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MicroRNAs in ankylosing spondylitis: Function, potential and challenges

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A B S T R A C T

Epigenetic mechanisms such as DNA methylation, histone modifications and non-coding RNA, are considered the essential connection between a disorder's onset and the environment, on a permissive genetic background. Among autoimmune and inflammatory-mediated disorders, Ankylosing Spondylitis (AS), a chronic arthritis of the spine, is a very good example for the weight of epigenetics' contribution. MicroRNAs (miRNAs) are single-stranded nucleotides which regulate gene expression and are involved in pathological and physiological processes. In this manuscript we provide a clarification on the role of microRNAs in AS, with a focus on the mechanisms of pathogenesis. In specific, we have examined the contribution of miRNAs in the processes of inflammation, new bone formation and T-cell function, and the pathways (i.e. Wnt, BMP, TGFβ signalling etc.) they regulate. The utility of miRNAs in better understanding AS pathogenesis is undisputed and their utility as therapeutic opportunity is strongly increasing.

1. Introduction

Ankylosing Spondylitis (AS) is a chronic autoimmune-mediated rheumatic disease, typically presenting before the age of 45, affecting the sacroiliac joints, the spine and the peripheral joints [1]. Inflammation is likely to occur primary at the entheses, the connective tissues between bones and tendons, where immune cells, particularly lymphoid cells, localize, expand, polarize and promote the production of interleukin (IL)-17, tumor necrosis factor-alpha (TNF-α), interferon gamma and other cytokines [2,3]. In addition to inflammation, new bone formation occurs, mediated by Wingless pathway proteins and other signalling cascades, possibly as an attempt to repair bone damage [4]. It typically involves entheses, leading to syndesmophytes formation and ligaments ossification. Distinctive manifestations of AS are inflammatory back pain, impaired motility and in more severe cases complete fusion of the spine occurs, the so called 'bamboo spine' seen on radiographs [5]. Extra-articular manifestations of AS include, among others, acute interior uveitis, inflammatory bowel disease and psoriasis. The diagnostic approach includes clinical, genetic, laboratory and imaging features [6], in order to allow an early diagnosis and a prompt treatment to control inflammation and prevent ankylosis. The appropriate management combines physical and pharmacological treatment [7]. Among available drugs, the blockade of TNF-α is the most-well established therapy in AS. In recent times, targeting IL-17A (i.e. secukinumab) confirms to be also valid in those patients which are unresponsive to TNF-α blockers. Thanks to immunogenetics studies the IL23/IL17A axis has been confirmed to have a key role in AS pathogenesis. This leads to the ongoing development of new compounds targeting different molecules of the pathway (i.e. the p40 subunit of IL-12/IL-23, antibodies blocking RORγt etc). However, a high proportion of patients still has an inadequate response, or loses efficacy after time, due to unknown mechanisms [8].

2. Immunopathogenesis of AS

AS has a strong genetic predisposition and, across common human diseases, has the highest rate of association with the class I human leukocyte antigen B27, HLA-B27. Several subtypes of HLA-B27, including B*2702, B*2703, B*2710, are reported to increase the risk to develop AS. The several causative variants of B27 are known to modify the biochemical structure of the protein and lead to an altered conformation of the HLA heavy chain or to a distorted presentation of peptides [9].

However, the pathogenic role of HLA-B27 remains unclear. Three main theories are supposed to explain the role of HLA-B27 in AS pathogenesis [10]: 1) the arthritogenic peptide theory, which proposes a central role of HLA-B27 in presenting peptides to autoreactive $CD8^+$ cytotoxic T-cells; 2) the second theory is the misfolding of HLA-B27 in the

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Endoplasmic Reticulum (ER), which causes ER stress and consequently activates the unfolded protein response (UPR), leading to an increase of inflammatory cytokines (including IL23); 3) the last theory proposes the aberrant expression of cell-surface HLA-B27 (abnormally folded to form homodimers or b2 microglobulin-free heavy chains) recognized by KIR-NK (killer-like receptor, Natural killer) cells or $CD4^+$ T-cells promoting type 17 immune response.

It is likely that these three theories may contribute together to develop AS, but other unknown mechanisms may contribute.

3. Genetic predisposition in AS

The positivity for HLA-B27 explains only the 30% for the heritability of the disease, which is estimated in roughly 90% [11]. This means that HLA-B27 contributes for a minority of the total genetic risk. In 2013 and 2015 respectively, an Immunochip study and a cross-disease phenotype study (performed in five clinically related immune mediated diseases) identified more than 100 genomic loci associated with AS: many of them are located in regulatory region of the genome and they may contribute to AS susceptibility [12,13]. Among these non-HLA genes, a strong association for the Endoplasmic Reticulum Aminopeptidase-1 (ERAP-1), ERAP-2 and the related LNPEP (leucyl/cystinyl aminopeptidase) was identified [14]. These aminopeptidases have a prominent role in trimming peptides (to an optimal length of 8/9 amino acids) which are then transported from the cytosol to the ER for loading on HLA class1 molecules, including HLA-B27 [15].

Th17 immunity and the IL23R/Il17 axis have a critical role in AS immunopathogenesis. IL23R, encoding for the heterodimeric Interleukin 23 Receptor, was the first non-MHC gene found to be associated with AS [16]. IL23R has a pivotal role in Th17 immunity, as it induces Th17 cells with a specific signature (positive for IL23R, IL17A, IL17F, CSF2 among the others).

Other associated genes are involved in the IL23/IL17 axis, such as STAT3 (Signal Transducer and Activator of Transcription 3) and TYK2 (Tyrosine Kinase 2), both in the downstream cascade of the IL23R pathway.

Many other genes have found to be strongly associated with AS including RUNX3, T-bet and *eomes* which are involved in T-cell development and regulation [17].

4. Epigenetic contribution in AS

The term epigenetics describes a plethora of functional mechanisms in the genome that do not exclusively result from the DNA sequence itself [18]. Epigenetic mechanisms may regulate gene expression, allow binding of regulatory proteins or transcription factors to specific DNA sequences, exert long-range chromatin interaction etc. [19,20].

Recently, several studies have demonstrated the involvement of epigenetic regulation, including DNA methylation, histone modifications and microRNAs (miRNAs) activity, in the development of AS [21,22]].

DNA methylation has been poorly investigated in AS: in 2017 a genome-wide DNA methylation profile on peripheral blood mononuclear cells obtained from 5 AS patients and 5 healthy controls, identified 1915 differentially methylated CpG sites mapped to 1214 genes, with the most significant signal found with *HLA-DQB1* [23].

Few works have investigated the contribution of histone modifications in AS. Reduced histone deacetylase (HDAC) and histone acetyl transferase (HAT) have been described in PBMCs from AS patients [24,25]; AS-associated IL23R genotype has been found associated with altered levels of lysine 4 mono-methylation levels (H3K4me1), possibly having a critical role in the disease.

MiRNAs are endogenous non-coding RNAs involved in gene expression regulation and in different steps of physiological and pathological processes [26]. The contribution of miRNAs to autoimmune disease has been widely demonstrated: they are involved in several aspects of innate and adaptive immunity, including the development, differentiation and function of lymphoid and myeloid cells [27,28]. In AS, miRNAs can target specific pathways potentially involved in the disease pathogenesis.

In the present manuscript we will review the role of miRNAs in AS, highlighting the pathways affected by miRNAs dysregulation, focusing on the recent discoveries and discuss the potential use of miRNAs as diagnostic/prognostic markers and plausible therapeutic targets for AS treatment (see Fig. 1).

5. miRNAs as biomarkers in rheumatic diseases

Immune-mediated rheumatic diseases are a class of heterogenous disorders, where systemic inflammation is a common hallmark. MiRNAs represent a potential strong candidate to become ideal biomarkers in autoimmune or immune-mediated rheumatic diseases [29]. Overall, miRNAs possess specific characteristics which make them reliable biomarkers, in particular: 1) stability, which make miRNAs more resistant and stable than mRNA; 2) tissue specificity, their expression is specific to tissue origin; 3) miRNAs are associated to specific physiological states vs pathological ones; 4) power of discrimination, in other words miRNAs can be used to increase the accuracy of diagnosis, not only in rheumatology, for instance in the oncology field miRNAs can help to distinguish the tissue origin of undifferentiated tumours.

MiRNAs can be easily measured in different biological sources, including saliva, urine, blood and tissue samples [30]. Of note, the reproducibility and the feasibility in measuring miRNAs in biological fluids make them really powerful non-invasive candidates as disease

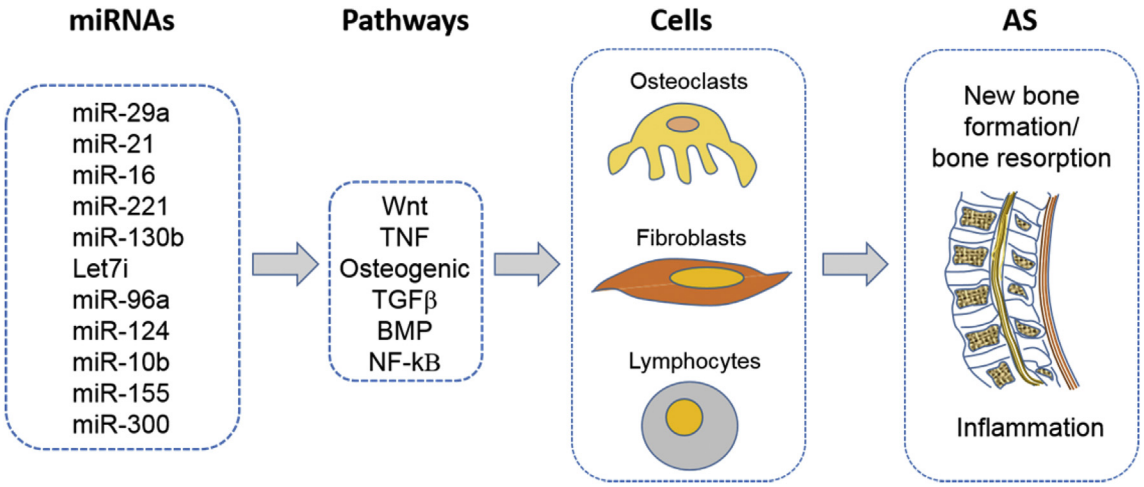

Fig. 1. Proposed functional miRNAs in AS.

biomarkers.

There is a limited number of data showing miRNAs as biomarkers in rheumatic disease like AS. As stated above, several studies have indicated miRNAs as potential biomarkers but almost all them have not been validated. This is now the goal for future research in the field.

6. miRNA biogenesis and mechanism of action

miRNAs are average 22 nucleotides single-stranded non-coding RNAs that negatively regulate target genes at post-transcriptional level allowing translational silencing with consequent repression of protein production [31].

The miRNA biogenesis starts with transcription by RNA pol II as primary miRNA (pri-miRNA), followed by the cleavage into an hairpin structure by a protein complex including the ribonuclease type III (RNase III) Drosha, into precursor-miRNA (pre-miRNA); which are then exported from the nucleus to the cytoplasm by an Exportin 5-dependent mechanism. In the cytoplasm the pre-miRNA is then processed by a second RNase III enzyme called Dicer, leading to the generation of a short RNA duplex. miRNA biogenesis is completed by the assembly of the mature, single-stranded miRNA from the duplex into the RNA-induced silencing complex (RISC) to bind to the target mRNA [32].

First identified in 1993 by Ambros and Ruvkun in *C. Elegans*; the interaction between lin-4, a gene regulating the timing of *C. Elegans* larval development, and the 3' untranslated region (UTR) of the lin-14 gene was the first evidence of miRNA-mRNA interaction [33,34].

Based on these first evidences it was believed that miRNAs exert their action through a perfect or imperfect matching with complimentary sequences only at the 3′UTR. In 2006 and 2008, respectively Miranda et al. and Tai et al. demonstrated that miRNAs can also target sequences at the 5′ UTR and at the coding sequence of target mRNAs (as for embryonic stem cells genes such as *Nanog*, *Sox2* and *Oct4*) [35,36].

As of March 10, 2020, based on miRBase record (http://www.mirbase.org/), a number of 2693 human miRNAs have been identified differentially expressed in both tissue and systemic circulation.

A growing body of evidence has shown the critical role of miRNAs in different pathological contexts; such as cancer, infectious diseases, bone homeostasis, apoptosis, cardiac development, and cardiac diseases. Recently, they have been emerged as new players in autoimmune and inflammatory diseases[37] including rheumatic disease [38]. miRNAs are able to modulate immune cell lineages differentiation and function: dysregulation of miRNAs can easily contribute to the breakdown of self-tolerance, leading to autoimmunity [29].

7. miRNA regulation in AS

At present, 47 studies in total have investigated the role of miRNAs in AS looking at their role as novel biomarkers and in modulating downstream targets associated with the progression of AS. These miRNAs were found to be involved in several processes: ossification, osteoblasts differentiation, inflammatory response, regulation of immune cells (lymphocytes, macrophages etc) etc. Here below we discussed the most relevant results in the field, with a view on miRNAs' role in AS physiological/pathological processes and pathways they regulate.

7.1. The role of miRNAs in new bone formation

In the context of AS, a first report from Yu et al., in 2011 indicated that fibroblasts, the most common cells in the connective tissue, which are involved in the process of ectopic ossification and osteogenesis in many diseases including AS [39], expressed a specific miRNA signature after exposure of osteoclast-like medium. In particular, miR-20a, miR-300, miR-185, miR-30d, miR-320a, miR-130b, miR-33a, miR-155, and miR-222 identified by miRNAs array, were significantly down regulated in fibroblasts, after conditioning with a pro-osteoclast medium. Target genes prediction software analysis revealed that the identified miRNAs were predicted to target, BMP2 (bone morphogenetic protein 2), Osteocalcin, and Runx2 (RUNT-related Transcription factor 2). These genes are involved in osteogenic differentiation, with a central role in AS pathophysiology, suggesting the fact that the cross talk between fibroblasts and osteoclasts, regulated by miRNAs, might be potentially relevant in AS disease progression [40].

More recently Huang and colleagues showed that the expression of mir-29a, a miRNA involved in osteoblast differentiation [41], is upregulated in peripheral blood mononuclear cells from patients with AS compared with patients with rheumatoid arthritis and healthy controls, suggesting a plausible implication of miR-29a as a diagnostic marker for new bone formation in AS. The miRNA expression has been demonstrated to not correlate with any disease activity index such as: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Bath ankylosing spondylitis disease activity index (BASDAI), bath ankylosing spondylitis function index (BASFI) and only a weak correlation was observed with the modified stoke ankylosing spondylitis spinal score (mSASSS) [42].

Another similar study performed on PBMCs investigated the levels of miR-29a and the mRNA levels of bone turnover markers with qPCR. Specifically, the authors confirmed increased mRNA levels of miR-29a in AS patients PBMCs, together with significant increase of DKK1, B-catenin and Runx2 mRNA. GSK3b, the glycogen synthase kinase 3 beta, an enzyme involved in WNT signalling [43] and in osteoblast differentiation, has been found conversely decreased in AS patients compared to controls. GSK3b was found positively correlated with B-catenin expression, indicating that Wnt signalling might contribute to new bone formation in AS patients [43].

MiR-29a expression has been reported to be increased in bones from AS patients ($n = 10$). It also promotes osteoblasts proliferation in hFOB1.19 (human osteoblast-like cell line) cells, inhibiting apoptosis and promoting invasion and migration, while DKK1 [44] one of miR-29a target acts conversely, suggesting an important pathological role in bone deposition, which is a hallmark of AS.

A report in 2014 from Huang et al., investigated the expression of mir-21 in whole blood of patients with AS. The expression of miR-21 has been shown to be upregulated in 122 patients compared with 122 controls; the authors showed an inverse correlation with the expression of the programmed cell death protein 4 (PDCD4), a repressor gene for osteoclastogenesis in particular in patients under treatment with nonsteroidal anti-inflammatory drugs (NSAID) and disease-modifying anti-rheumatic drugs (DMARD). Conversely, a positive correlation with PDCD4 was observed in patients treated with sulfasalazine. Furthermore, a positive correlation between miR-21 and collagen cross-linked C-telopeptide (CTX) levels, a marker of bone resorption, has been reported in patients with a disease duration < 7.0 years [45].

Together these results indicate that several miRNAs (in particular mir-29a) and Wnt signalling may provide interesting insights to better understand the mechanisms leading to new bone formation, stimulation of osteoblasts/osteoclasts and bone resorption. Further experiments are needed to fully address this biological question.

7.2. miRNAs as modulators of inflammation

An association study from Qi in 2013 in a cohort of Chinese patients, showed that a functional variant of miR-196a2 confers risk for Behcet's disease (a rare immune-mediated disease whose clinical manifestations include mucosal aphthae, spondyloarthritis and uveitis [46]), by modulating Bach1, and by regulating pro-inflammatory IL-1β and MCP-1 production. In specific the results showed that the TT genotype and T allele of miR-196a2/rs11614913 increase disease risk for Behcet's disease. Individuals carrying the TT genotype of rs11614913 showed a decreased miR-196a expression and an increased miR-196a target gene Bach1 expression in comparison with individuals carrying the CT or CC genotype. Interestingly, no significant correlations have been identified with the risk of anterior uveitis in patients with AS or of Vogt-Koyanagi-Harada syndrome, a disease characterized by uveitis [47].

Recently, Reyes-Loyola and colleagues assessed the mRNA levels of let-7i, miR-16 and miR-221 in AS patients' plasma from a Mexican cohort of 15 patients, naïve to TNF-α treatment and 13 controls [48]. The authors also measured the serum levels of CRP, MMP1 and MMP9 correlating the results obtained. Let7i was indicated as a possible biomarker for the diagnosis of AS, as it was found higher in patients compared to controls and with an AUC/ROC of 0.74. Moreover, plasma levels of miR-16 were associated with disease activity.

A very interesting work by Zhang and colleagues assessed the functional role of non-coding genes in AS mechanisms. They performed microarrays to compare lncRNA, mRNA and miRNA profile in hip joint ligament tissues from AS patients and controls. Specifically, the authors demonstrated through pathway analysis, gene prediction and network construction approach, that two miRNAs, miR-17–5p and miR-27b-3p might be crucial in enhancing the osteogenic differentiation potential in ligament fibroblasts and inflammation [49].

Ma and colleagues [50] demonstrated in a AS mouse model that the target gene of mir-96a is sclerostin, which is involved in chondrocytes and osteocytes function and is an indicator of the progression of the ossification process. Mir-96 is strongly increased in AS mice and overexpression of this miRNA increased the serum levels of inflammatory cytokines such as IL-6, Il-10 and TNFa, Wnt signalig and osteogenic markers (OPG, Runx2 and b-catenin).

The link between new bone formation and inflammation is clearly evident in AS: syndesmophytes developed more in inflamed vertebral edges compared to where no inflammation is present [51].

The exact pathways involved which link inflammation to new bone formation are still unknown. Li and colleagues have elegantly demonstrated that the intensity of the inflammation process affects the expression of Wnt proteins which are likely to be the main characters in bridging new bone formation and inflammation [52,53].

7.3. miRNAs as T-cells modulators

Back into 2013 Lai and co-workers profiled the expression of 270 miRNAs in T cells, comparing AS patients and healthy individuals. After validation, Let-7i, miR-16 and miR-221 were found higher in AS patients compared to controls, but only miR-221 and Let-7i correlated with the clinical parameters, Bath AS radiology index (BASRI) of lumbar spine. Let-7i has been reported having an effect on Toll like receptor 4 (TLR4) expression in T cell with a possible role in the production of IFNγ *in vitro* and in the immunopathogenesis of AS [54].

Hou and colleagues have demonstrated by luciferase reporter assay that insulin-like growth factor-1 receptor (IGF1R) is a direct target of Let-7i. In the presence of Let-7i the expression of IGF1R has been demonstrated to be down-regulated in AS T-cells and in a leukaemia T cell line, Jurkat [55]. Consequently, the inhibition of IGF1R influenced the expression of different downstream genes, including mTOR, Akt, PARP and BCL-2, leading to autophagy, as a mechanism to protect T-cells from apoptosis in a miRNA-mediated manner.

Looking at the role of miRNAs in the context of cells homeostasis in AS, a recent report has shown increased level of miR-124 in peripheral blood from AS patients; anthrax roxin receptor 2 (ANTXR2), a gene involved in now bone formation, and recently identified as a risk locus in AS in a genome-wide association studies (GWAS) [56], has also been confirmed as a target of miR-124 by luciferase reporter assay. The authors have reported that ANTXR2 inhibition by miR-124 promoted JNK activation and induced autophagy. Together the results from Hou and Xia, suggest the importance of miRNAs in the activation of protective mechanisms as autophagy, in the context of AS.

Using a bioinformatics approach Zhao and colleagues have investigated the expression of AS-related genes. The Limma package in R has been used to run multiple correlation between differentially expressed genes (DEGs) in a control group and AS. The up-regulated genes in AS samples were significantly enriched in oxidative phosphorylation and translational elongation. Meanwhile the authors investigated the possible interaction between differentially expressed genes and miRNAs using a combination of five miRNA prediction tool. Four miRNAs were identified as upregulated in AS and the four targets have been predicted, respectively and fifteen were identified to be down regulated. Finally, a screening on protein – protein interaction and a protein clustering analysis have been run resulting in the correlation of the DEGs with four domains related to ribosomal protein and proteasome [57].

Niu et al. have investigated miR-146, well known as an important player in the immune system and inflammation, in three different SNPs (*rs2910164*, *rs2431697* and *rs57095329*) in more than 600 Chinese people with AS and 600 healthy individuals, and any correlation with the disease was found, showing the same frequency in cases and controls for all the three SNPs [58].

Chen and colleagues have recently showed a specific miRNA signature for Th17 cells derived from AS patients' blood [59]. In particular the authors showed that miR-10b is upregulated and it regulates the production of cytokines in Th17 cells. Moreover, miR-10b can inhibit MAP3K7 expression in $CD4^+$ T-cells, leading to the inhibition of the pro inflammatory cytokine IL-17A.

Studies on serum and blood obtained from AS patients clearly showed the implication of miRNAs in AS pathogenesis. The aberrant expression of different miRNAs can affect specific subsets of immune cells, such as Th17 cells, altering the expression of downstream molecules leading to impaired function which might be relevant to AS.

7.4. Conclusions and future perspective

The examples we provided in this review showed that miRNAs regulation might play a pivotal role in AS, as drivers of inflammation, in participating in new bone formation process and tuning the function of different immune cells. Understanding how miRNAs can regulate the immune system will help us in clarifying the heterogeneity and the complexity of AS pathogenesis.

Application of targeting miRNAs in therapy has been successfully tested in preclinical trials with the aim to treat several cancers, ischemic heart diseases and heart failure [60]. MiRNA-122 inhibitor has been used in a phase II clinical trial to treat hepatitis C virus (HCV), with successful reduction of viral replication [61]. MRX34, a liposomal nanoparticle

Table 1
AS-related miRNAs: List of miRNAs with demonstrated implications in AS.

miRNA	Function	Regulation	Target genes	Tissue/sample	References
miR-29a	Osteoblast differentiation	Up-regulated	DKK1, GSK3b	PBMCs, hFOB1.19 (human osteoblast-like cells)	41, 43
miR-21	Osteoclastogenesis	Up-regulated	PDCD4	PBMCs	45
miR-20a, miR-300, miR-185, miR-30d, miR-320a, miR-130b, miR-33a, miR-155, miR-222	New bone formation, osteogenesis	Down-regulated after medium conditioning (not a direct proof for AS)	BMP2, Osteocalcin, Runx2	Fibroblasts	39
miR-124	T-cell homeostasis	Up-regulated	ANTXR2	PBMCs	56
Let 7i, mir-16, mir-221	T-cell modulation	Up-regulated	TLR4	T-cell	54
Let 7i	T-cell autophagy (?)	Up-regulated	IGF1R	Jukat (T-cell line)	55
mir-10b	Th17 regulation	Increased	MAP3K7	Th17 cells	59

Table 2
Clinical trials involving miRNAs: Trials targeting miRNAs in different diseases currently approved by FDA.

Drug	miRNA	Function	Phase trial	Disease
Miravirsen	miR-122	Inhibition	Phase II	Hepatitis C virus
MRX34	miR-34	Reducing expression	Phase I	Liver cancer, lymphoma
MRG110	miR-92	Inhibition	Phase I	Heart failure
MesomiR-1	miR-16	Targeting moiety	Phase I	Mesothelioma
Cobomarsen MRG106	miR-155	inhibition	Phase II	T-cell lymphoma
RG-012	miR-21	Inhibition	Phase I & II	Alport syndrome

which encapsulate double stranded miR-34, was tested in epithelial carcinomas and other cancers [62]. MRG110, an antisense oligonucleotide which inhibits the function of miR-92, was tested in a phase I trial with the aim to discover potential clinical application for heart failure [63] (see Table 2).

The clinical utility of miRNAs described in the examples above suggests a plausible usage of miRNAs also in rheumatic diseases: their association with peculiar clinical features make them valuable candidates as biomarkers for diagnosis and prognosis and can also offer new therapeutic options.

Osteoclasts, fibroblasts and lymphocytes are the main cell types affected by miRNAs dysregulation in AS. Refer to Table 1 for specific miRNA function.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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