Murine Animal Models in Osteogenesis Imperfecta

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Osteogenesis imperfecta (OI), also known as “brittle bone disease”, is a rare genetic disorder that encompasses a group of conditions affecting the connective tissue. It is characterized by a decreased bone-mineral-density (BMD) alongside increased susceptibility to bone fractures, due to an abnormality in the synthesis and/or processing of the main protein of the bone extracellular matrix (ECM), the type I collagen molecule. In approximately 85% of cases, it is caused by mutations in the *COL1A1* or *COL1A2* genes, encoding the α1 (I) and α2 (I) chains of type I collagen, respectively. In the remainder of the cases, mutations in up to 19 different genes related to type I collagen synthesis or processing have been identified. All these mutations contribute to two types of collagen I defects; quantitative (based on the reduction of type I collagen expression) and qualitative (structural alterations of the collagen I molecule). In addition to the genetic heterogeneity, OI exhibits clinical heterogeneity, mainly governed by the mutated gene, the type of mutation, the position of the mutation along the gene and the genetic background of the patient. Hence, genetic heterogeneity is translated into clinical phenotypes that range from mild (barely affected) associated with quantitative defects, to severe forms (qualitative ones), that in some cases (in the most severe phenotypes) result in perinatal mortality.

osteogenesis imperfecta | murine models | new therapies | preclinical studies | bone

1. Osteogenesis Imperfecta Murine Models Evaluated in Preclinical Research

In the quest for effective and safe therapeutic strategies for osteogenesis imperfecta (OI), there are a variety of mouse models available (although still insufficient), as expected and required for a disease as heterogeneous as OI. These murine models, carrying mutations in genes that encode collagen type I molecule, or molecules involved in collagen I processing, aim to emulate the genetic and phenotypic variability of OI patients. OI murine models encompass a wide range of severities depending on the mutation they carry, and although more than 20 different models for OI have been reported [1], herein, only the most commonly used OI murine models in preclinical research are recapitulated.

1.1. Osteogenesis Imperfecta Mice (oim)

The most commonly used OI murine model in preclinical studies is undoubtedly the osteogenesis imperfecta mice (oim). It was first described in 1993 by the Jackson Laboratory, when C3H/HeJ (a mice strain quite resistant to endotoxin) and C57BL/6JLe mice were bred, and a spontaneous mutation was generated [2]. Oim mice present a

deficiency of the pro-α2 chain of the type I collagen molecule, due to a guanine (G) deletion at nucleotide 3983 of the *Col1a2* gene, which alters the approximately 50 terminal amino acids of the pro α2 C-propeptide. This mutation prevents association with the pro-α1 chain, resulting in tissue accumulation of aberrant αl homotrimeric collagen in the extracellular matrix (ECM). However, it has recently been reported that mice lacking the α2 chain of the type I collagen molecule, do not present impaired bone biomechanical or structural properties, unlike oim homozygous mice [3]. This observation emphasizes the oim mutant allele, and consequently its protein product, as the cause of the bone fragility seen in oim mice and not, as previously thought, the absence of the type I collagen α2 chain.

The mutation that causes oim mice is very similar to the first identified mutation causing type III OI in humans, in which a 4 nucleotide deletion (c.4001_4004del) causes a frameshift (p.(Asn1334Serfs*34)) that alters the last 33 amino acids of the α2 chain of type I procollagen [4]. Moreover, novel mutations were identified in two additional patients with mild OI, causing a 48-amino-acid truncation of the α2 chain and a substitution of a cysteine residue important for interchain disulphide bonding, respectively [5]. In these cases, the mutations reported in humans also resulted in homotrimeric type I collagen synthesis, as the defective α2 (I) chain seemed to be unable to be incorporated into trimers [6]. Ultimately, homozygous oim mice resemble a severe, nonlethal, recessive form of human OI type III, presenting severe osteopenia, spontaneous fractures, small body size, and progressive skeletal deformities, along with very poor mechanical properties compared with their wild-type counterparts. These are features for which it is referenced as the only nonlethal murine model resembling the OI type III human disease.

Recent studies have focused on the understanding of different features of OI patients, extrapolating the results obtained with oim mice. As expected, the OI bone transcriptome of calvarial bone [7], together with the transcriptome of osteocytes isolated from the cortical femurs and tibias [8] of oim mice, was altered. I this process, genes involved in the Wingless-related-integration-site (Wnt) signaling and the transforming-growth-factor-beta (TGF-β) signaling were upregulated, compared to healthy counterpart animals. In terms of bone mineralization, oim bones are hypermineralized, which translates into a reduction in tissue elastic-modulus (the biomechanical parameter that measures stiffness). [9]. Recently, Zeng Z. and collaborators identified the deficient intrafibrillar mineralization in oim mice as a key contributor to OI-induced bone brittleness [10]. All in all, despite the fact that oim mice were spontaneously generated, and the mutation is not common in humans, they have proven to be undeniably useful, not only for studying collagen-matrix biology but for different preclinical studies in the context of type III OI.

1.2. Heterozygotic G610C Mice (Amish Mice)

In 2010, Daley E. and colleagues reported a novel translational model for moderate OI, the G610C OI knock-in mouse. This mouse presents a substitution of the gly-610 codon for cysteine, in the protein encoded by the *Col1a2* gene, causing a final structure alteration of the type I collagen molecule. It is important to emphasize that the glycine substitution is the most common cause of dominant OI. In heterozygosis, it is also known as the Amish mouse, as it modeled a large human Amish population of 64 individuals exhibiting OI type I/IV, and is, together with oim, one of the most popular mice in OI-research labs [11]. Phenotypic characteristics of the mutant mice include decreased body weight, BMD, and bone volume, in addition to a more highly mineralized cortical and trabecular

tissue, leading to decreased mechanical bone properties [12]. Cellular parameters have revealed that abnormally folded procollagen (due to glycine substitution) causes endoplasmic reticulum (ER) stress and osteoblast malfunction in Amish mice [13]. Indeed, misfolded procollagen in Amish osteoblasts indirectly triggers the integrated stress response (ISR) that is regulated, herein, by the mitochondrial heat shock protein 70 and activating transcription factor 5 [14]. These outcomes suggest that mitochondria might initiate the ISR upon disruption of ER-mitochondria connections, or might respond to the ISR activated by a sensor as yet unknown. Amish mice also exhibit an impairment in the early phase of bone repair compared to wild-type littermates, due to an increased amount of type II collagen (typical of cartilage) to the detriment of type I (characteristic of bone) [15].

1.3. Brittle Mice (Brtl)

The Brittle IV (Brtl) mouse was developed as a knock-in model for OI type IV, by introducing a Gly349Cys substitution into one *Col1a1* allele, the glycine substitution again being the most common cause of dominant OI. Puzzlingly, in spite of having the same mutation, the mice showed phenotypic variability encompassing two different severities: moderately severe (resembling human OI Type IV) or perinatal lethal (resembling human OI Type II) [16]. Phenotypic variability is not associated with differences in expression levels of the mutant allele in the total ribonucleic acid (RNA) nor with the expression of the mutant protein. However, the different ability to adapt to cellular stress due to mutant collagen retention was found to be responsible for the heterogeneity of the Brtl mice phenotype. Thus, lethal mice showed a downregulation in the expression of cellular chaperones, as well as cytoskeletal disorganization in contrast with the moderately severe ones, who showed chaperone up-regulation and normal cytoskeleton [17][18]. Moderately severe Brtl mice were smaller in size and showed reduced BMD, reduced cortical thickness, reduced cross-sectional area and reduced biomechanical properties until 6 months of age [19].Two-month old Brtl mice showed higher skeletal fragility compared with wild type (WT) littermates, since the Brtl mutation alters the collagen orientation [20], affecting the mechanical behavior of bone material [21]. However, when Brtl mice reach puberty, they show an improvement in bone strength and stiffness, due to an increase in the material properties of the ECM [22]. All in all, Brtl mice reproduce the variability described in some OI patients, becoming an excellent model to study the factors that affect human OI phenotypes. Herein, autophagy upregulation has also been demonstrated in mutant mesenchymal-stem-cells (MSCs) differentiated toward osteogenic lineage, as a consequence of ER stress due to anomalous mutant-collagen retention [23].

1.4. Jrt Heterozygous Mice

Chen F. and collaborators identified a mouse manifesting dual features of type IV OI and Ehlers–Danlos syndrome (EDS), named Col1a1$^{Jrt/+}$ (Jrt) mice [24]. EDS is a group of disorders that affect connective tissues supporting the skin, bones, blood vessels, and many other organs and tissues. Mutations in at least 20 genes (*COL5A1*, *COL5A2*, *COL1A1*, *TNXB*, *ADAMTS2*, *PLOD1* or *FKBP14*, among others) are associated with EDS. The combined phenotype presented relays on a mutation in a splice donor site of the *Col1a1* gene, which lead to the skipping of exon 9 and a consequent 18 amino-acid deletion within the amino (N)-terminal region of the triple helical domain of *Col1a1*. Jrt mice are smaller [25], have lower BMD, and worse trabecular microarchitecture, and therefore exhibit

mechanically weak, brittle, fracture-prone bones, the hallmarks of OI. Additionally, they exhibit some characteristics of EDS, such as reduced tensile-properties of skin, a rayed tail tendon, and curvature of the spine.

1.5. Heterozygous Abnormal Gait 2 (Aga2) Mice

The murine model termed Aga2 (abnormal gait 2) described in 2008 [26] is the consequence of a carboxyl (C)-terminal frameshift mutation in the *col1a1* gene. Although the mutation affects *col1a1*, and up to the present date an equivalent genetic mutation has not been identified in humans, it is considered an appropriate model to emulate OI types II and III. Heterozygous Aga2 mice exhibit reduced bone mass, multiple fractures, and early lethality (due to cardiorespiratory defects) [27]. Moreover, they show increased bone turnover and a disrupted native collagen network, in which abnormal pro-α1 chains accumulated intracellularly in dermal fibroblasts are poorly secreted extracellularly. Thus, unfolded protein accumulation leads to an induction of an ER stress-specific unfolded protein response.

1.6. Heterozygous Col1a1$^{\pm 365}$ OI Mouse

In order to emulate the OI type I phenotype, a heterozygous col1a1$^{\pm 365}$ OI mouse was newly generated by partial exons knockout (exon 2-exon 5; 365 nucleotides of mRNA) [28]. This deletion causes a frameshift mutation and premature chain termination, causing generally a large decrease in type I collagen synthesis. The OI murine model possesses significant femoral collagen reduction accompanied with sparse mineral scaffolds, bone loss, lowered mechanical strength and a broken bone-metabolism, pointing to a sustained skeletal weakness. The yes-associated protein (YAP), one of the key coactivators in the Hippo signaling pathway, known to play a pivotal role in osteoclasts and osteoblasts balancing in postnatal bone remodeling [29]) is altered in these mice. Hence, it is suggested that YAP down-regulation in both the femur and adipose-derived MSCs under osteogenic differentiation of col1a1$^{\pm 365}$ mice, may contribute to the reduced osteogenic potential and brittle bones.

1.7. Crtap Mouse

Besides mutations in genes that encode type I collagen molecule (*COL1A1* and *COL1A2*), OI is also caused, to a lesser extent, by alterations in genes that encode proteins involved in type I collagen processing, among others. This is the case of the cartilage associated protein (CRTAP), which is a member of the prolyl 3-hydroxylation complex, crucial for post-translational modifications and functionalization of collagen molecules [30]. In humans, *CRTAP* mutations are associated with the clinical spectrum of recessive OI, ranging from neonatal lethal cases (OI type II) to a milder phenotype (OI type VII), depending upon the nature of mutation. Using a homologous recombination approach, a strain of homozygous Crtap-deficient (Crtap) mice has been produced, which seems to properly model OI VII in humans [31].

The loss of *Crtap* in mice causes an osteochondrodysplasia (inherited abnormalities of growth and development of connective tissue, bone, and/or cartilage), with rhizomelia (discrepancy of the length of the proximal limb), kyphosis (curvature of the spine, which causes the top of the back to appear more rounded than normal), as well as severe osteoporosis, with defective osteoid formation. Due to loss of *Crtap*, mutant collagen shows evidence of

overmodification, presenting the collagen fibrils in mutant skin with increased diameter, consistent with altered fibrillogenesis. Crtap animals and OI type VII patients show an abnormally high mineral content and increased mineral densities in their bones [32]. The transcriptome of Crtap osteocytes have identified several dysregulated pathways already presented by other OI murine models, such as development and differentiation, ECM and collagen fibril organization, as well as Wnt and TGF-β signaling [8].

1.8. IFITM5 Transgenic Mice

The interferon induced transmembrane protein family 5 (Ifitm5), also known as the bone-restricted Ifitm-like protein, is known to regulate germ cell specification, its expression being prominent in osteoblasts. Humans with OI due to IFITM5 mutations mainly present a unique heterozygous replacement (c.-14C>T) of the 5′-untranslated region, which results in autosomal dominant OI type V. Accordingly, a transgenic mouse model with IFITM5 c.-14C>T mutation (IFITM5) exhibits severe skeletal malformation, as well as perinatal death [33][34]. Additionally, the limbs of the embryo exhibit a consistent delay in mineralization. Indeed, osteoblasts derived from embryo calvaria show decreased mineralization and reduced expression of osteoblast differentiation markers.

To sum up, taking into account the wide phenotypic and genotypic variability that patients with OI present, the OI mice models available to use in preclinical studies are limited. Although they present a phenotype that closely simulates bone fragility, the mutation is not strictly the same in most cases, which could be a serious handicap when developing certain therapeutic strategies, depending on their mechanisms of actions. Nevertheless, in the last five years new murine models for OI have been established, which present different mutations from previously used models, in an effort to expand the genetic background of OI models and thus increasing the possibilities in the search for new therapies for OI: the novel seal mouse, presenting a decrease in Col1a1 mRNA expression and consequently type I collagen reduction, which herein mimicks human type III OI [35]; homozygous lysyl hydroxylase 2 (LH2) mutant mice [36], resulting in embryos unable to develop normally that die at an early embryonic stage, due to cardiac problems; LH2 heterozygous mice, showing significant alterations in collagen crosslinking [37], as has been described in OI type XI patients [38]; the Swaying mouse model [39], presenting a spontaneous loss of function mutation in *Wnt1* and modeling OI type XV in humans [40]; the viable homozygous $Wnt1^{G177C/G177C}$ mice [41]; the Ifitm5 S42L knock-in mouse model, resembling the atypical OI type VI [42]; and the Tent5a KO mouse, displaying bone fragility and a skeletal hypomineralization phenotype, as a result of quantitative and qualitative collagen defects [43]. Moreover, new OI models are being developed in different OI research groups, such as Brtl Ser (which presents the mutation G349S in the *col1a1* gene), high bone-mass mice (HBM; a mutation in the *col1a1* gene; type I procollagen c-propeptide cleavage defect), $Tmem38b^{-/-}$ and Mbtps2. Finally, it remains to be demonstrated whether these new models are relevant to the search for new therapeutic strategies for OI.

2. Revealing Parameters in Preclinical In Vivo Studies

The main bone parameters that are considered when assessing potential treatments in OI murine models, and the different techniques that could facilitate the evaluation of the effects of these therapies, are discussed in the following lines (**Figure 1**). Histological and histomorphometric evaluation of bone are among the most popular and

long-established determinations in the field. Later on, the advancement of computational techniques promoted the development of image processing software to automate bone histomorphometry analysis [44][45]. These techniques provide quantitative information about remodeling and bone structure using various staining and immunohistochemistry methods, allowing the identification of several markers such as osteoclast activity (tartrate-resistant acid phosphatase (TRAP) staining), alkaline phosphatase (ALP) activity, receptor activator of nuclear factor kappa-beta ligand (RANKL) or osteoprotegerin expression, among others [19][46]. In addition to histology and histomorphometry, the development of new technologies has allowed the implementation of wider range of techniques, most of them coming from the field of materials physics, which can offer additional molecular and structural information about organic and mineral bone tissue.

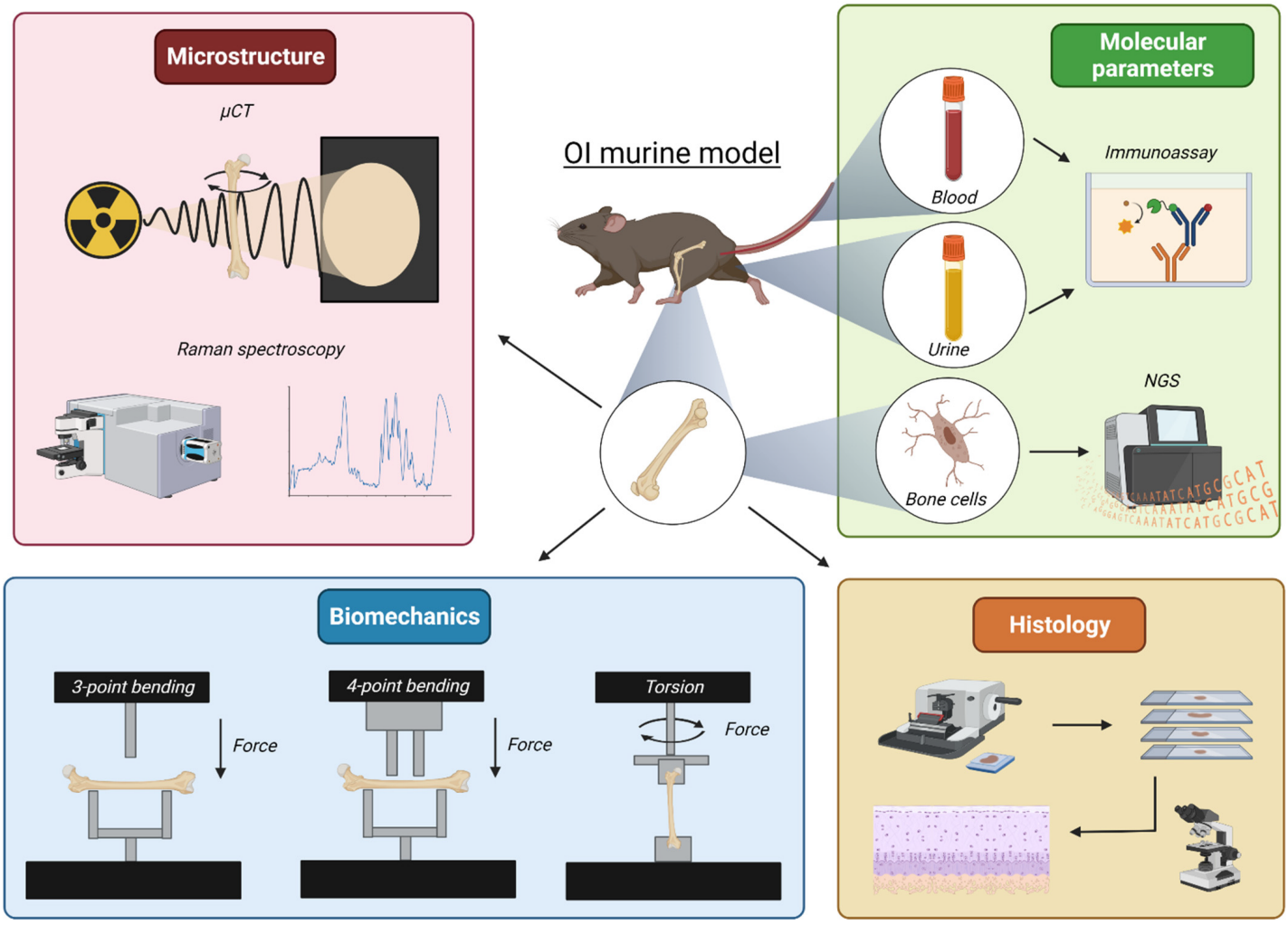

Figure 1. Bone properties evaluation. Different parameters are used to evaluate bone properties; histological and histomorphometric evaluation of bone; measurement of bone microstructure using image techniques (µCT and Raman spectroscopy), evaluation of biomechanics (3-/4-point bending test or torsion)) and analysis of molecular parameters (Immunoassay or NGS).

2.1. Bone Microarchitecture

Bone tissue is composed of hierarchically organized materials that ultimately confer its mechanical properties and strength. Thus, bone fragility being the main feature of OI, it is expected that one of the most studied parameters is going to bone microarchitecture. This requires the use of spatial-resolution methods at different length scales to fully understand the underlying mechanisms that lead to altered physiological and functional properties of the tissue. In fact, several imaging techniques such as Raman imaging or mainly microcomputed tomography (µCT), have proven their value in the study of the bone structure for different OI mouse models.

2.1.1. Microcomputed Tomography

µCT, a powerful non-destructive imaging methodology based on X-ray radiation, produces high-resolution three-dimensional (3D) images composed of several two-dimensional (2D) projections of the studied object. Samples can be imaged with pixel sizes as small as 1 µm. Over the years, this technique has gain popularity, being the gold standard in studies requiring morphological and quantitative bone analysis, such as the assessment of animal models of disease [47][48][49]. In addition to 3D reconstructions, µCT allows the calculation of several trabecular and cortical parameters that provide information on the quality of the bone [50]. Trabecular bone parameters include bone volume-fraction (BV/TV), the number, thickness, and spacing of trabeculae (Tb.N, Tb.Th and Tb.Sp, respectively), BMD and connectivity density (Cn. Den). The cortical parameters include cortical thickness (Ct.Th), cortical bone area (Ct.Ar), tissue mineral density (TMD), cortical fraction area (Ct.Ar/Tt.Ar), cortical porosity (Ct. Por) and bone area per total area (BA/TA).

2.1.2. Raman Spectroscopy

Raman Spectroscopy is also a non-destructive technique which provides detailed information (micron scale) about chemical structure, phase transition (when a substance changes into a different state, e.g., from solid to liquid) and polymorphy (the existence of solid material in more than one structure), crystallinity and molecular interactions of the sample. Since bone ECM presents a highly crystalline structure, this tissue exhibits very strong Raman scattering (the inelastic scattering of protons by matter). Therefore, this technique can be extremely useful in the characterization of both mineral and organic matrix components of bone. Bone Raman spectrum gives information about several components of the bone, allowing the identification of collagen secondary-structure changes, proteins, immature and mature mineral bone, proteoglycans, and aminoacids (tyrosine, phenylalanine, proline, cysteine) [51].

2.2. Bone Biomechanical Properties

During normal activities, bone tissue is subjected to tensile, compressive, and shear stresses, causing a fracture when the bone tissue is subjected to these forces in excess of its strength. Of the several methods for measuring the effects of forces in bone, the focus is on the three main techniques.

2.2.1. Three- and Four-Point Bending

Three- and four-point bending, a structural mechanical test that measures the properties of the whole bone as a unique structure, tests the mechanical properties of the mid-diaphysis, which is typically all cortical bone. Therefore, it is appropriate for testing long bones such as the femur or tibiae, in which the obtained data is usually combined with the bone geometric properties obtained from μCT [48], to ultimately estimate bone material properties. Three-point bending produces its peak stress at the material mid-point and reduced stress elsewhere, while four-point bending produces peak stresses along an extended region of the material. These point-bending techniques give a plot of the load vs. displacement of the bone, describing its structural properties using five basic parameters: stiffness (the resistance of bone to displacement), yield load (the amount of load it can sustain before permanent damage), maximum load (also called ultimate force of strength), post-yield displacement (measuring ductility) and work-to-fracture (the work that must be done to fracture the bone).

2.2.2. Torsional Loading to Failure

The torsional loading-to-failure test is also designed to measure the strength of the sample, but herein, it involves twisting the sample until it breaks. Torsional loading creates both transverse and longitudinal shear stresses in the bone, along with tensile and compressive stresses 45° from the shear direction. Ultimately, the bone will fail, creating a spiral fracture [52]. It is a useful test for acquiring several strength parameters such as torsional shear stress (the shear stress offered by the body against the torsional load), maximum torque (the maximum force applied at a distance that causes a change in angular momentum), shear modulus (the modulus of elasticity in shear, or the modulus of rigidity), torsional loading to failure (the amount of torsional load applied until fracture) and torsional ultimate strength (the maximum torsional stress-hold before rupture).

2.3. Markers in Biological Samples: Blood and Urine

There are several bone remodeling markers present in the biological fluids, able to correlate with the metabolic state of the bone; therefore they can be taken into consideration for disease and therapy monitoring. They can be divided into two main groups: bone-formation and bone-resorption markers. Bone-formation markers include osteocalcin (OST), ALP, N-terminal and C-terminal propeptide of type I procollagen (PINP and PICP, respectively). On the other hand, bone-resorption markers include N- and C-terminal cross-linking telopeptides of type I collagen (NTX and, CTX, respectively), pyridinoline and deoxypyridinoline cross-links (PYD and DPD, respectively), TRAP5b, bone sialoprotein (BSP) and Cathepsin K. All of these markers are usually measured using immunoassay techniques such as enzyme-linked immunoassay (ELISA), radioimmunoassay, and chemiluminescence, in addition to liquid chromatography—tandem mass spectrometry (LC–MS/MS) techniques.

Although many of these bone-turnover markers can be analyzed both in urine and blood, the best source depends on the marker of interest [53]. Furthermore, some of these markers, such as CTX or OST, may vary with the circadian rhythm or diet. Thus, it is critical to take into account the peculiarities of each marker in order to minimize the biological variability and choose the most adequate type of sample (serum, plasma or urine), accordingly. Moreover, there are other parameters that can affect these markers, such as age and sex [54]. On the other hand, since bone remodeling is a complex biological process sustained by balanced osteoblast osteogenesis and

osteoclast resorption, it is desirable to analyze more than one marker, in order to obtain a better overview of both processes [28][55].

2.4. Transcriptome Analysis

Transcriptome analysis permits the identification of genes that are differentially expressed between distinct conditions, leading to a deeper understanding of the genes and/or pathways related to those conditions. This knowledge is essential, not only when identifying potential therapeutic targets, but when evaluating the effects of new therapeutic attempts. In this context, RNA sequencing has revolutionized transcriptomics, thanks to the development of high-throughput next-generation sequencing (NGS). In fact, the transcriptomic analysis of different OI mice models has been crucial to better characterize the murine models and identify new altered signaling pathways. In this way, a transcriptomic study performed on the calvaria bone of oim and Jrt mice, showed that they shared 185 differentially expressed genes (106 upregulated and 79 genes downregulated in both models, with respect to their WT littermates). Among those shared 106 upregulated genes, several were involved in osteoblast function and ECM proteins, while others involved in Wnt and TGF-β signaling pathways were only upregulated in oim mice, but not, or to a lesser extent, in Jrt mice [7]. On the other hand, the study of the transcriptome of Crtap and oim osteocytes from femora and tibiae has shown similar alterations in both OI murine models, including type I collagen coding genes (*Col1a1* and *Col1a2*), bone-related transcripts *Bglap* and *Bglap2* (OST) and *Sparc* (osteonectin), and alterations in Wnt and TGF-β signaling pathways, suggesting a cellular attempt to increase osteoblast differentiation and function in response to pathogenic ECM [8].

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