HELDA
HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

UNIVERSITY OF HELSINKI

https://helda.helsinki.fi

Circulating miRNAs in bone health and disease

Grillari, Johannes

2021-04

Grillari , J , Mäkitie , R E , Kocijan , R , Haschka , J , Vazquez , D C , Semmelrock , E &
Hackl , M 2021 , ' Circulating miRNAs in bone health and disease ' , Bone , vol. 145 , 115787
. https://doi.org/10.1016/j.bone.2020.115787

http://hdl.handle.net/10138/341008
https://doi.org/10.1016/j.bone.2020.115787

cc_by_nc_nd
acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

Circulating miRNAs in bone health and disease

Johannes Grillari[1,2,3], Riikka E. Mäkitie[4,5,6], Roland Kocijan[7,8], Judith Haschka[7,9], David Carro Vázquez[10], Elisabeth Semmelrock[10], Matthias Hackl[3,10]

1. Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria
2. Institute for Molecular Biotechnology, BOKU – University of Natural Resources and Life Sciences, Vienna, Austria
3. Austrian Cluster for Tissue Regeneration
4. Folkhälsan Institute of Genetics and University of Helsinki, Helsinki, Finland
5. Research Program for Clinical and Molecular Metabolism, Faculty of Medicine, University of Helsinki, Helsinki, Finland
6. Molecular Endocrinology Laboratory, Department of Medicine, Hammersmith Campus, Imperial College London, London, United Kingdom
7. Hanusch Hospital of the WGKK and AUVA Trauma Center, 1st Medical Department at Hanusch Hospital, Ludwig Boltzmann Institute of Osteology, Vienna, Austria
8. Sigmund Freud University Vienna, School of Medicine, Metabolic Bone Diseases Unit
9. Karl Landsteiner Institute for Rheumatology and Gastroenterology, Vienna, Austria.
10. TAmiRNA GmbH, Vienna, Austria.

Highlights: 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Keywords: (maximum of 6 keywords)
circulating microRNAs
biomarkers
extracellular vesicles
bone metabolism
bone health and disease

Abstract:
microRNAs have evolved as important regulators of multiple biological pathways essential for bone homeostasis, and microRNA research has furthered our understanding of the mechanisms underlying bone health and disease.
This knowledge, together with the finding that active or passive release of microRNAs from cells into the extracellular space enables minimal-invasive detection in biofluids (circulating miRNAs), motivated researchers to explore microRNAs as biomarkers in several pathologic conditions, including bone diseases. Thus, several exploratory studies in cohorts representing various types of bone diseases have been performed.
In this review, we first summarize important molecular basics of microRNA function and release and provide recommendations for best (pre-)analytical practices and documentation standards for circulating microRNA research required for generating high quality data and ensuring reproducibility of results. Secondly, we review how the genesis of bone-derived circulating microRNAs via release from osteoblasts and osteoclasts could contribute to the communication between these cells. Lastly, we summarize evidence from clinical research studies that have investigated the clinical utility of microRNAs as biomarkers in various musculoskeletal disorders. While previous reviews have mainly focused on diagnosis of primary osteoporosis, we have also included studies exploring the utility of circulating microRNAs in monitoring anti-osteoporotic treatment and for diagnosis of other types of bone diseases, such as diabetic osteopathy, bone degradation in inflammatory diseases, and monogenetic bone diseases.

1. Introduction

1.1. Circulating microRNAs

The discovery of the first microRNA (miRNA), lin-4, in 1993 by the Ambros and Ruvkun groups in Caenorhabditis elegans [1,2] revolutionized the field of molecular biology and led to the recognition of a novel group of regulatory molecules partaking in fundamental signaling pathways. MiRNAs are short, non-coding RNA molecules that regulate gene expression. The canonical mature miRNA is 21 or 22 nucleotides in length and processed within the nucleus by RNA endonucleases drosha, DGCR8 from a primary miRNA (pri-miRNA) transcript into a precursor miRNA (pre-miRNA) encoded in intergenic or intronic regions of the genome. Dicer then removes the stem loop from the pre-miRNA forming a double stranded miRNA. While most miRNAs are "Dicer-dependent", for some miRNAs such as miR-451a it was shown that this step can be bypassed [3]. One of the two strands, the guide strand, is then incorporated into the RNA-induced silencing complex (RISC), while the passenger strand is degraded. When incorporated into RISC, the mature miRNA binds to target sequences in other RNA molecules that exhibit a certain degree of complementarity [4]. Interaction between RISC and mRNAs results in post-transcriptional silencing and down-regulation of encoded proteins.

To date, altogether 2654 human miRNAs have been reported [5]. Although many of these are ubiquitously expressed in most tissues and cell types, some are cell type and cell condition specific, tissue-specific (20-fold higher expression than the mean expression in other tissues) or tissue-enriched (mature miRNA expression is higher than in other tissues but less than 20-fold) [6]. Both the sequences and the cell-type specific expression patterns of miRNAs are highly conserved across a wide variety of organisms [7,8], underlining the importance of this evolutionarily conserved regulatory mechanism [9].

Besides their presence in cells, miRNAs have also been isolated from various cell-free matrices such as blood (serum and plasma), saliva, urine, cerebrospinal fluid (CSF), feces, follicular fluid, synovial fluid, pancreatic juice, bile, gastric juice, and other bodily fluids [10], all showing vast differences in their small RNA compositions. Contrary to cellular RNA species, extracellular miRNAs are highly stable, resisting RNase digestion as well as under harsh conditions including boiling, multiple freeze–thaw cycles, and high (pH=13) or low (pH=1) pH [11]. This unusual stability in 'liquid biopsies' is considered to stem from protection against ubiquitous, extracellular RNAses by either of two protecting mechanisms [12]. One is lipid membrane based ensheathing of miRNAs within extracellular vesicles (EVs) such as exosomes, microvesicles, or apoptotic bodies [12,13], while the other is by being complexed with proteins

and protein particles, especially AGO2 [12], high-density lipoprotein (HDL) [14,15] or nucleophosmin 1 (NPM1) [16–18].

In view of miRNAs and their 1) important regulatory roles, 2) their cross-species conservation, 3) tissue-specificity, and 4) presence in biofluids, they have been investigated as highly promising biomarker candidates in various disease areas, either as single miRNAs, miRNA signatures, or in combination with another known biomarker.
One prominent example is miR-122-5p, which is a liver-specific miRNA with important regulatory functions in cholesterol metabolism and hepatocyte differentiation. Significant increases in miR-122-5p plasma or serum levels have been observed in metabolic diseases such as diabetes [19], and it was since found to serve as a sensitive and specific biomarker for liver dysfunction resulting in validated clinical applications of miR-122-5p for the diagnosis of drug-induced liver injury [20], and prediction of liver dysfunction [21] in combination with miR-151a and miR-192. Another well-advanced clinical application of circulating miRNAs is as biomarkers in cardiovascular disease. Platelet-enriched miRNAs such as miR-223-3p can be applied to monitor platelet function *in vivo*, specifically in the context of monitoring anti-platelet therapy response [22], while muscle-enriched miRNAs (myomiRs) have been shown to serve as specific and sensitive biomarkers of cardiomyocyte injury as a consequence of ischemic events [23].
Here, we aim to summarize the technical challenges associated with circulating miRNA research, the present status of research investigating communication of bone cells via extracellular miRNAs, as well as the potential use of circulating miRNAs as biomarkers in bone diseases, especially in rare or secondary forms of osteoporosis.

1.2. Circulating miRNA as biomarkers – technical challenges

Following the initial excitement about the promise of circulating miRNA biomarkers it was observed that not all results could be reproduced in cohorts independent from the original discovery cohorts, even within the same laboratories [23]. This was in part based on several technical challenges related to the methods used for analyzing circulating miRNAs:
1) miRNA concentration in biofluids are significantly lower than in cells or tissues. Therefore, assays must be validated for their capability to detect and quantify extracellular miRNAs.
2) RNA composition in biofluids can be influenced by sample collection procedures and is therefore sensitive to pre-analytical variability and bias.

3) although stability of miRNAs in biofluids is presumably high, sample quality, such as the presence of enzyme inhibitors or contaminating cells, can easily confound circulating miRNA data.

Data variability has therefore been a long-standing issue and several studies have attempted to address sources of variability by investigating the effects of pre-analytical, analytical and biological variation, especially in blood-based samples, recapitulated in Table 1.

Table 1: Types, sources and mitigation of pre-analytical variability of cell-free miRNA quantitation using serum or plasma

Source of variability	Mitigation strategy	Report on	References
Sample type: serum collection refers to collection of fluid after blood coagulation, which results in release of platelet-stored miRNAs; plasma collection refers to collection of blood in the presence of an anticoagulant. Importantly, high molecular weight heparin (as used in Lithium-heparin plasma tubes) inhibits PCR analysis and is not suitable for miRNA analysis.	Plasma is recommended over serum due to reduced platelet-bias, but tubes containing heparin must be avoided. Serum can be used for studying miRNAs that are low abundant or not present in platelets. Specify and maintain the same cell-free blood sample type throughout a biomarker development project, or perform systematic testing of the impact of changing sample type.	Exact definition of the sample type used in the study.	[24,25]
Sample processing: centrifugation steps impact the purity of plasma, i.e. depletion of platelets and other cell types prior to the collection of supernatants.	Centrifugation is intended to separate cells from fluid. Lower g-forces do not remove platelets, i.e. will yield platelet-rich plasma (PRP), which is not cell-free. Double-centrifugation has been shown to result in removal of platelets (platelet poor plasma; PPP) and is therefore considered ideal for cell-free miRNA analysis in blood.	Exact definition of each processing step including sample incubation times, temperatures and g-force (not rpm).	[27]
Sample quality: sample quality can be impaired by contaminations with cells or impurities that inhibit assay performance. Besides platelets (see above), red blood cells represent a common contamination in the form of	Avoid: drawing blood from a hematoma; probing a traumatic venipuncture; drawing the plunger back too forcefully, if using a needle and syringe; small needles	Assess sample hemolysis using recommended methods and include the data as	[28,29]

hemolysis (lysis of red blood cells during phlebotomy). Hemolysis results in the release of red blood cell–enriched miRNAs into the cell-free fluid. Negatively affects both serum and plasma.	Make sure the venipuncture site is dry.	supplementary information.	
Sample storage: Prolonged incubation times prior to sample processing and storage can result in miRNA release due to platelet activation or decrease due to degradation.	Effects of incubation on miRNAs cannot be generalized, since they depend on the origin of miRNAs. Standardize and document sample incubation prior to processing using standard operating procedures (SOPs).	Sample storage conditions prior to the analysis.	[26]
Sampling time and circadian rhythm: cell-free blood levels of a subset of circulating miRNAs have been shown to undergo a day/night rhythm with changes of up to 1 Cq-value (100%; miR-375). In addition, there is evidence that food intake can alter circulating miRNAs levels.	Especially in bone biomarker research, where daytime dependent effects are usual, sampling should be standardized. We recommend collecting samples between 8 and 10am after an overnight fast.	Time of sample collection and fasting/non-fasting state.	[29,30]
Analytical variability: analysis of circulating miRNAs in serum/plasma is usually a multi-step process with potentially higher analytical variability. In addition, there might be unknown factors present in blood (e.g. pharmaceuticals) that might impact the analytical performance.	Several studies have reported the addition of synthetic spike-in controls to the sample matrix at each step of the workflow to monitor analytical variability and presence of inhibitors.	Assess analytical variability using spike-in controls and report the results.	[26]
Normalization strategy: there is not yet a common standard for the normalization of circulating miRNA experiments. Most studies have either used synthetic spike-in controls or endogenous RNAs (housekeeping RNAs) to account for analytical variability. In screening experiments, where a large number of RNAs is analyzed, so-called "global	Normalization strategy and rational need to be defined prior to the data collection and described in detail in the methods section.	Normalization strategy must be described.	

mean" normalizations can be applied, which are based on the hypothesis that the overall amount of spike-ins is constant.			

In order to increase reproducibility and meaning of circulating miRNA studies, we highly recommend to standardize reporting of circulating miRNA studies and to develop criteria for minimal information on circulating miRNA experimentation (MICmiRE), as has been done in the fields of qPCR (MIQE;[30]) microarrays (MIAME; [31]), next-generation sequencing (MINSEQE; [32]), and EVs (MISEV;[33]). Scientific journals could be recommended to mandatorily ask for these specific details. As a basis for MICmiRE, Table 1 might be considered.

2. Why miRNAs are relevant to bone health and disease

2.1. Importance of overall miRNA biogenesis for bone metabolism

The high impact of miRNAs on bone biology was initially observed by deletion of Dicer, which results in loss of mature miRNA generation and, as Gaur et al [34] observed, changes in the skeletons of mice after osteoblast-specific Dicer knock-out. Dicer ablation in fetal osteogenic mesenchymal cells using *Col1α1*-Cre driven Dicer$^{flox/flox}$ caused a deformed skeleton and defective bone formation in the fetal pup and was lethal after embryonic day 14.5 (E14.5). Deletion of Dicer during the later stage of osteoblast differentiation, specifically in osteoblasts expressing osteocalcin (*Ocn*-Cre; Dicer$^{flox/flox}$), delayed bone mineralization at birth, which however was reversed one month after. At the age of three months, and uniquely due to an effect on mature osteoblasts, the cortical bone thickness in these mice increased significantly together with enhanced synthesis and/or deposition of collagen in the extracellular matrix.
In similar studies, Bendre et al [35] observed that tamoxifen-dependent excision of Dicer1 in osterix+ preosteoblasts (*Sp7*-Cre/ERT2;Dicer$^{flox/flox}$) in both prepubertal and adult mice inhibited cortical but not trabecular bone development, corroborating the findings that Dicer-processed miRNAs play a critical role in postnatal control of cortical bone homeostasis. Furthermore, Liu et al [36] demonstrated that Dicer ablation in Runx2+ osteoblast lineage cells (*Runx2*-Cre;Dicer$^{flox/flox}$) during postnatal development leads to significant growth retardation, decreased bone formation and low bone density. In addition, it was shown that Runx2 regulates the Dicer-mediated miRNA processing pathway during osteoblast lineage commitment [37].
Taken together, these clearly indicate that Dicer-processed miRNAs are critical for normal pre-natal skeletal formation and post-natal regulation of bone growth. This appears especially

critical for osteogenesis and bone formation in early osteoprogenitors, whereas in mature osteoblasts Dicer-processed miRNAs have rather an anabolic effect. Conversely, a recent study [38] found that *in vivo* DGCR8 conditional deletion in Col1α1-expressing osteoblasts (*Col1α1*-Cre;DGCR8$^{flox/flox}$) produces increased osteoblast bone formation in mice, indicating that in a Dicer-independent manner the DROSHA/DGCR8-mediated miRNA processing pathway may adversely affect osteoblast function and bone formation. These results suggest that bone homeostasis might be controlled through regulation of miRNA biogenesis rates via Dicer expression.

Similar to osteoblasts, miRNAs appear to have a crucial role in osteoclast function as well. Sugatani and Hruska [39] identified that siRNA-mediated silencing of either DGCR8, Dicer or Ago in the macrophage/osteoclast lineage leads to suppression of osteoclastic transcription factors and their function in osteoclastic precursors, osteoclastogenesis and bone resorption. Their CD11b-Cre/Dicerfl/fl mice were Dicer-deficient during early differentiation in CD11b+ osteoclast precursors and displayed a moderate phenotype of osteopetrosis induced by impaired bone resorption and diminished osteoclast number associated with a decrease in the expression of the receptor for the osteoclastogenic mediator M-CSF (M-CSFR).

Consistently, a regulatory role for Dicer-dependent miRNAs in mature osteoclasts was proven by excision of the *Dicer*-gene by crossing with mice having Cre under regulation of the cathepsin K (CTSK) promoter [40]. Ctsk-Cre/Dicerfl/fl mice had increased bone mass due to a reduction in osteoclast development and *in vivo* bone resorption, as well as inhibited *in vitro* osteoclast function. Analogously, it was further observed by Sugatani and Hruska that Ctsk-Cre/DGCR8fl/fl mice with osteoclast-specific ablation of DGCR had inhibited osteoclast formation and bone resorption [150], leading to an *in vivo* decrease in bone development. Furthermore, they proved with in an *in vitro* experiment with cultured DGCR8fl/fl bone marrow macrophages infected with Cre-containing retroviruses that loss of DGCR8 inhibits osteoclastic phenotype-related genes and proteins during osteoclastogenesis. In summary, osteoclastogenesis and osteoclastic bone resorption essentially require both DGCR8-dependent and Dicer-dependent miRNA biogenesis.

2.2. Examples of individual miRNAs regulating osteoblast and osteoclast function

Several studies have shown the importance of single miRNAs for osteoblast differentiation and function *in vitro* and *in vivo* [41]. Below, we highlight only a few examples including microRNA families miR-34, miR-188, miR-21, miR-29, and miR-146a.

The miR-34 family members miR-34a, miR-34b, and miR-34c, can exert control over bone metabolism, specifically bone formation. Both, miR-34b and miR-34c suppress cell-autonomous differentiation of osteoblasts in cell lines and primary cells by directly targeting several osteoblast-related factors such as Runx2, Satb2, Notch1, and Notch2. Likewise, osteoblast specific-deletion of miR34b/c increases bone formation and bone mass *in vivo* while miR-34b/c overexpression produces an osteoporotic phenotype due to decreased osteoblast function [42,43]. In addition, miR-34a has been shown to play a role in bone resorption [43], but the study showing this role has been recently retracted due to anomalies in the bone histomorphometry data. This study would have provided evidence of a function of miR-34a in osteoclasts, where it could target TGFB-induced factor homeobox 2 (Tgif2), thereby acting as a potential osteoclastogenic inhibitor. Given the uncertainty of these results, the function of the miR-34 family in osteoclasts requires further evaluations.
miR-188 is a key regulator of the age-related switch between osteogenesis and bone marrow stem cell (BMSC) adipogenesis. In a study by Li et al [44] mice with miR-188-5p depletion displayed a substantial decrease in age-related bone loss and less bone marrow fat deposition. Vice-versa *in vivo* overexpression of miR-188-5p in osterix+ osteoprogenitors accelerated age-related bone loss and accumulation of bone marrow fat compared with control wildtype mice.
miRNAs also regulate the two main osteogenic signaling pathways Wnt/β-catenin and BMP. A study by Kapinas et al [45] showed that inhibitors of the Wnt signaling pathway, including Dikkopf-1 (DKK1), Kremen2, and secreted frizzled-related protein 2 (sFRP2), were directly targeted and suppressed by Wnt-enhancing miR-29a [45]. On the other hand, Sun et al showed that transfection of miR-375-3p represses two Wnt signaling mediators LRP5 and β-catenin, and thereby inhibits osteogenesis and cell apoptosis [46].
Furthermore, miR-214-3p is expressed both in osteoblasts and osteoclasts and activates the PI3K/AKT signaling pathway by suppressing phosphatase and tensin homologation (PTEN) and subsequently inducing osteoclast differentiation [47]. In line with the results from *in vitro* experiments, *in vivo* osteoclast-specific overexpression of miR-214 simultaneously stimulated bone resorption while also decreased osteoblast number and function leading to reduced bone formation [48]. As we will describe in the next section, Li et al showed that extracellular vesicles released from osteoclasts and carrying miR-214-3p can be taken up by local or distant osteoblasts, which explained this double effect of miR-214 in osteoclasts and osteoblasts.
In a different study, Sugatani et al [49] showed that miR-21 was activated by RANKL-induced c-Fos and, after being re-expressed, miR-21 rescued impaired RANKL-induced osteoclastogenesis in DGCR8- or Dicer-deficient mouse bone marrow–derived

monocyte/macrophage precursors (BMMs) [50]. They later demonstrated that estrogen-induced down-regulation of miR-21 biogenesis consequently enhanced the miR-21 target FasL, leading to increased osteoclast apoptosis [49]. *In vivo*, miR-21 global knockout mice confirmed the miR-21 pro-osteoclastogenic effect [51]. Finally, miR-21 also influences alveolar socket healing and morphometry of the skull in mice [52].

In several studies, controversial results from the manipulation of miR-29a/b/c (miR-29 family) or miR-29a indicated the independent regulatory role in osteoclasts of each miR-29 family member. For instance, Wang et al [53] observed an *in vivo* reduction in osteoclast surface, *ex vivo* osteoclast differentiation, and RANKL expression due to a gain of miR-29a function, and a consequent increase in *in vivo* osteoclast resorption and cortical bone porosity and fragility due to miR-29a/b/c knockdown. However, using miR-29a/b/c inducible knockdown Franceschetti et al [54] demonstrated that by mRNA targets, miR-29a/b/c is an inductor of osteoclast migration and differentiation. Consistently, among its direct targets are the mRNAs of cytoskeletal organization–associated molecules, macrophage lineage–associated proteins and the osteoclast survival and resorption regulator Ctr (calcitonin receptor).

Recently, it was observed that miR-146a$^{-/-}$ mice showed a strong and significant increase in trabecular bone volume with increasing age up to 16 months, while the wild-type mice experienced peak bone mass at months 3–4 followed by continuous bone loss [55]. It was further observed that the expression of miR-146a in total bone as well as osteoblasts increased with age in wildtype animals. Vice-versa, the loss of miR-146a expression in knockout mice increased osteoblast number and activity (but not osteoclast activity) resulting in increased levels of Wnt1 and Wnt5a and consequently Wnt signaling. Loss of miR-146a protected mice from OVX-induced bone loss, suggesting that silencing of miR-146a could be a novel strategy to counteract age-dependent bone loss.

In summary, there is accumulating evidence that miRNAs directly regulate bone physiology and homeostasis. The fact that compared to small molecules or biologics, which commonly only interfere with a single target, miRNAs produce their effect by interacting with multiple gene targets, has already established them as targets for therapeutic intervention in many disease areas such as cancer, inflammation or metabolic disease. Several ongoing clinical trials have provided evidence for the feasibility of modulating miRNA activity through administration of miRNA mimics [56,57] and antisense oligonucleotides (antagomiRs) that balance miRNAs' levels [58,59]. Thus, it seems plausible that miRNAs could also be targeted for the treatment of bone-related disorders [60].

3. Bone-derived circulating miRNAs: signaling and cell communication between osteoblasts and osteoclasts via miRNAs

The biological effects of miRNAs on osteoblasts and osteoclasts raise the question of whether these regulatory molecules serve as communicators between bone cells (as occurs with the information exchange at the protein level), producing physiological effects within the recipient cells, and affecting bone remodeling and homeostasis [61]. Recent papers have reported that the transmission of information via extracellular vesicles (EVs) or exosomes may provide a novel interaction mechanism between distant and different types of bone cells.

In the case of osteoblasts, Cui et al reported 43 miRNAs that were found highly abundant in mineralized exosomes of MC3T3-E1 pre-osteoblast cells [62] and are linked to osteoblast function and differentiation. They also showed in a coculture experiment that those osteogenic miRNAs contained in MC3T3-E1-derived exosomes can facilitate osteoblast differentiation of ST2 recipient cells and alter their miRNA expression levels not only indirectly but also directly by direct transference from the exosomes to the ST2 recipient cell content. Since this study reports the potential role of miRNA-containing exosomes in osteoblast–osteoblast communication, it also raises the possibility of osteoblast–osteoclast communication following a similar mechanism in which miRNAs contained in osteoblast lineage–derived exosomes could target key osteoclast differentiation factors, acting as a mechanism of intercellular communication between different bone cell types. In a later study following this hypothesis, the presence of miR-125b-5p was confirmed in osteoblast-derived vesicles, and was shown to be present in bone matrix, where it suppresses bone resorption in mice and plays a crucial role in osteoblast–osteoclast interactions [63]. Similarly, miR-503-3p from mineralized osteoblast-derived exosomes may inhibit RANKL-induced osteoclast differentiation by regulating RANK expression [64].

Regarding the potential role of EVs in the regulation of osteocytes, Sato et al demonstrated that osteocytes produce miRNA-containing exosomes by *in vitro* culture of MLO-Y4 osteocytic cells. In the same study, selective osteocyte ablation *in vivo* in mice also demonstrated that osteocytes generate and release miRNA-containing exosomes into systemic circulation [65]. Despite these, the extent to which miRNAs control osteocyte function, the exact downstream impacts on bone homeostasis and remodeling, and whether osteocyte-derived miRNAs could act in a paracrine manner on adjacent bone cells are still incompletely known [66].

Recent studies have shown that osteoclasts can communicate with osteoblasts via fusion of miRNA-containing exosomes [67]. By directly incubating osteoclast-related exosomes with osteoblast, Sun et al [68] and Li et al [69] have recently suggested that osteoclast-derived miR-

214 can be transmitted via exosomes and inhibit osteoblast activity. *In vivo* injection of exosomes obtained from the osteoclast supernatant drastically reduced bone formation [48]. These findings suggest that EVs derived from osteoclasts may be effective intercellular messengers controlling bone homeostasis and osteoclast–osteoblast communication to reduce osteoblastic bone formation.

In their miR-214 study, Sun et al also showed that the recognition and transfer of osteoclast exosomes to osteoblasts is facilitated by ephrinA2–EphA2 interaction [68]. Research by Wang et al [70] showed that miR-214 inhibits osteoblast activity by targeting ATF4, while their subsequent studies showed that miR-214 enhances osteoclastogenesis through the PI3K/Akt pathway [47]. Hence miR-214-containing osteoclast exosomes could play several functions that support bone degradation. These studies provide the first support for the hypothesis that functional genetic information may be transmitted between osteoblasts and osteoclasts via exosomes, although more research is required to validate these findings in humans [61].

Recent reviews [61,71] have noted that in addition to osteoclast-derived exosomes that can bind to the membrane of the recipient cell to deliver their content or to induce intracellular signaling though receptor interaction, also microvesicles released by osteoclasts and even osteoclast apoptotic bodies may have similar physiological and pathological functions in intercellular communication. However, more research is needed to verify that, for one, the transmission of miRNAs between bone cells can occur *in vivo*, and, secondly, that the physiological importance of this genetic transmission via exosomes is in fact comparable to protein-level communication, particularly in specific tissue or biological processes [72]. Newly developed methods for vesicle tracing can be used for this purpose such as imaging methods for extracellular vesicles using fluorescent lipid dyes [73] or bioluminescence tagging and others [74]. These methods will prove useful in studying the mechanisms and physiological roles of *in vivo* miRNA transmission via extracellular vesicles between different bone cell types.

Regarding the capability of miRNAs to mediate bone cell communication in the bone marrow niche, Davis et al showed an increase in miR-183-5p levels with age in bone-derived extracellular vesicles in the bone marrow, and that miR-183-5p is able to inhibit proliferation of BMSCs and promote stem cell senescence [66].

As pointed out by Xie et al and Yin et al [61,75] in their recent studies, the content of bone-derived exosomes, including proteins, mRNAs and miRNAs, changes from one donor cell to another. Different miRNAs contained within specific bone-derived exosomes are able to target many of the main factors controlling osteoclasts and osteoblasts, such as RUNX2, BMPs, and sclerostin [76]. Furthermore, exosomal miRNAs from the same parent cells may have opposing

roles with respect to osteoclast differentiation and osteoblast activity. Variations in miRNA levels found in the recipient cells do not match the amount of miRNA in the donor exosomes, indicating that in addition to miRNAs also other components of bone-derived exosomes can affect the recipient cell miRNA profile [62]. Interestingly, the impact that one specific type of miRNA may have on osteoblasts and osteoclasts can also be variable and even opposing between the two cell types [77].

Combined with the results mentioned earlier, these studies suggest that intercellular communication within the bone niche could occur through selective transmission of EVs and miRNA cargos. Thus, this type of communication might be a source of novel biomarkers that could be measured with minimal invasiveness in the peripheral blood.

4. Circulating microRNA biomarkers in osteoporosis

In vitro experiments have provided valuable insights into the regulation of bone metabolism through miRNAs (section 2), and evidence for the release of miRNAs from bone cells as part of intercellular communication (section 3). Together, these findings justify translational and clinical research to investigate the utility of circulating miRNAs as bone biomarkers. Based on the incidence of primary osteoporosis, and the lack of sensitive and specific biomarkers to support (prophylactic) treatment decision, it is with good reason that most research to date has been performed in the context of postmenopausal osteoporosis. Most studies have focused on diagnostic or prognostic application of microRNAs biomarkers in osteoporosis. Their application for monitoring treatment response has received comparatively little attention so far. In this section we provide a summary of clinical microRNA biomarker studies in the context of postmenopausal osteoporosis (4.1.) and monitoring of anti-osteoporotic treatment (4.2).

4.1. Circulating microRNAs as biomarkers for fracture risk in postmenopausal osteoporosis

Osteoporosis is a systemic skeletal disorder characterized by increased risk of bone fracture due to increased skeletal fragility, reduced bone mass and muscle weakness (sarcopenia). Fractures, particularly hip fracture, are a major health care concern due to the associated morbidity and mortality, mainly in elderly and postmenopausal women [78,79]. Therefore, fracture-risk screening in this group of patients is key to identify high risk individuals that could benefit from anti-osteoporotic treatment. In terms of population-based screenings, blood-based biomarkers have the advantage that they can be analyzed locally in central and basic laboratories, making their analysis scalable, cost-effective, and not requiring patient travel. A cost-utility analysis on an Austrian cohort showed that blood-based miRNA screening for fracture-risk assessment could lead to a significant improvement in health (reduced fracture-rates) while lowering the

economic burden stemming from fracture-care, if a prognostic performance (AUC = 0.85) can be met [80].

The search term "microRNA AND osteoporosis AND fracture AND (blood OR serum OR plasma) AND biomarker" in PubMed identified altogether 25 studies between the first study by Seeliger et al. in 2014 and November 1st in 2020. Of these, 18 provide original data on circulating miRNAs in the context of bone diseases (Table 2), while the other seven are reviews or non-human studies. We evaluated the level of information provided on pre-analytical and analytical methods and found that eight studies provided complete information, six sufficient (lacking for instance exact centrifugation parameters), while four studies did not provide any detailed information about sample collection. The majority of studies (14/18) have used serum for the analysis of circulating miRNAs. Two out of three studies using plasma described double-centrifugation and usage of EDTA as anticoagulant. One study used both serum and plasma, but on or no? details on comparability were given. Thirteen out of 18 studies reported the use of spike-ins to monitor RNA extraction and RT-qPCR performance, and 11 studies evaluated the degree of hemolysis (6 of which using the ratio of miR-23a/451a).

Predominately in early studies, published until 2018, so-called "discovery platforms" such as qPCR arrays that can cover between 96 to 768 different miRNAs [79,81,82] were applied to enable biomarker candidate selection, while subsequent studies have mostly relied on targeted RT-qPCR assays to measure up to 32 miRNA candidates per sample. All of the identified studies have used cross-sectional study designs comparing circulating miRNAs levels between controls or disease groups with specific fracture types. Half of the studies had recruited more than 100 samples for their analysis and used targeted RT-qPCR analysis of 1-32 miRNA candidates, which were selected based on literature as well as target prediction analysis.

Table 2 Pubmed search results for "microRNA AND osteoporosis AND fracture AND (blood OR serum OR plasma) AND biomarker" (excluding review articles)

Title	Year-Month	Primary Outcome	Population	Sample Size	Technology Platform	Number of miRNAs	Sample Type	Pre-analytical protocols	Hemolysis controls	Spike-In controls	Norm-alization	Main Finding	Ref
miR-27a-3p negatively regulates osteogenic differentiation of MC3T3 E1 preosteoblasts by targeting osterix.	2020-09	Bone mass	Women (definition is lacking)	137	RT-qPCR (unknown)	1	Serum	-	No	No	Not specified	miR-27a-3p levels decreased during osteogenic differentiation and increased in the serum of patients with osteoporosis.	[83]
Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures.	2020-01	Vertebral factures Bone mass	Postmenopausal women	126	RT-qPCR (LNA)	21	Serum	+++	Yes (miR-23a/451a)	Yes	Spike-Ins	Seven significantly up-regulated miRNAs were identified in patients with VerFx and low BMD compared to low BMD and healthy individuals.	[84]
Selected serum microRNA, abdominal aortic calcification and risk of osteoporotic fracture.	2019-05	Abdominal aortic calcification and risk of fracture	Postmenopausal women	434	RT-qPCR (TaqMan)	3	Serum	+++	Yes (macroscopic)	Yes	Endogenous miRNAs (miR-191-5p, miR-222-3p and miR-361-5p)	miR-26a-5p, -34a-5p and -223-5p are not significantly associated with incident fracture and AAC aggravation.	[85]
Evaluation of circulating miRNA-208a-3p, miRNA-155-5p and miRNA-637 as potential non-invasive	2020-03	Bone mass	Pre- and Postmenopausal women	140	RT-qPCR (miScript)	3	Serum	+	No	No	Endogenous small RNA (Snord68_11)	Differential expression suggests association with osteoporosis pathogenesis.	[86]

biomarkers […] pre- and postmenopausal osteoporotic females.												
Aberrant Expression of miR-100 in Plasma of Patients with Osteoporosis and its Potential Diagnostic Value	2019-09	Osteoporosis	Postmenopausal women	240	RT-qPCR (unknown)	1	Plasma	-	No	No	Not specified	miR-100 was abnormally increased in the plasma of osteoporotic patients	[87]
Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort.	2019-06	Bone Mass Bone Turnover Fractures	Pre- and Postmenopausal women	682	RT-qPCR (LNA)	32	Serum	+++	Yes (macroscopic)	Yes	Spike-Ins and Global Mean	No evidence that 32 preselected miRNAs were not associated with BTMs, BMD, microarchitecture, and or fragility fractures.	[88]
Circulating miR-103a-3p and miR-660-5p are associated with bone parameters in patients with controlled acromegaly.	2019-01	Acromegaly (ACRO)	Male/female patients and controls	54	RT-qPCR (LNA)	20	Serum	-	Yes (miR-23a/451a)	Yes	Global Mean	Circulating miR-103a-3p and miR-660-5p are differentially expressed in controlled ACRO patients.	[89]
Correlation of plasma microRNA-21 expression and bone turnover markers in postmenopausal women.	2018-12	Bone turnover markers and bone minearl density	Thai postmenopausal women	195	RT-qPCR (TaqMan)	1	EDTA-Plasma (2x)	+++	No	Yes	Not specified	No significant correlation between plasma miR-21-5p expression and BTMs. Higher expression of miR-21-5p in low BMD participants.	[90]

Serum miRNAs miR-140-3p and miR-23b-3p as potential biomarkers for osteoporosis and osteoporotic fracture in postmenopausal Mexican-Mestizo women.	2018-12	Bone mass Fractures	Postmenopausal women	40 + 97	RT-qPCR (TaqMan)	754	Serum	+	Yes (OD414nm)	No	Endogenous small RNA (RNU6)	Differences between osteopenic, osteoporosis and fracture patients observed for miR-23b-3p and miR-140-3p.	[91]
Circulating microRNAs as potential diagnostic biomarkers for osteoporosis	2018-05	Osteopenia/ Osteoporosis/ Fracture	Male/female 40y+	21 + 139 (serum) 134 (plasma)	RT-qPCR (miScript)	370	Serum or plasma	+	No	Yes	Endogenous small RNA (SNORD96A, RNU6)	Circulating hsa-miR-122-5p and hsa-miR-4516 could be potential diagnostic biomarkers for osteoporosis.	[92]
Bone-related Circulating MicroRNAs miR-29b-3p, miR-550a-3p, and miR-324-3p and their Association to Bone Microstructure and Histomorphometry.	2018-03	Bone Histo-morph-ometry	Male/female cohort with bone biopsies	36	RT-qPCR (LNA)	19	Serum	+	Yes (miR-23a/451a)	Yes	Spike-Ins	Bone-related circulating miRNAs miR-29b-3p, miR-550a-3p and miR-324-3p are associated to dynamic processes of bone, reflected by bone histomorphometry.	[93]
MiR-148a the epigenetic regulator of bone homeostasis is increased in plasma of osteoporotic postmenopausal women.	2016-12	Osteo-porosis	Postmenopausal women	74	RT-qPCR (miScript)	9	EDTA-Plasma (2x)	+++	Yes (not specified)	Yes	Endogenous miRNAs (let-7a-5p and miR-16-5p)	Expression of miR-148a-3p was significantly higher in the osteoporotic patient group compared to the controls.	[94]

Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures.	2017-02	Bone mass and vertebral fracture	Postmenopausal women	70	RT-qPCR (miScript)	14	Serum	+++	No	Yes	Endogenous small RNA (SNORD95, SNORD96A, RNU6)	Differential expression of miR-21-5p in the serum of women with low BMD and VFs.	[95]
Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures.	2016-11	Osteo-porosis	Pre- and Postmenopausal women and men	75	RT-qPCR (LNA)	187	Serum	+	Yes (miR-23a/451a)	Yes	Global Mean	Specific serum miRNAs are differentially expressed in osteoporosis.	[81]
Serum miRNA Signatures Are Indicative of Skeletal Fractures in Postmenopausal Women With and Without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose Tissue-Derived Mesenchymal Stem Cells In Vitro.	2016-12	Fracture	Postmenopausal women with/without type-2 diabetes	74	RT-qPCR (LNA)	375	Serum	+++	Yes (miR-23a/451a)	Yes	Spike-Ins	Circulating miRNAs are linked to fragility fractures in T2D postmenopausal women and are different to that linked to non-diabetic women with fractures.	[82]
Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture.	2015-11	Fracture	Postmenopausal women	27	RT-qPCR (LNA)	179	Serum	+	Yes (OD414nm)	Yes	Endogenous miRNAs (miR-93-5p)	miR-21-5p may be a biomarker of bone fracture.	[78]

Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation.	2015-10	Recent bone fracture	Postmenopausal women	37	RT-qPCR (LNA)	175	Serum	+++	Yes (miR-23a/451a)	Yes	Global Mean	Recent osteoporotic fractures are reflected by specific serum miRNA patterns. Changes could affect bonemetabolism or bone healing processes.	[96]
Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures.	2014-08	Fracture with low BMD vs fracture with normal BMD	Female and male patients	20 + 60	RT-qPCR (miScript)	not provided	Serum	-	No	No	Endogenous small RNA (SNORD96A, RNU6)	The first study to investigate circulating miRNAs in the context of bone fractures.	[79]

A panel of 20 miRNAs and five controls, referred to as osteomiR, has emerged from these studies. These miRNA bone biomarker candidates have been used as individual biomarkers in the context of low bone mineral density (BMD) and vertebral fractures [86] as well as in combined fashion using a multivariate model (that returns a fracture-risk score) to assess the prognostic performance for fracture-risk in older people [86]. Several of these osteomiRs have been described above in sections 2 and 3 (miR-214, miR-188, miR-133). In addition, miR-203a-3p should also be highlighted, as it has been identified to be elevated in serum of fracture patients [82], and induced in the bone and serum of ovariectomized rats [97]. *In vitro* data further supports a relevant role for this miRNA in bone pathology as it is induced by dexamethasone and silenced by BMP-2 signaling [98], and its up-regulation results in reduced osteogenesis via down-regulation of its direct targets DLX5 [99], RUNX2, and Smad [100].

Using a standardized assay and a panel of circulating miRNA enhances the comparability of results and increases the likelihood that results can be reproduced, ultimately resulting in the selection of few miRNA biomarker candidates to be used in large population-based studies to evaluate diagnostic and prognostic performances.

4.2.Effect of osteoporosis treatment on circulating miRNA levels

The follow-up of osteoporosis is currently done by monitoring BMD with dual energy X-ray absorptiometry (DXA) and measuring bone turnover markers (BTM) such as procollagen type-1 aminoterminal propeptide (P1NP) and carboxy-terminal telopeptide of type-I collagen (CTX). These, however, have proven insufficient in adequately reflecting bone health, disease progression or treatment response. For one, it has to be noticed that an increase in BMD only partly reflects the fracture risk reduction under anti-resorptive therapy [101] and does not mirror the magnitude of changes in bone organic matrix, mineral content or bone microstructure under osteoanabolic therapy [102]. Moreover, BMD changes during treatment are generally small and slow [103]. Consequently, short-term follow-up measurements by DXA are insufficient.

Evaluation of BTMs is a common and an establish practice for monitoring treatment response. Typically BTMs show a distinctive course in response to treatment with antiresorptive agents, such as bisphosphonates or denosumab, as well as osteoanabolic agents, such as teriparatide [104]. These changes in BTMs are also much faster and greater than changes in BMD [105].

The utility of circulating, bone-related miRNAs for the follow-up of osteoporosis and their response to anti-osteoporotic therapy is not fully known. Presently, only a few studies have investigated the effects of bisphosphonate treatment on miRNAs. The influence of alendronate (ALN) on miR-182-5p was examined by Pan et al. miRNA-182 is a regulator of osteoblast differentiation and apoptosis via the Rap1/mitogen-activated protein kinase (MAPK) signaling

pathway, targeting the adenylyl cyclase isoform 6 (ADCY6)–gene. OVX in rats resulted in a significant up-regulation of miR-182-5p, when compared to sham-operated rats. ALN led to a down-regulation of miR-182-p5 and consequently to an up-regulation of ADCY6 and the Rap1/MAPK signaling pathway [106].

Li et al investigated the role of ALN on miRNAs and osteoclastogenesis by inducing osteoclast differentiation in bone marrow-derived macrophage-like cells (BMMs) with RANKL and M-CSF. Results showed a significant decrease in miR-101-3p during osteoclastogenesis, whereas ALN led to a significant increase in miR-101-3p. TRAP positive osteoclasts were inhibited by both miR-101-3p and ALN. Furthermore, in an *in vivo* OVX mouse model, injection of miR-101-3p led to an inhibition of RANKL and the RANKL/OPG (osteoprotegerin) ratio. These data suggest, that ALN regulates the miR-101-3p/Rap1b (Ras-related protein) signaling pathway and thereby osteoclast differentiation [107].

Studies on serum miRNAs in osteoporosis patients have found numerous miRNAs to be up-regulated in patients with vertebral fractures, when compared with patients with low BMD without fractures or with healthy controls. In a subgroup of patients with vertebral fractures receiving ALN or risedronate (RIS), serum levels of miRNAs, such as miR-335-5p and miR-30e-3p, were slightly lower than in untreated patients [84].

The effect of zoledronic acid (ZOL) on miRNA expressions was investigated in breast cancer cells. In total, 21 miRNAs were differentially regulated by ZOL in comparison to untreated cells. Of these, miR-455, a regulator of osteoblast cell proliferation, apoptosis and oxidative stress via the HDAC2-Nrf2/ARE signaling pathway [108], was one of the most up-regulated miRNAs following ZOL treatment [109]. A similar effect was observed in our OVX-model [97]. Moreover, ZOL treatment also resulted in down-regulation of miR-133 in our animal model on postmenopausal osteoporosis, indicating a positive effect on RUNX2 and consequently on bone formation [97].

The influence of denosumab (DMAB), a monoclonal antibody to RANKL, on 16 circulating miRNAs was observed in postmenopausal women with low bone mass. Surprisingly, no significant change in the miRNA signature was seen after three or 12 months of DMAB therapy [110]. Recently, we initiated the MiDeTe-study (microRNA Levels Under Denosumab and Teriparatide Therapy in Postmenopausal Osteoporosis) in order to get more information on circulating miRNAs during DMAB therapy (ClinicalTrials.gov Identifier: NCT03472846). The follow-up period was set to 24 months with six clinical visits and timepoints for miRNA analysis.

Recently published investigations give evidence of an increased risk for vertebral fractures after discontinuation of DMAB therapy [111]. This might be due to increased bone turnover after the treatment period, reflected by an increase in BTMs such as CTX and PINP. Anastasilakis et al examined circulating miRNAs in three groups of women: postmenopausal women with vertebral fractures 8–16 months after the last administration of DMAB, DMAB-discontinuer without fracture, and treatment-naïve women with fracture. Results showed down-regulation in miR-222 and miR-503 in the DMAB-discontinuation group, with even lower levels for DMAB discontinuer with fracture. The mRNAs of RANK, which is negatively regulated by miR-503, and cathepsin K, which is negatively regulated by miR-222, were up-regulated, indicating an increased osteoclast formation after DMAB discontinuation [112].

Teriparatide (TPTD), a recombinant human parathyroid hormone–fragment (rhPTH1-34), is a potent osteoanabolic agent for treatment of severe osteoporosis. TPTD therapy is known to increase BMD and improve bone microstructure. Although, the exact mechanisms of PTH are incompletely known, PTH acts via different pathways including MAP kinase, phospholipase A and D, IGF-I and WNT, and thus stimulates osteoblastic signals, targets the extra- and intracellular regulators, influences osteoblast differentiation and prevents osteoblast apoptosis [113].

While the treatment response to TPTD is usually measured by evaluating BMD and BTMs, a few studies have also focused on the effect of teriparatide on miRNAs. Twelve months of TPTD treatment in postmenopausal women with osteoporosis resulted in a differential expression of several miRNAs [110]. After three months of treatment, six out of 16 miRNAs were differently regulated, and after one year two miRNAs significantly changed. miR-33-3p, a regulator of DKK1, and thereby of the WNT pathway, reached statistical significance after three months of therapy, and miR-133a-3p, an inhibitor of RUNX2 and thereby a key player in osteoblastogenesis, was significantly reduced after one year of therapy, reflecting the anabolic capacity of TPTD [110].

A recent study investigated the influence of TPTD on osteogenic differentiation of human marrow mesenchymal cells (hMSCs) with or without miR-375 overexpression. Higher levels of miR-375 were found in blood from osteoporosis patients, when compared with healthy controls. TPTD resulted in up-regulation of alkaline phosphatase (ALP) or RUNX2. In contrast, miR-375 overexpression led to down-regulation of the mentioned mRNAs. These data indicate a potential role for the miR-375/RUNX2 pathway in TPTD-induced osteogenic differentiation [114].

We have recently conducted an animal study on postmenopausal osteoporosis and tested the effects of anti-resorptive and osteoanabolic therapy on bone-related miRNAs [97]. Rats first underwent OVX or sham-operation and were then randomized to either ZOL, TPTD or placebo treatment. OVX led to significant changes in bone microstructure and miRNA transcription in bone. In total, 46 miRNAs were regulated by TPTD and ten by ZOL. Anti-osteoporotic treatment reversed the OVX-effects in untreated animals. The most promising miRNA candidate identified was miR-203a, which was up-regulated due to OVX, and rescued by TPTD and ZOL treatment. miRNA-203a is involved in the differentiation of mesenchymal stem cells and promotes osteogenic differentiation. miR-203a inhibits Dlx5 and thus the transcription factors Runx2 and Osx [99]. The significant correlation between miRNA-203a in bone tissue and serum supports its potential clinical utility as a bone biomarker for osteoporosis.
Of note, a similar pattern was also observed for miR-20a-5p, which was up-regulated following OVX and down-regulated after TPTD treatment [97]. miR-20a-5p promotes osteoblast differentiation and thus bone formation via Runx2, BMP2 and PPARγ [115].

5. Circulating miRNAs as emerging biomarkers for secondary, monogenic, and rare bone diseases

Secondary, monogenic, or rare forms of musculoskeletal disorders offer a unique platform for surveying miRNA regulation in relation to specific signaling pathways and unusual pathologic conditions. They also help clarify and expand our understanding on the underlying pathomechanisms in these diseases which, despite the rise in identified causative genes and contributing genetic loci, remains limited. From a clinical standpoint, miRNAs offer an innovative avenue for novel diagnostic means since 1) currently available metabolic markers are often normal in affected patients, despite severe osteoporosis or skeletal pathology [116,117], 2) the tools for diagnosing and monitoring disease progression or treatment response are limited for patients with rare and/or monogenic skeletal diseases; and 3) many of the molecular pathways and mechanisms that cause bone fragility in rare and/or monogenic diseases are still incompletely understood, and post-transcriptional regulation through miRNAs might provide insight and novel targets for therapeutic intervention.

5.1. Diabetic osteopathy

It is estimated that one in 11 people worldwide has diabetes (International Diabetes Federation 2017), correlated with increased morbidity, death, and health care costs [118]. Both type 1 (T1DM) and type 2 diabetes mellitus (T2DM) are associated with reduced bone strength and decreased risk of fracture [119,120] due to poor bone turnover. The pathways causing bone

fragility in diabetes mellitus are complex, and significantly different between type 1 and type 2 diabetes.
T1DM affects bone health more severely and leads to decreased BMD already in puberty, at a period of rapid skeletal development, leading to low peak bone mass [116]. As a result, the bone is weakened at a younger age and the negative effects during aging become much more severe. Bone regeneration is also poor in T1DM patients due to decreased bone formation and, to a lesser degree, bone resorption [121]. This is most likely attributed to impaired osteoblast function due to low concentrations of both insulin (caused by an almost complete β-cell failure) and insulin growth factor 1 (IGF-1) [122,123]. Other co-factors leading to decreased bone formation include bone microarchitecture changes, increased bone marrow fat content, low-grade inflammation, osteocyte dysfunction, and aggregation of advanced glycation end products (AGEs) in collagen due to hyperglycemia [124].
T2DM is much more common than T1DM and, due to the epidemic of obesity, its prevalence has further gradually increased in the recent years. T2DM affects bone health at the later stages of the disease, with less severe bone mass reduction and risk of fracture compared to T1DM [118]. In contrast to T1DM, BMD is not consistently reduced in patients with T2DM [125], linking the problem of decreased bone strength to degradation of bone microarchitecture. Degradation of bone microarchitecture is primarily attributed to hyperglycemia, and the subsequent accumulation of AGEs [126]. Nevertheless, the specific pathophysiological causes are unclear as obesity is very prevalent in T2DM patients and is itself linked to increased fracture risk.
Other causes behind bone fragility in T2DM include renal dysfunction, vitamin D deficiency, hypogonadism and systemic inflammation caused by fat-derived factors such as pro-inflammatory cytokines and adipokines, WNT pathway repression and, likely, bone microvascular dysfunction. These together contribute to impaired mechanostatic activity of osteocytes, bone turnover and bone matrix properties, leading to diminished bone strength. Fracture risk is further increased due to other diabetic complications, such as visual deterioration and neuropathy [127].

Many studies have shown that miRNAs and alterations in their expression patterns can be involved in the pathophysiology of osteoporosis and reduced fracture healing in both T1DM and T2DM. In a study by Takahara et al [128] using diabetic rats (with a T1DM induced by streptozotocin injection) with fractured femur, post-fracture microarray analysis revealed 17 miRNAs with more than two-fold change in the newly generated tissue at the fracture site when

compared to controls. Five miRNAs (miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p, and miR-222-3p) were selected based on literature review and analyzed by real-time PCR analysis to confirm changing patterns of expression during fracture healing in their diabetic rat model.

In a similar study using the same diabetic rat model, Tang et al [100] reported changes in a distinct signature of miRNAs in the diabetic rat mandibles compared to controls. The authors observed an overlap between these miRNAs and miRNAs identified by *in vitro* analysis of BMSCs cultured under different concentrations of glucose, including miR-181a-5p, miR-345-5p and miR-872-5p. However, other miRNAs did not overlap, indicating that high-glucose culture may not fully replicate diabetic conditions. As a result, they concluded that miR-203-3p can serve as a putative biomarker for diabetic bone loss, being up-regulated in the jaw bones of diabetic rats and in high-glucose cultured cells, and acting as a suppressor of osteogenesis by inhibiting the BMP/Smad pathway through Smad1 targeting.

A study by Heilmeier et al [82] evaluated the circulating miRNA profiles of 80 postmenopausal women with and without T2DM and osteoporosis to investigate the association between T2DM bone fracture and circulating miRNAs. With 48 significantly differentially expressed miRNAs, T2DM patients with fractures exhibited stronger shifts in circulating miRNAs relative to T2DM controls without fractures or the non-diabetic study arm (23 differentially regulated miRNAs). Interestingly, only a small overlap in the relative miRNA changes in T2DM and non-T2DM patients with fractures was observed (n=6); miR-382-3p, miR-1908, and miR-369-3p were down-regulated in both diabetic and non-diabetic fractured patients, while miRNA-203a-3p, miRNA-330-3p, and miRNA-550a-5p were up-regulated. The study also examined the impact of miR-550a-5p and miR-382-3p on bone metabolism by *in vitro* functional experiments, and discovered that miR-550a-5p hindered both adipogenic and osteogenic differentiation of human adipose-derived mesenchymal stem cells (hASCs), while overexpression of miR-382-3p enhanced osteogenic differentiation, but not adipogenic differention of hASCs.

So far, only one study has investigated circulating miRNA levels in T1DM patients. Grieco et al analyzed six miRNAs (miR-21-5p, miR-148a-5p, miR-24-3p, miR-27a-3p, miR-214-3p, and miR-375-3p) in 15 T1DM patients and 14 non-diabetic and age-matched controls. They found that miR-21-5p and miR-148a-5p were up-regulated in T1DM subjects, and correlated to markers of bone strength and metabolism [129].

Other studies have focused on the above-mentioned role of hyperglycemia in diabetic osteoporosis and bone fracture. You et al discovered that, by targeting CASP3 and inducing the PI3K/Akt pathway, miR-378 overexpression attenuates the high glucose-inhibition of

osteogenic differentiation. As mentioned before, hyperglycemia leads to the formation of AGEs, another co-factor for diabetic bone loss. AGEs have also been associated in DM patients or *in vitro* with miRNAs such as miRNA-146a [130], miRNA-214 [131] and miRNA-223. Of these, miRNA-233 has been proposed as an effective marker for antagonizing the AGE-induced damage to osteoblasts in DM by Qin et al [132], as they found that the AGE-promoted apoptosis in osteoblasts is induced by miR-223 via down-regulation of insulin-like growth factor 1 receptor.

5.2.Circulating miRNAs as biomarkers for bone degeneration in inflammatory disease

Several rheumatic diseases are known to affect bone metabolism. In seropositive rheumatoid arthritis (RA), the presence of Anti-citrullinated protein antibodies (ACPA) is associated with greater radiological joint damage and bone loss. However, no such biomarkers are available for spondyloarthropathies and other seronegative diseases, for which circulating miRNAs could offer a potential mean.

RA is a chronic systemic autoimmune disease characterized by inflammation of predominantly peripheral joints as well as local and systemic bone loss [133]. Dysregulated miRNA signatures in RA, their role in pathogenesis of the disease and their potential role in the development of autoimmunity have been elucidated repeatedly [134–137]. Studies have found elevated serum levels of miR-22, miR-38 and miR-486 in ACPA-positive subjects eventually developing RA [138]. Also, miRNA signatures of early RA patients showed lower levels of miR-16 and miR-223 compared with healthy controls [139], and miR-223 was reported to affect osteoclast and osteoblast differentiation, reflecting disease activity and treatment response in RA [140,141]. Furthermore, *in vitro* experiments showed that overexpression of miR-223 leads to an increased secretion of proinflammatory cytokines [142], while lentivirus-mediated silencing of miR-223 decreased the severity of arthritis, inhibited osteoclastogenesis and subsequent formation of bone erosions in collagen-induced arthritis [143]. Lastly, increased levels of miR-223 *in vitro* reduced osteoclastogenesis in inflamed synovia of RA patients [144]

WNT signaling plays a crucial role in bone homeostasis and on the osteoblast–osteoclast axis. The chronic inflammatory state in RA leads to an imbalance of this axis, resulting in increased bone resorption [145]. Analysis of inflamed synovia in RA patients showed a differential expression of 12 miRNAs participating in bone metabolism, specifically in osteoblast and chondrocyte differentiation via the WNT and BMP pathways [146]. For one, down-regulation of miR-133a, miR-145a and miR-204a affected RUNX2 and Osterix positively. Secondly, miR-145 was elevated in peripheral blood mononuclear cells (PBMCs) and synovium, favoring

osteoclastogenesis and aggravated bone erosions in collagen-induced arthritis [147]. Lastly, miR-99b-5p, miR-143-3p and miR-145-5p were also found to discriminate patients with and without bone erosions. While miR-99b-5p was an independent predictor of erosion progression, the combination of all three miRNAs had the highest accuracy [148].

Spondylarthropathies (SpA), including axial SpA (axSpA) and psoriatic arthritis (PsA), are characterized by local and systemic bone loss, deterioration of bone microstructure and new bone formation [149–151]. The pathophysiological role of the IL-23-Th17 axis in spondyloarthritis has been addressed in several studies [152]. For one, miR-155-5p, miR-210-3p and miR-10b have been reported to be up-regulated in Th17 cells of axSpA patients [153]. Zou et al investigated miR-21 in axSpA patients and correlated the findings to radiographic changes, BMD of lumbar spine and femoral neck. Results showed axSpA patients to have higher levels of miR-21 expression compared with controls. miR-21 correlated positively with more pronounced radiographic damage and negatively with BMD of lumbar spine and femoral neck [154]. The proinflammatory cytokine TNF-α has been reported to lead to impaired bone formation *in vivo* and *in vitro*, accompanied with suppression of miR-21-5p [155]. *In vitro* experiments on miR-21-5p showed that its expression and osteogenic activity were augmented with low TNF-α concentration and depressed with higher concentrations. Further, intravenous injection of miR-21-5p led to new bone formation and elevated expressions of STAT3, JAK2 and IL-12 in mice with proteoglycan-induced arthritis (PGIA). Therefore, miR-21-5p may be a potential mediator of new bone formation in axSpA [156] by promoting Th17 cell differentiation stimulated by IL-17 and IL-22, both of which are considered to lead to bone loss and new bone formation in AS [157,158].
Secondly, axSpA patients with pronounced radiographic damage showed nine differentially expressed miRNAs, of which six (miR-19a-3p, miR-24-3p, miR-27a-3p, miR-106a-5p, miR-223-3p, and miR-374a-5p) were associated with WNT-mediated bone formation [159]. miR-19a has been described as a negative regulator and miR-374a as an activator of WNT signaling pathway [160]. Further, overexpression of miR-24 is associated with an inhibition of osteogenic differentiation in osteoblastic cells [161], and miR-27 promotes osteoblast differentiation by modulation of WNT signaling and its expression is negatively regulated by RUNX2 [162].
Lastly, A differential expression of miRNAs was also found in PsA patients, differentiating between active and non-active diseases [163]. A higher expression of miR-146a-5p was found in PsA patients compared with both psoriasis patients and controls, and the increase resumed by successful clinical treatment after 28 weeks. The expression of miR-146a-5p was associated

with CRP level and osteoclast number, but not with the presence of enthesitis or the skin Psoriasis Area and Severity Index–score (PASI). [164]

5.3.Monogenetic bone disorders

While miRNAs are well-established in postmenopausal, and to a certain extent in secondary forms of osteoporosis, studies in monogenic forms remain scarce. We have previously conducted two studies in two types of monogenic osteoporosis, of which first was a survey in autosomal dominant WNT1 osteoporosis [116]. WNT1 is a ligand to the WNT/beta-catenin pathway in bone and heterozygous mutations in its encoding gene *WNT1* result in early-onset, low-turnover osteoporosis with reduced BMD, multiple peripheral and vertebral compression fractures, and subsequent increased thoracic kyphosis and loss of adult height [165,166]. We screened a custom-designed panel comprising 192 common miRNAs in a cohort of 12 subjects with a heterozygous missense mutation p.C218G in *WNT1* and distinguished a unique miRNA expression profile in the *WNT1* mutation-positive subjects with altogether nine differentially expressed miRNAs [116]. Of these, two were up-regulated (miR-18a-3p, miR-223-3p) and six down-regulated (miR-22-3p, miR-31-5p, miR-34a-5p, miR-143-5p miR-423-5p, miR-423-3p) and for three of them (miR-22-3p, miR-34a-5p, and miR-31-5p) binding sites in the 3’UTR of *WNT1* mRNA were bioinformatically predicted [116].

Following this, we profiled miRNA concentrations in 15 subjects with another form of monogenic osteoporosis—X-linked PLS3 osteoporosis [117]. Aberrant function of Plastin 3 (PLS3), arising from mutations in its encoding gene *PLS3*, results in severe and childhood-onset osteoporosis with multiple peripheral and compression fractures and markedly reduced BMD [167,168]. Due to its X chromosomal inheritance, *PLS3* mutation-positive males are typically more severely affected, while the phenotype in affected females varies from asymptomatic to osteoporosis with fragility fractures (5,6). With a similarly designed study setting and a pre-selected set of 192 miRNAs, we identified altogether seven statistically significantly differentially expressed miRNAs in the *PLS3* mutation-positive subjects with three up-regulated (miR-301b-3p, miR-181c-5p, miR-203a-3p) and four down-regulated (miR-532-3p, miR-590-3p, miR-93-3p, miR-133a-3p) miRNAs [117]. Two of these (miR-181c-5p and miR-203a-3p) have bioinformatically predicted targets in *PLS3* 3’UTR, which we further demonstrated for miR-181c-5p by *in vitro* analyses [117].

In neither cohort, the identified differentially regulated miRNAs correlated with age, subfamily division, type of mutation, or prior or ongoing osteoporosis medication [116]. There was also no overlap between the two studies, further underlining their specificity to the pertinent gene defects and disrupted molecular pathways. Studies in rare monogenic bone diseases are limited by small cohort sizes and clinically heterogenous phenotypes and, as in our studies, by their cross-sectional and exploratory natures lacking longitudinal data on dynamic changes in response to disease progression or therapeutic treatment. Although altered miRNA levels do not directly infer function, we hypothesized that the differentially regulated miRNAs mirror the interrupted signaling pathways in bone, elucidating yet unresolved underlying pathogenic mechanisms. Further investigations in larger cohorts and utilizing functional means are encouraged to delineate their functional roles and translate them into clinical and therapeutic applications.

6. Concluding remarks

MicroRNAs are important regulators of bone homeostasis and involved in the control of bone formation and resorption. Controlled release of miRNAs from bone cells suggests that miRNAs partake in cell communication between osteoblasts and osteoclasts. Blood levels of bone-derived miRNAs could therefore be used as biomarkers reflecting bone health and disease.

Importantly, laboratory methods for the analysis of circulating miRNAs require careful optimization of pre-analytical and analytical procedures and certain standards for reporting circulating miRNA data, to ultimately enhance the reproducibility of results.

Clinical research studies investigating circulating miRNA levels in patient populations with different types of bone diseases have shown that onset and progression of bone diseases lead to changes in circulating miRNA patterns. In monogenetic diseases, such as WNT1 or PLS3 osteoporosis, this pattern could reflect a molecular response to dampen the effects of aberrant gene expression. In primary and secondary forms of osteoporosis these circulating miRNA patterns might reflect the (complex) multifactorial nature of these disease involving not only bone but also other tissue types. Moving forward it is greatly encouraged for this research field to progress from exploratory studies to large, ideally population-based validation studies, to obtain clear evidence for or against the clinical utility of circulating miRNAs as clinical decision support tools.

7. Acknowledgements

We acknowledge funding from AWS Seed fund (P1405046-SZL01), the MSCA Innovative Training Network programme FIDELIO (860898), and the Orion Research Foundation, the Finnish ORL–HNS Foundation, the Juhani Aho Foundation.

8. References

1. Lee RC. The C Elegans Heterochronic Gene lin-4 Encodes Small RNAs With Antisense Complementarity To lin-14. *Cell*. 75, 843–854 (1993).

2. Wightman B, Ilho H, Ruvkun G. Posttranscriptional Regulation of the Heterochronic Gene lin-14 by W-4 Mediates Temporal Pattern Formation in C. elegans. *Cell*. (Vol. 75), 855–862 (1993).

3. Cheloufi S, Dos Santos CO, Chong MMW, Hannon GJ. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature*. 465(7298), 584–589 (2010).

4. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. *Cell*. 136(2), 215–233 (2009).

5. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. *Nucleic Acids Res.* 47(D1), D155–D162 (2019).

6. Lee EJ, Baek M, Gusev Y, Brackett DJ, Nuovo GJ, Schmittgen TD. Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA*. 14(1), 35–42 (2007).

7. Ludwig N, Leidinger P, Becker K, *et al.* Distribution of miRNA expression across human tissues. *Nucleic Acids Res.* , 1–13 (2016).

8. Bushel PR, Caiment F, Wu H, *et al.* RATEmiRs: the rat atlas of tissue-specific and enriched miRNAs database. *BMC Genomics* [Internet]. 19(1) (2018). Available from: https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-5220-x.

9. Griffiths-Jones S. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34(90001), D140–D144 (2006).

10. Godoy PM, Bhakta NR, Barczak AJ, *et al.* Large Differences in Small RNA Composition Between Human Biofluids. *Cell Rep.* 25(5), 1346–1358 (2018).

11. Chen X, Ba Y, Ma L, *et al.* Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 18(10), 997–1006 (2008).

12. Gallo A, Tandon M, Alevizos I, Illei GG. The Majority of MicroRNAs Detectable in Serum and Saliva Is Concentrated in Exosomes. *PLoS ONE*. 7(3), e30679 (2012).

13. Iftikhar H, Carney GE. Evidence and potential in vivo functions for biofluid miRNAs: From expression profiling to functional testing: Potential roles of extracellular miRNAs as indicators of physiological change and as agents of intercellular information exchange. *BioEssays*. 38(4), 367–378 (2016).

14. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are Transported in Plasma and Delivered to Recipient Cells by High-Density Lipoproteins. *Nat Cell Biol*. 13(4), 423–433 (2011).

15. Tabet F, Vickers KC, Cuesta Torres LF, *et al.* HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat. Commun.* [Internet]. 5(1) (2014). Available from: http://www.nature.com/articles/ncomms4292.

16. Wang K, Zhang S, Weber J, Baxter D, Galas DJ. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res.* 38(20), 7248–7259 (2010).

17. Arroyo JD, Chevillet JR, Kroh EM, *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci.* 108(12), 5003–5008 (2011).

18. Turchinovich A, Weiz L, Burwinkel B. Extracellular miRNAs: the mystery of their origin and function. *Trends Biochem. Sci.* 37(11), 460–465 (2012).

19. Willeit P, Skroblin P, Moschen AR, *et al.* Circulating MicroRNA-122 is associated with the risk of new-onset metabolic syndrome and type 2 diabetes. *Diabetes*. 66(2), 347–357 (2017).

20. Dear JW, Clarke JI, Francis B, *et al.* Risk stratification after paracetamol overdose using mechanistic biomarkers: results from two prospective cohort studies. *Lancet Gastroenterol. Hepatol.* 3(2), 104–113 (2018).

21. Starlinger P, Hackl H, Pereyra D, *et al.* Predicting Postoperative Liver Dysfunction Based on Blood Derived MicroRNA Signatures. *Hepatology* [Internet]. (2019). Available from: http://doi.wiley.com/10.1002/hep.30572.

22. Krammer TL, Mayr M, Hackl M. microRNAs as promising biomarkers of platelet activity in antiplatelet therapy monitoring. *Int. J. Mol. Sci.* .

23. Schulte C, Barwari T, Joshi A, *et al.* Comparative Analysis of Circulating Noncoding RNAs Versus Protein Biomarkers in the Detection of Myocardial Injury. *Circ. Res.* 125(3), 328–340 (2019).

24. Cheng HH, Yi HS, Kim Y, *et al.* Plasma Processing Conditions Substantially Influence Circulating microRNA Biomarker Levels. *PLoS ONE*. 8(6), e64795 (2013).

25. Mitchell AJ, Gray WD, Hayek SS, *et al.* Platelets confound the measurement of extracellular miRNA in archived plasma. *Sci. Rep.* 6(September), 1–11 (2016).

26. Mussbacher M. Impact of anticoagulation and sample processing on the quantification of human blood-derived microRNA signatures. *Cell*. (2020,).

27. Mussbacher M, Schrottmaier WC, Salzmann M, *et al.* Optimized plasma preparation is essential to monitor platelet-stored molecules in humans. *PLOS ONE*. 12(12), e0188921 (2017).

28. Kirschner MB, Kao SC, Edelman JJ, *et al.* Haemolysis during sample preparation alters microRNA content of plasma. *PLoS ONE*. 6(9) (2011).

29. Blondal T, Jensby Nielsen S, Baker A, *et al.* Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods*. 59(1), 164–169 (2013).

30. Bustin SA, Benes V, Garson JA, *et al.* The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55(4), 611–622 (2009).

31. Brazma A, Hingamp P, Quackenbush J, *et al.* Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. *Nat. Genet.* 29(4), 365–371 (2001).

32. MINSEQE: Minimum Information about a high-throughput Nucleotide SeQuencing Experiment - a proposal for standards in functional genomic data reporting [Internet]. . Available from: http://fged.org/projects/minseqe/.

33. Théry C, Witwer KW, Aikawa E, *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles*. 7(1), 1535750 (2018).

34. Gaur T, Hussain S, Mudhasani R, *et al.* Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse. *Dev. Biol.* 340(1), 10–21 (2010).

35. Bendre A, Moritz N, Väänänen V, Määttä JA. Dicer1 ablation in osterix positive bone forming cells affects cortical bone homeostasis. *Bone*. 106, 139–147 (2018).

36. Liu P, Baumgart M, Groth M, *et al.* Dicer ablation in osteoblasts by Runx2 driven cre-loxP recombination affects bone integrity, but not glucocorticoid-induced suppression of bone formation. *Sci. Rep.* [Internet]. 6(1) (2016). Available from: http://www.nature.com/articles/srep32112.

37. Zhou J, Hu Y, Chen Y, *et al.* Dicer-dependent pathway contribute to the osteogenesis mediated by regulation of Runx2. , 16.

38. Choi Y-J, Jeong S, Yoon K-A, *et al.* Deficiency of DGCR8 increases bone formation through downregulation of miR-22 expression. *Bone*. 103, 287–294 (2017).

39. Sugatani T, Hruska KA. Down-regulation of miR-21 biogenesis by estrogen action contributes to osteoclastic apoptosis. *J. Cell. Biochem.* 114(6), 1217–1222 (2013).

40. Mizoguchi F, Izu Y, Hayata T, *et al.* Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption. *J. Cell. Biochem.* , n/a-n/a (2009).

41. Lian JB, Stein GS, van Wijnen AJ, *et al.* MicroRNA control of bone formation and homeostasis. *Nat. Rev. Endocrinol.* 8(4), 212–227 (2012).

42. Bae Y, Yang T, Zeng H-C, *et al.* miRNA-34c regulates Notch signaling during bone development. *Hum. Mol. Genet.* 21(13), 2991–3000 (2012).

43. Wei J, Shi Y, Zheng L, *et al.* miR-34s inhibit osteoblast proliferation and differentiation in the mouse by targeting SATB2. *J. Cell Biol.* 197(4), 509–521 (2012).

44. Li C-J, Cheng P, Liang M-K, *et al.* MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. *J. Clin. Invest.* 125(4), 1509–1522 (2015).

45. Kapinas K, Kessler C, Ricks T, Gronowicz G, Delany AM. miR-29 Modulates Wnt Signaling in Human Osteoblasts through a Positive Feedback Loop. *J. Biol. Chem.* 285(33), 25221–25231 (2010).

46. Sun T, Li C-T, Xiong L, *et al.* miR-375-3p negatively regulates osteogenesis by targeting and decreasing the expression levels of LRP5 and β-catenin. *PLOS ONE*. 12(2), e0171281 (2017).

47. Zhao C, Sun W, Zhang P, *et al.* miR-214 promotes osteoclastogenesis by targeting Pten/PI3k/Akt pathway. *RNA Biol.* 12(3), 343–353 (2015).

48. Li Y-H, Tavallaee G, Tokar T, *et al.* Identification of synovial fluid microRNA signature in knee osteoarthritis: differentiating early- and late-stage knee osteoarthritis. *Osteoarthritis Cartilage*. 24(9), 1577–1586 (2016).

49. Sugatani T, Hruska KA. Down-regulation of miR-21 biogenesis by estrogen action contributes to osteoclastic apoptosis. *J. Cell. Biochem.* 114(6), 1217–1222 (2013).

50. Sugatani T, Vacher J, Hruska KA. A microRNA expression signature of osteoclastogenesis. *Blood*. 117(13), 3648–3657 (2011).

51. Hu C-H, Sui B-D, Du F-Y, *et al.* miR-21 deficiency inhibits osteoclast function and prevents bone loss in mice. *Sci. Rep.* [Internet]. 7(1) (2017). Available from: http://www.nature.com/articles/srep43191.

52. Strauss FJ, Stähli A, Kobatake R, *et al.* miRNA-21 deficiency impairs alveolar socket healing in mice. *J. Periodontol.* [Internet]. (2020). Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/JPER.19-0567.

53. Wang F-S, Chung P-C, Lin C-L, *et al.* MicroRNA-29a Protects Against Glucocorticoid-Induced Bone Loss and Fragility in Rats by Orchestrating Bone Acquisition and Resorption: MicroRNA-29a Ameliorates Bone Loss. *Arthritis Rheum.* 65(6), 1530–1540 (2013).

54. Franceschetti T, Kessler CB, Lee S-K, Delany AM. miR-29 Promotes Murine Osteoclastogenesis by Regulating Osteoclast Commitment and Migration. *J. Biol. Chem.* 288(46), 33347–33360 (2013).

55. Saferding V, Hofmann M, Brunner JS, *et al.* MiR-146a controls age related bone loss [Internet]. Molecular Biology. Available from: http://biorxiv.org/lookup/doi/10.1101/2020.03.20.000174.

56. Hanna J, Hossain GS, Kocerha J. The Potential for microRNA Therapeutics and Clinical Research. *Front. Genet.* [Internet]. 10 (2019). Available from: https://www.frontiersin.org/article/10.3389/fgene.2019.00478/full.

57. Bonneau E, Neveu B, Kostantin E, Tsongalis GJ, De Guire V. How close are miRNAs from clinical practice? A perspective on the diagnostic and therapeutic market. *EJIFCC*. 30(2), 114–127 (2019).

58. Mie Y, Hirano Y, Kowata K, *et al.* Function Control of Anti-microRNA Oligonucleotides Using Interstrand Cross-Linked Duplexes. *Mol. Ther. - Nucleic Acids*. 10, 64–74 (2018).

59. Lima JF, Cerqueira L, Figueiredo C, Oliveira C, Azevedo NF. Anti-miRNA oligonucleotides: A comprehensive guide for design. *RNA Biol.* 15(3), 338–352 (2018).

60. Puppo M, Taipaleenmäki H, Hesse E, Clézardin P. Non-coding RNAs in bone remodelling and bone metastasis: Mechanisms of action and translational relevance. *Br J Pharmacol.* (2019).

61. Yin P, Lv H, Li Y, Deng Y, Zhang L, Tang P. Exosome-Mediated Genetic Information Transfer, a Missing Piece of Osteoblast–Osteoclast Communication Puzzle. *Front. Endocrinol.* [Internet]. 8 (2017). Available from: http://journal.frontiersin.org/article/10.3389/fendo.2017.00336/full.

62. Cui Y, Luan J, Li H, Zhou X, Han J. Exosomes derived from mineralizing osteoblasts promote ST2 cell osteogenic differentiation by alteration of microRNA expression. *FEBS Lett.* 590(1), 185–192 (2016).

63. Minamizaki T, Nakao Y, Irie Y, *et al.* The matrix vesicle cargo miR-125b accumulates in the bone matrix, inhibiting bone resorption in mice. *Commun. Biol.* [Internet]. 3(1) (2020). Available from: http://www.nature.com/articles/s42003-020-0754-2.

64. Chen C, Cheng P, Xie H, *et al.* MiR-503 Regulates Osteoclastogenesis via Targeting RANK: MiR-503 REGULATES OSTEOCLASTOGENESIS. *J. Bone Miner. Res.* 29(2), 338–347 (2014).

65. Sato M, Suzuki T, Kawano M, Tamura M. Circulating osteocyte-derived exosomes contain miRNAs which are enriched in exosomes from MLO-Y4 cells. *Biomed. Rep.* 6(2), 223–231 (2017).

66. Plotkin LI, Davis HM. MicroRNA Regulation in Osteocytes. *Curr. Mol. Biol. Rep.* 4(4), 191–197 (2018).

67. Sun W, Zhao C, Li Y, *et al.* Osteoclast-derived microRNA-containing exosomes selectively inhibit osteoblast activity. *Cell Discov.* [Internet]. 2(1) (2016). Available from: http://www.nature.com/articles/celldisc201615.

68. Sun W, Zhao C, Li Y, *et al.* Osteoclast-derived microRNA-containing exosomes selectively inhibit osteoblast activity. *Cell Discov.* [Internet]. 2(1) (2016). Available from: http://www.nature.com/articles/celldisc201615.

69. Li D, Liu J, Guo B, *et al.* Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation. *Nat. Commun.* [Internet]. 7(1) (2016). Available from: http://www.nature.com/articles/ncomms10872.

70. Wang X, Guo B, Li Q, *et al.* miR-214 targets ATF4 to inhibit bone formation. *Nat. Med.* 19(1), 93–100 (2013).

71. Yuan F-L, Wu Q, Miao Z-N, *et al.* Osteoclast-Derived Extracellular Vesicles: Novel Regulators of Osteoclastogenesis and Osteoclast–Osteoblasts Communication in Bone Remodeling. *Front. Physiol.* [Internet]. 9 (2018). Available from: https://www.frontiersin.org/article/10.3389/fphys.2018.00628/full.

72. Mittelbrunn M, Sánchez-Madrid F. Intercellular communication: diverse structures for exchange of genetic information. *Nat. Rev. Mol. Cell Biol.* 13(5), 328–335 (2012).

73. Ter-Ovanesyan D, Kowal E, Regev A. Imaging of Isolated Extracellular Vesicles Using Fluorescence Microscopy. *Methods Mol Biol* . (1660), 233–241 (2017).

74. Chuo ST-Y, Chien JC-Y, Lai CP-K. Imaging extracellular vesicles: current and emerging methods. *J. Biomed. Sci.* [Internet]. 25(1) (2018). Available from: https://jbiomedsci.biomedcentral.com/articles/10.1186/s12929-018-0494-5.

75. Xie Y, Chen Y, Zhang L, Ge W, Tang P. The roles of bone-derived exosomes and exosomal microRNAs in regulating bone remodelling. *J. Cell. Mol. Med.* 21(5), 1033–1041 (2017).

76. Qin Y, Sun R, Wu C, Wang L, Zhang C. Exosome: A Novel Approach to Stimulate Bone Regeneration through Regulation of Osteogenesis and Angiogenesis. *Int. J. Mol. Sci.* 17(5), 712 (2016).

77. Chen J, Qiu M, Dou C, Cao Z, Dong S. MicroRNAs in Bone Balance and Osteoporosis: MicroRNAs IN BONE BALANCE AND OSTEOPOROSIS. *Drug Dev. Res.* 76(5), 235–245 (2015).

78. Panach L, Mifsut D, Tarin JJ, Cano A, Garcia-Perez MA. Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture. *Calcif. Tissue Int.* 97(5), 495–505 (2015).

79. Seeliger C, Karpinski K, Haug AT, *et al.* Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. *J. Bone Miner. Res.* 29(8), 1718–1728 (2014).

80. Walter E, Dellago H, Grillari J, Dimai HP, Hackl M. Cost-utility analysis of fracture risk assessment using microRNAs compared with standard tools and no monitoring in the Austrian female population. *Bone*. 108, 44–54 (2018).

81. Kocijan R, Muschitz C, Geiger E, *et al.* Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures. *J. Clin. Endocrinol. Metab.* 101(11), 4125–4134 (2016).

82. Heilmeier U, Hackl M, Skalicky S, *et al.* Serum miRNA Signatures Are Indicative of Skeletal Fractures in Postmenopausal Women With and Without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose Tissue–Derived Mesenchymal Stem Cells In Vitro. *J. Bone Miner. Res. Mon.* xx(xx), 1–20 (2016).

83. Xu Y, Li D, Zhu Z, *et al.* miR-27a-3p negatively regulates osteogenic differentiation of MC3T3-E1 preosteoblasts by targeting osterix. *Mol. Med. Rep.* 22(3), 1717–1726 (2020).

84. Zarecki P, Hackl M, Grillari J, Debono M, Eastell R. Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures. *Bone*. 130, 115105 (2020).

85. Pickering M-E, Millet M, Rousseau J-C, *et al.* Selected serum microRNA, abdominal aortic calcification and risk of osteoporotic fracture. *PLOS ONE*. 14(5), e0216947 (2019).

86. Ismail SM, El Boghdady NA, Hamoud HS, Shabayek MI. Evaluation of circulating miRNA-208a-3p, miRNA-155–5p and miRNA-637 as potential non-invasive biomarkers and the possible mechanistic insights into pre- and postmenopausal osteoporotic females. *Arch. Biochem. Biophys.* 684, 108331 (2020).

87. Ding W, Ding, S, Li J. Aberrant Expression of miR-100 in Plasma of Patients with Osteoporosis and its Potential Diagnostic Value. *Clin.Lab*. (65(9)) (2019).

88. Feurer E, Kan C, Croset M, Sornay-Rendu E, Chapurlat R. Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort. *J. Bone Miner. Res.* 34(6), 1074–1085 (2019).

89. Valassi E, García-Giralt N, Malouf J, *et al.* Circulating miR-103a-3p and miR-660-5p are associated with bone parameters in patients with controlled acromegaly. *Endocr. Connect.* 8(1), 39–49 (2019).

90. Perksanusak T, Panyakhamlerd K, Hirankarn N. Correlation of plasma microRNA-21 expression and bone turnover markers in postmenopausal women. *Climacteric.* (21(6)), 581–585 (2018).

91. Ramírez-Salazar EG, Carrillo-Patiño S, Hidalgo-Bravo A, *et al.* Serum miRNAs miR-140-3p and miR-23b-3p as potential biomarkers for osteoporosis and osteoporotic fracture in postmenopausal Mexican-Mestizo women. *Gene*. 679, 19–27 (2018).

92. Mandourah AY, Ranganath L, Barraclough R, *et al.* Circulating microRNAs as potential diagnostic biomarkers for osteoporosis. *Sci. Rep.* [Internet]. 8(1) (2018). Available from: http://www.nature.com/articles/s41598-018-26525-y.

93. Feichtinger X, Muschitz C, Heimel P, *et al.* Bone-related Circulating MicroRNAs miR-29b-3p, miR-550a-3p, and miR-324-3p and their Association to Bone Microstructure and Histomorphometry. *Sci. Rep.* 8(1), 1–11 (2018).

94. Bedene A, Mencej Bedrac S, Jese L, *et al.* MiR-148a the epigenetic regulator of bone homeostasis is increased in plasma of osteoporotic postmenopausal women. *Wien. Klin. Wochenschr.* , 1–8 (2016).

95. Yavropoulou MP, Anastasilakis AD, Makras P, Tsalikakis DG, Grammatiki M, Yovos JG. Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures. *Eur. J. Endocrinol.* 176(2), 169–176 (2017).

96. Weilner S, Skalicky S, Salzer B, *et al.* Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. *Bone*. 79, 43–51 (2015).

97. Kocijan R, Weigl M, Skalicky S, *et al.* MicroRNA levels in bone and blood change during bisphosphonate and teriparatide therapy in an animal model of postmenopausal osteoporosis. *Bone*. 131, 115104 (2020).

98. Laxman N, Rubin C-J, Mallmin H, Nilsson O, Tellgren-Roth C, Kindmark A. Second generation sequencing of microRNA in Human Bone Cells treated with Parathyroid Hormone or Dexamethasone. *Bone*. 84, 181–188 (2016).

99. Laxman N, Mallmin H, Nilsson O, Kindmark A. miR-203 and miR-320 Regulate Bone Morphogenetic Protein-2-Induced Osteoblast Differentiation by Targeting Distal-Less Homeobox 5 (Dlx5). *Genes* [Internet]. 8(1) (2016). Available from: http://www.ncbi.nlm.nih.gov/pubmed/28025541 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5294999.

100. Tang Y, Zheng L, Zhou J, *et al.* miR-203-3p participates in the suppression of diabetes-associated osteogenesis in the jaw bone through targeting Smad1. *Int. J. Mol.*

Med. [Internet]. (2018). Available from: http://www.spandidos-publications.com/10.3892/ijmm.2018.3373.

101. Delmas PD, Seeman E. Changes in bone mineral density explain little of the reduction in vertebral or nonvertebral fracture risk with anti-resorptive therapy. *Bone*. 34(4), 599–604 (2004).

102. Paschalis EP, Krege JH, Gamsjaeger S, *et al.* Teriparatide Treatment Increases Mineral Content and Volume in Cortical and Trabecular Bone of Iliac Crest: A Comparison of Infrared Imaging With X-Ray-Based Bone Assessment Techniques: TERIPARATIDE INCREASES BONE MATRIX/MINERAL CONTENT IN ILIAC CREST. *J. Bone Miner. Res.* 33(12), 2230–2235 (2018).

103. Bhattoa HP. Laboratory aspects and clinical utility of bone turnover markers. , 12.

104. McClung MR, San Martin J, Miller PD, *et al.* Opposite Bone Remodeling Effects of Teriparatide and Alendronate in Increasing Bone Mass. *Arch. Intern. Med.* 165(15), 1762 (2005).

105. Lee J, Vasikaran S. Current Recommendations for Laboratory Testing and Use of Bone Turnover Markers in Management of Osteoporosis. *Ann. Lab. Med.* 32(2), 105 (2012).

106. Pan B-L, Tong Z-W, Li S-D, *et al.* Decreased microRNA-182-5p helps alendronate promote osteoblast proliferation and differentiation in osteoporosis via the Rap1/MAPK pathway. *Biosci. Rep.* [Internet]. 38(6) (2018). Available from: https://portlandpress.com/bioscirep/article/doi/10.1042/BSR20180696/98219/Decreased-microRNA1825p-helps-alendronate-promote.

107. Li J, Li Y, Wang S, Che H, Wu J, Ren Y. miR-101-3p/Rap1b signal pathway plays a key role in osteoclast differentiation after treatment with bisphosphonates. *BMB Rep.* 52(9), 572–576 (2019).

108. Zhang S, Wu W, Jiao G, Li C, Liu H. MiR-455-3p activates Nrf2/ARE signaling via HDAC2 and protects osteoblasts from oxidative stress. *Int. J. Biol. Macromol.* 107, 2094–2101 (2018).

109. Fanale D, Amodeo V, Bazan V, *et al.* Can the microRNA expression profile help to identify novel targets for zoledronic acid in breast cancer? *Oncotarget*. 7(20), 29321–29332 (2016).

110. Anastasilakis AD, Makras P, Pikilidou M, *et al.* Changes of Circulating MicroRNAs in Response to Treatment With Teriparatide or Denosumab in Postmenopausal Osteoporosis. *J. Clin. Endocrinol. Metab.* 103(3), 1206–1213 (2018).

111. Tripto-Shkolnik L, Fund N, Rouach V, Chodick G, Shalev V, Goldshtein I. Fracture incidence after denosumab discontinuation: Real-world data from a large healthcare provider. *Bone*. 130, 115150 (2020).

112. Anastasilakis AD, Yavropoulou MP, Makras P, *et al.* Increased osteoclastogenesis in patients with vertebral fractures following discontinuation of denosumab treatment. *Eur. J. Endocrinol.* 176(6), 677–683 (2017).

113. Canalis E, Giustina A, Bilezikian JP. Mechanisms of Anabolic Therapies for Osteoporosis. *N. Engl. J. Med.* 357(9), 905–916 (2007).

114. Teriparatide alleviates osteoporosis by promoting osteogenic differentiation of hMSCs via miR-375/RUNX2 axis. *B Lei.* , 8.

115. De-Ugarte L, Serra-Vinardell J, Nonell L, *et al.* Expression profiling of microRNAs in human bone tissue from postmenopausal women. *Hum. Cell.* 31(1), 33–41 (2018).

116. Riikka Mäkitie, Hackl M, Niinimäki R, Kakko S, Grillari J, Mäkitie O. Altered MicroRNA Profile in Osteoporosis Caused by Impaired WNT Signaling. *JCEM*. (2018).

117. Mäkitie RE, Hackl M, Weigl M, *et al.* Unique, Gender-Dependent Serum MICRORNA Profile in *PLS3* Gene-Related Osteoporosis. *J. Bone Miner. Res.* [Internet]. (2020). Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jbmr.4097.

118. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic Review of Type 1 and Type 2 Diabetes Mellitus and Risk of Fracture. *Am. J. Epidemiol.* 166(5), 495–505 (2007).

119. Bouillon R. Diabetic bone disease. , 6 (1991).

120. Wang H, Ba Y, Xing Q, Du J-L. Diabetes mellitus and the risk of fractures at specific sites: a meta-analysis. *BMJ Open*. 9(1), e024067 (2019).

121. Kalaitzoglou E, Popescu I, Bunn RC, Fowlkes JL, Thrailkill KM. Effects of Type 1 Diabetes on Osteoblasts, Osteocytes, and Osteoclasts. *Curr. Osteoporos. Rep.* 14(6), 310–319 (2016).

122. Guerra-Menéndez L, Sádaba MC, Puche JE, *et al.* IGF-I increases markers of osteoblastic activity and reduces bone resorption via osteoprotegerin and RANK-ligand. *J. Transl. Med.* 11(1), 271 (2013).

123. Zhang N, Jiang H, Bai Y, *et al.* The molecular mechanism study of insulin on proliferation and differentiation of osteoblasts under high glucose conditions. *Cell Biochem. Funct.* 37(5), 385–394 (2019).

124. Cortet B, Lucas S, Legroux-Gerot I, Penel G, Chauveau C, Paccou J. Bone disorders associated with diabetes mellitus and its treatments. *Joint Bone Spine*. 86(3), 315–320 (2019).

125. Ma L, Oei L, Jiang L, *et al.* Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. *Eur. J. Epidemiol.* 27(5), 319–332 (2012).

126. Picke A-K, Campbell G, Napoli N, Hofbauer LC, Rauner M. Update on the impact of type 2 diabetes mellitus on bone metabolism and material properties. *Endocr. Connect.* 8(3), R55–R70 (2019).

127. On behalf of the IOF Bone and Diabetes Working Group, Napoli N, Chandran M, *et al.* Mechanisms of diabetes mellitus-induced bone fragility. *Nat. Rev. Endocrinol.* 13(4), 208–219 (2017).

128. Takahara S, Lee SY, Iwakura T, *et al.* Altered expression of microRNA during fracture healing in diabetic rats. *Bone Jt. Res.* 7(2), 139–147 (2018).

129. Grieco GE, Cataldo D, Ceccarelli E, *et al.* Serum Levels of miR-148a and miR-21-5p Are Increased in Type 1 Diabetic Patients and Correlated with Markers of Bone Strength and Metabolism. *Non-Coding RNA*. 4(4), 37 (2018).

130. Yang Y, Fang S. Small non-coding RNAs-based bone regulation and targeting therapeutic strategies. *Mol. Cell. Endocrinol.* [Internet]. (2016). Available from: http://linkinghub.elsevier.com/retrieve/pii/S0303720716304877.

131. Li L-M, Hou D-X, Guo Y-L, *et al.* Role of MicroRNA-214–Targeting Phosphatase and Tensin Homolog in Advanced Glycation End Product-Induced Apoptosis Delay in Monocytes. *J. Immunol.* 186(4), 2552–2560 (2011).

132. Qin Y, Ye J, Wang P, Gao L, Wang S, Shen H. miR-223 contributes to the AGE-promoted apoptosis via down-regulating insulin-like growth factor 1 receptor in osteoblasts. *Biosci. Rep.* [Internet]. 36(2) (2016). Available from: https://portlandpress.com/bioscirep/article/doi/10.1042/BSR20150271/56172/miR223-contributes-to-the-AGEpromoted-apoptosis.

133. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *The Lancet*. 388(10055), 2023–2038 (2016).

134. Ammari M, Jorgensen C, Apparailly F. Impact of microRNAs on the understanding and treatment of rheumatoid arthritis: *Curr. Opin. Rheumatol.* 25(2), 225–233 (2013).

135. Singh RP, Massachi I, Manickavel S, *et al.* The role of miRNA in inflammation and autoimmunity. *Autoimmun. Rev.* 12(12), 1160–1165 (2013).

136. Salehi E, Eftekhari R, Oraei M, Gharib A, Bidad K. MicroRNAs in rheumatoid arthritis. *Clin. Rheumatol.* 34(4), 615–628 (2015).

137. Chen J-Q, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun. Rev.* 15(12), 1171–1180 (2016).

138. Ouboussad L, Hunt L, Hensor EMA, *et al.* Profiling microRNAs in individuals at risk of progression to rheumatoid arthritis. *Arthritis Res. Ther.* [Internet]. 19(1) (2017). Available from: https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-017-1492-9.

139. Lenert A, Fardo DW. Detecting Novel Micro RNAs in Rheumatoid Arthritis With Gene-Based Association Testing. *Clin Exp Rheumatol* . (Jul-Aug 2017;35(4):586-592. Epub 2017 Jan 27).

140. Castro-Villegas C, Pérez-Sánchez C, Escudero A, *et al.* Circulating miRNAs as potential biomarkers of therapy effectiveness in rheumatoid arthritis patients treated with anti-TNFα. *Arthritis Res. Ther.* [Internet]. 17(1) (2015). Available from: http://arthritis-research.com/content/17/1/49.

141. Filková M, Aradi B, Šenolt L, *et al.* Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis. *Ann. Rheum. Dis.* 73(10), 1898–1904 (2014).

142. Ogando J, Tardáguila M, Díaz-Alderete A, *et al.* Notch-regulated miR-223 targets the aryl hydrocarbon receptor pathway and increases cytokine production in macrophages from rheumatoid arthritis patients. *Sci. Rep.* [Internet]. 6(1) (2016). Available from: http://www.nature.com/articles/srep20223.

143. Li Y-T, Chen S-Y, Wang C-R, *et al.* Brief Report: Amelioration of collagen-induced arthritis in mice by lentivirus-mediated silencing of microRNA-223. *Arthritis Rheum.* 64(10), 3240–3245 (2012).

144. Shibuya H, Nakasa T, Adachi N, *et al.* Overexpression of microRNA-223 in rheumatoid arthritis synovium controls osteoclast differentiation. *Mod. Rheumatol.* 23(4), 674–685 (2013).

145. Busse B, Hahn M, Schinke T, Püschel K, Duda GN, Amling M. Reorganization of the femoral cortex due to age-, sex-, and endoprosthetic-related effects emphasized by osteonal dimensions and remodeling. *J. Biomed. Mater. Res. A*. 9999A, NA-NA (2009).

146. Maeda Y, Farina NH, Matzelle MM, Fanning PJ, Lian JB, Gravallese EM. Synovium-Derived MicroRNAs Regulate Bone Pathways in Rheumatoid Arthritis: SYNOVIUM-DERIVED MiRNAs REGULATE BONE PATHWAYS IN RHEUMATOID ARTHRITIS. *J. Bone Miner. Res.* 32(3), 461–472 (2017).

147. Chen Y, Wang X, Yang M, *et al.* miR-145-5p Increases Osteoclast Numbers In Vitro and Aggravates Bone Erosion in Collagen-Induced Arthritis by Targeting Osteoprotegerin. *Med. Sci. Monit.* 24, 5292–5300 (2018).

148. Yue J, Lau TCK, Griffith JF, *et al.* Circulating miR-99b-5p as a novel predictor of erosion progression on high-resolution peripheral quantitative computed tomography in early rheumatoid arthritis: A prospective cohort study. *Int. J. Rheum. Dis.* 22(9), 1724–1733 (2019).

149. Baraliakos X, Listing J, Rudwaleit M, Sieper J, Braun J. The relationship between inflammation and new bone formation in patients with ankylosing spondylitis. *Arthritis Res. Ther.* 10(5), R104 (2008).

150. Magrey MN, Khan MA. The Paradox of Bone Formation and Bone Loss in Ankylosing Spondylitis: Evolving New Concepts of Bone Formation and Future Trends in Management. *Curr. Rheumatol. Rep.* [Internet]. 19(4) (2017). Available from: http://link.springer.com/10.1007/s11926-017-0644-x.

151. Neumann A, Haschka J, Kleyer A, *et al.* Cortical bone loss is an early feature of nonradiographic axial spondyloarthritis. *Arthritis Res. Ther.* [Internet]. 20(1) (2018). Available from: https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-018-1620-1.

152. Smith JA, Colbert RA. Review: The Interleukin-23/Interleukin-17 Axis in Spondyloarthritis Pathogenesis: Th17 and Beyond: IL-23/IL-17 Axis in SpA. *Arthritis Rheumatol.* 66(2), 231–241 (2014).

153. Chen L, Al-Mossawi MH, Ridley A, *et al.* miR-10b-5p is a novel Th17 regulator present in Th17 cells from ankylosing spondylitis. *Ann. Rheum. Dis.* 76(3), 620–625 (2017).

154. Zou Y-C, Gao Y-P, Yin H-D, Liu G. Serum miR-21 expression correlates with radiographic progression but also low bone mineral density in patients with ankylosing spondylitis: a cross-sectional study. *Innate Immun.* 25(5), 314–321 (2019).

155. Yang N, Wang G, Hu C, *et al.* Tumor necrosis factor α suppresses the mesenchymal stem cell osteogenesis promoter miR-21 in estrogen deficiency-induced osteoporosis. *J. Bone Miner. Res.* 28(3), 559–573 (2013).

156. Zou Y-C, Yan L-M, Gao Y-P, Wang Z-Y, Liu G. miR-21 may Act as a Potential Mediator Between Inflammation and Abnormal Bone Formation in Ankylosing Spondylitis Based on TNF-α Concentration-Dependent Manner Through the JAK2/STAT3 Pathway. *Dose-Response*. 18(1), 155932581990123 (2020).

157. Murugaiyan G, da Cunha AP, Ajay AK, *et al.* MicroRNA-21 promotes Th17 differentiation and mediates experimental autoimmune encephalomyelitis. *J. Clin. Invest.* 125(3), 1069–1080 (2015).

158. Sherlock JP, Joyce-Shaikh B, Turner SP, *et al.* IL-23 induces spondyloarthropathy by acting on ROR-γt+ CD3+CD4−CD8− entheseal resident T cells. *Nat. Med.* 18(7), 1069–1076 (2012).

159. Prajzlerová K, Grobelná K, Hušáková M, *et al.* Association between circulating miRNAs and spinal involvement in patients with axial spondyloarthritis. *PLOS ONE*. 12(9), e0185323 (2017).

160. Landskroner-Eiger S, Qiu C, Perrotta P, *et al.* Endothelial miR-17∼92 cluster negatively regulates arteriogenesis via miRNA-19 repression of WNT signaling. *Proc. Natl. Acad. Sci.* 112(41), 12812–12817 (2015).

161. Zhao W, Wu C, Dong Y, Ma Y, Jin Y, Ji Y. MicroRNA-24 Regulates Osteogenic Differentiation via Targeting T-Cell Factor-1. *Int. J. Mol. Sci.* 16(12), 11699–11712 (2015).

162. Hassan MQ, Gordon JAR, Beloti MM, *et al.* A network connecting Runx2, SATB2, and the miR-23a 27a 24-2 cluster regulates the osteoblast differentiation program. *Proc. Natl. Acad. Sci.* 107(46), 19879–19884 (2010).

163. Pelosi A, Lunardi C, Fiore PF, *et al.* MicroRNA Expression Profiling in Psoriatic Arthritis. *BioMed Res. Int.* 2018, 1–15 (2018).

164. Lin S-H, Ho J-C, Li S-C, Chen J-F, Hsiao C-C, Lee C-H. MiR-146a-5p Expression in Peripheral CD14+ Monocytes from Patients with Psoriatic Arthritis Induces Osteoclast Activation, Bone Resorption, and Correlates with Clinical Response. *J. Clin. Med.* 8(1), 110 (2019).

165. Laine CM, Joeng KS, Campeau PM, *et al.* *WNT1* Mutations in Early-Onset Osteoporosis and Osteogenesis Imperfecta. *N. Engl. J. Med.* 368(19), 1809–1816 (2013).

166. Mäkitie RE, Haanpää M, Valta H, *et al.* Skeletal Characteristics of WNT1 Osteoporosis in Children and Young Adults. *J. Bone Miner. Res.* 31(9), 1734–1742 (2016).

167. van Dijk FS, Zillikens MC, Micha D, *et al.* *PLS3* Mutations in X-Linked Osteoporosis with Fractures. *N. Engl. J. Med.* 369(16), 1529–1536 (2013).

168. Laine CM, Wessman M, Toiviainen-Salo S, *et al.* A Novel Splice Mutation in *PLS3* Causes X-linked Early Onset Low-Turnover Osteoporosis: *PLS3* MUTATION AND EARLY-ONSET OSTEOPOROSIS. *J. Bone Miner. Res.* 30(3), 510–518 (2015).