Proteomic and Transcriptomic Approaches for Studying Bone Regeneration in Health and Systemically Compromised Conditions

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List of abbreviations

BMP	bone morphogenetic protein
CSD	critical size defect
ECM	extracellular matrix
ePTFE	expanded Polytetrafluoroethylene
FGF	fibroblast growth factor
GBR	guided bone regeneration
IGF	insulin-like growth factor
IL	interleukin
MAPK	mitogen-activated protein kinase
MSC	mesenchymal stem cell
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
OVX	ovariectomy
PDGF	platelet-derived growth factor
RAP-1	Ras-proximate-1 or Ras-related protein 1
RT-PCR	reverse transcription- polymerase chain reaction
TGF	transforming growth factor
TMT	tandem mass tags
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

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Abstract

Bone regeneration is a complex biological process, where the molecular mechanisms are only partially understood. In an ageing population, where the prevalence of chronic diseases with an impact on bone metabolism is increasing, it becomes crucial to identify new strategies that would improve regenerative outcomes also in medically compromised patients. In this context, *omics* are demonstrating a great potential, as they offer new insights on the molecular mechanisms regulating physiologic/pathologic bone healing and, at the same time, allow the identification of new diagnostic and therapeutic targets.

This review provides an overview on the current evidence on the use of transcriptomic and proteomic approaches in bone regeneration research, particularly in relation to type 1 diabetes and osteoporosis, and discusses future scenarios and potential benefits and limitations on the integration of multi-omics.

It is suggested that future research will leverage the synergy of *omics* with statistical modelling and bioinformatics to prompt our understanding of the biology underpinning bone formation in health and medically compromised conditions.

With an eye towards personalized medicine, new strategies combining the mining of large datasets and bioinformatic data with a detailed characterization of relevant phenotypes will need to be pursued to further our understanding of disease mechanisms.

Introduction

During the past years, *omics* technologies have started to reveal as powerful tools to investigate the molecular mechanisms behind biological processes such as bone healing, as well as to discover unique disease-related proteins that could offer new targets for therapies or to monitor disease progression. The main *omics* platforms that have been applied to characterize bone regeneration in healthy and compromised conditions are transcriptomics, proteomics and epigenomics [1].

The world of "*omics'* is continuously evolving (Figure 1). The first *omics* discipline to appear was *genomics*, which investigates the structure, function, evolution, mapping and editing of genomes. On the other hand, e*pigenomic*s focuses on the characterization of reversible modifications of DNA or DNA-associated proteins (e.g. DNA methylation or histone acetylation). *Transcriptomics* moves from DNA to RNA and focuses on the RNA transcripts that are produced by the genome under specific circumstances or in a specific cell, while *proteomics* studies the full set of proteins that are actually produced by cells/organisms. More recently, *metabolomics* has emerged, which is the comprehensive analysis of metabolites in a biological specimen and, therefore, better represents the molecular phenotype. According to the type of metabolite investigated, metabolomics can be further differentiated into lipidomics, glycomis and fluxomics.

Omics investigations are increasingly being used not only to understand the molecular mechanism of physiologic and pathologic bone healing, but also in drug discovery and assessment of drug toxicity and efficacy. Moreover, the possibility of investigating biomaterials through computational biology (*materiomics*) is now a reality and is progressively changing our approach to biomedical science [2].

This literature review aims to summarise current evidence on the use of transcriptomic and proteomic approaches to study bone regeneration in health and systemically compromised conditions, to discuss relevant and appropriate regenerative models, as well as future challenges and possibilities related to the integration of *omics* data.

Considering the large number of diseases that can impact on bone formation, it would be impossible to cover them all in a single review. Hence, we decided to focus our attention on diabetes mellitus type 1 and osteoporosis, which are good examples of complex and multifactorial systemic diseases whose understanding might be improved by the integration of clinical and histological data with high-throughput omics insights.

Medline via Ovid was thoroughly searched by combining MeSH terms and free text (Table 1). Our search strategy returned 4417 records, which reduced to 2613 when the Cochrane filter for human studies was applied. An evident trend of increase in the number of publications on this topic emerged, with 67% of the papers published in the past 5 years (since 2014).

Molecules/proteins involved in bone regeneration

In the past years, several key molecules regulating osteo-precursor cell recruitment, proliferation and commitment, as well as molecules directing the ossification process were identified. Particularly, three categories of signalling molecules proved to play a crucial role: pro-inflammatory cytokines, growth factors and bone morphogenetic proteins [3]. Amongst pro-inflammatory cytokines, Interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-α) are particularly expressed during initial healing stages and they play an important role in initiating downstream responses starting from bone resorption and leading to bone regeneration [4]. Several growth factors are known to regulate osteogenic cells and osseous formation, namely transforming growth factor-beta (TGF-beta), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF). Within the TGF-beta family, bone morphogenetic proteins (BMPs) are secreted signalling molecules that are expressed during embryonic development and regulate the growth, differentiation and apoptosis of different cell types [5]. In particular, BMP-2, BMP-4, BMP-7 and BMP-9 present significant osteoinductive activity and they promote the differentiation and proliferation of mesenchymal stem cells (MSCs) into osteoblasts [5]. The cross-talk between TGF-beta/BMP signalling and other major signalling pathways, such as mitogen-activated protein kinase (MAPK), Wnt, Hedgehog, Notch and FGF is considered to be critical for the coordination of osteoblast differentiation, bone homeostasis and skeletogenesis [6].

Despite our understanding of the bone regeneration process has significantly advanced within the past years, there are still important knowledge gaps to fill and we are far from being able to control and modulate the bone regenerative process. For instance, we know which are some of the key cells playing a role in the osseous wound, but it is unclear how they communicate between each other, what is the role of the different lymphocytes subtypes at the different stages of bone healing and what is the final impact of the interaction between the different cell lineages on MSC osteogenic cells [7]. Moreover, the precise spatial and

temporal impact of immune cells and their cytokines on bone healing remains largely unknown.

Evidence is now accumulating on a close relationship between bone metabolism and the immune system, which offers new perspective for the prevention and treatment of pathological bone loss (osteoimmunology)[8]. However, in order to ensure predictable bone regeneration outcomes even in challenging clinical situations or in the presence of underlying systemic conditions that negatively affect the osteogenesis process, novel multidisciplinary strategies need to be developed that will allow new insights in the complexity of the homeostatic mechanisms regulating bone metabolism.

Omics approaches to characterize bone tissue samples in health and disease. Where are we?

In contrast to other organs, the human skeleton is accessible for *omics* studies, as researchers can take advantage of biopsies from orthopaedic surgeries.

Several pre-clinical and clinical studies investigated the expression of specific genes within bone tissue samples by employing reverse transcription (RT) PCR and using a defined list of primers. Despite those studies provide valuable information, the evaluation of few, predetermined genes carries the obvious limitation that only a partial and biased information can be obtained, with the risk of losing the “global picture” of a biological process and any information related to the up/down regulation of signalling pathways.

On the contrary, with the development of microarray technologies and whole genome sequencing, changes in the expression level of thousands of genes can now be examined simultaneously, and a comprehensive analysis of the dysregulated genes can be performed to obtain information regarding physiologic and pathogenic mechanisms.

Moreover, it is possible to move forward and investigate not only the complete set of RNA transcripts that are produced by the genome under specific circumstances or in a specific cell, but also the actual set of proteins that have been produced (proteomics). Although bone tissue represents a challenge for protein extractions, thanks to the continuous advancements in techniques and methodologies, several studies have now been able to characterize bone samples in terms of protein expression. As a matter of fact, standardised protocols for protein extraction that minimise thermal protein degradation and allow to obtain reproducible

results also in high-density cortical bones have been published [9, 10]. In addition, demineralization-free approaches have been developed and a recent study documented a solid-digestion approach that would allow the characterization of insoluble proteins and post-translational modifications that would otherwise be missed by traditional bone protein extraction [11].

- ***Transcriptomic studies on bone tissue samples***

Several studies applied transcriptomics to cells extracted from bone samples, for instance to unravel the genes directing the differentiation and commitment of mesenchymal stem cells (MSC) [12-14], or to identify the genes differently expressed in the presence of systemic diseases (e.g. osteoporosis or diabetes type 1) [15, 16]. Remarkably, using RNA sequencing, Helbling et al. [17] performed transcriptional analyses on the dynamic changes in transcriptional landscape of four major bone marrow stromal cell types from mice, from early postnatal to late aging stages. They were able to demonstrate molecular fingerprints defining cell-specific anatomical and functional features, a re-programming of pro-hematopoietic, immune, and matrisomic transcriptional programs during the transition from juvenile stages to adulthood, as well as an ageing-driven progressive upregulation of pro-inflammatory gene expression in stroma.

The majority of the studies on bone tissue samples focused only on the expression of specific sets of genes (e.g. by applying probe-based RT-PCR systems). Nevertheless, few studies have also applied full transcriptomics approaches.

Single-cell and spatially resolved transcriptomics were combined by Baccin et al. [18] to systematically map the molecular, cellular and spatial composition of distinct bone marrow niches from the femurs, tibiae, hips and spines of mice. This study allowed to transcriptionally profile all major bone marrow-resident cell types, determine their localization and clarify sources of pro-haematopoietic factors. They demonstrated that Cxcl12-abundant-reticular (CAR) cell subsets (Adipo-CAR and Osteo-CAR) differentially localize to sinusoidal and arteriolar surfaces, act locally as ‘professional cytokine-secreting cells’ and henceestablish peri-vascular micro-niches. Interestingly, the 3D bone-marrow organization could be accurately inferred from single-cell transcriptome data using the RNA-Magnet algorithm described by the authors.

A genome-wide gene expression approach by means of microarrays was also employed to identify new candidate genes involved in the physiopathology of experimental osteoporosis in mice [19]. Moreover, Chai et al. [20] described the mRNA profiling in the femur and muscle of ovariectomized (i.e. osteoporotic) rats using RNA sequencing and they showed 440 mRNAs differentially expressed in femur samples, with the major enriched pathways being the ribosome, phosphatidylinositol signaling system and protein processing in endoplasmic reticulum.

When looking at human data, one of the largest studies on human bone was done in a cohort ("Oslo cohort") of post-menopausal women (osteoporotic and an age-matched healthy control group), from whom 84 trans-iliac bone biopsies were obtained. Gene Affymetrix microarray expression analysis showed that 8 transcripts significantly correlated to total hip BMD (5% false discovery rate), explaining 62% of the bone mineral density variation expressed as T-score, and 53% when adjusting for the influence of age [21]. Within the same cohort, gene expression profiles (via microarray analysis) of human trabecular bone derived from 24 biopsies taken from two different skeletal sites that experience different degree of mechanical loading (iliac crest and lumbar spinal lamina) were compared [22]. The study provided evidence that gene transcripts that are markers of osteocyte, as well as osteoblast and osteoclast- related genes, were up-regulated in the spine and allowed the identification of a number of transcripts which had never earlier been associated with bone growth and remodelling.

Two studies [23, 24] compared intertrochanteric biopsies from osteoporotic patients with fracture with surrogate osteoarthritis controls, and autopsies from controls with normal bone mineral density ones. Global transcriptional profiling identified 150 differentially expressed genes in osteoporotic bone, of which 75 had known or suspected roles in bone metabolism.

Moreover, RNA sequencing was performed on iliac crest needle biopsies from 58 non-osteoporotic healthy women, of which 20 received estrogen therapy for 3 weeks [25]. The study provided information on how molecular pathways and gene activities changed from young to elderly women and on the effects of estrogen therapy. For instance, in postmenopausal vs young women 12 canonical pathways were differently expressed, including Notch signaling, Inhibition of matrix metalloproteinases and eNOS signaling.

Remarkably, within the context of the Human Protein Atlas project, Andersson et al. [26] recently described the gene expression profiles in bone marrow and compared it to secondary

lymphoid tissues by combining high throughput transcriptomics with affinity-based proteomics.

- **Proteomics studies on bone tissue samples**

Few animal studies have characterized the proteome of healthy and osteoporotic bone. In ovariectomized (OVX) osteoporotic-like rats compared to sham-operated ones, 2D SDS-PAGE and MS showed a reduction in non-collagenous proteins, although no selective loss of particular proteins was observed [27]. Another study in OVX rats identified three distinctly proteins named thioredoxin peroxidase 1, myosin light polypeptide 2 and ubiquitin-conjugating enzyme E2-17 kD as osteoporosis-related [28], while a study in mice suggested that proteins related to cytoskeleton and to energy pathways might be important estrogen-regulated proteins in bone [29].

In human bone samples, SDS-PAGE combined with MALDI-TOF/TOF MS and tandem MS was employed to analyse bone-matrix proteins from 24 osteonal and 24 interstitial tissue samples of a healthy cadaver [30]. The study showed statistically relevant differences between younger osteonal and older interstitial bone tissue regarding the expression of three major bone matrix proteins, collagen, osteocalcin, and osteopontin. In another study, SDS-PAGE combined with nano LC-MS/MS enerated a library of proteins expressed in 4 samples of healthy cancellous bone fragments obtained from patients undergoing hip replacement surgery [31].

LC-MS/MS was also applied to characterize the proteins expressed in the femur of osteopenic patients with osteoarthritis and age-matched controls and the results indicated that carbonic anhydrase I and phosphoglycerate kinase 1 increased in the presence of osteopenia, while apolipotrotein A-1 reduced[32].

The proteins expressed within dental cementum and alveolar bone were also assessed by LC-MS/MS with the aim to identify tissue-specific markers [10]. Interestingly, COL14A1 resulted to be strictly associated with alveolar bone, while SERFINF1 and SOD3 were markers of dental cementum.

MS/MS-based proteomics strategies using tandem mass tags (TMT) were recently employed to characterize the vertebral body-derived bone marrow supernatant fluid of osteoporotic and non-osteoporotic patients [33]. Proteins belonging to splicing, translation, protein

degradation, cytoskeletal organization and lipid metabolism were indicated as differentially regulated between osteoporotic and healthy patients.

In recent years, exosome proteomic analyses, including analyses of exosomes derived from bone cells [34, 35], have received increasing attention. Exosomes are 40-100-nm diameter endocytic membrane vesicles that have been involved in multiple activities, from immune response, antigen presentation, regulation of cell commitment and differentiation, intracellular communication and the transfer of RNA and proteins. There is significant evidence demonstrating the role of exosomes in regulating osteogenesis and angiogenesis both *in vitro* and *in vivo* [36]. For instance, exosomes secreted by bone marrow mesenchymal stem cells (BMSCs), osteoclasts, and osteoblasts have been shown to participate in the regulation of bone homeostasis. Moreover, osteoclast-secreted exosomes can inhibit osteoblast activity and suppress osteoblastic bone formation [37, 38], while exosomes secreted from osteoblasts and BMSCs are able to enhance osteoblastogenesis [39]
Another interesting field of application of bone proteomic is the profiling of archaeological bone, with the aim to provide clues to diseases from ancient times and to study bone proteome changes overtime and across species [40-42].

Omics approaches to characterize the bone regeneration process in health and disease

Since the characterization of the bone regeneration process with *omics* approaches requires the collection of tissue samples at multiple healing times, animal models have been preferentially employed to serve this purpose. Conversely, and for obvious ethical reasons, only a very limited number of clinical studies have been able to describe the biological events occurring during bone regeneration in humans.

- **Animal models of bone regeneration**

Different regeneration models have been proposed to study the process of bone regeneration, going from union and non-union fractures, to critical and non-critical size defects, to post-extraction sockets, distraction osteogenesis and models of *de novo* bone

formation, in the presence or absence of different barrier membranes and bone grafts [43]. Herein, an overview of the most widely used models is presented.

Fracture model: Most of today's knowledge on bone regeneration comes from studies on fracture healing, mainly in small rodents [44]. The femur is usually the preferred site, as it is larger than the tibia and is covered by thick muscles that help stabilization [45]. Closed fractures (e.g. closed midshaft femoral fracture) can be achieved with the insertion of an intramedullary pin and subsequent fracturing the bone by means of a blunt guillotine driven by a dropped weigh [46]. As an alternative, a surgical osteotomy can be performed and in this technique a midshaft osteotomy is made with an oscillating saw and then the periosteum is stripped around the fracture site [47]. Usually fractures present a combination of endochondral and intramembranous ossification. At the periphery of the callus, where blood perfusion is reduced, there is always a chondrogenic differentiation of the progenitor cells and a cartilage template is initially created. The newly formed cartilage then undergoes calcification and is ultimately replaced by bone (endochondral ossification) [3]. Conversely, where the blood supply is better preserved, the osteoprogenitor cells can differentiate directly into osteoblasts and synthetize bone (intramembranous ossification) [48].

Both in mice and humans the process of fracture healing, with intramembranous or endochondral ossification depends on the type of fracture, soft tissue trauma and stabilization. Therefore, several bone-healing models with different experimental setups have been developed to mimic the clinical situation (for review see [47, 49]).

Socket healing and ridge preservation models: The healing of an alveolar socket after tooth extraction has been extensively characterized in different animals, as well as in human studies [50, 51]. In summary, after a tooth extraction haemorrhage occurs and the blood clot develops in the wound area, which provides a scaffold for cell migration and is a source of important signalling molecules and nutrients directing the migration, proliferation and differentiation of stem cells into the alveolar socket.

It is possible to test the regenerative effect of different biomaterials on alveolar ridge preservation. The most studied animal model for ridge preservation is the dog, where the mandibular third and fourth premolars (P3 and P4) are usually the preferred teeth to be extracted. It is suggested to consider healing times of at least 2/3 months to assess if a procedure can reduce alveolar resorption and of 6 months for long-term effects [52, 53]. The ridge preservation model in dogs has the advantage that the alveolar socket has a similar

shape and same healing process than in humans, although it should be considered that dogs have a faster healing.

Critical size defect (CSD) model: CSDs are challenging defects that would not heal spontaneously with bone tissue for the lifetime of the animal, so they allow to test the regenerative ability of a biomaterial. One of their advantages is that they involve healing of orthotopic bone sites, like the mandible or the calvaria, making the results more relevant than those obtained from heterotopic models [54]. One of the most popular CSD is the calvarial 5-mm of diameter defect in rats [55], as it is easy to perform, relatively low invasive for the animal and it offers support for implantation of regenerative materials. In addition, the possibility of performing bilateral defects allows to reduce the number of animals employed (following the reduction and refinement principles in animal research) and to have simultaneously test and control samples or to perform different types of analyses (e.g. histological and proteomic/genomic analyses) on the same animal [56]. The regeneration of CSDs in calvaria (or mandible), for instance by applying the guided bone regeneration (GBR) principle, recapitulates the steps of intramembranous bone formation [57, 58], hence it can be considered a suitable model to test regenerative strategies for this type of ossification.

Dome model: Space-maintaining domes, capsules and cylinders have been used to promote bone regeneration in several animal studies. The idea is to apply a rigid hollow device that can withstand the collapse and maintain the space for bone formation. Remarkably, this model allows to study not only the regeneration of a bone defect, but also the process of neo-osteogenesis, i.e. *de novo* bone formation, on top of the genetically determined skeletal borders. Can be applied in different animal species and skeletal sites. In particular, for its anatomical characteristics the calvarial bone is one of the preferred sites for the application of the dome model [59-64], although it has also been applied to the mandibular ramus [65-67] and tibia [68]. Rat and rabbit calvarial dome models are the most widely used.

Moreover, the process of osseointegration and the influence of different implant surfaces have been investigated by collecting peri-implant tissues and cells [43]. Animal models also allow to study the effect of systemic diseases on the bone formation process and might serve as important proof-of-principle systems before moving to clinical practice [45, 56, 69].

Every bone regeneration model has advantages and limitations. When selecting a model, several factors should be considered, including the similarity to the human model of interest,

the animal lifespan and type of remodelling, as well as the expertise of the team, ethical, economic and legislative implications. Moreover, the type of planned analyses should not be disregarded, as different analyses may require different amounts of samples, which should be collected in an easy and reproducible way.

- **Transcriptomic studies on bone regeneration**

- *Physiologic bone regeneration*

A part from studies that applied RT quantitative PCR to test the expression of specific gene sets [70-72], more comprehensive analyses (e.g. microarrays or subtractive hybridization) have described the sequence of gene activation at different stages of the bone regenerative process. In femur fracture and femur ablation models in rats, microarray analyses showed that the immediate healing response (day 1) was characterized by genes with energy derivation, transporter and binding activities, followed by an increased expression of genes regulating cell proliferation, protein metabolism and immune-inflammatory response (at 3-4 days). Between 5 and 14 days, genes and pathways related to neurogenesis, skeletogenesis, cell motility, cell adhesion and angiogenesis were the most expressed ones, including Wnt and TGF-beta/BMP related genes [73-76]. Moreover, when comparing fractured to non-fractured sites, a study suggested that that CCL2, NOS2, CSF2, and DLC1 may be important in regulating bone overgrowth via the anti-apoptosis of osteoblasts [77]. In 3 mm deep alveolar bone defects in rats, the gene expression pattern confirmed an high energy metabolism during the early days of healing, followed by an upregulation of pro-α-2 type I collagen at 2.5 weeks, during the stage of granulation tissue formation [80].

In order to measure the change in gene expression during the juvenile growth period, the femoral head, enclosing the proximal femoral physis, primary spongiosa, and articular cartilage, was collected from both femora of 16 rats between 4 and 10 weeks of age and one femur of each rat had a mid-diaphyseal femoral fracture at 4 weeks of age [78]. The study showed an up-regulation with age of genes related to cartilage, blood vessels, osteoprotegerin, osteomodulin, and most ribosomal proteins, while there was down-regulation of genes related to bone, growth-promoting cytokines, G proteins, GTPase-mediated signal transduction factors, cytokine receptors, mitosis, integrin-linked kinase, and the cytoskeleton. When comparing standard to non-union fracture, the latter was characterized by a downregulation of several BMPs [79].

Our group has extensively studied gene expression profile during bone regeneration in rat calvarial CSDs. More specifically, in a series of pre-clinical studies we described the genes differently regulated at 7 and 15 days of healing when calvarial CSDs were treated according to the GBR principle with an intra and extra cranial expanded polytetrafluoroethylene (ePTFE) membrane, an intracranial ePTFE membrane and an extracranial polished titanium disc, or an intracranial ePTFE membrane and an extracranial micro-rough titanium disc [81-83]. We showed that the regeneration of bone under a barrier membrane recapitulated the cascade of events occurring during normal intramembranous osteogenesis. Overall, the transcriptome at 7 days reflected an immature wound, with an upregulation of inflammatory and immune response. Conversely, at 14 days a more complex cellular and metabolic activity was evident, with an upregulation of several genes encoding growth factors, enzyme activity and extracellular matrix formation. Remarkably, when applying a moderately rough compared to a polished titanium disc to cover the defect, a different regulation of relevant gene ontology groups involved in skeletal development, angiogenesis and neurogenesis was observed [82, 83].

While it is beyond the remit of this review to discuss in details studies on implant osseointegration, it is worth mentioning that animal and human studies applying whole-genome analyses on peri-implant tissues and cells have also contribute to elucidate the sequence of gene activation during bone formation and they have confirmed that, while immuno-inflammatory response- and ECM- associated genes are the ones most highly expressed during the early days, the later stages of organization of the granulation tissue and formation of woven involve the upregulation of angiogenesis, osteogenesis and neurogenesis genes [84-91]. We previously published transcriptomic data from trephine bone core specimens retrieved together with cylindrical titanium implants from the retromolar areas of healthy volunteers at different time points (4, 7 and 14 days) Our results indicated that immune-inflammatory pathways, such as the I-kB kinase/NF-kB cascade, were up-regulated during the first healing days, whilst TGF-β/BMP, Notch and Wnt signalling pathways were significantly expressed at the later healing time [88, 89]. Interestingly, hydrophilic compared to hydrophobic titanium implants accelerated osteogenesis by upregulating pro-osteogenic and pro-angiogenic pathways at 7 days of healing. Hence, early transcriptomic analyses indicated a clear effect of hydrophilicity of biomaterials such as titanium on the bone formation cascade.

- *Type 1 diabetes mellitus*

Several studies have employed the streptozicin-induced model in small rodents to elucidate the impact of type 1 diabetes mellitus on bone formation. In the tibia bone marrow ablation model and distraction osteogenesis model, diabetes type 1 negatively affected the expression of transcription factors regulating osteoblast differentiation (DLX5, Osterix and CBFA1/RUNX-2), and of bone matrix genes (osteocalcin and collagen type 1) [92, 93]. Fracture healing studies also indicated that diabetes type 1 reduced the number of MSC and increased the levels of inflammatory cytokines in the wound area [94, 95], while it reduced the gene expression of PDGF [96] and delayed the expression of TGF-β1 and BMP-2 [97].

In a GBR model, our group reported the effect of uncontrolled and insulin-controlled streptozocin-induced diabetes by correlating histology and transcriptomics findings [98]. A clear delayed and prolonged inflammatory response was observed in diabetic animals, which was reversed by insulin treatment. A differential expression of genes associated with the ossification process (BMP4, ITBP4, THRA and CD276) was evident at 15 days of healing between the healthy controls and diabetic animals.

In a study where implants were placed in the tibia of healthy and diabetic type 1 rats, comprehensive RNA gene expression profiling was performed on bone tissue samples harvested 3 months after implant placement and revealed that osteoblast-related gene expressions was decreased in diabetic rats, whereas lipid metabolism pathway-related gene expression was increased [99].

- *Post-menopausal osteoporosis*

By employing the ovariectomized (OVX) model, few pre-clinical studies assessed the effect of experimental osteoporosis on gene expression during bone regeneration. For instance, in a fracture model, a number of genes were found to be differentially regulated in case of rigid or non-rigid fixation and in case of treatment with alendronate [100], while another study suggested that THBS2, SDC2, FKBP10, OASL2, IFIT1 and IFIT2 may serve important roles during the fracture healing process in osteoporosis [101]. Bioinformatic analyses of gene expression profile in callus tissues of osteoporotic mice also indicated that dysregulated collagen metabolic process, ECM-receptor interaction and p53 signaling pathway may be responsible for impaired fracture healing in these animals [102].

Since Lrp 5 deficiency (Lrp5-/-) as well as osteoblast-specific overexpression of Krm2 (Col1a1-Krm2) result in severe osteoporosis occurring at young age, a study evaluated the influence of these proteins in a fracture fixation model in mice [103]. Using microarray analysis they indicated a reduced expression of genes mainly involved in osteogenesis that seemed to be responsible for the observed stronger impairment of healing in Col1a1-Krm2 mice compared to Lrp5-/- mice.

Transcriptomics has been also used to study osteogenesis in the presence of different bone substitutes [104] and to characterize the osseointegration process in osteoporotic conditions [105].

- **Protein expression during bone regeneration**

Limited studies have characterized the proteome during bone regeneration.

- *Physiologic bone regeneration*

Two studies used the palatal expansion model in small rodents to characterize the early molecular events of bone regeneration. They indicated that at 2-3 days of healing there was an increased expression of proteins involved in angiogenesis, cell proliferation, differentiation cytoskeleton function, stress reaction and energy metabolism during expansion compared to controlled sites [106, 107]. The proteins expressed in gingival tissue and alveolar bone following tooth extraction were described in mini-pigs [108]. Overall, a crosstalk between proteins expressed in soft and hard tissue with respect to cellular assembly, organization, and communication emerged. In a femoral bone defect, the possibility of monitoring in situ wound healing by proteomic and metabolomic analyses of wound fluid collected with a microdialysis catheter was also described [109].

Our group has recently characterized the proteome of early stages of bone formation (4, 7 and 14 days) in a model of *de novo* bone formation (titanium domes applied to the calvaria of rabbits) [59]. While proteins of the complement, coagulation cascade and inflammatory-immune response were the most represented at day 4, starting form day 7 the proteome indicated a maturing osseous wound, with an increasing prevalence of metabolic and cellular activity and an increased expression of pathways involved in osteoblast differentiation and skeletogenesis (e.g. Wnt, Notch, Rap1, Tgf-β). Since the study provided also information on the proteins differently regulated by hydrophilic and hydrophobic titanium surfaces we managed to combine, for the first time, these proteomic data with genomic data obtained

from the same surfaces and healing points in a previous human study [88, 89]. By applying advanced bioinformatic tools and focusing specifically on bone formation-related signalling pathways, we identified specific signalling pathways, such as Wnt, vascular endothelial growth factor (VEGF) and MAPK, as differentially modulated by titanium surface hydrophilicity both at a genomic and proteomic level [110]. The study suggested that hydrophilic surfaces might particularly enhance bone formation by promoting angiogenesis and osteogenesis coupling.

- *Post-menopausal osteoporosis*

Proteomic approaches might be particularly useful to identify proteins and pathways that are dysregulated in the presence of pathological conditions, as they may become target of future therapeutic approaches. In line with this consideration, our group was the first to evaluate the proteins expressed during different stages of bone formation in healthy and osteoporotic-like conditions [58, 111]. Calvarial CSDs treated with an intra and extra cranial collagen membrane and an osteoconductive graft, were assessed both histological and in terms of protein expression at 7, 14 and 30 days of healing. Gel electrophoresis and MALDI-TOF/MS and LC-MS/MS of the regenerated bone indicated a tendency for an enhanced inflammatory and stress response and a delayed organization and maturation of the granulation tissue in osteoporotic animals. Moreover, specific proteins (apolipoprotein E and apolipoprotein A-IV) were significantly overexpressed at all healing points in OVX animals.

To the best of our knowledge, no studies characterizing the proteome during bone regeneration in diabetic type 1 conditions are available.

Concluding remarks

High-throughput technologies have revolutionized bone research. Each type of omics data provides a list of differences (in genes, proteins, metabolites) associated with a physiologic/pathologic process that hold the potential of becoming useful markers of a disease process or a possible therapeutic target. Available evidence seems to suggest that omics might be particularly helpful to characterize early molecular events of bone regeneration and to intercept dysregulated mechanisms in pathologic conditions. However,

data from only one omic type risk to be partial and they might not necessarily reflect a true causative association. While single-level omics approaches have the merit to have contributed to the identification of disease-specific mutations and epigenetic alterations, they lack the ability to establish the casual relationship between molecular signatures and the phenotypic manifestation of disease hallmarks. As a consequence, research is now moving towards the integration of multi-omics approaches to better understand the multi-layered molecular basis of complex physiologic/pathologic mechanisms (Figure 1). As we live in the 'postgenomic' era of bone research, it becomes increasingly evident that future progresses in bone research will largely rely upon collaborations across multidisciplinary groups, such as the Big Data initiatives and similar approaches focused at facilitating the accessibility and exchange of biomedical digital data and promoting biological discovery [112-114]. The ultimate goal of future omic research will be the development of precision treatment options, with the possibility of improving bone regenerative outcomes by tailoring the treatment according to the patient's needs.

In order to promote multi-layered analyses in bone regeneration research (and in research in general), a prerequisite is the necessity to organize genetic, biological and functional data relating to the musculoskeletal system into easily searchable and accessible databases.

However, multi-omics data analysis poses several challenges and one of the biggest is the manipulation of large dataset and the risk that, if not properly powered and designed, studies might produce associations that turn out to be false positives [115]. Moreover, multi-omics require the creation of a pipeline that integrates data generated from different platforms, with potential issues related to the heterogeneity of data that are collected, prepared and measured under different conditions [116].

Despite new knowledge is starting to unravel the cascade of events of bone formation and new potential regulators of bone formation have been validated, it is interesting to notice that this knowledge has so far not translated into predictable regeneration of challenging scenarios, like vertical augmentation in the maxilla- facial region or non-union fractures, and we are still unable to tailor treatment protocols according to the patient's needs. Unfortunately, human studies are affected by a multitude of confounding factors, such as diet and lifestyle choices, that are almost impossible to control for, and there might be limitations in terms of samples availability. Animal models, provided they are representative of the medical condition under investigation, can partially overcome these issues and present

several advantages, including reproducibility, easy access to relevant tissues, accurate phenotyping and control of environmental factors [115]. Nevertheless, a careful selection of bone regeneration models needs to take place for future studies, in order to ensure that enough and adequate/appropriate samples are available to perform multi-layered analyses. Moreover, it is important to recognize that animal models may not necessarily recapitulate the biology of human diseases and differences in bone metabolism and composition need to be considered.

A new concept of "trans-omics" is now emerging that integrates multiomics with clinical phenomes (Figure 1). While multiomics investigates the networks and correlations amongst genes, proteins, and metabolites, it is believed that trans-omics will provide the full picture of patient phenome-based molecular networks, making a step forward towards personalized medicine [117]. While trans-omics is still in its early days, it represents a step forward in integrative medicine and it is anticipated that it will help characterizing patients, identifying new disease-specific biomarkers and targets and uncovering mechanisms underlying drug responsiveness.

Conflict of interest statement

The authors have no conflict of interest to declare related to this article.

Figure legend

Figure 1 The flow of genetic information, starting from genes, moving to proteins and metabolites is represented. The integration of multi-omics approaches is considered essential to understand complex physiological/pathological mechanisms. In recent years, the possibility of integrating multi-omics with phenomes has emerged (trans-omics). This step forward in integrative medicine will likely bring to the development of new prediction models, diagnostic markers and therapeutic targets, as well as the discovery of novel pathways/networks.

Table legend

Table 1 Despite this is not a systematic review, a thorough search strategy was performed in Medline via Ovid to identify all the studies that investigated gene expression or protein

expression in bone specimens in condition of health or in the presence of diabetes type 1 or osteoporosis.

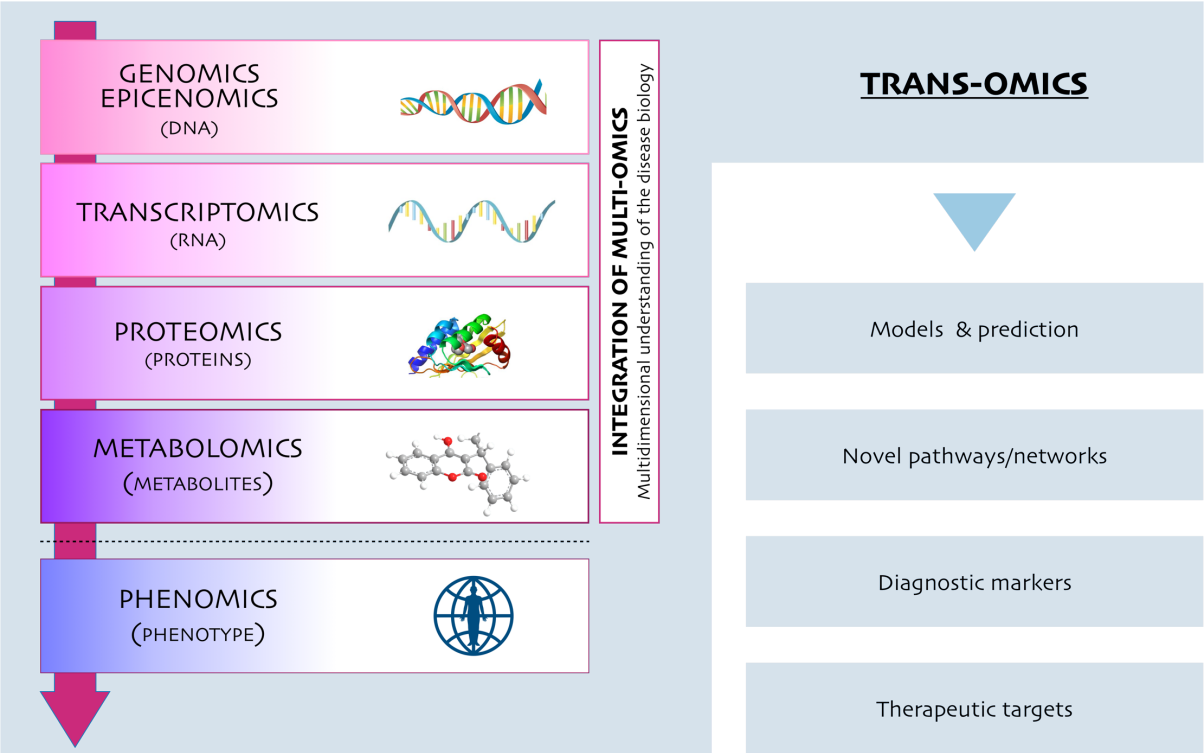

Figure 1

MeSH terms	Free text
Population	
diabetes mellitus/ OR exp diabetes mellitus, type 1/ OR exp diabetes mellitus, type 2/ OR exp hyperglycemia/ OR bone disease, metabolic/ OR exp bone demineralization, pathologic/ OR exp osteoporosis	(Diabetes adj3 mellitus) OR (diabetes and type 1) OR (diabetes and type 2) OR hyperglycaemia OR osteoporo$ OR osteopeni$
Intervention	
exp "Bone and Bones"/ genomics/ OR exp proteomics/ OR exp gene expression/ OR exp gene expression regulation/ OR exp Gene Expression	Bone OR (bone adj3 biops$) OR (bone adj3 sample) Genom$ OR proteom$ OR (gene adj3 expression) OR (gene adj3 profiling) OR

Profiling/ OR exp Microarray Analysis/ OR exp Computational Biology/	(protein adj3 expression) OR transcriptom$ OR (microarray adj3 analys$s) OR (computational adj3 biology) OR bioinformatic$
Final Result NOT (exp animals/ not humans.sh.)	

Table 1

References

[1] S. Reppe, H.K. Datta, K.M. Gautvik, Omics analysis of human bone to identify genes and molecular networks regulating skeletal remodeling in health and disease, Bone 101 (2017) 88-95.
[2] S.W. Cranford, J. de Boer, C. van Blitterswijk, M.J. Buehler, Materiomics: an -omics approach to biomaterials research, Adv Mater 25(6) (2013) 802-24.
[3] R. Dimitriou, E. Jones, D. McGonagle, P.V. Giannoudis, Bone regeneration: current concepts and future directions, BMC medicine 9 (2011) 66.
[4] R. Dimitriou, E. Tsiridis, P.V. Giannoudis, Current concepts of molecular aspects of bone healing, Injury 36(12) (2005) 1392-404.
[5] F.J. Hughes, W. Turner, G. Belibasakis, G. Martuscelli, Effects of growth factors and cytokines on osteoblast differentiation, Periodontol 2000 41 (2006) 48-72.
[6] G. Chen, C. Deng, Y.P. Li, TGF-beta and BMP signaling in osteoblast differentiation and bone formation, Int J Biol Sci 8(2) (2012) 272-88.
[7] J.J. El-Jawhari, E. Jones, P.V. Giannoudis, The roles of immune cells in bone healing; what we know, do not know and future perspectives, Injury 47(11) (2016) 2399-2406.
[8] M. Rauner, W. Sipos, S. Thiele, P. Pietschmann, Advances in osteoimmunology: pathophysiologic concepts and treatment opportunities, Int Arch Allergy Immunol 160(2) (2013) 114-25.
[9] X. Jiang, M. Ye, X. Jiang, G. Liu, S. Feng, L. Cui, H. Zou, Method development of efficient protein extraction in bone tissue for proteome analysis, J Proteome Res 6(6) (2007) 2287-94.
[10] C.R. Salmon, D.M. Tomazela, K.G. Ruiz, B.L. Foster, A.F. Paes Leme, E.A. Sallum, M.J. Somerman, F.H. Nociti, Jr., Proteomic analysis of human dental cementum and alveolar bone, J Proteomics 91 (2013) 544-55.
[11] T.P. Cleland, Solid Digestion of Demineralized Bone as a Method To Access Potentially Insoluble Proteins and Post-Translational Modifications, J Proteome Res 17(1) (2018) 536-542.
[12] M. Scheideler, C. Elabd, L.E. Zaragosi, C. Chiellini, H. Hackl, F. Sanchez-Cabo, S. Yadav, K. Duszka, G. Friedl, C. Papak, A. Prokesch, R. Windhager, G. Ailhaud, C. Dani, E.Z. Amri, Z. Trajanoski, Comparative transcriptomics of human multipotent stem cells during adipogenesis and osteoblastogenesis, BMC Genomics 9 (2008) 340.
[13] X. Xu, X. Li, R. Yan, H. Jiang, T. Wang, L. Fan, J. Wu, J. Cao, W. Li, Gene expression profiling of human bone marrow-derived mesenchymal stem cells during adipogenesis, Folia Histochem Cytobiol 54(1) (2016) 14-24.
[14] E.Z. Taskiran, B. Karaosmanoglu, Transcriptome analysis reveals differentially expressed genes between human primary bone marrow mesenchymal stem cells and human primary dermal fibroblasts, Turk J Biol 43(1) (2019) 21-27.
[15] Y.J. Choi, I. Song, Y. Jin, H.S. Jin, H.M. Ji, S.Y. Jeong, Y.Y. Won, Y.S. Chung, Transcriptional profiling of human femoral mesenchymal stem cells in osteoporosis and its association with adipogenesis, Gene 632 (2017) 7-15.
[16] K.A. de Lima, G.L. de Oliveira, J.N. Yaochite, D.G. Pinheiro, J.T. de Azevedo, W.A. Silva, Jr., D.T. Covas, C.E. Couri, B.P. Simoes, J.C. Voltarelli, M.C. Oliveira, K.C. Malmegrim, Transcriptional profiling reveals intrinsic mRNA alterations in multipotent mesenchymal stromal cells isolated from bone marrow of newly-diagnosed type 1 diabetes patients, Stem Cell Res Ther 7(1) (2016) 92.

[17] P.M. Helbling, E. Pineiro-Yanez, R. Gerosa, S. Boettcher, F. Al-Shahrour, M.G. Manz, C. Nombela-Arrieta, Global Transcriptomic Profiling of the Bone Marrow Stromal Microenvironment during Postnatal Development, Aging, and Inflammation, Cell Rep 29(10) (2019) 3313-3330 e4.
[18] C. Baccin, J. Al-Sabah, L. Velten, P.M. Helbling, F. Grunschlager, P. Hernandez-Malmierca, C. Nombela-Arrieta, L.M. Steinmetz, A. Trumpp, S. Haas, Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization, Nat Cell Biol 22(1) (2020) 38-48.
[19] B. Pineda, E. Serna, A. Laguna-Fernandez, I. Noguera, L. Panach, C. Hermenegildo, J.J. Tarin, A. Cano, M.A. Garcia-Perez, Gene expression profile induced by ovariectomy in bone marrow of mice: a functional approach to identify new candidate genes associated to osteoporosis risk in women, Bone 65 (2014) 33-41.
[20] S. Chai, L. Wan, J.L. Wang, J.C. Huang, H.X. Huang, Systematic analysis of long non-coding RNA and mRNA profiling using RNA sequencing in the femur and muscle of ovariectomized rats, J Musculoskelet Neuronal Interact 19(4) (2019) 422-434.
[21] S. Reppe, H. Refvem, V.T. Gautvik, O.K. Olstad, P.I. Hovring, F.P. Reinholt, M. Holden, A. Frigessi, R. Jemtland, K.M. Gautvik, Eight genes are highly associated with BMD variation in postmenopausal Caucasian women, Bone 46(3) (2010) 604-12.
[22] S.S. Varanasi, O.K. Olstad, D.C. Swan, P. Sanderson, V.T. Gautvik, S. Reppe, R.M. Francis, K.M. Gautvik, H.K. Datta, Skeletal site-related variation in human trabecular bone transcriptome and signaling, PLoS One 5(5) (2010) e10692.
[23] B. Hopwood, A. Tsykin, D.M. Findlay, N.L. Fazzalari, Gene expression profile of the bone microenvironment in human fragility fracture bone, Bone 44(1) (2009) 87-101.
[24] B. Hopwood, A. Tsykin, D.M. Findlay, N.L. Fazzalari, Microarray gene expression profiling of osteoarthritic bone suggests altered bone remodelling, WNT and transforming growth factor-beta/bone morphogenic protein signalling, Arthritis Res Ther 9(5) (2007) R100.
[25] J.N. Farr, M.M. Roforth, K. Fujita, K.M. Nicks, J.M. Cunningham, E.J. Atkinson, T.M. Therneau, L.K. McCready, J.M. Peterson, M.T. Drake, D.G. Monroe, S. Khosla, Effects of Age and Estrogen on Skeletal Gene Expression in Humans as Assessed by RNA Sequencing, PLoS One 10(9) (2015) e0138347.
[26] S. Andersson, K. Nilsson, L. Fagerberg, B.M. Hallstrom, C. Sundstrom, A. Danielsson, K. Edlund, M. Uhlen, A. Asplund, The transcriptomic and proteomic landscapes of bone marrow and secondary lymphoid tissues, PLoS One 9(12) (2014) e115911.
[27] J. Huang, X. Wang, T.L. Zhang, K. Wang, Alterations of ovariectomized rat bone and impact of non-collagenous proteins on mineralization, Joint Bone Spine 76(2) (2009) 176-83.
[28] Y. Fan, J. Liu, S. Wang, H. Wang, F. Shi, L. Xiong, W. He, X. Peng, Functional proteome of bones in rats with osteoporosis following ovariectomy, Life Sci 76(25) (2005) 2893-901.
[29] R. Pastorelli, D. Carpi, L. Airoldi, C. Chiabrando, R. Bagnati, R. Fanelli, S. Moverare, C. Ohlsson, Proteome analysis for the identification of in vivo estrogen-regulated proteins in bone, Proteomics 5(18) (2005) 4936-45.
[30] G.E. Sroga, L. Karim, W. Colon, D. Vashishth, Biochemical characterization of major bone-matrix proteins using nanoscale-size bone samples and proteomics methodology, Mol Cell Proteomics 10(9) (2011) M110 006718.
[31] R.D. Alves, J.A. Demmers, K. Bezstarosti, B.C. van der Eerden, J.A. Verhaar, M. Eijken, J.P. van Leeuwen, Unraveling the human bone microenvironment beyond the classical

extracellular matrix proteins: a human bone protein library, J Proteome Res 10(10) (2011) 4725-33.
[32] C.D. Chaput, L.J. Dangott, M.D. Rahm, K.D. Hitt, D.S. Stewart, H. Wayne Sampson, A proteomic study of protein variation between osteopenic and age-matched control bone tissue, Exp Biol Med (Maywood) 237(5) (2012) 491-8.
[33] Q. Zhou, F. Xie, B. Zhou, J. Wang, B. Wu, L. Li, Y. Kang, R. Dai, Y. Jiang, Differentially expressed proteins identified by TMT proteomics analysis in bone marrow microenvironment of osteoporotic patients, Osteoporos Int 30(5) (2019) 1089-1098.
[34] M. Ge, R. Ke, T. Cai, J. Yang, X. Mu, Identification and proteomic analysis of osteoblast-derived exosomes, Biochem Biophys Res Commun 467(1) (2015) 27-32.
[35] O.G. Davies, S.C. Cox, I. Azoidis, A.J.A. McGuinness, M. Cooke, L.M. Heaney, L.M. Grover, Osteoblast-Derived Vesicle Protein Content Is Temporally Regulated During Osteogenesis: Implications for Regenerative Therapies, Front Bioeng Biotechnol 7 (2019) 92.
[36] Y. Qin, R. Sun, C. Wu, L. Wang, C. Zhang, Exosome: A Novel Approach to Stimulate Bone Regeneration through Regulation of Osteogenesis and Angiogenesis, Int J Mol Sci 17(5) (2016).
[37] W. Sun, C. Zhao, Y. Li, L. Wang, G. Nie, J. Peng, A. Wang, P. Zhang, W. Tian, Q. Li, J. Song, C. Wang, X. Xu, Y. Tian, D. Zhao, Z. Xu, G. Zhong, B. Han, S. Ling, Y.Z. Chang, Y. Li, Osteoclast-derived microRNA-containing exosomes selectively inhibit osteoblast activity, Cell Discov 2 (2016) 16015.
[38] D. Li, J. Liu, B. Guo, C. Liang, L. Dang, C. Lu, X. He, H.Y. Cheung, L. Xu, C. Lu, B. He, B. Liu, A.B. Shaikh, F. Li, L. Wang, Z. Yang, D.W. Au, S. Peng, Z. Zhang, B.T. Zhang, X. Pan, A. Qian, P. Shang, L. Xiao, B. Jiang, C.K. Wong, J. Xu, Z. Bian, Z. Liang, D.A. Guo, H. Zhu, W. Tan, A. Lu, G. Zhang, Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation, Nat Commun 7 (2016) 10872.
[39] Y. Cui, J. Luan, H. Li, X. Zhou, J. Han, Exosomes derived from mineralizing osteoblasts promote ST2 cell osteogenic differentiation by alteration of microRNA expression, FEBS Lett 590(1) (2016) 185-92.
[40] R. Sawafuji, E. Cappellini, T. Nagaoka, A.K. Fotakis, R.R. Jersie-Christensen, J.V. Olsen, K. Hirata, S. Ueda, Proteomic profiling of archaeological human bone, R Soc Open Sci 4(6) (2017) 161004.
[41] C. Wadsworth, M. Buckley, Proteome degradation in fossils: investigating the longevity of protein survival in ancient bone, Rapid Commun Mass Spectrom 28(6) (2014) 605-15.
[42] E. Cappellini, L.J. Jensen, D. Szklarczyk, A. Ginolhac, R.A. da Fonseca, T.W. Stafford, S.R. Holen, M.J. Collins, L. Orlando, E. Willerslev, M.T. Gilbert, J.V. Olsen, Proteomic analysis of a pleistocene mammoth femur reveals more than one hundred ancient bone proteins, J Proteome Res 11(2) (2012) 917-26.
[43] E. Calciolari, N. Donos, The use of omics profiling to improve outcomes of bone regeneration and osseointegration. How far are we from personalized medicine in dentistry?, J Proteomics 188 (2018) 85-96.
[44] K. Lybrand, B. Bragdon, L. Gerstenfeld, Mouse models of bone healing: fracture, marrow ablation, and distraction osteogenesis, Curr Protoc Mouse Biol 5(1) (2015) 35-49.
[45] E. Calciolari, N. Donos, N. Mardas, Osteoporotic Animal Models of Bone Healing: Advantages and Pitfalls, J Invest Surg 30(5) (2017) 342-350.
[46] F. Bonnarens, T.A. Einhorn, Production of a standard closed fracture in laboratory animal bone, J Orthop Res 2(1) (1984) 97-101.

[47] A. Schindeler, R.J. Mills, J.D. Bobyn, D.G. Little, Preclinical models for orthopedic research and bone tissue engineering, J Orthop Res 36(3) (2018) 832-840.
[48] C. Sfeir, L. Ho, B.A. Doll, K. Azari, J.O. Hollinger, Fracture repair, in: G.E.F.M. Jay R. Lieberman MD (Ed.), Bone Regeneration and Repair2005, pp. 21-44.
[49] M. Haffner-Luntzer, A. Kovtun, A.E. Rapp, A. Ignatius, Mouse Models in Bone Fracture Healing Research, Current Molecular Biology Reports 2(2) (2016) 101-111.
[50] G. Cardaropoli, M. Araujo, J. Lindhe, Dynamics of bone tissue formation in tooth extraction sites. An experimental study in dogs, J Clin Periodontol 30(9) (2003) 809-18.
[51] M.H. Amler, P.L. Johnson, I. Salman, Histological and histochemical investigation of human alveolar socket healing in undisturbed extraction wounds, J Am Dent Assoc 61 (1960) 32-44.
[52] M.G. Araujo, J. Lindhe, Ridge preservation with the use of Bio-Oss collagen: A 6-month study in the dog, Clin Oral Implants Res 20(5) (2009) 433-40.
[53] M.G. Araujo, J. Lindhe, Ridge alterations following tooth extraction with and without flap elevation: an experimental study in the dog, Clin Oral Implants Res 20(6) (2009) 545-9.
[54] J. Wang, M.J. Glimcher, Characterization of matrix-induced osteogenesis in rat calvarial bone defects: I. Differences in the cellular response to demineralized bone matrix implanted in calvarial defects and in subcutaneous sites, Calcif Tissue Int 65(2) (1999) 156-65.
[55] A. Vajgel, N. Mardas, B.C. Farias, A. Petrie, R. Cimoes, N. Donos, A systematic review on the critical size defect model, Clin Oral Implants Res 25(8) (2014) 879-93.
[56] N. Mardas, X. Dereka, N. Donos, M. Dard, Experimental model for bone regeneration in oral and cranio-maxillo-facial surgery, J Invest Surg 27(1) (2014) 32-49.
[57] N. Donos, N.P. Lang, I.K. Karoussis, D. Bosshardt, M. Tonetti, L. Kostopoulos, Effect of GBR in combination with deproteinized bovine bone mineral and/or enamel matrix proteins on the healing of critical-size defects, Clin Oral Implants Res 15(1) (2004) 101-11.
[58] E. Calciolari, N. Mardas, X. Dereka, N. Kostomitsopoulos, A. Petrie, N. Donos, The effect of experimental osteoporosis on bone regeneration: Part 1, histology findings, Clin Oral Implants Res 28(9) (2017) e101-e110.
[59] E. Calciolari, N. Mardas, X. Dereka, A.K. Anagnostopoulos, G.T. Tsangaris, N. Donos, Protein expression during early stages of bone regeneration under hydrophobic and hydrophilic titanium domes. A pilot study, J Periodontal Res 53(2) (2018) 174-187.
[60] A. Linde, C. Thoren, C. Dahlin, E. Sandberg, Creation of new bone by an osteopromotive membrane technique: an experimental study in rats, J Oral Maxillofac Surg 51(8) (1993) 892-7.
[61] D. Lundgren, A.K. Lundgren, L. Sennerby, S. Nyman, Augmentation of intramembraneous bone beyond the skeletal envelope using an occlusive titanium barrier. An experimental study in the rabbit, Clin Oral Implants Res 6(2) (1995) 67-72.
[62] H. Zigdon, D. Lewinson, T. Bick, E.E. Machtei, Vertical bone augmentation using different osteoconductive scaffolds combined with barrier domes in the rat calvarium, Clinical implant dentistry and related research 16(1) (2014) 138-44.
[63] H. Zigdon-Giladi, D. Lewinson, T. Bick, E.E. Machtei, Mesenchymal stem cells combined with barrier domes enhance vertical bone formation, J Clin Periodontol 40(2) (2013) 196-202.
[64] N. Mardas, F. Schwarz, A. Petrie, A.-R. Hakimi, N. Donos, The effect of SLActive surface in guided bone formation in osteoporotic-like conditions, Clinical Oral Implants Research 22(4) (2011) 406-15.

[65] L. Kostopoulos, T. Karring, R. Uraguchi, Formation of jawbone tuberosities by guided tissue regeneration. An experimental study in the rat, Clin Oral Implants Res 5(4) (1994) 245-53.
[66] N. Donos, D. Bosshardt, N. Lang, F. Graziani, M. Tonetti, T. Karring, L. Kostopoulos, Bone formation by enamel matrix proteins and xenografts: an experimental study in the rat ramus, Clinical Oral Implants Research 16(2) (2005) 140-6.
[67] N. Mardas, L. Kostopoulos, A. Stavropoulos, T. Karring, Denaturation of demineralized bone matrix significantly reduces bone formation by guided tissue regeneration, Clin Oral Implants Res 14(6) (2003) 804-11.
[68] E. Hjorting-Hansen, M. Helbo, M. Aaboe, K. Gotfredsen, E.M. Pinholt, Osseointegration of subperiosteal implant via guided tissue regeneration. A pilot study, Clin Oral Implants Res 6(3) (1995) 149-54.
[69] N. Donos, X. Dereka, N. Mardas, Experimental models for guided bone regeneration in healthy and medically compromised conditions, Periodontol 2000 68(1) (2015) 99-121.
[70] I. Elgali, A. Turri, W. Xia, B. Norlindh, A. Johansson, C. Dahlin, P. Thomsen, O. Omar, Guided bone regeneration using resorbable membrane and different bone substitutes: Early histological and molecular events, Acta Biomater 29 (2016) 409-423.
[71] Z. Lin, H.F. Rios, S.L. Volk, J.V. Sugai, Q. Jin, W.V. Giannobile, Gene expression dynamics during bone healing and osseointegration, J Periodontol 82(7) (2011) 1007-17.
[72] C.I. van Houdt, C.R. Tim, M.C. Crovace, E.D. Zanotto, O. Peitl, D.J. Ulrich, J.A. Jansen, N.A. Parizotto, A.C. Renno, J.J. van den Beucken, Bone regeneration and gene expression in bone defects under healthy and osteoporotic bone conditions using two commercially available bone graft substitutes, Biomed Mater 10(3) (2015) 035003.
[73] J.K. Wise, K. Sena, K. Vranizan, J.F. Pollock, K.E. Healy, W.F. Hughes, D.R. Sumner, A.S. Virdi, Temporal gene expression profiling during rat femoral marrow ablation-induced intramembranous bone regeneration, PLoS One 5(10) (2010).
[74] C.H. Rundle, H. Wang, H. Yu, R.B. Chadwick, E.I. Davis, J.E. Wergedal, K.H. Lau, S. Mohan, J.T. Ryaby, D.J. Baylink, Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair, Bone 38(4) (2006) 521-9.
[75] X. Li, R.J. Quigg, J. Zhou, J.T. Ryaby, H. Wang, Early signals for fracture healing, J Cell Biochem 95(1) (2005) 189-205.
[76] M. Hadjiargyrou, F. Lombardo, S. Zhao, W. Ahrens, J. Joo, H. Ahn, M. Jurman, D.W. White, C.T. Rubin, Transcriptional profiling of bone regeneration. Insight into the molecular complexity of wound repair, J Biol Chem 277(33) (2002) 30177-82.
[77] C. Liu, Y. Liu, W. Zhang, X. Liu, Screening for potential genes associated with bone overgrowth after mid-shaft femur fracture in a rat model, J Orthop Surg Res 12(1) (2017) 8.
[78] R.A. Meyer, Jr., M.H. Meyer, N. Ashraf, S. Frick, Changes in mRNA gene expression during growth in the femoral head of the young rat, Bone 40(6) (2007) 1554-64.
[79] T. Niikura, D.J. Hak, A.H. Reddi, Global gene profiling reveals a downregulation of BMP gene expression in experimental atrophic nonunions compared to standard healing fractures, J Orthop Res 24(7) (2006) 1463-71.
[80] T. Ohira, F. Myokai, N. Shiomi, K. Yamashiro, T. Yamamoto, Y. Murayama, H. Arai, F. Nishimura, S. Takashiba, Identification of genes differentially regulated in rat alveolar bone wound healing by subtractive hybridization, J Dent Res 83(7) (2004) 546-51.

[81] R. Al-Kattan, M. Retzepi, E. Calciolari, N. Donos, Microarray gene expression during early healing of GBR-treated calvarial critical size defects, Clin Oral Implants Res 28(10) (2017) 1248-1257.
[82] S. Ivanovski, S. Hamlet, M. Retzepi, I. Wall, N. Donos, Transcriptional profiling of "guided bone regeneration" in a critical-size calvarial defect, Clin Oral Implants Res 22(4) (2011) 382-9.
[83] N. Donos, M. Retzepi, I. Wall, S. Hamlet, S. Ivanovski, In vivo gene expression profile of guided bone regeneration associated with a microrough titanium surface, Clin Oral Implants Res 22(4) (2011) 390-8.
[84] N. Kojima, S. Ozawa, Y. Miyata, H. Hasegawa, Y. Tanaka, T. Ogawa, High-throughput gene expression analysis in bone healing around titanium implants by DNA microarray, Clin Oral Implants Res 19(2) (2008) 173-81.
[85] E.C. Kim, R. Leesungbok, S.W. Lee, J.Y. Hong, E.J. Ko, S.J. Ahn, Effects of static magnetic fields on bone regeneration of implants in the rabbit: micro-CT, histologic, microarray, and real-time PCR analyses, Clin Oral Implants Res 28(4) (2017) 396-405.
[86] C.M. Mengatto, F. Mussano, Y. Honda, C.S. Colwell, I. Nishimura, Circadian rhythm and cartilage extracellular matrix genes in osseointegration: a genome-wide screening of implant failure by vitamin D deficiency, PLoS One 6(1) (2011) e15848.
[87] A. Thorfve, A. Bergstrand, K. Ekstrom, A. Lindahl, P. Thomsen, A. Larsson, P. Tengvall, Gene expression profiling of peri-implant healing of PLGA-Li+ implants suggests an activated Wnt signaling pathway in vivo, PLoS One 9(7) (2014) e102597.
[88] N. Donos, S. Hamlet, N.P. Lang, G.E. Salvi, G. Huynh-Ba, D.D. Bosshardt, S. Ivanovski, Gene expression profile of osseointegration of a hydrophilic compared with a hydrophobic microrough implant surface, Clin Oral Implants Res 22(4) (2011) 365-72.
[89] S. Ivanovski, S. Hamlet, G.E. Salvi, G. Huynh-Ba, D.D. Bosshardt, N.P. Lang, N. Donos, Transcriptional profiling of osseointegration in humans, Clin Oral Implants Res 22(4) (2011) 373-81.
[90] G.N. Thalji, S. Nares, L.F. Cooper, Early molecular assessment of osseointegration in humans, Clin Oral Implants Res 25(11) (2014) 1273-85.
[91] G. Thalji, C. Gretzer, L.F. Cooper, Comparative molecular assessment of early osseointegration in implant-adherent cells, Bone 52(1) (2013) 444-53.
[92] J.L. Fowlkes, R.C. Bunn, L. Liu, E.C. Wahl, H.N. Coleman, G.E. Cockrell, D.S. Perrien, C.K. Lumpkin, Jr., K.M. Thrailkill, Runt-related transcription factor 2 (RUNX2) and RUNX2-related osteogenic genes are down-regulated throughout osteogenesis in type 1 diabetes mellitus, Endocrinology 149(4) (2008) 1697-704.
[93] H. Lu, D. Kraut, L.C. Gerstenfeld, D.T. Graves, Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate osteoblast differentiation, Endocrinology 144(1) (2003) 346-52.
[94] K.I. Ko, L.S. Coimbra, C. Tian, J. Alblowi, R.A. Kayal, T.A. Einhorn, L.C. Gerstenfeld, R.J. Pignolo, D.T. Graves, Diabetes reduces mesenchymal stem cells in fracture healing through a TNFalpha-mediated mechanism, Diabetologia 58(3) (2015) 633-642.
[95] J. Alblowi, R.A. Kayal, M. Siqueira, E. McKenzie, N. Krothapalli, J. McLean, J. Conn, B. Nikolajczyk, T.A. Einhorn, L. Gerstenfeld, D.T. Graves, High levels of tumor necrosis factor-alpha contribute to accelerated loss of cartilage in diabetic fracture healing, Am J Pathol 175(4) (2009) 1574-85.

[96] W.A. Tyndall, H.A. Beam, C. Zarro, J.P. O'Connor, S.S. Lin, Decreased platelet derived growth factor expression during fracture healing in diabetic animals, Clin Orthop Relat Res (408) (2003) 319-30.
[97] M.T. Xu, S. Sun, L. Zhang, F. Xu, S.L. Du, X.D. Zhang, D.W. Wang, Diabetes mellitus affects the biomechanical function of the callus and the expression of TGF-beta1 and BMP2 in an early stage of fracture healing, Braz J Med Biol Res 49(1) (2016) e4736.
[98] M. Retzepi, E. Calciolari, I. Wall, M.P. Lewis, N. Donos, The effect of experimental diabetes and glycaemic control on guided bone regeneration: histology and gene expression analyses, Clin Oral Implants Res (2017).
[99] X.X. Wang, Y. Ma, Q. Li, B.Q. Jiang, J. Lan, [Differential expression genes of bone tissues surrounding implants in diabetic rats by gene chip], Shanghai Kou Qiang Yi Xue 21(5) (2012) 521-5.
[100] M. Hauser, M. Siegrist, I. Keller, W. Hofstetter, Healing of fractures in osteoporotic bones in mice treated with bisphosphonates - A transcriptome analysis, Bone 112 (2018) 107-119.
[101] F. Gao, F. Xu, D. Wu, J. Cheng, P. Xia, Identification of novel genes associated with fracture healing in osteoporosis induced by Krm2 overexpression or Lrp5 deficiency, Mol Med Rep 15(6) (2017) 3969-3976.
[102] C. Wang, T. Yu, L. Tan, J. Cheng, Bioinformatics analysis of gene expression profile in callus tissues of osteoporotic phenotype mice induced by osteoblast-specific Krm2 overexpression, Int J Rheum Dis 19(12) (2016) 1263-1271.
[103] A. Liedert, V. Rontgen, T. Schinke, P. Benisch, R. Ebert, F. Jakob, L. Klein-Hitpass, J.K. Lennerz, M. Amling, A. Ignatius, Osteoblast-specific Krm2 overexpression and Lrp5 deficiency have different effects on fracture healing in mice, PLoS One 9(7) (2014) e103250.
[104] R. Zhao, S. Chen, B. Yuan, X. Chen, X. Yang, Y. Song, H. Tang, X. Yang, X. Zhu, X. Zhang, Healing of osteoporotic bone defects by micro-/nano-structured calcium phosphate bioceramics, Nanoscale 11(6) (2019) 2721-2732.
[105] Z. Du, Y. Xiao, S. Hashimi, S.M. Hamlet, S. Ivanovski, The effects of implant topography on osseointegration under estrogen deficiency induced osteoporotic conditions: Histomorphometric, transcriptional and ultrastructural analysis, Acta Biomater 42 (2016) 351-363.
[106] J. Ma, Y. Wu, W. Zhang, R.J. Smales, Y. Huang, Y. Pan, L. Wang, Up-regulation of multiple proteins and biological processes during maxillary expansion in rats, BMC Musculoskelet Disord 9 (2008) 37.
[107] W.B. Zhang, L. Wang, Label-free quantitative proteome analysis of skeletal tissues under mechanical load, J Cell Biochem 108(3) (2009) 600-11.
[108] H.-Y. Yang, J. Kwon, M.-S. Kook, S.S. Kang, S.E. Kim, S. Sohn, S. Jung, S.-O. Kwon, H.-S. Kim, J.H. Lee, T.-H. Lee, Proteomic analysis of gingival tissue and alveolar bone during alveolar bone healing, Molecular & cellular proteomics : MCP 12(10) (2013) 2674-88.
[109] S. Kalkhof, Y. Forster, J. Schmidt, M.C. Schulz, S. Baumann, A. Weissflog, W. Gao, U. Hempel, U. Eckelt, S. Rammelt, M. von Bergen, Proteomics and metabolomics for in situ monitoring of wound healing, Biomed Res Int 2014 (2014) 934848.
[110] E. Calciolari, S. Hamlet, S. Ivanovski, N. Donos, Pro-osteogenic properties of hydrophilic and hydrophobic titanium surfaces: Crosstalk between signalling pathways in in vivo models, J Periodontal Res 53(4) (2018) 598-609.

[111] E. Calciolari, N. Mardas, X. Dereka, A.K. Anagnostopoulos, G.T. Tsangaris, N. Donos, The effect of experimental osteoporosis on bone regeneration: part 2, proteomics results, Clin Oral Implants Res 28(9) (2017) e135-e145.
[112] S.A. Cushman, Grand challenges in evolutionary and population genetics: the importance of integrating epigenetics, genomics, modeling, and experimentation, Front Genet 5 (2014) 197.
[113] N. Savage, Bioinformatics: Big data versus the big C, Nature 509(7502) (2014) S66-7.
[114] D. Karasik, F. Rivadeneira, M.L. Johnson, The genetics of bone mass and susceptibility to bone diseases, Nat Rev Rheumatol 12(8) (2016) 496.
[115] Y. Hasin, M. Seldin, A. Lusis, Multi-omics approaches to disease, Genome Biol 18(1) (2017) 83.
[116] F.R. Pinu, D.J. Beale, A.M. Paten, K. Kouremenos, S. Swarup, H.J. Schirra, D. Wishart, Systems Biology and Multi-Omics Integration: Viewpoints from the Metabolomics Research Community, Metabolites 9(4) (2019).
[117] X. Wang, Clinical trans-omics: an integration of clinical phenomes with molecular multiomics, Cell Biol Toxicol 34(3) (2018) 163-166.