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## Epigenetics of spondyloarthritis

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## **ABSTRACT**

Spondyloarthritis (SpA) is a chronic inflammatory disorder resulting from a combination of genetic predisposition and environmental factors. Despite recent advances, a substantial fraction of its genetic basis remains poorly understood. Several mechanisms have been proposed to account for this unexplained heritability, including epigenetics which can play a role at the interface between genetic and environmental susceptibility factors. Epigenetics refers to changes in gene expression that are not encoded in the DNA sequence itself. Such mechanisms may include DNA methylation, histone modifications and non-coding RNAs. Disruption of one of these systems can lead to inappropriate gene expression, which in turn might favour the development of disease. Thanks to recent technological progress, there has been a growing interest in the field of epigenetics in complex diseases, including SpA. However, epigenetic studies face some methodological limitations that hamper interpretation of their results: small sample size, absence of biological replication, lack of adequate controls for potential confounders, studies not performed in the most relevant cell/tissues. In the future, integration of epigenetics with other "omics" data will probably be necessary to improve our understanding of SpA pathogenesis. These issues need to be addressed before considering the use of epigenetic marks in clinical routine, as biomarkers or as drug targets.

Keywords: spondyloarthritis, ankylosing spondylitis, epigenetics, DNA methylation, micro-RNA, histone modification.

## 1) INTRODUCTION

Complex diseases such as spondyloarthritis (SpA) result from a combination of genetic predisposition and environmental factors. SpA has a strong genetic component dominated by the HLA-B27 allele of the major histocompatibility complex (MHC). Several large genome-wide association studies (GWAS) have uncovered more than 40 other susceptibility loci outside the MHC [1]. However, only about 25% of the heritability is likely to be explained by currently known risk loci [2]. Several mechanisms have been proposed to account for the unexplained heritability, including epigenetic variations. Notably, epigenetic effects may be affected by the environment and thus potentially represent a major interface between genetic and environmental susceptibility factors.

Introduced in the early 1940s by Conrad Waddington, the term epigenetics initially refers to *“the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being”* [3]. Over the years, the meaning of the word has gradually narrowed and may be defined today as *“all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself”* [4]. Epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNAs, thereby regulating gene expression (**Figure 1**). Epigenetic modifications can occur during development, under endogenous stimulations, or be environmentally induced. They are also reversible and can be inherited during cell divisions [5].

Epigenetic modifications are key regulators of gene activation states. Thus, disruption of one of these interacting systems can lead to inappropriate gene expression, which in turn might favour the development of diseases. Aberrant epigenetic profiles have been described, mainly in cancer [6], and more recently in several chronic inflammatory diseases [7]. Investigating epigenetic modifications is expected to provide new insights into SpA pathogenesis that can also lead to the development of innovative diagnostic or prognostic tools as well as the identification of new drug targets. In this review, we summarize and discuss the current state of research and knowledge on epigenetic modifications occurring in SpA.

## 2) EPIGENETIC PROCESSES

### a) DNA methylation

DNA methylation refers to the addition of a methyl group to a cytosine to form 5-methylcytosine. It predominantly occurs on cytosine phosphate guanine (CpG)-paired bases, often clustered in particular regions of the genome, called CpG islands, located in the promoter of nearly 60% of genes [8]. The process of methylation establishment and maintenance is carried out by the DNA

methyltransferase (DNMT) proteins family: DNMT3A, DNMT3B and DNMT3L for *de novo* methylation and DNMT1 to maintain DNA methylation during cellular replication.

DNA methylation is frequently described as a ‘silencing’ epigenetic mark (**Figure 1A**). However, it has been recently shown that the effect of DNA methylation on gene expression varies with context and with the position of the methylation relative to the transcriptional unit. Methylation in the immediate vicinity of the transcription start site blocks transcription initiation, leading to a decrease of messenger RNA (mRNA) production. On the contrary, methylation in the gene body has been correlated with transcription elongation and might be involved in splicing mechanism [8].

#### b) Post-translational modifications of histones

The basic element/unit of chromatin is the nucleosome, a nucleoprotein complex composed by DNA wrapped around a histone octamer (two copies of H2A, H2B, H3 and H4). The poorly structured N-terminal ‘tails’ of histone proteins that protrude from the nucleosome are subjected to addition of a diversity of chemical groups also termed as histones marks [9]. Methylation, acetylation, phosphorylation and ubiquitination are among these modifications. They can act as synergistic, complementary or antagonistic signals to dynamically modulate transcription outcome in the cell by altering chromatin structure and regulating DNA accessibility to transcription factors (**Figure 1B**) [10]. Histone marks can be either deposited or removed by different sets of histone-modifying enzymes called “writers” and “erasers” respectively [11]. This particular aspect implies that histone marks are reversible in order to activate or silence gene expression. Moreover, histones can bear multiple marks on different residues influencing mutually each other. This observation early suggested the existence of crosstalk that fine-tunes transcription regulation in a context-dependent manner. It is the interplay between these modifications that inspired Jenuwein and Allis to propose almost 20 years ago the hypothesis of the histone code [12].

#### c) MicroRNAs

Among non-coding RNAs, microRNAs (miRNAs) have been the most studied – especially in translational research – and are predominantly involved in gene expression modulation. Other types of non-coding RNAs, including long non-coding (lncRNAs), have been recently involved in the pathogenesis of inflammatory diseases [13].

MiRNAs are small noncoding, single stranded, endogenously expressed RNAs molecules [14]. The canonical biogenesis of miRNA is predominant and requires several steps. First DNA sequence is transcribed into primary miRNA (pri-miRNA) which is secondary cleaved into precursor miRNAs (pre-miRNA) by the microprocessor complex comprised of Drosha and DGCR8. The pre-miRNA is then

exported to the cytoplasm and processed by Dicer, resulting in a mature miRNA duplex which is loaded into the Argonaute family of proteins to form the minimal miRNA-induced silencing complex (miRISC). Finally miRISC can interact with complementary sequences on the 3'UTR of target mRNAs to induce translational repression through mRNA deadenylation and decapping leading to 5'–3' degradation. [15]. Generally, miRNAs do not completely silence their target genes, but rather decrease their expression.

A given miRNA may have hundreds of different mRNA targets, and a given target might be regulated by multiple miRNAs. This leads to a complex dynamic modulation of gene expression, which has been shown to play a critical role in numerous cellular processes including development, proliferation, invasion, and apoptosis. Moreover, numerous studies have demonstrated that miRNAs can be released into extracellular fluids, and might act as autocrine, paracrine, or endocrine regulators to modulate cellular activities [16].

### **3) FACTORS INFLUENCING EPIGENETIC MODIFICATIONS**

Epigenetic marks can be affected by a variety of factors described below. These factors are important to keep in mind when considering an experimental design to identify specific alterations of the epigenome in a given disease to avoid spurious findings.

#### **a) Cell type**

On the contrary to the primary sequence of the human genome which is largely preserved in all human cell types and tissue, the epigenome varies considerably among cell types, as extensively studied by the NIH Roadmap Epigenomics Program and ENCYClopedia Of DNA Elements (ENCODE) project [17,18].

This cell specificity must be considered in analytic perspectives, especially to critically evaluate epigenomic studies, taking into account that a high proportion has been performed in peripheral blood with a mixture of different cell types. Thus, differences observed between conditions might be related to a difference in the proportion of different cell types rather than to the studied condition.

#### **b) Individual characteristics**

Among demographic factors, age seems to have a strong influence on epigenetic marks [19]. Epigenome is also influenced by ethnicity [20,21] and by sex [22,23].

#### **c) Lifestyle and environmental factors**

All epigenetic marks can be altered by exposure to lifestyle factors such as smoking, medication, alcohol and diet and environmental factors such as air pollution [24,25].

d) Interindividual variations of the DNA sequence

Interindividual variations of the DNA sequence (such as single nucleotide polymorphism) are a substantial determinant of epigenetic marks. Indeed, DNA polymorphism can influence both DNA methylation state [26], miRNA expression [27] and histone post-translational modifications magnitude [28]. It may be particularly useful to identify the functional consequences of disease associated variants [29].

#### **4) MEASUREMENT AND ANALYSIS OF EPIGENETIC MARKS**

a) DNA methylation

DNA methylation information is erased by standard molecular biology techniques, such as polymerase chain reaction (PCR). Therefore, almost all DNA methylation analysis techniques rely on a 2-step process: a methylation-dependent pre-treatment of the DNA, followed by amplification or hybridization [30]. DNA pre-treatment relies on three main approaches: methylation-sensitive endonuclease digestion, affinity enrichment or bisulphite conversion (considered as the gold standard). Once genomic DNA has been treated with one of the methylation-dependent steps, probe hybridization or sequencing can be used to reveal the location of the methylated residues. The combination of these two steps has resulted in the development of numerous techniques, details of which can be found elsewhere [30]. All these techniques were initially restricted to limited regions, but many of them can now be used on a genome-wide scale. To date, the most common method of DNA methylation assessment relies on Illumina® BeadChip® arrays because they offer a good compromise between cost and number of measured sites.

b) Post-translational modifications of histones.

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) extensively contributed to our understanding of the relationship between histone marks and transcription regulation occurring during cell differentiation [31]. Histone mark enrichment profiles, often referred as epigenome maps, are used to segment and annotate whole-genome regions into distinct “chromatin states”, which represent more detailed characteristic epigenetic signatures (e.g., weak transcription and poised promoter) [32]. Analysis of the distribution of either H3K4me3 active or H3K27me3 repressive marks allows reliable prediction of transcription states at promoters genome-wide.

But most of all, histone marks allow *ab initio* prediction of non-coding regulatory sequences such as enhancers. Enhancers are key elements involved in cell-specific transcription regulation through long

distance interactions with promoter sequences [33]. They have been involved in many genetic disorders and developmental diseases as their malfunction can lead to aberrant gene expression. If their prediction remains highly challenging, enhancers are known to be associated with certain epigenetic signatures, the best known being H3K27ac and H3K27ac/H3K4me1 that respectively describe primed and active elements [34].

#### c) Micro-RNAs

MiRNAs can be isolated from cells, tissues – but also body fluids (such as serum, plasma, tears, or urine), from which they are much harder to extract (due to a lower quantity). A significant improvement has been observed in miRNAs isolation methods, from the traditional phenol-chloroform extraction followed by RNA precipitation, with high level of contaminants, to column-based RNA adsorption method and complementary specific techniques – including protocol for separation of small RNAs [35].

Detection of miRNA is challenging because of their small size (21 to 24 nucleotides), low levels of expression and high sequence similarity with other expressed miRNAs. A suitable method should be chosen based on the requirements of the investigation (e.g. single-target investigation or genome-wide study) and the experimental conditions (amount of RNA, level of expression, sample type...). Current methods used for detecting miRNAs include quantitative PCR (qPCR) or in situ hybridization for detection of specific miRNA, micro-arrays or RNA sequencing for genome-wide detection.

Identifying target genes and functional effects of a defined miRNA is another challenge. Different methods can be used, each with varying benefit/limitation ratio: *in silico*-based target prediction; transient transfection of a miRNA mimic or inhibitor followed by whole transcriptome sequencing; CRISPR/Cas9 strategy; cross-linking of RNA-protein complexes followed by immunoprecipitation and RNA sequencing to detect miRNA/mRNA binding [35].

### 5) EPIGENETIC VARIATIONS IN SPONDYLOARTHRITIS

To date, most of the epigenetic studies in SpA have been focused on miRNAs. Less than 10 studies investigating DNA methylation have been published and even less investigated histone modifications.

#### a) DNA methylation profile in SpA

Variants in *DNMT3A*, *DNMT3B* and *DNMT3L* genes have been recently associated with ankylosing spondylitis (AS) [2], implying that DNA methylation could be important for AS pathogenesis.

However, only a few studies have investigated this epigenetic mechanism in SpA, all of them focused on AS. Their designs and main results are summarized in **Table 1**.

Only two such studies were performed genome-wide [43,44]. The first one published in 2017 compared a limited number of AS patients with healthy controls (5 in each group) and identified 1,915 differentially methylated positions (DMPs). The most significant DMP was located in *HLA-DQB1*, a MHC class II locus often in linkage disequilibrium with *HLA-B* [43]. Thus, given the HLA-B27 frequency bias between cases and controls, it cannot be excluded that such a difference is linked to HLA-B27 rather than to the disease status itself. More recently, the influence of the HLA-B27 on the genome-wide DNA methylation profile was studied by comparing three groups of patients: 12 HLA-B27+ AS, 12 HLA-B27- AS patients and 12 patients with HLA-B27- osteoarthritis (OA) [44]. Interestingly, more DMPs were detected between HLA-B27-positive and -negative AS patients (n=159) than between AS and OA (n = 67), suggesting a strong influence of HLA-B27 on the DNA methylation profile.

Other studies were centred on known AS-genetically associated regions and/or inflammatory-related genes [36,37,45-47]. Almost all of them examined correlation between methylation levels and parameters such as disease activity or clinical manifestations, but due their small sample sizes they were rather underpowered. Regarding studies of previously reported AS-associated genes, it is noteworthy that none of them investigated whether the associated SNPs at these *loci* operated through effects on methylation.

#### b) Histone modifications in SpA

As for DNA methylation, only few studies have addressed so far alterations of histone marks in SpA and none of them has been performed at genome-wide level. Most of them concerned AS-associated SNPs whose positions correlated with enhancers signature. By integrating prediction of candidate causal variants based on dense genotyping data with enhancer mapping in different immune cell types using H3K27ac distribution, Farh *et al.* detected enrichment of associated SNPs in active enhancers from Th0, Th1 and Th17 cells [42]. Studying H3K4me1 mark allowed the identification of an enhancer close to the *IL23R* gene that encodes a component of the heterodimeric IL-23 receptor. This could potentially account for the effect of the AS-risk allele of SNP *rs11209032* on increasing Th1 proliferation [41]. Surprisingly however, if the level of H3K4me1 in this region was decreased in patients homozygous for the risk allele, no change in *IL23R* mRNA was observed, suggesting a more complex regulatory mechanism involving this enhancer. A similar strategy led to explain the putative role of *rs4648889*, an AS-associated SNP that falls in a H3K27ac/H3K4me1-enriched enhancer of *RUNX3* [40]. Both H3K4me1 and *RUNX3* mRNA levels were consistently decreased in CD8+ T cells

from AS patients carrying the risk allele. As RUNX3 is known to enhance CD8 T-cell development, the lower expression of RUNX3 induced by *rs4648889* might explain the lower number of CD8+ T cells previously reported in AS patients [39]. Despite the lack of comprehensive data from large cohort of patients, these data suggest a strong association between SpA and cis-regulatory genetic polymorphisms inducing chromatin remodeling.

Mutations in histone modifying enzymes that regulate global level of histone modifications were pointed out in numerous diseases, such as cancers, due to their impact on the expression level of many genes simultaneously [29]. To date, only few of them were correlated with SpA. The global level of acetylated histones in the cell depends on a balance between Histone Acetyl Transferase (HAT) and Histone Deacetylase (HDAC) that regulate transcription factors and RNA polymerase recruitment on promoters. A HAT/HDAC activity change was assayed in AS patients treated with TNF $\alpha$  inhibitor [38]. Treatment was associated with a significant increase in HAT activity that can explain similarity between the anti-inflammatory effects of TNF $\alpha$  inhibitors and that of some HDAC inhibitors [48]. An increased level of HDAC3, that regulates the c-Jun N-terminal kinase pathway and NF- $\kappa$ B activity was also detected in peripheral blood mononuclear cells from AS patients [49].

#### c) MicroRNAs in SpA

There is growing evidence that aberrant miRNA expression is linked to inflammatory diseases, such as rheumatoid arthritis (RA) or inflammatory bowel diseases. During the last decade, involvement of miRNAs has been studied in the context of AS [50] with two main approaches: (i.) miRNAs as actors of SpA pathogenesis, as discussed in the paragraph below or (ii.) miRNAs as diagnostic, prognostic or therapeutic biomarkers, as discussed in the next section.

MiRNA expression profile of SpA patients has been studied in nearly 40 studies to date. More than two third of them concerned bulk samples (whole blood, peripheral blood mononuclear cells, serum, plasma) with non overlapping thus inconclusive results. The others focused on sorted cell subsets, such as the circulating CD14<sup>+</sup> monocytes or CD4<sup>+</sup> T cells, two cell types thought to be critically involved in disease pathogenesis. In SpA patients, both cell types were shown to harbor significant modifications in miRNAs expression profile (with 13 and 11 differential expressed miRNAs, respectively). These miRNAs were predicted to be involved in disease relevant pathways such as inflammation, intestinal permeability, or bone formation [51,52].

Some variations are consistent with the pro-inflammatory cytokine environment reported in SpA. For instance, IL-23 was shown to induce a differential miRNAs expression profile in T cells from AS patients, including overexpression of miR-29b-1-5p [53]. Moreover, Perez-Sanchez *et al.* identified

several miRNAs overexpressed in SpA patient with an influence on the production of inflammatory cytokines such as IL-1 [54]. The role of T helper 17 (Th17) cells is well described in SpA pathophysiology. Interestingly, miR-10b was significantly increased in AS Th17 cells, as compared to cells from healthy controls. This overexpression seemed to be induced by TNF- $\alpha$  and IL-6 and inhibited the production of IL17A [55].

Several studies searched for a correlation between miRNAs expression profile and markers of disease-activity (C-reactive protein [CRP], erythrocyte sedimentation rate [ESR], Bath AS Disease Activity [BASDAI], AS Disease Activity Score [ASDAS]), inflammatory cytokines production, or ossification related-parameters. The most significant results are summarized in **Table 2**. Of note, one study compared miRNAs between HLA-B27 positive and -negative axial SpA patients and observed significantly different expression profiles [48].

Despite the strong influence of DNA sequence on miRNA expression, few studies have tried to determine whether miRNAs deregulation might be an independent genetic determinant of AS/SpA. Niu *et al.* and Xu *et al.* tried to identify a specific association between miR-146a polymorphisms, known to be involved in immune responses, and SpA [61,62]. However, no strong association has been clearly identified. Moreover, none of the GWAS hits map to a miRNA gene [2].

## 5) FUTURE DIRECTIONS FOR EPIGENETIC STUDIES IN SPA

### a) How to improve quality of studies?

Studies examining DNA methylation, miRNA and histone modifications in SpA have suffered from several methodological limitations, which hamper interpretation of their results: small sample size, absence of biological replication, lack of adequate controls for potential confounders, studies not performed in the most relevant cell/tissues. These issues need to be addressed to increase their reliability and to consider the potential use of epigenetic marks in clinical routine. **Table 3** summarizes key points which should be taken into account in the design of future epigenetic studies in the context of SpA.

### b) Integration with other -omics data

No single technology can capture the complexity of the molecular mechanisms that lead to human disease. Thus, the combination of several technologies such as dense genotyping, transcriptomics, epigenomics, metabolomics and proteomics could help to provide a deeper comprehension of the disease pathogeny. However, this multi-omics approach introduces new computational and interpretation challenges [63]. To date no systematic multi-omics approach has been applied to SpA but efforts have been made in this direction, for example by linking together AS associated variants

and epigenetic marks [43–45]. Integration with microbiota data could also be very useful due to the strong pathophysiologic link between gut and joint in SpA [64].

c) From bench to bedside

Epigenetic marks provide new insights into disease pathogenesis but might also be useful in clinical practice, with two main domains of application: biomarkers and drug development.

Epigenetic biomarkers refer to any epigenetic mark or altered epigenetic mechanism which can be measured in the body fluids or tissues and which is able to contribute to diagnose or predict the outcome of a disease or to predict/monitor responses to therapy [65]. In SpA, the lack of relevant studies in the field strongly hampers their translation into clinical application. Some tried to use circulating miRNAs expression profiling as a diagnosis tool for AS but performance on validation cohorts still need to be assessed [54,57]. Few studies also tried to define the relation between miRNAs expression profile and treatment response in the perspective of using miRNAs as a therapeutic strategy tool but the process of validation has not been performed yet [66,67].

During the last 20 years, the perspective of epigenome reversible intervention to treat human diseases has emerged, historically beginning in the field of cancerology. Some encouraging results and promising developments have been observed in numerous indications, including haematopoietic and solid cancers: for example, DNMT and HDAC inhibitors, and more recently small molecules targeting specific genetic alterations [68]. More recent studies have been carried for the discovery of epigenetic drugs to treat inflammatory disease, especially in RA, systemic lupus and systemic-onset juvenile idiopathic arthritis [69]. Advances in SpA epigenetic treatment strategy is ongoing: an example of promising targeted-strategy drug development has been recently published by Cribbs et al., showing the potential *in vitro* effect on Th17 cells differentiation of an H3K27 demethylases inhibitor [70].

#### **DISCLOSURE STATEMENT**

None of the authors has any conflicts of interest to declare.

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**Figure 1. Epigenetic mechanisms and their influence on gene expression**

(A) DNA methylation refers to the addition of a methyl group to a cytosine. It is described as a 'silencing' epigenetic mark. (B) Nucleosomes, composed of DNA wrapped around histone proteins, can change conformation to modulate DNA accessibility. Histone tails are post-translationally modified by the addition or removal of chemical groups (also called 'histone marks', such as H3K27Me3 – associated with chromatin compaction and repression of transcription, or H3K4Me3 – associated with chromatin opening and enabling of transcription). (C) Noncoding RNAs, especially micro-RNA, can mediate the recognition and binding of RNAs that become targeted for degradation.

**Table 1. Design and main results of DNA methylation studies in SpA.**

Study	Region	Population	Methods	Sample type	Main results
Hao <i>et al.</i> [36]	China	5 AS 5 HC	Genome-wide Illumina® array	PBMC	1,915 DMPs Best association in <i>HLADQB1</i> gene
Coit <i>et al.</i> [37]	Europe	12 HLA-B27+ AS 12 HLA-B27- AS 12 OA	Genome-wide Illumina® array	Whole blood	AS vs OA: 67 DMPs HLA-B27+ vs HLA-B27- AS: 159 DMPs
Lai <i>et al.</i> [38]	Taiwan	43 HLA-B27+ AS 6 HLA-B27+ HC	Candidate gene qRT-PCR	Serum	Hypermethylation of <i>SOCS1</i> in AS vs HC
Aslani <i>et al.</i> [39]	Iran	40 AS 40 HC	Candidate gene qRT-PCR	PBMC	Hypermethylation of <i>DNMT1</i> promoter and lower DNMT1 mRNA expression in AS vs HC
Karami <i>et al.</i> [40]	Iran	50 AS 50 HC	Candidate gene qRT-PCR	PBMC	Hypermethylation of <i>BCL11B</i> promoter and lower BCL11B mRNA expression in AS vs HC
Chen <i>et al.</i> [41]	China	99 AS 99 HC	Candidate gene NGS-based	PBMC	Hypermethylation of <i>IRF8</i> promoter and lower <i>IRF8</i> mRNA expression in AS vs HC
Zhang <i>et al.</i> [42]	China	99 AS 99 HC	Candidate gene qRT-PCR	PBMC	Hypermethylation of two <i>IL12B</i> CpG islands and higher IL12B mRNA expression in AS vs HC

AS: ankylosing spondylitis; HC: healthy controls; PBMC: peripheral blood mononuclear cells; DMPs: differentially methylated positions; OA: osteoarthritis; qRT-PCR: quantitative real-time polymerase chain reaction

**Table 2. Correlations between miRNA and SpA-related clinical, biological or radiological parameters**

Study	miRNA	Region	SpA subtype	Sample size	Sample type	Disease-related parameters	Sense of correlation
Wang <i>et al.</i> [56]	miR-199a-5p	China	AS	41	CD3 <sup>+</sup> cells	ASDAS, m-SASSS	Negative
Qian <i>et al.</i> [57]	miR-155	China	AS	80	Serum	BASDAI	Positive
Kook <i>et al.</i> [58]	miR-214	Korea	AS	65	Serum	ASDAS	Positive
Lai <i>et al.</i> [52]	miR-221	China	AS	27	CD3 <sup>+</sup> cells	BASRI	Positive
Li <i>et al.</i> [59]	miR-27a	China	nr-axSpA	26	PBMC	ASDAS, CRP	Negative
Prajzlerova <i>et al.</i> [60]	miR-221-3p	Czech Republic	AS	48	Serum	CRP	Positive
	miR-181a-5p					BASDAI	Positive
	miR-146a-5p						
	miR-151a-3p						
	miR-99b-5p						
	miR-133a-3p						
	miR-625-3p						
miR-885-5p							
Perez-Sanchez <i>et al.</i> [54]	miR-146a-5p miR-125a-5p miR-22-3p	Spain	AS	53	Serum	CRP, TNF- $\alpha$ levels	Positive
Fogel <i>et al.</i> [51]	mir-146a-5p	France	AS	81	CD14 <sup>+</sup> PBMC	CRP	Negative

AS: ankylosing spondylitis; ASDAS: ankylosing spondylitis disease activity score; m-SASSS: modified stoke ankylosing spondylitis spinal score; BASDAI: Bath ankylosing spondylitis disease activity index; BASRI: Bath ankylosing spondylitis radiologic index; nr-axSpA: non radiographic axial spondyloarthritis; PBMC: peripheral blood mononuclear cell; CRP: C-reactive protein; TNF: tumor necrosis factor

**Table 3. Methodological points to consider when conducting or interpreting epigenetic studies**

<b>Topic</b>	<b>Potential issue</b>	<b>Points to consider</b>
<b>Cohort selection</b>	Spurious associations Lack of reproducibility	<ul style="list-style-type: none"> <li>• Match age, sex and ethnicity between cases and controls</li> <li>• Consider potential confounding factors: smoking, treatment, HLA-B27 distribution</li> <li>• Discuss the possibility to include patients with other inflammatory diseases as controls</li> </ul>
<b>Sample type</b>	Mix of cells with different proportions between groups No disease-relevance of sample type	<ul style="list-style-type: none"> <li>• Whenever possible, use of sorted cells is preferable</li> <li>• Choose the most relevant cell types considering disease pathogenesis (tissue resident and/or circulating cells)</li> </ul>
<b>Sample size</b>	Lack of statistical power	<ul style="list-style-type: none"> <li>• Estimate the sample size requirement depending on the number of tests performed and of the expected effect size</li> </ul>
<b>Replication/validation</b>	Lack of reproducibility	<ul style="list-style-type: none"> <li>• Depending on the method of detection initially used, consider the interest of a technical validation (same samples assessed with a different method, most often PCR)</li> <li>• Replication of the results in an independent cohort is highly recommended.</li> </ul>

