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REVIEW

Post-genomics of bone metabolic dysfunctions and neoplasias

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Post-genomic research on osteoblastic and osteoclastic cells, in contrast to that on many other cell types, has only been undertaken recently. Nevertheless, important information has been gained from these investigations on the mechanisms involved in osteoblast differentiation and on markers relevant for tissue regeneration and therapeutic validation of drugs, hormones and growth factors. These protein indicators may also have a diagnostic and prognostic value for bone dysfunctions and tumors. Some reviews have already focused on the application of transcriptomics and/or proteomics for exploring skeletal biology and related disorders. The main goal of the present review is to systematically summarize the most relevant post-genomic studies on various metabolic bone diseases (osteoporosis, Paget's disease and osteonecrosis), neoplasias (osteosarcoma) and metabolic conditions that indirectly affect bone tissue, such as alkaptonuria.

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1 Introduction

Bone is a specialized form of connective tissue that serves both as a tissue and an organ system. Its basic functions include locomotion, protection, mineral homeostasis and hematopoiesis. The whole skeletal system is an extremely dynamic environment undergoing a constant remodeling process in which bone is renewed to maintain strength and mineral homeostasis. This is achieved through a balance of two tightly regulated and complex processes, namely bone formation mediated by osteoblasts (OBs) and bone resorption mediated by osteoclasts (OCs). These two processes are controlled by numerous paracrine and endocrine factors, including OB- and OC-specific signaling proteins and transcription factors, as well as hormones and growth factors [1].

Bone tissue structure and functionality can be affected by various pathogenic conditions, due to both metabolic (osteoporosis (OP), osteonecrosis (ON) and Paget's disease (PD)) and genetic (osteogenesis imperfecta and osteopetrosis) disorders as well as neoplastic transformation of bone cells. Apart from these disorders, there are other metabolic conditions that can indirectly have negative and severe effects at the bone level such as alkaptonuria (AKU), which is a rare, autosomal recessive disorder of tyrosine and phenylalanine metabolism. One of the most debilitating symptoms of AKU, appearing around the third/fourth decade of life, is a progressive form of arthritis of the spine and large joints, significantly reducing patients' quality of life [2].

Despite our increasing knowledge of the individual molecular mechanisms of OB/OC activation, how these mechanisms are orchestrated to maintain normal bone structural integrity is poorly understood. The identification of intrinsic and extrinsic factors responsible for the maintenance of bone homeostasis has been the focus of extensive

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Abbreviations: AKU, alkaptonuria; **BMD,** bone mineral density; **GC,** glucocorticoid; **OB,** osteoblast; **OC,** osteoclast; **ON,** osteonecrosis; **ONFH,** ON of the femoral head; **ONJ,** ON of the jaw; **OP,** osteoporosis; **OS,** osteosarcoma; **OVX,** ovariectomized; **PD,** Paget's disease; **PKA,** cAMP-dependent protein kinase; **PKC,** protein kinase C; **PTH,** parathyroid hormone

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research over the decades, but the complex nature of all the involved processes has severely hampered a thorough understanding. This is because conventional reductionist approaches examine a limited number of biological factors based on very specific signaling or metabolic pathways. Conversely, the recent advent of high-throughput post-genomic technologies (epigenomics, transcriptomics and proteomics) provided researchers with the opportunity to finally get a comprehensive insight into bone physio-pathology by qualitative and quantitative mapping of the whole ''omics'' repertoires in large-scale studies. For example, in the area of bone research, global expression-profiling approaches have been applied to determine the basal and key events in skeletal biology, development and remodeling. For exhaustive reviews on expression-profile studies on OBs and OCs differentiation, regulation and interaction processes, readers can refer to Refs. [3–6]. The main aim of the present review is to systematically summarize the most relevant post-genomic studies related to several metabolic diseases and neoplasias of bone, taking osteosarcoma as a case study (Table 1). Finally, the potential future impacts of so-far-unexplored proteomic and sub-proteomic analyses are discussed to highlight their role in finding new therapeutic targets or in providing new insights into the molecular mechanisms of bone diseases.

2 Metabolic bone diseases

2.1 Osteoporosis

OP is the most common chronic metabolic disease of bone in industrialized countries. OP is a degenerative skeletal disease characterized by low bone mass or increased bone porosity. The deterioration of bone architecture leads to an increased bone fragility and, consequently, to an increased fracture risk involving especially the hip, spine and wrist, although any bone can be affected.

OP is classified as either primary or secondary according to the pathogenic mechanisms. Primary OP is caused by estrogen and/or calcium deficiency and aging, frequently occurring in postmenopausal women and older men. Secondary OP is associated with other diseases such as multiple myelomatosis, hyperparathyroidism and hyperthyroidism, or with pharmacological treatments, like in the case of chronic glucocorticoid (GC) treatment. OP, either primary or secondary, is the results of an imbalance in OB/OC functions, numbers and/or activity.

Although many genetic, metabolic, hormonal and environmental factors have been identified as taking part in the etiology of OP, the complex physiology of the bone tissue makes the study of the molecular mechanisms of OP an extremely challenging task [7]. At the bone level, post-genomics approaches have been used to investigate the molecular mechanisms of both primary and secondary OP and to study the effect of therapeutic agents. Current pharmacological treatment strategies for reducing the incidence of osteoporotic fractures can be separated into two categories, anti-resorptive or anti-catabolic agents that target OCs, and bone-forming or anabolic agents that target OBs. Although many pharmacological agents have been developed and commercialized in recent years, none of them is able to fully restore the correct balanced bone-remodeling process or is free from side effects [8].

The first studies addressing OP were carried out in rat models where the loss of estrogens and the onset of OP were shown to be correlated. In particular, bone tissue and OB progenitor cells from ovariectomized (OVX) rats were investigated by both gene and protein expression profiling [9, 10]. Comparative proteomic analyses, using 2-DE coupled to MALDI-TOF/MS, of bone tissue from OVX rats identified three proteins overexpressed in estrogen-deficient OP animals, peroxiredoxin 2, myosin light polypeptide 2 and ubiquitin-conjugating enzyme E2-17 kDa, none of which had been previously associated with OP [9]. In a preliminary comparative study of gene expression, profiling of bone marrow stromal cells (BMSCs) among juvenile, adult, OVX-induced osteoporotic and aged rats, Xiao et al. [10] identified a high number of genes with altered profiles associated with OP induced by genetic factors and not related to ageing. Among these, genes with increased expression were related to different biological processes, including skeletal development (*Alpl* and *Zic1*), basal metabolism (*Uchl1*, *Crabp2* and *Mmp8*), signaling pathways (G proteins and neuropeptide pathways: *Hcrtr1*, *Htr2b*, *Ucn*, *Trrp6*, *Cirl3*), transcription factors (*Crabp2*) and bone cell growth and maintenance (*Crabp2*, *Alpl*, *Csrp2* and *Cdkn2b*), whereas down-regulated genes were found to be related to hormone activity (*Npy*, *Prlpb*, *Plpc-b*).

With the same intent of identifying OP-related genes, the transcriptomic approach was applied to human bone tissues from OP women [11, 12]. In both studies, the authors presented huge amounts of data. However, like many other microarray-based studies, the study by Hopwood and colleagues [11] showed poorly mined data, lacking an adequate statistical and bioinformatic analysis; consequently, the biological relevance of the study is difficult to discern. In contrast, the study performed by Balla et al. [12] represents an interesting example of how extensive and focused multivariate statistical analysis can allow data to be refined, enabling the selection of a few genes that highly discriminate non-OP and OP postmenopausal women.

Comparative proteome analyses between OP and non-OP individuals have also been performed [13]. Relying on SELDI-TOF/MS analysis of serum samples from individuals classified as high or low/normal bone turnover according to the urine N-telopeptide of type I collagen (NTX) scores, Bhattacharyya et al. [13] found a set of proteins that successfully discriminated the two patient groups (80% sensitivity and 100% specificity), and that were also significantly correlated with bone mineral density (BMD). Four of these proteins were identified as fragments of

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Table 1. Summary of the most important findings from the discussed papers

Ref.	Experimental approach	Samples	Main findings	Validation
Osteoporosis				
[8]	Proteomics (2DE and PMF)	Bone tissue from OVX rats	OE: peroxiredoxin 2, myosin light polypeptide 2 and ubiquitin-conjugating enzyme E2-17 kDa	No
[9]	Transcriptomics	BMSC from juvenile, adult, OVX and aged rats.	UR: *Alpl*, *Zic1* (skeletal development), *Uchl1*, *Crabp2*, *Mmp8* (basal metabolism), *Hcrtr1*, *Htr2b*, *Ucn*, *Trrp6*, *Cirl3* (signaling pathways), *Crabp2*, *Alpl*, *Csrp2* and *Cdkn2b* (bone cell growth and maintenance). DR: *Npy*, *Prlpb*, *Plpc-b* (hormone activity)	No
[10]	Transcriptomics	Bone tissue from OP and OA women	OC differentiation and function-related genes: UR: **TREM2**, **MARCO**, *CCL3*, *CCL2*, *CCL18*, *IL1B*, *IL11*, *and IL6*, **IL10**, **CD14.** DR: *SHIP1*, **NF1**, **YAF2 and DARC** OB differentiation and function-related genes: UR: *CTSB*, *FST*, *SPP1*, *CTGF*, *ECM1*, *MGP*, **TGFBI**, **ANXA2**, *ST14*, *SPARC*, **PRRX1 LRP12**, and **KFL10**. DR: *SLC14A1*, *GHSR*, **TGFB1** and **ADM**, *ATP7A and NIPBL* Adipocite differentiation and function-related genes: UR: *ADFP*, *GFP4*, *AEBP1*, *SPARC*, *FST*, *HSD11B1*, **IL10** and **CTGF** DR: *ADM*, *SLC14A1*, *TAZ and NIK*	Yes (RT-PCR)
[11]	Transcriptomics	Bone tissue from OP women	UR: *CD36* and *TWIST2* DR: *ALPL*, *COL1A1*, *MMP2*, *MMP13*, *MMP9*, *PDGFA*, *NFKB1*	No
[12]	Proteomics (SELDI-ToF/MS)	Sera from individuals classified as high or low/normal bone turnover	UE: interalphatrypsin-inhibitor heavy chain H4 precursor (4 frgments)	No
[14]	Proteomics (2DE and PMF)	CMCs from women at high or low BMD levels	OE: **Ras suppressor protein 1, gelsolin, manganese-containing superoxide dismutase, glutathione peroxidase 1, protein disulfide-isomerase**	Yes (WB)
[15]	Transcriptomics	CMCs from women at high or low BMD levels	UR in the low BMD group: **CCR**, **HDC**, **GCR**, **SCYE1**, **FCER1A** DR in the low BMD group: **RAMP3**	Yes (RT-PCR)
[21]	Transcriptomics	Primary human OBs exposed to dexamethasone	DKK1-mediated inhibition of the WNT signaling pathways	No
[22]	Transcriptomics	Bone tissue from mice exposed to prednisolone	UR: *Csf1*, *Itgb3*, *Adam8*, *Trem2*, *Oscar* (OC differentiation and function), *c-Src*, *Syk*, *Vav3* (cytoskeleton organization), *ATPase*, *Mmp9*, *Ctsk*, *Prss22* (matrix degradation), *Cebpb*, Pparg, AdipoQ (adipocyte differentiation and function) DR: c-fms, Ibsp (OC differentiation and function), Plcg1, c-Fos, Nfatc1 (signal transduction), *Cebpa*, *Ppard* (adipocyte differentiation and function) DKK1-mediated inhibition of the WNT signaling pathways	No
[26]	Transcriptomics	Bone tissue from OVX mice treated with PTH(1–34) or E2	PTH(1–34) UR: *Vdr*, *Tnfsf11*, *Ctsk* E2 UR: *Bag3*, *Bmp1*, *Bmp8a*, *Ccnd1*, *Pth1r* and *Ctsl*	No
[27]	Transcriptomics	Bone tissue from rats continuously or intermittently treated with PTH(1–34)	PTH (1–34) intermittent: Upregulation of binding, catalysis and immune response-related genes. PTH (1–34)continuous: Upregulation of bone turnover and OC-related genes. PTH (1–34) both treatment: Upregulation of skeletal development-associated genes	No
[28]	Transcriptomics	Bone tissue from rats continuously or intermittently treated with PTH(1–34), PTH(1–31) and PTH(3–34)	PTH (1–31) exhibits better bone anabolic properties	Yes (RT-PCR)

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Table 1. Continued

Ref.	Experimental approach	Samples	Main findings	Validation
[29]	Proteomics (2DE and PMF)	BMC from mouse treated with PTH(1–84)	OE: calreticulin, protein disulfide isomerase associated 6 UE: vimentin	Yes (RT-PCR)
[30]	Proteomics (SELDI-ToF/MS)	Serum from mice treated with PTH(1–34)	OE: hemoglobin-α, hemoglobin-β	No
Paget's disease				
[32]	Transcriptomics	OB and BMSC from pagetic and nonpagetic bone	UR: *IL-6*, *IL-1B*, *ALPL* DR: *IBSP*, *BGLAP*, *Wnt signal pathway*	No
[33]	Transcriptomics	OC progenitors from pagetic patients	UR: *IFN,A IFNB1*, *IFNG*, *p38* β *2 MAPK*, *IFNGR1* and *IFNGR2*, *STAT1* DR: *TNFA*	No
[34]	Transcriptomics	OC from pagetic patients with or without the p62-P392L substitution	Mutation-independent DR: *CASP3*, *TNFRSF10-A* (apoptosis), *TNFRSF11A* (cell signaling), *ACP5*, *CTSK* (bone resorption) and *MAPT*	No
Osteonecrosis				
[37]	Transcriptomics	Bone cell progenitors from multiple myeloma patients on biphosphonate therapy with or without ONJ	UR: *IL-18* DR: *NFAT2*, *NFAT3*, *p49/p100*, *FUBP1*, *MIF*, *CCL5/RANTES* (signalling, activation, or differentiation of OCs), *NF-Y*, *BMAL1*, *E2F*, *PPARG, PRKCH* (signalling, activation, or differentiation of OBs)	No
[38]	Proteomics (2DE and PMF)	Sera from ONFH patients	OE: kininogen variant 1, complement factor C3 precursor and complement factor H UE: apolipoprotein A-IV precursor, antitrombin III chian B and gelsolin isoform α precursor	No
[39]	Proteomics (2DE and PMF)	Albumin-depleted sera from ONFH patients	OE: **plasminogen activator inhibitor type 1, crossLaps, anti-p-3 antibody** UE: **tissue-type plasminogen activator**, bone-carboxyglutammate protein, C-sis	Yes (ELISA)
[40]	Transcriptomics	Bone tissue from a murine model of ON Bone tissue from ONFH and OA for validation	UR: **p53**	Yes (IHC)
Osteosarcoma				
[45]	Transcriptomics	Chemonaive OS cells and paired OB cells	UR: *EBF2*	Yes (IHC)
[46]	Transcriptomics	OS tissues from definitive surgery classified as good/poor responders	Selection of 45 predictors genes as basis for a Support Vector Machine classifier able to classify initial OS biopsy samples according to their chemoresponsiveness	
[47]	Transcriptomics	Chemonaive OS tissues before classification as good/poor responders	Transcriptional fingerprint of OS chemoresistance consting of 104 genes (e.g.: **DSP**, OPG, **SERPINE1**, **BGN**, **ANXA2**, PLA2G2A, and **SPARCL1**).	Yes (RT-PCR)
[48]	Transcriptomics	Chemonaive OS tissues before classification as good/poor responders	UR in poor responders: **HSD17B10**, *HECT*, **HECW1** DR in poor responders: **ARFIP1**, **IFITM3**, **IFITM2**, **RPL8** *HSD17B10* predictive markers for OS chemoresistance and therapeutic target	Yes (RT-PCR, IHC)
[49]	Transcriptomics	Chemonaive OS tissues before classification as good/poor responders	Selection of 60 genes able to discriminate between good and poor responders, among these genes *AKR1C4*, *GPX1* and *GSTTLp28* showed the most significant correlation with poor responders	Yes (RT-PCR)
[51]	Transcriptomics	Human OS xenograft submitted to single-agent chemotherapy (doxorubicin, cisplatin and ifosfamide)	Poor responders to doxorubicin UR: *HSP27*, *HSP70*, **BAG2** and *SRI* DR: *COL12A1*, *INHBA*, *MGP* and *TGFBI* Poor responders to cisplatin UR: *RTN3* DR: *GALC* and *PHLDA1*	Yes (siRNA)

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Table 1. Continued

Ref.	Experimental approach	Samples	Main findings	Validation
			Poor responders to ifosfamide: UR: *AXNA4*, *AVEN*, **IER3**, *GADD45A*, *SH3KBP1*, *FHL2*, *MLF1*, *CUTL1*, *TCF8*, *PLAU*, *SERPINE1* and *CTSL* DR: *BMP4*, *MADH6*, *ID3*, *MSX2*, *MEF2C*, *COL11A2* and *ALPL*.	
[56]	Transcriptomics	Chemonaive OS tissues with/without pulmonary metastasis in a 4 years follow-up	DR of *ASS* with the highest statistical significance	Yes (IHC)
[68]	Proteomics (iTRAQ-LC-MS/MS)	Plasma membrane proteins from MG63 OS cell line and hFOB1.19 OB cells	OE: CD151	Yes (IHC)
[73]	Proteomics (2DE and PMF)	Total protein extracts from 3 OS cell lines (U2OS, SaOS-2 and IOR/OS9) and hFOB1.19 OB cells	OE: activator of 90 kDa heat-shock protein, ATPase homolog 1 AHA1, and stomatin-like protein 2. UE: glutathione *S*-transferase omega-1, E3 ubiquitin-protein ligase TRIM21, phosphoacetylglucosamine mutase and interferon-induced GTP-binding protein Mx1	No
[74]	Proteomics (2DE and PMF)	Total protein extracts from SaOS-2 cell line and primary OB cell culture	OE: pyruvate kinase M1, L-lactate dehydrogenase B chain, triose phosphate isomerase 1, creatine kinase B chain, heat-shock protein 90, 150 kDa oxygen-regulated protein, retinoblastoma-binding protein 4, alkaline phosphatise. UE: galectin 1, annexin I, osteonectin, cathepsin D, tropomyosin 3, heat-shock protein 27, superoxide dismutase, glutamate dehydrogenase 1, UMP-CMP kinase, enoyl-CoA hydratase-like protein	
[75]	Proteomics (2DE and PMF)	Total protein extracts from SaOS-2 cell line and primary OB cell culture	OE: heat-shock protein 70, mthsp75, ATP synthase, **cytochrome *b-c*1 complex subunit 1**, Ras-related nuclear protein, actin capping protein, **ubiquitin carboxyl-terminal hydrolase isozyme L1, peroxiredoxin-4,** UE: Pyruvate dehydrogenase E1, Annexin V, Prohibitin,	Yes (WB)
[77]	Proteomics (SELDI-ToF/MS)	Plasma samples from OS and osteochondroma	Protein signature distinguishing OS and osteochondroma. Identification of serum amyloid A	Yes (WB)
[78]	Proteomics (2DE and PMF)	OS and benign bone tumor (chondroblastoma, osteoblastoma and giant cell tumor of bone) tissues	OE: vimentin, tubulin-α1c, lamin B2, coatomer protein complex, subunit epsilon, zinc finger protein 133, ferritin light polypeptide, myosin, light chain 6, ezrin, transferrin, α1-antitrypsin, chaperonin-containing TCP1. UE: adenylate cyclase 1, actin-β, tubulin-β, ATP synthase mitochondrial F1 complex β polypeptide, reticulocalbin 3 EF-hand calcium-binding domain, tyrosine 3-monooxygenase.	
[79]	Proteomics (2DE and nano-LC-ESI-MS/MS)	Chemonaive OS and primary OB cell cultures	OE: heat-shock protein β 6, heme-binding protein 1, ubiquitin carboxyl terminal hydrolase isozyme L1, **ezrin**, LIM and SH3 domain protein 1, thioredoxin reductase 1, peroxirredoxin 6, α **crystallin β chain**, septin 11 UE: 40S ribosomal protein SA, nucleophosmin, heat-shock cognate 71 kDa protein, α-enolase, fascin, pyruvate kinase isozymes M1/M2, actin cytoplasmic 2	Yes (RT-PCR, WB)
[80]	Proteomics (2D-DIGE and LC-LTQ ion trap MS/MS)	Chemonaive OS tissues before classification as good/poor responders	Differentially expression of 55 protein spots **(e.g. peroxiredoxin 2)**	Yes (WB)

UR: upregulated; DR: Downregulated; OE: Overexpressed; UR: Underexpressed. Genes and/or proteins in bold character are those submitted to validation.

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inter-α-trypsin-inhibitor heavy chain H4 precursor, previously reported as a biomarker in different types of cancers [14], and whose decreased presence in the high bone turnover group probably reflects an increased OC activity.

Discordant BMD values were also used to separate premenopausal and/or postmenopausal women into two groups representing different risks of future OP [15, 16]. As OC progenitors, circulating monocytes (CMCs) from individuals of these two groups were submitted to comparative 2-DE [15] or microarray analysis [16] to investigate the role of CMCs in OP pathogenesis and to potentially select novel early OP biomarkers. Among the 38 differentially expressed proteins identified, 5 (Ras suppressor protein 1, gelsolin, manganese-containing superoxide dismutase, glutathione peroxidase 1 and protein disulfide-isomerase) were validated by Western blotting. The up-regulation of Ras suppressor protein 1 and gelsolin, and the concomitant up-regulation of manganese-containing superoxide dismutase and the down-regulation of glutathione peroxidase 1 seen in the low BMD group have already been shown to have a role in OC differentiation and activity [17–20]; the role of protein disulfide-isomerase in OC physiology and OP progression is still under investigation. On the other hand, in the low BMD group, microarray analysis revealed the up-regulation of genes involved in migration (*CCR3*), histamine production (*HDC*) and sensitivity to GC (*GCR*).

GCs strongly affect bone cell physiology, mainly through the inhibition of both osteoblastogenesis and osteoclastogenesis, and prolonged steroid therapy is a well-established cause of secondary type OP [21]. Database interrogations produced only two papers on the topic of a functional genomic approach applied to secondary OP. Hurson et al. [22] investigated the global transcriptomic response in primary human OBs exposed to dexamethasone. The major finding of this study was the altered expression in developmental-dedicated genes in OBs, in particular the *DKK1*-mediated inhibition of the WNT signaling pathways. Yao et al. [23] used a mouse animal model to investigate the effects of GC excess (prednisolone) on bone gene expression. GCs were demonstrated to activate genes involved in OC maturation and function in a time-dependent manner, and the intensity of alteration correlated with GC-induced bone loss observed in clinical studies. These authors also revealed both an increased expression of transcription factors promoting adipocyte differentiation and markers for mature adipocyte (*AdipoQ*) and the *Dkk1*-mediated suppression of OB differentiation and maturation. This latter result confirmed previously reported findings for the human cellular model [22].

Post-genomic technology offers new perspectives for the characterization of transgenic animal models that have been developed to clarify the mechanisms of action together with the primary type of bone cells involved in GC-induced bone loss [24, 25]. Kim [24], using transgenic mice in which the gene for the GC receptor was conditionally disrupted in the OC lineage, revealed an inhibitory effect of GCs on bone resorption by disarranging the OC cytoskeleton in vitro. In addition, these transgenic mice were found to be protected from inhibition of bone formation caused by GCs, suggesting a major role of OCs in GC-induced OP. However, this latter finding was contradicted by Rauch et al. [25], who found that OB-specific transgenic mice were fully protected against GC-induced bone loss, while OC-specific transgenic mice, similar to the control animals, suffered from GC-induced bone loss. As yet there is no clear explanation of this discrepancy. The authors also demonstrated that GCs induced bone loss by inhibiting OB differentiation, as confirmed by the down-regulation of OB phenotypic markers (*Runx2*, *Akp1*, *Col1a1* and *Bglap2*), through interaction with GC receptors in their monomeric form. This mechanism of action is only partially superimposable on the mechanism responsible for their anti-inflammatory action, thus opening up new opportunities for the development of more selective GC receptor agonists [25].

Post-genomic attention has also focused on the exploration of the mechanism of action or the effects of anti-osteoporotic treatments, such as genistein [26], calcium implementation [27], biphosphonate [28], 17-β-estradiol (E2) and parathyroid hormone PTH(1–34) [29–33]. Among these, PTH(1–34) has been the most intensively studied, with particular attention to its anabolic or catabolic effects according to the administration regimen.

In a first paper, large-scale transcriptional profiling was performed on femoral metaphysis and diaphysis bone tissue from OVX mice treated with PTH(1–34) (an anabolic agent) and E2 (an anti-resorptive agent) alone or in combination [29]. To address the intracellular signaling pathways regulated by PTH(1–34) and E2, the authors performed a time course analysis coupled to a very stringent selection of the candidate genes. The presence of *Vdr*, *Tnfsf11* and *Ctsk* among the genes up-regulated by PTH(1–34), and the presence of *Bag3*, *Bmp1*, *Bmp8a*, *Ccnd1*, *Pth1r* and *Ctsl* among the genes up-regulated by E2, confirmed the pivotal role of these bone-related molecules in mediating the therapeutic response.

Onyia et al. [30] performed a microarray analysis of bone tissue from rats continuously or intermittently treated with PTH(1–34). Genes up-regulated following both regimens included mainly genes related to skeletal development, in particular matrix-associated and mineralization-related genes (*Col1αI*, *Col1α2*, *Bgalap*, *Sparc*, *Dnc*, *ColVα2*, *Timp1* and *Serpinh1*). Intermittent administration of PHT up-regulated the expression of binding, catalysis and immune response-related genes, whereas continuous PTH treatment induced the highest number of genes involved in bone turnover and OC-associated genes. Li et al. [31] conducted similar research and applied a knowledge-based approach to data analysis to guarantee an optimal exploitation of microarray data. The results presented in these two papers are only partially superimposable, mainly due to differences in the methodological (treatment duration, sampling time) and technical (different rat chips)

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approaches used. In addition, the contribution of the protein kinase C (PKC) and the cAMP-dependent protein kinase (PKA) pathways in regulating the anabolic effect of PTH in bone was evaluated using the two truncated forms of the parathyroid hormone, PHT(3–34) and PHT(1–31), which selectively and independently activate such pathways. A comparative gene expression analysis showed that a coordinated action of both signaling systems (PKC and PKA) is needed to induce the catabolic effect. Nevertheless, the activation of the PKA pathway is sufficient for the anabolic effect, suggesting that PTH(1–31) could serve as a better anabolic therapeutic agent than PTH(1–34).

Bone and systemic responses to PTH have also been investigated using both gel-based and gel-free proteomic approaches [32, 33]. Unfortunately, none of the studies utilized a cellular or an animal model of OP. 2-DE coupled to PMF succeeded in identifying an overexpression of calreticulin and protein disulfide-isomerase associated 6, and the underexpression of vimentin in PTH(1–84)-treated mouse bone marrow cells [32]. Furthermore, SELDI-TOF analysis, used to evaluate the systemic response to PTH treatment in mice, revealed a number of altered protein peaks, two of which were identified as hemoglobin-α and hemoglobin-β [33].

2.2 Paget's disease

PD is the second most common metabolic bone disease after OP. This pathology is characterized by focal areas of disorganized increases in bone turnover with abnormalities in all phases of the remodeling process. In these focal areas, an initial lytic phase due to intense OC activity is followed by a sclerotic phase characterized by OB over activity and increased deposition of bone matrix and mineral, suggesting a pivotal role of pagetic OCs in PD progression. Although various hypotheses on the development of PD including both genetic and viral factors have been postulated, the exact molecular mechanisms of PD pathogenesis are largely unknown [34].

Post-genomic approaches for the study of this pathology are clearly unexploited. To the best of our knowledge, only three papers have taken into account the gene expression profile in PD, while no studies have been performed aimed at exploring other ''omics'' repertoires for this pathology. Naot et al. [35], driven by the focal nature of the condition, investigated the gene expression of OBs and BMSCs to address differences in mRNA cellular profiles in both pagetic and non-pagetic bone, the latter sourced from both pagetic and normal individuals. Unexpected results were first reported for the *RANKL/OPG* ratio, which was consistent with a decreased bone turnover, suggesting alternative pathways/mechanisms for OC over activation in the onset of the pathology. Expression profile analyses revealed that the overexpression of *IL-6*, *IL-1B*, *ALPL*, the underexpression of *IBSP* and *BGLAP*, and the *DKK-1*-mediated inhibition of the Wnt signal pathway in a limited group of OBs could be responsible for the altered number, morphology and functionality of OCs leading to pagetic lesion progression.

Nagy et al. [36] analyzed the global expression changes in 15 selected genes, relevant to bone metabolism and osteoclastogenesis, in peripheral blood cells as OC progenitors. Significant alterations were found in the gene expression profile of the interferon pathway members (up-regulated genes: *IFNA*, *IFNB1*, *IFNG*, *p38* β *2 MAPK*, *IFNGR1* and *IFNGR2*, *STAT1*; down-regulated gene: *TNFA*). Even though the number of analyzed genes is limited, the central role of the interferon pathway in controlling many aspects of cellular physiology makes this study an interesting starting point for further analyses aimed at elucidating the pathomechanisms of altered bone turnover in PD.

A data set comprising 48 selected OC-related genes was used by Michou et al. [37] to monitor changes in gene expression in cultured OCs from PD patients all carrying the p62-P392L substitution, the SQSTM1 mutation most frequently associated with PD [38]. The authors revealed significant down-regulation of MAPT and of some genes involved in apoptosis (*CASP3*, *TNFRSF10-A*), cell signaling (*TNFRSF11A*), bone resorption (*ACP5*, *CTSK*), only in a mutation-independent manner. They ascribed the failure in detecting p62-P392L mutation-induced alterations of gene expression to the low number of analyzed samples as well as to the restricted data set used.

2.3 Osteonecrosis

Osteonecrosis of the jaw (ONJ) is a painful condition with exposed bone in the oral cavity. The development of ONJ has been associated with long-term therapy with a high dose of biphosphonates in patients with multiple myeloma, and as a complication of radiotherapy in patients with head and neck cancer [39].

To investigate the role of prolonged biphosphonate treatments in the etiopathogenesis of ONJ, a comprehensive study was performed in multiple myeloma patients taking into account different clinical and biochemical parameters [40]. Comparative molecular profiling of expressed genes in peripheral blood mononucleated cells from multiple myeloma patients on biphosphonate therapy with or without ONJ and healthy controls showed a global inhibition of osteoblastogenesis. In particular, the significant down-regulation of genes involved in signaling, activation or differentiation of both OCs (*NFAT2*, *NFAT3*, *p49/p100*, *FUBP1*, *MIF*, *CCL5/RANTES*) and OBs (*NF-Y*, *BMAL1*, *E2F*, *PPARG*, *PRKCH*) as well as the up-regulation of *IL-18*, an OC-inhibiting factor, were detected. To the best of our knowledge, this is the only paper describing the changes occurring at the transcriptome level in biphosphonate-treated ONJ patients, despite some minor concerns about the cellular model used (bone cell progenitors instead of fully

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differentiated OCs or OBs) and the concomitance of a bone-affecting malignancy (multiple myeloma).

Osteonecrosis of the femoral head (ONFH) is a very common disease resulting from a temporary or permanent ischemia of the femoral head. The exact etiology of ONFH is still unknown, although excessive GC administration, alcohol abuse, thrombotic disorders, collagen II gene mutations and endothelial nitric oxide gene polymorphism have been found to strongly contribute to the onset of the pathology [41].

Recently, two studies were performed using serum proteomics to identify protein biomarkers correlated to the development of this pathology [41, 42]. Tan et al. [42], using comparative 2-DE analysis of serum proteins from ten ONFH patients and healthy controls, detected seven differentially expressed proteins (three up-regulated and four down-regulated), which were also validated in 46 sera from patients affected by ONFH, osteoarthritis (OA), rheumatoid arthritis (RA) and fractures. The use of sera from other bone-related diseases allowed the selection of four biomarkers (plasminogen activator inhibitor type 1, crossLaps, anti-p-3 antibody and tissue-type plasminogen activator) that were highly discriminating for ONFH. Based on the results, the authors hypothesized that an imbalance of the coagulation process, the action of proto-oncogenes and anti-oncogenes involved in cell growth and apoptosis, and the loss of OBs activity may contribute to the development of ONFH. In addition, the up-regulation of p-53 was confirmed by a bone transcriptional profiling study in an ON animal model [43].

In a second study [41], the same approach revealed that kininogen variant 1, complement factor C3 precursor and complement factor H were significantly more abundant in ONFH sera, while apolipoprotein A-IV precursor, antithrombin III chain B and gelsolin isoform α precursor were more abundant in healthy sera.

The discrepancies in the findings in these two studies can be ascribed to differences in the technical approaches and patient enrolment. In the first study, albumin-depleted sera were used to obtain a better detection of low concentration proteins. There was an age difference between the cohorts of the two studies, with older patients being enrolled in the second study (16.1 ± 3.1 versus 41.5 ± 3.3 years). This may indicate possible age-dependent mechanisms in the development of ONFH.

2.4 Osteosarcoma

Osteosarcoma (OS) is the most commonly diagnosed primary malignant tumor of bone and the third most common cancer in childhood and adolescence. It is characterized by the production of osteoid or immature bone by tumor cells. OS mainly affects the long bone and is usually aggressive with a high tendency to metastasize, mainly to the lung. Histologically, OS is subdivided into osteoblastic, chondroblastic and fibroblastic variants according to the predominant extracellular matrix [44].

Over the last 20 years, the introduction of combination treatments (neoadjuvant chemotherapy, surgery and adjuvant chemotherapy) has raised the 5-year survival, for non-metastatic patients, to 70% [45]. However, patients who exhibit metastasic disease at the time of diagnosis or poor response to chemotherapy are at higher risk of relapse and worse prognosis. Therefore, it is crucial to identify new molecular biomarkers for early diagnosis, prognosis and chemotherapy responsiveness, as well as to select new molecular targets as a basis for the development of more effective therapeutics. For these reasons, great effort has been made to elucidate the molecular mechanisms underlining the development of OS. Post-genomic approaches have been extensively applied to OS research, in particular gene expression profiling. Transcriptomic studies performed on human OS have been successfully applied to investigating tumor phenotype and biology, as well to identifying predictive biomarkers of OS metastatic potential or chemoresponsiveness.

For instance, broad transcriptomic profile approaches were applied to the OS cell lines SaOS-2 and U2OS [46, 47] to explore the basal mRNA content in these two widely used cellular models [46]. These studies investigated the influence of RANKL [47], and how genetic differences could reflect on the phenotypic behavior through changes at the transcriptomic level [46]. In the study by Patiño-García et al. [48], the author compared the expression profile of seven chemonaive OS tissue-derived cells with paired normal OBs, and the transcriptional factor *EBF2* was found to be up-regulated in tumor samples. *EBF2* was further validated by immunohistochemistry in 46 OS samples (70% of positivity). mRNA fingerprints were also generated to search for a transcriptional signature able to discriminate the likelihood of a poor or good response to chemotherapy [49–53]. The main concern arising from these latter studies concerns the sampling time. Four studies chose to compare a priori the global mRNA repertoire from chemonaive OS biopsies, and then correlated the results using the Huvos grading system [50–53]. In another study biomarker candidates were selected from samples from definitive surgery and their reliability was validated retrospectively on chemonaive biopsy specimens to enhance the sensitivity and sensibility of the detection system [49].

Bruheim et al. [54] investigated alterations in the global expression profiles in ten murine xenograft models of human OS, and classified them as good or poor responders to deoxirubicin, cisplatinum and ifosfamide. The analysis of the differentially expressed genes allowed a common signature for deoxirubicin and cisplatinum treatments (21 overlapping genes) to be established, whereas treatment with ifosfamide was found to share only two genes with cisplatinum and none with deoxirubicin treatments. The identification of responsiveness-prediction biomarkers specific for each of the elements of the

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neoadjuvant chemotherapy cocktail could have remarkable consequences in the clinical management of OS by developing individually adjustable pharmacological intervention protocols.

The metastatic potential of OS was also evaluated in a variety of biological samples. OS cell lines and sublines of different metastatic potential were extensively used as cellular models of metastatic human OS in transcriptional comparative analyses [55–58]. However, as demonstrated by Lisle et al. [55], data from in vitro experiments need to overcome a strong in vivo validation step prior to any possible application in clinical practice.

Kobayashi et al. [59] investigated clinical OS samples using a comparative gene expression analysis. These authors investigated the alterations in global mRNA profiles of chemonaive OS biopsies from patients who had or had not developed pulmonary metastasis after a 4-year follow-up. Among genes displaying differential expression levels between the groups, the gene encoding argininosuccinate synthetase had the highest statistical significance and was submitted to a further validation phase. Interestingly, this latter phase was performed at the protein level by immunohistochemistry, thus improving the robustness and potential translational application of this candidate biomarker. Moreover, since argininosuccinate synthetase was found to be down-regulated in the non-metastatic group, the authors also suggested an innovative therapeutic strategy for patients with OS having higher risk of metastasis based on systemic arginine deprivation in combination with neoadjuvant chemotherapy.

OS is characterized by a high level of genomic instability, highly heterogeneous karyotypes both intra- and inter-tumor, and significantly altered gene expression. Although both genetic alterations and gene expression changes have been extensively researched in OS [60], genetic and epigenetic factors, in particular disorders in DNA methylation, and their interplay in the development of OS are still largely unexplored. Recently, the research group of Zielenska and Squire set up an integrative approach for analyzing genetic (DNA copy number) and epigenetic (promoter methylome) factors and their cumulative role in gene expression alterations in both OS cell lines and tissue samples [61, 62]. Al-Romain and colleagues [63] carried out a comprehensive study to investigate the action of decitabine, a DNA methylation inhibitor, in the U2OS cell line. Decitabine showed an anti-proliferative activity both in vitro and in vivo, resulting from a decrease in methylation of the promoter region and the consequent up-regulation of pro-apoptotic genes such as *GAD45A*, *HSPA9B*, *PAWR* and *PDCD5*.

Within the bone cancer field, proteomic studies addressing the analyses of human OS are far less abundant compared to other ''omics'' studies. However, the proteome level of different types of human biological samples, such as cell lines, primary cell cultures, and OS bone tissue or serum, have been characterized. Proteomics have been applied to OS cell lines in several approaches: to produce well-characterized 2–DE reference maps (U2-OS [64] and SaOS-2 [65]); to investigate the cellular response to deficiencies in the oxidative phosphorylation systems (through the inhibition of the complexes I and IV or the lack of mithocondrial DNA) [66]; to investigate the role of the transcription factor E2F1 [67]; and to evaluate the action of prenyl phenol compounds such as ascofuranone [68], ascochlorin [69] or the garlic derivate diallyl trisulfide [70] as new anti-cancer therapeutic agents for OS. In addition, some proteomic research has focused on elucidating the molecular mechanisms involved in OS development. As an example, plasma membrane proteins from OS (MG63) and human osteoblastic cell lines (hFOB1.19) were submitted to comparative proteomics using iTRAQ-based LC-MS/MS analysis [71]. Among the identified up-regulated proteins, the authors selected and validated CD-151, an important regulator of communication between tumor and endothelial cells. This had been already evaluated as a diagnostic or prognostic marker for other types of cancer [72–75].

Comparative 2-DE was applied to total protein extracts of one osteoblastic (hFOB1.19) and three OS (U2OS, SaOS-2 and IOR/OS9) cell lines to identify potential OS biomarkers [76]. The activator of the 90-kDa heat shock protein ATPase homolog 1 (AHA1) and stomatin-like protein 2 were found to be commonly up-regulated in OS, while glutathione S-transferase omega-1, E3 ubiquitin-protein ligase TRIM21, phosphoacetylglucosamine mutase and interferon-induced GTP-binding protein Mx1 were found to be commonly down-regulated.

Comparative 2-DE analysis was also performed using primary human OB cell cultures [77, 78] instead of SV-40 immortalized osteoblastic cell lines [76]. In the studies by Spreafico [77] and Liu [78], the authors followed the same protocol for bone-derived primary cells culturing, protein extract preparation and 2-DE. Both studies reported a list of proteins whose expression was found altered in SaOS-2 with respect to normal human OBs. However, since an anatomical site-related transcriptional signature has been demonstrated in bone [79], a comparison of the results is not revealing, as the primary osteoblastic cells came from different anatomical sources: the hip bone of a 35-year-old patient and cranial bone of an abortus.

Proteomic investigations have also been conducted on samples derived from OS patients. Serum amyloid A was identified as a member of the plasma proteomic signature able to discriminate OS from benign osteochondroma [80].

Li et al. [81] applied 2-DE to samples of bone tissues from OS and benign bone tumor (chondroblastoma, osteoblastoma and giant cell tumor of bone) patients to identify specific markers of OS. Twelve proteins were found to be up-regulated and six proteins down-regulated. Among these, the identification of structural proteins like vimentin, tubulin-α1c and tubulin-β suggests an important role for the cytoskeleton and microtubules structures in the development and progression of OS and provides a possible explanation for the specific biological behavior.

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Folio et al. [82] investigated specific alterations in cancer-related proteins in chemonaive OS patients. Comparative analysis of the proteomic maps of normal and paired tumor-derived OBs showed the up-regulation of ezrin and crystalline α/β in OS transformed cells.

Kikuta et al. [83], using 2-D-DIGE, identified by a proteomic signature for chemotherapy responsiveness in OS tissues. Moreover, these authors validated peroxiredoxin 2 as a useful biomarker for distinguishing a priori good from poor responders.

3 Concluding remarks

Altogether, post-genomic technologies provide a holistic approach and present researchers both challenges and opportunities. Although the identification of endpoints is only the first critical analytical step, the understanding gained of the global responses of biological systems is high, given the broad impressions on systems' responses that are obtained. Nevertheless, there is a crucial need to understand both the biological and the methodological contexts [84]. The technology is not fool proof, as recently highlighted for microarray technology [85]. Post-genomics often provide researchers with a daunting set of data to be analyzed; for instance, the lack of robust quality control procedures and adequate data analysis have caused problems with array-based transcriptomic investigations [85]. Undoubtedly, the integration of -omic technologies will also require a careful validation of results through detailed functional studies.

While drafting this review, we became aware that proteomic approaches to bone research were less extensively used than gene- or mRNA-based approaches. Consequently, a lot of potential applications of proteomic techniques remain unexploited. Besides global protein expression profiling, proteomics represents the elective tool for identifying changes in a wide variety of dynamic cellular processes, such as protein–protein interactions, critical PTMs, specific cellular and subcellular localizations, that ultimately affect and determine cell functioning.

In the field of sub-proteomics, secretome investigations are gaining importance as the systematic analysis of extracellular matrix components and proteins released from a given type of cell. Secreted proteins account for approximately 10–15% of the proteins encoded by the human genome, and are involved in numerous biological processes. Notably, the analysis of alterations of the secretomic profiles as a consequence of pathological status unambiguously represents a so-far poorly explored source of biomarkers and potential therapeutic targets [86]. For bone research, the complex nature of the extracellular matrix (both in its chemical composition and functionality) at the same time represents both an opportunity and a major challenge for secretome analyses. Nevertheless, as recently reviewed [87], interesting potential applications of imaging mass spectrometry (TOF-SIMS/MS) for the simultaneous structural analysis of both the mineral and organic portion of bone tissue have been described.

Another under-investigated sub-proteomic repertoire is that represented by PTMs of proteins, including phosphorylation, glycosylation and reversible/irreversible oxidation. Relying on both gel-based and gel-free approaches, several techniques are available and offer tremendous opportunities for analyzing PTMs, helping in the elucidation of pathophysiological mechanisms in diseased or stressed cells. In this light, we recently undertook the proteomic and redox-proteomic characterization of several AKU cells (unpublished data). AKU is a rare, autosomal recessive disorder of tyrosine and phenylalanine metabolism due to a deficient 1,2-homogentisate dioxygenase activity. As a result, the intermediate metabolite homogentisic acid (HGA) is accumulated and probably oxidized to a quinone metabolite (1,4-benzoquinone-2-acetic acid, BQA) with concomitant production of ROS and induction of oxidative stress. Late symptoms of AKU, appearing around the fourth decade of life, include the deposition of melanin-like pigments in connective tissues (ochronosis) and involve a progressively debilitating form of arthritis of the spine and large joints. Hitherto, the mechanisms leading to ochronotic arthropathy have not been investigated to any significant degree, and the set-up and characterization of AKU models have been addressed only recently [88–90].

To investigate the molecular basis of AKU and ochronotic arthropathy, we analyzed the proteome profiles of AKU OBs and found that levels of specific proteins were altered in AKU, such as

(i) proteins involved in cell organization, indicating an impaired production of bone matrix and alterations of bone structure;
(ii) molecular chaperones, indicating defects in protein folding/refolding;
(iii) proteins involved in protection from oxidative stress, indicating a lowered ability to face the HGA-mediated oxidative stress.

These findings were also corroborated by the redox-proteomic analysis of carbonylated proteins in alkaptonuric OBs. The study of protein carbonylation, but more generally protein oxidation, in bone-related cells has so far not received much attention. For instance, the first redox-proteomic analysis providing the identification of carbonylated proteins in a chondrocytic cell line appeared only recently [81]. Nevertheless, the analysis of protein carbonylation proved to be a powerful tool in discriminating AKU cells from their control, pointing out an increased protein oxidation and the existence of a very specific "carbonylation" signature in AKU, which was directed against structural proteins and proteins involved in the oxidative stress response (unpublished data).

Finally, proteomic and sub-proteomic analyses might also provide fundamental insights into novel therapeutic targets and/or predictive and prognostic biomarkers [91].

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Also in this case, the understanding of protein PTMs might help the identification of new therapeutics, as recently demonstrated for OS for which selective inhibitors of Src phosphorylation proved to posses anti-proliferative and pro-apoptotic activities both in vitro and in vivo [92, 93].

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