterizing the properties of the cements used in the present study, an increase of the gallium release was observed over time in the presence of RAW264.7 cells seeded on the surface of cement pellets and stimulated with 20 nM RANKL [41]. It should be noted that, compared to the human primary cells we used for the present

study, RAW264.7 is a mouse cell line which required only the presence of RANKL to differentiate into osteoclasts. We can hypothesize that human primary monocytes are more demanding in terms of environment to initiate osteoclastic differentiation. Indeed, co-culture conditions in the presence of primary human osteoblasts proved to be sufficient for osteoclastogenesis in the absence of any exogenous effectors.

In co-culture experiments, it is difficult to comment Ga impact on marker genes which expression is found also in osteoblasts alone. Osteoblastic expression has been reported for transcription factors NFATC1 and AP1 family members (Fos/Jun) [42, 43], as well as for ITGB3 and CTSK [44, 45]. However, regarding genes restricted to osteoclasts such as *ACP5* and *MMP9*, Ga exerts an unambiguous and pronounced inhibitory action. Interestingly, Ga impact on *ACP5* expression was further support at the protein level, as attested by ACP5 histochemical staining presented in Fig.3. Lastly, we do not observe any Ga-induced variation of *RANKL*, *MCSF* or *OPG* genes expression, suggesting that Ga inhibition is not mediated through a modulation of the RANKL/OPG ratio.

Considering the complexity of the molecular regulations impacting bone reconstruction *in vivo*, a 23% positive impact on bone defect filling is a very promising result. This is probably linked to the dual effect Ga exerts, i.e. a decrease of osteoclastogenesis together with an increased production by osteoblasts of factors favouring bone construction. Indeed, Ga stimulates *COL1A1* expression, which obviously contributes to the new tissue formation as the most abundant bone extracellular matrix protein [46]. We also observe that the expression of genes encoding for non-collagenous proteins was enhanced including *IBSP*, *ON*, and *POSTN*. Bone sialoprotein II (IBSP or BSP II) is a phosphorylated glycoprotein, which is involved in the initial steps of bone tissue mineralization [47, 48]. Osteonectin (ON; or SPARC for "secreted protein, acidic and rich in cysteine") is also linked to tissue mineralization along development, remodelling and bone repair [49]. Regarding periostin (POSTN, originally known as "osteoblast-specific factor" or OSF2), this glutamate-containing protein is preferentially located in the periosteum, and its expression is enhanced during bone fracture repair [50]. These data are reminiscent with results we published earlier. In this previous study, using the MC3T3-E1 osteoblast precursor murine cell line, we shown that Ga treatment enhanced the expression of marker genes including alkaline phosphatase (*Alp*), osterix (*Osx*) and runt-related transcription factor 2 (*Runx2*) [29].

Furthermore, in a study published in 1993, Bockman *et al.* reported that Ga increased type I collagen and fibronectin mRNA, as well as collagen protein levels in primary cultures of rat calvarial osteoblasts [51]. The same year, Jenis *et al.* published results indicating that Ga increased basal type I collagen mRNA levels and reduced basal osteocalcin mRNA levels in normal rat osteoblasts *in vitro* [52]. They also reported that systemic Ga administration decreased serum calcium levels and reduced osteocalcin steady-state mRNA levels, while having no effect on type I collagen mRNA levels or bone mineral density *in vivo*.

Taken together, and in addition to Ga inhibitory action on osteoclastogenesis, our data support the hypothesis that Ga-induced enhancement of bone repair we measure *in vivo* could also be linked to the stimulated expression of genes involved in bone tissue formation. In addition, the comparison with the results from Jenis *et al.* [52] point out how crucial could be the delivery mode of a pharmacological agent. Indeed, using systemic administration of a therapeutic dosage used for the treatment of Paget's disease, they did not observe any impact on bone mineral density of left femur in normal rats treated with gallium, while we measured a positive effect. This discrepancy could be related to the local delivery mode of Ga we used, this leading to higher local concentrations of an active molecule released continuously. In addition, and these hypothesis are not exclusive, Jenis *et al.* reported Ga effect on normal bone remodelling, while we addressed the issue of bone reconstruction following the creation of a defect. Thus, these different physiological situations could also account for our respective observations.

Lastly, using calcium deficient apatite (CDA), we demonstrated that the presence of Ga induced a significant stimulation of bone reconstruction, as attested by two types of quantification, i. e. 3D micro-CT scan and SHG analysis. Regarding SHG, these results are in agreement with the data we obtained measuring *COL1A1* gene expression. Actually, SHG analysis allows the quantification of fibrillar collagen (Fig. 5B) and we also observed that Ga stimulated *COL1A1* gene expression in primary osteoblasts (Fig. 2C). Together, these data reinforce the correlation between molecular data we obtained *in vitro* and bone reconstruction we measured *in vivo*.

Importantly, it should be stressed that to our knowledge, this is the first time that the use of SHG is reported in the field of bone reconstruction quantification within a bone substitute implanted *in vivo*.

Indeed, concerning osteo-articular applications, SHG analysis has been used to evaluate osteogenic cell differentiation *in vitro* [53-57], or to study tissue samples retrieved from normal or pathological situations [40, 58-66]. Based on the consistency we observe with gene expression and 3D micro-CT scan quantification data, we demonstrate in the present report that SHG analysis is also fully suitable to monitor the reconstructive properties of a bone substitute upon its implantation *in vivo*.

## 5. Conclusions

We propose that Ga positive impact we report in this study is linked to a decreased osteoclastic differentiation coupled to an increased new bone tissue formation. In terms of mechanisms, this is understated by *in vitro* data obtained at the molecular level through genes expression analysis. This is confirmed *in vivo* by the Ga-induced enhancement of bone reconstruction we observe using CDA/Ga to fill bone defects generated in a rat model.

Interestingly, in addition to the positive impact on bone formation we report here, Ga offers other worthwhile prospects. Indeed, as detailed in the Introduction section above, Ga displays anti-neoplastic, anti-infectious and anti-inflammatory properties [16]. Further studies are required to fully evaluate the potentialities of Ga-loaded bone substitutes used for the filling of bone defects for which tumour development and infections represent critical issues.

Lastly, as a beneficial side-effect of our quantification experiments, we provide strong data supporting the use of SHG technique to address the issue of *in vivo* tissue reconstruction within a bone substitute.

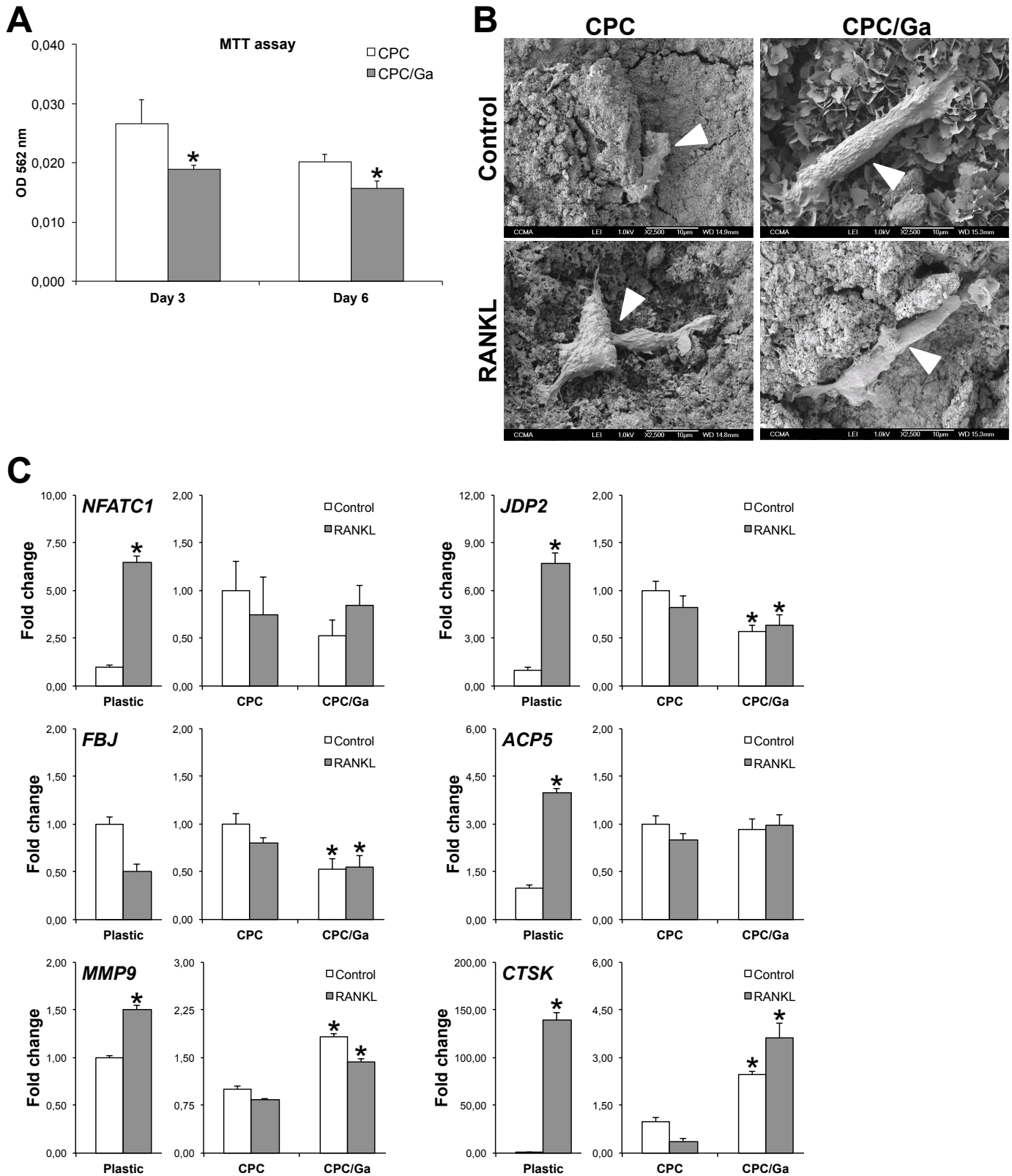
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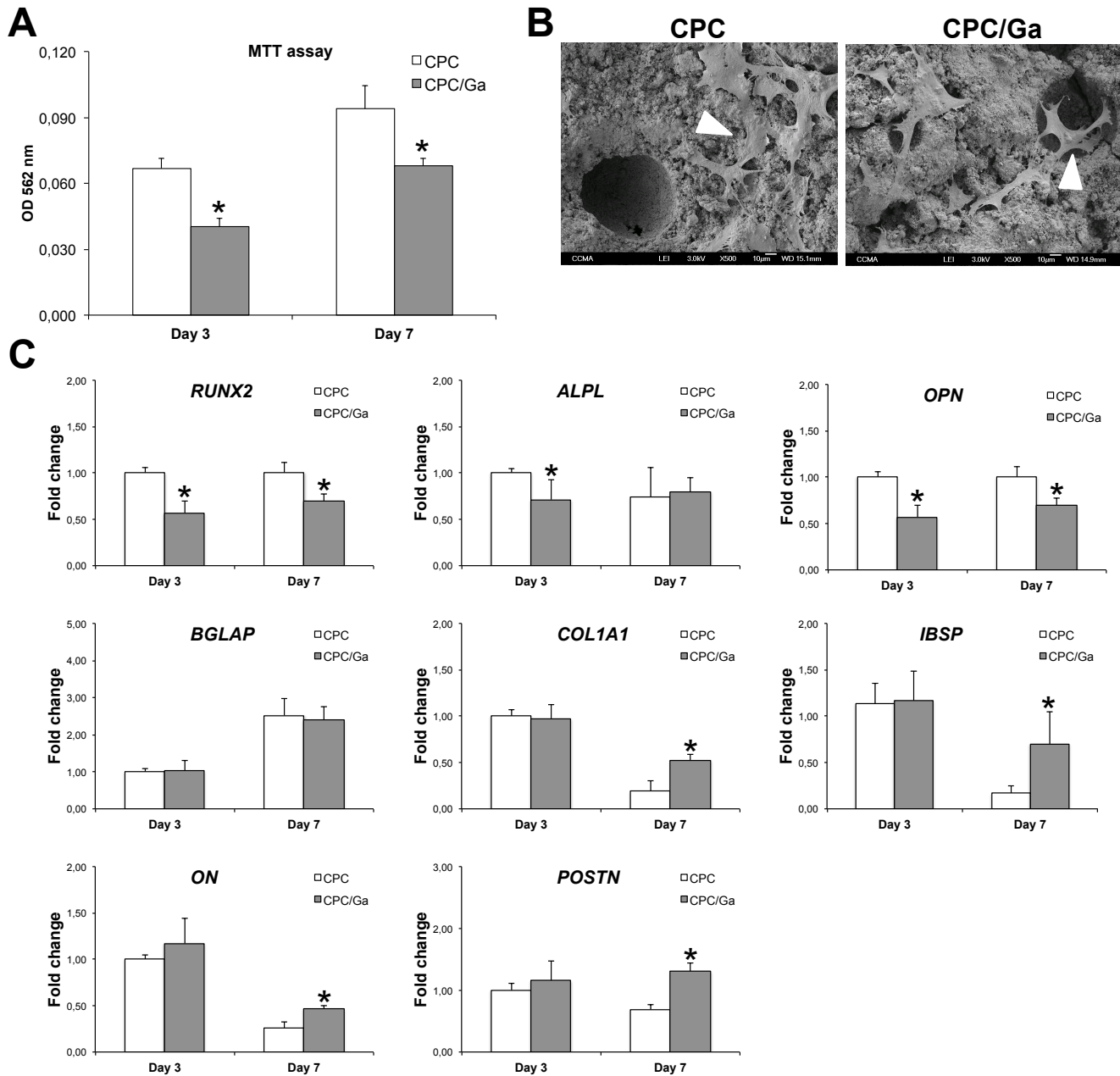
Table 1.

Human primers for qPCR.

| Gene          | Forward primer (5'→3')     | Reverse primer (5'→3')      | GI        |
|---------------|----------------------------|-----------------------------|-----------|
| <i>ACP5</i>   | gaccaccttggaatgtctctg      | tggctgaggaagtcactctgagttg   | 161377454 |
| <i>ALPL</i>   | gtacgagctgaacaggaacaacg    | cttggcttttccttcatggtg       | 294712525 |
| <i>BGLAP</i>  | ggcgcagcctttgtgtccaa       | ggctcccagccattgatacag       | 314122170 |
| <i>COL1A1</i> | ctcctgacgcacggcc           | ccgttctgtacgcaggtgatt       | 110349771 |
| <i>CTSK</i>   | tgaggcttctcttgggtgtccatac  | aaaggggtgtcattactgcggg      | 315075295 |
| <i>FBJ</i>    | tgcctctcctcaatgaccctga     | ataggtccatgtctggcacgga      | 254750707 |
| <i>IBSP</i>   | ctgtgccactcactgccttg       | caaatgctgagcaaaattaaagc     | 167466186 |
| <i>ITGB3</i>  | cattactctgcctccactacca     | aacggattttcccataagca        | 47078291  |
| <i>JDP2</i>   | cttcttcttgttccggcatc       | cttctggagggtgaaactgg        | 205277420 |
| <i>MCSF</i>   | tgacgaggctgattgaca         | gatctttcaactgttcttggctaca   | 166235151 |
| <i>MMP9</i>   | cctggagacctgagaaccaatc     | ccacccgagtgtaaccatagc       | 74272286  |
| <i>NFATC1</i> | gcatcacaggggaagaccgtgtc    | gaagttcaatgtcggagtttctgag   | 27502384  |
| <i>ON</i>     | accagcacccttgcagc          | aggtcacaggctcgaataaagc      | 48675809  |
| <i>OPG</i>    | cctccaagcccctgaagggtt      | cctgggtgggtccacttaatgg      | 148743792 |
| <i>OPN</i>    | agaagtttcgcagacctgacatc    | gatggccttgtatgcaccatt       | 91598938  |
| <i>POSTN</i>  | ttgaaggtgggtgatggctatttatt | cgtcttctagatccttgaactttttgt | 209862910 |
| <i>RANKL</i>  | ctcagccttttctcactcact      | ccaagaggacagactcactttatgg   | 197927084 |
| <i>RPLP0</i>  | tgcacagtagcccttctatcat     | aggcagatggatcagccaaga       | 49087144  |
| <i>RUNX2</i>  | gcttcttgcctcaccga          | tcagtgagggatgaaatgcttg      | 226442790 |

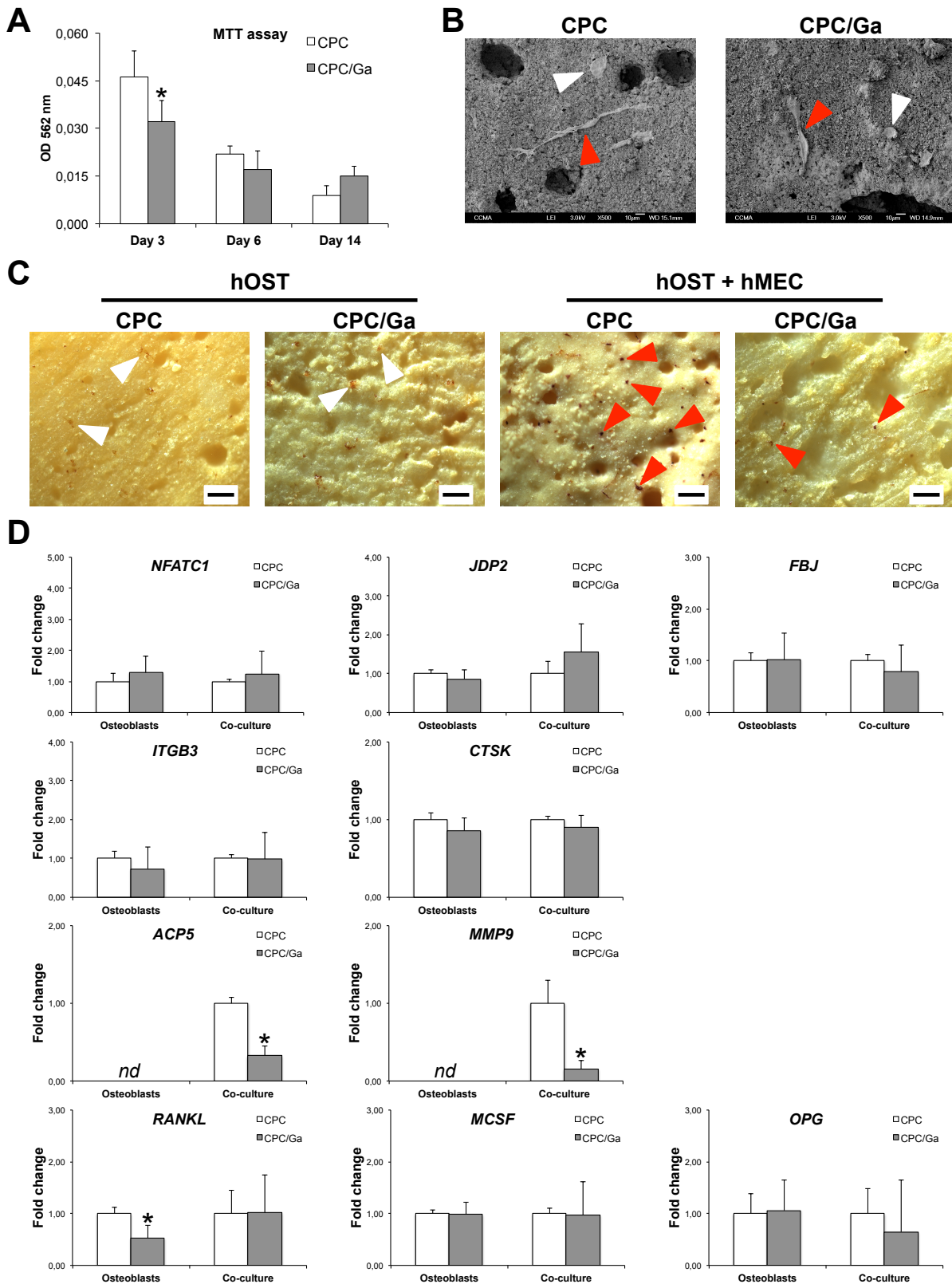


**Fig. 1. Cellular proliferation, morphology and differentiation of human primary monocytes on CPC or CPC/Ga pellets.** (A) MTT assay. 90 000 monocytes were cultured during 3 or 6 days. Data represent the mean  $\pm$  SD of 3 independent experiments, with each including five MTT measures. (B) Field-emission scanning electron microscopy analysis of pellets seeded with monocytes, and cultured during 5 days in the presence of hM-CSF (33 ng/mL; upper panels) or in the presence of hM-CSF/hRANKL (33 ng/mL and 66 ng/mL respectively; lower panels). White arrowheads indicate cells (scale bars: 10  $\mu$ m). (C) Real-time PCR quantification of osteoclastogenesis markers. Monocytes were cultured during 2 days (*NFATC1*, *JDP2*) or 5 days (*ACP5*, *MMP9*, *CTSK*), in the presence of 33 ng/mL hM-CSF (Control) or 33 ng/mL hM-CSF and 66 ng/mL hRANKL (RANKL). Data represent the mean  $\pm$  SEM of 3 independent experiments, each performed in triplicate. Data were normalized with respect to gene expression on plastic/control or on CPC/control for cell culture on cements pellets. \* Statistically significant compared to plastic/control or to CPC/control ( $p < 0.05$ , bi-directional analysis).

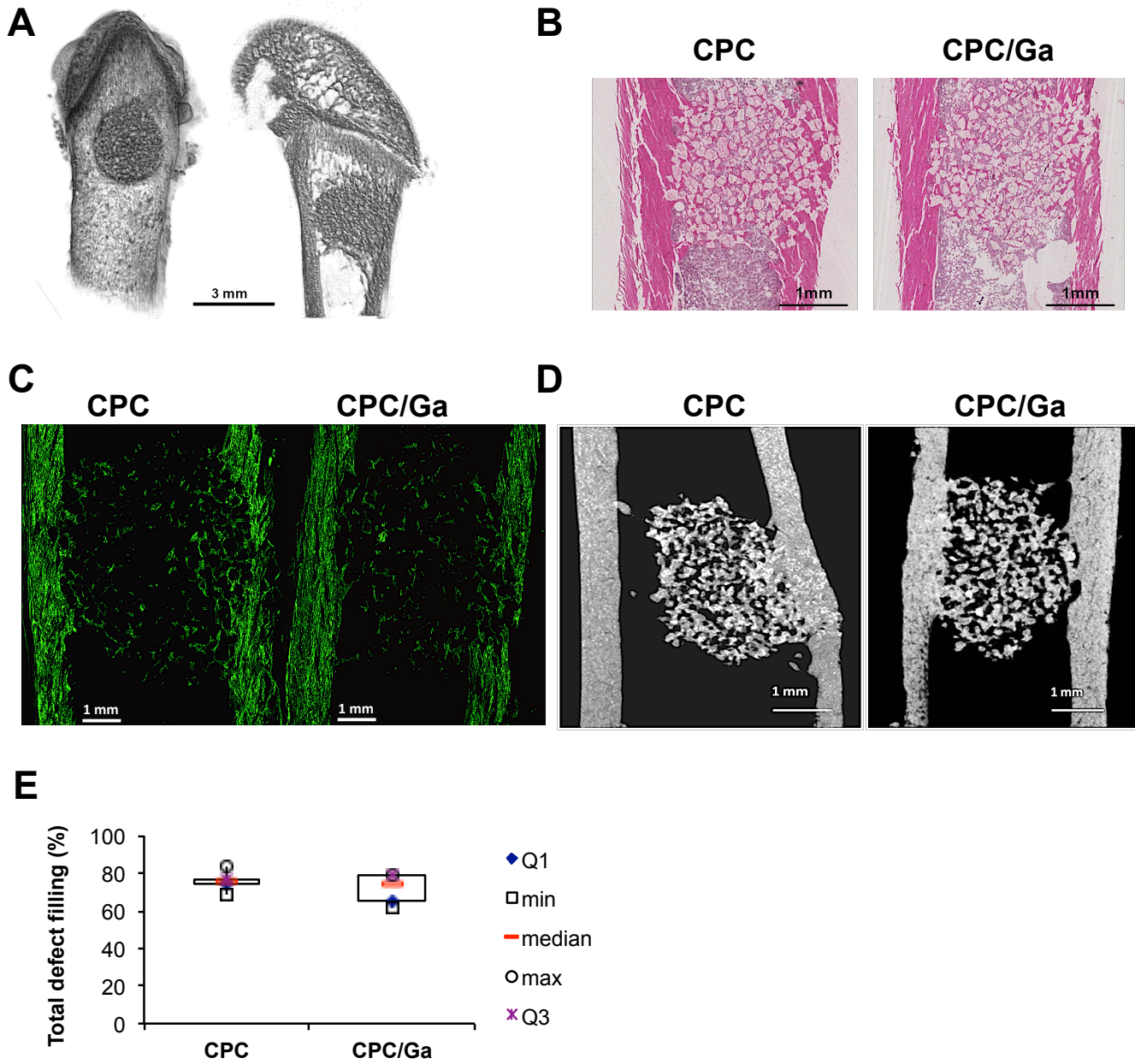


**Fig. 2. Cellular proliferation, morphology and differentiation of human primary osteoblasts on CPC or CPC/Ga pellets.** (A) MTT assay. 50 000 osteoblasts were cultured during 3 or 6 days. Data represent the mean  $\pm$  SD of 3 independent experiments, with each including five MTT measures. (B) Field-emission scanning electron microscopy analysis of osteoblasts cultured during 5 days on pellets. White arrowheads indicate cells (scale bars: 10  $\mu$ m). (C) Real-time PCR quantification of osteoblastic markers after 3 or 7 days of cell culture. Data represent the mean  $\pm$  SEM of 3 independent experiments, each performed in triplicate. Data were normalized with respect to gene expression on CPC at day 3. \* Statistically significant compared to CPC/day 3 ( $p < 0.05$ , bi-directional analysis).

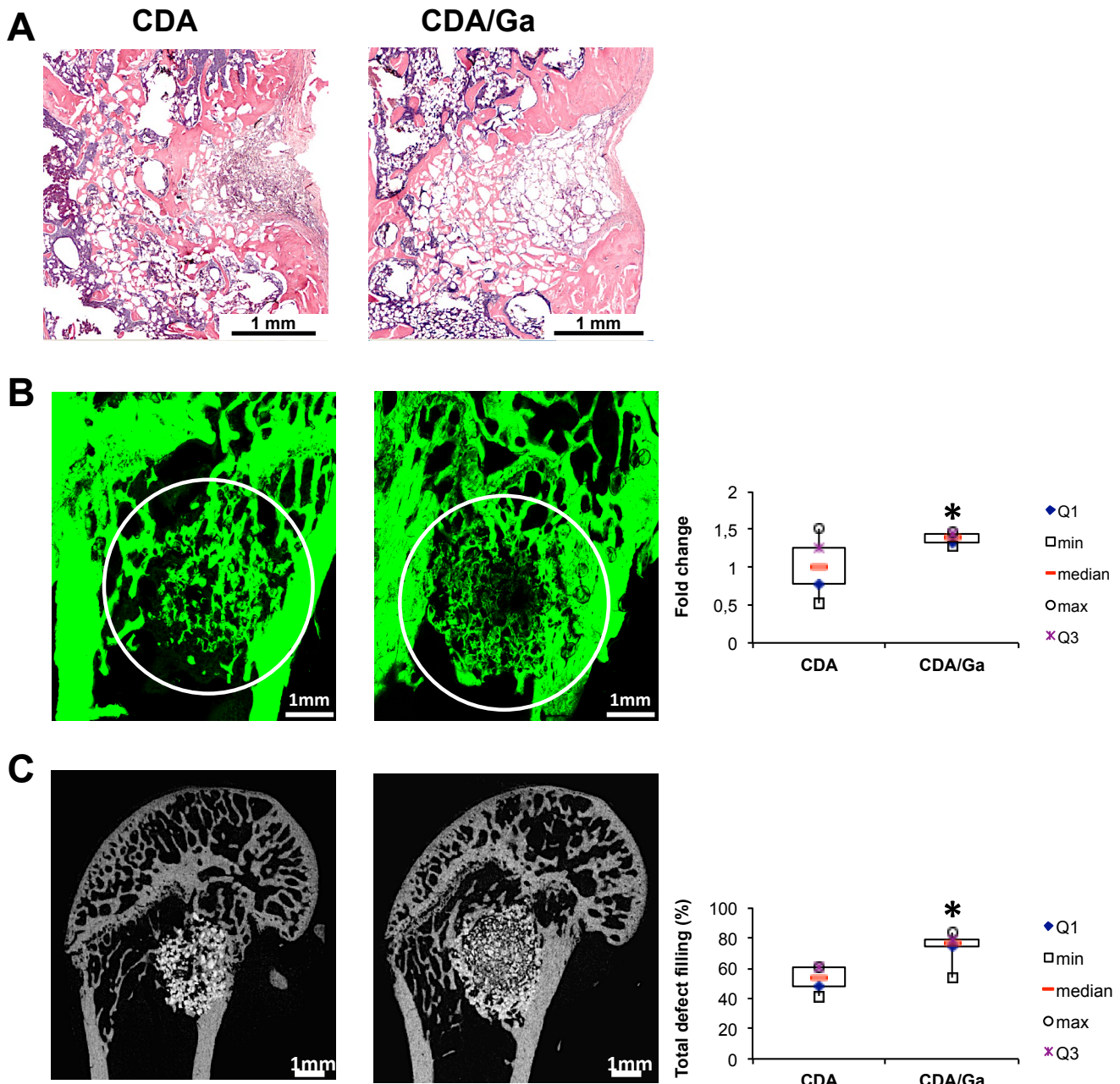




**Fig. 3. Cellular proliferation, morphology and differentiation of human primary monocytes co-cultured with human primary osteoblasts on CPC or CPC/Ga pellets.** (A) MTT assay. 100 000 osteoblasts and 200 000 monocytes were co-cultured during 3, 6 or 14 days. Data represent the mean  $\pm$  SD of 3 independent experiments, with each including five MTT measures. (B) Field-emission scanning electron microscopy analysis of osteoblasts and monocytes co-cultured during 14 days on pellets. White and red arrowheads indicate osteoblasts or osteoclasts respectively (scale bars: 10  $\mu$ m). (C) Histochemical staining of differentiated monocytes positive (red arrowheads) for ACP5 activity. 100 000 osteoblasts were cultured alone or in the presence of 200 000 monocytes during 14 days (scale bars: 100  $\mu$ m). (D) Real-time PCR quantification of osteoclastic markers after 6 or 14 days of osteoblast culture or osteoblasts/monocytes co-culture. Data represent the mean  $\pm$  SEM of 3 independent experiments, each performed in triplicate. For each condition (osteoblasts or co-culture) data were normalized with respect to gene expression on CPC. \* Statistically significant compared to CPC ( $p < 0.05$ , bi-directional analysis).



**Fig. 4. Femoral implantation in rat of CPC and CPC/Ga.** 2 months after implantation: (A) qualitative 3D micro-CT; (B) HES histological staining; (C) second harmonic generation analysis (SHG); (D) quantitative 3D micro-CT analysis; (E) total defect filling quantification. Results are expressed as total defect fill rate.



**Fig. 5. Femoral implantation in rat of CDA and CDA/Ga.** 2 months after implantation: (A) HES histological staining; (B) second harmonic generation (SHG) sections (lefts panels, white circles point implant area), and fibrillar collagen quantification (right panel). Data are normalised with respect to mean SHG intensity per pixel in CDA condition; (C) quantitative 3D micro-CT analysis. \* Statistically significant compared to CDA ( $p < 0.05$ , bi-directional analysis).

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## PART ONE B:

# Development of quantitative second-harmonic generation microscopy for bone tissue engineering applications

## 1. Context and objectives of the study

Classical histomorphometry is a gold standard in bone tissue assessment despite its major limitation only to 2D analysis. More recent 3D micro-CT quantification tools allowed the study of whole implant volumes. Nevertheless, this technique is poorly resolute in assessment of new bone ingrowth within calcium phosphate biomaterials implanted *in vitro*. On the other hand, second-harmonic generation (SHG) microscopy was recently proposed as a powerful tool for bone imaging, due to its intrinsic specificity for organized collagen fibrils.

In the present study we embarked on confirming SHG specificity for bone collagen by direct comparison with classical HES histological slides. Our previous quantitative micro-CT results showed a significant increase in total defect filling when CDA/Ga was implanted, compared to control. Since discriminative thresholding with micro-CT was not possible, we decided to use the novel SHG microscopy technique in order to quantitatively assess bone reconstruction induced by calcium phosphate biomaterials.



## 2. Methods

### *Sample preparation and SHG imaging*

As stated before, SHG microscopy has intrinsic specificity for collagen fibres, which results in high contrast images, with no additional staining needed and no additional steps in sample preparation. Upon retrieval, rat femoral implants were fixed for 24 h in neutral formalin solution (pH 7.2), and then decalcified in 10% EDTA (pH=8) for four weeks. Next, samples were embedded in paraffin and 5- $\mu$ m histological sections were prepared and stained with HES. The same histological slides were used for SHG imaging (Zeiss LSM 710 microscope, Germany). Mai Tai laser excitation wavelength was set to 880 nm and the SHG signal was detected in transmission and reflection mode.

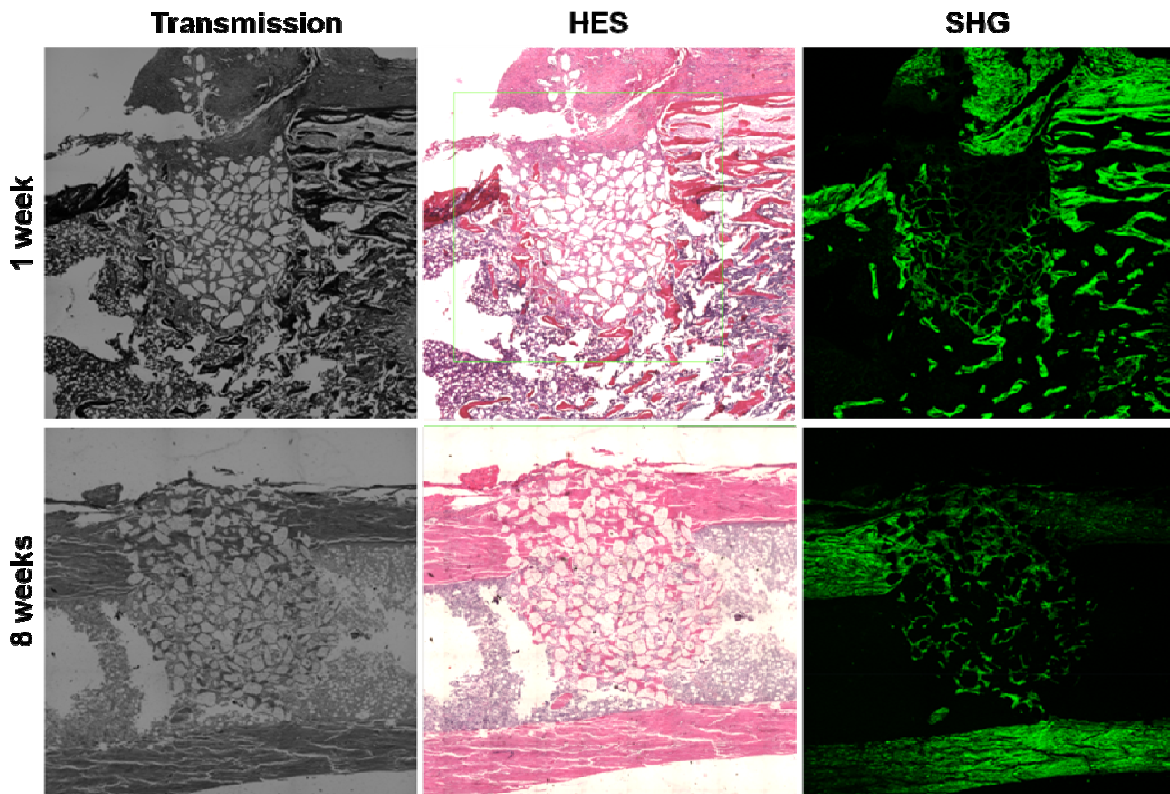
### *SHG analysis*

For CPC and CPC/Ga samples at 1 and 8 weeks postoperatively, 5- $\mu$ m sections were qualitatively assessed and for CDA and CDA/Ga samples, 100- $\mu$ m sections were prepared in order to do quantification as well. Quantitative comparison of collagen signal between Ga and control implants was performed on whole implant area (excluding the cortical bone) using an in-house quantification tool developed in "Mathlab" programming language by S. Schaub from iBV PRISM platform (Platform of Resources in Imaging and Scientific Microscopy).

## 3. Results and discussion

Firstly, we compared CPC samples retrieved 1 and 8 weeks after implantation. As expected (Figure 17) due to its specificity for organized collagen fibrils, SHG signal completely coincided with mature bone tissue areas observed on HES-stained histological slides. Furthermore, we observed an uneven distribution of SHG signal in 1-week implants, while the 8-week CPC exhibited homogenous signal distribution with complete cortical bone reconstruction. These findings are in accordance with the well-established hypothesis that upon calcium phosphate biomaterial implantation,

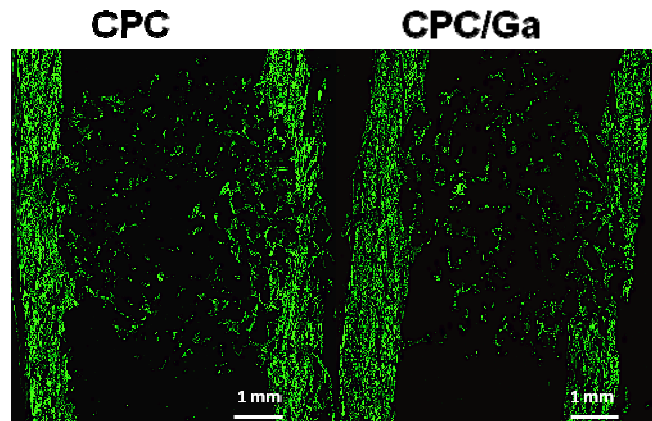
bone reconstruction begins on the defect border and overtime progresses towards the implant center.



**Figure 17. Bone reconstruction in a femoral defect in rat.**

At 1 and 8 weeks postoperatively, in the presence of 80-200  $\mu\text{m}$  CPC microparticles, newly formed bone is observed (rose zones) between biomaterial microparticles (white zones in the implant area) on 5- $\mu\text{m}$  HES-stained histological sections and by SHG imaging (green signal in the implant area).

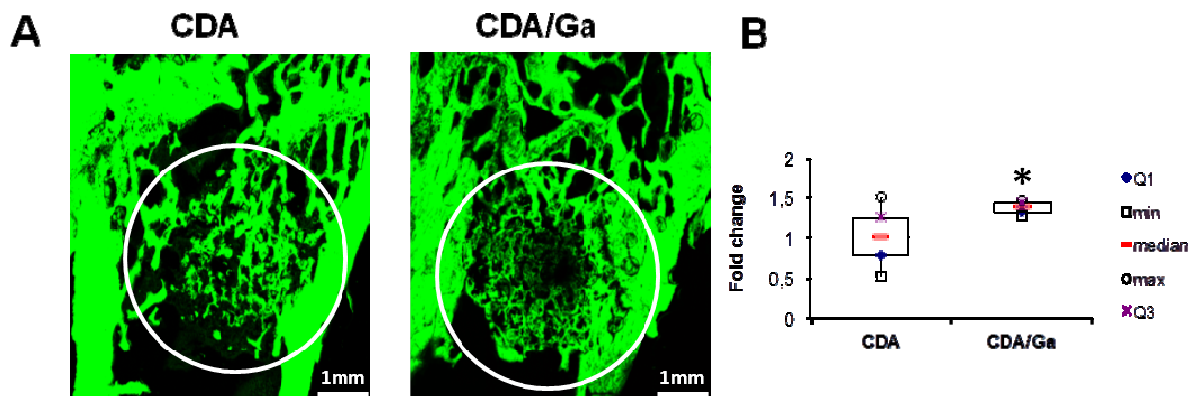
Secondly, SHG images of 8-week samples of CPC were compared with CPC/Ga implants. As for the CPC, successful new bone ingrowth was observed on the implant area and this was accompanied by complete cortical reconstruction as well. In addition, no apparent differences were observed in SHG signal distribution between Ga-loaded and control CPC (Figure 18).



**Figure 18. SHG images of bone reconstruction in femoral defect in rat.**

At 8 weeks post-operatively, in the presence of 80-200  $\mu\text{m}$  CPC and CPC/Ga microparticles, newly formed bone is observed (green) in the implant area (5- $\mu\text{m}$  sections).

Thirdly, 100- $\mu\text{m}$  thick histological sections of CDA and CDA/Ga were prepared and imaged by SHG in order to do 3D quantification of collagen signal (Figure 19). We chose particular CDA and CDA/Ga implants based on the results of previous non-destructive analysis by micro-CT that showed a significant increase in total defect filling in the presence of Ga, compared to control CDA.



**Figure 19. SHG quantitative analysis of bone reconstruction in femoral defect in rat.**

At 8 weeks postoperatively, in the presence of 80-200  $\mu\text{m}$  CDA and CDA/Ga microparticles.

A) Typical SHG images of CDA and CDA/Ga used for quantitative analysis (100- $\mu\text{m}$  sections).

B) Mean SHG intensity/pixel.

Indeed, quantitative SHG analysis (using the in-house quantification tool) confirmed the effect observed in 3D micro-CT assessment (Figure 19). Concerning

CDA samples analysis, it is important to notice that the use of 100  $\mu\text{m}$  sections instead of 5  $\mu\text{m}$  sections increased by a factor of 20 the power of the quantification. However, this is not without certain problems related to the thickness of the section, which make sometimes difficult an accurate delimitation of the implant area.

For future developments, we envision the use of more sections per implant but with a thickness limited to 25  $\mu\text{m}$ , which seems to us a very workable compromise between the power of the analysis and the accuracy of the ROI (region of interest) delimitation.

#### **4. Conclusions**

Microscopy techniques based on non-linear optical processes allow for specific imaging of unmodified samples at high spatial resolution in three dimensions. By doing so, they provide powerful tools for the specific characterization of bone reconstruction within calcium phosphate biomaterials. Furthermore, quantitative assessment of collagen fiber organization confirmed the results observed in micro-CT analysis, suggesting that SHG can function as a complementary quantifying tool in bone-tissue engineered constructs. It would be further interesting to apply SHG microscopy in studying the *in vitro* 3D tissue-engineered constructs, as well as subcutaneous biomaterial implants.

All in all, the results of this work show that quantitative SHG microscopy can serve as a valuable tool for evaluating the organized collagen fibers within a biomaterial, this providing an easy-to-use, accurate, and powerful technique to score the performance of bone substitutes under development upon their implantation *in vitro*.



## PART TWO:

### Effects of gallium in a bone metastatic model

#### 1. Context and objectives of the study

Bone tissue is a common metastatic soil for a great number of solid tumours, including breast cancer. Upon homing to bone, cancer cells intensely secrete factors that stimulate osteoclastic differentiation and subsequent excessive bone resorption, leading to the release of high amount of TGF- $\beta$ , the most prominent cytokine in tumour cells activation. This vicious cycle between tumour cells and osteoclasts results in painful osteolytic lesions that further induce severe skeletal events, e.g. fractures or spinal cord compression. Given the burden on patients' life quality, the development of treatments targeting the disruption of the crosstalk between tumour cells and bone cells appears as a promising strategy to attenuate bone metastasis expansion.

Due to its chemical affinity for bone tissue, its established antiresorptive potential, and its antineoplastic properties, gallium (Ga) represents an attractive candidate for bone metastases treatment and/or prevention. Given that no study has yet assessed Ga effects on bone metastases, we embarked on investigating if Ga can successfully disrupt the vicious cycle driving osteolytic lesions.

We used a co-culture model that mimicks physiological metastatic bone environment to study if Ga can reverse cancer cell-induced excessive osteoclastic differentiation. Next, taking advantage of a bone-seeking clone derived from an aggressive breast cancer cell line, we assessed Ga possible effect on proliferation, viability and marker genes expression.

## **2. Methods and results**

Article II (in preparation): Gallium, a promising candidate to disrupt the vicious cycle driving osteolytic metastases

# Gallium, a promising candidate to disrupt the vicious cycle driving osteolytic metastases

Authors: Ivana Strazic <sup>a, b, c, d</sup>, Iva Guberovic<sup>e</sup>, Blanka Didak<sup>e</sup>, Heidy Schmid <sup>a, b, c, d</sup>, Annie Schmid-Alliana <sup>a, b, c, d</sup>, Jean-Michel Boulerf, Jean-Claude Scimeca <sup>a, b, c, d</sup>, Elise Verrone<sup>e</sup>

<sup>1</sup> INSERM U791, Faculté de Chirurgie Dentaire, Nantes, France

<sup>a</sup> Université Nice Sophia Antipolis, iBV, UMR7277, 06100 Nice, France

<sup>b</sup> CNRS, iBV, UMR7277, 06100 Nice, France

<sup>c</sup> Inserm, iBV, U1091, 06100 Nice, France

<sup>d</sup> GRAFTYS SA, 13854 Aix en Provence, cedex 3, France.

<sup>e</sup> Université Nantes, INSERM U791, 44042 Nantes, France

<sup>f</sup> Université Nantes, CNRS, Nantes, France

JCS and EV contributed equally to the supervision of the study

Corresponding author: E Verron

[elise.verron@univ-nantes.fr](mailto:elise.verron@univ-nantes.fr)

INSERM U791

Faculté de Chirurgie Dentaire

1, place Alexi Ricordeau

44042 Nantes

France

Tel : +33 2 40 41 29 16

Fax: +33 2 40 08 37 12



## **Abstract**

Bone metastases of breast cancer typically lead to a severe osteolysis due to an excessive osteoclastic activity. Considering the inhibitory resorption activity of Ga and its bone affinity, we decided to explore the *in vitro* effect of Ga on osteoclastogenesis in an aggressive bone metastatic environment based on a co-culture model. Ga inhibits in a dose-dependent manner the formation of mature osteoclasts, which in turn may block the release of TGF- $\beta$ 1 from bone matrix preventing the vicious circle between bone cells and tumour cells. In addition, Ga strongly affects proliferation, viability and expression of critical markers of MDA-231BO cells, a specific bone metastatic clone of MDA-MB-231 cells line.. This is the first time that antitumor properties of Ga have been studied especially in the context of bone metastases. These data strongly suggest that Ga may be a promising candidate for the prevention and/or treatment of bone metastases in patients with breast cancer.

**Keywords:** breast cancer, bone metastasis, gallium, osteoclastogenesis

## 1. Introduction

Bone tissue is one of the most favoured sites for metastasis of breast tumours and bone lesions are preferentially localized in spine and pelvic bone (Verron *et al.*, 2014). Up to 80% of all patients diagnosed with breast cancer will develop bone metastases characterized by severe osteolytic lesions. Subsequently, bone metastases lead to severe bone pain, bone instability, fractures, spinal cord compression, hypercalcemia and bone marrow aplasia (Mercadante, 1997). As described in a retrospective study including 617 women with breast cancer, 52% experienced at least one of these skeletal-related events (SREs) (Delea *et al.*, 2006). Focussing on spinal, metastasis, about 20% of cases exhibited neurological deficit symptoms due to: (i) mechanical compression of spinal cord directly by the tumour or caused by the displacement of bone fragments, kyphotic deformity; (ii) vascular insufficiency as a consequence of segmental artery occlusion by tumour emboli, venous thrombus, and spinal cord injury due to oedema caused by internal haemorrhage of spinal cord. Consequently, it appears crucial to prevent or delay the onset of SREs since their development considerably affects patients' quality of life and in some cases increases the risk of death (Saad *et al.*, 2007).

Bone homeostasis is regulated through a balanced communication between the major cells forming and remodelling bone tissue, i.e. osteoclasts, osteoblasts and osteocytes. Breast tumour cells invasion into bone shifts the balance of bone turnover to favour resorption processes through an enhanced osteoclastic activity (Roodman, 2004). Indeed, breast cancer cells promote the formation and the activity of osteoclasts by secreting osteolytic cytokines including parathyroid hormone-related peptide (PTHrP), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins (such as IL-8 and IL-11), as well as matrix metalloproteinases that are involved in bone protein matrix destruction (Akhtari *et al.*, 2008). In addition, breast cancer cells negatively affect osteoblasts in terms of proliferation and activity leading to a decrease of bone formation (Chen *et al.*, 2010a). Consequently, due to the development of these breast cancer cells-induced osteolytic lesions, regulatory growth factors stored in large quantities within bone tissue are massively released. Among these factors locally available, transforming growth factor- $\beta$  (TGF- $\beta$ 1 is the most implied in tumour cells activation (Chen *et*

*al.*, 2010b; Buijs *et al.*, 2011a; Buijs *et al.*, 2011b; Juarez *et al.*, 2011). As postulated by Stephen Paget, this microenvironment can be considered as a soil that provides a favourable niche for the growth and the development of tumour cells (Ribatti *et al.*, 2006). In turn, tumour cells produce angiogenic factors such as vascular endothelial growth factor (VEGF-A) (Kakonen *et al.*, 2003; Roodman, 2004; Sterling *et al.*, 2011). This unfavorable phenomenon stimulates tumour neovascularization, which favor oxygen and nutriment supply, as well as tumour cells dissemination.

The disruption of the vicious cycle described above represents a promising therapeutic strategy. This could be achieved through interfering with the cross talk between cancer cells and osteoclasts, with a view to reduce osteolytic bone lesions and to limit tumour cell growth at the bone metastatic site. In this attempt, Gallium (Ga) could be a promising candidate for the prevention or the treatment of bone metastasis due to (i) its chemical affinity for biological hydroxyapatite, (ii) its antiresorptive activity (Verron *et al.*, 2012a) and (iii) its potential antitumor properties. Indeed, Ga significantly decreases osteoclasts differentiation and activity, without negatively interacting with osteoblasts (Verron *et al.*, 2010a), and we also demonstrated at the molecular level that Ga decreased the RANKL-induced initial expression and auto-amplification of NFATc1, the major factor of osteoclast differentiation (Verron *et al.*, 2012b). Moreover, through transferrin-dependent and independent mechanisms, Ga accumulates at sites of accelerated cellular proliferation including malignant tissue (Bernstein, 1998). Once internalized into tumor cells, Ga blocks DNA synthesis and alters plasma membrane permeability and mitochondrial functions, these events leading to cell apoptosis (Bernstein, 1998; Collery *et al.*, 2002).

Ga antineoplastic activity has been demonstrated in lymphoma and bladder cancer (Chitambar, 2012). However, it has never been studied in the context of breast carcinoma and its severe bone metastasis. Considering Ga antineoplastic potential together with its inhibitory action on osteoclastogenesis and bone resorption, we were interested in evaluating its effects on one of the most aggressive human breast carcinoma cell line (MDA-MB-231), characterized by the absence of expression of receptor of oestrogen and its high ability to metastasize to bone tissue. In order to achieve this goal, and taking advantage of a model

established by Guo *et al.* (Guo 2008), we designed experiments associating adenocarcinoma cells and RAW 264.7 monocytes. Based on this system, we studied Ga impact on the cross talk occurring between cancer cells prone to spread to bone (MDA-MB-231) and precursor cells able to resorb bone tissue upon differentiation into osteoclasts (RAW 264.7).

## 2. Materials and Methods

### *Materials*

Alpha Minimum Essential Medium ( $\alpha$ -MEM) and Dulbecco's Modified Eagle's Medium (DMEM), glutamine, antibiotics (penicillin: 100 units/mL and streptomycin: 100  $\mu$ g/mL), Phosphate Buffered Saline (PBS) and trypsin/EDTA were obtained from Gibco (Paisley, UK). Foetal bovine serum (FBS) for MDA-MB-231 cellular culture was purchased from Pan Biotech GmbH (Aidenbach Germany) and Foetal bovine serum (FBS, Hyclone) for RAW 264.7 cellular culture was obtained from Thermo scientific (Braunschweig, Germany). Ga nitrate, TGF- $\beta$ 1, zoledronic acid and TRAP staining kit (cat. no. 386) were provided by Sigma (Saint-Quentin Fallavier, France). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was obtained from Sigma-Aldrich, USA. Mouse effectors were produced as fusion proteins with GST. GST-Rankl was produced as previously described (Beranger *et al.*, 2007). For all the experiments using cells of murine origin, a GST protein, produced and purified using the same protocol, was used as a control. Co-culture experiments were performed in 24-well transwell plate (3.0  $\mu$ m pore size, Cat No 3399, Corning, France). qRT-PCR experiments were performed using: NucleoSpin RNA II kit provided by Macherey- Nagel (Duren, Germany); RT Mix containing primers, oligo dTs, RT buffer and RT enzyme from Invitrogen Corp. (Paisley, UK); nuclease-free water (Amidon, USA); MasterMix from Applied Biosystems (USA).

### *Cell culture*

The mouse monocyte cell line RAW264.7 was obtained from ATCC (Ref. # TIB-71; LGC Standards, Molsheim, France). Cells were cultured in DMEM containing 5% foetal bovine

serum (Hyclone serum, Thermo Fisher Scientific, Brebière, France). For osteoclastic differentiation experiments, RAW264.7 cells were seeded at 5,000 cells/cm<sup>2</sup> in  $\alpha$ -MEM containing 5% Hyclone serum and effectors were added immediately. RANKL (Receptor Activator of Nuclear Factor- $\kappa$  B Ligand) was used at 20 nM. Cells were cultured for four days with a renewal of the medium at day 2.

The oestrogen-independent human breast adenocarcinoma cell line MDA-MB-231 was obtained from ATCC. Cells were cultured in DMEM medium supplemented with 10% of foetal bovine serum and 1% of antibiotics (Chen *et al.*, 2001).

The bone-seeking (MDA-231BO) clone established from MDA-MB-231 cell line (Yoneda *et al.*, 2001) was kindly provided by Dr. Toshiyuki Yoneda (University of Texas Health Science Center at San Antonio). Cells were cultured in glutamine-supplemented DMEM medium plus 5% FCS and 1% of antibiotics.

#### *RAW 264.7 cells differentiation in presence of conditioned medium*

For the preparation of conditioned medium (CM), MDA-MB-231 cells were plated in T-75 tissue culture flasks and cultured as described previously. When cells reach confluency, the medium was collected, filtrated using a 0.22  $\mu$ m pore filter, and stored at -80°C. A second type of conditioned medium was collected from MDA-MB-231 cells treated with TGF- $\beta$ 1. Briefly, when cells reach confluency, they were deprived of growth factors by an overnight incubation in the presence of 0.1% serum, before a 20 hours treatment with 10 ng/ml of TGF- $\beta$ 1. This conditioned medium was then collected and treated as described above.

For differentiation experiments, RAW 264.7 cells were washed with pre-warmed PBS prior a 4 days treatment with RANKL. Depending on the experiment, cell cultures were supplemented at the same time with conditioned medium from MDA-MB-231 cells (up to 20%) and gallium (up to 100  $\mu$ M).

#### *Co-culture of RAW 264.7 cells with MDA-MB-231 cells*

For co-culture experiments, insert systems (0.4  $\mu$ m pore-size transwell inserts in 24 well plates) were used. RAW 264.7 cells were seeded at cell density of 5 000 cells/cm<sup>2</sup> on the lower

chamber and were treated with 20 nM RANKL for 2 days, prior to combining with MDA-MB-231 cells that were preliminary grown for 2 days on separate upper chambers (inserts) at cell density of 10 000 cells/cm<sup>2</sup>. In basal condition, MDA-MB-231 cells were pre-cultured in DMEM medium supplemented with 10% FBS and 1% P/S. In stimulated conditions mimicking bone metastases microenvironment, 10 ng/mL of TGF- $\beta$ 1 was added during this pre-culture phase, and maintained after combining cell lines for 2 days. For both conditions (basal or TGF- $\beta$ 1-treated conditioned medium), Ga was added at the beginning of the co-culture period.

#### *Culture of MDA-231BO cells for Ga/TGF- $\beta$ experiments*

MDA-231BO cells were seeded in F-75 flask at a density of  $1.5 \times 10^6$ . Cells were then cultured 4 days, with medium renewal at day 2, in presence of Ga (100 and 300  $\mu$ M) or vehicle. Alternatively, MDA-231BO cells were cultured 4 days, with medium renewal at day 2, then serum-starved overnight and treated 20h with TGF-  $\beta$ 1 at 10 ng/mL or vehicle. Lastly, MDA-231BO cells were cultured 4 days in the presence of Ga or vehicle, with medium renewal at day 2, then serum-starved overnight and treated 20 hours with TGF-  $\beta$ 1 at 10 ng/mL or vehicle. For cells initially treated with Ga, it was maintained during starvation and TGF- $\beta$ 1 treatment.

#### *Tartrate resistant acid phosphatase (TRAP) staining*

RAW 264.7 cells were rinsed gently with pre-warmed PBS before fixation, and TRACP staining was performed using the leukocyte acid phosphatase kit according to the manufacturer's instructions to detect the presence of TRAP-positive cells. Stained cells were observed using a light microscope (Axioplan 2, Zeiss, Germany). TRAP-positive multinucleated cells containing at least three nuclei were counted as osteoclasts.

#### *Proliferation Assay*

MDA-231BO cells were seeded at a density of 20 000 cells/cm<sup>2</sup> and treated with 0, 100, 200 and 300  $\mu$ M of Ga during three days. Cellular proliferation was quantified by scoring cells

manually after Trypan blue staining. Results were expressed as a mean  $\pm$  SD of three independent counting.

#### *Viability Assay*

MDA-231BO cells were seeded at a density of 20 000 cells/cm<sup>2</sup> and treated with 0, 100, 200 and 300  $\mu$ M of Ga during three days. At days 1, 2 and 3 of culture, cells were rinsed two times in phosphate-buffered saline (PBS) and incubated in 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1h in cell incubator. MTT was aspirated delicately and 0.3 ml of lysis buffer (SDS 10%, HCl 0.01 N) was added/well and left overnight in dark at room temperature. Supernatants were then centrifuged 2 min at 20 000 g, and absorbance at 562 nm was measured in 100  $\mu$ l.

#### *Quantitative real-time PCR*

For RNA isolation, cells were lysed with lysis buffer provided by manufacturer of kit and RNA was isolated with NucleoSpin RNA II according manufacturer instructions. Quantity of RNA and purity of samples were measured on NanoDrop 1000 (Thermo Scientific, (Courtaboeuf, France)). Integrity of RNA was analyzed on BioAnalyser 2100 (Agilent Technologies, Austria).

For reaction of reverse transcription, 1  $\mu$ g of isolated RNA and mixture, which contained random primers, StrataScript enzyme, oligo dT and nuclease free water, were used. Conditions of reaction were 25°C for 10 minutes, 50°C for 30 minutes and 85°C for 5 minutes in Thermocycler (Eppendorf, Le Pecq, France). PCR was conducted with 25 ng of cDNA and mixture of ROX and SYBER GREEN dyes, gene specific primers and nuclease free water. Controls consisted in samples without cDNA (NTC) and samples without RT enzyme (NRT). The following temperature profile was used: 40 cycles of 30 s at 95°C, 1 min at 60°C and 30 s at 72°C. Amplification curves were analysed using the MxProV3 software (Stratagene). PCR products with Ct over 35 cycles were considered as undetectable. The delta Ct (dCt) (cycle threshold) method was used to calculate relative expression levels. Cycle thresholds were normalized against Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) gene expression, in

order to control for cDNA quantification differences. Results are reported as the fold change in gene expression relative to control conditions.

Primers used in qPCR. Gene symbols (abbreviations) are as follows: *CXCR4*, Chemokine (C-X-C Motif) Receptor 4; *HPRT1*, Hypoxanthine Phosphoribosyltransferase 1; *MMP9*, Matrix Metalloproteinase 9; *MMP13*, Matrix Metalloproteinase 13; *PTH1H*, Parathyroid Hormone-Like Hormone; *TGFB1*, Transforming Growth Factor Beta Receptor 1; *VEGFA*, Vascular Endothelial Growth Factor A.

### *Statistical Analysis*

The data shown is representative of at least three independent experiments. Results are expressed as mean +/- standard deviation of three determinations. The statistical differences between two independent groups were evaluated using the Mann & Whitney test (bi-directional analysis). Comparative analysis of more than two independent groups was performed using the Kruskal-Wallis test (bi-directional analysis). The differences measured were considered to be statistically significant for  $p < 0.05$ .

## **3. Results**

### *3.1 Effect of Ga on breast cancer cells-induced osteoclastic formation*

#### *3.1.1 RAW 264.7 cells differentiation in presence of conditioned medium*

##### *Conditioned medium from MDA-MB-231 cells*

Raw 264.7 cells were cultured 4 days in presence of RANKL (20 ng/mL) or vehicle, and the number of multinucleated TRAP<sup>+</sup> cells was quantified (Fig. 1A). The result obtained in the presence of RANKL was used as control condition (100%). In parallel, the same differentiation experiments were performed in the presence of 10% or 20% conditioned medium (CM) isolated from MDA-MB-231 cells culture. As shown in Fig. 1A, we observed that 10% CM significantly increased osteoclast formation by 62% as compared to control condition (cells treated with RANKL during 4 days in absence of CM). By contrast, this stimulatory effect disappeared when 20% CM was tested.



Based on these results, we next evaluated Ga impact on conditioned medium-induced osteoclastic differentiation in the presence of 10% CM from MDA-MB-231 cells. As shown in Fig. 1B, a 4 days treatment with Ga decreased in a dose-dependent manner the number of mature osteoclasts measured in presence of 10% CM. Using 100  $\mu$ M Ga, we observed that the number of multinucleated TRAP+ cells decreased by approximately 90% as compared to the control condition.

#### *Conditioned medium from TGF- $\beta$ 1-treated MDA-MB-231 cells*

In order to mimic an aggressive bone metastatic environment, MDA-MB-231 were cultured as described under Experiment Procedures, in the presence of 10 ng/mL TGF- $\beta$ 1 or vehicle. Increasing amounts of this conditioned medium were used as an adjuvant during RAW 264.7 cells differentiation. As shown in Fig. 1C, incubating RAW 264.7 cells with CM from TGF- $\beta$ 1-treated MDA-MB-231 cells significantly increased the number of osteoclasts as compared to control condition. The addition of 20% CM was the most effective dose and increased the number of osteoclasts by 236 % as compared to cells treated with RANKL alone.

Based on these results, we next evaluated Ga impact on conditioned medium-induced osteoclastic differentiation in the presence of 20% CM from TGF- $\beta$ 1-treated MDA-MB-231 cells. As shown in Fig. 1D, a 4 days treatment with Ga decreased in a dose-dependent manner the number of mature osteoclasts measured in presence of 20% CM. Using 10  $\mu$ M Ga, we observed that the number of multinucleated TRAP+ cells decreased by 68% as compared to the control condition, and this effect further increased with higher doses of Ga. Interestingly, when compared to the results obtained with CM from MDA-MB-231 cells (Fig. 1B), Ga impact was more pronounced in the presence of CM from TGF- $\beta$ 1-treated MDA-MB-231 cells.

### 3.1.2 Co-culture of RAW 264.7 cells with MDA-MB-231 cells

#### *Basal condition*

We first performed co-culture of MDA-MB-231 cells with RAW-264.7 cells previously primed with 20 nM of RANKL. As compared to RAW 264.7 cells alone, co-culture with MDA-MB-231 cells almost doubled the number of mature osteoclasts (data not shown). We next tested Ga impact on RAW 264.7 cells osteoclastogenesis occurring in the presence of MDA-MB-231. As shown in Fig. 2A, a 48 hours Ga treatment significantly inhibited in a dose-dependent manner the formation of multinucleated TRAP<sup>+</sup> cells, and Ga at 100  $\mu$ M induced the most effective inhibition (approximately 85%).

#### *Treatment with TGF- $\beta$ 1*

In an effort to mimic bone metastases microenvironment conditions, MDA-MB-231 cells were treated 10 ng/mL TGF- $\beta$  before and during the co-culture with RANKL-primed RAW 264.7 cells. As shown in Fig. 2B, the formation of multinucleated TRAP<sup>+</sup> cells was increased significantly by 15% as compared to the control condition. Considering more qualitative aspects, we observed that in the presence of TGF- $\beta$ , multinucleated TRAP<sup>+</sup> cells were bigger and contained more nuclei (Fig. 2B, right panels).

Using these co-culture conditions in the presence of TGF- $\beta$ , we then tested Ga on RAW 264.7 cells osteoclastogenesis. As shown in Fig. 2C, a 48 hours Ga treatment inhibited the formation of multinucleated TRAP<sup>+</sup>. Similarly to the results obtained in the absence of TGF- $\beta$  (Fig. 2A), Ga at 100  $\mu$ M induced the most effective inhibition (approximately 85%).

### 3.2 Effect of Ga on bone-seeking (MDA-231BO) clone

To determine whether Ga may also affect metastatic tumour cells with a bone tropism, we quantified Ga impact on the bone-seeking MDA-231BO clone, which has been established by repeating sequential passages, in nude mice and *in vitro*, of MDA-MB-231 tumour cells isolated from bone metastases.

### 3.2.1 Effect of Ga on MDA-231BO cells proliferation and viability

Cellular proliferation scoring was based on cell number quantified along three days of culture in the presence of increasing Ga concentrations. As shown in Fig. 3A, Ga inhibited in a dose-dependent manner MDA-231BO cells proliferation and at day 3, 300  $\mu$ M Ga decreased by 84% cell proliferation as compared to untreated cells. We also observed that Ga affected MDA-231BO cells viability, as illustrated by the 64 % inhibition measured at day 3 in the presence of 300  $\mu$ M Ga (Fig. 3B).

### 3.2.2 Effect of Ga on tumoral markers expression

To determine Ga impact on the osteolytic potential of MDA-231BO cells, we quantified the expression of selected marker genes upon Ga treatment, in the absence or presence of TGF- $\beta$ 1. As shown in Fig. 4, in the absence of TGF- $\beta$ 1 (left part of histograms), *PTH1H* gene expression was reduced by 71 % in the presence of 100  $\mu$ M Ga, with no further inhibition with a higher dose. In the presence of TGF- $\beta$ 1, we observed in basal condition a non-significant decrease of *PTH1H* gene expression. Adding Ga led to an inhibition that reached 66 % in the presence of 300  $\mu$ M Ga.

Treating MDA-231BO cells with 100  $\mu$ M and 300  $\mu$ M Ga inhibited also *MMP9* gene expression by 69% and 84% respectively. When TGF- $\beta$ 1 was added, a strong up regulation (5- to 6-fold) of basal expression was measured. The addition of Ga abolished this increase and restored the level of *MMP9* gene expression in the absence of any treatment.

Concerning *MMP13*, TGF- $\beta$ 1 or 100  $\mu$ M Ga treatments did not modify gene expression. By contrast, regardless the absence or presence of TGF- $\beta$ 1, 300  $\mu$ M Ga stimulated the expression of *MMP13* gene.

We also observed that Ga enhanced *VEGFA* gene expression (approximately 2-fold stimulation for both concentrations), while TGF- $\beta$ 1 by itself also induced the same increase. Adding 300  $\mu$ M Ga to TGF- $\beta$ 1 further stimulated *VEGFA* gene expression by 50%.

Similarly to *PTH1H*, *TGFBR1* gene expression was down regulated by Ga treatment in the absence or presence of TGF- $\beta$ 1, reaching respectively 66% and 48% with 300  $\mu$ M Ga.

Lastly, regardless the absence or presence of TGF- $\beta$ 1, CXCR4 gene expression was not modified by a 100  $\mu$ M Ga treatment. By contrast, either for control or TGF- $\beta$ 1 condition, incubating cells with 300  $\mu$ M Ga induced a 2.5-fold stimulation of gene expression.

#### 4. Discussion

Bone metastases of breast cancer typically lead to a severe osteolysis due to an excessive osteoclastic activity. Considering its bone affinity, as well as its inhibitory action on resorption, we decided to explore *in vitro* Ga effects on osteoclastogenesis, using conditions mimicking an aggressive bone metastatic environment. To address this issue, we used the human breast cancer cell line MDA-MB-231, as well as a bone-seeking clone (MDA-231BO cells), which has been isolated from bone metastases of MDA-MB-231 tumour cells.

First of all, and similarly to Guo *et al* (Guo *et al.*, 2008), we observed that factors derived from breast cancer cells supported mature osteoclasts formation from RANKL-primed precursors in the absence of supporting cell types. We firstly stimulated RAW264.7 cells with breast cancer-derived factors by using conditioned medium (CM) isolated from MDA-MB-231 cell culture. According to Gallet *et al*, MDA-MB-231 cells release several soluble factors such as M-CSF and PTHLH, which both act on osteoclastogenesis and mature osteoclasts survival (Gallet *et al.*, 2004). After 4-days incubation in the presence of conditioned medium, Ga caused a significant decrease in mature osteoclasts number. Interestingly, this inhibitory effect was more pronounced when MDA-MB-231 cells were previously treated with TGF- $\beta$ 1, a factor present within an aggressive bone metastatic environment□.

These preliminary experiments focused on the direct impact of Ga on osteoclast and did not ascertain whether Ga may disrupt the vicious circle between osteoclasts and tumour cells. In this attempt, RAW264.7 cells were co-cultured with MDA-MB-231 cells, in the absence or presence of TGF-  $\beta$ 1. As compared to using conditioned medium, co-culture conditions closely mimics the *in vivo* situation by producing a dynamic and continuous renewal of growth factors released by tumours cells. In line with this, Salamanna *et al.* demonstrated that co-culture of RAW264.7 cells with MRMT-1 breast cancer cells induced a higher formation of multinucleated TRAP+ cells than by using conditioned medium alone (Salamanna *et al.*, 2013). In addition, since Ga was administrated 48 hours after the stimulation of MDA-MB-231 cells and RAW264.7 cells with TGF-  $\beta$ 1 and RANKL respectively, this co-culture model may document Ga actions when the metastatic process is in progress. Using this model, we

demonstrated that 48-hours treatment with Ga significantly diminished osteoclastogenesis in an aggressive bone metastatic environment.

These results suggested that Ga could also impact on tumour cells. To address this question, we used a bone-seeking clone (MDA-231BO cells), which has been isolated from bone metastases of MDA-MB-231 tumour cells. We first confirmed previous results published by Yoneda *et al.* concerning the greater production of parathyroid hormone-related protein (PTHrP) by MDA-231BO as compared to the parental MDA-MB-231 cells (data not shown). This phenotypic change allows breast cancer cells to survive, proliferate into bone and promote osteoclastic bone resorption, which in turn leads to the establishment of severe osteolytic bone metastases. Interestingly, we observed that Ga inhibited in a dose-dependent manner both the proliferation and the viability of MDA-231BO cells, further supporting the interest of using this cell clone in experiments related to a bone metastases context.

To ascertain whether Ga may also affect the activity of these aggressive cells in terms of angiogenesis, invasion, and osteolysis, we next embarked on measuring the expression of major critical marker genes by performing real-time PCR experiments. To our knowledge, this is the first time that TGF- $\beta$ 1 action on the expression by MDA-231BO cells of these critical marker genes is quantified.

Firstly, we evaluated Ga impact on the major osteolytic marker, PTHrP, which is produced by bone metastatic cells. Reducing by 71% PTHrP expression in basal conditions, Ga may directly impact MDA-231BO cells osteolytic properties. Indeed, PTHrP up regulates the expression of RANKL in bone marrow stromal cells, which in turn stimulates the differentiation and activation of osteoclasts, hence favouring tumour progression and bone destruction (Nakamura *et al.*, 2008). Consequently, TGF- $\beta$ 1 that is among the most abundant growth factor stored in bone (Hauschka *et al.*, 1986) is released continually into the bone marrow cavity in active form. In response to TGF- $\beta$ 1, increased PTHrP production may be one of the most critical properties for breast cancer cells to accelerate osteolytic bone metastasis (Yin *et al.*, 1999; Kakonen *et al.*, 2003). Mimicking these conditions, we used TGF- $\beta$ 1-treated MDA-231BO cells and we found that the expression of PTHrP gene was inhibited by 300  $\mu$ M Ga. Thus, Ga antitumor activity could be mediated in part through the downregulation of

*PTHLH* gene expression by tumoral cells, and in other part by disturbing the RANK signalling pathways in osteoclasts, as described in our previous study (Verron *et al.*, 2010b). Taken together, Ga may disrupt the vicious cycle by interfering with the cross talk between breast cancer cells and osteoclasts, which consequently may reduce osteolytic bone lesions and affect tumour cells growth in bone metastases.

By hydrolysing components of the extracellular matrix (ECM), Matrix MetalloProteinases (MMPs), which are a family of zinc-dependent endopeptidases, play a pivotal role in the migration, the invasion and the development within bone of breast carcinoma cancer cells (Kakonen *et al.*, 2003). It has been shown that MDA-MB-231 cells expressed high levels of MMP1, 3 and 9 during the degradation of the ECM, whereas MMP2 and 7 were not detectable (Bachmeier *et al.*, 2001; Barrett *et al.*, 2002). Interestingly, MMP1 and 9 expressions were much higher in bone-seeking clone as compared to parental cells (Yoneda *et al.*, 2001). In preliminary experiments, we found that MDA-231BO cells expressed higher level of MMP9 and 13 as compared to MDA-MB-231 (data not shown). Concerning MMP13, we did not show any significant action due to TGF- $\beta$ 1 treatment, while 300  $\mu$ M Ga stimulated MMP13 gene expression in both conditions. Next, treating MDA-231BO cells with Ga, we first observed an inhibition of *MMP9* gene expression. Interestingly, even when *MMP9* expression was strongly up-regulated by TGF- $\beta$ 1, Ga was able to restore the basal expression level in the absence of any treatment. It should be noted that the lowest Ga concentration (100  $\mu$ M) was sufficient to inhibit *MMP9* expression, while it did not impact *MMP13* expression. Moreover, performing real time PCR quantifications, we observed that *MMP13* was ten-fold less expressed than *MMP9*. Taken together, these data suggest that Ga could exert positive actions on matrix proteases expression and cancer cells invasion.

Anti-angiogenic therapies have raised major interests and promises in cancer therapy in general, and more specifically for the treatment of hyper-vascularized skeletal metastases that are observed in patients with renal carcinoma. Indeed, cutting the blood supply to a tumour is a pertinent approach. Ga induced *VEGFA* expression regardless of the condition (absence or presence of TGF- $\beta$ 1), and this could be prejudicial for the patient. Nevertheless, this specific clinical context requires bone reconstruction after tumour resection. In this

attempt, sufficient vascularization is essential to favour resorption/substitution of the implanted-bone substitutes leading to neof ormation of bone tissue, and angiogenic properties of Ga may be desired. Moreover, before concluding on the putative angiogenesis properties of Ga, further experiments performed on endothelial vascular cells are required.

Similarly to anti-angiogenic therapies, targeting TGF-  $\beta$ 1 has also been applied to cancer (Korpál *et al.*, 2010; Buijs *et al.*, 2011a). Given that TGF-  $\beta$ 1 has been described to stimulate angiogenesis and suppress immune surveillance of tumour cells (Buijs *et al.*, 2011b), these treatments may also reduce bone invasion by tumour cells. In this way, different classes of TGF-  $\beta$ 1 inhibitors have been tested in clinical trials of bone metastases including monoclonal neutralizing TGF-  $\beta$ 1 antibodies (Edwards *et al.*, 2010; Juárez *et al.*, 2011). Considering this therapeutic target of bone metastases, we wanted to decipher whether Ga could disturb TGF-  $\beta$ 1 signalling. Similarly to *PTH1H*, *TGFBR1* gene expression was down regulated by Ga treatment in the absence or presence of TGF- $\beta$ 1, reaching respectively 66% and 48% with 300  $\mu$ M Ga. It is well documented that TGF- $\beta$ 1 is able to promote cancer metastases acting at different levels (tumour microenvironment and immune response) (Padua *et al.*, 2009). Thus, Ga action on one of the TGF- $\beta$ 1 receptor (*TGFBR1*) could represent a substantial benefit in terms of disrupting the vicious cycle involved in bone metastases development. However, TGF- $\beta$ 1 is also involved in tumour-inhibiting pathways (Padua *et al.*, 2009), and anti-TGF- $\beta$ 1 therapies should be carefully evaluated. In this line, and this may be of great interest to avoid drawbacks due to systemic administration, it should be noted that Ga coupled to a bone substitute could exert its action locally and directly on cancer cells, as demonstrated by this report.

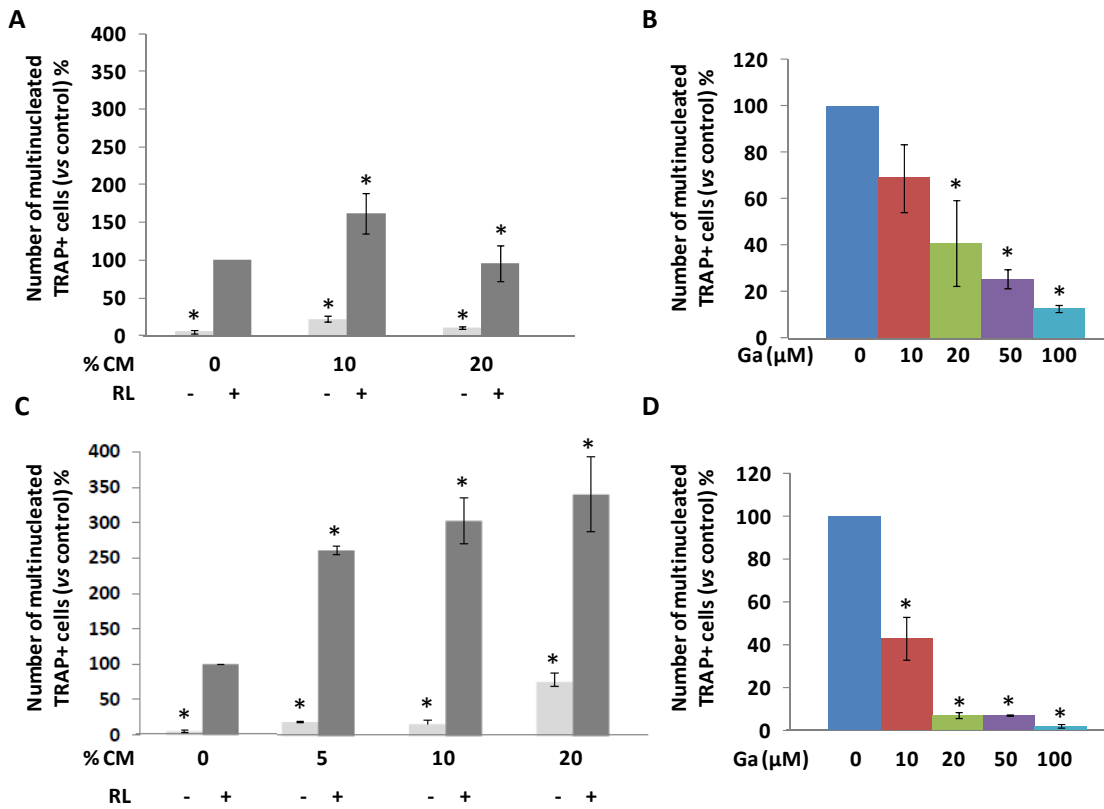
As mentioned in the Introduction section, interactions between breast tumour cells and bone microenvironment are implied in the establishment of bone metastases. Among chemokine receptors expressed by osteotropic cancer cells to mediate their adhesion to endothelium, chemokine (C-X-C motif) receptor 4 (*CXCR4*), the receptor for SDF-1 (stromal cell derived factor-1, or *CXCL12*) has been shown to play a pivotal role in tumour cell homing to bone (Muller *et al.*, 2001; Smith *et al.*, 2004). However, even if *CXCR4* has been found to



closely correlate with the incidence, development, treatment and prognosis of breast cancer (Sterling *et al.*, 2011), overexpression of individual proteins was insufficient to accelerate bone metastases (Kang *et al.*, 2003). By contrast, up regulating the expression of CXCR4, Ga would favour tumour cells retention within CXCL12-rich bone microenvironment, this preventing their dissemination towards other organs. Moreover, keeping metastatic cells in close contact with Ga, which displays a strong affinity for bone mineral, should favour Ga direct actions on cancer cells that are reported above.

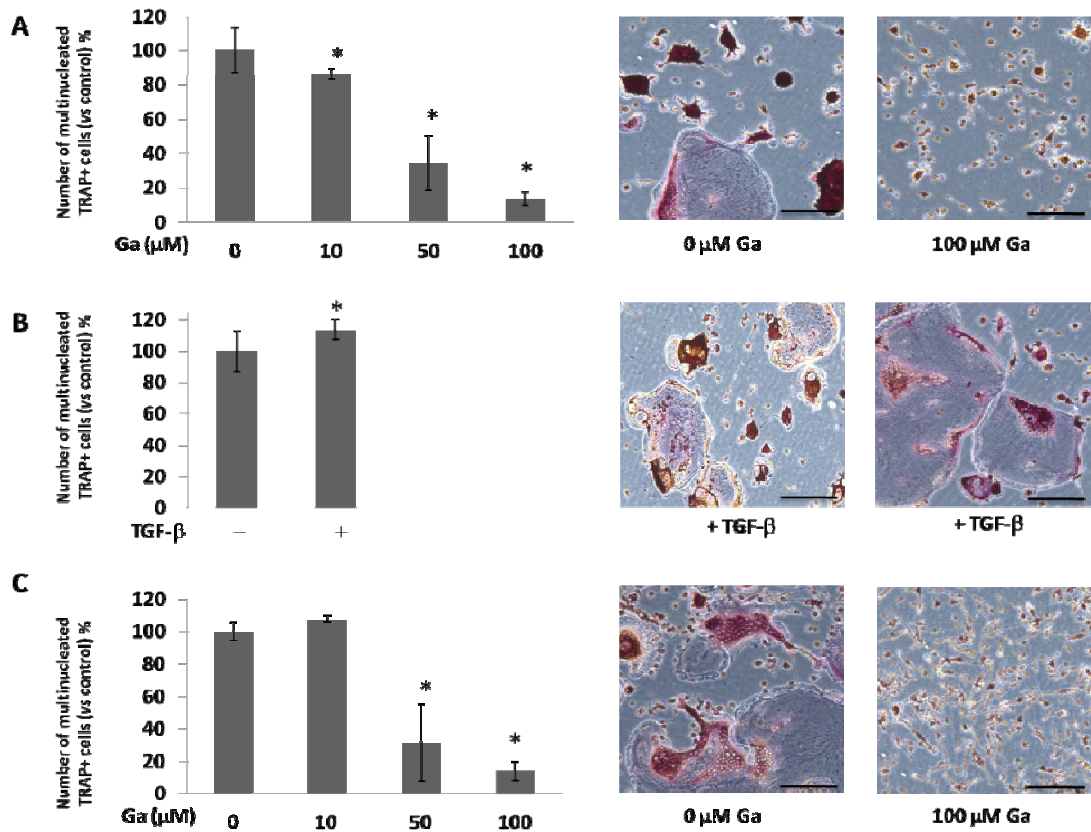
To summary, Ga regulates excessive osteoclastogenesis by acting directly on osteoclasts differentiation. Indirectly, Ga inhibits of PTHLH and MMP9 secretion by tumour cells, and consequently blocks TGF- $\beta$ 1 release from the bone matrix. Together with the inhibition Ga exerts on TGFBR1 expression, these actions could lead to the disruption the vicious circle involving bone cells and tumour cells (Fig. 5). In addition, we demonstrate that Ga affects both proliferation and viability of bone metastatic cells. This is the first time that antitumor properties of Ga have been studied in the context of bone metastases, and our data strongly suggest that Ga is a promising candidate for the treatment of patients with bone metastases from breast cancer.

Fig 1



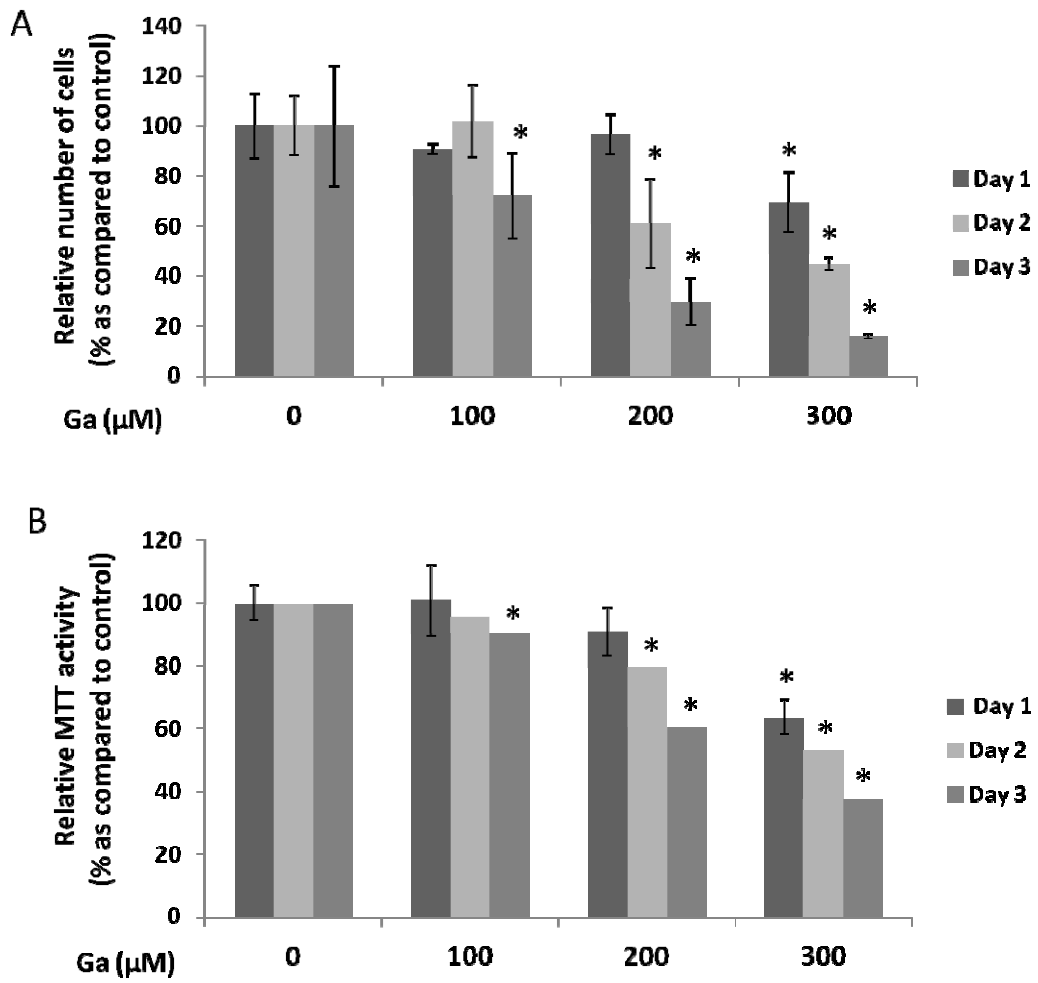
**Figure 1. Ga effect on RAW 264.7 cells differentiation in the presence of conditioned medium from MDA-MB-231 cells.** (A) RAW 264.7 cells were treated during 4 days with 20 nM RANKL (RL) or vehicle, in the presence of conditioned medium (CM; 10% or 20%) isolated from untreated MDA-MB-231 cells. (B) Ga effect on RAW 264.7 cells differentiation measured in the presence of 10% conditioned isolated from untreated MDA-MB-231 cells. (C) RAW 264.7 cells were treated during 4 days with 20 nM RANKL (RL) or vehicle, in the presence of conditioned medium (CM; 5%, 10% or 20%) isolated from MDA-MB-231 cells treated with 10 ng/mL TGF- $\beta$ . (D) Ga effect on RAW 264.7 cells differentiation measured in the presence of 20% conditioned medium isolated from MDA-MB-231 cells treated with 10 ng/mL TGF- $\beta$ 1. \* $p < 0.05$ , statistically significant compared to untreated cells (Kruskall-Wallis test, bidirectional analysis). For A and C experiments, results are normalised according to TRAP+ cell number in 0%CM/+RL condition. For B and D experiments, results are normalised according to TRAP+ cell number in 0  $\mu$ M Ga condition.

**Fig 2**



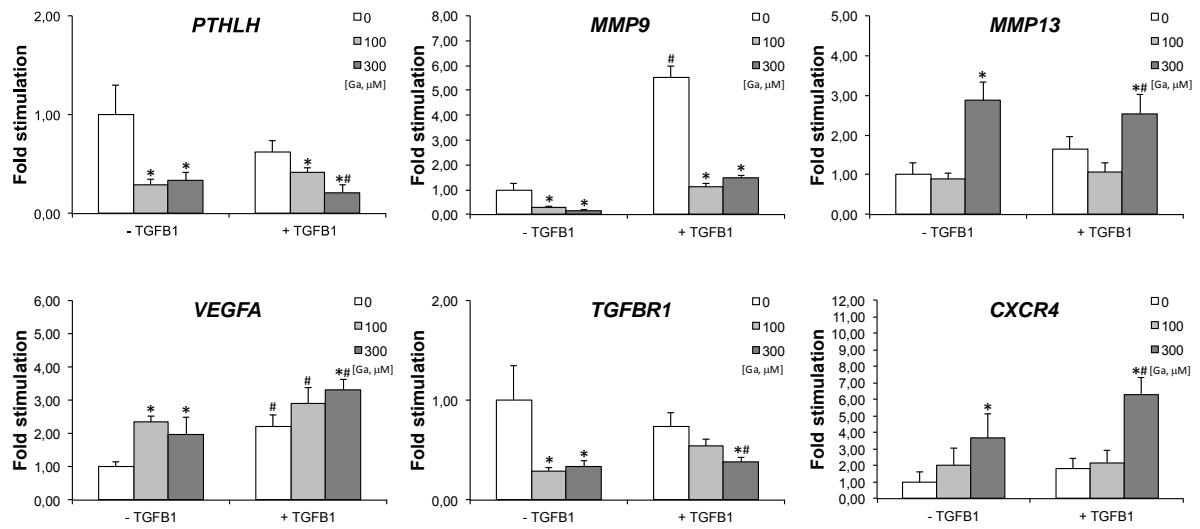
**Figure 2. Ga effect on the differentiation of RAW 264.7 cells co-cultured with MDA-MB-231 cells.** (A) Ga impact on RAW 264.7 cells differentiation in the presence of MDA-MB-231 cells. (B) TGF- $\beta$ 1 (10 ng/mL) impact on RAW 264.7 cells differentiation in the presence of MDA-MB-231 cells. (C) Ga impact on RAW 264.7 cells differentiation in the presence of MDA-MB-231 cells treated with 10 ng/mL TGF- $\beta$ 1. \* $p < 0.05$ , statistically significant compared to untreated cells (Kruskal-Wallis test, bidirectional analysis). In A, B, and C, right panels show light microscopy of TRAP staining (scale bars, 100  $\mu\text{m}$ ). For A, and C experiments, results are normalised according to TRAP+ cell number in 0  $\mu\text{M}$  Ga condition. For B experiment, results are normalised according to TRAP+ cell number in TGF- $\beta$ 1- condition.

**Fig 3**



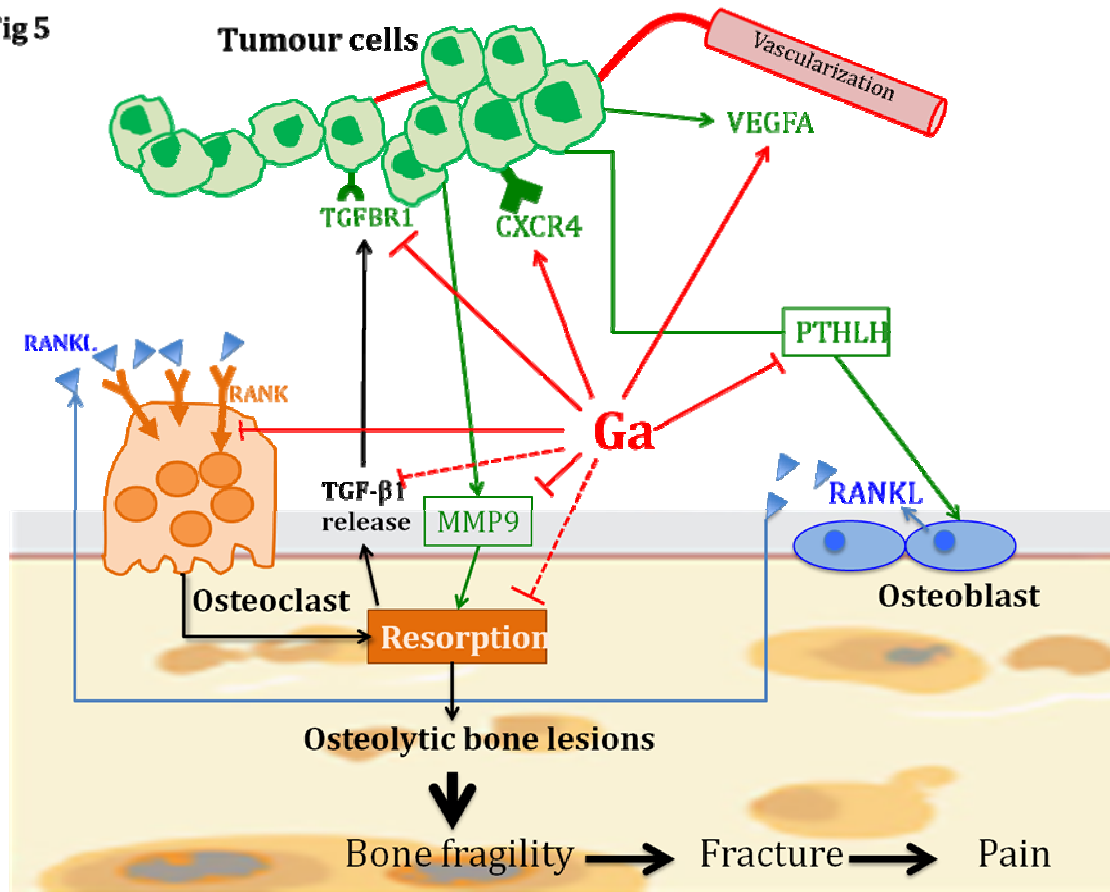
**Figure 3. Ga effect on MDA-231BO cells proliferation and viability.** (A) Effect of increasing doses of Ga on MDA-231BO cells proliferation (A) and viability (B). Results are normalised according to cell number in the untreated condition. \* $p < 0.05$ , statistically significant compared to untreated cells (Kruskal-Wallis test, bidirectional analysis).

**Fig 4**



**Figure 4. Gene expression in MDA-231BO cells: Ga effect in absence or presence of TGF-β1.** MDA-231BO cells were cultured 4 days in the presence of Ga or vehicle, with medium renewal at day 2, then serum-starved overnight and treated 20h with TGF-β1 (10 ng/mL) or vehicle. For cells initially treated with Ga, it was maintained during starvation and TGF-β1 treatment. Results are reported as fold change in gene expression relative to untreated cells (0 μM Ga/-TGF-β1) after normalization against HPRT-1. \* p < 0.05, statistically significant compared to "0 μM Ga" condition; # p < 0.05, statistically significant compared to "0 μ Ga/-TGF-β1 " condition (Kruskall&Wallis test, bidirectional analysis).

Fig 5



**Figure 5. Ga actions in the context of bone metastases.** Ga effects are schematized with lines ended by arrows (stimulation) or bars (inhibition). Dotted lines represent the consequences of Ga actions on osteoclastogenesis and bone resorption.

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## **IV GENERAL THESIS DISCUSSION AND** **PERSPECTIVES**



The introduction of calcium phosphate-based biomaterials in 1980s has revolutionized the field of orthopaedic surgery. Although their mechanical resistance and reconstructive properties fall behind the autograft performance, CaPs are still considered as one of the best alternatives to autologous bone grafting. Their ability to be resorbed and gradually replaced by new bone tissue at the implantation site has promoted their extensive use in clinical practice.

Different pathological situations, such as bone remodelling disorders, infections, excessive inflammation, or residual metastatic cells after tumour resection, can compromise the local bone neoformation and result in considerable postoperative pain. In this view, it is advantageous if an implanted biomaterial, besides its basic mechanical and reconstructive properties, can partially or completely counteract the pathological situation. Indeed, the investigation of different modes of integration of pharmacological compounds into biomaterial structure is one of the main efforts in current development of bone substitutes. As a beneficial side effect of this approach, the strategy of local drug delivery circumvents the major drawbacks of systemic administration namely, poor drug bioavailability, important side effects and in some cases poor patient compliance. Moreover, considering the particular situation of non load-bearing orthopaedic indications, injectable CaP cements (CPC) can easily adapt to complex irregular bone defects providing mechanical support at the injury site and also an osteoconductive surface that promotes bone ingrowth. After implantation, a CPC setting occurs through negligible exothermic reaction, which doesn't damage the surrounding bone tissue or the possible loaded therapeutic agent.

The mentioned properties point out CPCs as highly convenient vectors for local drug delivery in pathological bone context.

#### *Bone tissue reinforcement*

Some of the studied biomaterial-active molecule combinations include: bisphosphonates-loaded CaP for osteoporotic fracture prevention (Elise Verron, Gauthier, Janvier, Pilet, *et al.*, 2010); CaP loaded with analgesics for postoperative pain management (Elise Verron, Gauthier, Janvier, Le Guen, *et al.*, 2010); orthopaedic prosthesis coatings with antibiotics (Tran *et al.*, 2015); CPC coupled to

chemotherapeutic agents for residual metastases eradication (Lopez-Heredia *et al.*, 2011). As an additional example, and in line with the use of this type of bone substitutes loaded with active molecules, Tian *et al.* have studied a calcium phosphate biomaterial loaded with strontium, which displayed anti-osteoporotic properties (Tian *et al.*, 2009).

Osteoporosis is definitely one of the most prominent bone diseases today, as attested by the constant increase in incidence of osteoporotic fractures that tend to heal slowly and can result in considerable chronic pain and reduced patient's mobility. These fractures mainly occur in the conditions of excessive osteoclastic resorption and diminished osteoblastic bone-forming activity. Several small-scale clinical trials reported on positive effects of Ga administration on excessive bone resorption and concomitant hypercalcaemia. Later, it was shown that Ga antihypercalcemic effect stems from its inhibitory action on osteoclasts and consequently on bone resorption. Even though Ga presents a strong affinity to bone, upon oral administration it is poorly absorbed and mainly concentrated in the kidneys and liver (E. Verron, Bouler, & Scimeca, 2012), possibly resulting in organ-specific toxicity. Therefore, it would be preferable to increase Ga bioavailability at the bone site, without increasing the total administrated dose. This could be achieved by local Ga delivery directly to the aimed tissue, taking into account that it is crucial to control the timing and kinetics of Ga *in situ* release. Indeed, after the delivery to the bone site, a burst Ga release would display short-term effects, and a loss of long-term beneficial actions on bone tissue. Moreover, although osteoporosis is a systemic disease, a high percentage of fractures occurs at specific sites, i.e. femoral neck, spine and wrist, which justifies the local approach in fracture prevention and/or treatment. Having these requirements in mind, calcium phosphate cements (CPCs) seem good vectors for local Ga delivery, due to their progressive *in vitro* resorption that assures the sustained and controlled release of the loaded drug.

Considering this objective, Mellier *et al.* (C Mellier *et al.*, 2011; Charlotte Mellier, Fayon, Boukhechba, Verron, LeFerrec, *et al.*, 2015) showed that Ga could be loaded into a CPC structure via substitution with calcium. Given the multiple possible Ga therapeutic effects, this CPC/Ga combined device presents interesting properties for implantation in pathological situations, such as fragilized osteoporotic bone, or in cavities generated by bone tumours resection.

To examine its properties *in vitro*, we implanted CPC/Ga in a critical rat femoral defect. Contrary to our speculations that Ga could enhance bone reconstruction by decreasing osteoclastic bone resorption, Ga-loaded CPC did not exhibit any effect on defect healing compared to its commercially available non-loaded version. To explain this lack of effect, we speculate that the slow CPC dissolution rate *in vitro* did not permit a sufficient Ga release into the implant microenvironment. Thus, the local Ga concentration was suboptimal to have any significant impact on bone cells, including osteoclasts. In order to increase Ga concentration at the implantation site, we used another type of CaP-based substitute, i.e. calcium deficient apatite (CDA) microparticles, which contained a higher amount of Ga, and which were more resorbable. Using CDA/Ga, and compared to the control CDA, we observed an increase in total defect filling and fibrillar collagen amount as shown by our SHG analysis. These results suggest that enhancing Ga concentration at the implantation site can have beneficial effects on bone reconstruction. Although CDA microparticles are not yet a suitable bone substitute for clinical application, these results clearly established the proof of concept demonstrating the advantage of Ga incorporation into the structure of a bone substitute. Certainly, further developments of CPCs with higher Ga content are envisioned, as well as their characterization in osteoporotic animal models (rat, ewe) in order to assess their performances in a pathological bone environment.

To further decipher the mechanisms of Ga action on bone cells observed *in vitro*, we used a model of human osteoblasts and monocytes co-cultured on calcium phosphate pellets. Compared to classical monoculture on plastic surface, the concomitant culture of bone forming and bone resorbing cells on a calcium phosphate substrate resembles more closely to the physiological bone environment. Indeed, as underlined by Nakamura *et al.* and Boukhechba *et al.*, there is a difference in osteoclastic actin ring organisation (M. Nakamura *et al.*, 2013) between cells cultured on plastic or in the presence of calcium phosphate substrates. In our model, osteoblast-monocyte crosstalk on CPC pellets allowed for the complete osteoclastic differentiation in absence of any exogenous ligands, i.e. M-CSF and RANKL, as attested by osteoclast-characteristic TRAP staining. Ga-loaded CPC was able to reduce the number of TRAP<sup>+</sup> multinuclear cells, compared to control CPC, and this was also confirmed by a significant inhibition of typical osteoclastic markers ACP5 (TRAP) and MMP9. Since MMP9 is an important proteinase involved in bone

matrix degradation, its inhibition would possibly result in a decreased bone resorption *in vitro*. In addition, Hayman *et al.* reported disrupted endochondral ossification and mild osteopetrosis in ACP5-deficient mice (Hayman *et al.*, 1996). As a whole, our *in vitro* observations strongly suggest that Ga released from CPC structure is able to interfere with osteoclastic differentiation, which is in accordance with previously reported Ga antiosteoclastic effects (Blair *et al.*, 1992; T. J. Hall & Chambers, 1990; Elise Verron, Masson, Khoshniat, Duplomb, Wittrant, Baud&apos;Huin, *et al.*, 2010), which may account for a shift of the resorption/construction balance towards bone formation.

On the other hand, we found that CPC/Ga possibly stimulated osteoblastic activity by up regulating the expression of several marker genes involved in bone matrix synthesis and mineralization, i.e. collagen, bone sialoprotein II, osteonectin and periostin. These results corroborate with the increase in total bone defect filling and collagen synthesis we observed *in vitro* within Ga-loaded biomaterial implants. In addition, we showed that Ga could reduce osteoblastic proliferation *in vitro*, possibly suggesting that osteoblasts enter in their final maturation phase. This is supported by the upregulated expression of *IBSP*, *ON*, and *POSTN*, which are characteristic markers of later phases of osteoblastic maturation. In contrast to our study, it was previously reported that Ga does not affect osteoblastic viability and proliferation of primary murine cells and MC3T3 –E1 cell line (Elise Verron, Masson, Khoshniat, Duplomb, Wittrant, Baud&apos;Huin, *et al.*, 2010). This discrepancy could be explained by the fact that the mentioned results were obtained with murine cell line models, while our study was conducted on human primary osteoblasts. Secondly, osteoblasts cultured on plastic surface may respond differently from those cultured on bone-mineral resembling substrate, which is the case for CPC pellets. Interestingly, Boukhechba *et al.* showed that, compared to plastic seeding, osteoblastic marker genes are differentially expressed in cells seeded on calcium phosphate microparticles (Boukhechba *et al.*, 2009). This puts in advance the importance of relevant culture models, which more accurately predict *in vitro* responses to therapeutic agents. Altogether, the exact molecular mechanisms of Ga action on bone forming osteoblasts are still unknown, and further research is needed to elucidate the signalling pathways governing Ga effects on this bone cell type.

Concerning the very first step of Ga interaction with cells, it is now well accepted that Ga cellular internalization is mediated by transferrin receptor (Collery

*et al.*, 2002; E Verron *et al.*, 2012). Nevertheless, some authors proposed calcium channels as alternative mechanisms for Ga cellular entry (Abe *et al.*, 2007; Sohn *et al.*, 1993). It would be interesting to further document on Ga interaction with Ca<sup>2+</sup> channels having in view their importance in osteoclastic differentiation. Lastly, when it comes to the Ga intracellular localization, an early study on very small number of Paget's disease patients reported almost exclusive nuclei localization in osteoclasts, which is different from tumour cells where a high association of Ga with the lysosome fraction within the cytoplasm was reported (Mills, Masuoka, Graham, Singer, & Waxman, 1988). These sub-cellular localisation data suggest areas for future research on Ga antiresorptive and antitumor properties.

Another gap in our knowledge about Ga action on bone is the osteocyte. Osteocytes are involved in the control of both osteoblasts and osteoclasts, and they have a remarkable role in the orchestration of bone remodelling. Given that no study has already evaluated Ga effect on osteocytes, it is important to establish whether Ga can interact as well with this ultimate stage of osteoblastic differentiation. The lack of good *in vitro* cell models, together with the difficulties to isolate primary osteocytes, have hampered the research in osteocyte biology in general and *a fortiori*, the investigation about possible Ga effects on the most abundant cell type in bone. As mentioned above, we found that Ga upregulated the expression of several rather late osteoblastic marker genes, and it would be interesting to see if this effect would persist in further osteoblastic maturation towards osteocyte. Furthermore, it would be suitable to study the possible Ga effects on osteocyte formation in a 3D culture systems including calcium phosphate substrates, such as described in several reports (Boukhechba *et al.*, 2009), (Vazquez *et al.*, 2014) or (Quent, Theodoropoulos, Hutmacher, & Reichert, 2015). Even more, the development of dynamic 3D osteoblast-osteocyte models, with mechanical forces applied to the cells, promises to yield relevant data more comparable to *in vitro* physiological situation (Takai, Mauck, Hung, & Guo, 2004).

In addition to develop new models suitable for bone cells study, the use of accurate and reliable techniques to quantify bone tissue formation constitutes an issue. Considering more specifically CaP-based biomaterials, the precise 3D quantification of newly formed bone within calcium phosphate substitutes was so far hindered by difficulties in micro-CT thresholding between biomaterial and bone mineral phase. Considering that type I collagen is the main constituent of bone



organic matrix, its molecular organisation permits for second-harmonic generation (SHG). Indeed, this peculiar microscopy mode can be employed to quantify the organized collagen fibrils in different samples, ranging from *in vitro* engineered bone tissue constructs to osseous biomaterial implants. In the present work, we report for the first time the use of SHG microscopy to quantitatively assess osteogenesis after a bone substitute implantation. This powerful technique opens the door to non-destructive and highly specific 3D quantification of bone reconstruction in the field of bone tissue engineering.

Taken all together, our data promote Ga-CaP biomaterial combined devices as interesting candidates for local osteoporotic fracture prevention. In decades to come, increasing life expectancy will probably lead to a higher number of osteoporotic patients and even greater medical and economical toll for osteoporotic management. In such a situation, the development of efficient treatments that delay osteoporotic bone loss and/or prevent fractures without important side-effects is a major public health challenge.

#### *Bone metastasis treatment*

Given the great number of solid tumours that metastasises to bone, leading to devastating and painful complications associated with bad patient outcome, another important challenge is to successfully block the metastatic process and tumour expansion in bone tissue. In the past years, advances in bone metastasis treatment, including surgical approaches, radiotherapy, and targeted pharmacological therapy, have somewhat improved life quality of the patients. Nevertheless, for the moment, these strategies remain only palliative and do not significantly improve overall patient survival (Krzyszinski & Wan, 2015).

In the case of breast cancer, up to 80% of patients develop bone metastases characterized by severe osteolytic lesions. Nowadays, the main approach in their treatment consists of surgical excision, in some cases accompanied by appropriate chemotherapy. The remaining bone cavity is filled by a suitable bone substitute, which provides mechanical support for the fragilized bone site and also contributes to reduce pain that could be due to the bone void after the resection surgery. Then, depending on its formulation, the substitute can be gradually resorbed and replaced by new bone tissue. However, residual metastatic cells can hamper this bone

reconstruction process. Indeed, tumour-resection surgeries aim to remove the diseased tissue with a minimal damage to the surrounding healthy tissue. This sometimes results in incomplete tumour removal leading to cancer recurrence either at the same site or in a distant organ. To counteract this risk, a local delivery of chemotherapeutic agents via bone substitutes constitute an attractive perspective, as proposed by Lopez-Heredia *et al.* (Lopez-Heredia *et al.*, 2011) who developed and characterized a calcium phosphate cement (CPC) loaded with chemotherapeutic agent paclitaxel. By seeding osteosarcoma U2OS and metastatic breast cancer MDA-MB-231 cells on CPC/paclitaxel composite biomaterial, they observed that CPC-released paclitaxel could efficiently decrease tumour cell viability. These results strongly support the concept of local delivery of anticancer agents at the bone metastatic site.

Among the different therapeutic actions of Ga compounds, antineoplastic properties are the most extensively studied. Numerous studies are in favour of Ga antiproliferative and antimitogenic actions on cancer cells. It was previously demonstrated that Ga is capable of interfering with cancer cells DNA synthesis, chromatin function and protein synthesis (Collery *et al.*, 2002). Due to its chemical similarity with  $Fe^{3+}$  ion, Ga can bind to different  $Fe^{3+}$ -binding proteins and thus, disrupt substantial iron-dependent intracellular pathways, as well as mitochondrial function. Furthermore, Ga was found to alter enzymatic activity of ATPases, DNA polymerases, ribonucleotide reductase, and tyrosin-specific protein phosphatases, as well as to provoke a cell cycle arrest (Collery *et al.*, 2002) directing the tumour cell into apoptosis.

Having in mind the mentioned antitumor potential of Ga compounds, we hypothesize that delivering Ga into bone metastatic environment can inhibit tumour cells proliferation and abolish or delay metastasis progression. In one similar attempt, Zeimaran *et al.* synthesized poly(octanediol citrate)/gallium-containing bioglass scaffolds as a possible prevention tool of metastatic relapse in tumour resection cavities (Zeimaran *et al.*, 2014).

Given that no study has investigated Ga effect on bone metastases, we decided to use MDA-MB-231, as one of the most aggressive human breast carcinoma cell lines, and its bone-seeking clone MDA-231BO, in order to assess Ga anti-metastatic potential. Breast cancer cells are shown to induce osteoclast maturation (Salamanna *et al.*, 2013), this leading to the resorption of bone matrix

and consequently, the release of high amounts of TGF- $\beta$ 1, which is the most implied cytokine in tumour cells activation. Thus, this crosstalk between osteoclasts and tumour cells is the very basis of bone metastasis vicious cycle, and targeting this dialogue represents a promising therapeutic strategy for metastasis attenuation. In our co-culture model, we demonstrated that Ga treatment was able to dose-dependently reverse the MDA-MB-231-mediated differentiation of osteoclast precursor cells. To further mimic physiological bone metastatic environment, these experiments were reproduced in the presence of TGF- $\beta$ 1 cytokine. In this more aggressive condition, Ga treatment remarkably abolished MDA-MB-231-mediated osteoclastogenesis. We demonstrate here that, in addition to a direct action on osteoclasts, Ga can possibly reduce bone osteolytic lesions by affecting tumours cells viability and proliferation, as well as through an inhibition of critical markers genes directly responsible of the vicious cycle, *i.e.* *PTH1LH*, *MMP9* and *TGFBR1*.

A step closer to the real pathological situation would be to perform *in vitro* studies on a bone-resembling substrate, such as calcium phosphate, loaded with Ga. In the same way that calcium phosphate cement (CPC) was employed for *in vitro* investigation of Ga effect on human primary osteoclasts and osteoblasts in non-pathological context, it could be used to study Ga effect on bone metastatic cells alone or in co-culture with osteoclast precursors. It would be interesting to see if CPC-released Ga could interfere with bone metastasis vicious cycle, as observed when Ga was administered in solution. Further studies of vicious cycle markers, like TNF- $\alpha$  and SDF-1, are needed to better understand and therapeutically target the osteoclasts- tumour cells interactions between osteoclasts and tumour cells.

Envisioning *in vitro* studies, a rat model of bone metastases resection was recently developed by Gouget-Surmenian *et al.* (unpublished data). This model represents an interesting approach to assess *in vitro* CPC/Ga potential in a metastatic environment, and to establish if delivering Ga in this environment via a bioresorbable bone substitute is beneficial in fighting the residual metastatic cells.

Regarding the metastatic relapse, some preclinical evidence suggest that metastatic tumour cells can home to and localize in the HSC niche of the bone and survive in a dormant state for long periods (Weillbaecher *et al.*, 2011). Due to the gradual CPC degradation *in vitro*, sustained Ga release could also act on cancer cells in dormancy over a longer period of time.

At last, studies using different tumour cell lines showed that Ga could potentiate the antitumor activity of some putative chemotherapeutics and circumvent drug resistance of tumour cells. This is of particular interest in treatment of multiresistant tumour cell subpopulations (Collery *et al.*, 2002). Thus, combining the chemotherapy with Ga-loaded bone substitutes could decrease the total administrated dose of the chemotherapeutic agent, mitigating the major side effects that usually accompany these treatments.

Taken together, our data indicate that Ga is a plausible candidate for coupling with bone substitutes implanted after tumour resection, due to its potent disruption of vicious cycle by inhibiting on one side the excessive osteoclastogenesis, and on the other side metastatic cells expansion and activity.

#### *Prevention of postoperative infections*

As discussed above, multiple benefits from the presence of Ga in bone pathological environment strongly support also its use as an adjuvant in orthopaedic implants. Besides its antiresorptive and antineoplastic properties, Ga can participate in the prevention of postoperative risks due to its antimicrobial and anti-inflammatory action. Indeed, the first described therapeutic use of Ga compounds was the treatment of syphilis in a rabbit model and the eradication of *Trypanosoma evansi* in a mouse model (Bernstein, 1998). To date, Ga was proved efficient against several pathogens including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* (Rangel-Vega *et al.*, 2015). An interesting recent approach, which takes advantage of Ga antibacterial properties, is gallium and silver ions coating of titanium bone implants to counteract the biofilm formation after implantation (Cochis *et al.*, 2014).

#### *Concluding remarks*

On the whole, we propose that Ga incorporation enhances biological properties of calcium phosphate substitutes for bone tissue engineering. In terms of applications, gallium could be exploited for: (i) its antiresorptive potential for the prevention of osteoporotic fractures; (ii) its antitumor effects that may be beneficial

to reconstructing bone tissue after tumour resection; (iii) its antimicrobial action that could counteract postoperative infections.

## **ANNEX**

Article III : Molecular effects of gallium on osteoclastic differentiation of mouse and human monocytes





## Molecular effects of gallium on osteoclastic differentiation of mouse and human monocytes

E. Verron<sup>a,b,d,\*</sup>, A. Loubat<sup>c</sup>, G.F. Carle<sup>a</sup>, C. Vignes-Colombeix<sup>d</sup>, I. Strazic<sup>d</sup>, J. Guicheux<sup>d</sup>, N. Rochet<sup>a</sup>, J.M. Boulter<sup>d</sup>, J.C. Scimeca<sup>a</sup>

<sup>a</sup> GÉPITOs, Université de Nice, CNRS, UMR 6235; UFR Médecine, 28 avenue de Valombrose, 06107 NICE, cedex 2, France

<sup>b</sup> GRAFTYS SA, Aix En Provence, France

<sup>c</sup> IFR50, Université de Nice, Service de Cytométrie Pasteur; UFR Médecine, 28 avenue de Valombrose, 06107 NICE, cedex 2, France

<sup>d</sup> INSERM U791, Faculté de Chirurgie Dentaire, Nantes, France

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### ABSTRACT

We had previously reported that gallium (Ga) inhibited both the differentiation and resorbing activity of osteoclasts in a dose-dependent manner. To provide new insights into Ga impact on osteoclastogenesis, we investigated here the molecular mechanisms of Ga action on osteoclastic differentiation of monocytes upon Rankl treatment. We first observed that Ga treatment inhibited the expression of Rankl-induced early differentiation marker genes, while the same treatment performed subsequently did not modify the expression of late differentiation marker genes. Focusing on the early stages of osteoclast differentiation, we observed that Ga considerably disturbed both the initial induction as well as the autoamplification step of *Nfatc1* gene. We next demonstrated that Ga strongly up-regulated the expression of *Traf6*, *p62* and *Cyld* genes, and we observed concomitantly an inhibition of IκB degradation and a blockade of NFκB nuclear translocation, which regulates the initial induction of *Nfatc1* gene expression. In addition, Ga inhibited *c-Fos* gene expression, and subsequently the auto-amplification stage of *Nfatc1* gene expression. Lastly, considering calcium signaling, we observed upon Ga treatment an inhibition of calcium-induced Creb phosphorylation, as well as a blockade of gadolinium-induced calcium entry through TRPV-5 calcium channels. We identify for the first time *Traf6*, *p62*, *Cyld*, IκB, NFκB, *c-Fos*, and the calcium-induced Creb phosphorylation as molecular targets of Ga, this tremendously impacting the expression of the master transcription factor *Nfatc1*. In addition, our results strongly suggest that the TRPV-5 calcium channel, which is located within the plasma membrane, is a target of Ga action on human osteoclast progenitor cells.

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## 1. Introduction

Gallium (Ga) was discovered in 1875 by the French chemist Paul-Emile Lecoq de Boisbaudran, who chose this name in honor of Gallia (France) [1]. In the early 1970s, it was demonstrated that Ga had several therapeutic uses including (i) the decrease of accelerated bone mineral resorption, which occurs during osteolytic bone diseases, and the subsequent lowering of elevated plasma calcium levels associated with these pathologies; (ii) the inhibition of neoplastic proliferation; (iii) the treatment of some intracellular pathogens such as species of *Mycobacterium* [2]. Studies on Ga, which were mainly focused on its antitumor

activities [3], have shown that Ga caused a mild hypocalcemia [4]. In this context, Ga has been used to treat hypercalcemia resulting from malignant tumors such as parathyroid carcinoma and Paget's disease of bone [5–7]. This antihypercalcemic effect of Ga results from an inhibition of bone resorption rather than an increase of urinary calcium excretion [5,8]. Indeed, due to its chemical characteristics, Ga presents an affinity for biological apatite that explains the presence of Ga deposits in bone tissue, and preferentially at sites of rapid bone remodeling such as active metaphyseal growth plate and healing fractures [9–11].

Despite these observations, few studies have explored the effect of Ga on bone cells [8,12,13]. Hall et al. demonstrated that Ga inhibited bone resorption in a dose-dependent manner, and for example, at 100 μg/mL, Ga reduced the resorption activity by 64% without inducing modifications to the morphology or number of osteoclasts [12]. In contrast to these findings, Blair et al. showed a cytotoxic effect of Ga on osteoclasts [13]. Considering this discrepancy, we previously investigated the biological effect of

\* Corresponding author at: INSERM U791, Faculté de Chirurgie Dentaire, 1, place Alexi Ricordeau, 44042 Nantes, France. Tel.: +33 2 40 41 29 16; fax: +33 2 40 08 37 12.

E-mail address: [elise.verron@univ-nantes.fr](mailto:elise.verron@univ-nantes.fr) (E. Verron).



Ga on bone cells. We reported that Ga inhibited both the differentiation and the resorbing activity of osteoclasts in a dose-dependent manner (0–100  $\mu\text{M}$ ) [14]. Furthermore, Ga did not affect the viability or proliferation of osteoblasts. Despite these results strongly suggesting a therapeutic potential of Ga, many unknowns remain concerning the mechanism of Ga action. With this in mind, we examined the molecular mechanisms of Ga action on osteoclasts.

Osteoclasts are giant multinucleated cells of monocyte-macrophage origin that degrade bone matrices [15]. RANKL (receptor activator of NF $\kappa$ B ligand) and M-CSF (macrophage colony stimulating factor) are the critical factors that regulate osteoclastic differentiation [16]. RANKL is expressed by osteoblasts and triggers pre-osteoclast differentiation [17] whereas M-CSF, secreted by osteoblasts, provides differentiated cells with a survival signal [18]. RANKL strongly induces the expression of nuclear factor of activated T cells, cytoplasmic 1 (Nfatc1), a transcription factor that is a master regulator of osteoclast differentiation. Nfatc1 induction is dependent on both NF $\kappa$ B and *c-Fos* pathways [19–22]. More specifically, the initial induction of Nfatc1 synthesis requires the activation of the NF $\kappa$ B pathway through the signaling molecule Traf6 (tumor necrosis factor receptor-associated factor 6), as well as the nuclear translocation of the transcription factor Nfatc2, that is constitutively expressed in precursor cells. Upon RANKL binding to its receptor RANK, the complex cooperates with costimulatory receptors to activate calcium signaling that stimulates both the initial induction and the auto-amplification phase of Nfatc1 expression [23]. Indeed, Nfatc1 binds to its own promoter, thus leading to robust induction of Nfatc1 protein synthesis [20,24]. An activator protein (AP)-1 complex containing RANKL-activated *c-Fos* is also required for the auto-amplification of Nfatc1 [23]. Eventually, AP-1/Nfatc1 complexes turn on a transcriptional program leading to the expression of osteoclast-specific genes, and the acquisition of mature phenotypic markers such as tartrate-resistant acid phosphatase (Acp5), calcitonin receptor (Ctr), matrix metalloproteinase 9 (Mmp9), integrin- $\beta$ 3 (Itg- $\beta$ 3) or a specific osteoclastic proton pump namely ATP6V0A3.

In this study, we investigated Ga effects on RANKL-induced osteoclastic differentiation of monocytes, comparing first the early and late stages of the process. We next focused on molecular events governing osteoclastic differentiation in its early phase, including the regulation of *Nfatc1* gene expression. Lastly, we hypothesized that Ga may act on calcium uptake and we tested this hypothesis.

## 2. Materials and methods

### 2.1. Materials

Alpha minimal essential medium ( $\alpha$ -MEM), Dulbecco's Modified Eagle's Medium (DMEM), antibiotic mixture (P/S; 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin), phosphate-buffered saline (PBS), Hanks' Balanced Salt Solution (HBSS), ionomycin, Fluo-3AM and Fura-RedAM were purchased from Invitrogen Corporation (Paisley, UK). Fetal calf serum (FCS), culture plates and plastics were obtained from Dominique Dutscher (Brumath, France). Gallium nitrate and gadolinium nitrate were obtained from Sigma (Saint Quentin Fallavier, France). Recombinant human M-CSF and RANKL were provided by PeproTech (Rocky Hill, NJ, USA).

### 2.2. Cell culture

RAW 264.7 cell line (Ref. # TIB-71) was obtained from ATCC (LGC Standards, Molsheim, France). Cells were cultured in DMEM containing 5% fetal bovine serum (Hyclone serum, Thermo Fisher

Scientific, Brebière, France). For osteoclastic differentiation experiments, RAW 264.7 cells were seeded at 5000 cells/cm<sup>2</sup> in  $\alpha$ -MEM containing 5% Hyclone serum and effectors were added immediately. RANKL (Receptor Activator of Nuclear Factor- $\kappa$  B Ligand) was used at 20 nM. Cells were cultured for four days with a renewal of the medium at day 2.

Human osteoclast precursors were purified from blood samples from healthy donors (obtained from the EFS, Etablissement Français du Sang, Marseille, France). Human CD11b<sup>+</sup> cells were purified using a CD11b MicroBeads kit (catalogue # 130-049-601) from Miltenyi Biotec (Paris, France) according to the manufacturer's instructions. For osteoclastic differentiation experiments, CD11b<sup>+</sup> cells were seeded at 30,000 cells/cm<sup>2</sup> in  $\alpha$ -MEM containing 10% Hyclone serum, and cultured in the presence of 33 ng/mL hM-CSF and 66 ng/mL hRANKL as previously described [25].

### 2.3. Human cytokines and mouse effectors production (GST control protein, RANKL)

Human recombinant cytokines were purchased from Pepro-Tech (Recombinant Human M-CSF, #300-25; Recombinant Human soluble RANK Ligand, #310-01). Mouse effectors were produced as fusion proteins with GST. GST-Rankl was produced as previously described [26]. For all the experiments using cells of murine origin, a GST protein, produced and purified using the same protocol, was used as a control.

### 2.4. Real-time PCR experiments

Total RNA samples were prepared using NucleoSpin RNA II kit (Macherey Nagel, Hoerd, France), and reverse transcription (Superscript II/Rnase H<sup>-</sup>/Reverse transcriptase, Invitrogen) was performed with 1  $\mu\text{g}$  of RNA and random primers. A 10-fold dilution of cDNAs was used in amplification reactions. PCR experiments reported in Table 1 were performed using an ABI PRISM 7000 system (Applied Biosystems, Life Technologies SAS, Villebon-sur-Yvette, France), and qPCR Mastermix Plus was purchased from Eurogentec (Eurogentec France SASU, Angers, France). Reactions were performed in a 20  $\mu\text{L}$  final volume using 5  $\mu\text{L}$  of diluted cDNAs. Amplification conditions were as follows: 50 °C, 2 min; 95 °C, 10 min; (95 °C, 15 s; 60 °C, 1 min) cycled 40 times. The 36B4 housekeeping gene (Acidic Ribosomal Phosphoprotein P0) was used for normalization of the results. Real-time PCR reactions were performed using mouse and human gene-specific primers listed in Tables 1 and 2.

### 2.5. Immunolabeling

Cells were grown on glass coverslips in 24-well plates. After fixation and blocking with PBS containing 10% normal goat serum

**Table 1**  
Mouse gene-specific primers used for real time RT-PCR analysis.

| Mouse gene   | Primer sequence                      | GI        |
|--------------|--------------------------------------|-----------|
| 36B4         | Forward 5'-tccaggctttgggcatca-3'     | 118131200 |
|              | Reverse 5'-cgctgggaacactcgcgatagg-3' |           |
| Nfatc1       | Forward 5'-tgaggctggtctctccgagtt-3'  | 118131200 |
|              | Reverse 5'-cgctgggaacactcgcgatagg-3' |           |
| Traf6        | Forward 5'-aacgtgctgtgtccatggc-3'    | 38348245  |
|              | Reverse 5'-cagttctcatgtgcaactggg-3'  |           |
| Sqstm1 (p62) | Forward 5'-atgtggaacatggagggaaga-3'  | 118130186 |
|              | Reverse 5'-ggagttcaccctgtatgggt-3'   |           |
| Cylid        | Forward 5'-caacatggatgccaggttgc-3'   | 28972434  |
|              | Reverse 5'-gcctgaactcattgtgacagta-3' |           |
| c-Fos        | Forward 5'-gggacagccttctactacat-3'   | 31560587  |
|              | Reverse 5'-gatctgcgcaaaagtctgtg-3'   |           |

**Table 2**  
Human gene-specific primers used for real time RT-PCR analysis.

| Human gene | primer sequence  | GI        |
|------------|--|-----------|
| 36B4       | Forward 5'-tgcatcagtagcccccattatcat-3'<br>Reverse 5'-aggcagatggatcagccaaga-3'    | 49087144  |
| NFATC1     | Forward 5'-gcatcacagggaagaccgtgtc-3'<br>Reverse 5'-gaagttcaatgtcggagtcttctgag-3' | 27502392  |
| JDP2       | Forward 5'-cttctctgttccggcagc-3'<br>Reverse 5'-cttctcggagggtgaaactgg-3'          | 205277415 |
| JUND       | Forward 5'-gtctacgcgaacctgagcagcta-3'<br>Reverse 5'-ctcgtctctgagcgcagccaggc-3'   | 169234622 |
| FRA2       | Forward 5'-tagatagctgctcaggcag-3'<br>Reverse 5'-ggttggacatggaggatcac-3'          | 44680151  |
| C-FOS      | Forward 5'- tgcctctcctaatgacctga-3'<br>Reverse 5'-ataggtccatgtctggcagcga-3'      | 6552332   |
| ATP6VOA3   | Forward 5'-gaagaggaaatgagcagcc-3'<br>Reverse 5'-ccgtaccaggagggtcaac-3'           | 19924144  |
| ACP5       | Forward 5'-gaccacctggcactgtctctg-3'<br>Reverse 5'-tggtgaggaagtcatctgagttg-3'     | 161377452 |
| MMP9       | Forward 5'-gtgctgggctgctgcttctgct-3'<br>Reverse 5'-gtcgcctcaaggtttggaat-3'       | 74272286  |
| CTR        | Forward 5'-tggtgccaaccactatccatgc-3'<br>Reverse 5'-cacaagtccgccatgacag-3'        | 46361988  |
| ITGB3      | Forward 5'-cattactctgctccactacca-3'<br>Reverse 5'-aacggattttcccataagca-3'        | 47078291  |
| BIM        | Forward 5'-atcccccttttcatcttta-3'<br>Reverse 5'-aggacttgggtttgtgttg-3'           | 116734657 |
| BCLXL      | Forward 5'-atggcagcagtaaagcaagc-3'<br>Reverse 5'-cggaaagagttcattcactacctgt-3'    | 20336334  |
| BCL2       | Forward 5'-atgtgtgtggagagcgtcaacc-3'<br>Reverse 5'-tgagcagagcttccagagacacc-3'    | 72198188  |
| BAX        | Forward 5'-gctgttgggctggatccaag-3'<br>Reverse 5'-tcagccctcttctccaga-3'           | 163659848 |
| BAD        | Forward 5'-cgagtgagcaggaagactcca-3'<br>Reverse 5'-aggagtccacaactcgtcact-3'       | 197116381 |

for 20 min at room temperature, cells were incubated with a 1/50 dilution of an anti-Nfat1 antibody (Cat. # sc-7294, Santa Cruz Biotechnology, Heidelberg, Germany) or with a 1/50 dilution of an anti-NFκB antibody (Cat. # 4764S, Cell Signaling Technology, Danvers, MA 01923) in PBS containing 1.5% normal goat serum (Rockland, Gilbertsville, USA), for 60 min at room temperature. After washes in PBS, cells were subsequently incubated with a 1/500 dilution of a secondary antibody coupled to FITC (Cat. # sc-2010, Santa Cruz Biotechnology, Heidelberg, Germany), in PBS containing 3% normal goat serum, for 45 min at room temperature in the dark. Cells were counterstained with DAPI (Fig. 3A) or Topo-3 (Fig. 3B) for nuclear staining. Immunofluorescence was visualized using a confocal laser scanning microscope (LSM5, Zeiss).

## 2.6. Western-blot

RAW 264.7-cells were lysed in lysis buffer containing 0.2% Nonidet 40, 50 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0, 1 mM DTT, including a protease inhibitor cocktail ("Complete Mini", Roche Diagnostics, Meylan, France) and phosphatase inhibitors (50 mM NaF and 1 mM Na<sub>3</sub>VO<sub>4</sub>). Following a centrifugation step at 15,000 × g for 15 min, protein concentration in the supernatant was determined using a BCA Protein Assay Kit from Pierce (Perbio Science). Proteins were separated by SDS-PAGE on 10% gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% BSA in Tris-buffered saline (TBS) containing Tween-20 (TTBS) at room temperature for 1 h 30 and then incubated for 2 h with primary antibodies: mouse anti-phospho-IκB (Cat. # 9246), mouse anti-IκB (Cat. # 4814), mouse anti-phospho-Akt (Cat. # 4051), mouse anti-Akt (Cat. # 2967), mouse anti-phospho-CREB (Cat. # 9196), mouse anti-β-Actin (Cat. # 3700) (all primary antibodies were from Cell Signaling Technology, Danvers, MA 01923). Horseradish peroxidase-conjugated anti-mouse antibody (Cat. # sc-2005, Santa Cruz Biotechnology, Heidelberg, Germany) was used as a secondary

antibody (45 min incubation). The antigen–antibody complexes were visualized using ECL Plus Kit (Amersham Biosciences, Piscataway, USA).

## 2.7. Measurement of intracellular Ca<sup>2+</sup> oscillations

CD11<sup>+</sup> cells suspended at 1.5 × 10<sup>6</sup> cells/mL in HBSS were preincubated with 100 μM Ga for 1 h at 37 °C. Cells were loaded during 30 min with cell-permeable calcium indicators, in HBSS medium containing 2.6 μM Fluo-3 AM (Cat. # F-1242) and 5.5 μM Fura-Red AM (Cat. # F-3021) (calcium indicators were from Invitrogen Corporation, Paisley, UK). Cells were next washed once and resuspended in HBSS medium (1.5 × 10<sup>6</sup> cells/mL). Where noted, reagents including ionomycin, EGTA, gallium, and gadolinium were added to the medium after an equilibration period of 1 min. Using specific filters, the Fluo-3/Fura-Red emission ratio of fluorescence was analyzed by flow cytometry (FACS Aria cytometer, BD Biosciences, Le Pont de Claix, France).

## 2.8. Statistical analysis

The data shown is representative of at least three independent experiments. Results are expressed as mean ± standard deviation of three determinations. The statistical differences between two independent groups were evaluated using the Mann & Whitney test (bi-directional analysis). Comparative analysis of more than two independent groups was performed using the Kruskal–Wallis test (bi-directional analysis). The differences measured were considered to be statistically significant for  $p < 0.05$ .

## 3. Results

### 3.1. Effects of Ga on differentiation and survival of human osteoclasts

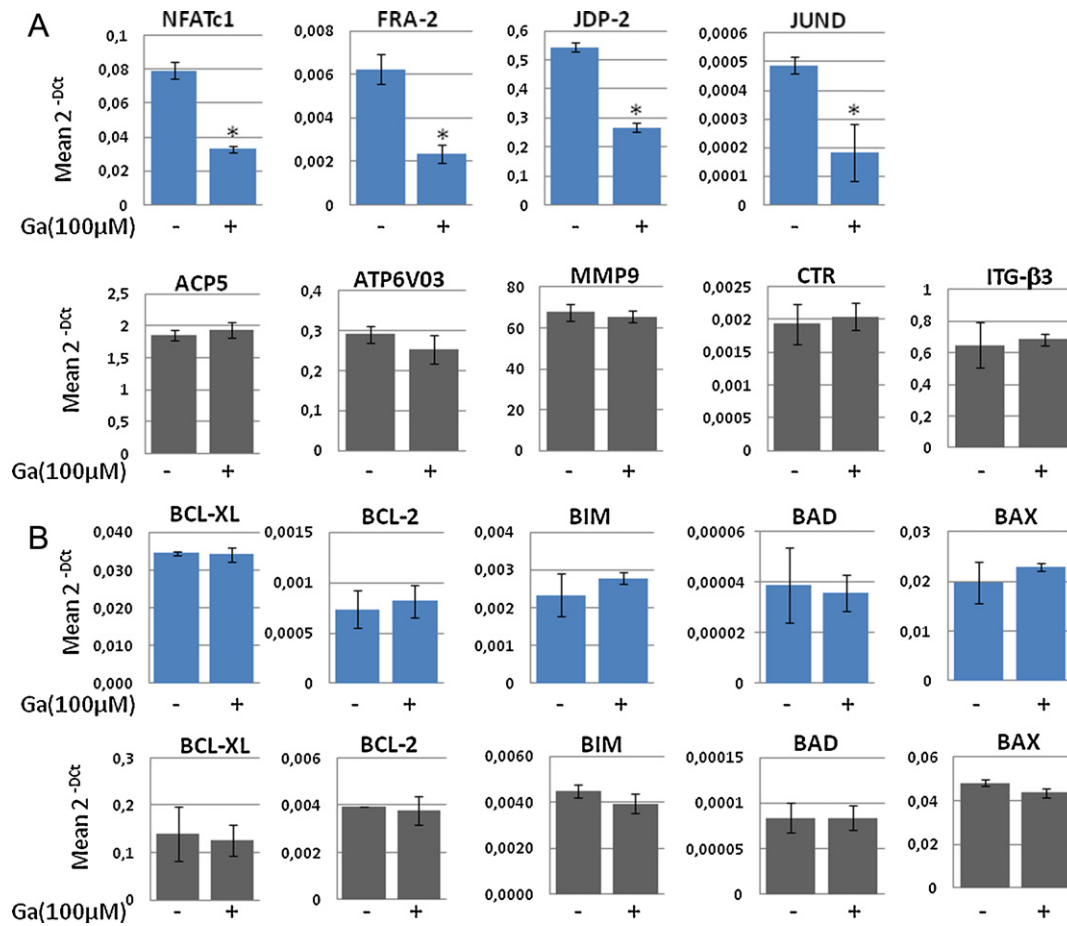
Human CD11b<sup>+</sup> cells isolated from peripheral blood were cultured in differentiating medium for 2 or 7 days, before a 12-h incubation in the presence of 100 μM Ga, the pharmacological dose which was demonstrated to be optimal in our previous study [14]. As depicted in Fig. 1A, 100 μM Ga significantly down-regulated the expression of specific osteoclastic differentiation early marker genes including *NFATC1*, *FRA2*, *JDP2* and *JUND*. Conversely, when cells were incubated in the presence of 100 μM Ga after seven days of differentiation, Ga did not modify the expression of specific osteoclastic differentiation late marker genes such as *ACP5*, *ATP6VOA3*, *MMP9*, *CTR* and *ITGB3* (Fig. 1A).

On the whole, a 12-h Ga treatment inhibited the induction of early differentiation marker gene expression, while the same treatment performed subsequently did not modify the expression of late differentiation marker genes.

As RANKL and M-CSF are also largely involved in osteoclastic survival, we wanted to determine whether Ga may have an effect on apoptosis induction both in precursor and mature osteoclasts. Thus, we explored Ga impact on the expression of pro- and anti-apoptotic genes. Human CD11b<sup>+</sup> cells were differentiated in the presence of hM-CSF and hRANKL. After 2 or 7 days, cells were treated for 12 h with 100 μM Ga (Fig. 1B), and the expression of pro- and anti-apoptotic genes was quantified by RT-PCR. As shown in Fig. 1B, Ga treatment did not affect the expression of the most prominent pro- and antiapoptotic factors, as measured in both osteoclastic precursor cells and in mature osteoclasts.

### 3.2. Ga effect on *Nfatc1* expression

Given that Ga disturbed expression of early marker genes (Fig. 1A), we next focused on this early phase of osteoclastic differentiation, and more particularly on the initial induction of



**Fig. 1.** Ga effect on differentiation (A) and apoptosis (B) of osteoclasts. Human CD11b<sup>+</sup> cells were cultured in the presence of 33 ng/mL hM-CSF and 66 ng/mL hRANKL. After two days (blue) or seven days (gray), cells were treated for 12 h with 100 µM Ga (+) or its vehicle (-). Quantitative real-time PCR analysis was performed using *36B4* as housekeeping gene. \**p* < 0.05, statistically significant compared to untreated cells (Mann & Whitney test, bidirectional analysis). (A) Real time PCR analysis of the main osteoclastic differentiation marker genes. (B) Real time PCR analysis of the main cellular survival and apoptosis marker genes. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

*Nfatc1* gene expression, and the auto-amplification step that leads to *Nfatc1* protein level upregulation.

We had previously demonstrated that Ga reduced, in a dose-dependent manner, the initial induction of *Nfatc1* gene in RAW 264.7 cells incubated in the presence of Rankl for 12 and 24 h [14]. To investigate Ga impact on the auto-amplification stage, which occurs subsequent to the initial induction, we measured *Nfatc1* gene expression after a 48 h incubation period of cells with both Rankl and Ga. As shown in Fig. 2A, Ga induced a downregulation of *Nfatc1* gene expression, and this inhibition reached 40% when 100 µM Ga was used.

To correlate these effects of Ga at the transcript level with *Nfatc1* protein content, we performed immunolabeling of RAW 264.7 cells treated with Rankl/Ga for 48 h (Fig. 2B). In the absence of Rankl, we detected a weak labeling of cells (Fig. 2B, b), while Rankl treatment induced a strong expression of *Nfatc1* protein expression (Fig. 2B, c and d). Lastly we observed that a 100 µM Ga treatment prevented Rankl-induced *Nfatc1* protein expression (Fig. 2B, e and f).

In summary, we found that in addition to the initial induction of *Nfatc1* (12–24 h), Ga treatment impacted latter amplification step (48 h), both at the transcript and protein levels.

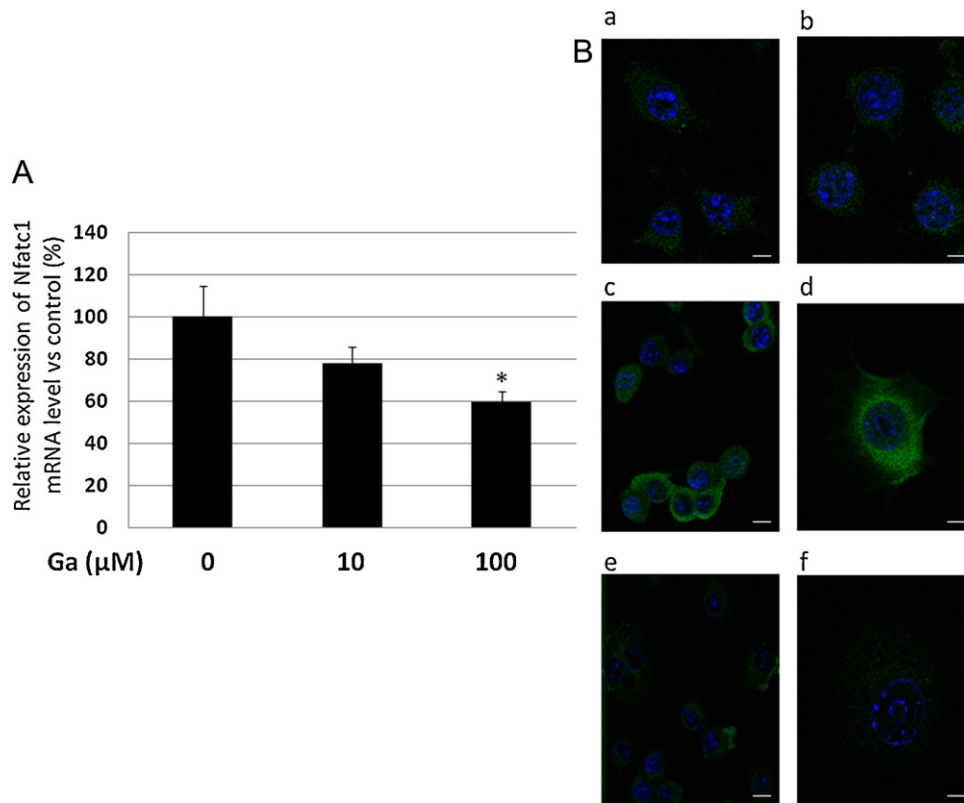
### 3.3. Ga effect on initial induction of *Nfatc1*

As NFκB activation plays a critical role in initiating a robust induction of *Nfatc1*, we next wanted to decipher the effect of Ga on the NFκB signaling pathway.

The phosphorylation of IκB is a marker for ubiquitination and subsequent proteasome-mediated degradation. The degradation of inhibitory κB (IκB) unmasks the nuclear localization signal motif of NFκB, thus allowing its nuclear translocation and participation in the initial induction of *Nfatc1* gene expression. As shown in Fig. 3A, Rankl induced IκB phosphorylation within 15 min. Concomitantly, IκB protein was degraded, as shown at 30 min, before resynthesis between 45 and 60 min. Ga did not block IκB phosphorylation, but rather weakly stimulated it at 45 min. In contrast, we observed that 100 µM Ga partially inhibited Rankl-induced IκB degradation detected at 30 and 45 min.

Overall, while Ga treatment did not reduce IκB phosphorylation within differentiating osteoclast progenitor cells, Ga partially and transiently blocked Rankl-induced IκB degradation. To deepen Ga effect on NFκB signaling pathway, we determined whether this blockage had repercussions on nuclear NFκB translocation by performing immunostaining of NFκB protein. As shown in Fig. 3B(a), NFκB, which is preferentially located in the cytoplasm following a 15 min Rankl treatment, is progressively translocated within the nucleus as observed after a longer incubation period with Rankl. In contrast, a 100 µM Ga treatment blocked NFκB nuclear translocation, at least up to 45 min following Rankl addition (Fig. 3B, b).

Upstream signaling of NFκB by Rankl involves the recruitment of Traf6 following the binding of Rankl to its receptor Rank. In addition, NFκB nuclear translocation is mediated by the interaction of Traf6 with p62 and IKK [27]. Moreover, p62 interacts with deubiquitinating enzyme Cyld to negatively regulate Rank signaling [28]. Thus, we next wanted to evaluate Ga impact on



**Fig. 2.** Ga effect on the expression of *Nfatc1* gene and Nfatc1 protein. (A) Real-time PCR analysis of *Nfatc1* gene expression. RAW 264.7 cells were differentiated for 48 h with 20 nM Rankl, in the presence of 10  $\mu\text{M}$  or 100  $\mu\text{M}$  Ga. Results are expressed as a percentage of *Nfatc1* gene expression level in control condition. \* $p < 0.05$ , statistically significant compared to untreated cells (Kruskall–Wallis test, bidirectional analysis). (B) Immunostaining analysis of Nfatc1 protein expression. (a) Control immunostaining without primary antibody; (b) control differentiation without Rankl treatment; (c–f) RAW 264.7 cells were cultured for 48 h with 20 nM Rankl, in absence (c, d) or in presence (e, f) of 100  $\mu\text{M}$  Ga. (bar = 10  $\mu\text{m}$ ).

Traf6, p62 and Cyld. As depicted on Fig. 4, we observed that 100  $\mu\text{M}$  Ga significantly increased *Traf6*, *p62* and *Cyld* gene expression when compared to the quantification upon cells treatment with Rankl alone.

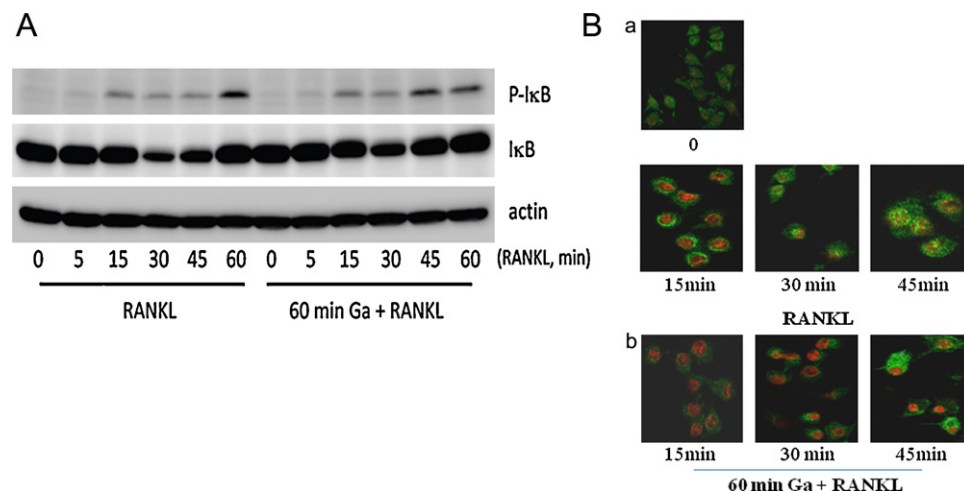
#### 3.4. Ga effect on *Nfatc1* auto-amplification

*Nfatc1* positively regulates its own gene expression through an auto-amplification mechanism involving its recruitment to its own

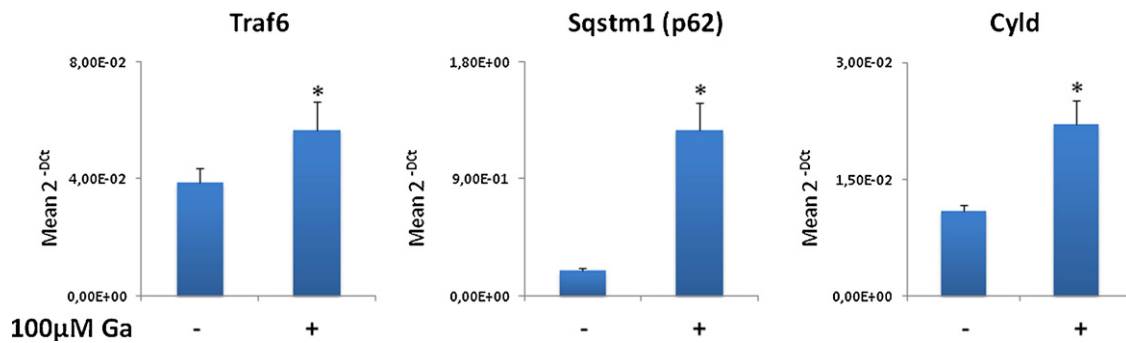
promoter as an AP-1 complex containing c-Fos [19,20,23]. Using RAW 264.7 cells, we observed that a 100  $\mu\text{M}$  Ga treatment reduced Rankl-induced *c-Fos* gene expression by 53% in comparison to control condition (Fig. 5).

#### 3.5. Ga effect on Rankl-induced Akt and Creb phosphorylation

Akt signaling is one of the pathways that interact with NF $\kappa$ B and *c-Fos* in response to Rankl [29,30]. Rankl stimulation triggers



**Fig. 3.** Ga effect on Rankl-induced NF $\kappa$ B signaling pathway. (A) Ga effect on Rankl-induced I $\kappa$ B phosphorylation. RAW 264.7 cells were cultured for 16 h in serum-depleted medium containing 0.2% BSA, pretreated with 100  $\mu\text{M}$  Ga (+) or its vehicle (–) for 1 h, and stimulated with 20 nM Rankl for the indicated time. Western-blot analysis of I $\kappa$ B phosphorylation. Cell extracts were analyzed using antibodies directed against the total form or the phosphorylated form of I $\kappa$ B (p-I $\kappa$ B).  $\beta$ -actin detection was used as a loading control. (B) Ga effect on Rankl-induced nuclear NF $\kappa$ B translocation. RAW 264.7 cells were cultured as described in (A). Next, cells were pretreated with 100  $\mu\text{M}$  Ga (b) or its vehicle (a) for 1 h, and stimulated with 20 nM Rankl for the indicated time. Immunostaining analysis of NF $\kappa$ B protein expression was performed. (bar = 10  $\mu\text{m}$ ).



**Fig. 4.** Ga effect on Rankl-induced *Traf6*, *p62* and *Cyld* gene expression in mouse cells. Real-time PCR analysis of *Traf6*, *p62* and *Cyld* gene expression. RAW 264.7 cells were differentiated for 48 h with 20 nM Rankl, in the presence or in the absence of 100 μM Ga. Quantitative real-time PCR analysis was performed using *36B4* as housekeeping gene. \**p* < 0.05, statistically significant compared to untreated cells (Mann & Whitney test, bi-directional analysis).

Akt phosphorylation, and p-Akt regulates the activity of IκB kinase (IKK), which phosphorylates IκB [31]. Phospho-Akt is also involved in the phosphorylation of the transcription factor Creb (cyclic AMP-responsive element-binding), and Creb-mediated induction of *c-Fos* is implied in the transcriptional control of *Nfatc1* during osteoclastogenesis [32]. Thus, we investigated Ga effect on Rankl-induced Akt- and Creb phosphorylation.

Using RAW 264.7 cells, and as shown in Fig. 6A, Rankl treatment induced the phosphorylation of both Akt and Creb. In addition, Ga pretreatment did not modify Rankl-induced phosphorylation of both Akt and Creb.

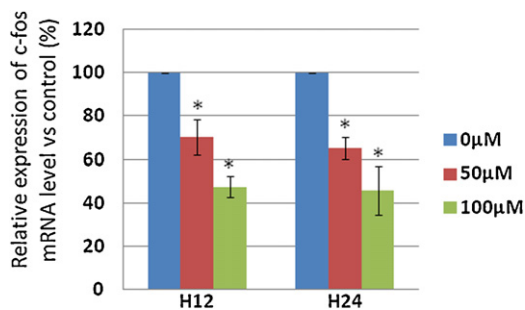
### 3.6. Ga effect on calcium signaling pathway

Ga has been shown to substitute calcium in biological apatite [9]. Considering the essential role of calcium signaling during osteoclastic differentiation, as well as the chemical similarity between Ga and calcium, we examined the effects of Ga on calcium-induced intracellular signaling and on calcium entry within osteoclast precursor cells.

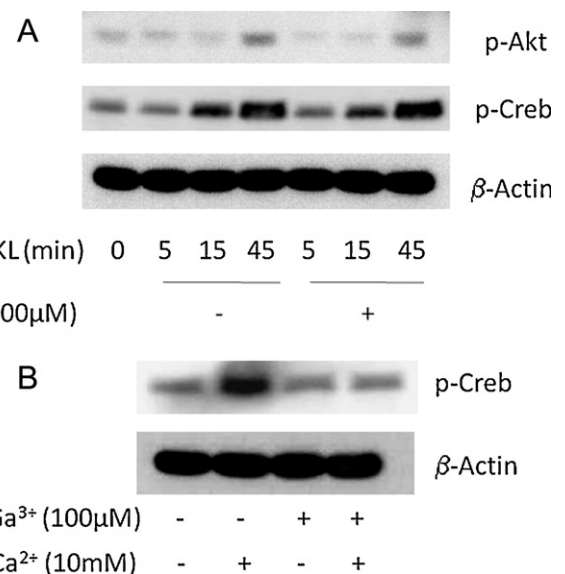
Rankl activates calcium signaling, which triggers the CaMK-Creb pathway. As shown in Fig. 6A, Rankl stimulated Creb protein phosphorylation, and in these experimental conditions, a treatment with 100 μM of Ga did not exert any inhibitory effect. In addition to the release of stores coming from the endoplasmic reticulum, intracellular calcium oscillations may also result from the entry of extracellular calcium through specific calcium channels present at the cell surface. Thus, we studied Creb phosphorylation in response to extracellular calcium, in the presence or in the absence of gallium. As shown in Fig. 6B, a 10 min treatment of RAW 264.7 cells with 10 mM Ca<sup>2+</sup> induced a

strong upregulation of Creb phosphorylation. In contrast, a 1 h cells pretreatment with 100 μM Ga completely reversed the calcium-induced Creb phosphorylation.

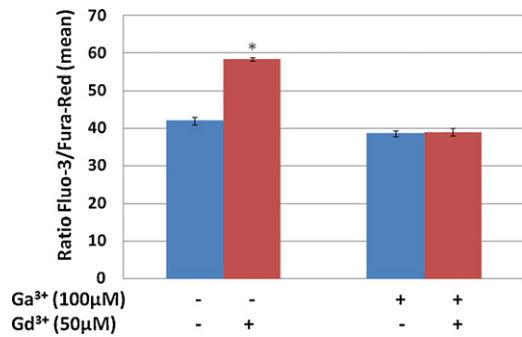
Lastly, we wanted to determine whether Ga could interfere with extracellular calcium uptake through a specific calcium channel, i.e. TRPV-5A (Transient Receptor Potential cation channel subfamily V member 5A). TRPV-5A is a calcium channel highly expressed in human osteoclasts [33], and which is largely involved in intracellular calcium oscillations. Using gadolinium (Gd) as a specific agonist of this channel, as well as human CD11b<sup>+</sup> cells, we examined whether Ga may block calcium entry through this channel. As shown in Fig. 7, the addition of 50 μM Gd induced a significant increase in intracellular calcium in CD11b<sup>+</sup> cells. In contrast, when cells were simultaneously treated with 50 μM Gd and 100 μM Ga, Gd did not trigger anymore intracellular calcium increase.



**Fig. 5.** Ga effect on Rankl-induced *c-Fos* gene expression in mouse cells. Real-time PCR analysis of *c-Fos* gene expression in mouse cells. RAW 264.7 cells were cultured with 20 nM Rankl, and 50 μM or 100 μM of Ga for 12 and 24 h. Results are expressed as a percentage of *c-Fos* gene expression level in control condition. \**p* < 0.05, statistically significant compared to untreated cells (Kruskall–Wallis test, bidirectional analysis).



**Fig. 6.** Ga effect on Akt and CREB phosphorylation in mouse cells. (A) Western-blot analysis of Rankl-stimulated Akt and Creb phosphorylation. RAW 264.7 cells were cultured for 16 h in serum-depleted media containing 0.2% BSA, pretreated with 100 μM Ga (+) or its vehicle (-) for 1 h, and stimulated with 20 nM Rankl for the indicated time. Cell extracts were analyzed using antibodies directed against the phosphorylated forms of Akt (p-Akt) or Creb (p-Creb). β-actin detection was used as a loading control. (B) Western-blot analysis of Ca<sup>2+</sup>-stimulated Creb phosphorylation. RAW 264.7 cells were starved for 5 h in DMEM containing 0.2% BSA, preincubated with 100 μM Ga (+) or its vehicle (-) for 1 h, and treated (+) or not (-) with 10 mM Ca<sup>2+</sup> for 10 min. Cell extracts were analyzed using antibodies directed against the phosphorylated form of creb (p-creb). β-actin detection was used as a loading control.



**Fig. 7.** Ga effect on  $\text{Ca}^{2+}$  uptake in human cells. Flow cytometry analysis of gadolinium-stimulated calcium uptake. Human  $\text{CD11b}^+$  cells were loaded with Fluo-3 and Fura-Red for 30 min. Cells were treated with (+) or without (-) 100  $\mu\text{M}$  Ga and 50  $\mu\text{M}$  Gd. After 5 min,  $\text{Ca}^{2+}$  uptake was monitored for 1 min. Results are expressed as the mean of Fluo-3/Fura-Red emission ratio ( $n = 7$ ).

On the whole, using osteoclast precursors pretreated with Ga, we observed an inhibition of calcium-induced Creb phosphorylation, as well as a blocking of gadolinium-induced calcium entry through the TRPV-5A calcium channel.

#### 4. Discussion

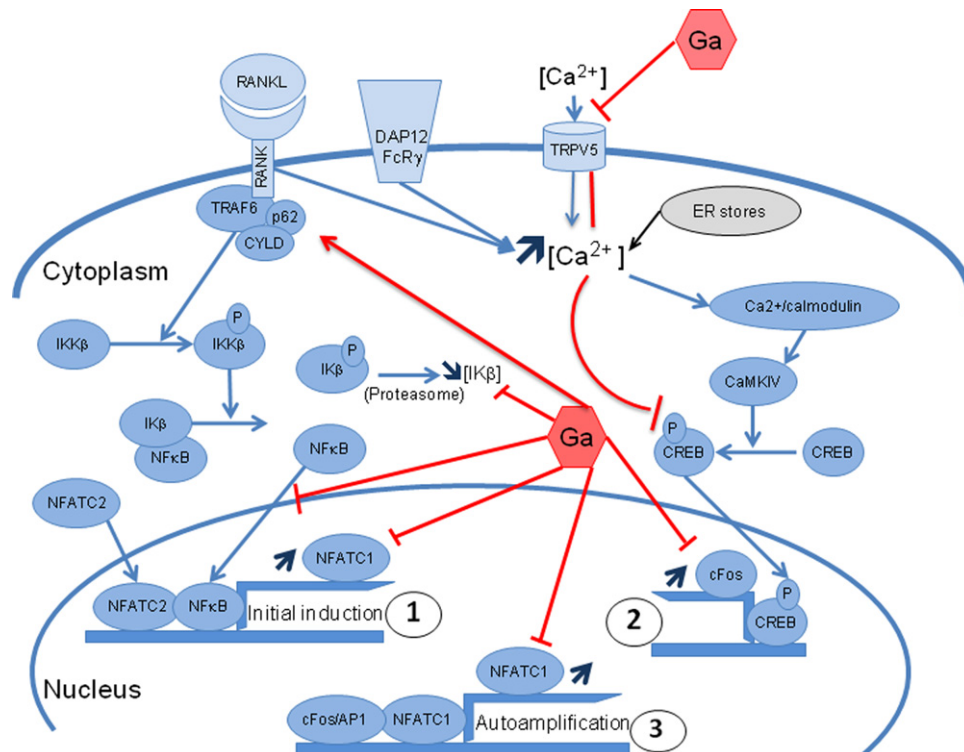
In our previous studies, we demonstrated that Ga inhibited the process of osteoclastic differentiation in a dose-dependent manner, which results in blocking osteoclast resorptive activity [14]. To obtain pertinent results about a potential distinct sensitivity to Ga of precursor and mature cells respectively, we investigated this issue using human  $\text{CD11b}^+$  cells isolated from peripheral blood as a source of osteoclast progenitors [25]. We observed that Ga specifically affected the expression of marker genes within differentiating osteoclastic precursors rather than in mature osteoclasts. Furthermore, we observed that Ga did not disturb the expression of pro and antiapoptotic markers both in

precursor and mature osteoclasts. These data confirm our preliminary experiment performed on mature osteoclasts obtained upon RAW 264.7 cells differentiation, a cell line that is considered more resistant than human primary cells. Results obtained from a human model of osteoclast-like cells strengthen the relevance of our basic data. For practical reasons, we continued using the murine RAW 264.7 cells.

We had previously reported that Ga reduced the expression of *Nfatc1* gene in a dose-dependent manner at the early stages (12–24 h) of osteoclastic differentiation in the murine RAW 264.7 cell line [14]. Using the same model, we show here that this inhibitory effect of Ga on *Nfatc1* gene expression persists for 48 h in Rankl-treated cells (Fig. 2A), and that this transcriptional effect impacts *Nfatc1* protein content of the cells (Fig. 2B). From these results, we conclude that Ga inhibits both the initial and auto-amplification steps of *Nfatc1* induction (Fig. 8).

Focusing on NF $\kappa$ B activation, we have documented Ga impact on the initial induction of *Nfatc1*. NF $\kappa$ B forms a complex with its inhibitory element (I $\kappa$ B) that prevents its translocation into the nucleus. Phosphorylation of I $\kappa$ B induces its own degradation resulting in activation of NF $\kappa$ B. As shown in Fig. 3A, Ga partially protect I $\kappa$ B from degradation without inhibiting its phosphorylation. This result, suggesting that Ga interacts with I $\kappa$ B degradation, is in accordance with a study from Chen et al. [34], which describes an inhibition of the proteasome activity by gallium(III) complexes. Furthermore, Ang et al. reported in 2009 [35] that proteasome inhibitors modulate NF- $\kappa$ B activation and osteoclast formation. We hypothesize that by blocking proteasome-mediated I $\kappa$ B degradation, p-I $\kappa$ B protein accumulates in the cytoplasm (Fig. 3).

Further supports to this hypothesis come from our data on *Traf6* and *p62*. *Traf6* is known to interact with *p62* and IKK, thereby mediating I $\kappa$ B degradation [36]. We demonstrate that 100  $\mu\text{M}$  Ga increases *Traf6* and *p62* gene expression (Fig. 4), and this is interesting considering that the accumulation of *p62* appears to impact the ubiquitine/proteasome system by delaying the delivery of ubiquitinated proteins to the proteasome [27]. Thus, signaling



**Fig. 8.** Molecular targets of Ga action on osteoclast precursor cells differentiation.

repression observed upon p62 overexpression in NFκB reporter assays might be explained by an impaired clearance of IκB via the ubiquitine proteasome system [36]. Ga-induced *Cyld* overexpression further documents the molecular mechanisms of Ga action on NFκB signaling pathway (Fig. 4). *Cyld* is a deubiquitinating enzyme which is considered as a crucial negative regulator of osteoclastogenesis [28]. Indeed, *Cyld* appears to be recruited through a direct interaction with the p62, and this negatively regulates NFκB pathway by removing the ubiquitin modification from TRAF6 [36]. As a whole, these data contribute to explain how NFκB signaling pathway, and more specifically IκB degradation, is impaired upon Ga treatment.

To determine whether IκB degradation inhibition had an impact on nuclear NFκB translocation, we studied cellular localization of NFκB following Ga treatment by performing immunostaining analysis (Fig. 3B). In the absence of Ga treatment, NFκB translocates within the nuclear compartment following a 45 min Rankl treatment. By contrast, the treatment with 100 μM Ga, maintained preferentially NFκB within the cytoplasm. Overall, the inhibition of IκB degradation observed with 100 μM Ga treatment was accompanied by a blockage of Rankl-induced NFκB nuclear translocation, this leading to the disruption of *Nfatc1* initial induction (Fig. 8).

We also observe that Ga down-regulates *c-Fos* gene expression within differentiating osteoclast progenitors. Thus, we conclude that Ga also impacts the auto-amplification of *Nfatc1*, since *c-Fos* is known to be critical for this stage of osteoclastic differentiation [23].

To further investigate the molecular mechanisms of Ga action, we were interested in finding intracellular targets that could interact with both NFκB and *c-Fos*. We focused on Akt, a downstream target of PI3K, which is also activated by Rankl [29,30].

In the canonical pathway, Akt activates the IKK complex that phosphorylates and degrades IκB [31], and Sugatani et al. have shown that Akt regulates NFκB activity through an Akt phosphorylation site present within the IKK complex [37]. Our data clearly indicate a lack of Ga effect on Akt activation, this indicating that Ga effect on IκB phosphorylation we observe at 45 min (Fig. 3) is not mediated through Akt. Akt phosphorylates also the transcription factor Creb, which regulates *c-Fos* expression [32]. However, according to the literature, Rankl-induced *c-Fos* expression is only partially understood. In our study, pretreatment with 100 μM Ga for 1 h did not alter the activation of either Akt or Creb in response to Rankl. From these data, it is unlikely that Ga directly modulates *c-Fos* expression via Creb.

NFκB activation occurs through two distinct mechanisms. The most important mechanism is based on IκB phosphorylation that governs the proteasome-mediated IκB degradation. This mechanism includes both classical (canonical) and alternative (non-canonical) NFκB signaling pathways. In contrast to this phosphorylation process, another NFκB activation pathway through the polymerization-mediated depletion of free IκB has been recently described [38]. This pathway is mediated by transglutaminase 2 (TGase 2)/μcalpain. Briefly, TGase 2 is a crosslinking enzyme involved in cellular processes such as cell migration, adhesion, and differentiation. TGase 2 induces polymerization of IκB which becomes a substrate for μcalpain, a non-lysosomal thiolprotease [39]. This proteolytic pathway is independent of the ubiquitin-proteasome degradation system. It will be interesting to investigate the effects of Ga on this pathway, especially considering that the activity of these enzymes (TGase 2 and μcalpain) depends on calcium ions.

Although many unknowns remain concerning how Ca<sup>2+</sup> signaling triggers *Nfatc1* activation, Ca<sup>2+</sup> is critical for osteoclastic proliferation, differentiation and survival. For example, the

Ca<sup>2+</sup>-CaMK-Creb pathway is not only important for the initial induction of *c-Fos* but it also regulates the expression of osteoclast-specific genes in cooperation with *Nfatc1* [23]. In addition, it is now well-established that osteoclasts can sense environmental level of Ca<sup>2+</sup> through the Ca-sensing receptor (CaR) leading to intracellular signaling which notably stimulates Creb phosphorylation and therefore differentiation of osteoclasts [40]. Using mouse RAW 264.7 cells stimulated with an elevated extracellular Ca<sup>2+</sup> concentration, we observe that Ga completely inhibits calcium-induced Creb phosphorylation. This result suggests an interaction between Ga and calcium signaling.

Given the chemical similarity of Ga and calcium, we hypothesized that Ga may interact with Ca<sup>2+</sup>-signal by blocking Ca<sup>2+</sup> entry. Extracellular Ca<sup>2+</sup> entry is of particular importance for human osteoclasts since it is responsible for a significant increase in intracellular Ca<sup>2+</sup> in response to RANKL. Indeed, in a human osteoclast model, Chamoux et al. showed that none of the traditional calcium-channels was involved in the RANKL-induced calcium spike [33]. They described that RANKL treatment opens a specific calcium-channel, namely TRPV-5, which belongs to the TRP channel family. This phenomenon allows extracellular Ca<sup>2+</sup> entry, and thus intracellular oscillations of Ca<sup>2+</sup> concentration independently from the signaling pathway involving PLCγ and intracellular calcium stores. In an effort to determine whether Ga may block this critical Ca-channel, we used gadolinium as a specific agonist of TRPV-5 in primary human osteoclast precursors isolated from peripheral blood, and we report that Ga completely abolishes intracellular Ca<sup>2+</sup> oscillations induced by gadolinium. This result strongly suggests that Ga could also act by blocking a specific Ca-channel in the plasma membrane of osteoclasts, thus disturbing intracellular Ca<sup>2+</sup> oscillations (Fig. 8).

## 5. Conclusion

In summary, we identify for the first time *Traf6*, p62, *Cyld*, IκB, NFκB, *c-Fos*, as well as calcium-induced Creb phosphorylation, as molecular targets of Ga during osteoclast differentiation, this tremendously impacting the expression of the master transcription factor *Nfatc1*. In addition, our results strongly suggest that the TRPV-5 calcium channel, which is located within the plasma membrane, is a target of Ga action on human osteoclast progenitor cells.

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