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CKJ REVIEW

MicroRNAs: emerging biomarkers and therapeutic targets of bone fragility in chronic kidney disease

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ABSTRACT

Bone fragility is highly prevalent, yet underdiagnosed in patients with chronic kidney disease. Incomplete understanding of the pathophysiology and limitations of current diagnostics contribute to therapeutic hesitation, if not nihilism. This narrative review addresses the question of whether microRNAs (miRNAs) may improve therapeutic decision making in osteoporosis and renal osteodystrophy. miRNAs are key epigenetic regulators of bone homeostasis and show promise as both therapeutic targets and as biomarkers, primarily of bone turnover. Experimental studies show that miRNAs are involved in several osteogenic pathways. Clinical studies exploring the usefulness of circulating miRNAs for fracture risk stratification and for guiding and monitoring therapy are few and, so far, provide inconclusive results. Likely, (pre)analytical heterogeneity contributes to these equivocal results. In conclusion, miRNAs are promising in metabolic bone disease, both as a diagnostic tool and as therapeutic targets, but not yet ready for clinical prime time.

LAY SUMMARY

Bone fragility is a very common complication in patients with chronic kidney disease. Incomplete understanding of the mechanisms and insufficient diagnostic tools lead to undertreatment of bone fragility. MicroRNAs may help to close this treatment gap, which is especially large in patients with advanced chronic kidney disease. MicroRNAs are key epigenetic regulators of bone homeostasis and show promise both as therapeutic targets and as biomarkers of bone diseases. However, more evidence is required before microRNAs are ready for clinical prime time.

Keywords: bone fragility, CKD-MBD, microRNA, osteoporosis, renal osteodystrophy

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BONE FRAGILITY IN CHRONIC KIDNEY DISEASE

Patients with chronic kidney disease (CKD) experience an excessively high fracture burden [1–3]. For instance, the hip fracture risk in patients on dialysis is 4- to 6-fold higher than in age- and gender-matched controls [4, 5]. The fracture risk further increases during the first 3 years after kidney transplantation [6, 7]. Fractures in patients with CKD associate with increased morbidity, mortality and costs, even more so than in non-CKD counterparts [8].

Bone fragility in CKD is the composite of age-related bone loss, and drug-induced and CKD-related bone abnormalities. Primary age-related or postmenopausal osteoporosis may manifest itself at a younger chronological age in patients with CKD, consistent with the notion that CKD is a state of accelerated/premature ageing. Patients with CKD are often treated with a multitude of drugs, many of them with proven or putative detrimental effects in bone. Finally, the uremic environment, characterized by (micro) inflammation, metabolic acidosis, accumulation of uremic toxins, and disturbances in calcium, phosphate, parathyroid hormone (PTH) and vitamin D metabolism causes renal bone disease, commonly referred to as renal osteodystrophy (ROD). ROD encompasses abnormalities in bone turnover (remodeling), mineralization and volume, which alone, or in combination, may impair bone strength. High turnover bone disease, which is essentially the histological expression of secondary hyperparathyroidism, has long been the predominant type of renal osteodystrophy, but in the last two decades low turnover bone disease has become increasingly prevalent in patients on dialysis [9]. Both high and low bone turnover may impair bone strength, though via different mechanisms [10]. Mineralization defects have waned over time and are rather uncommon in contemporary adult patients on dialysis.

The gold standard to evaluate bone disease in CKD is the histomorphometric analysis of a transiliac bone biopsy. Due the invasive nature of the bone biopsy and the limited availability of histomorphometric expertise, bone biopsies are not routine clinical practice. Biomarkers, such as PTH and alkaline phosphatase, can predict the bone turnover status with reasonable accuracy, but with poor precision, leaving much room for improvement.

The incomplete understanding of the complex pathophysiology of bone fragility and limitations of available diagnostics deter the clinician from starting preventive therapy in CKD patients presenting with bone fractures, often resulting in therapeutic nihilism.

The field of epigenetics is rapidly evolving. Beyond assisting in the understanding of disease biology, epigenetics may improve clinical management by providing valuable diagnostic biomarkers and therapeutic targets [11]. Among epigenetic posttranslational modulators, the small non-coding microRNAs (miRNAs) represent a promising group of molecules, since they are involved in virtually all (patho)physiological processes. This review aims to provide an update on the current evidence on the role of miRNAs in bone (patho)biology from a clinical perspective, focusing on their potential as biomarkers and therapeutic targets.

MIRNAS: PROMISING THERAPEUTIC TARGETS AND BIOMARKERS

miRNAs are non-coding, single-stranded RNA molecules composed of approximately 20–24 nucleotides that negatively regulate gene expression. miRNAs bind to the 3′-untranslated region (3′-UTR) region of complementary messenger RNA (mRNA), thereby blocking protein translation or stimulating mRNA degradation (Fig. 1) [12]. Identification of miRNAs follows a standardized nomenclature (Fig. 2). A single miRNA can target hundreds of mRNAs and, inversely, one mRNA can be targeted by many miRNAs [13]. Hence, a complex network between miRNAs and mRNAs fine-tunes genetic expression. Patterns of miRNA expression seem to be tissue-specific and highly conserved between species, highlighting their evolutionary importance [14, 15]. So far, over 2600 mature miRNAs have been identified from the human genome (miRBase V22, http://mirbase.org/) which regulate over half of the ∼30 000 human mRNA transcripts. Dysregulation of miRNAs are reported in and may drive multiple diseases [16–20]. Not only are miRNAs vital intracellular regulators of gene expression, they also exert paracrine and endocrine effects following active cellular uptake of miRNA [21, 22]. Recently, miRNAs have been described as mediators of interorgan crosstalk [23–25]. In the setting of CKD, it is important to notice that circulating miRNAs are not eliminated by hemodialysis [26].

miRNA interference as therapeutic strategy

RNA interference (RNAi) holds tremendous therapeutic potential [27–29]. Insights into the mechanism of miRNA have given rise to synthetic miRNA agonists and antagonists as therapeutics for intravenous, subcutaneous or topical administration [30–32]. Although a vast number of *in vitro* and animal studies have shown high efficacy, clinical implication has been hampered by issues related to stability of the administrated oligonucleotides, delivery to the target cells, and avoiding off-target effects [33]. Molecule stability and cellular delivery have significantly improved with novel techniques, e.g. by modifying the molecular structure, by packaging in nanoparticles or liposome-like particles or by conjugation with cell-specific molecules [34, 35]. However, safety issues related to off-target effects due to the multitude of potential mRNA targets remain a major concern. These safety issues have caused most clinical trials for miRNA therapeutics to be halted already in phase I studies. Recently, phase II clinical trials for promising miR-122 antagomiRs (Miravirsen and RG-101) to treat hepatitis C have been put on hold due to rare cases of hyperbilirubinemia [36]. Currently, miRNA therapeutics for Alport's disease and keloid formation are being investigated in phase II clinical trials [36].

Small interfering RNAs (siRNAs) are similar to miRNAs in size and also silence gene expression through mRNA binding and cleaving. The main differences, however, are that siRNAs are double-stranded RNAs and are more specific to a single mRNA strand with 100% complementarity. Patisiran is the first siRNA to be approved by the US Food and Drug Administration, in August 2018, acting on the liver for the treatment of Hereditary Transthyretin Amyloidosis with polyneuropathy [37]. Recently, other several clinical trials with RNAi by siRNA have shown excellent safety and efficacy in treating several diseases, including acute intermittent porphyria (Givosiran [38, 39]), primary hyperoxaluria type 1 (Lumasiran [40]) and hypercholesterolemia (Inclisiran [41, 42]). The difference in number of targets between siRNA and miRNA may explain the success of siRNA therapeutics. The surging bioinformatics tools for miRNA target prediction and enrichment analyses is expected to improve selecting miRNA for therapeutic implications.

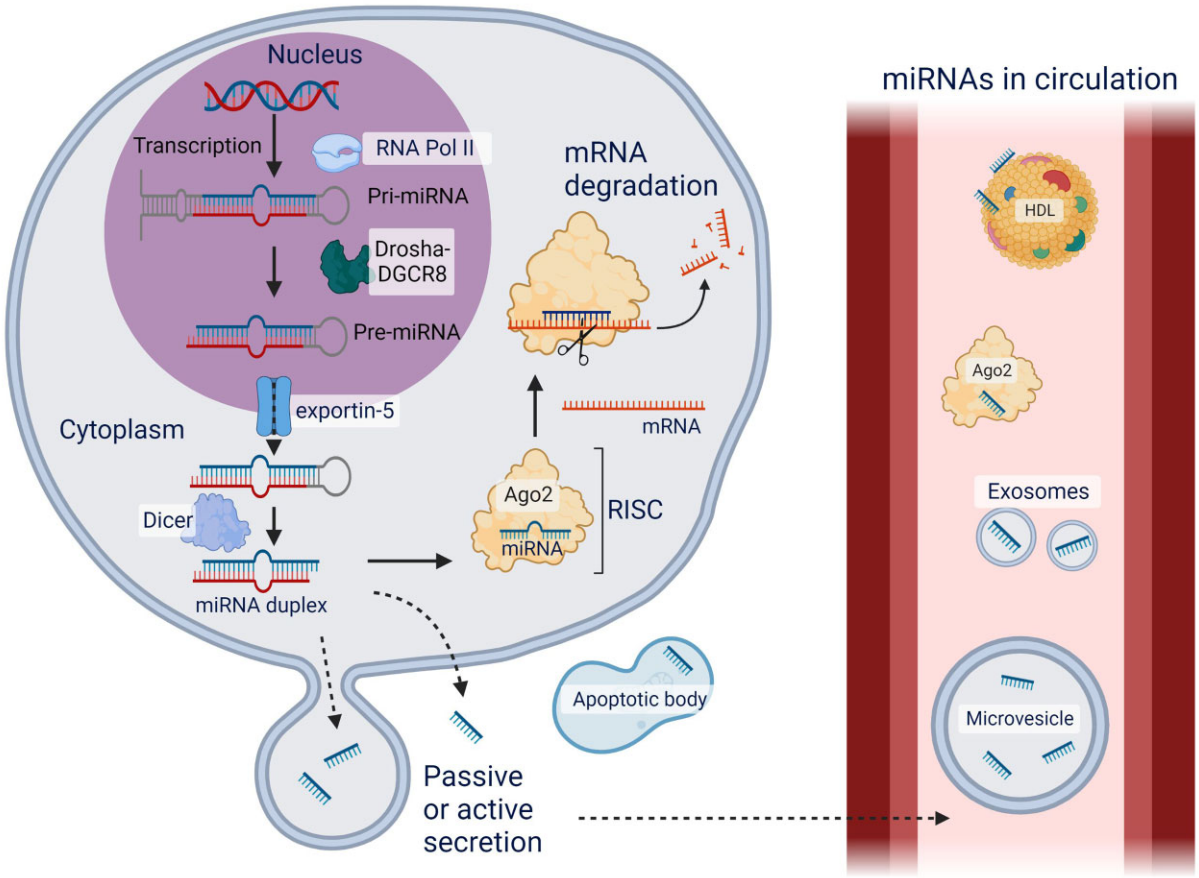

Figure 1: miRNA biogenesis and secretion. The biogenesis of miRNAs starts in the nucleus, where miRNA encoding genes are transcribed by RNA polymerase II into a primary miRNA (pri-miRNA) of several kilobases, containing local stem–loop structures. Pri-miRNA is cleaved at the stem of the hairpin by an RNase III enzyme called Drosha together with its cofactor DiGeorge Syndrome Critical Region 8 (DGCR8), releasing a hairpin structure of approximately 60–70 nucleotides long, termed precursor miRNA (pre-miRNA). Pre-miRNA is then transported by exportin-5 to the cytoplasm and cleaved into the double-stranded miRNA by a second RNase III enzyme called Dicer. The miRNA strand with the less thermodynamically stable 5′ end, termed the guide strand, is selected by the protein Argonaute 2 and integrated into the RNA-induced silencing complex (RISC). The other strand, known as the passenger strand, is degraded by RISC. The mature miRNA in the RISC complex binds to its target mRNA based on sequence-specific binding of 5–7 complementary nucleotides at the 3′-UTR region. This binding mediates mRNA degradation or translational inhibition [137]. Created with BioRender.com.

miRNAS as circulating biomarkers

miRNAs are found in biofluids, packaged within extracellular vesicles (exosomes and microvesicles) or bound to lipoproteins and ribonucleoproteins. Selective secretion and uptake via active cellular transport mechanisms largely determine the composition of circulating miRNAs, while passive leakage of miRNAs from damaged or dead cells also contributes to the pool of extracellular miRNAs [43–48].

Several properties of miRNA advocate circulating miRNAs as excellent candidates for clinical biomarkers. miRNAs are stable in blood as they are protected from endogenous RNases by carriers, can be reliably detected in samples stored for prolonged time, and are resistant to repetitive freeze–thaw cycles [49–52]. Circulating miRNAs, overall, represent tissue-specific levels of expression [53–57]. miRNAs can be quantified with simple, sensitive RT-qPCR assays, while high-throughput techniques such as micro-arrays and RNA sequencing allow to screen for miRNAs of interest or to rapidly analyze patterns of larger miRNA panels. Quick and cheap multi-miRNA panels allow for more accurate risk prediction, diagnostics, treatment guidance and monitoring.

During the last decade, miRNAs have been established as a novel class of highly sensitive circulating biomarkers for multiple metabolic and age-related diseases [46]. Licensed diagnostic miRNA panels are already available for thyroid, pancreatic and breast cancer, cardiovascular disease and Alzheimer, and some are even covered by major insurance companies [34]. OsteomiR™, a panel of 19 plasma miRNAs, has been proposed as a risk predictor for osteoporotic fractures postmenopausal women that is independent of bone mineral density (BMD) [58–60].

Several pitfalls in the analyses of circulating miRNAs have to be acknowledged. Most errors in a clinical chemistry laboratory are due to preanalytical errors. Preanalytical variability of biospecimens can have significant effects on downstream analyses, and controlling such variables is therefore essential [61]. This also holds true for circulating miRNAs. The impact of patient characteristics such as age, sex and body size as well as fluctuations due to diurnal rhythm, food intake and exercise remain to be established. Variability increases with longer bench time before processing, likely due to hemolysis or leakage from blood cells. Therefore, it is of the utmost importance to

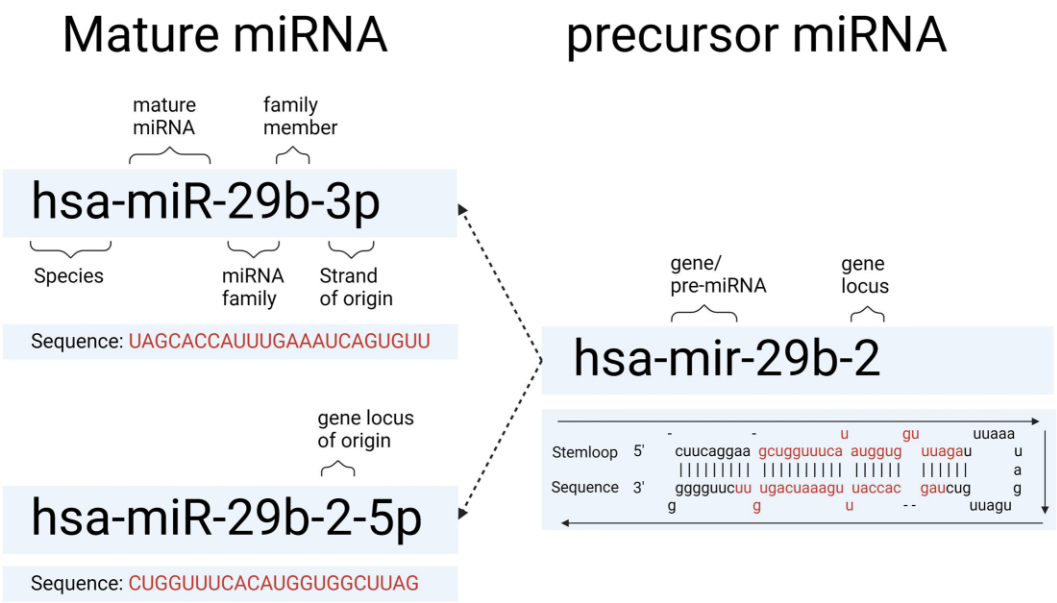

Figure 2: Nomenclature of miRNAs. Both the gene locus and precursor of miRNAs are referred as "mir," while the mature miRNA product is designated "miR." Each miRNA has a unique number in order of discovery, sometimes accompanied by a letter to distinguish miRNAs of the same family (e.g. miR-29a and miR-29b). When the same mature miRNA sequence originates from different loci, an additional number is added to the name of the non-identical precursor miRNA and complementary miRNA strand referring to the locus of origin. Additionally, a three-letter prefix specifies the species (e.g. "hsa" stands for *Homo sapiens*) and a suffix, -3p or -5p, reflects from which strand of the double-stranded pre-miRNA the mature miRNA originates (e.g. hsa-miR-29a-3p). Adapted from miRbase.

standardize the collection, processing, transport and storage to limit variability. Further, analytical variability should be accounted for. Heterogeneity between available commercial assays and the use of different normalization strategies impair reproducibility [62–65]. Awaiting harmonization and standardization of the analytical procedure, extensive reporting of relevant biological, environmental and technical factors should be mandatory.

ROLE OF MIRNAS IN BONE (PATHO)BIOLOGY

Experimental evidence

Bone remodeling encompasses bone formation by osteoblasts and bone resorption by osteoclasts. Bone remodeling is orchestrated by mechanosensory osteocytes to adapt bone to physical stressors. In healthy bone, formation and resorption are in a dynamic equilibrium. Slight deviations in intracellular processes or intercellular communication can impair bone development and remodeling.

The crucial role of miRNAs in bone development and remodeling is demonstrated experimentally by skeletal defects in conditional knockouts of the miRNA-processing enzymes Drosha and Dicer [66–73]. Numerous miRNAs have been found to play a key role in the differentiation of osteoblasts and osteoclasts, as has been described in recent reviews [19, 74, 75]. However, only few miRNAs have been described in clinical osteoporosis or renal osteodystrophy. Here we describe how these miRNAs are involved in established molecular pathways of bone cell development and function (Fig. 3).

Osteoblastogenesis

Osteoblastogenesis is the differentiation of bone marrow–derived pluripotent mesenchymal stem cells (BMSCs) into mature osteoblasts. This differentiation process is orchestrated by upregulation of master transcription factors Osterix (Osx) and Runt-related transcription factor 2 (Runx2) and their downstream signaling cascades including Transforming growth factor-beta (TGF-β)/bone morphogenic protein (BMP) and Wnt/β-catenin pathways. The vital role of miRNAs during osteoblastogenesis and involvement in osteogenic pathways has been well documented [75].

Runx2 is targeted by several miRNAs resulting in repression of osteoblastogenesis. *In vitro*, overexpression of miR-30c [76], miR-133a [77], miR-204/211 [78] or miR-23b [79] has been shown to repress the Runx2 protein expression and osteoblast maturation of BMSCs while inhibition of these miRNAs showed opposite effects. Additionally, luciferase reporter assays demonstrated binding of these miRNAs to the *Runx2* 3′-UTR. *In vivo*, miR-23b agonists impeded osteogenic differentiation of human BMSCs and induced severe osteoporosis in mice [79]. TNF-α, an established inhibitor of osteoblast differentiation, induces miR-23b expression [79]. Thus, miR-23b may be involved in TNF-α-mediated reduction of BMSC osteogenesis by targeting *Runx2*.

Conversely, miRNAs may promote osteoblastogenesis by targeting inhibitors of the Runx2 pathway. For example, miR-29a tempered Runx2 degradation by targeting Histone deacetylase 4 (*HDAC4*) and as such promoted osteogenic differentiation of murine preosteoblasts [80] and human MSCs [81].

Some miRNAs have been shown to affect WNT/β-catenin signaling, regulating key cellular functions including proliferation, migration and apoptosis. miR-23a inhibits osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by targeting the Wnt receptor *LRP5* [82]. Other miRNAs target inhibitors of WNT/β-catenin signaling such as Dikkopf-1 (*DKK1*) and secreted frizzled-related protein 1 (*SFRP1*). Targeting of *DKK1* by miR-203 [83, 84], miR-335–5p [85, 86], and miR-433-5p [87] promoted osteogenic differentiation and survival *in vitro* and *in vivo*.

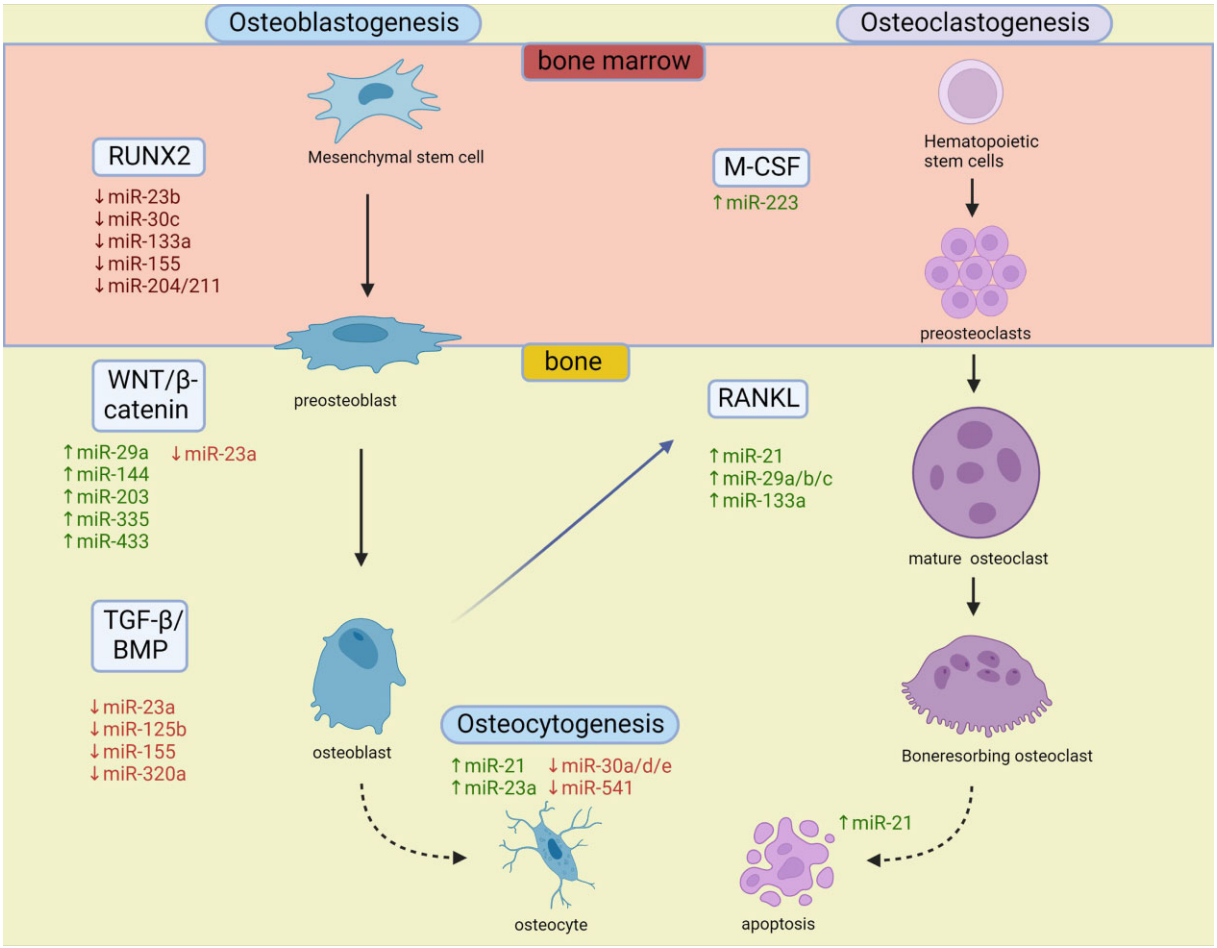

Figure 3: miRNA-mediated regulatory network of cellular differentiation. This figure displays a non-exhaustive list of miRNAs that have been experimentally shown to be involved in molecular pathways in the differentiation of mesenchymal stem cells into osteoblasts (osteoblastogenesis) and further differentiation into osteocytes (osteocytogenesis) and in the differentiation of hematopoietic stem cells into osteoclasts (osteoclastogenesis). RANKL: receptor activator of nuclear factor kappa-B ligand; RUNX2: Runt-related transcription factor 2; TGF-β: Transforming growth factor beta; WNT: Wingless/Integrated. Created with BioRender.com.

miR-144 promoted proliferation and osteoblastic differentiation of BMSCs by targeting *SFRP1* [88].

TGF-β/BMP signaling is crucial for osteoblastic differentiation and homeostasis. Binding of a BMP to the type II receptor (BMP2R) results in recruitment and phosphorylation of a BMP type I receptor, which in turn phosphorylates an R-SMAD transcription factor to mediate downstream pathways. miR-155 inhibited BMP-induced osteogenic differentiation of MSCs both *in vitro* and in nude mice by targeting *BMPR2*, but also *Runx2* [89]. BMP receptor type 1b (BMPR1b) was downregulated by miR-125b, hampering osteogenic differentiation of human BMSCs (hBMSCs) [90]. Additionally, transfection of hBMSCs with a miR-125b inhibitor improved skeletal regeneration in rats [90]. miR-23a [91] and miR-320a [92] were reported to inhibit osteogenic differentiation of hMSCs by silencing downstream mediators of the BMP pathway Mitogen-activated protein kinase 13 (MAPK13) and Homeobox a10 (HOXA10), respectively.

Osteocytogenesis

Osteocytes are terminally differentiated cells of osteoblasts and are the most abundant cell type in bone. Osteocytes reside in bone lacunae and act as essential mechanosensory regulators of bone remodeling. Molecular mechanisms behind osteocytogenesis and osteocyte function remains poorly understood, mainly due to the difficulty of accessing osteocytes deeply entrapped in the mineralized bone matrix and their low mitogenic activity [93]. With recent advances of high-resolution microscopic technologies and high-throughput molecular screening, as well as the development of cell- and tissue-specific transgenic animals, molecular pathways, including miRNAs, are beginning to be uncovered.

Depending on stage of osteogenic differentiation, the action of miRNAs may differ. For instance, miR-30b/c inhibited early osteoblastic differentiation, while miR-30a/d/e inhibited late osteocytogenesis in murine and human MSCs [94]. Gain-of-function mutation of miR-23a cluster (miR-23a~27a~24-2) stimulated osteocytogenesis, by silencing *Prdm16*, a negative regulator of TGF-β, while reducing bone mass and osteoblast count in mice [95]. Apoptosis of osteocytes, a sign of aging bone, was shown to be induced by connexin43 deficiency mediated by miR-21 downregulation and subsequent upregulation of its target phosphatase and tensin homolog (PTEN), an apoptotic gene [96].

Osteoclastogenesis

Osteoclastogenesis encompasses the fusion of bone marrow monocyte-macrophage precursors to form the bone-resorbing

Table 1: Bone tissue miRNAs in postmenopausal and idiopathic osteoporosis.

Ref.	Study type	Country	Study population	Tissue sample	Outcomes	No. of miRNAs	Correlation miR—outcome
Seeliger *et al.* 2014 [55]	Cross-sectional	Germany	OP hip Fx ($n = 20$) Non-OP Fx ($n = 20$)	Femoral bone	Osteoporosis	13	↑ miR-21, miR-23a, miR-24, miR-25, miR-100, and miR-125b
Kelch *et al.* 2017 [112]	Cross-sectional	Germany	OP hip Fx ($n = 14$) (male $n = 7$) OA undergoing HRS ($n = 14$) (male $n = 7$)	Femoral bone	OP hip Fx vs osteoarthritis	9	↑ miR-21-5p,miR-24-3p, miR93-5p, miR-100-5p, miR-125b-5p—Fx ↑ miR-21-5p, miR-23a-3p, miR-24-3p, miR93-5p, miR-100-5p, miR-125b-5p—low BMD
De-Ugarte *et al.* 2016 [111]	Cross-sectional	Spain	PMOP hip Fx ($n = 6$) Non-OP OA undergoing HRS ($n = 6$)	Femoral bone, osteoblasts	PMOP hip Fx vs OA	Screening: 1932 Validation: 8	82 miRs differentially expressed. ↑ miR-320a and miR-483-5p in validation PCR test and expressed in osteoblasts from human knee tissue
Garmilla-Ezquerra *et al.* 2015 [110]	Cross-sectional	Spain	Screening: PMOP hip Fx ($n = 8$) OA undergoing HRS ($n = 8$) Validation: PMOP hip Fx ($n = 19$) OA undergoing HRS ($n = 19$)	Femoral bone	PMOP hip Fx vs OA	Screening: 760 Validation: 6	Screening: ↑ 7 miRs and ↓ 5 miRs Validation: ↑ miR-518f and ↓ mir-187
Gautvik *et al.* 2020 [113]	Cross-sectional	Norway	PM women ($n = 84$)	Iliac bone ($n = 84$) Femoral bone ($n = 18$)	Total hip BMD, previous Fx	758	Iliac: 75 ncRNAs—BMD (TH) 28 ncRNAs—previous Fx Femoral: 94 ncRNAs—BMD (TH)

Fx: fracture; HRS: hip replacement surgery; OA: osteoarthritis; OP: osteoporosis; PM: postmenopausal; PMOP: postmenopausal osteoporosis; TH: total hip.

osteoclasts. This differentiation process is driven by macrophage colony-stimulating factor-1 (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). Osteoclast overactivity triggers a negative bone balance and can lead to osteoporosis, while osteoclast dysfunction can lead to brittle bone. The role of miRNAs in osteoclastogenesis and bone resorption has been extensively described [97].

During RANK-L-induced osteoclastogenesis, miR-21 [98] and miR-133a [99] are upregulated. miR-21 extends the life span of osteoclasts by targeting programmed cell death 4 (*PDCD4*) [98] and Fas ligand [100], both involved in osteoclast apoptosis. *In vivo*, miR-21 deficiency attenuated bone resorption in mice [101]. Injection of a miR-133a antagonist increased lumbar BMD in ovariectomized rats [99, 102]. miR-223 promoted osteoclastogenesis by targeting the transcriptional repressor Nuclear Factor IA (*NFIA*), an inhibitor of M-CSF [103]. Suppression of miR-223, either indirectly by exposure to high phosphate levels or directly by an antagomiR, inhibited osteoclastogenesis in monocyte/macrophage-like cells and human osteoclastic-differentiated peripheral blood mononuclear cells [103]. miR-155 is induced by TGF-β/Smad4 [104] and Interferon-β [105] and in turn inhibits osteoclastogenesis by targeting suppressor of cytokine signaling-1 (*SOCS1*) and microphthalmia-associated transcription factor (*MITF*), two essential regulators of osteoclastogenesis. Members of the miR-29 family overall stimulate osteoclastogenesis by various interactions [106]. However, discrepant findings have been reported [107, 108]. For example, a miR-29a gain-of-function reduced osteoclast surface and RANKL expression and protected against glucocorticoid-induced bone loss in rats. Most likely, differences in experimental set-up and interspecies variability explain these heterogeneous findings [109].

Clinical evidence

To define the role of miRNAs in clinical bone (patho)biology, studies investigating miRNA expression in human bone tissue are necessary. These studies are scarce, most probably due to the invasive nature of human bone sampling and need for specific tissue preservation (Table 1) [55, 110–113]. Of note, studies exploring bone tissue miRNAs in patients with CKD are lacking at present.

Seeliger *et al.* collected femoral bone tissue during surgical treatment for hip fractures in patients >50 years with or without underlying osteoporosis and analyzed a panel of 11 miRNAs that were upregulated in serum of osteoporotic patients and two additional miRNAs, miR-93 and miR-637, which are known to be associated with bone development [55]. Underlying osteoporosis correlated with upregulation of six miRNAs on bone tissue level, namely miR-21, miR-23a, miR-24, miR-25, miR-100 and miR-125b. In a separate cohort evaluating nine of the upregulated circulating miRNAs, Kelch *et al.* confirmed upregulation of miR-21-5p, miR-93-5p, miR-100-5p and miR-125b-5p in serum, in trabecular tissue of the femoral neck, and in isolated osteoblasts and osteoclasts from patients with osteoporotic hip fractures, compared with patients undergoing hip replacement surgery for osteoarthritis [112]. Furthermore, these four miRNAs correlated negatively with BMD and, except for miR-125b-5p, were gender-independent [112].

Gautvik *et al*. compared miRNA profiles of weight-bearing femoral bone with non-weight-bearing iliac bone. Comparison between the two sites revealed large discrepancies with minor overlap in miRNAs correlated with total hip BMD and calculated fracture risk (FRAX) score [113]. These findings suggest a differential bone metabolism in weight bearing and non-weight bearing bone and highlight the importance of the sampling site when interpreting results.

While these pilot studies paved the way for miRNA epigenetics in osteoporosis, results should be interpreted cautiously. First, the abovementioned studies recruited patients undergoing surgery for a hip fracture. It should be noted that the impact of a recent fracture on miRNA profiles is unknown. It thus remains an open question whether the discriminating miRNA (signatures) are in the causal pathway of bone fragility (and as such represent interesting targets of therapy), or whether they merely reflect the physiological bone response to fracture. Second, patients with osteoarthritis are not ideal controls as this condition in itself may be hypothesized to associate with a specific bone miRNA phenotype. Furthermore, these studies investigated whole bone samples, and miRNA profiles may differ according to bone type, location and compartment (bone marrow, cortical bone, trabecular bone). Future studies should investigate miRNA expressions in different bone compartments and sampling sites. Bone marrow is often seen as an intermediate between circulating miRNA and bone miRNA, and warrants extra investigation. A uniform method of tissue sampling and storage needs to be established.

Recent technological advancements can further advance miRNA research in bone pathology. Newer generations of trephines allow for an easily accessible and minimally invasive method to retrieve bone tissue [114]. Further refinement of molecular diagnostics, such as single cell transcriptomics and multi-omics, will undoubtedly advance the field and allow to expand pathophysiological knowledge. This pathophysiological knowledge will potentiate the first clinical studies regarding miRNA therapeutics in bone disease.

CIRCULATING MIRNAS AS BIOMARKERS IN METABOLIC DISEASE

Circulating miRNAs in osteoporosis

Specific circulating miRNAs (signatures) have been repeatedly associated with osteoporosis [55, 115, 116] and BMD [59, 99, 117, 118] (summarized in Table 2). Circulating miRNAs discriminate fracture status [60, 112, 118, 119], some with high accuracy (area under the curve > 0.9) [59, 120]. Prospective studies exploring the miRNAs as risk biomarkers for incident fractures are scanty and have so far yielded disappointing results [121, 122].

In a comprehensive study, Kocijan *et al*. investigated miRNA signatures in premenopausal and postmenopausal women, as well as in men [59]. They identified 19 miRNAs that consistently discriminated fracture status. Some of these miRNAs showed significant correlations with bone biomarkers (miR-29b-3p with P1NP and miR-365-5p with iPTH, TRAP5b, P1NP and Osteocalcin), and with lumbar spine BMD (miR-19b-3p, miR-324-3p, miR-532-5p and miR-93-5p) [59]. In a follow-up study, some of these miRNAs also correlated with histomorphometric parameters of bone formation and with bone microstructure; miR-9b-3p, miR-324-3p and miR-550a-3p were most informative [123].

Circulating miRNAs may also be of interest to monitor the response to pharmacological therapy. Both anabolic and antiresorptive therapy may modify circulating miRNA profiles, sometimes parallel to changes in biochemical bone turnover markers and BMD [124]. Antiresorptive treatment with denosumab induced changes in circulating miRNAs and, as would be expected, the response was most pronounced in the subgroup of patients previously treated with anabolic teriparatide [125]. An *in silico* study suggests that the effect of teriparatide on genes is most likely mediated by miR-146a-5p, miR-205-3p, miR-33a-3p, miR-338-5p and miR-410-3p [126].

Results, overall, are not unequivocal as is illustrated by the case of miR-21. Both positive [55, 112, 119, 120] and negative associations [59, 115, 116] between miR-21 and osteoporosis have been reported. miR-21 shows positive correlations with inflammation, ageing and various cancers, and is often seen as a universal biomarker of disease state [127]. Mechanistically, miR-21 functions as a modulator in multiple pathways maintaining inflammation while simultaneously preventing overwhelming inflammatory activation [127], but more specifically, miR-21 mediates RANKL-induced osteoclastogenesis [98] and inhibits osteoclast apoptosis [100]. Consistently, antiresorptive treatment reduced serum levels of miR-21, while miR-21 was upregulated in patients treated with teriparatide [125].

Circulating miRNAs in renal osteodystrophy

The clinical need for biomarkers predicting fracture risk and turnover in CKD is high. Studies exploring the role of miRNAs in ROD are scanty (Table 3).

In a pilot study, Jeong *et al*. investigated miRNAs with unselected micro-array in peritoneal dialysis patients with normal/high PTH (>150 pg/mL) or low PTH (<150 pg/mL). They found 165 miRNAs to be differentially expressed between low and normal/high PTH [128]. As the main driver for bone remodeling, PTH is often used as a surrogate marker for bone turnover. Whether these miRNAs truly reflect bone turnover remains to be demonstrated in studies assessing bone turnover by specific biomarkers or, ideally, bone histomorphometry.

In a case–control study comparing hemodialysis patients with matched controls, Yavropoulou *et al*. investigated a panel of seven circulating miRNAs that were dysregulated in their previous postmenopausal osteoporosis cohort (cfr. [116]). This study showed significantly lower serum levels of miR-21-5p, miR-23a-3p and miR-124-3p in hemodialysis patients compared with control counterparts [129]. Further analyses revealed an independent correlation between miR-23a-3p and trabecular bone score measured with dual energy X-ray absorptiometry, after adjustment for BMD values and parameters of calcium metabolism. miR-124-3p was correlated with low BMD showing 78% sensitivity and 83% specificity. Interestingly, miR-124-3p was also correlated with BMD in postmenopausal osteoporosis [124].

To the best of our knowledge, only Nickolas *et al*. have investigated the correlation of circulating miRNAs with parameters of bone turnover as assessed by bone histomorphometry [56]. In patients with CKD 3–5D, a panel of four miRNAs involved in osteoblast (miR-30b, miR-30c and miR-125b) or osteoclast (miR-155) development outperformed a panel of traditional biochemical biomarkers, including PTH, bone-specific alkaline phosphatase and CTx, in differentiating between low and normal/high bone turnover in cortical bone. In clinical practice, bone turnover is not measured in cortical bone, but in trabecular bone only, for which the traditional biomarker panel performed better. However, whether cortical or trabecular bone histomorphometry should be the clinical gold standard is an ongoing discussion. The authors advocate that cortical bone may have more biological validity in ROD, as hyperparathyroid

Table 2: Circulating miRNAs in postmenopausal and idiopathic osteoporosis.

Ref.	Study type	Country	Study population	Tissue sample	Outcomes	No. of miRNAs	Correlation miR—Outcome
Li *et al.* 2014 [115]	Cross-sectional	China	120 PM women: osteoporosis ($n = 40$), osteopenia ($n = 40$), control ($n = 40$)	Plasma	Low vs normal BMD (LS/TH)	3	↑ miR-133a—low BMD ↓ miR-21–low BMD miR-146a no correlation
Mandourah *et al.* 2018 [118]	Cross-sectional	England	Osteoporosis ($n = 51$), osteopenia ($n = 76$), control ($n = 12$)	Plasma and serum	Fragility Fx, BMD	Screening (pooled): 370 Validation: 29	↓ miR-122-5p and miR-4516—low BMD, Fx in history
Yavropoulou *et al.* 2017 [116]	Cross-sectional	Greece	PM women, low BMD ($n = 70$), controls ($n = 30$)	Serum	Low vs normal BMD (LS/TH/FN), Fx in history	14	↑ miR-124-3p, miR-2861—low BMD ↓ miR-21-5p, miR-23a, miR-29a-3p—low BMD ↓ miR-21-5p—vertebral Fx
Kocijan *et al.* 2016 [59]	Cross-sectional	Austria	36 patients with fragility Fx (peripheral/vertebral, >6 mo): premenopausal ($n = 10$), postmenopausal ($n = 10$), male ($n = 16$), matched controls ($n = 39$)	Serum	Fragility Fx in history, BMD, BTMs	187	↑ miR-152-3p, miR-335-5p, miR-320a—Fx ↓ 16 miRNAs—Fx ↑ miR-29b-3p—P1NP ↑ miR-365-5p—iPTH, TRAP5b, P1NP and Osteocalcin ↑ miR-19b-3p, miR-324-3p, miR-532-5p and miR-93-5p—BMD (LS)
Feichtinger *et al.* 2018 [123]	Cross-sectional	Austria	Cohort from Kocijan *et al.* 2016	Serum	Bone microstructure (μCT) and histomorphometry based (bone biopsy)	19	↑ miR-29b-3p, miR-324-3p and miR-550a-3p—MAR ↑ miR-29b-3p—BFR/BS ↑ miR-550a-3p, miR-7-5p—BS/BV ↓ miR-550a-3p and let-7b-5p—BV/TV ↑ miR-335-5p and miR-140-5p—ct.po. ↓ miR-29b-3p and miR-324-p—anti-resorptive therapy
Seeliger *et al.* 2014 [55]	Cross-sectional	Germany	Women >50 y, surgery for hip Fx Screening: with OP ($n = 10$), controls ($n = 10$) Validation: with OP ($n = 30$), controls ($n = 30$)	Serum	OP vs no OP in patients with hip Fx	Screening (pooled): 83 Validation: 11	↑ miR-21, miR-23a, miR-24, miR-93, miR-100, miR-122a, miR-124a, miR-125b, and miR-148a—osteoporosis
Li *et al.* 2018 [99]	Cross-sectional	China	PMOP ($n = 10$), PM controls ($n = 10$)	Serum	Correlation BMD (LS)	1	↓ miR-133a—lumbar BMD (Spearman $r^2 = 0.84$)
Bedene *et al.* 2016 [117]	Cross-sectional	Slovenia	PMOP ($n = 17$), PM control ($n = 57$)	Plasma	BMD, TBS, FRAX	9	↑ miR-148a—osteoporosis ↑ miR-126-3p—BMD of distal radius ↓ miR-423-5p—FRAX
Weilner *et al.* 2015 [60]	Cross-sectional	Austria	Screening: PMOP hip Fx ($n = 7$), PM control ($n = 7$) Validation: PMOP hip Fx ($n = 12$), PM control ($n = 11$)	Serum	Recent OP hip Fx	Screening: 175 Validation: 6	Screening: ↑ miR-10a-5p, miR-10b-5p, miR-22-3p ↓ miR-133b, miR-328-3p, let-7g-5p Validation: ↓ miR-22-3p, miR-328-3p, let-7g-5p
Heilmeier *et al.* 2016 [120]	Cross-sectional	USA	PM women: DM+ Fx+ ($n = 20$), DM+ ($n = 20$), Fx+ ($n = 20$), control ($n = 20$)	Serum	Fragility Fx in PM women with and without DM type 2	375	↑43 miRs, ↓3 miRs—Fx in DM+ ↑ 4 miRs, ↓19 miRs—Fx in DM– ↑ miR-550a-5p, miR-203a and miR330-3p, ↓ miR-382-3p, miR-1908 and miR-369-3p—Fx in both groups

Table 2: Continued.

Ref.	Study type	Country	Study population	Tissue sample	Outcomes	No. of miRNAs	Correlation miR—Outcome
Panach *et al.* 2015 [119]	Cross-sectional	Spain	Screening: PMOP hip Fx ($n = 8$), HRS for OA ($n = 5$) Validation: PMOP hip Fx ($n = 15$), HRS for OA ($n = 12$)	Serum	PMOP hip Fx vs osteoarthritic HRS	Screening (pooled): 179 Validation: 6	Screening: ↑ 7 miRs, ↓ 5 miRs Validation: ↑ miR-122-5p, miR-125b-5p, and miR-21-5p—PMOP Fx
Feurer *et al.* 2019 [121]	Longitudinal	France	Premenopausal ($n = 99$), postmenopausal ($n = 583$)	Serum	Fragility Fx (prevalent and incident), BTMs, BMD, microarchitecture (HRpQCT)	32	22 miRNAs—prevalent Fxs 21 miRNAs—osteocalcin 26 and 19 miRNAs—BMD (FN and hip, resp.) 27 and 29 miRNAs—radius and tibia vBMD, resp. ↓ miR-145-5p and miR-503-5p—incident Fxs All associations were lost after correction for age
Anastasilakis *et al.* 2018 [124]	Longitudinal	Greece	PMOP women: treated with teriparatide ($n = 30$), treated with denosumab ($n = 30$)	Serum	Relative expression changes during treatment and association with BMD and BTMs	16	Teriparatide: ↓ miR-33-3p and miR-133a ↓ miR-124a—aBMD ↓ miR-24 and ↑ miR-27a—β-CTX ↓ miR-24–P1NP Denosumab: ↓ miR-21-5p, miR-23a-3p, miR-26a-5p, miR-27a, miR-222-5p, and miR-335-5p—β-CTX
Yavropoulou *et al.* 2020 [125]	Longitudinal	Greece	PMOP women treated with denosumab for 12 months and previously: treatment-naïve ($n = 11$), on zoledronate ($n = 6$), on teriparatide ($n = 20$)	Serum	Relative expression changes during treatment	7	At baseline, ↓ miR-21a-5p in zoledronate group and ↑ miR-21a-5p, miR-23a-3p, miR-29a-3p, and miR-338-3p in teriparatide group Denosumab treatment: ↓ miR-21a-5p, miR-338-3p and miR-2861 in the whole cohort and additionally ↓ miR-29a-3p in the subgroup previously on teriparatide
Kelch *et al.* 2017 [112]	Cross-sectional	Germany	OP hip Fx ($n = 14$) (male $n = 7$) HRS for OA ($n = 14$) (male $n = 7$)	Serum	OP hip Fx vs osteoarthritis	9	↑ miR-21-5p, miR-23a-3p, miR-24-3p, miR93-5p, miR-100-5p, miR-122-5p, miR-124-3p, miR-125b-5p, and miR-148a-3p—Fx miR-125b-5p—gender-dependent

BFR: bone formation rate; BS: bone surface; BTM: bone turnover marker; BV: bone volume; ct.po.: cortical porosity; FN: femoral neck; FRAX: fracture risk assessment tool; Fx: fracture; LS: lumbar spine; MAR: mineral apposition rate; OA: osteoarthritis; OP: osteoporosis; PM: postmenopausal; PMOP: postmenopausal osteoporosis; TH: total hip; TV: total volume.

bone disease affects trabecular and cortical bone differently with important cortical porosity and thinning [9, 130–133]. Additionally, normative reference data for cortical bone is lacking, therefore the authors arbitrarily defined cortical low turnover by lowest tertile of intracortical bone formation rate/bone surface (BFR/BS).

In aggregate, circulating miRNAs emerge as promising candidate biomarkers for diagnosis, prognosis or response to therapy in metabolic bone diseases. Nevertheless, evidence remains fragmentary, and thus should be considered premature. Furthermore, most studies suffer from major limitations including small sample size, missing or inappropriate controls, lack of a validation cohort or failure to correct for multiple testing. While several panels of circulating miRNAs are suggested as alternative or adjunct to BMD or FRAX score as risk predictor of osteoporotic fractures, robust validation is still missing. In the

Table 3: Circulating miRNAs in renal osteodystrophy.

Ref.	Study type	Country	Study population	Tissue sample	Outcomes	No. of miRNAs	Correlation miR—outcome
Nickolas *et al.* 2020 [56]	Cross-sectional	USA	Patients with CKD 3–5D ($n = 23$)	Serum	Histomorphometric parameters of low bone turnover, BTMs	4	↓ miR-125b, miR-155, miR-30b, miR-30c—low cortical bone turnover vs non-low turnover
Yavropoulou *et al.* 2020 [129]	Cross-sectional	Greece	Hemodialysis patients ($n = 30$), controls ($n = 30$)	Serum	CKD vs non-CKD, TBS, BMD	7	↓ miR-21-5p, miR-23a-3p, miR-124-3 in CKD ↓ miR-23a-3p—TBS ↓ miR-124-3p predictive for low BMD (FN)
Jeong *et al.* 2017 [128]	Cross-sectional	South Korea	Patients on PD Screening: low iPTH ($n = 7$), high iPTH ($n = 8$) Validation: low iPTH ($n = 11$), high iPTH ($n = 41$)	Whole blood	iPTH	Screening: 1918 Validation: 3	Screening: 165 miRNAs differently expressed Validation: ↓ miR-3680-5p in low iPTH

BTM: bone turnover marker; FN: femoral neck; iPTH: intact parathyroid hormone; PD: peritoneal dialysis; TBS: trabecular bone score.

setting of renal osteodystrophy, circulating miRNA may fill an unmet clinical need as biomarker for bone turnover. Moreover, circulating miRNAs may also elucidate aspects of bone pathology that are not reflected by current biomarkers. A panel of circulating miRNAs in adjunct to current biochemical bone turnover markers may markedly improve clinical insight in bone health and turnover status of an individual patient with CKD and help direct therapeutic options without the need of bone biopsy. Future studies with larger cohorts, prospective data and unselected miRNA screening using micro-arrays or next genome sequencing are needed as a next step to identify miRNAs of interest in ROD. Analysis of miRNA in bone tissue is much needed to help understand the complex bone pathobiology in ROD.

MIRNA AS TREATMENT TARGET IN METABOLIC BONE DISEASE

One of the largest challenges in developing miRNA-based therapeutics is to identify the best miRNA candidates for a specific condition. While current knowledge of the microRNome in osteoporosis and renal osteodystrophy is fragmentary, emerging high-throughput analytical techniques and bioinformatics for pathway analyses will enable revelation of which miRNAs are "drivers" and which miRNAs are "bystanders" in these disease states. Another challenge is organ-specific targeting. Delivery systems targeting bone formation [134, 135] and bone resorption surfaces [136] have found success in experimental settings but have yet to be clinically validated. Alternatively, taking inter-organ communication into account, miRNAs originating from other organs may be targeted to alleviate bone health. Especially in renal osteodystrophy, where current therapies fail to come to the expectations, novel therapeutic pathways are worth exploring.

CONCLUSION

Epigenetics and more specifically miRNAs rapidly gain interest in basic and clinical research, also in the field of metabolic bone diseases and in CKD. miRNAs may close knowledge gaps in the pathophysiology of osteoporosis and ROD, and may identify novel therapeutic targets. Current evidence, though fragmentary, is promising and calls for additional studies. Tackling the analytical heterogeneity thereby should be a priority. miRNAs have the potential to advance clinical decision making and ultimately improve outcomes in CKD-mineral and bone disorders, but are not ready yet for clinical prime time.

DATA AVAILABILITY STATEMENT

No new data were generated or analysed in support of this research.

CONFLICT OF INTEREST STATEMENT

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