GENETIC STUDIES ON SPONDYLOARTHRITIS: FROM DISEASE PREDICTORS TO THERAPEUTIC TARGETS

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EDITORIAL
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Editorial: Genetic Studies on Spondyloarthritis: From Disease Predictors to Therapeutic Targets

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Editorial on the Research Topic

Genetic Studies on Spondyloarthritis: From Disease Predictors to Therapeutic Targets

Seronegative spondyloarthropathies (SpA) are a group of chronic inflammatory diseases and ankylosing spondylitis (AS) is the prototype (Linden et al., 1984). In the last decade the clinical diagnosis of axial SpA (axSpA) has been changed, mainly, in the early disease stage for the heterogenous manifestations of the diseases (Ortolan et al., 2021). The Assessment of Spondyloarthritis International Society (ASAS) classification criteria consider axSpA into radiographic (r-axSpA) and non-radiographic (nr-axSpA) (Rudwaleit et al., 2009). SpA main clinical manifestation is the inflammation of the spine and the entheses, but also extra-skeletal manifestations are common, including the inflammation of peripheral joints, the eye (uveitis), the skin (psoriasis) and the gut (inflammatory bowel diseases, IBD) (Elewaut and Matucci-Cerinic, 2009; Bridgewood et al., 2020).

AS is highly heritable (s ~ 50), with a complex genetic background only partially clarified, undoubtedly dominated by the Human Leukocyte Antigen (HLA-B27) allele, but with >100 genetic loci significantly incriminated by genome-wide association studies (GWAS) (The Wellcome Trust Case Control Consortium, 2007; Ellinghaus et al., 2016). It is now evident that the AS genetic background is shared between the different SpA subfamilies (i.e., psoriatic arthritis, IBD and acute anterior uveitis) contributing to their excess incidence not only in individuals with AS but also in their relatives (Ellinghaus et al., 2016; Robinson et al., 2016; Costantino et al., 2018).

This Research Topic will provide an insight on the current advances in the genetics of SpA, highlighting the candidate genes, pathways and cell types involved in SpA pathogenesis that can be taken forward for designing novel therapeutic approaches and stimulating researchers to investigate the role of genes in the treatment response (Costantino et al., 2018). Furthermore, the impact of ethnicity, genetic role, gender, and environment will be discussed.

Genetics studies are not only those with large datasets (i.e., GWAS). Costantino et al. highlighted the importance and the success obtained from SpA family-based studies, as they have the potential in identifying the different genetic factors involved in SpA. Interest in SpA family-based approaches is renowned as they might be crucial for the identification of rare variants through next-generation sequencing.

Recent GWAS highlighted the different results obtained following the ethnic differences of the cohorts analysed, between Europe and East Asia. Wu et al. illustrated the different genetic distribution (in particular for HLA alleles and IL23R polymorphisms) for Europe and Asia. In addition, the authors emphasized the importance to use a polygenic risk score approach (PRS). Potentially, with PRS it will be possible to have an early diagnose of AS, as PRS will use genotype data

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from thousands of genetic variants and quantify an individual's genetic risk. Besides ethnicity, also gender might have an impact on the genetics and epigenetics of SpA susceptibility. Chimenti et al. comprehensively described the genetic associations, the cytokines, the epigenetic mechanisms and the clinical phenotypes which have sex related differences in SpA subfamilies. Ortolan et al. provided a very elegant systematic literature review confirming how genetics might have a major role in predicting response to therapy in SpA, after the analysis of cross-sectional, case-control and cohort studies.

There is an urgent need in the definition of more specific targets for therapy and personalized medicine in SpA. This unmet need is well described by two well-structured articles in our topic. Hromadova et al. discuss the potential in targeting Tyrosine Kinase 2 (TYK2) gene in SpA, with selective TYK2 inhibitors. The authors covered the different aspects of TYK2 from basic biology to therapeutic targeting, especially in SpA. The current available treatment in axial psoriatic arthritis (axPsA) is the focus of the work by Floris et al. The authors summarized the recent findings in axPsA focusing on the efficacy of the currently available treatments including TNFα, IL17/IL23 and JAK inhibitors.

Simone et al. explored the dysregulated "type17" response in SpA. The authors elegantly summarised the multifaceted role of Th17 cells as possible trigger occurring in SpA, covering the genetics, environment and microbiome aspects of these cells.

The specific functional role of SpA-associated Single Nucleotide Polymorphisms (SNPs) has been analysed in two works from our collection. Pimenta et al. analysed four SNPs in Alpha-actinin-3 (ACTN3) and Vitamin D receptor (VDR) genes to demonstrate association between axSpA with muscle performance, analysing clinical and epidemiological data. Finally, Cohen et al. used a functional genomics approach, including *in silico* analysis, electrophoretic mobility shift assay and chromosome conformation capture, to define the functional role of a strongly AS-associated SNP located at the genomic locus encompassing *RUNX3*, a transcription factor involved in the development of $CD8^+$ lymphocytes.

In conclusion, we can firmly affirm that all the articles included in this collection provided a clear evidence of the importance of genetics studies in SpA and the need of genetics in the definition of new targets for therapy and improve drug development.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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What Have We Learned From Family-Based Studies About Spondyloarthritis?

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Spondyloarthritis (SpA) is a chronic inflammatory disorder with a high familial aggregation, emphasizing the existence of genetic susceptibility factors. In the last decades, family-based studies have contributed to better understand the genetic background of SpA, in particular by showing that the most likely model of transmission is oligogenic with multiplicative effects. Coexistence of different SpA subtypes within families also highlighted the complex interplay between all subtypes. Several whole-genome linkage analyses using sib-pairs or multiplex families were performed in the 1990s to try to identify genetic susceptibility factors besides HLA-B27. Unfortunately, no consistent results were obtained and family-based studies have been progressively set aside in favor of case-control designs. In particular, case-control genome-wide association studies allowed the identification of more than 40 susceptibility regions. However, all these loci explain only a small fraction of disease predisposition. Several hypotheses have been advanced to account for this unexplained heritability, including rare variants involvement, leading to a renewed interest in family-based designs, which are probably more powerful in the detection of such variants. In this review, our purpose is to summarize what has been learned to date regarding SpA genetics from family-based studies, with a special focus on recent identification of rare associated variants through next-generation sequencing studies.

Keywords: spondyloarthritis, ankylosing spondylitis, genetics, family-based studies, rare variant

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INTRODUCTION

Spondyloarthritis (SpA) is a chronic inflammatory disorder which encompasses several presentations characterized by axial and/or peripheral joint inflammation, often in association with extraarticular inflammation of the eye, skin, or gut (Taurog et al., 2016). One of the striking features of the disease is its high familial aggregation. In this review, our purpose is to summarize what has been learned to date regarding SpA genetics from family-based studies. We also highlight the potential interest of family-based designs for identification of rare associated variants.

GENETIC EPIDEMIOLOGY OF SpA

Classical genetic epidemiology approach is based on family studies with three sequential steps addressing the following questions: (1) "does disease cluster in families?," (2) "is familial clustering

related to genetic, environmental, or cultural risk factors?," and (3) "how is genetic susceptibility inherited?" (King et al., 1984). This approach has been applied to determine and quantify genetic influence in SpA. As for most of the studies in the field of SpA genetics, our knowledge on genetic epidemiology mainly comes from studies restricted to ankylosing spondylitis (AS) phenotype. Restriction to this well-defined prototypical phenotype of SpA, requiring an advanced radiological sacroiliitis, aimed at increasing the genetic homogeneity of the studied cohorts, and thus improving reliability and power of the analyses. However, as discussed later in this review, other subtypes of SpA have been found among family members of patients with AS suggesting shared genetic factors. Taking these cases into considerations may help to provide a comprehensive picture of the genetic factors involved in SpA.

Do AS Cluster in Families?

Familial aggregation studies are commonly the first step in the identification of genetic determinants of disease. Their objective is to determine if the risk to develop a disease is increased if one of the relatives already has this disease. Aggregation studies may be separated into three categories according to their design: population-based, case-control, or family-based studies (Matthews et al., 2008). The most popular approach is to sample independent (i.e., unrelated) patients, called probands, and to obtain their detailed family history of disease. Potential biases include ascertainment bias and/or overreporting of affected cases which might lead to overestimation of familial aggregation (Wickramaratne, 1995; Guo, 1998).

In AS, several family-based approaches have been used to assess recurrence risk ratio (RRR). By compiling data from six studies comprising 4,924 siblings and 466 parents of AS probands, Brown et al. (2000) reported a RRR of 82 in siblings and 79 in parent-child. This estimate was based on a disease prevalence of 0.1% in the general population, lower to that now commonly admitted in European population [0.25%, 95% confidence interval (CI): 0.18–0.33%] which might have led to an overestimation of the risk (Stolwijk et al., 2016). However, similar ratios were obtained in population-based studies in Iceland with a first-degree RRR ranging from 75 to 94 (Thjodleifsson et al., 2007; Geirsson et al., 2010). In contrast, two register-based case-control studies of AS patients in Sweden reported a substantially lower RRR with a sibling risk between 15 and 20 (Sundquist et al., 2008; Morin et al., 2019).

Despite some discrepancies in the magnitude of the risk, familial aggregation studies all demonstrated that AS clusters in families more frequently than expected by chance.

Is Familial Clustering Related to Genetic, Environmental, or Cultural Risk Factors?

Three general mechanisms, not mutually exclusive, may explain such familial clustering: genetic factors, environmental factors to which related individuals may be exposed together or cultural inheritance of risk factors related to lifestyle. Heritability refers to the proportion of the variation in a trait due to genetic factors (Tenesa and Haley, 2013). Classical study designs to assess it are family-based, mainly twin studies. More recently, SNP-based heritability assessment methods using unrelated cases and controls have been developed (Yang et al., 2010, 2017).

Twin Studies

Genetic contributions to a disease may be estimated from twin studies by comparing the phenotypic similarity of monozygotic (MZ) to that of dizygotic (DZ) twins. Indeed, both types of twins are raised together and therefore share a large proportion of environmental exposures and cultural risk factors. However, MZ twins are genetically identical whereas DZ twins share only 50% of their genome. Thus, comparison of the concordance for a trait between MZ and DZ twins allows to estimate its genetic heritability. However, twin design relies on several assumptions which may lead to an overestimation of heritability if not met. In particular, they assume that shared environmental factors are identical in MZ and DZ pairs, which has been questioned (Felson, 2014).

In AS, two twin studies have estimated heritability. The first one was a compilation from four studies including 83 twin pairs (Brown et al., 1997). Concordance rate was higher in the 27 MZ pairs (63%) than in the 56 DZ ones (12.5%). Heritability was estimated to 97% (95% CI: 92–99.2%) based on a disease prevalence of 0.1%. The second one, compiling one Norwegian and two Danish nationwide twin surveys, found lower concordance rates (maybe because of the nationwide population design which minimizes the risk of ascertainment bias) but again with striking differences between MZ (40%) and DZ twins (4.3%) (Pedersen et al., 2008a). In that study, heritability was estimated to 61% but with a broad 95% confidence interval ranging from 0 to 99% because of the small sample size of the study (28 twin pairs only). A similar Danish nationwide study focused on psoriatic arthritis, one of the SpA subtypes, found a lower heritability (34%) (Pedersen et al., 2008b).

Single Nucleotide Polymorphism-Based Heritability Studies

With the development of high-throughput genotyping methods, it became possible to estimate the genetic similarity between individuals through the use of whole-genome single nucleotide polymorphism (SNP) array data. Different statistical methods have been developed to test this SNP-based heritability (Yang et al., 2017). A major asset of this approach is the possibility to use unrelated subjects with no risk of confusion due to shared environmental factors and thus to use very large datasets from genome-wide association studies (GWAS). SNP-based heritability assessment detects only the additive effects of causal variants tagged by common SNPs present in the SNP microarray used. Thus, this type of heritability is expected to be lower than twin-based heritability, and the difference between the two methods may reflect the contribution of rare variants or structural variants not included in the microarrays or non-additive effects (Yang et al., 2010).

In AS, SNP-based heritability has been estimated at 60.8% using all the SNPs of Immunochip array and at 27.8%

using only the 244 independent association signals significant at genome-wide level (Ellinghaus et al., 2016). This gap suggests that common variants associated with AS are yet to be identified.

More recently, heritability of more than 2,000 traits has been estimated in the UK Biobank (UKBB) cohort using a genome-wide microarray with a better coverage than Immunochip (Abbott and Neale, 2020). In this cohort, AS heritability was estimated at 39.9%. However, this estimate should be interpreted cautiously for several reasons. First, the sample size of only 584 AS patients was too low to yield robust estimate. Moreover, identification of AS cases in this cohort relied on medical records and probably lacked accuracy. Finally, UKBB cohort cannot be considered representative of the United Kingdom population, with a selection bias toward healthier individuals (Fry et al., 2017). This may explain the very low prevalence of AS in this cohort (0.04%) which might inflate the estimated heritability.

Altogether, heritability studies suggest a strong genetic contribution to SpA. The difference observed between the heritability assessments from twins and those from unrelated case–control studies also suggests a potential contribution of rare or structural variants not captured by the Immunochip.

How Is Genetic Susceptibility Inherited?

Given evidence for genetic influence on disease susceptibility, the next step is to determine how genetic susceptibility is inherited. As for most common diseases, AS does not appear to be inherited as a simple Mendelian dominant or recessive trait. Segregation analyses aimed at determining how susceptibility segregates in families. By comparing observed disease incidence in each relative class with that expected based on a specified model, it is possible to test various genetic hypotheses. Determination of the mode of inheritance of a complex disease is, however, challenging because of numerous potential confounders including genetic heterogeneity, ascertainment bias, and incomplete penetrance (King et al., 1984).

To assess the most likely mode of inheritance of AS, Brown et al. (2000) compared recurrence risk ratios estimated from several genetic models to those observed in different class of relatives of affected subjects according to previously published data. Among the five tested models ("single locus," "polygenic multiplicative," "two locus multiplicative," "HLA, residual polygons," and "five locus multiplicative"), the best fitting model was a five-locus model with multiplicative interaction between loci. The precise number of genes involved cannot be accurately modeled, and models with three to nine genes were equally consistent with the observed data.

PHENOTYPIC FAMILY-BASED APPROACHES

Studies of familial cases have also helped to better understand the relationships between SpA subtypes and to refine the clinical description of familial SpA.

Can We Study Together All SpA Subtypes for Genetic Purpose?

Spondyloarthritis consists of several closely related disorders, including AS, psoriatic arthritis, inflammatory bowel disease-associated SpA, reactive arthritis, and undifferentiated SpA. Although each of these entities is defined by specific characteristics, they share several major epidemiological, clinical, and imaging features, as well as an association with HLA-B27, leading to the unified concept of SpA (Moll et al., 1974). A critical question regarding the concept of SpA concerns the extent to which distinct manifestations belonging to the SpA spectrum depend on identical factors, including genetic predisposition.

Two alternative models have been proposed. The first one hypothesized a genetic heterogeneity, with different combinations of several independent predisposing factors leading to a variety of phenotypic expressions of disease. This assumption was supported by studies suggesting that different disease forms bred true within families (Hochberg et al., 1978; Calin et al., 1984). The alternative model of phenotypic diversity postulated that there is a predominant predisposing component common to most forms of SpA, but different manifestations occur because of the additional influence of minor factors. To test these two models, the French Group for Genetic Research on SpA (GFEGS) has extensively studied SpA manifestations in families with multiple cases of SpA. They showed that distinct SpA subtypes can coexist within families (Said-Nahal et al., 2000) and similar observations were also made in Chinese families (Chou et al., 2005). They also demonstrated that all the articular and extra-articular manifestations belonging to the spectrum of SpA segregated together (Said-Nahal et al., 2001). Finally, they estimated the recurrence risk ratio at 35 for parent/child and 45 for siblings (Dernis et al., 2009). Altogether, these results suggested that SpA subtypes might be considered phenotypic variations of a unique disease and could be studied together in genetic studies.

Is Disease Severity Genetically Determined in SpA?

Little is known about the genetic control of disease severity in SpA. The concept of severity in itself is not well defined in SpA as severity can include multiple aspects of the disease such as pain, disease activity, impaired physical function, or radiographic structural damage.

By studying AS-affected sib pairs, Calin and Elswood (1989) showed a high familiality of pain and disability indices, as well as radiographic damages. Hamersma et al. (2001) estimated the heritability of disease activity [through Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)] and functional impairment [through Bath Ankylosing Spondylitis Functional Index (BASFI)] in AS. Strong heritability was observed for both indexes (51% for BASDAI and 68% for BASFI) but not for the age at disease onset (18%). High heritability has also been demonstrated for radiographic damages assessed by Bath Ankylosing Spondylitis Radiology Index (Brophy et al., 2004).

However, to date, no genetic factors of disease severity has been consistently reported.

Is There Any Phenotypic Difference Between Familial and Sporadic SpA?

Several studies investigated whether clinical presentation of familial cases differed from that of sporadic cases (i.e., without known affected relative). However, the definition of a "familial" form is not as straightforward as it might appear because the probability of reporting an affected relative is highly dependent on the number of ascertained relatives, i.e., the sibship size.

Factors described as associated with familial disease differed between studies, except for a higher prevalence of HLA-B27 identified in most of them (Almodóvar et al., 2011, 2016; Joshi et al., 2012; Kim et al., 2014). There was also a trend to a milder disease in familial forms (Calin et al., 1993; Almodóvar et al., 2016), which might have been caused by a selection bias among familial cases (the probability to diagnose a patient with mild symptoms might be higher in the presence of a family history of SpA).

Thus, differences of clinical presentation between sporadic and familial cases of SpA seem to be minor.

FAMILY-BASED APPROACHES FOR IDENTIFICATION OF GENES OF SUSCEPTIBILITY TO SpA

Historically, family-based designs were the preferred tool used to identify not only genetic factors of susceptibility, especially in Mendelian diseases but also in complex traits with familial aggregation, such as SpA. More recently, there was a renewed interest in family-based approaches for their potential interest in the detection of rare variants.

Linkage Analyses

Linkage analysis is a genetic method that searches for chromosomal segments that cosegregate with the disease phenotype through families. In SpA, three genome-wide linkage studies using microsatellites were published, two in AS and one in SpA as a whole (Laval et al., 2001; Miceli-Richard et al., 2004; Zhang et al., 2004). In all of them, major histocompatibility complex (MHC) region was highly linked to SpA. However, only two loci besides the MHC reached significance threshold: one on 16q in AS (Laval et al., 2001) and the other on 9q31-34 in SpA (Miceli-Richard et al., 2004).

These findings have been followed up in order to identify more precisely the gene involved in SpA susceptibility in the significantly linked regions. In the first published AS GWAS (Wellcome Trust Case Control Consortium et al., 2007), a suggestive association was found between AS and two SNPs close to the 16q region. This association was further replicated and refined to a region including the gene *TRADD*, involved in NFκB signaling and the regulation of proinflammatory cytokines (Pointon et al., 2010). On the other hand, comprehensive study of the 9q31-34 region, including fine mapping of the whole region and systematic sequencing of a large number of genes in that region led to the identification and replication of a protective haplotype of six SNPs near the *TNFSF15* gene, a Crohn's disease susceptibility gene (Zinovieva et al., 2009), and of a rare SNP located in *TNFSF8*, a gene which plays a critical role in Th17 cell differentiation (Sun et al., 2010), as significantly associated with SpA (Zinovieva et al., 2008, 2011).

One limitation of linkage studies is that they cannot locate disease-associated loci on a fine scale. To try to circumvent this

TABLE 1 | Family-based studies investigating rare variants in SpA.

Study	Design	Ethnicity	Variant (gene)	Discovery sample	Validation sample	Functional consequences
Uddin et al. (2013)	Whole-genome CNV	Caucasian	7 kb duplication (UGT2B17)	1 family (6 AS)	587 AS/584 HC ($p = 0.09$)	No functional data
Rong et al. (2015)	Targeted sequencing	Chinese Han	Arg580Gly (IRS1)	1 family (8 AS)	Not detected in 309 AS and 210 HC	No functional data
O'Rielly et al. (2016)	Whole-exome sequencing	Caucasian	9 bp deletion (SEC16A) and 20 bp deletion (MAMDC4)	1 family (9 AS)	944 AS/1134 HC ($p = 0.92$)	Conformational change (SEC16A) and RNA-mediated decay (MAMDC4)
Tan et al. (2018)	Whole-genome linkage + exome sequencing	Chinese Han	Leu87Val (ANKDD1B)	1 family (5 AS)	Not detected in 500 HC	No functional data
Feng et al. (2018)	Whole-genome linkage + exome sequencing	Chinese Han	Arg213Try (TREML2)	1 family (23 AS)	331 AS/487 HC ($p = 0.4$)	No functional data
Garshasbi et al. (2020)	Whole-exome sequencing	Iranian	Ser2486Gly (RELN)	1 family (7 AS)	Not detected in 50 HC	No functional data
Liu et al. (2020)	Exome sequencing	Chinese Han	Lys132Asn (RNF123)	1 family (2 AS)	994 AS/999 HC ($p = 0.03$)	Decreased ability of monocytes to differentiate into osteoclasts

CNV, copy number variant; AS, ankylosing spondylitis; HC, healthy controls; p, p value.

issue, a more recent linkage analysis used a high-density panel of SNPs and identified a new locus significantly linked with SpA was identified on 13q13 (Costantino et al., 2016). However, despite the higher density of marker, the disease interval could not be restricted to less than 1.4 Mb and further investigations are needed to identify causal variant(s) in this region.

Family-Based Association Analysis

Familial approach can also be applied to genetic association study. To date, only one family-based association analysis has been published in SpA (Costantino et al., 2017). None of the tested SNPs reached genome-wide significance. However, combined analysis including two independent family-based replication cohorts identified an association close to genome-wide significance between an intronic SNP of *MAPK14* and SpA. Moreover, nominal associations for polymorphisms in several *loci* previously associated with AS through case-control GWAS reinforcing the evidence of shared genetic background between SpA as a whole and AS.

Rare Variants

To date, case-control genome-wide association studies allowed the identification of more than 40 susceptibility regions outside of the major histocompatibility complex. However, all these loci, including HLA-B27, explain less than 30% of AS heritability (Ellinghaus et al., 2016). Several hypotheses have been advanced to account for this unexplained heritability, including structural variants, gene-gene and gene-environment interactions, and rare variants (Bodmer and Bonilla, 2008; Eichler et al., 2010). However, case-control studies often lack statistical power to detect the latter accurately. As an example, despite a rather large sample size (5,040 patients and 21,133 healthy controls), the only reported case-control study investigating the role of rare variants in AS had a power estimated to 9% for variants with a minor allele frequency (MAF) of 1% and close to zero for variants with MAF of 0.02%, corresponding to the median of the study (Robinson et al., 2016).

Family-based approaches can help in the detection of rare variants potentially involved in SpA. Indeed, variant filtering process is easier in families because of the possibility to analyze the cosegregation of variants with the phenotype under study. Moreover, rare variants are more prone to be population specific and family-based designs are more robust to population stratification than case-control design (Laird and Lange, 2006).

There is an increasing number of studies combining family-based design and next-generation sequencing in SpA (Uddin et al., 2013; Rong et al., 2015; O'Rielly et al., 2016; Feng et al., 2018; Tan et al., 2018; Garshasbi et al., 2020; Liu et al., 2020). They all showed a perfect or at least a high degree of cosegregation of one or several rare variants with SpA in large families with multiple cases of SpA (**Table 1**). Interestingly, none of these variants or their corresponding genes has been previously identified through GWAS approaches. However, because of the low frequency of these variants, independent replication of associations was challenging and all the studies except one failed to validate the association in an independent cohort (Liu et al., 2020). Experimental validation might be an alternative to genetic replication but should be investigated.

CONCLUSION

Family studies have been critical to demonstrate the genetic background of SpA and to model the transmission of the disease. They also highlighted the connection between all SpA subtypes and reinforced the unified concept of SpA. Recent studies have revealed their potential in the identification of genetic factors involved in SpA susceptibility. Thus, the recent gain of interest in the role of rare variants in complex diseases might lead family-based approaches to return to the front stage.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Genetics of Ankylosing Spondylitis—Focusing on the Ethnic Difference Between East Asia and Europe

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Ankylosing spondylitis (AS) is a common, highly heritable inflammatory arthritis affecting the mainly axial joints in both East Asia and Europe. To date, the pathogenesis of AS is still unknown, although we know that genetics play a vital role in it. The HLA-B27 allele is found in over 85% of AS patients. However, strong evidence suggests that other major histocompatibility complex (MHC) and non-MHC genes are also involved in the pathogenesis. In addition, current data showed that there were significant differences in both genomics and metagenomics among the different ethnic populations. The investigation of the key role of the microbiome in AS pathogenesis also highlighted the host–microbiome genetic interactions. Here, we systematically review current AS genetic research data and further compare genetic differences, especially between East Asian and European groups, which may highlight the challenge in future genetic studies.

Keywords: ankylosing spondylitis, genetics, polygenetic risk score, East Asia, Europe

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INTRODUCTION

Ankylosing spondylitis (AS) is one of the commonest rheumatic diseases in both Asia and Europe. It is a highly heritable chronic inflammatory disease that mainly affects the axial joints, but also has peripheral joints and various organs involvement (Brown et al., 1997, 2000). Pathogenesis of AS is still unknown. The worldwide distribution of AS is closely related to the carrier rate of human leukocyte antigen (HLA)-B27 in population. The prevalence of the disease is about 0.55% of Caucasian population (Braun et al., 1998) and 0.26% in Chinese (Wigley et al., 1994) but less common in Japanese and Africans, mostly attributing to the parallel carrier status of HLA-B27 alleles in these ancestry groups. While the HLA-B27 allele is found in over 85% of patients (Caffrey and James, 1973), there is strong evidence indicating that other major histocompatibility complex (MHC) and non-MHC genes also jointly play roles in the pathogenesis of the disease. Interestingly, current data showed an obvious disease-associated genetic discrepancy across different populations. The observed genetic heterogeneity across divergent populations at several risk loci is by differences in allele frequencies, linkage disequilibrium patterns, effect sizes of associated polymorphisms, or a combination of these factors (International Genetics of Ankylosing Spondylitis Consortium et al., 2013). The objective of this review is mainly to summarize the currently available genetic data and to further make comparisons of this disease genetically between East Asian and European populations.

THE DIFFERENCE IN HLA ALLELES

Associations between *HLA-B27* and AS were first reported in 1972 (which were some of the earliest described genetic associations), and it remains the most substantial risk factor for AS (Stokes et al., 1972). There are significant differences in the worldwide distribution of *HLA-B27* and its subtypes (Khan et al., 2007; Gragert et al., 2013). The prevalence of *HLA-B27* positivity in the Chinese and Korean populations has been reported to be from 4 to 8% and 2.3 to 7%, respectively (Kim and Kim, 2010; Yang et al., 2013), which is lower than that in Caucasians but much higher than that in the Japanese population (1%) (Feltkamp et al., 2001).

HLA-B27 plays a pivotal role in the pathogenesis of AS. To date, in both Europeans and Asians, the most accurate tag SNP of *HLA-B27* is rs116488202 (International Genetics of Ankylosing Spondylitis Consortium et al., 2013), which is superior to previously reported tag SNP rs4349859 and rs13202464 in Asian populations (Lin et al., 2011). To date, there are 213 known alleles of *HLA-B27* at the nucleotide sequence level, while at the translated protein level, there are 160 known subtypes based on one or more amino acid sequence differences (Khan, 2017). The frequencies of *HLA-B27* alleles vary in different race groups (**Supplementary Table 1** and **Figure 1**; Khan et al., 2007; Gragert et al., 2013). Like other ethnic groups, more than 80% of Chinese AS patients are *HLA-B27* positive, but the primary subtype is *HLA-B*27:04*, followed by *HLA-B*27:05*, which is a predominate subtype for Caucasian cases (Liu et al., 2010). The distribution of *HLA-B27* subtypes also reveals substantial demographic and geographic diversity in China. Although *HLA-B*27:04* is a major subtype in Chinese individuals, it has been reported that the proportion of *HLA-B*27:04* carriers in AS patients were higher in southern China than in northern China, whereas *HLA-B*27:05* positivity was the reverse (Rong and Jieruo, 2013). Being consistent with Mainland Chinese Han Cases, Taiwan Han population is also dominated by *HLA-B*27:04* (Yang et al., 2004; Liu et al., 2010). As for Korea being up north of China geographically, it makes sense that *B*27:05* is the predominant subtype in Koreans, which is similar to Caucasians but different from other Asians down south (Park et al., 2009).

*HLA-B*27:06* has a relatively weak and negative association with AS compared to the *HLA-B*27:04* subtype in Southeast Asia (Nasution et al., 1997; Garcia-Fernandez et al., 2001; Chen et al., 2002), which is more prevalent in Malay descendants (Lopez-Larrea et al., 1995; Gonzalez-Roces et al., 1997; Ren et al., 1997; Hou et al., 2007). In addition, *B*27:09* was found not associated with AS in Sardinia (D'Amato et al., 1995). Mathieu et al. (2008a,b) have proposed a possible evolutionary effect of genetic selection by malaria infection, which could explain the absence of risk haplotypes for AS where malaria was endemic.

Recently, a large-scale study in European case–control cohorts has been initially genotyped by Illumina Immunochip (International Genetics of Ankylosing Spondylitis Consortium et al., 2013), taking the lead in fine-mapping MHC region of HLA classic alleles, SNP, and amino-acid residues in European AS cases and controls. This study suggested the associations with *HLA-B40* and multiple other class I and II alleles (Cortes et al., 2015). It also demonstrated that in that Caucasian the amino-acid sequence of *HLA-B* at position 97 in the B-peptide-binding pocket is the crucial determinant of HLA associations with AS. After controlling for the associated haplotypes in HLA-B, independent associations with variants in the *HLA-A*, *HLA-DPB1*, and *HLA-DRB1* loci were observed.

As for East Asian populations, several loci associated with AS in the Chinese population have been identified, including *HLA-B60* and MHC I chain-related gene A (*MICA*) (Ho and Chen, 2013; Zhou et al., 2014). A recent case–control study in Korean additionally identified the association with AS at HLA-C*15:02 (Kim et al., 2015). In our recent study, we analyzed the associations of AS across the MHC aiming to identify potentially causal SNPs, amino acids, or haplotypes using an extended cohort of East Asian ancestry (1,637 Chinese, Taiwanese, and Korean AS cases and 1,589 ethnically matched controls). We have assessed the MHC association of AS in an expanded East Asian cohort. Imputation of the MHC region was conducted in order to assess the variants, HLA classical alleles, and amino-acid residue HLA proteins. This study suggests that the HLA-B associations (B27 and B40) are mainly driven by the amino acids at positions 70 and 97, which locate in the B-pocket of HLA-B peptide-binding groove. Except for HLA-B associations, previously reported East Asian-specific association at *HLA-C*15* had been validated. In addition, a novel association at *HLA-DQB1*04* has been identified (Wang et al., 2020). However, our study needs further validation in larger cohort and conduct with better MHC imputation reference panel of the Pan-Asian population.

THE DIFFERENCE IN NON-MHC GENETICS

As discussed above, AS is strongly associated with variants in the MHC region and HLA alleles. However, *HLA-B27* and other MHC genes contribute no more than one-third of the genetic risk. It has been intensely investigated that genetic factor non-MHC variants contribute to disease susceptibility. So far, at least 36 genetic variants in non-MHC regions have been identified as associated with AS in genome-wide association study (GWAS) (Brown et al., 2016).

A large-scale multi-ethnic case–control association study performed with Illumina Immunochip microarray provided a new perspective on the similarities and differences in AS susceptibility between East Asian and Caucasians. A total of 13 loci had at least nominal levels of association in East Asians (Chinese, Koreans, Taiwanese), whereas 23 achieved genome-wide significance in white Europeans (International Genetics of Ankylosing Spondylitis Consortium et al., 2013; Ellinghaus et al., 2016; Robinson et al., 2016). Additional studies have been performed in European cohorts. An exome-wide study further identified a novel genome-wide significant association at *CDKAL1*, and several suggestive and secondary loci (Robinson et al., 2016). Ellinghaus et al. (2016) conducted a case–control Immunochip study of five closely associated conditions, including AS, primary sclerosing cholangitis, psoriasis, Crohn's disease, and ulcerative colitis, in a cohort of European ancestry,

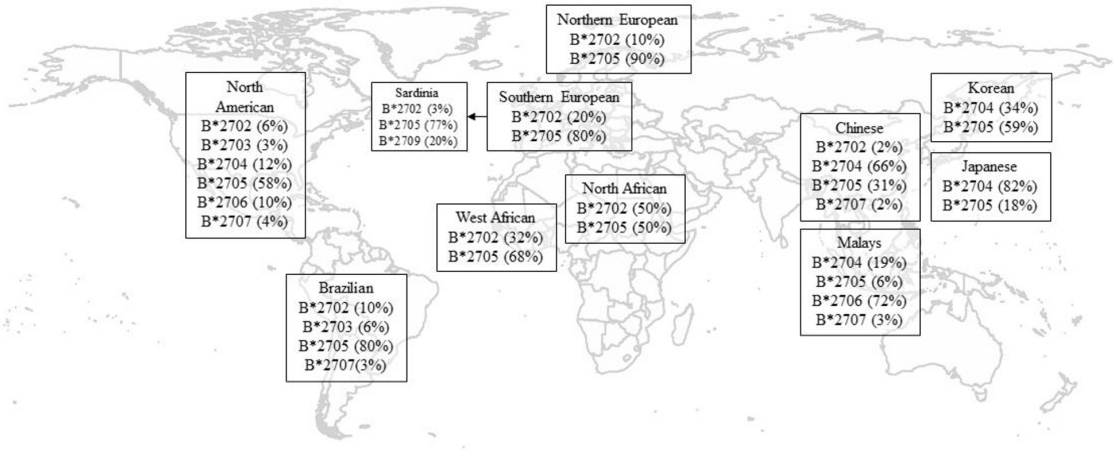

FIGURE 1 | Geographical distribution of HLA-B27 subtypes. Only the most frequent alleles were shown, and statistics are rounded off for simplicity and indicate percentage in healthy controls. Data of North American were adapted from Gragert et al. (2013; **Supplementary Table 1**). Data for Korean were adapted from Park et al. (2009). Data of other populations were adapted from Khan et al. (2007).

delineated the genetic overlap between the conditions, and identified 17 new genome-wide significant susceptibility loci of AS. It also showed that comorbidities between AS and the other chronic inflammatory diseases were mostly attributed to genetic pleiotropy. However, the promising findings of the susceptibility and pleiotropy in Caucasians need to be validated in Asian cohorts.

On account of the limited sample size of the East Asian cohort in the Immunochip study, the power of the East Asian cohort was much inferior to that of the European cohort. However, there is hitherto more than 40% overall of the associated loci that have been validated in East Asian (**Figure 2**), including *ERAP1*, *GPR35*, *HHAT*, *HLA-B*, *ICOSLG*, *IL23R*, *IL27*, *NOS2*, *NPEPPS*, *RUNX3*, *TBX21*, *TYK2*, *UBE2E3*, *UBE2L3*, and *ZMIZ*1, and two intergenic regions (2p15 and 21q22) (Brown et al., 2016). Besides, a GWAS study in Han Chinese identified two AS associated loci (*HAPLN1-EDIL3* and *ANO6*) (Lin et al., 2011). However, few signals of the association have been observed on these two loci in an independent Immunochip study of both East Asians and Caucasians.

Even though most large-scale GWASs have been disproportionately investigated in cohorts of European descent and similar patterns of predisposition were observed between East Asians and Europeans, which is consistent with a shared ancestor origin of the disease-associated SNP, genetic differences exist between ethnic groups pointing to differences in ethnicity-specific etiopathogenesis. Taking the interleukin-23 receptor (IL23R) gene, for example, the primarily associated variants indicated diversity between Europe and Asia. rs11209026, a critical non-synonymous SNP in *IL23R* associated with AS in Caucasians, was not polymorphic in East Asians (Davidson et al., 2009). No other common SNPs on *IL23R* found to be significantly associated with AS in Caucasian European populations. It was suggested that *IL23R* might be a Caucasian-specific associated gene. However, a low-frequency visitant in IL23R, rs76418789, has been reported to potentially attenuate the protective effects of *IL23R* against AS in Han Chinese (Davidson et al., 2013). The same SNP was also nominally associated with AS in Europeans. On the contrary, the minor allele frequency (MAF) of rs76418789 was about 3.7% in East Asians, while only 0.34% in Europeans. In addition to the rare variants on *IL23R*, the SNPs on *STAT3* were also found associated with AS in the Chinese population, which is a downstream molecule of IL-23R in the IL-23 signaling pathway involving the differentiation of Th17 cell populations (Davidson et al., 2011). It may indicate that the sharing effect on Th17 cells is attributed to different mechanisms of disease pathogenesis. Among the other AS-associated loci defined in Europeans but not in East Asians, only the primary associated variant rs17765610 on *BACH2* presents a different frequency of over six times greater in Europeans than in East Asians (East Asians, 1.8%; Europeans, 11.8%) (International Genetics of Ankylosing Spondylitis Consortium et al., 2013). The findings might indicate that the different associations between East Asia and Europe were not attributed to the various frequencies of associated variants at most loci.

METAGENOMICS

It has been drawing increasing attention that gut inflammation plays a pivotal role in the pathogenesis of AS. IBD, as a paradigm for microbiota effects on the pathogenesis of the immune-mediated disease, is strongly related and significantly overlaps with AS in genetic predisposition. HLA-B27 transgenic rats did not develop gut and joint inflammation when bred in a germ-free environment, while inflammations present when they were exposed to healthy gut bacteria (Rath et al., 1996).

Several studies have investigated how the microbiome as a key role in driving the pathogenesis of AS. Intestinal dysbacteriosis may affect the permeability of the intestinal wall, the expression of related inflammatory factors, the intestinal mucosal immune status in AS patients, and molecular mimicry

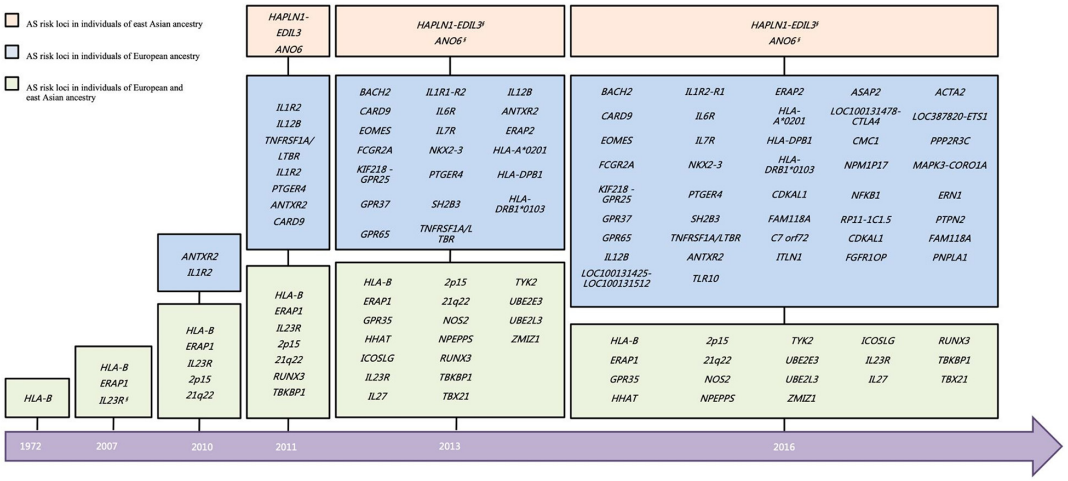

FIGURE 2 | Historical overview of disease susceptibility polymorphisms identified in AS. Risk loci selected with a genome-wide significance threshold of $P < 5 \times 10^{-7}$ as well as secondary associations ($P < 5 \times 10^{-4}$ after conditioned for the primary associated SNP), in the previous studies are included. *But different SNP. §No independent support for the two loci in other East Asian cohort. AS, ankylosing spondylitis.

of HLA-B27 (Ebringer and Ghuloom, 1986; Ciccia et al., 2009, 2014, 2015; Wright et al., 2016). Intestinal flora imbalance can also mediate host metabolism and immune function imbalance *via* a series of cytokines, thus participating in the pathogenesis and progress of AS.

Studies have revealed several notable differences in bacterial species and in abundance. Costello et al. (2015) have compared the terminal ileum microbial communities in AS patients with healthy controls in Australia, and Lachnospiraceae, Veillonellaceae, Ruminococcaceae, Rikenellaceae, Bacteroidaceae, Porphyromonadaceae, and Prevotellaceae were significantly enriched or decreased in abundance in AS patients. Wen et al. (2017) have reported a quantitative metagenomics study suggesting that discrete gut microbial signature is associated with the pathogenesis of AS in Chinese, suggesting consistent findings with the report of Costello, such as Prevotellaceae. It also showed some discordance with previous reports in Europeans, like Bacteroidetes, and identified other novel biomarkers that might be involved in the development of AS in the Chinese population (Wen et al., 2017). To further investigate the roles of microbiome in AS pathogenesis, Yin et al. (2019) conducted a case–control metagenomic analysis of 250 Han Chinese. In addition to confirmation of previously reported gut dysbiosis and species differences in AS, the results also indicate that treatment with TNF inhibitor (TNFi) normalizes the gut microbiome. The AS gut microbiome is enriched for bacterial peptides that have previously been shown to be presented by HLA-B27, and that this enrichment is also normalized by TNFi treatment. Bacterial peptides presented by HLA-B27 have been found enriched in gut microbiome in AS patients, which is also normalized by TNFi treatment. Relative to untreated patients, TNFi therapy of AS patients was also associated with a reduction of potentially arthritogenic bacterial peptides, which are enriched bacterial peptides homologous to HLA-B27-presented epitopes. Host-bacteria genetic interactions were also observed between an AS-associated SNP (*RUNX3*) and microbiome, highlighting a non-MHC host genotype influencing AS *via* the microbiome potentially (Yin et al., 2019).

Over the last few decades, Asia, especially China, has experienced rapid urbanization, resulting in massive changes of dietary habits, which directly links to the changes of the microbiome (Yang et al., 2016). Gut microbiota is both an important therapeutic target for AS and an essential biomarker for disease surveillance. The reconstruction of gut microbiota has a potential therapeutic effect on AS patients, and the relationship between intestinal flora and AS deserves further study.

CLINICAL BENEFITS AND PROSPECTS

To date, clinical practice data show that the average diagnostic delay of AS is 6–10 years and early treatment, such as anti-TNFα biological agents, have been proved to improve disease outcomes. So, the early prediction of AS is challenging, causing noteworthy. However, the genome-wide significant associated SNPs only represent a trivial fraction of total heritability. A polygenic risk score (PRS) is aiming to use genotype data from thousands of genetic variants to quantify an individual's genetic risk for specific diseases and has potential as a diagnostic and screening test for common heritable diseases, including AS (Torkamani et al., 2018). In our recent research (Li et al., 2021), we used GWAS data from 15,083 AS cases and 20,902 controls and then developed and validated PRS in European-descent and East Asian ethnicity subjects. PRS showed greater discriminatory capacity and accuracy than HLA-B27 testing, MRI scanning, or CRP testing, and could be used to assist in diagnosing AS among chronic back pain patients, as well as screening populations to identify subjects at increased risk of the disease. When the PRS was derived and tested in individuals of primarily their

ancestries, the area under the curves (AUC) of the European and East Asian GRSs were 0.92 and 0.95, respectively. The discriminant capacities were attenuated cross-ethnic validations (AUC = 0.79 for European model in the East Asian cohorts, AUC = 0.88 for East Asian model in the European cohorts). It suggests that the performance of the PRS does vary between ethnic groups. The PRS developed specifically for the East Asian population performed considerably better in that population than did the European PRS, elucidating the different genetic landscapes between East Asian and European.

The similarities and differences in the genetic features of AS between East Asia and other ethnic populations have demonstrated the utility of gene mapping in probing the genetic diversity among different ancestry groups. It is of great importance to confirm the associated loci in populations of different ancestries, which is a crucial indicator of the overall significance of defining the true disease-causing variant. The identification of genetic signatures in both the East Asian and European populations will provide additional details for unraveling the genetic basis of AS and other autoimmune diseases.

PRS will be of clinical use, particularly for a disease of low prevalence and high heritability, like AS. Given the low cost of microarray, even next-generation sequencing, PRS makes it possible for population screening. Modified PRS models of ethnic specificity and multi-omics, including epigenomics and metagenomics, need more comprehensive training cohorts and more accurate evidence for better disease prediction of AS. To date, most genetics studies have been undertaken in European ancestry. Therefore, further studies of multi-omics analyses, microbiota, and environmental factors will require undertaking or expanding trans-ethnic cohorts.

AUTHOR CONTRIBUTIONS

XW, GW, LZ, and HX made substantial contributions to draft and revise the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.671682/full#supplementary-material

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frontiers in Genetics

MINI REVIEW
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Targeted Therapies in Axial Psoriatic Arthritis

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Specific and high-quality evidence on the efficacy of the current targeted therapies for axial disease in psoriatic arthritis (axPsA) is still scarce. Indeed, almost all the cohorts investigated in clinical trials on PsA consisted of patients with peripheral arthritis, where a small number of them also had axial involvement. Only one randomized controlled trial was so far specifically designed to assess the efficacy of a biological disease-modifying antirheumatic drug (DMARD) in axPsA. For other biological and synthetic targeted DMARDs, the most specific evidence for treatment in axPsA is extrapolated from *post-hoc* analyses based on PsA patients with concomitant peripheral and axial manifestations. Furthermore, the current trials and *post-hoc* analysis on axPsA are affected by major limitations, including the lack of a widely accepted definition of axPsA and the lack of specific and validated outcome measures. Finally, poor data are available on the genetics of axPsA, although alleles differentially expressed in different patterns of axPsA might offer advantages in the prospective of personalized medicine in axPsA patients. Overall, this review suggests that there is an urgent need for more reliable evidence derived from studies specifically designed for axPsA and based on a validated definition of axPsA and on specific outcome measures.

Keywords: psoriatic arthritis, targeted therapy, spondylarthritis, axial psoriatic arthritis, treatment

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INTRODUCTION

Psoriatic arthritis (PsA) is a heterogenous systemic inflammatory disease with different subtypes of joint manifestations, including peripheral arthritis, dactylitis, enthesitis, and axial disease (Moll and Wright, 1973). The prevalence of axial disease in PsA (axPsA) widely varies depending on its definition and disease duration (Gladman, 2007). Indeed, isolated spondylitis was described by Moll and Wright in only 5% of patients with PsA (Moll and Wright, 1973), but when axial involvement is defined by inflammatory back pain and/or radiography along with peripheral involvement, its prevalence ranges from 5 to 28% in early PsA to 25–70% in longstanding PsA (Gladman, 2007; Chandran et al., 2009).

Despite the relatively high occurrence of axPsA, several unresolved issues persist with regard to its definition, classification, and management (Gladman et al., 2021). An evidence-based and widely accepted definition of axPsA does not exist yet (Feld et al., 2018). Furthermore, although significant epidemiological clinical and prognostic differences between axPsA and ankylosing spondylitis (AS) have been highlighted in several studies (Jadon et al., 2017; Feld et al., 2018), it is still a matter of debate whether axPsA and AS are different phenotypes on the spectrum of the same disease, or they are different diseases with overlapping features (Feld et al., 2018). Genetic studies were called into question to understand the basis of such differences and overlapping

aspects (Rahmati et al., 2020). In particular, the prevalence of HLA-B27, the key genetic marker of AS, was demonstrated to be lower in patients with PsA (20 vs. 95%), although among PsA patients, those with axPsA were significantly more frequently HLA-B27-positive compared with those without axial involvement ($P < 0.001$). Moreover, when different patterns of axial involvement were separately investigated, HLA-B27 was recorded in only 22% of PsA patients with unilateral sacroiliitis, which is more typical in axPsA, compared with 85% of patients with bilateral sacroiliitis (Vecellio et al., 2021). Conversely, in a study on 282 PsA patients, HLA-B*0801 was significantly more frequent in patients with asymmetrical sacroiliitis (Winchester et al., 2016).

In this context of uncertainty in the definition and classification of axPsA, the current recommendations for the management of PsA patients with axial involvement are mainly extrapolated from studies on AS or non-radiographic axial spondylarthritis SpA (nr-axSpA), and poor specific evidence exists regarding the use of the available biologic (b-) and targeted synthetic (ts-) disease-modifying antirheumatic drugs (DMARDs) in ax-PsA (Gossec et al., 2012; Coates et al., 2016; Singh et al., 2019).

The objective of this work was to summarize current evidence regarding the efficacy of the currently available b- and ts-DMARDs, including tumor necrosis factor-alpha (TNFα) inhibitors, interleukin (IL)-17 inhibitors, IL-23 and IL-12/23 inhibitors, and Janus kinase (JAK) inhibitors, in treatment of axPsA.

Tumor Necrosis Factor-Alpha Inhibitors

Infliximab is a chimeric human–murine monoclonal antibody (MoAb) that binds with high affinity to both soluble and transmembrane forms of TNF (Antoni, C. E. et al., 2005; Antoni, C. et al., 2005). It is approved for the treatment of PsA and AS. In the pivotal randomized controlled trials (RCTs) submitted for its approbation in PsA (IMPACT 1-2), <5% of patients had axial involvement along with peripheral arthritis, and no specific sub-analyses on axial outcomes were performed (Antoni, C. E. et al., 2005; Antoni, C. et al., 2005). No RCTs were specifically implemented in axPsA patients treated with infliximab.

Etanercept is a soluble human TNF-α receptor p75 Fc fusion protein that inhibits the biological activity of TNF by competitively binding to its cell surface receptors (Mease et al., 2004). It is licensed for treatment of PsA, AS, and non-radiographic axial spondylarthritis (nr-axSpA). In the RCT submitted for approbation in treatment of PsA, only 3% of patients had axial involvement, and respective outcomes were not analyzed. No RCTs have specifically tested the efficacy of etanercept in axPsA (Mease et al., 2004).

Adalimumab is a human immunoglobulin G (IgG)1 MoAb targeting TNF (Mease et al., 2005; Genovese et al., 2007). It is licensed for the treatment of PsA, AS, and nr-axSpA. In the two RCTs (ADAPT, M02-570 Study) submitted for approbation in treatment of PsA, <2% of patients had axial involvement, and axial outcomes were not assessed (Mease et al., 2005; Genovese et al., 2007). No RCTs have been implemented in adalimumab-treated axPsA patients.

*Golimuma*b is a human monoclonal IgG1 antibody that forms high-affinity, stable complexes with both the soluble and transmembrane TNFs (Kavanaugh et al., 2009). It is licensed for the treatment of PsA, AS, and nr-axSpA. In the RCT (GO-REVAL) assessing the efficacy of golimumab in PsA, 10% of patients were reported to have axial involvement; however, no specific definition on the axial disease was provided, and analysis on the outcomes of axial involvement was not assessed (Kavanaugh et al., 2009). No RCTs were specifically implemented in axPsA patients treated with golimumab.

Certolizumab pegol is a humanized antibody Fab' fragment against TNF conjugated to polyethylene glycol (Cauli et al., 2015). It is licensed for the treatment of PsA, AS, and nr-axSpA. In the RCT (PsA001) assessing its efficacy in the treatment of PsA, no data were reported on prevalence and outcomes of axial involvement (Mease et al., 2014). No RTCs have specifically assessed the efficacy of certolizumab in axPsA.

Several RCTs demonstrated the efficacy of different TNF inhibitors in reducing clinical disease activity in AS and nr-ax-SpA, as assessed by composite indices, including the Assessment in AS (ASAS) criteria and the Bath AS Disease Activity Index (BASDAI) (van der Heijde et al., 2005; Haibel et al., 2008). Albeit less univocally, efficacy was also recorded in reducing the radiographic progression in these conditions (van der Heijde et al., 2008). No sub-analyses were performed in patients with concomitant psoriasis. A meta-analysis confirmed a significant association between HLA-B27 and a higher rate of response to TNF-α inhibitors in AS, as assessed by the ASAS-40 [odds ratio 1.83, 95% confidence interval (CI) 1.39–2.42] and the BASDAI-50 (odds ratio 1.81, 95% CI 1.35–2.42). Furthermore, with regard to pharmacogenomic, no association was shown between −308 TNF gene polymorphism and BASDAI response (Maneiro et al., 2015).

Interleukin-17 Inhibitors

Secukinumab is a fully human IgG1κ MoAb that selectively binds to IL-17A with high affinity. It is approved for the treatment of both PsA and axSpA.

Of three RCTs (FUTURE 1, 2, and 5) submitted for approval of secukinumab in treatment of PsA, all were conducted in patients with active peripheral arthritis, according to the inclusions criteria, and none provided data on axial involvement (McInnes et al., 2015; Mease et al., 2015; van der Heijde et al., 2020).

A recent phase 3 double-blind, randomized trial evaluated the efficacy and safety of secukinumab in managing axial manifestations in PsA patients, who have failed to respond to non-steroidal anti-inflammatory drugs and were bio-naïve (Baraliakos et al., 2020). Patients treated with secukinumab 300 and 150 mg, when compared with placebo-treated patients, had a significantly higher rate of ASAS-20 (63 and 66 vs. 31%) and ASAS-40 (44 and 40 vs. 12%) achievement at week 12. Furthermore, the least-squares means of treatment difference vs. placebo in change from baseline to week 12 in total Berlin magnetic resonance imaging (MRI) score for the entire spine were −0.4 (0.1) and −0.4 (0.1) for secukinumab 300 and 150 mg, respectively ($p < 0.05$) (**Table 1**) (Baraliakos et al., 2020).

Three RCTs (MEASURE 1, 2, and 3) assessed the effectiveness and safety of secukinumab in AS, but the

TABLE 1 | Summary of studies specifically designed to assess the effectiveness of different biologic DMARDs in patients affected by psoriatic arthritis with axial involvement.

Treatment	References	Definition of axial involvement	Type of study	N	Main findings
Secukinumab (SEC)	Baraliakos et al., 2020	- Clinician-diagnosed axial involvement - Spinal pain VAS > 40/100 - BASDAI >4 (despite ≥2 NSAIDs)	Phase III double blind RCT (MAXIMASE)	498	• SEC 300 and 150 mg significantly improved ASAS20 response vs. PBO at week 12 (63% and 66 vs. 31%). • SEC 300 and 150 mg significantly improved ASAS40 response vs. PBO at week 12 (44% and 40 vs. 12%). • Least square means (LSM) of treatment difference of SEC 300 and 150 mg vs. PBO from baseline in total Berlin MRI score for the entire spine at week 12 was −0.4 (<0.001) and −0.4 ($p < 0.05$).
Ixekizumab (IXE)	Deodhar et al., 2019a	- Self-reporting axial pain starting before the age of 45 years at baseline	*Post-hoc* integrated analysis of two phase III RCTs (SPIRIT-P1/P2)	105	• Pain and stiffness significantly improved at Weeks 16 and 24 in patients with PsA treated with IXEQ4W or IXEQ2W vs. PBO ($p < 0.05$). • Fatigue significantly improved at Week 16 in patients treated with IXEQ4W or IXEQ2W vs. PBO and at Week 24 with IXEQ2W vs. PBO ($p < 0.05$). • Total BASDAI scores significantly improved at Weeks 16 and 24 in patients treated with IXEQ4W or IXEQ2W vs. PBO ($p < 0.01$). • Physical function significantly improved at Weeks 16 and 24 in patients treated with IXEQ4W or IXEQ2W vs. PBO when assessed by HAQ-DI or SF-36 PCS ($p < 0.05$).
Ustekinumab (UST)	Kavanaugh et al., 2016	- Clinician-diagnosed spondylitis	*Post-hoc* integrated analysis of two phase III RCTs (PSUMMIT-1 and 2)	256	• At week 24, significantly more patients achieved BASDAI20/50/70 responses (54.8/29.3/15.3% vs. 32.9/11.4/0%; $p \leq 0.002$). • Higher improvement in BASDAI question 2 concerning axial pain in UST-treated vs. PBO 1.85 vs. 0.24 ($p < 0.001$) and mean per cent ASDAS-CRP improvements (27.8 vs. 3.9%; $p < 0.001$) for UST vs. PBO.
Guselkumab (GUS)	Helliwell et al., 2020	- Imaging-confirmed sacroiliitis	*Post-hoc* integrated analysis of two phase III RCTs (SPIRIT-P1/P2)	312	• In GUS 100 mg q4w, q8w, and PBO groups, the LS mean change at 24w in BASDAI was −2.7, −2.7, −1.3 ($p < 0.001$); in modified BASDAI was −2.6, −2.7, −1.4 ($p < 0.001$), in spinal pain was −2.5, −2.7, −1.4 ($p < 0.001$); in ASDAS was −1.4, −1.4, −0.7 ($p < 0.001$). • The rate of BASDAI 50 achievement was 38, 40, 19% ($p < 0.01$).
Upadacitinib (UPA)	Deodhar et al., 2020a,c	- Clinician-diagnosed spondylitis	*Post-hoc* integrated analysis of two phase III RCTs (SELCET-PsA1-2)	541–626	• Mean delta-BASDAI at week 12: 1–75 and −2.22 in UPA 15 and 30 mg vs. PBO −0.56 ($p < 0.001$). • Mean delta-BASDAI at week 24: −2.61 and −2.71 in UPA 15 and 30 mg vs. PBO −1.00 ($p < 0.001$).

PBO, placebo; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; ASAS, Assessment of Spondylarthritis International Society; ASDAS, Ankylosing Spondylitis Disease Activity Score.

proportion of patients with PsO was minimal (<10%), and sub-analysis on this subgroup of patients was not performed (Baeten et al., 2015; Pavelka et al., 2020). In a *post-hoc* analysis aimed to assess the impact of HLA-B27 status on clinical outcomes in AS patients treated with secukinumab, it was recorded that secukinumab was effective regardless of HLA-B27 status, although HLA-B27-positive patients may derive increased therapeutic benefit (Deodhar et al., 2020a). In the only RCT (PREVENT) demonstrating the effectiveness of secukinumab in nr-ax-SpA, no data on psoriasis were reported (Deodhar et al., 2021).

Ixekizumab is a recombinant IgG4κ MoAb that binds with high affinity to and neutralizes IL-17A. It is approved for the treatment of both PsA and axSpA. Of two RCTs (SPIRIT-P1 and -P2) assessing effectiveness and safety of ixekizumab in the treatment of PsA, all had the presence of peripheral arthritis as an inclusion criterion, and none provided data on the proportion of patients with axial involvement nor assessed axial outcomes (Mease et al., 2017; Nash et al., 2017). A *post-hoc* integrated analysis of these two trials was conducted in a subset of 105 PsA patients with self-reporting axial pain starting before the age of 45 years (axial imaging not included). When compared with placebo, ixekizumab 80 mg every 4 weeks was associated with significantly higher improvement of axial pain (BASDAI question #2 −3.25 vs. −1.26, $p < 0.001$), stiffness (BASDAI question #4/5 −2.5 vs. 0.32, $p < 0.01$), fatigue (−1.84 vs. −0.53, $p < 0.05$), and total BASDAI score (−2.8 vs. 0.78, $p < 0.01$) at 16 weeks. Similar results were recorded at 24 weeks and for ixekizumab every 8 weeks (**Table 1**) (Deodhar et al., 2019a). Of the three RCTs evaluating the efficacy of ixekizumab in the treatment of AS (COAST-V and -W) nr-axSpA (COAST-X), none reported data on occurrence on concomitant psoriasis (Deodhar et al., 2020b; Dougados et al., 2020). In the *post-hoc* analysis of COAST-X based on HLA-B27 status and disease duration, patients treated with ixekizumab saw improvement in signs and symptoms of nr-axSpA as assessed by ASAS-40 and BASDAI50 responses regardless of HLA-B27 status (positive or negative) or disease duration (<5 or ≥5 years) (Navarro-Compán et al., 2020).

Brodalumab is a fully human MoAb that binds to the IL-17 receptor subunit A with high affinity. It has recently been approved for the treatment of PsA. Results from two recently published phase II RCTs (AMVISION 1 and 2) showed a significant improvement in signs and symptoms of PsA in patients treated with brodalumab vs. placebo. However, axial involvement was not assessed (Mease et al., 2021). No RCTs are available in axPsA and axSpA.

Interleukin-12/23–IL23 Inhibitors

Ustekinumab is a fully human MoAb with a high affinity for the p40-subunit shared by IL-12 and IL-23. It is approved for the treatment of moderate to severe psoriasis and PsA but not for axSpA. Two phase 3 RCTs (PSUMMIT 1 and 2) demonstrated the efficacy of ustekinumab in the treatment of multiple domains of PsA, including peripheral arthritis, enthesitis and dactylitis, and radiographic progression of joint damage (Kavanaugh et al., 2016). Approximately 30% of patients from both the trials had physician-reported spondylitis, and ustekinumab demonstrated significant improvements in axial symptoms at week 24, as assessed by the BASDAI, regardless of prior TNF inhibitor use. A *post-hoc* integrated analysis of these RCTs was conducted in 223 PsA patients with peripheral arthritis and physician-reported spondylitis. At week 24, significantly more patients achieved BASDAI 20, 50, and 70 responses (54.8, 29.3, and 15.3 vs. 32.9%, 11.4 and 0%; $p \leq 0.002$), improvement in BASDAI question #2 concerning axial pain (−1.85 vs. −0.24; $p < 0.001$), and mean percent ASDAS-CRP improvements (27.8 vs. 3.9%; $p < 0.001$) for ustekinumab vs. placebo recipients, respectively (**Table 1**) (Kavanaugh et al., 2016). On the other hand, three RCTs assessing efficacy and safety of ustekinumab in the treatment of AS and nr-axSpA were discontinued because the primary and major secondary endpoints (ASAS-20, 40, and ASDAS improvement) were not met (Deodhar et al., 2019b).

Guselkumab is a human IgG1λ MoAb that binds to the IL-23p19 subunit and inhibits the downstream signaling of IL-23. It is approved for the treatment of PsA but not axSpA. Two RCTs (DISCOVER 1 and 2) demonstrated the efficacy of guselkumab in the treatment of PsA, as assessed by ACR 20, 50, and 70 (Deodhar et al., 2018). A *post-hoc* integrated analysis of these studies was carried out in 312 PsA patients with peripheral arthritis and imaging-confirmed axial involvement consistent with sacroiliitis (both bio-naïve and not). In patients treated with guselkumab 100 mg every 4 weeks vs. placebo, a greater LS mean changes from baseline to week 24 in BASDAI (−2.67 vs. −1.35, $p < 0.001$), spinal pain (BASDAI question #2 −2.73 vs. −1.30, $p < 0.001$), modified BASDAI (−2.16 vs. −1.13, $p < 0.001$), and ASDAS-CRP (1.43 vs. −0.71, $p < 0.001$) were recorded (Helliwell et al., 2020). Moreover, a greater proportion of guselkumab-treated patients achieved BASDAI-50 (40.5 vs. 19.1%, $p < 0.01$) and ASDAS responses of inactive disease (17.4 vs. 1.7%, $p < 0.001$), major improvement (27.9 vs. 8.7, $p < 0.01$), and clinically important improvement (53.5 vs. 28.7%, $p < 0.01$) at week 24. Improvements in axial symptoms were observed irrespective of HLA-B27 status (**Table 1**) (Helliwell et al., 2020). No RCTs were conducted on axial-SpA.

Risankizumab (BI 655066/ABBV-066) is a humanized IgG1 MoAb that selectively inhibits IL-23 by specifically targeting the p19 subunit 18. It is approved for the treatment of moderate to severe psoriasis (Mease et al., 2018b). In a phase II RCT, it showed efficacy in the treatment of PsA, but no data are specifically reported on axPsA patients. In another phase II RCT, risankizumab failed to meet the study's primary endpoint and showed no evidence of clinically meaningful improvements compared with placebo in patients with active AS (Baeten et al., 2018).

Janus Kinase Inhibitors

Tofacitinib is a JAK1/JAK3 inhibitor approved for the treatment of PsA. Two phase III RCTs (ORAL-Beyond and ORAL Broaden) demonstrated the efficacy of tofacitinib in the treatment of peripheral involvement, but the effect on the

axial domain was not specifically investigated (Gladman et al., 2017). In a phase II RCT in AS bio-naïve patients (<4% with PsO), tofacitinib 5 and 10 mg twice daily demonstrated greater clinical efficacy vs. placebo in reducing signs, symptoms, and objective endpoints of active AS in adult patients with a similar 12-week safety profile as reported in other indication (van der Heijde et al., 2017). A *post-hoc* analysis on the same study showed that approximately one-third of tofacitinib-treated AS patients experienced clinically meaningful reductions in spinal MRI inflammation at week 12. Patients achieving myelin imaging compound for MRI inflammation had a greater clinical response (Maksymowych et al., 2018).

Upadacitinib is another selective JAK1 inhibitor. It is approved for the treatment of PsA and recently also for AS. In two phase III RCTs (SELCET PsA1 and PsA2) in PsA with peripheral arthritis, upadacitinib was demonstrated effective in the achievement of ACR20, 50, and 70 (Genovese et al., 2020; Mcinnes et al., 2020). In a *post-hoc* analysis of these two studies, involving ∼400 PsA patients with physician-diagnosed spondylitis, treatment with UPA 15 and 30 mg resulted in significantly greater improvements from baseline in the overall BASDAI, BASDAI questions #2 (neck/back/hip pain) and #3 (joint swelling/pain), and ASDAS-CRP endpoints at weeks 12 and 24 vs. placebo. Similarly, significantly higher percentages of pts on UPA 15 and 30 mg achieved BASDAI 50, ASDAS ID, LDA, MI, and CII at weeks 12 and 24 vs. placebo (**Table 1**) (Deodhar et al., 2020c). The effectiveness of upadacitinib was reported in phase II/III RCT on AS, where data on psoriasis were not reported (van der Heijde et al., 2019).

Filgotinib is a selective JAK1 inhibitor. In a phase II RCT (EQUATOR) on 131 PsA patients with peripheral arthritis, 80% in the filgotinib group vs. 33% in the placebo group achieved ACR20 at week 16 ($p < 0.0001$) (Mease et al., 2018a). No data on axial involvement were reported. In a phase II RCT (TORTUGA) on 116 patients with AS, the mean ASDAS change values from baseline to week 12 were −1.47 in the filgotinib group and −0.57 in the placebo group, with an LS mean difference between groups of −0.85 (95% CI −1.17 to −0.53; $p < 0.0001$) (Mease et al., 2018a).

Deucravacitinib is a novel oral agent that selectively inhibits tyrosine kinase 2. In a phase II RCT, it demonstrated efficacy in peripheral PsA, but there are no data on axial disease (Mease et al., 2020).

DISCUSSION

The results of this review suggest that, although the interest in the axial disease in PsA has progressively increased in the last years, specific and high-quality evidence on the efficacy of the current targeted therapies in this subset of patients still represents an unmet need.

Almost all PsA patients recruited in clinical trials had peripheral arthritis, and only a small number had axial involvement. Only one RCT was specifically designed to assess the efficacy of a b-DMARD in axPsA, demonstrating significant improvements across multiple clinical and imaging endpoints in a population with high activity of inflammatory back pain treated with secukinumab. For other b- and ts-DMARDs (ixekizumab, ustekinumab, guselkumab, and upadacitinib), the most specific evidence for potential efficacy in axPsA treatment are derived from *post-hoc* analysis of RCTs based on PsA patients with concomitant peripheral and axial involvement.

Current studies on axPsA are affected by major limitations, starting from the lack of a validated and widely accepted definition of axPsA that prevents identifying a homogeneous group of patients in which reliable clinical trials can be performed. This is why a steering committee including members from the Group for Research and Assessment of Psoriasis and Psoriatic Arthritis and the Assessment of SpA International Society is working toward developing an evidence-based and widely accepted definition of axPsA (the AXIS trial) (Gladman et al., 2021). A further major limitation in the studies on axPsA is that in the absence of specific outcome measures for axPsA, all studies have borrowed assessment tools from AS (i.e., BASDAI and ASDAS). However, such instruments are not validated for axial disease in PsA, and the burden of peripheral arthritis may impact them. Indeed, improvements in other domains may result in an overall improvement of such outcome measures, even if there are no significant changes in the axial disease activity. This could partially explain the conflicting evidence regarding ustekinumab, which demonstrated efficacy in a *post-hoc* analysis on axPsA but not in an RCT in AS, or it may explain why guselkumab was found to be useful in axPsA, but risankizumab, another IL-23 inhibitor, did not meet the primary endpoint in AS patients. Finally, extremely poor data are available on pharmacogenetic of PsA, particularly for axPsA. Although substantive advances have been made in the understanding of genetics in psoriatic disease over the last decade, we are still at an early stage. The rapid emergence of affordable high-throughput technology will likely lead to the discovery of genetic factors that may lead to a more in-depth comprehension of mechanisms underlying the different phenotypical patterns of PsA and more precise profiling of axPsA patients. Such findings may hopefully support clinicians in choosing for each patient the treatment with higher likelihood efficacy (Rahmati et al., 2020).

In conclusion, there is an urgent need for more reliable data derived from studies specifically designed for axPsA and based on a validated definition of axPsA and on specific outcome measures. Moreover, in the prospective of personalized medicine, a further effort should also be made to develop specific studies aimed to identify genetic biomarkers of axPsA.

AUTHOR CONTRIBUTIONS

AF and AC conceptualized the review. AF, MC, EC, and MA contributed to analyzing the data and wrote the manuscript. AC and MP supervised the work. All authors reviewed the article and approved the submitted version.

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REVIEW
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From Science to Success? Targeting Tyrosine Kinase 2 in Spondyloarthritis and Related Chronic Inflammatory Diseases

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Spondyloarthritis (SpA) is a family of inflammatory arthritic diseases, which includes the prototypes of psoriatic arthritis and ankylosing spondylitis. SpA is commonly associated with systemic inflammatory diseases, such as psoriasis and inflammatory bowel disease. Immunological studies, murine models and the genetics of SpA all indicate a pathogenic role for the IL-23/IL-17 axis. Therapeutics targeting the IL-23/IL-17 pathway are successful at providing symptomatic relief, but may not provide complete protection against progression of arthritis. Thus there is still tremendous interest in the discovery of novel therapeutic targets for SpA. Tyrosine kinase 2 (TYK2) is a member of the Janus kinases, which mediate intracellular signaling of cytokines via signal transducer and activator of transcription (STAT) activation. TYK2 plays a crucial role in mediating IL-23 receptor signaling and STAT3 activation. A plethora of natural mutations in and around TYK2 have provided a wealth of data to associate this kinase with autoimmune/autoinflammatory diseases in humans. Induced and natural mutations in murine Tyk2 largely support human data; however, key inter-species differences exist, which means extrapolation of data from murine models to humans needs to be done with caution. Despite these reservations, novel selective TYK2 inhibitors are now proving successful in advanced clinical trials of inflammatory diseases. In this review, we will discuss TYK2 from basic biology to therapeutic targeting, with an emphasis on studies in SpA. Seminal studies uncovering the basic science of TYK2 have provided sound foundations for targeting it in SpA and related inflammatory diseases. TYK2 inhibitors may well be the next blockbuster therapeutic for SpA.

Keywords: TYK2, spondyloarthritis, IL-23, JAK inhibitor, JAK, clinical trials

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INTRODUCTION

Spondyloarthritis (SpA) is a family of seronegative chronic inflammatory arthritic diseases, united by shared clinical features and the association with HLA-B27 (Taurog et al., 2016; Ritchlin et al., 2017). Both the axial and peripheral joints can be affected, with arthritis progression characterized by paradoxical bone erosion and new bone formation. In extreme forms, the

axial joints, including the sacroiliac and intervertebral joints, can completely fuse. Inflammation of the skin, gastrointestinal tract and eyes are often present in SpA patients in the forms of psoriasis, inflammatory bowel disease (IBD) and uveitis, respectively. Intestinal inflammation, often subclinical, is common in axial forms of the disease, including the most common form, ankylosing spondylitis (AS) (Gracey et al., 2020b). Psoriasis on the other hand, is a diagnostic feature of psoriatic arthritis (PsA) (Taylor et al., 2006), which typically affects, but is not restricted to, the peripheral joints. Considerable overlap exists in the clinical phenotype of PsA and AS (Jadon et al., 2017), with modern classification criteria labeling them as peripheral SpA (pSpA) and axial SpA (axSpA), respectively (Sieper et al., 2009; Rudwaleit et al., 2011). Under these modern classification criteria, AS is a form of radiographic axSpA; however, the term AS is still in common use, so will be used throughout the manuscript where appropriate.

Therapeutic options for SpA are limited. Non-steroidal anti-inflammatory drugs (NSAIDs) remain a frontline therapy, particularly for AS (Van Der Heijde et al., 2017), with biologic and targeted synthetic disease modifying anti-rheumatic drugs (bDMARDs/tsDMARDs) acting as second line therapies when the response to NSAIDs is insufficient. For PsA, additional conventional synthetic (csDMARDs), such as methotrexate, are also recommended as frontline therapies prior to bDMARDs/tsDMARDs (Ogdie et al., 2020). Approved bDMARDS include TNF inhibitors (TNFi) and IL-17 inhibitors (IL-17i) for both AS and PsA, with IL-23 inhibitors (IL-23i) additionally approved for PsA (Al-Mossawi et al., 2019; Fragoulis and Siebert, 2020). For PsA, two types of tsDMARDs are approved: the PDE4 inhibitor, apremilast, and the Janus kinase (JAK) inhibitor, tofacitinib (Al-Mossawi et al., 2019). No tsDMARDs are currently approved for AS. With short term use, approximately 60–65% of SpA patients achieved a 20% reduction in symptoms (ASAS20) in most randomized controlled trials with current bDMARDs. With long term use, evidence exists for bDMARDs slowing joint fusion, but they may not prevent it (Haroon et al., 2013; Braun et al., 2017; Mease et al., 2018b; Koo et al., 2020).

Therapies approved for related inflammatory disease are more extensive and sometimes more effective than those approved for SpA. For rheumatoid arthritis (RA), three JAK inhibitors and a range of bDMARDs are approved, including those targeting TNFα, IL-6, IL-1β, B cells (CD20), and T cells (CD80/CD86). For IBD, the TNFi (except for etanercept) and IL-12/23i have remission rates comparable to those seen in SpA (Hanauer et al., 2002; Feagan et al., 2016). In contrast to SpA, IL-17i do not work and may worsen IBD (Hueber et al., 2012). Further, IBD has two integrin inhibitors approved, which do not appear to be effective in and may worsen SpA (Varkas et al., 2017; Dubash et al., 2018). Tofacitinib is also approved for IBD, with remission rates of around 40% (Panés et al., 2017; Sandborn et al., 2017). For psoriasis, the range of bDMARDs and tsDMARDs available is comparable to SpA, with the newer therapies, such as the IL-23i, achieving almost complete remission of skin disease in most patients (Kaushik and Lebwohl, 2019). By comparison, the same approved therapeutics for SpA generally achieve a 50% reduction in symptoms in around half of treated patients (Mease et al., 2020b).

The therapeutic armaments against SpA, and their efficacy, reflects our knowledge of the underlying immunopathology of the disease. Indeed, all approved bDMARDs and tsDMARDs for SpA were first trialed in diseases with extensively characterized immune components, such as RA or psoriasis, before being tested in SpA. In these diseases, animal models have been long established and widely used (Brand et al., 2007; van der Fits et al., 2009), and the latest omics techniques have been applied to the target tissue in humans (Zhang et al., 2019; Hughes et al., 2020). By comparison, there are few animal models of SpA that faithfully recapitulate the disease (Vieira-Sousa et al., 2015), and human target tissue, especially of the axial skeleton, remains largely inaccessible for research.

We know at the immunological level that type 3 immunity (also known as "Type-17 immunity" or the "Th17-axis"), namely IL-17A and IL-17A-producing cells, play a critical role in SpA (Taams et al., 2018; Gracey et al., 2020b). This is highlighted by the success of IL-17i across major forms of SpA. While IL-23 was long suspected to be the key driver of IL-17A in SpA, this does not seem to be the case, at least in patients with axSpA, who fail to respond to IL-23i (Baeten et al., 2018). IL-1β and IL-6 are IL-23 independent inducers of IL-17A, but biologics targeting both of these cytokines also failed clinical trials in AS (Haibel et al., 2005; Sieper et al., 2015). By comparison, IL-23 plays an essential role in all stages of psoriasis and blocking it is clinically effective (Papp et al., 2017), whereas IL-23 only seems to play a role in early experimental RA (Pfeifle et al., 2017), with its blockade providing no clinical protection against symptomatic disease in patients (Smolen et al., 2017).

HLA-B27 is a major histocompatibility complex (MHC) class I molecule that presents peptides to CD8+ T cells, allowing for their activation. By virtue of the strong association of HLA-B27 with SpA, CD8+ T cells likely play a central role in SpA pathogenesis; however, conclusive evidence from humans and animal models does not yet exist. As a result, alternative theories for how HLA-B27 may mediate a CD8+ T cell independent role in SpA have been put forward, such as the endoplasmic reticulum stress theory, and HLA-B27 interaction with killer inhibitory receptors (KIR) (Bowness, 2015). Nevertheless, a clear disturbance of cytotoxicity in CD8+ T cells of AS patients was recently revealed (Gracey et al., 2020c), and IL-17A+ CD8+ T cells expressing *IL23R* were found to be enriched in the synovial fluid of PsA patients (Steel et al., 2020). A better understanding of CD8+ T cells in SpA may facilitate the development of novel therapeutics against the diseases.

Genetic studies of SpA support the role of type 3 immunity and CD8+ T cells. Genome-wide association studies (GWAS) of AS implicate a number of genes involved in the production of, and response to, IL-17, and also many genes in MHC class I peptide processing and CD8+ T cell function (Cortes et al., 2013). PsA GWAS have not advanced as quickly as the AS GWAS, likely due to the strong overlap with psoriasis making it difficult to determine risk factors for PsA alone *vs* those shared with psoriasis. Nonetheless, a similar pattern of type 3 immunity and CD8+ T cell-related genes are linked to

PsA (Bowes et al., 2015; Stuart et al., 2015). Importantly, various single-nucleotide polymorphisms (SNPs) in and around tyrosine kinase 2 (*TYK2*) were linked to both AS and PsA in these studies (Cortes et al., 2013; Bowes et al., 2015; Stuart et al., 2015; Ellinghaus et al., 2016). As TYK2, a member of the JAK family, is known to mediate intracellular signaling for the IL-23R, it is assumed that *TYK2* is one of the many genes leading to a perturbed type 3 immune response in SpA patients.

Here, we discuss the biology of TYK2 and advances made in targeting it. While this article is included in a special publication on the genetics of SpA, only a handful of genetic studies have implicated *TYK2* in the context of SpA (Cortes et al., 2013; Dendrou et al., 2016; Ellinghaus et al., 2016), and only one functional study of TYK2 has focused on SpA patients and animal models (Gracey et al., 2020a). In addition, TYK2 inhibitors are still relatively early in their development compared to other JAK inhibitors (JAKi), which is reflected in the lower number of publications on TYK2 compared to the other JAK family members (**Figure 1**). For these reasons, this article will more broadly discuss the biology and therapeutic targeting of TYK2 relevant to SpA, with direct citations of SpA research made when available.

JAKS ARE ESSENTIAL INTRACELLULAR CYTOKINE SIGNALING MOLECULES

The JAK family of non-receptor kinases is a small but influential set of intracellular signaling molecules. Here, we illustrate key concepts of JAK mediated signaling using the IL-23R as an example to keep with the theme of this article (**Figure 2**). The JAK family shares the four functional domains: four-point-one, ezrin, radixin, moesin (FERM), and Src Homology 2 (SH2) domains, followed by pseudokinase and kinase domains. The peptide sequence of the FERM and SH2 domains dictate which specific cytokine receptors each JAK can bind (Haan et al., 2006; Ferrao and Lupardus, 2017). JAKs bind to their respective cytokine receptors constitutively, with JAK binding often being essential for surface expression of its cognate receptor chain (Ragimbeau et al., 2003; Kumar et al., 2008; Carbone and Fuchs, 2014). The pseudokinase and kinase domains are closely associated, with the former inhibiting the kinase activity in the resting state (Lupardus et al., 2014). The intimate association of these two kinase domains led to them being named after the Roman god, Janus, who has two faces looking in opposite directions (Wilks, 2008). The binding of a ligand to its corresponding JAK-associated receptor complex leads to conformational changes, which results in the activation of the associated JAKs through *trans*- and auto-phosphorylation, and subsequent phosphorylation of tyrosine residues of the signal transducing cytokine receptor chain (Villarino et al., 2017; Morris et al., 2018). These phospho-tyrosines act as docking sites for STAT transcription factors, which subsequently become phosphorylated by the JAKs, and translocate to the nucleus as homo- or heterodimers. Thus, JAKs are a critical link in the chain of events from cytokines to cellular responses.

There are four members of the family, namely JAK1, JAK2, JAK3, and TYK2, which mediate selected hormone, colony stimulating factor and cytokine signaling (O'Shea et al., 2015). These JAK family members partner predictably with specific cytokine receptor subunits, resulting in a consistent pairing of homo- or heterodimer JAK-receptor subunits (Morris et al., 2018). For example, the IL-12 family utilizes TYK2 through its interaction with the common receptor subunit IL-12RB1. JAK2 binds to the partner receptors, IL-23R and IL-12RB2, to provide specificity to IL-23 and IL-12, respectively. TYK2 also associates with the type I interferon receptor 1 (IFNAR1) to mediate IFN-I signaling; IL-10R2 to mediate IL-10 family signaling (IL-10, IL-22); IL-13RA1 to mediate IL-13 and in some circumstances IL-4 signaling; and gp130, the common chain of the IL-6 receptor. Despite binding to gp130, TYK2 is not required for mediating IL-6R family signaling (Wöss et al., 2019).

While JAK-STAT signaling is often portrayed as a linear path, there are multiple levels of complexity and control. JAK signaling is negatively regulated in a number of ways. Constitutively expressed phosphatases act to restrain the duration of JAK signaling, such as CD45 and SHP1/SHP2 (Morris et al., 2018), but no conclusive evidence exists if this negative regulation plays a role in IL-23R signaling. Inducible negative regulation occurs through the suppressor of cytokine signaling (SOCS) family, which can specifically block kinase activity of JAK (Liau et al., 2018), block STAT docking (Yamamoto et al., 2003) and can also target the activated cytokine receptor for degradation via ubiquitination (Kamizono et al., 2001; Durham et al., 2019). Some evidence exists that SOCS1 is able to negatively regulate IFN-I signaling via TYK2 (Piganis et al., 2011), and SOCS3 may be an important negative regulator of IL-23R signaling (Chen et al., 2006).

The STAT specificity of a given cytokine receptor is driven not by the JAKs themselves, but by the peptide sequence of the cytokine receptor's intracellular domain providing a docking site for a given STAT (Stahl et al., 1995; Naeger et al., 1999). For example the IL-12RB2 binds STAT4, while IL-23R binds STAT3 (Teng et al., 2015; Floss et al., 2020). While a given cytokine receptor preferentially induces the activation of selected STATs, there is no clear understanding of what role the specific receptor-associated JAKs play in activating a given STAT. Our poor understanding of how these intracellular signaling pathways work is also reflected by the fact that not all phosphorylations of STATs are activating, a fact that can be overlooked in research. As an example, while STAT3 tyrosine (Y)705 is generally considered to be an indication of activation, serine (S)727 and Y640 phosphorylation negatively regulate STAT3 activity (Chung et al., 1997; Mori et al., 2017). The STAT molecules also undergo other post-translational modifications that alter their function. For example, STAT3 undergoes non-degradative ubiquitination that promotes Y705 phosphorylation (Cho et al., 2019). STAT3 also undergoes palmitoylation to promote its recruitment to the cell membrane and de-palmitoylation to release STAT3 for nuclear translocation (Zhang et al., 2020).

An additional aspect of the JAK family of kinases relevant to inhibition by small molecules is that they may also undertake kinase-independent functions. TYK2 is described to have a scaffold function in cytokine signaling receptor complexes, whereby JAK binding to a cytokine receptor chain can be essential

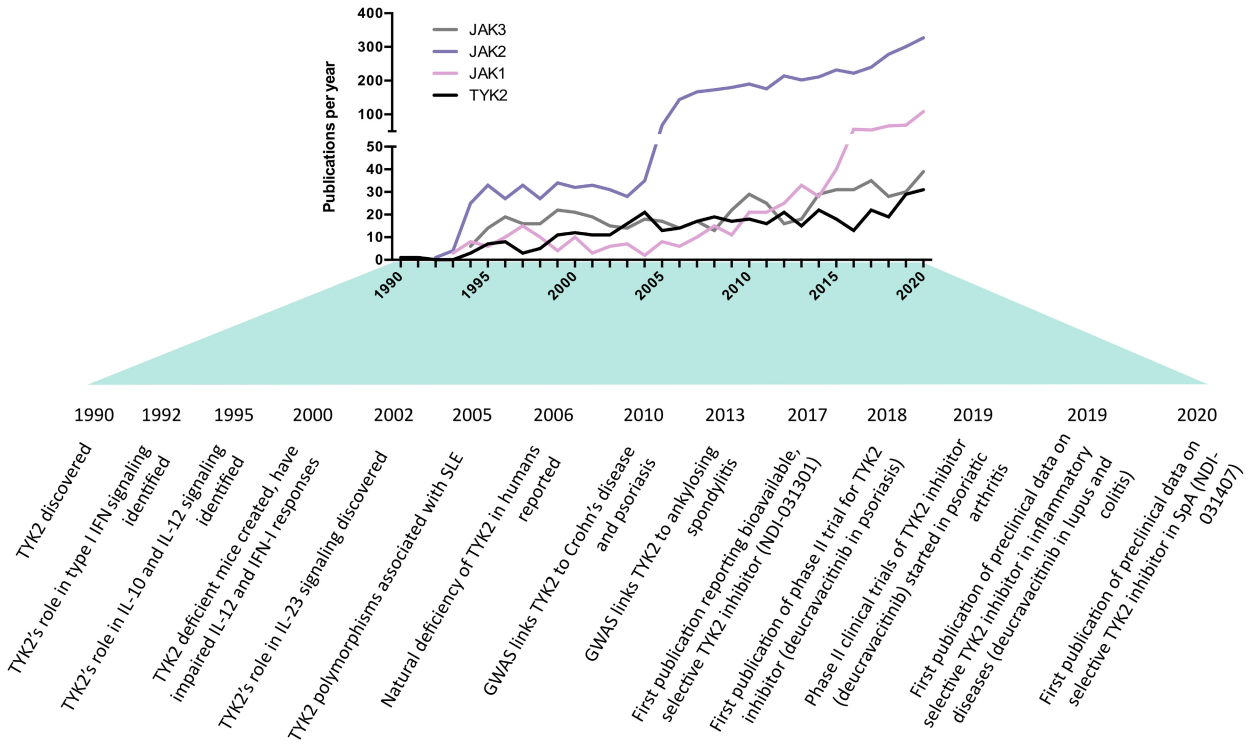


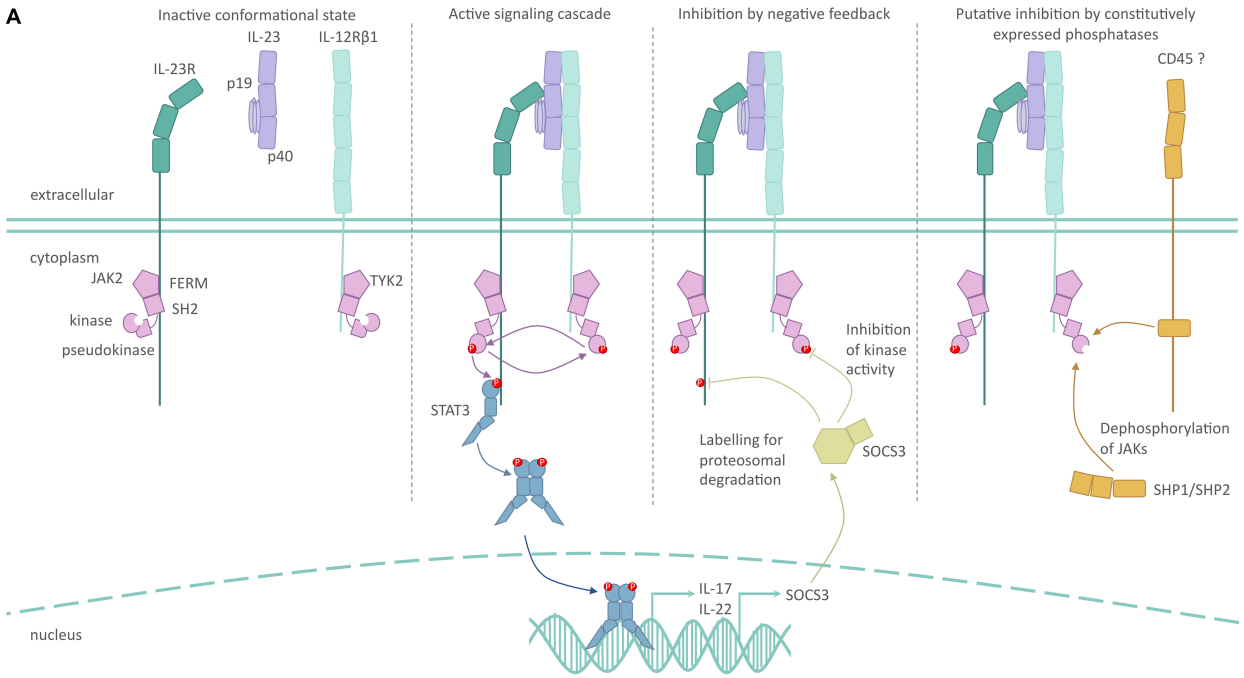
FIGURE 1 | History and milestones of Janus kinase (JAK) research, with an emphasis on tyrosine kinase 2 (TYK2) in the context of SpA. Data obtained from SCOPUS database using searching the abstract and title for short name (e.g., "TYK2") or long name (e.g., "tyrosine kinase 2"). The increase in JAK2 publications was likely driven by the discovery of JAK2 mutations in myeloproliferative disorders in 2005. The increase in JAK1 publications coincided with the discovery of its role in leukemia.

for its surface expression (Ragimbeau et al., 2003; Kumar et al., 2008; Carbone and Fuchs, 2014). Specifically, kinase inactive (mutated) TYK2 stabilizes the receptor subunit it binds, such as IL-10R2, IL-12RB1, and IFNAR1, facilitating sustained surface expression and cytokine binding. The kinase active JAK on the partner cytokine receptor subunit can then initiate the signaling cascade (Li et al., 2013; Kreins et al., 2015). Finally, TYK2 might also act on non-STAT substrates; in the context of IFN-I signaling, phosphoinositide 3 kinase appears to be activated by TYK2 independent of its kinase activity (Rani et al., 1999). Thus, while the textbook depiction of JAK-STAT signaling often portrays a linear pathway, the truth is considerably more complicated, as is often the case in biology.

TYROSINE KINASE 2 DEFICIENCY AND POLYMORPHISMS IN MAN

A plethora of functional mutations have been identified at the *TYK2* locus in man, ranging from complete inactivation, to mild modification of function (**Figure 3** and **Table 1**). These "experiments of nature" have revealed the roles that TYK2 plays in specific cytokine signaling cascades, guided the development of targeted mutations in mice, and ultimately have provided the rationale for therapeutic targeting for inflammatory diseases. While most TYK2 biology is conserved between mouse and man, some important differences do exist, which will be highlighted in the next section.

Tyrosine kinase 2 mutations that cause deficiency are rare, but impart a strong immune phenotype on carriers through the introduction of a premature stop codon in the *TYK2* transcript (Minegishi et al., 2006; Kreins et al., 2015; Fuchs et al., 2016). It is estimated that homozygous complete inactivation of TYK2 occurs in less than 1 in 600,000 people (Boisson-Dupuis et al., 2018). The first report of a TYK2 deficient person arose from an investigation into the cause of a patient with hyper IgE syndrome (HIES) and repeated fungal, viral and mycobacterial infections (Minegishi et al., 2006). A molecular investigation found the patient to have signaling deficiencies for IFN-I, IL-6, IL-10, IL-12, and IL-23. In particular, the patient's T cells displayed no STAT phosphorylation or gene induction in response to IFN-I, and were incapable of making IFNy in response to IL-12. This later point was proposed as the reason why the patient had elevated Th2 and reduced Th1 cell frequency. It is important to note that IL-23-induced IL-17 in CD4+ T cells had only recently been discovered at the time of this publication in 2006 (Cua et al., 2003; Park et al., 2005), which may have been why Th17 cells were not explored. A series of case reports, published in 2015, detailed seven additional TYK2-deficient patients from five families (Kreins et al., 2015). These patients all presented with recurrent mycobacterial and viral infections, without recurrent fungal infections or HIES. As with the index TYK2 deficient



B

Cytokine	IL-23		IL-12		IFN-I		IL-10		IL-22		IL-13	
Receptor chain	IL-12Rβ1	IL-23R	IL-12Rβ1	IL-12Rβ2	IFNAR1	IFNAR2	IL-10R2	IL-10R1	IL-10R2	IL-22R	IL-13Rα1	IL-4Rα
Associated JAK	TYK2	JAK2	TYK2	JAK2	TYK2	JAK1	TYK2	JAK1	TYK2	JAK1	TYK2	JAK1
Major transducer	STAT3		STAT4		STAT1 and STAT2		STAT3		STAT3		STAT6	

FIGURE 2 | Role of TYK2 in IL-23 receptor signaling. **(A)** Inactive JAK2/TYK2 associate with the intracellular domain of their respective cytokine receptor subunits in the steady-state through FERM and SH2 domains. JAK inactivity is mediated in part by pseudokinase domain inhibition of the kinase domain. The binding of IL-23 unites its respective cytokine receptor subunits, which in turn causes a conformational change in JAK2/TYK2, allowing for their kinase activity. Phosphorylation of JAKs and the intracellular domain of the cytokine receptors allows for the recruitment and activation of the STAT3 transcription factor and subsequent induction of gene expression. Inducible inhibition of IL-23 is mediated in part by the SOCS family of proteins, which can bind to JAKs to block their cytokine activity, and can tag JAKs for ubiquitin mediated degradation. While not specifically researched in the context of IL-23R, constitutive inhibition of JAKs is mediated by phosphatases such as SHP1 and CD45. **(B)** Selected TYK2-associated cytokines, their receptor subunits and associated intracellular signaling molecules. The dominant STAT activated by the respective cytokine is indicated, but STAT usage can be cell- and context-dependent.

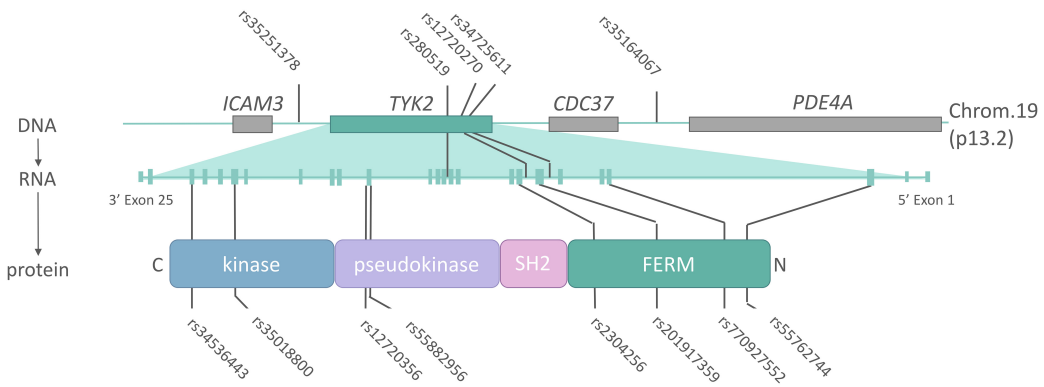


FIGURE 3 | The *TYK2* locus (DNA), transcript (mRNA) and protein annotated with single-nucleotide polymorphisms (SNPs) associated with AS or related inflammatory diseases. Non-coding SNPs (intronic or intergenic) lie on top of the *TYK2* DNA locus, while non-synonymous (protein changing) SNPs indicated below TYK2 protein. Details on the indicated SNPs can be found in **Table 1**.

TABLE 1 | Tyrosine kinase 2 (*TYK2*) locus single-nucleotide polymorphisms (SNPs) associated with ankylosing spondylitis (AS) or related autoimmune diseases.

SNP	AA change	Location	Functional effect	MAF European	MAF Asian	OR (AS)	Association	References
rs35251378	–	Intergenic		0.2840	0.5090	N/A	PsA	Stuart et al. (2015)
rs34536443	P1104A	Exon23 kinase	Loss of kinase activity	0.0420	0.0003	0.70	Ps, RA, SLE, T1D, AS, CD, UC, and MS	Dendrou et al. (2016)
rs35018800	A928V	Exon20 kinase	Loss of kinase activity	0.0075	0.0000	0.60	RA, SLE, AS, CD, and UC	Diogo et al. (2015)
rs12720356	I684S	Exon15 pseudokinase	Loss of kinase activity	0.0863	0.0000	1.09	Ps, RA, SLE, T1D, AS, CD, and UC	Dendrou et al. (2016)
rs280519	–	Intron 11		0.5032	0.5740	N/A	Ps and SLE	Strange et al. (2010) and Cunninghame Graham et al. (2011)
rs55882956	R703W	Exon15 pseudokinase	Loss of kinase activity	0.0011	0.0280	N/A	RA (suggestive association)	Motegi et al. (2019)
rs2304256	V362F	Exon8 FERM	Promotion of exon 8 inclusion	0.2836	0.4430	N/A	Ps, SLE, and T1D	Wallace et al. (2010), Diogo et al. (2015), Enerbäck et al. (2018), and Li et al. (2020)
rs12720270	–	Intron 7	Promotion of exon 8 inclusion	0.1832	0.4700	N/A	SLE	Graham et al. (2007), Hellquist et al. (2009), and Li et al. (2020)
rs201917359	R231W	Exon7 FERM		0.0010*	0.0030*	N/A	RA	Motegi et al. (2019)
rs34725611	–	Intron 6		0.2768	0.5060	N/A	PsA	Bowes et al. (2015)
rs770927552	GCTT deletion	Exon4 FERM	Truncation (nonsense mutation)	0.0000	0.0000	N/A		Minegishi et al. (2006) and Kreins et al. (2015)
rs55762744	A53T	Exon3 FERM	Impact on protein function	0.0109	0.0006	N/A	MS	Dyment et al. (2012)
rs35164067	–	Intergenic		0.2001	0.4690	1.14	T1D and AS	Cortes et al. (2013)

*Functional effect, OR (for AS only) and disease associated found in indicated reference. Minor allele frequency (MAF) obtained from ALFA project data of over 100,000 individuals on NCBI's SNP database. *ALFA project SNP data not available, so 1,000 genomes used instead.*

patient, these subsequent individuals all had impaired responses to IFN-I, IL-10, IL-12, and IL-23, but unlike the index case, they responded normally to IL-6. Moreover, this study showed that impaired IL-6 signaling in the first patient is not due to the TYK2 deficiency. Of importance to SpA, this paper showed that despite a loss of response to IL-23, circulating Th17 cell frequencies appeared normal in TYK2 deficient individuals. A final case report, published in 2016, confirmed the essential role of TYK2 in IFN-I responses and a limited role in IL-10 activity through reduced IL-10R2 expression (Fuchs et al., 2016). The effect of this mutation on IL-12 and IL-23 signaling was not assessed.

Mutations that cause, or associate with, a reduction in TYK2 function are relatively common and can be found in and around the *TYK2* gene. Small genetic studies that focused on the *TYK2* locus itself were the first to link variants to autoimmune disease. Specifically, IFN-I related genes were targeted in studies of systemic lupus erythematosus (SLE), a rheumatic disease strongly linked to altered IFN-I activity (Sigurdsson et al., 2005). Here, the SNP rs2304256 (V362F) was found to be strongly associated with SLE (OR 1.6, corrected *P*-value 3.4×10^{-7}). Large, unbiased genome wide association studies (GWAS) later found *TYK2* to be associated to a range of autoimmune diseases, including rs12720356 (I684S) with Crohn's disease [odds ratio (OR) 1.12] (Franke et al., 2010) and psoriasis (OR 1.4) (Bowes et al., 2015), while AS was associated with an intergenic SNP near *TYK2* (rs35164067, OR 1.16) (Cortes et al., 2013). A large meta-analysis utilizing the GWAS data from five autoimmune diseases, including AS, confirmed the link with rs12720356 (I684S), but highlighted that the direction of association (i.e., risk *vs* protection) was not the same for each disease (Ellinghaus et al., 2016). This paradox was further explored in a meta-analysis specifically for *TYK2* by Dendrou et al. (2016), in which rs12720356 (I684S) was found to be a risk factor for AS, CD, and ulcerative colitis (UC), but not other autoimmune diseases such as RA, psoriasis, SLE and type I diabetes. The biological reason for this clustering is not clear, but may reflect the relative contribution of the kinase *vs* pseudokinase domains in the target tissues of the respective diseases. Dendrou et al. (2016) further commented that rs12720356 may not be a causative SNP in all association studies, with linked SNPs potentially providing disease risk by modulating the expression of adjacent genes. Nonetheless, the broad pattern of disease clustering based on rs12720356 resembles that seen previously in a GWAS meta-analysis of all risk factors, whereby AS was genetically closer

to CD, UC, and psoriasis than to RA, SLE or diabetes (Farh et al., 2015). In addition, the aforementioned meta-analysis of *TYK2* showed the *TYK2* variant with the strongest link to all tested autoimmune diseases was rs34536443 (P1104A), whereby homozygosity imparted an OR in AS of 0.1. Below, we will give examples of how these and other natural mutations across different TYK2 domains can affect its function.

One common non-synonymous mutation in *TYK2* occurs in the pseudokinase domain, namely I684S (rs12720356). It has been reported that I684S reduces IL-12 signaling (Enerbäck et al., 2018) and that IFNy production in CD4+ T cells and NK cells, but not IL-17 production, correlates with I684S genotype (Gracey et al., 2020a). It should be mentioned that the results as to the biological effects of I684S are conflicting, with some reports disputing an effect in cell-based assays (Dendrou et al., 2016; Boisson-Dupuis et al., 2018). It is noteworthy to mention here that rs12720356 (I684S) heterozygosity was found to correlate with reduced spinal fusion in AS patients (Gracey et al., 2020a).

One of the most frequent and well-studied TYK2 mutations, P1104A (rs34536443), can be found in 4% of Europeans (NCBI dbSNP), with one in 600 Europeans being homozygous (Boisson-Dupuis et al., 2018). This missense mutation in the catalytic domain causes a loss of TYK2 kinase activity, yet the protein is fully translated (Li et al., 2013). Akin to the TYK2 deficiency discussed above, individuals homozygous for P1104A are susceptible to mycobacterial infections and do have impaired IL-23 signaling (Dendrou et al., 2016; Boisson-Dupuis et al., 2018; Kerner et al., 2019). Data on the functional effects of P1104A beyond IL-23 are conflicting: One paper reported P1104A transduced EBV T and B cells and primary human cells have only a modest interference with IFN-I and IL-10 signaling and no alteration in IL-12 signaling (Boisson-Dupuis et al., 2018). Another study of P1104A reported an effect on IFN-I and IL-12 induced STAT phosphorylation, but no effects on IL-10 or IL-6 signaling in primary human cells (Dendrou et al., 2016). Both studies reported no impact of P1104A on the surface receptor expression of IFNAR1, IL-12R, and IL-23R.

The IL-23 specific effect of TYK2 was recently replicated in a study of two siblings with compound heterozygous mutations in the TYK2 FERM domain (Nemoto et al., 2018). These patients were heterozygous for both the index truncation mutation discussed above (Minegishi et al., 2006) (now designated rs770927552), and a novel non-synonymous mutation (rs201917359, R231W). It was estimated that the protein levels of TYK2 were reduced by 35% in these subjects; however, IFN-I, IL-6, IL-10, and IL-12 signaling remained intact. It is likely that R231W results in altered binding of TYK2 to the cytokine receptors given its location in the FERM domain. Indeed a recent study demonstrated reduced IL-23 signaling activity in a cell based assay for R231W (Motegi et al., 2019), yet in neither study was surface cytokine receptor expression assessed.

Finally, it is possible that common variants in the *TYK2* locus can also affect *TYK2* splicing. One recent study addresses this concept, in which two SNPs were found to cause alternative splicing of TYK2 (Li et al., 2020). Specifically, the intronic rs12720270 and exonic rs2304256 (V362F) were found to promote the inclusion of exon 8 in the *TYK2* transcript. The same study showed exon 8 not to affect kinase activity of TYK2, but to be essential for cell responsiveness to IFN-I. These results can be best explained by location of exon 8 in the FERM domain, thus these splice variants impact on association of TYK2 with cytokine receptors, but not its catalytic activity.

In summary, nonsense mutations of *TYK2* resulting in complete loss of TYK2 protein have strong immunological phenotypes by affecting the intracellular signaling of a range of cytokines both directly through kinase activity of TYK2, and indirectly through its scaffolding function. On the other hand, missense mutations which keep TYK2 protein largely intact, but selectively affect its function, appear to have a more restricted effect on cytokine signaling, with the strongest known effects being on IFN-I, IL-12, and IL-23.

USING MICE TO STUDY TYK2: PROS AND CONS

Mice play a crucial role in biomedical research, yet approximately 96 million years of divergent evolution have resulted in biological discrepancies when using animals to understand human biology (Nei et al., 2001; Mestas and Hughes, 2004). While many biological roles of TYK2 between mouse and man are conserved, there are some crucial differences that must be considered. Here, the development of mice to study TYK2 will be discussed, before comparing the biological function and effects of TYK2 in mice *vs* man.

Human TYK2 and murine Tyk2 share approximately 80% sequence identity at the mRNA level and 85% at the protein levels (NCBI blast). While this may seem low, it is on par with the average conservation of orthologous genes between the species (Makałowski et al., 1996). To this point, one recent study examined the inter-species sequence homology of the Tyk2 ATP binding site in the kinase domain, revealing only one amino acid substitution out of 42 amino acids between species (Gerstenberger et al., 2020b). Here, human isoleucine (I) 960 was found to be substituted by a valine in mouse at the equivalent position (V980). While the functional effect of this single amino acid change on the activity of Tyk2 *in vitro* and *in vivo* is not known, it was found to have a clear effect on the potency of selective TYK2 inhibitors as discussed in the following section.

The first manipulations of Tyk2 in mice were complete knockouts. Tyk2 KO mice were shown to have defective IFN-I and IL-12 responses, and were susceptible to viral infection, albeit not as severely as IFNAR1 KO mice (Karaghiosoff et al., 2000; Shimoda et al., 2000). IL-10 signaling was shown to be impaired only in certain experimental conditions (Shaw et al., 2006) and no effect was seen on IL-6 signaling (Karaghiosoff et al., 2000; Shimoda et al., 2000). These initial studies preceded the discovery of IL-23, with subsequent studies using Tyk2 KO mice revealing a crucial role for this JAK in IL-23 signaling (Nakamura et al., 2008), including an essential role in models for both dermatitis and colitis (Ishizaki et al., 2011).

Following the characterization of complete Tyk2 KO mice, loss of function mutations were reported. The first was a spontaneous mutation in the B10.Q/J strain (Shaw et al., 2003). This mouse

strain was noted for its lack of response to IL-12 (Yap et al., 2001), leading to genomic studies pinpointing the mutation to a SNP in the pseudokinase domain rendering Tyk2 non-functional (Shaw et al., 2003). Importantly, this pseudokinase mutation completely blocked STAT3 phosphorylation in response to IL-23. The second loss of function mutation to be reported was kinase inactivation through targeted single base pair mutation (K923E) (Prchal-Murphy et al., 2012). These mice have defective IFN-I and IL-12 response akin to complete Tyk2 KO mice (Prchal-Murphy et al., 2015). Importantly, it was observed that the Tyk2-K923E mice also had drastically reduced protein levels of Tyk2 despite transcript levels being unaffected, suggesting an important role for kinase activity of Tyk2 on its stability in mice (Prchal-Murphy et al., 2012), an effect not seen in humans. A third targeted mutation of Tyk2 was designed to mimic P1104A (rs34536443) through mutating the orthologous proline at 1124 (P1124A) (Dendrou et al., 2016). Unlike the Tyk2-K923E mice, Tyk2-P1124A mice had normal transcript and protein levels of Tyk2. Despite this, the Tyk2-P1124A mice also had defective IFN-I, IL-12, and IL-23 responses.

The take home message from these murine models of altered Tyk2 function is that, as with humans, IFN-I, IL-12 and IL-23 pathways are undeniably reliant on Tyk2. Care must be taken when selecting Tyk2 mutated mice as to which particular model to use for the question to be answered; complete knockout, kinase silencing, or loss of function through targeted mutation. For certain applications, inter-species differences may also need to be considered, such as that occurring in the ATP binding site of Tyk2.

TYROSINE KINASE 2, A ONCE NEGLECTED THERAPEUTIC TARGET

The hunt for selective inhibitors for JAKs began in the mid 1990's as a collaboration between Pfizer and the NIH (Garber, 2013). The general strategy at the time was to generate ATP-mimics that would block the kinase domain of JAKs, and thus inhibit their function. The first generation inhibitors were relatively non-specific, blocking all members of the JAK family with various potencies; tofacitinib mainly targeted JAK1/JAK3, baricitinib JAK1/JAK2, and ruxolitinib JAK1/JAK2 (Banerjee et al., 2017). The second generation of JAKi for inflammatory diseases were designed to avoid JAK2, as inhibition of this molecule was found to induce cytopenia, a serious adverse event. JAK1 was the favored target, owing to its broad involvement in signaling by inflammatory disease-relevant cytokines, including the IL-2, IL-6, IL-13, type I and type II IFN families (Schwartz et al., 2017). Two promising examples of JAK1 inhibitors approved for use in rheumatic diseases are filgotinib and upadacitinib (Choy, 2019). TYK2 has long been touted as an ideal therapeutic target for inflammatory diseases due to its association with a small number of cytokine receptors relative to the other JAKs, and central role in blocking IL-23 (Shaw et al., 2003; Ishizaki et al., 2014). Despite this, the development of selective TYK2 inhibitors lagged behind the progress made by other second generation JAKi. That is not to say that attempts at inhibiting TYK2 have not been made: the patent history for TYK2 reveals a number of approaches made by academic groups and pharmaceutical companies to target this JAK (Menet, 2014; He et al., 2019), but this activity is not reflected in the literature, with relatively few papers published for TYK2 inhibitors.

Targeting of TYK2 by small molecule initially followed the same strategy as with the other JAKs, namely targeting of the kinase domain ("catalytic inhibitors"). A notable example of such a kinase domain inhibitor is NDI-031407, which effectively blocked IL-23-induced skin inflammation and SpA-like arthritis in mice (Gracey et al., 2020a). In addition, there have been publications involving dual TYK2 and JAK1 catalytic inhibitors; SAR-20347, blocked psoriasis-like skin inflammation in mice (Works et al., 2014) and PF-06700841 (brepocitinib), was found to be protective against adjuvant-induced arthritis in rats (Fensome et al., 2018). Despite this apparent success, this line of TYK2/JAK1 dual inhibitors were found to have drastically reduced potency against murine and rat Tyk2 compared to human TYK2 due a single amino acid substitution in the ATP binding site (Gerstenberger et al., 2020b), as discussed above. Using the humanized Tyk2-V980I mouse, a TYK2-selective inhibitor, PF-06826647, was shown to be effective against murine dermatitis (Gerstenberger et al., 2020a). Thus, it is possible that the relatively slow progress in targeting catalytic domain of TYK2 was in part caused by this previously overlooked inter-species variation.

A novel approach was recently taken to target not the kinase domain, but the pseudokinase domain of TYK2. These so called "allosteric" inhibitors act by stabilizing the pseudokinase domain of TYK2, thus promoting auto-inhibition of kinase domain of TYK2 (Tokarski et al., 2015; Wrobleski et al., 2019). BMS-986165 (deucravacitinib) was the first and most advanced molecule to take this approach (Burke et al., 2019; Wrobleski et al., 2019), revealing unprecedented specificity for TYK2 over the other JAKs ($>10{,}000\times$). The preclinical and clinical trials involving this molecule will be discussed in the following section. A second pair of pseudokinase inhibitors, TYK2iA and TYK2iB, have been developed with available data demonstrating strong inhibition of IL-12 and IFN-I in cellular assays in the context of type I diabetes (Coomans de Brachène et al., 2020). These allosteric inhibitors have yet to be tested for their ability to block IL-23, or inhibit the rheumatic diseases.

Taken together, TYK2 inhibition by small molecule appears to have come of age. Initial targeting of the kinase domain yielded promising lead molecules, and provided preclinical evidence that TYK2 is a valid target. However, taking the novel approach of targeting the pseudokinase domain revolutionized the field. This is evident in the success of early stage clinical trials, and the initiation of phase III trials in various inflammatory diseases that will be discussed in the next section.

PRECLINICAL AND CLINICAL TRIALS OF TYK2 INHIBITORS

While there have been a number of publications detailing preclinical *in vitro* and *in vivo* experiments with a range of

TABLE 2 | Janus kinase (JAK) inhibitors and their specificity for TYK2.

Name	Other name	Primary JAKs	Selectivity (cell free kinase assay)		References
Tofacitinib	CP-690550	JAK3/JAK2	21× (JAK3 *vs* TYK2)		Meyer et al. (2010)
Baricitinib	INCB-28050/ LY-3009104	JAK2/JAK1	10× (JAK2 *vs* TYK2)		Fridman et al. (2010)
Upadacitinib	ABT-494	JAK1	100× (JAK1 *vs* TYK2)		Parmentier et al. (2018)
Filgotinib	GLPG0634	JAK1	12× (JAK1 *vs* TYK2)		Van Rompaey et al. (2013)
Brepocitinib	PF-06700841	JAK1/TYK2	3.3× (TYK2 *vs* JAK2)	282× (TYK2 *vs* JAK3)	Fensome et al. (2018)
Deucravacitinib	BMS-986165	TYK2	5× (TYK2 JH2 *vs* JAK1 JH2)	>10,000× (all JAK JH1 domains, JAK2, and JAK3 JH2)	Wrobleski et al. (2019)

For non-TYK2 selective JAKi, selectivity of primary targeted JAK vs TYK2 given. For TYK2-selective inhibitors, selectivity of TYK2 vs off target inhibition of other JAKs given. Note that for first generation JAKs (tofacitinib and baricitinib), secondary JAKs are effectively blocked at working JAK concentrations, and thus these JAKi are often referred to as "pan-JAK" inhibitors.

TYK2 inhibitors, few have progressed to clinical trials. Of those in trials, the primary indication has been psoriasis, owing to its strong link to IL-23, and unambiguous primary clinical endpoint, namely resolution of skin inflammation. Here we will discuss the preclinical and clinical data pertaining to TYK2 inhibitors that have focused on the type 3 immunity in SpA and related inflammatory diseases. For completeness, we will also discuss first generation JAKi that block TYK2 as a secondary target, and second generation non-TYK2 targeting JAKi that have completed advanced clinical trials in SpA (**Table 2**).

As early as 2015, the first TYK2-selective inhibitors were displaying exciting preclinical results. Conference abstracts on the catalytic inhibitors, NDI-031407 and its predecessor NDI-031301, revealed potency for IL-12 signaling blockade *in vitro*, and IL-23-induced dermatitis *in vivo* (Miao et al., 2015, 2016). NDI-031407 was recently shown to block both human and murine IL-23R signaling and IL-17 induction *in vitro*, and was successful in limiting Th17 cell expansion and halting the development of experimental murine SpA *in vivo* (Gracey et al., 2020a). Importantly, despite TYK2 being essential for IL-23-induced STAT3 phosphorylation and IL-22 production, TYK2 was only partially responsible for IL-23-induced IL-17. Successors to these molecules are now in phase I clinical trials (Bristol Myers Squibb, 2021).

Pfizer, who developed one of the most widely used JAKi to date, tofacitinib, has been active in developing TYK2 catalytic inhibitors. As early as 2014, phase I clinical trials (NCT02310750) had begun on the TYK2/JAK1 dual inhibitor brepocitinib (PF-06700841), although preclinical or clinical data was not published until 2018 (Banfield et al., 2018; Fensome et al., 2018). Preclinical data on brepocitinib showed it to be effective at blocking IL-23 signaling in human *in vitro* cellular assays, and blocked adjuvant-induced arthritis in rats (Fensome et al., 2018). Brepocitinib has since done well in both phase I and phase II trials, whereby it reduced C-reactive protein (CRP) by almost 50% and almost completely resolved skin inflammation in psoriasis patients (75% reduction in their psoriasis symptoms [PASI75] in ∼80% of subjects) (Banfield et al., 2018; Forman et al., 2020). Of importance to SpA, brepocitinib is currently in phase II trials for PsA (NCT03963401). In addition, Pfizer has also developed a TYK2-specific catalytic inhibitor, PF-06826647. This molecule has been shown to block IL-23 *in vitro*, and can suppress imiquimod-induced dermatitis in Tyk2-V980I mice (Gerstenberger et al., 2020a). While phase I trials only started in 2017 (NCT03210961), they revealed a reasonable safety profile, and a clinical effect against psoriasis (Tehlirian et al., 2020). Phase II trials are ongoing for PF-06826647 in various inflammatory diseases, yet no forms of SpA are currently being investigated as targets.

The most advanced TYK2 inhibitor is deucravacitinib (BMS-986165). As previously discussed, this inhibitor targets the pseudokinase domain. As early as 2016, conference abstracts of preclinical data on deucravacitinib revealed it to be effective at blocking IL-23 signaling, and showed that it could prevent experimental colitis in mice (Gillooly et al., 2016). These preclinical results were recently published, demonstrating the ability of deucravacitinib to block IL-23-induced STAT3 phosphorylation in human CD4+ T cells and IL-17 production in murine CD4+ T cells (Burke et al., 2019). The same publication showed that this TYK2 inhibitor could prevent colitis in two distinct murine models and a partner publication demonstrated the ability of deucravacitinib to prevent IL-23-induced skin inflammation in mice (Wrobleski et al., 2019). There are no publications that detail efficacy of deucravacitinib in preclinical models of SpA or rheumatoid arthritis, but it is effective in blocking IFN-I dependent lupus-like disease in mice (Burke et al., 2019). Phase I trials with deucravacitinib began in 2017, with phase II trials cumulating in astounding results for the treatment of psoriasis (Papp et al., 2018). Specifically, 75% of patients achieved a PASI75 and almost 50% achieved a 90% reduction in skin scores (PASI90). Of considerable importance to SpA, the results of a phase II trial of 200 PsA patients treated with deucravacitinib were recently presented, in which 63% of patients achieved a 20% improvement in rheumatic symptoms (ACR20), compared to 32% in the placebo group (NCT03881059) (Mease et al., 2020).

While not yet compared head-to-head against biologics or other JAKi, deucravacitinib looks to be a solid candidate for treatment of inflammatory diseases. Specifically, deucravacitinib achieved primary endpoints comparable to the TNFi trials for psoriasis, yet is less effective than anti-IL23 blockers (Reich et al., 2019), which achieve PASI90 in 90% of patients. In addition,

deucravacitinib appears to be more effective against psoriasis than existing JAK inhibitors such as tofacitinib and baricitinib, likely due to more specific targeting of IL-23 (Papp et al., 2016a,b). Importantly, this phase II trial of deucravacitinib did not report any of the common side effects of current JAKi, such as cytopenia, likely due to avoidance of JAK2 inhibition.

There are currently no clinical trials reported for TYK2 inhibition in axSpA. Given IL-23i failed to demonstrate a meaningful effect against AS (radiographic axSpA) (Deodhar et al., 2019) and broad axSpA (Baeten et al., 2018), it is not clear if blocking this pathway by TYK2 will be beneficial for this group of diseases. The failure of IL-23i for axSpA has created an enigma in the field as anti-IL-23 is effective against pSpA (PsA) (Mease et al., 2020b), and related skin (Reich et al., 2019) and gut diseases (Feagan et al., 2016, 2017). While there are many possible explanations for this discrepancy (Siebert et al., 2018; McGonagle et al., 2021), there has been no conclusive evidence at the molecular level as to why IL-23i failed in axSpA. The use of TYK2 inhibitors against axSpA to target the IL-23 axis from a different angle, may shed light on this currently unexplainable conundrum.

One concern with blocking TYK2 is the occurrence of viral and mycobacterial infections as predicted by individuals with TYK2 deficiency (Kreins et al., 2015; Kerner et al., 2019). While mycobacteria infections were not reported in the trials of deucravacitinib, there was a higher incidence of respiratory and nasopharyngeal infections, indicating enhanced susceptibility to viral infection as an adverse event to TYK2 blockade. Of note, an expert committee was recently formed to evaluate existing data on JAKi in order to provide guidance for the use of JAKi in the treatment of the rheumatic diseases (Nash et al., 2020). This report concluded that given the available safety profiles, serious infections do occur with JAKi at a rate comparable to the TNF inhibitors. Only tofacitinib presents an enhanced risk for infection relative to the TNFi in patients over 65 (Lortholary et al., 2020; Nash et al., 2020).

For completeness, it is important to mention that non-TYK2 targeting JAKi have been generating considerable interest in SpA, with many achieving encouraging results in clinical trials. Proof of JAKi effectiveness in axSpA came from an off-label, proof of concept phase II trial of tofacitinib in AS (van der Heijde et al., 2017). This pan-JAK inhibitor achieved 20% improvement in AS symptoms (ASAS20) in 80% of patients at the higher dose (*vs* 40% in placebo), and was able to reduce MRI scores of the sacroiliac joint. Based on the success of this trial, tofacitinib entered phase III trials, which were recently completed (NCT03502616). Conference proceedings of these advanced trials appear to have confirmed the successes of the phase II trial, with 56% achieving ASAS20 *vs* 29% in the placebo group, and 40% achieving a 40% improvement in symptoms (ASAS40) *vs* 12.5% in the placebo (Deodhar et al., 2020). These levels are slightly lower than phase III trials with IL-17i, which report an ASAS20 of 70% (Marzo-Ortega et al., 2017). PsA was also proven to be effectively treated with tofacitinib, achieving an ACR20 of 50% *vs* 24% in the placebo group (Gladman et al., 2017). On the basis of such results, tofacitinib is approved for use in PsA, with applications submitted to regulatory agencies for use in AS.

The success of tofacitinib in SpA spurred interest in treating SpA with second generation JAKi. The JAK1 selective inhibitor, filgotinib, reached primary endpoints in phase II trials of both AS and PsA (Mease et al., 2018a; van der Heijde et al., 2018). In both forms of SpA, this JAK1 inhibitor appear on par with, if not superior to, tofacitinib, achieving an ASAS20 of 76% for AS and an ACR20 of 80% for PsA. Head-to-head trials are required to confirm this. Phase III trials recently begun for filgotinib treatment of AS (NCT04483700 and NCT04483687). Upadacitinib, also a selective JAK1 inhibitor, displays similar efficacy against SpA. In PsA, a phase III trial revealed reasonable success, with 64% of the treated group reaching an ACR20 (Mease et al., 2020a). In AS, a combined phase II/III trial demonstrated an ASAS20 of 65%, and significant improvements in both spine and sacroiliac MRI scores (van der Heijde et al., 2019). Based on these successes, approval for upadacitinib treatment of axSpA has been requested to both the FDA and EMA, with a decision expected in 2021. Without a doubt, the therapeutic armament against SpA will expand over the next 5 years with the addition of multiple JAKi.

CONCLUSION

With current approved therapeutics for SpA failing to fully prevent disease progression despite providing symptomatic relief and some modulation of progression of structural damage with TNFi (Haroon et al., 2013; Koo et al., 2020; Sepriano et al., 2021), there is a clear unmet medical need for novel DMARDs. TYK2 is implicated in multiple chronic inflammatory diseases, including SpA, through genetic associations and a central role in IL-23 activation of innate, innate-like and adaptive immune cells. Further, TYK2 is an attractive therapeutic target as it mediates more selective intracellular cytokine signaling compared to other JAKs. Despite depictions of TYK2 as an on/off switch, biochemical and immunological studies in mouse and man reveal considerable complexity in the regulation of TYK2, and kinase-dependent *vs* -independent functions. This complexity is highlighted by diverse functional and immunological effects of natural mutations throughout the *TYK2* gene, with selected SNPs having opposing risk *vs* protective effects in different autoimmune diseases. While the tools exist to understand the functional complexity of TYK2 in mice, caution must be taken when selecting the correct mouse model for the question at hand, given both the biological differences between murine and human TYK2, and TYK2 kinase-dependent and -independent functions.

Janus kinase inhibitor appear set to disrupt the 15-years dominance that cytokine-blocking mAb have had on the treatment of SpA. Blocking JAK1 and all its associated cytokines appear to be as effective as the current approved biologics for SpA. Time will tell if the oral availability of such a JAK inhibitors will provide a competitive advantage against established injectable biologics. While TYK2 inhibitors are relatively early in their development as compared to other JAKi, they appear to now be coming of age. Available data suggests that TYK2 blockade will be effective at treating forms of SpA such as PsA, with a safety profile comparable, if not superior to, other

JAK inhibitors. Preclinical data strongly supports a role for TYK2 in axSpA, and thus TYK2 inhibitors are poised to resolve the paradoxical failure of the IL-23i in axSpA. Time will tell if the science behind TYK2 inhibition will prove successful in SpA.

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EG and DH planned and wrote the manuscript. DE, RI, and BS critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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The Genetic Contribution to Drug Response in Spondyloarthritis: A Systematic Literature Review

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Objective: Spondyloarthritis (SpA) are a group of diseases with a high heritability, whose pathogenesis is strongly determined by an interplay between genetic and environmental factor. Therefore, the aim of our study was to determine whether genetic variants could also influence response to therapy in SpA.

Methods: A systematic literature review (SLR) was conducted in PubMed and Web of Science core collection, without publication-year restrictions (Last search 8th April 2021). The search strategy was formulated according to the PEO format (Population, Exposure, Outcome) for observational studies. The population was adult (≥18 years) patients with SpA. The exposure was inheritable genetic variations of any gene involved in the disease pathogenesis/drug metabolism. The outcome was response to the drug, both as dichotomous (response yes/no) and as continuous outcomes. Exclusion criteria were: (1) languages other than English, (2) case series, case reports, editorials, and reviews, (3) studies reporting genetic contribution to drug response only limited to extra-musculoskeletal features of SpA, (4) epigenetic modifications. Quality of the included study was independently assessed by two authors.

Results: After deduplication, 393 references were screened by two authors, which led to the final inclusion of 26 articles, pertinent with the research question, that were considered for qualitative synthesis. Among these, 10 cohort, one cross-sectional, and five case-control studies were considered of at least good quality according to Newcastle-Ottawa Scale (NOS). In studies about TNF-blockers therapy: (1) polymorphisms of the TNF receptor superfamily 1A/1B (*TNFRSF1A/1B*) genes were most frequently able to predict response, (2) −238 and −308 polymorphisms of *TNFα* gene were studied with conflicting results, (3) *TNFα* polymorphism rs1799724, rs1799964, −857, −1,013, +489 predicted drug response in non-adjusted analysis, (4) *PDE3A* rs3794271 had a linear relationship with DAS28 reduction after anti-TNFα therapy. *DHFR* polymorphism +35,289 was able to predict response to methotrexate.

Conclusions: Our SLR highlighted the existence of a genetic component in determining drug response. However, further studies are warranted to better define quantify it.

Keywords: spondyloarthritis, genes, polymorphism, drug, therapy

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INTRODUCTION

Spondyloarthritis (SpA) is a group of systemic inflammatory diseases with common clinical characteristics and a shared genetic background (Costantino et al., 2018). The typical clinical features include (1) musculo-skeletal manifestations, with axial skeleton (spine and sacroiliac joints) involvement, peripheral arthritis, enthesitis, dactylitis, and (2) extra-musculoskeletal manifestations (EMMs) such as inflammatory bowel disease (IBD), psoriasis, and anterior uveitis. Depending on the main clinical and radiological presentation, the following disease subset have been identified and included under the umbrella term of SpA: ankylosing spondylitis (AS), psoriatic arthritis (PsA), arthritis associated with IBD, reactive arthritis, and undifferentiated SpA (Costantino et al., 2018). Spondyloarthritis have a high heritability, with a complex genetic background that has only been partially elucidated, but which is surely dominated by the Human Leukocyte Antigen (HLA-B27) allele: positive individuals have a relative risk of SpA onset of about 40 compared to those who are HLA-B27 negative. HLA-B27 is part of the Major Histocompatibility Complex class I and it accounts for 20% of the SpA heritability (Costantino et al., 2018). Thus, as strong as its association with the disease might be, HLA-B27 is not the only responsible for SpA genetic susceptibility, as genome wide studies have highlighted in 2007 (Wellcome Trust Case Control Consortium et al., 2007). In particular, among the non-MHC loci, endoplasmic reticulum amino peptidase (*ERAP)1* and Interleukin-23 receptor (*IL23R) genes* were found to be strongly associated with SpA (Wellcome Trust Case Control Consortium et al., 2007). This discovery even led to new pathogenetic hypothesis, with important therapeutic implications (Gaffen et al., 2014).

The importance of genetic factors in the disease susceptibility, prompted researchers to investigate the role of genes in response to therapy as well (Song et al., 2015; Costantino et al., 2018). In fact, heterogeneity in drug response, even with the most effective drugs, has been observed in different disease phenotypes or - in general- in different patients (Ferraccioli et al., 2007). As an example, IL-23 inhibitors are effective in peripheral but not axial manifestations of SpA (Deodhar et al., 2019). Moreover, many patients do not experience adequate disease control with first-line therapy, such as non-steroidal anti-inflammatory drugs or conventional synthetic Disease Modifying Rheumatic Drugs (csDMARDs) and there are no clear indicators to predict this (van der Heijde et al., 2017; Gossec et al., 2020). Furthermore, a consistent proportion of patients (up to one-third) does not even respond to the first biotechnological drug (representing second-line therapy), whichever this might be (Merola et al., 2017). Thus, genetic variants of genes involved in both SpA pathogenesis and phenotypic expression, as well as in the drug metabolism, could play a role in determining drug response (Ferraccioli et al., 2007).

Therefore, the aim of the present study was to collect existing evidence supporting the role of genetics in predicting response to therapy in SpA.

MATERIALS AND METHODS

Literature Search

A systematic literature review (SLR) in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) was conducted (Moher et al., 2009). PubMed and Web of Science core collection were searched, without publication year restrictions. Last search was on 8th April 2021.

The research question was formulated according to the PEO format (Population, Exposure, Outcome) for observational studies. The population (P) of interest was considered to be adult ($\geq$18 years) patients with SpA. Studies including patients with other rheumatic diagnoses were considered eligible only if the results for SpA were presented separately. The exposure (E) was represented by genetic predisposition, meaning specific inheritable genetic variations of any gene that could be involved in the disease pathogenesis, or in drug metabolism. The outcome of interest was drug response, both as dichotomous outcome (response yes/no according to various disease activity status criteria or response criteria) and as continuous outcomes. Examples of dichotomous outcomes were: the Assessment of SpondyloArthritis international Society (ASAS)-based indices ASAS 20, ASAS 40, Bath Ankylosing Spondylitits Disease Activity Index (BASDAI) 50, BASDAI $\geq$ 4 (Anderson et al., 2001; Rudwaleit et al., 2004). Among continuous outcomes the following were considered: tender/swollen joint count, BASDAI, percentage of patients, Disease Activity Score on 28-joints count (DAS28) (van der Heijde et al., 1990; Garrett et al., 1994).

Inclusion criteria regarding population were: (1) adult axSpA patients as defined by: clinical diagnosis, ASAS criteria for axSpA or modified NY criteria for AS (van der Linden et al., 1984; Rudwaleit et al., 2009); (2) PsA patients as defined by rheumatologist diagnosis or ClAssification criteria for Psoriatic ARthritis (CASPAR) criteria (Taylor et al., 2006); (3) SpA associated to IBD, reactive arthritis or undifferentiated arthritis (if included).

Exclusion criteria were: (1) studies in languages other than English, (2) case series, case reports, editorials, and reviews, (3) studies reporting genetic contribution to drug response only limited to EMMs, such as IBD or psoriasis, and not presenting data for patients with SpA separately, (4) epigenetic modifications (e.g., DNA methylation and miRNA).

We checked MeSH terms for SpA, genetics, drug response to identify search terms in an attempt to capture all possible synonyms. In the final search, however, MeSH terms were not used to avoid excluding more recent works. The detailed search strategy is indicated in the Supplementary File.

Study Selection, Data Extraction, and Risk of Bias Assessment

Two reviewers (AO, GC) assessed titles and abstracts on suitability for inclusion, according to the inclusion/exclusion criteria, followed by a full-text review if necessary. Discrepancies were resolved by consensus. The following information was

extracted from the study: author, year, study design, number of included patients, characteristics of the study population (disease classification, gender, age, disease duration), of the exposure (gene where a variation was detected, and type of variation), and outcome measures. The quality of the extracted studies was then evaluated by Newcastle-Ottawa Scale (NOS) for cross-sectional, cohort, and case-control studies (Wells et al., 2021). Newcastle-Ottawa Scale study quality was then graded according to the total score. Cross-sectional studies were graded as: very good = 9–10; good = 7–8; satisfactory = 5–6; unsatisfactory = 0–4 (Modesti et al., 2016). Cohort and case–control studies were graded as: very good = 8–9; good = 7; satisfactory = 5–6; unsatisfactory = 0–4.

A PRISMA flowchart was generated for the final selection of the studies to be included (see Results section for details).

Data Extraction

Exposure was expressed as presence or absence of a specific genetic variation. Outcome was expressed according to the analysis presented in the study. If analysis were adjusted, odds ratio (95% Confidence Interval-CI), hazard rate (95%CI), or beta (95%CI) were reported for logistic regression, Cox regression or linear regression, respectively. Otherwise, only *p*-value was reported for descriptive statistics. Due to heterogeneity of the included population, exposure, and outcomes a meta-analysis could not be performed.

RESULTS

Study Selection

A total of 524 references were retrieved by the databases search. After removing duplicates, titles, and abstracts of the remaining 393 references were screened for eligibility, which led to the elimination of 330 articles. This was mainly due to wrong target population (e.g., rheumatoid arthritis, psoriasis, gout), wrong exposure (e.g., monocytes expression profile, long non-coding mRNA as inflammatory modulators), or wrong outcome (e.g., disease onset or severity instead of response to therapy); two papers were not in English. The full-text of 61 articles was examined, resulting in the exclusion of 35 further articles that did not fulfill inclusion/exclusion criteria: 29 were reviews or book chapters, one did not present data for SpA separately, one did not specify treatment, four were congress abstracts with insufficient information to extract. The remaining 26 articles were considered for qualitative evaluation.

The PRISMA flowchart is displayed in **Figure 1**.

Study Characteristics

The 26 studies that were included in the qualitative assessment were thoroughly examined to identify: author, year, study design, number of participants, definition of population, exposure, outcome. The main characteristics of the studies are displayed in **Table 1**. There were 15 cohort studies (Tutuncu et al., 2005; Seitz et al., 2007; Chandran et al., 2010; Eder et al., 2010; Morales-Lara et al., 2010, 2012; Ramírez et al., 2012; Julià et al., 2014; Schiotis et al., 2014; Fabris et al., 2016; Chen, 2017; Yan et al., 2017; Liu et al., 2019; Ovejero-Benito et al., 2019; Polo Y La Borda et al., 2019), eight case-control studies (Manolova et al., 2014; Murdaca et al., 2014; Ma et al., 2017; Wang et al., 2017; Zhao et al., 2017; Aita et al., 2018; Xing-Rong et al., 2018; Xu et al., 2020; Sokolik et al., 2021) and one cross sectional study (Nossent et al., 2014). The definition of the populations was heterogeneous, with studies conducted in Europe, USA, and China, and mainly including AS and PsA patients (**Table 1**). Exposure was also heterogeneous, as several genetic polymorphisms were evaluated, with target genes implicated in the pathogenesis (e.g., C Reactive Protein—CRP, Tumor Necrosis Factor α–TNFα), drug metabolism (e.g., Cytocrome P450), drug immunogenicity (e.g., Fc receptor). The response to therapy was variably evaluated by validated outcomes of the following types: (1) dichotomous: ASAS 20, ASAS 40, BASDAI 50, American College of Rheumatology (ACR) 20, Psoriatic Arthritis Response Criteria (PsARC) (2) categorical: EULAR response criteria; (3) continuous: tender or swollen joint count, DAS28, BASDAI change score, morning stiffness. Some studies used non-validated but clinically significant outcomes, among which (1) a $\geq$70% improvement in physician global assessment (PhGA) and SJC/TJC plus a $\geq$50% improvement in two of: erythrocyte sedimentation rate, CRP, patient global assessment (PGA) (Tutuncu et al., 2005) (2) BASDAI $\leq$ 4 (Aita et al., 2018) (3) a $\geq$50% in a Numerical Rating Scale (NRS) for pain (Ovejero-Benito et al., 2019), (4) necessity of therapeutic switch yes/no (Fabris et al., 2016), (5) actively inflamed joint count (meaning tender and/or swollen joints; Chandran et al., 2010).

Risk of Bias Assessment

According to the NOS for cohort studies, 11 studies were graded as very good or good (Chandran et al., 2010; Eder et al., 2010; Morales-Lara et al., 2012; Ramírez et al., 2012; Julià et al., 2014; Schiotis et al., 2014; Fabris et al., 2016; Chen, 2017; Yan et al., 2017; Liu et al., 2019; Polo Y La Borda et al., 2019), and were therefore included in the qualitative synthesis. One study was deemed unsatisfactory (Morales-Lara et al., 2010) and three were only satisfactory (Tutuncu et al., 2005; Seitz et al., 2007; Ovejero-Benito et al., 2019), thus their results are not discussed in detailed. The lone cross-sectional study was considered of good quality according to NOS (Nossent et al., 2014). Among the case-control studies, four were only satisfactory (Manolova et al., 2014; Wang et al., 2017; Xu et al., 2020; Sokolik et al., 2021), one was unsatisfactory (Ma et al., 2017), and five good (Tong et al., 2012; Zhao et al., 2017; Aita et al., 2018) or very good (Murdaca et al., 2014; Xing-Rong et al., 2018). The latter were the ones that were taken into consideration for the qualitative synthesis. A common reason for higher grades in the cohort studies was the fact that the exposure (genetic polymorphism) was surely present at the start of the study and likely unbiased, resulting from the same laboratory test applied for the whole sample. In general, across all study designs, comparability grading was not always optimal as a minority of studies applied proper correction for several covariates, while the majority only corrected for one important factor or reported unadjusted analysis. **Table 2** reports the detailed grading of each study.

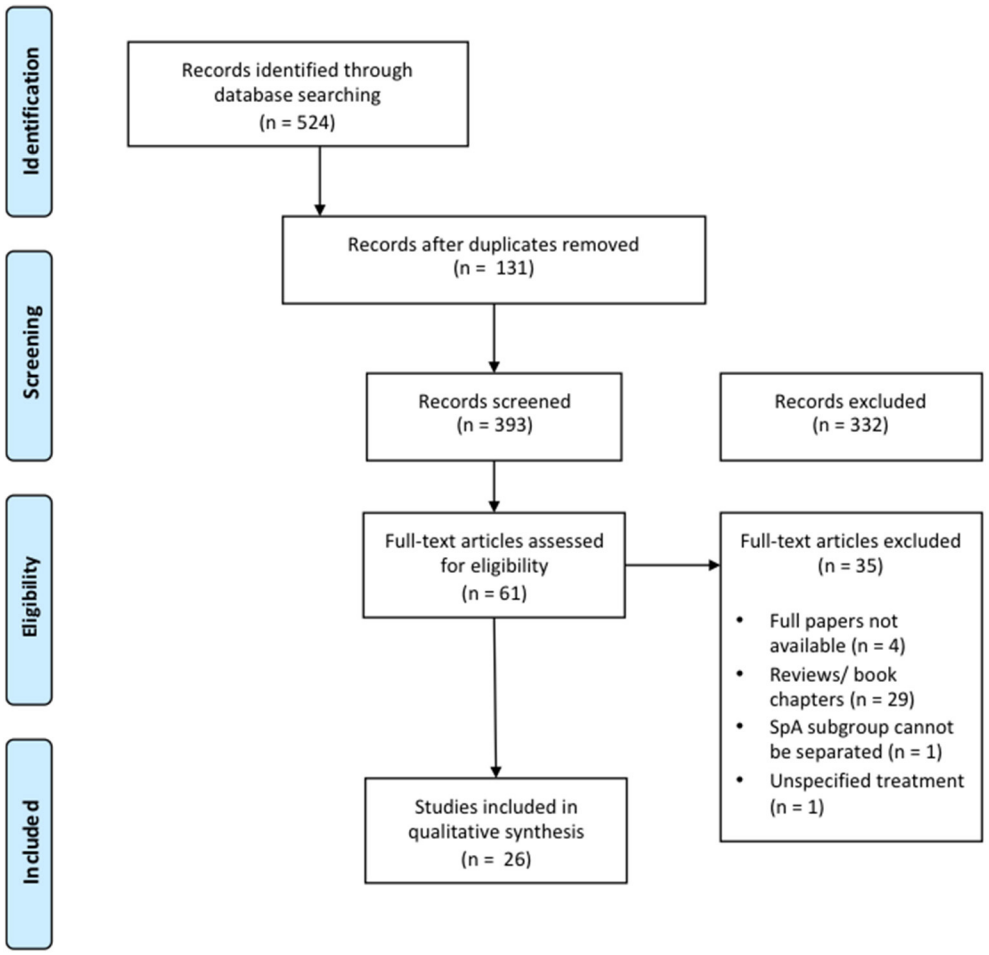

FIGURE 1 | PRISMA flow diagram of studies' inclusion.

Result Synthesis

In order to synthetize results regarding the influence of genetic variants on response to therapy, only data from studies that were deemed of good or very good quality were extracted and are presented in **Table 3**.

Genes Involved in SpA Pathogenesis

Most of the studies focused on anti-TNFα therapy. Several of them investigated genes involved in SpA pathogenetic mechanisms, in particular TNFα, TNFα receptors, and several interleukins (IL) both with pro inflammatory (e.g., IL-6) and anti-inflammatory (e.g., IL-10) effects.

Polymorphisms of the TNF receptor superfamily 1 A and 1B (*TNFRSF1A/1B*) genes were those that most frequently were able to predict response (*TNFRSF1A* rs767455 genotype AA, *TNFRSF1A* rs1800693 genotype GG, *TNFRSF1B* rs1061622 genotype TT and GG) according to various criteria such as BASDAI 50, EULAR response, or ASAS 20, ASAS 40 (Morales-Lara et al., 2010; Julià et al., 2014; Ovejero-Benito et al., 2019). Notably, Schiotis et al., who also investigated the *TNFRSF1B* polymorphism rs1061622, found that the GG genotype was associated with non-response, thus reaching opposite conclusion compared to the previously mentioned studies despite a fair numerosity and correcting for other polymorphisms (Schiotis et al., 2014). Other authors simply could not demonstrate any association to response according to the ASAS 20 for the polymorphisms they investigated in the *TNFRSF1A* gene (rs2234649, rs4149570, rs4149621, rs4149569; Zhao et al., 2017; Xing-Rong et al., 2018). Notably, among the investigated genetic variations, also *TNFRSF1A* rs767455 was present, and its association with clinical response was therefore not confirmed by all authors (Zhao et al., 2017; Xing-Rong et al., 2018).

The TNFα gene was also frequently studied in relation to therapy response, with two studies failing to demonstrate an association of the −238G>A (rs361525) and −308G>A (rs1800629) polymorphisms and clinical response according to ACR 20 and BASDAI (Murdaca et al., 2014; Nossent et al., 2014). These studies, however, did not correct for any confounding factor. Conversely, Fabris et al., correcting the association of the same −308 A polymorphism to therapy

TABLE 1 | Characteristics of the studies satisfying inclusion and exclusion criteria for the SLR, with particular reference to study design and characteristics of population, exposure, outcome.

References	Study design	Number of SpA patients	SpA subtype	Disease definition	Males	Age ± *SD*	Country	Exposure: candidate gene/s	HWE checked	Therapy	Follow up (weeks)	Response to therapy definition
Xu et al., 2020	Case-control	232	AS	mNY criteria	52,5%	62.3 ± 8.2	China	CRP	Yes, tested variants in HWE	Etanercept	12 w	ASAS20/ASAS40
Morales-Lara et al., 2010	Cohort	49 (33 AS, 16 PsA)	AS/PsA	ND	ND	ND	Spain	Fc receptor	No	Infliximab	48 w	ACR20 or BASFI20
Manolova et al., 2014	Case-control	58	AS	mNY criteria	79,3%	38.1±8.6	Bulgaria	TNFα	Yes, tested variants in HWE	anti-TNFα	24 w	ASAS20
Schiotis et al., 2014	Cohort	121	AS	mNY criteria	73,5%	47.7 ± 9.5	Spain	190 genes among which IL-23 R ERAP 1	Yes, tested variants in HWE	anti-TNFα	12-20 w	BASDAI50
Chen, 2017	Cohort	312	AS	mNY criteria	55,7%	35.2 ± 5.83	China	CYP P450	Yes, tested variants in HWE	Etanercept	24 w	ASAS20, BASDAI50
Morales-Lara et al., 2012	Cohort	55	PsA	CASPAR	56,3%	51.4 ± 10.8	Spain	TNFRSF10A TNFRSF1A	Yes, tested variants in HWE	anti-TNFα	24 w	EULAR criteria
Tutuncu et al., 2005	Cohort	5	PsA	ND	ND	ND	USA	Fc gamma receptor type IIIA	No	anti-TNFα	12 w	≥70% PhGA and SJC/TJC and ≥50% improvement in 2 of: ESR, CRP, PGA, MS
Ramírez et al., 2012	Cohort	103	PsA	CASPAR	52,4%	49.7 ± 13.5	Spain	Fc gamma receptor	No	anti-TNFα	24 w	EULAR criteria
Aita et al., 2018	Case-control	137 (55 AS, 82 PsA)	AS/PsA	mNY criteria / CASPAR	61,3%	51.6 ± 12.6	Italy	TNFα TNF-RSF1A MEFV	Yes, tested variants in HWE	anti-TNFα	144 w	BASDAI ≤ 4
Liu et al., 2019	Cohort	79	AS	mNY criteria and ASAS	88,6%	36.0 ± 11.5	China	MYOM2 VPS13B DISP1 IL27	No	etanercept	12 w	ASAS40
Julià et al., 2014	Cohort	81	PsA	CASPAR	53.0%	48.9 ± 12.7	Spain	PDE3A	No	anti-TNFα	12 w	ΔDAS28
Ovejero-Benito et al., 2019		20	PsA	CASPAR	ND	ND	Spain	TNFRSF1A/1B TNFAIP3 TNIP1 TNF TRAF3IP2	Yes, tested variants in HWE	anti-TNFα	24 w	NRS-Pain50
Polo Y La Borda et al., 2019	Cohort	118 (49 AS, 24 nr-axSpA, 45 p-SpA)	SpA	ASAS	61,8%	53.0 ± 11.2	Spain	36 genes involved mainly in pathogenesis	Yes, tested variants in HWE	anti-TNFα	252 w	Decrease ≥50% or reduction of at least two BASDAI points; EULAR criteria
Xing-Rong et al., 2018	Case-control	215	AS	mNY criteria	82,7%	28.2 ± 9.3	China	TNFRSF1A /1B	Yes, tested variants in HWE	etanerceptSASP celecoxib	48 w	ASAS20, ASAS40
Yan et al., 2017	Cohort	185	AS	mNY criteria	69,1%	37.4 ± 6.2	China	ABCB1	Yes, tested variants in HWE	etanercept	12 w	BASDAI50/ASAS20

(Continued)

TABLE 1 | Continued

References	Study design	Number of SpA patients	SpA subtype	Disease definition	Males	Age ± *SD*	Country	Exposure: candidate gene/s	HWE checked	Therapy	Follow up (weeks)	Response to therapy definition
Fabris et al., 2016	Cohort	187 (66 AS, 74 nrSpA/pSpA, 47 uSpA)	SpA	ASAS	66,3%	52.0 ± 30.0	Italy	*TNFRSA1B*, *TNFα*, *FCGR3A, IL-6, IL-6R, TGF-β*	No	anti-TNFα	272 ± 224 w	Non-Switch vs. Switch
Seitz et al., 2007	Cohort	33 (22 AS, 10 PsA)	AS, PsA	mNY criteria, ACR	ND	ND	Switzerland	*TNFα*	No	anti-TNFα	24 w	ΔBASDAI, ΔDAS28
Zhao et al., 2017	Case-control	200	AS	mNY criteria	77,5%	45.8 ± 11.7	China	*TNFRSF1A NLRP3*	Yes, tested variants in HWE	Etanercept, csDMARD	12 w	ASAS20
Tong et al., 2012	Case-control	106	AS	mNY criteria	77,3%	41.6 ± 15.8	China	*TNFα*	Yes, tested variants in HWE	anti-TNFα	12 w	ASAS40-50-70
Murdaca et al., 2014	Case-control	57	PsA	CASPAR	43,8%	50.0 ± 7.0	Italy	*TNFα*	Yes, tested variants in HWE	anti-TNFα	24 w	ASAS20
Nossent et al., 2014	Cross-sectional and cohort	335	AS	mNY criteria	70,1%	45.0± 12.6	Norway	*TNFα*	No	anti-TNFα	340 w (mean)	ΔBASDAI
Ma et al., 2017	Case-control	68	AS		55,8%	32.4 ±12.6	China	*NAT1*	No	MTX	up to 26 w	Morning stiffness, tender joints
Chandran et al., 2010	Cohort	119	PsA	CASPAR	56,3%	44	Canada	*MTHFR DHFR SLC19A1*	Yes, some variants (rs1051266 and rs180113) were in HWE	MTX	24 w	Actively inflamed joint count
Wang et al., 2017	Case-control	130	AS	mNY criteria	75,3%	30.81 ± 6.92	China	*CYP P450 COX-2*	No	NSAIDs	12 w	ΔBASDAI, ASAS 20, ASAS40
Eder et al., 2010	Cohort	133	PsA	ND	59,4%	45.6± 12.3	Canada	*MIF*	Yes, tested variants in HWE	IAI	12-24 w	Presence/absence of tenderness or effusion
Sokolik et al., 2021	Case-control	74	PsA	CASPAR	41,8%	46 ± 10.9	Poland	*IL-6*	Yes, tested variants in HWE	MTX	ND	ACR20 PSARC

SpA, spondyloarthritis; HWE, Hardy-Weingberg equilibrium; AS, ankylosing spondylitis; PsA, psoriatic arthritis; nrSpA, non-radiographic axial spondyloarthritis; pSpA, peripheral spondyloarthritis; uSpA, undifferentiated spondyloarthritis; SD, standard deviation; TNFα, tumor necrosis factor α; IL-23, interleuchin 23; ERAP1, endoplasmic reticulum aminopeptidase 1; CYP P450, cytochrome P450; MYOM2, myomesin 2; VPS13B, vacuolar protein sorting 13 homolog B; DISP1, dispatched RND transporter family member 1; IL-27, interleukin; PDE3A, phosphodiesterase 3A; TNFAIP3, TNFα induced protein 3; TNFRSF1A/1B, Tumor necrosis factor receptor superfamily member 1A/1B; TNFRSF10A, TNF receptor superfamily member 10a; TNIP, TNFα interacting protein 1; TRAF3IP2, TRAF3 interacting protein 2; ABCB1:ATP binding cassette subfamily B member 1; IL-6/IL-6R interleukin 6/ receptor; TGF-β, transforming growth factor β; NLRP3, NLR family pyrin domain containing 3; NAT1, N-acetyltransferase 1; MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; SLC19A1, solute carrier family 19 member 1; MIF, macrophage migration inhibition factor; IQR, interquartile range; mNY criteria, modified New York criteria; CASPAR, classification criteria for psoriatic arthritis; ASAS: assessment in ankylosing spondylitis; ACR, American College of Rheumatology; BASFI, bath ankylosing spondylitis function index; BASDAI, bath ankylosing spondylitis disease activity index; EULAR, European league against rheumatism; DAS28, disease activity score for 28 joints; PhGA, physician global assessment; SJC, swollen joint count; TJC, tender joint count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; PGA, patient global assessment; MS, morning stiffness; NSR-pain, numeric rating scale for pain; PSARC, psoriatic arthritis response criteria; csDMARDs, conventional synthetic and targeted synthetic; MTX, methotrexate; NSAIDs, non-steroidal anti-inflammatory drugs; IAI, intra articular injection; w, weeks; ND, not defined.

TABLE 2 | Application of Newcastle-Ottawa quality assessment scale (NOS) for cohort, cross-sectional and case control studies.

References	Score in each Newcastle-Ottawa quality assessment scale item			Total score	Study quality
	Selection	Comparability	Outcome		
Cohort Studies					
Morales-Lara et al., 2010	1	0	2	3	Unsatisfactory
Schiotis et al., 2014	4	1	3	8	Very good
Chen, 2017	4	1	3	8	Very good
Morales-Lara et al., 2012	4	2	3	9	Very good
Tutuncu et al., 2005	2	1	3	6	Satisfactory
Ramírez et al., 2012	4	1	3	8	Very good
Liu et al., 2019	4	1	2	7	Good
Julià et al., 2014	4	1	3	8	Very good
Ovejero-Benito et al., 2019	2	1	3	6	Satisfactory
Polo Y La Borda et al., 2019	4	1	3	8	Very good
Yan et al., 2017	4	1	3	8	Very good
Fabris et al., 2016	4	3	3	8	Very good
Seitz et al., 2007	3	0	3	6	Satisfactory
Chandran et al., 2010	4	1	3	8	Very good
Eder et al., 2010	4	1	3	8	Very good
	Selection	**Comparability**	**Outcome**		
Cross-sectional studies					
Nossent et al., 2014	3	1	3	7	Good
	Selection	**Comparability**	**Exposure**		
Case-control studies					
Xu et al., 2020	2	1	3	6	Satisfactory
Manolova et al., 2014	2	1	3	6	Satisfactory
Aita et al., 2018	3	1	3	7	Good
Xing-Rong et al., 2018	4	2	2	9	Very good
Zhao et al., 2017	3	1	3	7	Good
Tong et al., 2012	3	1	3	7	Good
Murdaca et al., 2014	4	2	3	9	Very good
Ma et al., 2017	0	0	1	1	Unsatisfactory
Wang et al., 2017	2	1	3	6	Satisfactory
Sokolik et al., 2021	3	0	3	6	Satisfactory

Note of the use of NOS: a cohort study can be awarded a maximum of four stars for the selection category, a maximum of two stars can be given for comparability and a maximum of three stars for the outcome category. A cross-sectional study can be awarded a maximum of five stars for the selection category, a maximum of two stars can be given for comparability and a maximum of three stars for the Outcome category. A case-control study can be awarded a maximum of four stars for the Selection category, a maximum of two stars can be given for comparability and a maximum of three stars for the Exposure category.

response (according to ASAS0 20) for age, gender, disease duration, and diagnosis, found a significantly positive association (Fabris et al., 2016). Other TNFα gene polymorphism described to be associated to either ASAS 40, ASAS 50, or ACR 20 response were −857C>T (rs1799724), −1031T>C (rs1799964), while +489G>A (rs80267959) was associated to ACR 20 response. All these findings derived, however, from non-adjusted analysis and were not confirmed by all studies (Tong et al., 2012; Murdaca et al., 2014; Aita et al., 2018).

Furthermore, genes encoding for molecules implicated in the signaling transduction cascade (including inflammatory cascade), such as phosphodiesterase *(PDE)3A*, were shown to have a linear relationship with DAS28 reduction after anti-TNFα therapy (Julià et al., 2014). Other polymorphisms implicated in SpA pathogenesis that were found to be independently associated to non-response were: rs755622 in macrophage migration inhibition factor (*MIF*), rs917997 in IL18-receptor accessory protein (*IL18-RAP*), rs3740691 in ADP Ribosylation Factor GTPase Activating Protein 2 (*ARFGAP2*), rs1800896 in *IL-10*, 2rs4240847 in Mitogen-Activated Protein Kinase-Activated Protein Kinase (*MAPKAPK2*), rs11096957 in Toll like receptor-10 (*TLR-10*), rs11541076 in Interleukin 1 Receptor Associated Kinase 3 (*IRAK-3*) (Schiotis et al., 2014; Polo Y La Borda et al., 2019).

TABLE 3 | Studies included in the qualitative synthesis.

References	Therapy	Follow up	Exposure: candidate gene/s	Polymorphism	Risk genotype/allele	Effect size (95%CI) or *p*-value	Outcome	Effect size adjusted	Correction for multiple testing
Schiotis et al., 2014	Anti-TNFα	12–20 w	*MIF*	rs755622	GG+CG	OR 3.14 (1.19–8.22)	Non-response according to BASDAI50	Yes, the candidate polymorphism were all included in a multivariate model and effect sizes of independent predictors of non-response are included	No
			IL18RAP	rs917997	AA+AG	OR 3.35 (1.38–8.15)			
			TNFRSF1B	rs1061622	GG+TG	OR 2.46 (1.00–6.04)			
			ACE	rs4343	–	ns			
			UQCC1	rs6060369	–	ns			
			ARFGAP2	rs3740691	AA+AG	OR 2.90 (1.12–7.51)			
			ASPN	rs331377	–	ns			
			CALM1	rs3213718	–	ns			
			IL10	rs1800896	AA	OR 3.09 (1.04–9.15)			
			CYP2D6	rs764481	–	–			
			CALM1	rs2300496	–	–			
			CALM1	rs2300500	–	–			
Chen, 2017	Etanercept	24 w	*CYP2C9*	rs1057910	–	ns	Percentage of responders according to BASDAI50 and/or ASAS20	No	No
			CYP2D6	rs1065852	CC	$p < 0.05$ vs. CT			
			CYP3A5	rs776746	3/3	$p < 0.05$ vs. 1*/3*			
Morales-Lara et al., 2012	Anti-TNFα	12–24 w	*TNFRSF10A*	rs20575	–	ns	EULAR response	No	No
			TNFRSF1A	rs767455	AA	$p = 0.04$			
Ramírez et al., 2012	Anti-TNFα	12-24 w	*FCGR2A*	rs1801274	–	ns	EULAR response	No	Yes
			FCGR3A	rs396991	RR	$p = 0.03$			
Aita et al., 2018	Anti-TNFα	40–144 w	*TNF*	rs1799964	–	ns	BASDAI ≤ 4	No	No
			TNFRSF1A	rs1799724	–	ns			
				rs1800750	–	ns			
				rs1800629	–	ns			
				rs361525	–	ns			
				rs1800693	G	$p = 0.03$			
Liu et al., 2019	Etanercept	12 w	*MYOM2*	rs2294066	CC	$p < 0.0001$	ASAS40	No	No
			VPS13B	rs7460625	–	ns			
			DISP1	rs2609383	–	ns			
			DISP1	rs2789975	–	ns			
			IL27	rs17855750	–	ns			
Julià et al., 2014	Anti-TNFα	12 w	*PDE3A*	rs3794271	AA	Beta = −0.71; $p < 0.0001$	ΔDAS28	Yes, for DAS28 baseline value	No
Polo Y La Borda et al., 2019	Anti-TNFα	252 w	*MAPKAPK2*	rs4240847	A	HR 1.63 (1.08,2.44)	Non response defined as decrease<50% BASDAI or reduction <1.2 of DAS28	No	No
			TLR10	rs11096957	T	HR 1.49 (1.10,2.04)			
			IRAK3	rs11541076	T	HR 1.49 (1.00–2.17)			
			+other 38 (ref [26])						
Xing-Rong et al., 2018	Etanercept + sulfasalazyne + colecoxib	48 w	*TNFRSF1A*	rs767455	–	ns	ASAS20 ASAS40	No	No
			TNFRSF1A	rs2234649	–	ns			
			TNFRSF1B	rs1061622	TT/GG	$p = 0.041$ for ASAS20 $p = 0.021$ for ASAS 40			

(Continued)

TABLE 3 | Continued

References	Therapy	Follow up	Exposure: candidate gene/s	Polymorphism	Risk genotype/allele	Effect size (95%CI) or *p*-value	Outcome	Effect size adjusted	Correction for multiple testing
Yan et al., 2017	Etanercept	12 w	*ABCB1* *ABCB1* *ABCB1*	rs2032582 rs1128503 rs1045642	GG+GA CT+TT –	$p < 0.05$ $p < 0.05$ –	ASAS20 (no differences in ASAS50 e ASAS70)	No	No
Fabris et al., 2016	Anti-TNFα	272 ± 224 w	*TNF* *TNFR2* *IL6* *IL6R* *FCGR3A* *TGF-β*	rs1800629 rs1061622 rs1800795 rs2228145 rs396991 rs19822073	A – GG – – –	OR 4.40 (1.50–13.10) ns ns ns ns ns	EULAR response criteria or BASDAI50 or rheumatologist opinion whether to continue therapy	Yes, covariates were: age, gender, disease duration, diagnosis	No
Zhao et al., 2017	Etanercept, csDMARD	12 w	*TNFRSF1A* *TNFRSF1A* *TNFRSF1A* *TNFRSF1A* *NLRP3* *NLRP3* *NLRP3* *NLRP3*	rs4149570 rs767455 rs4149569 rs4149621 rs4612666 rs10925019 rs3806265 rs3806268	– – – – – – – G	ns ns ns ns ns ns ns OR 2.17 (1.03-4.56)	ASAS20	Yes, correction for age and gender	No
Tong et al., 2012	Anti-TNFα	12 w	*TNF*	rs1799724 rs1799964 rs1800629 rs361525	T T – –	$p = 0.0021$ $p = 0.0004$ ns ns	ASAS40 and/or ASAS50 and/or ASAS70	No	No
Murdaca et al., 2014	Anti-TNFα	24 w	*TNF*	rs361525 rs1800629 rs80267959	– – G	– – $p = 0.021$**	ACR20	No	Yes
Nossent et al., 2014	Anti-TNFα	340 w (mean)	*TNF*	rs361525 rs1800629	– –	ns ns	ΔBASDAI	No	No
Chandran et al., 2010	MTX	24 w	*MTHFR* *DHFR* *DHFR* *SLC19A1*	rs1801131 rs1650697 rs1232027 rs1051266	– A – –	– OR 2.99 (1.20, 7.55) – –	50% reduction in "actively" inflamed joint (tender and/or swollen)	Yes, adjustment for concomitant medication	No
Eder et al., 2010	IAI	12–24 w	*MIF*	rs755622	GG + GC	ns	No tenderness or effusion in the injected joint	Yes, adjustment for age, sex, duration of PsA. disease activity	No

**Only significant genotypes or risk alleles, among those tested, are indicated.*

***Nominal p significance.*

Ns, not significant; OR, odds ratio; IAI, intra articular injection; MTX, methotrexate; ACR, American College of Rheumatology; ASAS, assessment in ankylosing spondylitis; BASDAI, bath ankylosing spondylitis disease activity index; EULAR, European League Against Rheumatism; DAS28, disease activity score for 28 joints; PsA, psoriatic arthritis; MIF, macrophage migration inhibitory factor; IL18RAP, interleukin 18 receptor accessory protein; TNFRSF1B, TNF receptor superfamily member 1B; ACE, angiotensin I converting enzyme; UQCC1, ubiquinol-cytochrome C reductase complex chaperone 1; ARFGAP2, ADP ribosylation factor GTPase activating protein 2; ASPN, asporin; CALM1, calmodulin 1; IL10, interleukin 10; CYP2C9, cytochrome P450 family 2 subfamily C member 9; CYP2D6, cytochrome P450 family 2 subfamily D member 6;CYP3A5, cytochrome P450 family 3 subfamily A member 5; TNFRSF10A, TNF receptor superfamily member 10a; FCGR2A, Fc fragment Of IgG Receptor IIa; FCGR3A, Fc fragment Of IgG receptor IIIa; TNFRSF1A, tumor necrosis factor receptor superfamily member 1A; MEFV, mediterranean fever; MYOM2, myomesin 2; VPS13B, vacuolar protein sorting 13 homolog B; DISP1, dispatched RND transporter family member 1; IL27, interleukin 27; PDE3A, phosphodiesterase 3A; MAPKAPK2, mitogen activated protein kinase activated protein kinase 2; TLR10, Toll-like receptor 10; IRAK-3, interleukin 1 receptor associated kinase 3; TNFRSA1B, tumor necrosis factor receptor superfamily member 1B; ABCB1:ATP binding cassette subfamily B member 1; TNF, tumor necrosis factor alpha; TNFR2, tumor necrosis factor receptor 2; IL6, interleukin 6; IL6R, interleukin 6 receptor; TGF-β, transforming growth factor beta; DHFR, dihydrofolate reductase; NLRP3, NLR family pyrin domain containing 3; MTHFR, methylenetetrahydrofolate Reductase; SLC19A1, solute carrier family 19 member 1; RFC, reduced folate carrier.

Finally, one study, among those of good quality investigating pathogenetic genes, explored the role of *MIF* polymorphism rs755622 in predicting clinical response to intra-articular steroid injections in PsA; the analysis failed to show any association when correcting for age, sex, disease duration, and activity (Eder et al., 2010).

Genes Involved in Drug Metabolism or Immunogenicity

Fewer authors took into consideration genes that might be involved in drug metabolism or immunogenicity. Amid these, genes encoding for enzymes that are part the cytochrome (CYP) P450 superfamily have been tested: the allele variants *CYP2D6*10* and *CYP3A*3* were more frequently found in BASDAI50 responders than non-responders to etanercept (Chen, 2017). Other works examined genes encoding for the Fc fragment receptor 2A and 3A, under the hypothesis that polymorphisms resulting in a higher/lower affinity to the Fc region of TNFα blockers may modulate both their half-life and cellular effects, and may therefore produce differential therapeutic effects in individuals (Ramírez et al., 2012). Ramirez et al. found that *FCGR3A* was indeed associated to EULAR response, although in a non-adjusted analysis (Ramírez et al., 2012). Fabris et al. were not able to confirm this finding after adjusting for age, gender, disease duration, and diagnosis (AS/PsA) (Fabris et al., 2016).

One study investigated response to methotrexate in terms of reduction of at least 50% of "actively inflamed joints," meaning tender and/or swollen joints, highlighting that *DHFR* polymorphism +35289A>G (rs1232027) was able to predict response to methotrexate (even when correcting for concomitant medications; Chandran et al., 2010).

A synthesis of the genes that have been found to be associated to drug response is represented in **Table 4**.

DISCUSSION

The results of our SLR highlighted that the genetic component is surely one of the determinants of drug response in SpA. However, the heterogeneity existing in present literature prevented us to quantify the genetic contribution to therapy response, particularly regarding anti-TNFα biological drugs, which were the most studied.

Admittedly, there are several challenges in conducting predictions studies about genetic variants in drug response in SpA. Firstly, given that most studies focused on genes involved in the disease pathogenesis, it must be remembered that several pathways have been implied in this process. Dysregulation of the IL-17/23 axis and the activation of innate immunity, with effectors like gamma-delta T cells, type 3 innate lymphoid cells (ILCs), neutrophils, macrophages, and lately also cytotoxic B lymphocytes have been described in SpA (Tang and Inman, 2021). In addition, interaction with environmental triggers is fundamental for disease onset and perpetuation. As an example, polymorphisms of TLR-2 and−4, key receptors in pathogen recognition expressed by macrophages or dendritic cells, have been associated to SpA onset at an early age (Perica et al., 2015). When certain genetic variants are associated to disease onset or severity, it is logical to suspect they might be involved in drug response as well. However, since pathogenesis is not solely driven by one of these mechanisms, it is unlikely that a single gene, or a narrow spectrum of gene within a particular pathway, might significantly explain the tendency to respond to a certain targeted therapy. Furthermore, several aspects of SpA pathogenesis are still unknown: one above all, it is not clear how HLA-B27 exerts its pathogenetic effect. For this reason, comprehensive genetic approaches, such as genome-wide association studies (GWAS) have been undertaken in order to uncover unknown factors of susceptibility (Jung et al., 2014; Robinson et al., 2016). This kind of studies might also have therapeutic implications, and has the advantage, compared to the classic candidate-gene(s) design, of being hypothesis-free. Both candidate and whole genome strategies have limitations, however, candidate gene approach lacks the objectivity of genome-wide screening in the process of choosing specific candidates from numbers of potential possibilities; the choice of genes depends on the prior knowledge of the illness, which often remains partly unknown (Sabourin et al., 2019). In addition, in order to be clinically useful, a quite strong relation between a certain genetic variant and clinical outcomes has to be highlighted. Not to mention the candidate gene should also have a demonstrated added value, compared to clinical predictors of response (e.g., male sex), to be of interest (Ni et al., 2013; Ramonda et al., 2021). In practice, it is often the case that certain polymorphisms are only weakly associated to drug response. This can clearly be seen from the adjusted OR, along with their wide 95% CI, represented in **Table 3** (Schiotis et al., 2014; Fabris et al., 2016; Zhao et al., 2017; Polo Y La Borda et al., 2019).

A second, but not less important, issue is represented by the reproducibility of results. Even studies investigating the same polymorphism, such as TNFα −308, which has been associated to anti-TNF response both in adult and juvenile SpA (Scardapane et al., 2012), often have contrasting results (Murdaca et al., 2014; Nossent et al., 2014; Fabris et al., 2016). In part, this could be due to the small sample size of some of these studies or to the diversities in the included ethnicities (e.g., Asian vs. Caucasian). On the other hand, the outcomes of drug response are also not standardized across studies. Moreover, analysis are carried out very differently, adjusting for different sets of factors, or without any/with very little adjustment. All these factors add up to the challenge of detecting significant and reproducible genetic markers of drug response.

Thirdly, it has been highlighted how genetic research is particularly prone to type I error (i.e., the risk of falsely rejecting a true null hypothesis or, in other words, to identify a significant association when indeed no association exists; Sabourin et al., 2019). This might happen because of the highly non-independent nature of the variants in a genome, which implies that the assumptions underlying the commonly used statistical methods are often not met (Sabourin et al., 2019). Furthermore, more commonly type I error may stem from multiple testing (comparison of several variants), genotyping errors, and population stratification, that can result in spurious associations (Jorgensen et al., 2009). One of the most obvious, yet important, remedies for this, would be to correct for multiple

TABLE 4 | Synthesis of genes that have been studied in relation to treatment response in spondyloarthritis, and summary of results.

Candidate gene	Polymorphism	References	Risk genotype/Allele	Significant association with clinical response to drugs
TNF	rs1799724	Tong et al., 2012	T	Yes, positively associated to ASAS40 and/or ASAS50 and/or ASAS70
		Aita et al., 2018	–	No
	rs1799964	Tong et al., 2012	T	Yes, positively associated to ASAS40 and/or ASAS50 and/or ASAS70
		Aita et al., 2018	–	No
	rs1800629	Tong et al., 2012	–	No
		Fabris et al., 2016	A	Yes, positively associated to EULAR response criteria or BASDAI50 or rheumatologist opinion whether to continue therapy
		Murdaca et al., 2014		No
		Nossent et al., 2014	–	No
		Aita et al., 2018	–	No
	rs361525	Tong et al., 2012	–	No
		Murdaca et al., 2014	–	No
		Nossent et al., 2014	–	No
		Aita et al., 2018	–	No
	rs80267959	Murdaca et al., 2014	G	Yes, positively associated to ACR20
	rs1800750	Aita et al., 2018	–	No
TNFRSF1A	rs4149570	Zhao et al., 2017	–	No
	rs767455	Zhao et al., 2017	–	No
		Xing-Rong et al., 2018	–	No
		Morales-Lara et al., 2012	AA	Yes, positively associated to EULAR response criteria
	rs4149569	Zhao et al., 2017	–	No
	rs4149621	Zhao et al., 2017	–	No
	rs2234649	Xing-Rong et al., 2018	–	No
	rs1800693	Aita et al., 2018	G	Yes, positively associated to BASDAI
TNFRSF1B	rs1061622	Xing-Rong et al., 2018	TT/GG	Yes, positively associated to ASAS20/ASAS40
		Polo Y La Borda et al., 2019	–	No
		Schiotis et al., 2014	GG+TG	Yes, negatively associated with BASDAI50
	rs3397	Polo Y La Borda et al., 2019	–	No
	rs976881	Polo Y La Borda et al., 2019	–	No
PDE3A	rs3794271	Julià et al., 2014	AA	Yes, positively associated to ΔDAS28
		Polo Y La Borda et al., 2019	–	No
HFR	rs1650697	Chandran et al., 2010	A	Yes, positively associated to 50% reduction in "actively" inflamed joint (tender and/or swollen)
	rs1232027	Chandran et al., 2010	–	No

ACR, American College of Rheumatology; ASAS, assessment in ankylosing spondylitis; BASDAI, bath ankylosing spondylitis disease activity index; EULAR, European League Against Rheumatism; DAS28, disease activity score for 28 joints; TNF, tumor necrosis factor alpha; TNFRSF1B, TNF receptor superfamily member 1B; TNFRSF1A, tumor necrosis factor receptor superfamily member 1A; PDE3A, phosphodiesterase 3A; DHFR, dihydrofolate reductase.

testing, especially in the candidate-gene approach studies where several variants are tested. Unfortunately, only a slight minority of the retrieved studies applied this correction (**Table 3**), although this might be less impactful in those studies which tested a limited number of variants (e.g., 3–4). Another way it has been found to limit this problems is replication or cross-validation within the same sample (Liu et al., 2019).

Certainly, however, the fact that several polymorphisms, mainly implicated in the disease pathogenesis, were able to predict to some extent the treatment response, even in adjusted analysis and with a fair numerosity in study populations, points toward the real existence of a genetic determination of drug response (Julià et al., 2014; Schiotis et al., 2014; Fabris et al., 2016; Zhao et al., 2017). This was especially seen with TNFα-blockers

therapy, which is also the most frequently used effective therapy for SpA (van der Heijde et al., 2017). Studies investigating polymorphisms involved in drug metabolism in anti-TNFα were less consistent. Interestingly, also response to methotrexate seemed to be predicted by a polymorphism of a gene involved in drug metabolism (*DHFR* +35289), which is somehow more expected than for anti-TNFα as methotrexate is a traditional csDMARD, with a prevalent liver metabolism.

Our study had the methodological strength of being a SLR, and therefore we were able to capture all relevant literature pertaining our research questions, as well as providing a quality assessment of each study. The potential limitations are linked to the design of included studies, which all used a candidate-gene approach: this kind of research is more prone to type I error and to publication bias (i.e. the presentation of mostly positive results, neglecting studies with negative findings). To this regard, GWAS studies could be at a lower risk of bias. Moreover, no RCT taking genetic variants into consideration was retrieved, but only observational studies. Other issues were heterogeneity in the description of population, exposure and outcome. The latter prevented us to perform a meta-analysis to quantify the genetic contribution to drug response in SpA.

In conclusion, we were able to identify a genetic component in drug response across all the included study. Incorporating genetic analysis into clinical studies could help to predict responses to different treatment options, aiming toward personalized medicine. However, further studies are warranted to better define the genotypes that are most involved in contributing to response to therapy and to describe the magnitude of this phenomenon, especially in comparison with the most commonly used clinical predictors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AO and GC participated in study design, data extraction, analysis and synthesis, and drafted the manuscript. ML and PG helped in data collection, critical interpretation of data, and revised the manuscript for important intellectual content. AD and RR conceived the study, analyzed the results critically, and revised the manuscript for important intellectual content. All authors approved the final version to be published.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.703911/full#supplementary-material

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REVIEW
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Genetics, Epigenetics, and Gender Impact in Axial-Spondyloarthritis Susceptibility: An Update on Genetic Polymorphisms and Their Sex Related Associations

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Spondyloarthritis (SpA) is a group of chronic inflammatory rheumatic disease that can be divided into predominantly axial or predominantly peripheral involvement, with or without associated psoriasis, inflammatory bowel disease or previous infection. Axial SpA (axSpA) encompasses ankylosing spondylitis (AS) with radiological sacroiliitis, and a type without radiographic sacroiliitis, called "non-radiographic axial SpA" (nr-axSpA). Males and females show large differences in their susceptibility to SpA, such as distinctions in clinical patterns, phenotypes and in therapeutical response, particularly to TNF inhibitors (TNFi). Several studies indicate that AS women have doubled risk to failure TNFi compared with males. This diversity in drugs' efficacy among women and men may be caused by differences in the balance of sex hormones and in gene-specific expression likely triggered by X-chromosome instability and gene-specific epigenetic modifications. Evidence reported that polymorphisms in microRNAs on X- and other chromosomes, such as miR-146a, miR-155, miR-125a-5p, miR-151a-3p and miR-22-3p, miR-199a-5p could be involved in the different clinical presentation of SpA, as well as disease activity. In addition, association with non–response to TNFi treatment and presence of IRAK3 and CHUCK genes in SpA patients was recently detected. Finally, polymorphisms in genes involved in IL-23/IL-17 pathway, such as in drug pharmacodynamics and pharmacokinetics may have a role in response to TNFi, IL17i, and IL23i. A major understanding of genomic variability could help in the development of new therapeutic targets or in taking advantages of different mechanisms of action of biological drugs. Moving from the multifactorial etiology of disease, the present review aims at evaluating genetic and epigenetic factors and their relationship with sex and bDMARDs response, helping to investigate the different expression among males and females of genes on

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X- and other chromosomes, as well as mi-RNA, to highlight relationships between sex and occurrence of specific phenotypes and symptoms of the disease. Moreover, the role of the epigenetic modification in relation to immune-regulatory mechanisms will be evaluated.

Keywords: Spondyloarthritis, genetic predisposition and association, precision medicine, gender predisposition, gender medicine

INTRODUCTION

Spondyloarthritis (SpA) is a heterogeneous group of inflammatory chronic diseases characterized by common clinical features, as inflammatory back pain, peripheral joint involvement, dactylitis and enthesitis (Chimenti et al., 2020b). SpA can be divided into predominantly axial or predominantly peripheral form, with or without psoriasis (PsO), inflammatory bowel disease (IBD) or previous associated infection. Axial SpA (axSpA) encompasses ankylosing spondylitis (AS) with radiological signs of sacroiliitis, and a type without radiographic sacroiliitis, called "non-radiographic axial SpA" (nr-axSpA) (Chimenti et al., 2019a; Ward et al., 2019). All the clinical subtypes share common pathogenic, clinical, and radiologic features such as the genetic predisposition and the association with HLA-B27, the presence of extra-articular involvement and bone remodeling associated with bone resorption and osteoproductive lesions called syndesmophytes (Chimenti et al., 2020b). In axial-SpA the sex prevalence has dramatically changed during the last two decades: SpA were linked to male gender with a ratio of 10:1 with respect to female gender, nowadays the ratio has reduced to 3:1 for AS/axSpA (West, 1949). However, in contrast to AS, nr-axSpA male and female patients present the same prevalence (van der Horst-Bruinsma et al., 2013). Recent evidence supports the hypothesis of different clinical subtypes among males and females, suggesting a genetic and hormonal based pathogenesis. In this direction, the pathogenesis of SpA is not clearly understood to date and the role of immunological and genetic data showed clear sex dimorphisms in SpA patients (Rusman et al., 2018). Certainly, pathogenic mechanisms of SpA comprise a complex interplay among genetic background, environmental triggers, and mechanical stress that leads to the overall activation of inflammation and autoimmunity (Pedersen and Maksymowych, 2018). The role of genetic susceptibility in SpA was strongly associated with the presence of human leukocyte antigen (HLA) and non-HLA alleles that surely takes part in the predisposition to the disease (Brown et al., 2000). The class I HLA allele HLA–B27 is strongly linked with the development of SpA due to its role in the pathogenesis of the disease (Hacquard-Bouder et al., 2007). The revolution of the genome-wide association study (GWAS) era has identified hundreds of genes associated with SpA, mainly IL23R, ERAP1 (Endoplasmic Reticulum Aminopeptidase-1), ERAP2, and MEFV (Mediterranean fever) linked to innate and acquired immune response and cytokines production (Brown et al., 2000). The current interest in Precision Medicine, in order to identify preventive and therapeutic interventions, is increasing in the management of SpA patients. Clinical effect of biological DMARDs (bDMARDs) is now known to be affected by gender, as well as the clinical, genetic, and psychosocial life-style context (Garrido-Cumbrera et al., 2021). This different efficacy in women and men is due to biological differences which may be caused by sex-specific gene expression likely triggered by gene-specific epigenetic modifications. However, in SpA patients, a series of factors may interfere in epigenetic modifications: age, smoke, diet, and environmental factors (Chimenti et al., 2020b). In this context, the clinical phenotype and the response to drugs can be influenced by gender and considered as complex traits in SpA. The study of genetic and epigenetic mechanisms involved in the pathogenesis of SpA may be of help to define novel targets for more effective therapy. The aim of this review was to highlight the role of genetics and epigenetics in the susceptibility and clinical pattern of SpA as well as in bDMARDs treatment response variability with a focus on gender differences and predisposition.

GENETIC PREDISPOSITION TO SPONDYLOARTHRITIS

Over the past decades a growing interest in the pathogenesis of SpA has provided rapid advances in understanding the genetic basis of the disease. A family aggregation to SpA has long been recognized and studies of concordance in twins and families of patients affected by AS indicate that susceptibility of the disease is widely due to genetic factors (Brown et al., 1997). More recent studies reported that the major histocompatibility complex (MHC) region gives a contribution of about 20% to the heritability (Ellinghaus et al., 2016).

HLA B27

The HLA-B27 is the allele most associated with AS, as well as with other types of SpA (Breban et al., 2015; Lin and Gong, 2017). Most AS patients express HLA-B27 (90%) and this allele is responsible for up to 28% of the etiology of SpA. Nevertheless, less than 5% of HLA-B27 positive people in the general population develop this disease (Reveille, 2011). Much research has shown a higher prevalence of HLA-B27 in males than in females. Recently, a link between HLA-27 expression and high concentrations of testosterone was demonstrated (Akassou and Bakri, 2018). HLA-B27 is highly polymorphic and several sub-alleles with different contributions to susceptibility to SpA have been identified. Of these subtypes, B*2705 is found most frequently in the Caucasian population and has a strong association with AS and PsA; on the contrary, HLA-B*2706, detected in Southeast Asia, and

B*2709, present in Sardinia, do not seem to be related to SpA (Haroon et al., 2017).

The main function of HLA-B27 is the presentation of intracellular peptides to cytotoxic (CD8-positive) T lymphocytes and multiple theories about its pathogenic mechanisms have been suggested. These hypotheses include the presentation of "arthritogenic" peptide recognized by the T-cell receptor (TCR) of autoreactive $CD8^+$ T cells; cell surface HLA-B27 homodimers recognition by natural killer (NK) receptors; accumulation of misfolded HLAB27 in the endoplasmic reticulum during protein biosynthesis leading to inflammatory response; failed elimination of bacteria or virus with consequent intracellular microbial survival and prolonged abnormal immune system activation (Haroon et al., 2017). Recently, it has been described that HLA-B27 could perturbate the composition of the gut microbiota, leading to loss of mucosal tolerance and activation of aberrant inflammatory response. Significant differences in microbiota composition were detected between HLA-B27-positive and HLA-B27-negative SpA patients, with increased *R. mucilaginosa* and *E. lenta*, and low levels of *Bifidobacterium* and *Odoribacter* in HLA-B27 positive patients, similar to what has been reported in patients with ileal Crohn's disease (CD) and ulcerative colitis (UC) (Chimenti et al., 2018; Lim et al., 2018). Moreover, a recent metabolome analysis in HLA-B27 transgenic rats has shown a perturbation in levels of short-chain fatty acids and other microbial metabolites during gut inflammation (Asquit et al., 2017). Microbial dysbiosis and perturbed microbial metabolic function are associated with inflammatory pathways (IFNg, TNF, and IL-23/IL-17) and with imbalance between Tregs, Th1, Th2, and Th17 cells, which may lead to chronic inflammation in the joint, skin, or gut as well as a loss of tolerance for non-pathogenous (Asquit et al., 2017; Gill et al., 2019). Even though HLA-B27 plays an undisputedly critical role in AS pathogenesis, only 1–3% of HLA-B27-positive people develop the disease, and not all AS patients carry the HLA-B27 antigen, advising that other genes may be involved in the development of the pathology (Chimenti et al., 2018; Gill et al., 2019).

Other HLA Genes

Different mechanisms have been advanced to explain SpA susceptibility, such as large variants (deletions, duplications, inversions), single nucleotide polymorphisms (SNPs), gene-gene, and gene-environment interactions. In the last decades, the advent of genome-wide association studies (GWASs) has improved our understanding of SpA disease pathogenesis. HLA-B51, mostly associated with Behçet's disease, seems to be a risk variant for AS, as well as HLA-B40, most specifically the B*40:01 allele (a DNA-defined allele that corresponds to HLA-B60 at the serologically defined or protein level) was increased in B27-positive patients with AS (Clarke and Vyse, 2009; Dougados and Baeten, 2011). HLA-B27 and HLA-B60 genes are located on the same chromosome but they could be involved in different antigen-triggered pathologic pathways. Therefore, the epistatic effects between HLA-B27 and HLA-B60 may be due to the similar downstream T-cell mediated immune response (Wei et al., 2015). Moreover, HLA-Cw*0702 is associated with axial PsA, HLA-DQ3 is involved in both PsA disease and its progression (Chimenti et al., 2020b). Although some HLA-B/C haplotypes are ancestral, explaining their over-expression in SpA, the role of these alleles in determining the risk and the clinical expression of disease is still under investigation (Queiro et al., 2006). Recent studies investigated the MHC class I chain A related (MICA), which mediates the activation of natural killer cells, γδT cells, and $\alpha\beta CD8^+$ T cells. MICA polymorphisms could be associated with the susceptibility to both PsO and PsA, AS, IBD, and Behçet's disease (Wang and Zhou, 2015). MICA-129 Val/Val polymorphism has been identified as protective against radiographic axial PsA, probably due to a low affinity for its receptor and a lower inflammation on sacroiliac joint (Wang and Zhou, 2015; Fechtenbaum et al., 2019). Nevertheless, a defining contribution of MICA, as well as other non-HLA MCH genes (e.g., TNF, TAP1, TAP2, and LMP2) to AS susceptibility has not yet been established and could be confounded by the linkage disequilibrium known to exist in this region (Fechtenbaum et al., 2019).

Non-HLA Genes

Outside the MHC region, other genes have been identified as risk factor for SpA. Among them, evidence from the literature highlights the ERAP1 and 2, that show epistasis with HLA-B*27. ERAP1 is associated with AS only in individuals carrying the HLA-B27 or HLA-B*40:01 alleles (Wang and Zhou, 2015), while interaction between ERAP2 and AS is present in both HLA-B27 positive and negative disease, suggesting their different functional mechanism in causing AS (Reveille, 2014). The main function of ERAP1 and 2 products is to trim peptides to an optimal length for MHC class I binding and presentation (Cortes et al., 2015). ERAP1 variants cause a modification in three-dimensional structure of protein and could also influence gene expression. It was shown a higher expression of ERAP1 in dendritic cells of AS patients compared to healthy controls, suggesting that overexpression of ERAP1 could promote the disease (Robinson et al., 2015). Loss-of-function variants of ERAP1 lead to an aberrant peptides' presentation, influencing dimerization or misfolding of HLA-B27 and contributing to disease pathogenesis, although to date the exact mechanism is unclear. The second function of ERAP1 is the cleavage of cell surface receptors for the proinflammatory cytokines IL-1 (IL-1R2), IL-6 (IL-6Rα) and TNF (TNFR1), downregulating their signaling (Chimenti et al., 2018). ERAP1 and ERAP2 haplotype (concerning *rs27044*, *rs30187,* and *rs2549782* SNPs) are associated with familial AS (Saveanu et al., 2005; Evans et al., 2011) while *rs27037*, *rs27044*, and *rs30187* SNPs are involved in syndesmophytes formation and AS severity (Saveanu et al., 2005; Tsui et al., 2010; Evans et al., 2011). In contrast, ERAP2 variant *rs2248374* seems to cause a loss of ERAP2 protein by reducing MHC class I surface expression in cell lines, being potentially protective for AS (Wang et al., 2012; Paladini et al., 2019). Another gene described as involved in AS susceptibility is RUNX3, involved in differentiation of cytotoxic-lineage T cells into phenotypically mature $CD8^+$ T cells, which have been implicated in the pathogenesis of AS. Moreover, RUNX3 regulated other immunological cells and through TGF-β signaling pathway could drive the imbalance of Th17/Treg

in AS (Vecellio et al., 2019). Three other AS–associated genes (EOMES, IL7R, and ZMIZ1) were also identified as impacting on variation in $CD8^+$ lymphocyte counts and differentiations. Probably other mechanisms besides the effect on lymphocytes are underlying but they are still unknown (Evans et al., 2011). RUNX3 polymorphism (*rs6600247*) is associated with lower $CD8^+$ T cell counts, causing an altered antigen presentation, while the opposite occurred with IL7R haplotype rs991570, suggesting an unknown mechanism related to the IL7/RUNX3 pathway (Roberts et al., 2016; Vecellio et al., 2019). Previously, it was identified that other AS-associated SNPs, *rs4648889* and *rs4265380*, located upstream of the RUNX3 gene, might have a regulatory impact respectively on $CD8^+$ T cell and monocytes and similar genetic associations have been also described in PsA (Di Meglio et al., 2011). In AS, such as in IBD, genetic associations have also been reported at TBX21, encoding the transcriptional factor T-bet that control the functional differentiation of many cell types and is overexpressed in AS CD8 T-cells and NK cells. Moreover, expression of TBX21 and T-bet is higher in AS patients than controls and homozygosity for the rs11657479 risk allele increases T-bet expression (O'Rielly and Rahman, 2013; Lau et al., 2017). Associations with other genes (DEFB4, CDKAL1, KIF21B, ORMDL3, MST1, and PSMG) have been proposed but their relevance in AS has not been confirmed (Reveille et al., 2010; Aita et al., 2018).

Cytokines

Several lines of evidence suggested a relevant role of IL-17 and IL-23 in the pathogenesis of AS. association between the IL-23R locus and AS have been demonstrated, as well as genes related to the IL-17 pathway and risk alleles for AS. The identification of protective alleles in IL23R that result in reduced phosphorylation of signal transducer and activator of transcription 3 (STAT3) and in impairment production of IL-17 supports the hypothesis that activation of the IL-23–IL-17 pathway is controlled at a genetic level (Gravellese and Schett, 2018). Association between IL23R variants and SpA, reported by GWAS studies, has strengthened the importance of IL-17-IL23 axis SpA pathogenesis. For example, the *rs11209032* SNP, located within the intergenic region between IL23R and IL12RB2, might influence Th1-cell number and correlates with disease susceptibility (Roberts et al., 2016). Also, the variant allele of *rs11209026* SNP, previously associated with IBD, modifies the interaction between IL23R and its signaling partner, JAK-2 kinase, with a protective effect. Indeed, carriers of this allele showed a decreased IL-17 and IL-22, as well as a reduction of circulating Th17 cells (Di Meglio et al., 2011). Other variants within genes encoding proteins crucial for Th-17 signaling (TRAF3IP2, TYK2, STAT3, SOCS1, IRF4, and KLF4) have been investigated but further functional studies are needed to explain how these variants contribute to susceptibility and phenotypic expression of SpA. Further SpA-associated gene pathways include IFNs, IL-1, and TNFα. IFN is a key early mediator of inflammation, involved in production of proinflammatory cytokines, e.g., TNFα and IL-1, as well as in activation of NFkB signaling (Gravellese and Schett, 2018). Dysregulated NFkB activation may lead to the transcription of several target genes contributing to SpA pathogenesis. Multiple genetic loci involved in NFkB signaling (TNFRSF1A-LTBR, TRADD, TBKBP1, CARD9, and PTGER4) have been investigated in AS. Particularly, CARD9 could promote the production of IL-17 and IL-23 and, through the indirect activation of PTGER4, influence the bone ossification and radiographic progression observed in AS (Di Meglio et al., 2011; Roberts et al., 2016). Variations within some genes encoding proteins for IFN signaling have been evidenced in GWAS analysis, mostly in patients affected by psoriasis but could have a significance also in PsA and AS (Di Meglio et al., 2011). INF is a key early mediator of inflammation, determining the production of proinflammatory cytokines, as TNF-α and interleukin (IL)-1, and influencing the activation of NFkB signaling. SNPs in TNFα gene have been identified as potentially associated with SpA. In particular, a specific *rs1799964*/*rs1800629* haplotype exerts a protective role for SpA, mainly for AS and in HLA-B27 positive subjects, as demonstrated by the reduction of TNFα release. However, the relation between SNPs of genes involved in TNFα signaling and AS has shown controversial results, maybe related to the differences in the ethnic origin or to the number of the individuals under study (Roberts et al., 2016). SNPs in IL-1 gene cluster were reported to be associated with AS, with greater focus on IL-1A and IL-1R2 genes. Nevertheless, the contribution of IL-1 in AS susceptibility is likely to be limited, as evidenced by inefficacy of IL-1 receptor antagonist in AS treatment (Sims et al., 2008; Aita et al., 2018). Based on previous published data on the association of TLRs genes with autoimmune disease, research work has been carried out on their role in AS. Toll-like receptors (TLRs) have an important role in the mechanism of innate immunity and may influence inflammatory responses (Oliveira-Toré et al., 2019). Furthermore, they are involved in the activation of adaptive immune system upregulating costimulatory molecules of the antigen-presenting cells and play a role in the self-sustained inflammatory cycle and progression of chronic diseases (Oliveira-Toré et al., 2019). Polymorphisms in TLR genes could contribute to the susceptibility to SpA. A recent study pointed out that TLR2 gene *rs5743708∗A* polymorphism increased the chance of developing SpA. In addition, high levels of IL-12 were found in the presence of polymorphisms in TLR2 (*rs5743708*) and TLR9 (*rs5743836*) genes, while TLR9 *rs187084* was associated with increased production of IFNγ and TNFα. These polymorphisms contribute to potentiate the Th1, Th2, and Th17 immune response seen in SpA, which may confer to individuals carrying the variant alleles a predisposition to the development of SpA (Oliveira-Toré et al., 2019).

EPIGENETIC MECHANISMS IN SPONDYLOARTHRITIS

Not only genetic variants but also epigenetic mechanisms, such as DNA methylation, histone modification and non-coding RNAs, have shown in the last years to be particularly relevant to explain the SpA pathogenesis. The term epigenetics refers to all mechanisms that produce a change in the gene expression, without modifying the DNA sequence. In recent

decades, the interest in epigenetic mechanisms increased due to the evidence that their alterations are present in many diseases, including autoimmune diseases. In SpA, alterations of histone H3 (H3K27ac and H3K4me1) seem to be correlated with RUNX3 expression and the decrease of $CD8^+$ T cell in the presence of *rs4648889* SNP variant (Cherqaoui et al., 2020). In addition, H3K4me1 methylation regulates the activity of IL23R gene. Among epigenetic mechanisms, miRNAs represent one of the most interesting examples. These small molecules of non-coding RNAs can regulate the expression of multiple genes at a post-transcriptional level by inhibiting translation or inducing messenger RNA degradation. Significant alterations in miRNA expression have been observed in axSpA patients, showing a lower expression of 14 miRNAs in comparison with healthy controls (Prajzlerová et al., 2017; Cherqaoui et al., 2020). Interestingly, most of these miRNAs are involved in osteoblast differentiation or the Wnt signaling pathway while only miR-625-3p was significantly different in nr-AxSpA patients compared to controls. Moreover, also the genetic variability of miRNAs seems to be involved with autoimmune disorders susceptibility, and several associations were already reported (Latini et al., 2017). Until now, however, there is only little evidence of associations with PsA. For example, a Chinese study reported an association between common polymorphisms in mir-146a and mir-499 genes and ankylosing spondylitis (Xu et al., 2015). As already mentioned, epigenetics could play a role in the interactions between genetic and environmental susceptibility factors and might favor SpA development. DNA methyltransferase (DNMT) 3A and 3B are involved in genomic imprinting and X-chromosome inactivation, which may in part explain the different distribution of SpA according to gender (Hanson and Brown, 2017; Hao et al., 2017). Variants in DNMT3A, DNMT3B, and DNMT3L genes have been recently associated with AS, and different methylated positions (DMPs), that can influence HLA-B27, are identified (Ellinghaus et al., 2016; Cherqaoui et al., 2020). In addition, an increased level of Histone Deacetylase 3 (HDAC3), which regulates NF-kB activity, was detected in AS patients (Jiang and Wang, 2016). Additional genetic and epigenetic mechanisms, such as genomic imprinting, could explain the gender difference in inheritance of SpA (Rahman et al., 1999). Genomic imprinting is a normal process that differentially regulates the expression of specific genes depending on the sex of the transmitting parent. Already more than twenty years ago, a higher penetrance of PsO was reported if the father was affected and a similar phenomenon was observed among PsA patients (Burden et al., 1998; Rahman et al., 1999; Karason et al., 2003). Later a significant linkage on chromosome 16q was noted only after conditioning for paternal transmission, suggesting that genetic imprinting may play a role in the inheritance pattern of psoriasis and PsA (Karason et al., 2003; Eder et al., 2012). However, despite some encouraging advances in SpA epigenetic studies, data are still lacking and further studies are needed. **Table 1** summary of genes whose variability is involved in SpA. **Figure 1** summarizes the interaction among genetic and epigenetic factors and sex in SpA.

GENDER DIFFERENCES IN CLINICAL PHENOTYPES IN AXIAL SpA

Several sex related differences have been described among the various subtypes of SpA. These can be divided based on joint manifestations. Differences related to gender on joint manifestations are summarized in **Table 2**. Regarding axial-SpA, relevant differences can be highlighted among gender (Rudwaleit et al., 2009; Conigliaro et al., 2016). First of all, a male predominance (3:1) has been found in the radiographic form while an equal sex distribution is registered in the nr-axSpA (1,03:1) (van der Linden et al., 1984; Rudwaleit et al., 2009). In this condition, disease burden appears to be similar for both sexes (Kennedy et al., 1993; Ward et al., 2009; Baumberger, 2017; Rusman et al., 2020). However, differences emerged concerning clinical and disease activity distribution. Indeed, men seem to display a higher radiological damage and a higher radiological progression (as highlighted with Bath Ankylosing Spondylitis Radiology Index and modified Stoke Ankylosing Spondylitis Spine), showing more marked radiographic spinal changes and worse hip involvement than women (Kennedy et al., 1993; Rusman et al., 2018). Confirming this, Lee et al. (2007) demonstrated a more severe radiographic damage among men who showed higher median BASRI−spine scores both in unadjusted and adjusted analyses for age and disease duration. On the other hand, the signs of peripheral involvements such as dactylitis, enthesitis, or swollen joint count appear to be significantly more prevalent in women (Kennedy et al., 1993; Rusman et al., 2020). Extra-articular manifestations also appear with a higher rate in female patients (Landi et al., 2016). Concerning acute anterior uveitis, a recent review reported a slightly higher predominance in women (M/F = 28/33%) (Zeboulon et al., 2008), as in patients affected by IBD (Stolwijk et al., 2015; Landi et al., 2016) or PsO (M/F = 25/33%) (Stolwijk et al., 2015; Landi et al., 2016) studies report a higher predominance in female patients. Regarding comorbidities such as cardiovascular diseases (CVD) and osteoporosis (Op), non-conclusive data are available on gender differences; evidences on CVD are scarce while a study on young males reported that 51% had a low bone mineral density (BMD) and 15% had Op (Zarco et al., 2015), another study concluded that male patients have a 4 times greater risk for low BMD compared with females (van der Weijden et al., 2012). These latter data are in contrast with those regarding the general population, where Op is typically prevalent in post-menopausal women (van der Weijden et al., 2012). Furthermore, CRP levels appear to be significantly higher, at baseline levels, in male patients compared with females, while no conclusive sex differences have been found for ESR (Jordan and Cooper, 2002; van der Weijden et al., 2011). Finally, women show higher disease activity, mostly in terms of subjective measures, reporting worse baseline BASDAI scores (in particular total and nocturnal back pain, duration of morning stiffness, and fatigue) as widely described in many studies (van der Horst-Bruinsma et al., 2013; Rubio Vargas et al., 2016; Kilic et al., 2017) while just a few studies described differences for baseline scores, being concordantly lower in

TABLE 1 | Genes whose variability is involved in Spondyloarthritis.

Genes	Chromosome	Function	Evidence of association	References
HLA-B27	6p21.3	Antigen presentation	AS, axial-PsA, enthesitis, dactylitis, uveitis	Ito et al., 2001; Reveille, 2011; Tyagi et al., 2012; Vecellio et al., 2019; Chimenti et al., 2020a
HLA-B27:05:02	6p21.3	Antigen presentation	AS, symmetric sacroiliitis, enthesitis, dactylitis, uveitis	Ito et al., 2001; Reveille, 2011; Vecellio et al., 2019; Chimenti et al., 2020a
HLA-B38	6p21.31	Antigen presentation	Peripheral PsA	Clarke and Vyse, 2009
HLA-B39	6p21.31	Antigen presentation	Peripheral PsA	Clarke and Vyse, 2009
HLA-B60 (B*4001)	6p21.3	Antigen presentation	AS, PsA	Wei et al., 2015
HLA-B15	6p21.31	Antigen presentation	AS, CD	Clarke and Vyse, 2009
HLA-C06:02	6p21.3	Antigen presentation	PsA, PsO	Chimenti et al., 2018
HLA-C07:01:01	6p21.3	Antigen presentation	Axial PsA, sacroiliitis, dactylitis	Reveille, 2011
MICA	6p21.33	NK and T cells activation	PsA, PsO	Wang and Zhou, 2015
IL-1R2	2q11-12	Interferon signaling	AS, uveitis, UC	O'Rielly and Rahman, 2013
IL-1 complex (IL-1A, IL-1B, IL1-RN)	2q14.1	Th1 response	AS, peripheral PsA	Clarke and Vyse, 2009
IL-12B	5q33.3	Th17 signaling	AS, PsA, PsO	Burden et al., 1998; Vecellio et al., 2019
IL-17A	6p12.2	Th17 signaling	AS, PsA	Burden et al., 1998; Vecellio et al., 2019
IL-17F	6p12.2	Th17 signaling	AS, PsA	Burden et al., 1998; Vecellio et al., 2019
IL-23R	1p31.3	Th17 signaling	AS, peripheral and erosive PsA, PsO, uveitis, CD, UC	Reveille et al., 2010; Gravellese and Schett, 2018
IL-23A	12q13.3	Th17 signaling	PsA, PsO, uveitis	Burden et al., 1998
IL-7R		Antigen presentation	AS, UC	Tsui et al., 2010
TNF-A	6q21.3	NFkB signaling	AS, PsA	Tsui et al., 2010
TBKBP1	17q21.32	NFkB activation and signaling	AS	Vecellio et al., 2019
TNFRSF1A-LTBR	12p13	NFkB activation and signaling	AS	Vecellio et al., 2019
ERAP1	5q15	Antigen presentation	AS, PsA, PsO, uveitis, CD, UC	Reveille et al., 2010
TBX21	17q21.32	Th1 cell expression	AS, CD	Reveille et al., 2010
FBXL19	16p11.2	NFkB activation and signaling	PsA, PsO	Vecellio et al., 2019
KIF21B	1q31	Unknown	AS, uveitis, CD, UC	Vecellio et al., 2019
CARD9	9q34.3	NFkB activation and signaling	AS, CD, UC	Gravellese and Schett, 2018
PTGER4	5p13.1	Th17 signaling	AS, CD	Vecellio et al., 2019
IRAK1	Xq28	NFkB signaling	PsA	Chimenti et al., 2019b
ANTXR2	4q21	Bone remodeling	AS	Vecellio et al., 2019
STAT3	17q21	Th17 signaling	AS, CD, UC	Berlinberg and Kuhn, 2020
CYP2D6	22q13.1	unknown	AS, CD, UC	Berlinberg and Kuhn, 2020
ANKH	5p15.2	Bone remodeling	AS	Queiro et al., 2013
TLR4	9q32-33	NFkB signaling	AS, PsA, uSpA	O'Rielly and Rahman, 2013
TLR2	4q31.3	NFkB signaling	AS, PsA, uSpA	O'Rielly and Rahman, 2013
TLR9	3p21.2	NFkB signaling	AS, PsA	O'Rielly and Rahman, 2013
TYK2	19P13.2	IFN and NFkB activation and signaling	PsA, PsO	Vecellio et al., 2019
KIR3DL1	19q13.4	NK and T cells activation	AS, PsO, PsA	Chimenti et al., 2019b
ADAM33	20p13	Cell-cell and cell-matrix interactions	PsA, PsO	Chimenti et al., 2018
TNIP1	5q32- q33.1	NFkB activation and signaling	PsA, PsO	Vecellio et al., 2019
RUNX3	1p36	Antigen presentation	AS, PsO	Vecellio et al., 2019
EOMES	3p24.1	Unknown	AS	Tsui et al., 2010
ZMIZ1	10q22.3	Unknown	AS, PsO, CD, UC	Tsui et al., 2010
ANO6	12q12	Bone remodeling	AS	Vecellio et al., 2019
DMP1	4q22.1	Bone remodeling	AS	Mori et al., 2000
HAPLN1	5q14	Bone remodeling	AS	Vecellio et al., 2019

HLA, human leukocyte antigen; NK, Natural Killer cells; AS, Ankylosing Spondylitis; PsA, psoriatic arthritis; PsO, psoriasis; UC, ulcerative colitis; CD, Crohn disease.

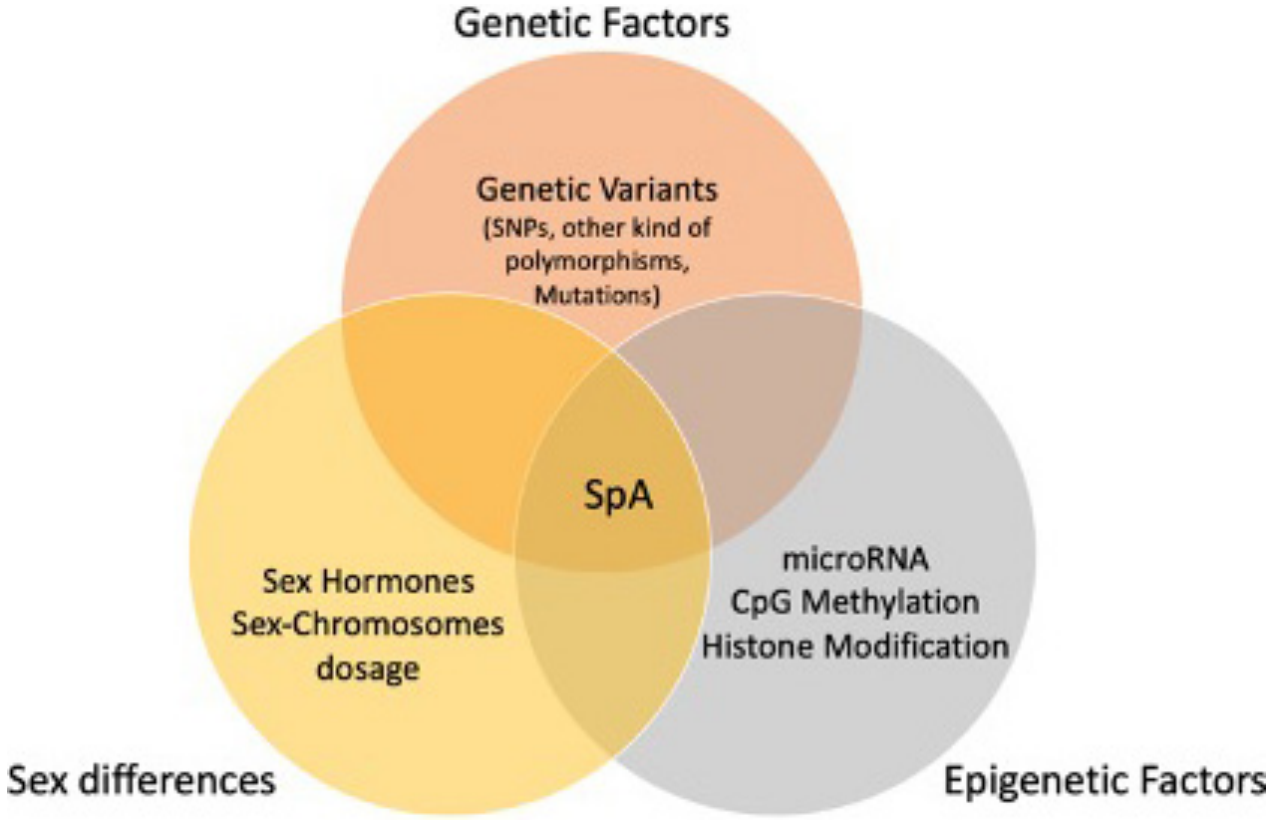

FIGURE 1 | Interaction among genetic and epigenetic factors and sex in Spondyloarthritis (SpA).

females, of BASFI (van der Horst-Bruinsma et al., 2013; Rubio Vargas et al., 2016) and ASDAS-CRP (Webers et al., 2016). At the same time, female patients display less improvement in BASDAI, BASFI, and ASDAS-CRP scores after treatment (van der Horst-Bruinsma et al., 2013; Neuenschwander et al., 2020). In addition, women report worse quality of life when the Ankylosing Spondylitis Quality of Life questionnaire (ASQoL) and the Assessment of SpondyloArthritis international Society Health Index (ASA-HI) were used while no gender differences were found using the EuroQoL and the SF-36 Health Survey (Kennedy et al., 1993; Roussou and Sultana, 2011; Tournadre et al., 2013; Lubrano et al., 2017; Ibáñez Vodnizza et al., 2020; Neuenschwander et al., 2020). In summary, men with ax-SpA present higher objective markers of inflammation associated with radiological progression, although severe ankylosis also occurs in females. Nonetheless, women generally have higher subjective indicators of disease activity and, in addition, more peripheral involvement and extra-articular manifestations. As for comorbidities, osteoporosis has an unexpectedly high prevalence in young male patients. Concerning sex related differences in Entheropatic arthritis (EA), few data are available in literature and a small number of studies explored this topic, reporting axial manifestations as more frequently present in male patients than in females and women appear to be more likely to develop both type 1 and type 2 arthropathy (Dougados et al., 1991; Orchard et al., 1998; Turkcapar et al., 2006; Rodríguez-Reyna et al., 2009; Yüksel et al., 2011; Peluso et al., 2013; Picchianti-Diamanti et al., 2020). On the contrary, several findings suggest the presence of sex-related differences in PsA patients. It is reported that male patients have a high prevalence of axial disease and a high frequency of HLA-B27 antigens positivity, while women tend to have a more erosive disease, higher number of swollen joint count and a higher prevalence of disability (Gladman et al., 1992; Queiro et al., 2001; Wallenius et al., 2009; Queiro et al., 2013). Eder et al. (2013) demonstrated, on a large cohort of PsA patients, that axial involvement is more frequent in men as well as radiographic damage in both axial and peripheral joints (assessed with the modified Stokes Ankylosing Spondylitis Spine Score and the modified Steinbrocker score), while women reported with a higher frequency functional limitation and impaired quality of life (reporting higher scores in HAQ and BASFI and lower in the SF-36-PCS). In addition, differences were found in the pattern of arthritis at onset: women presented more frequently with the polyarticular subtype while men presented with olygoarthritis. On the other hand, the authors reported no significant differences in active and swollen joint counts, the presence of dactylitis, inflammatory spinal pain, and PASI score even if psoriatic nail lesions were more frequently found in men (Queiro et al., 2001).

TABLE 2 | Clinical differences related to gender in Ankylosing Spondylitis.

Sex related differences in AS	
Radiological progression Radiological damage	↑In men
Axial involvement	↑In men
Peripheral involvement	↑ In women
Swollen joint count	↑In women
Osteoporosis	↑ In men
Disease activity scores	
BASDAI, baseline	↑ In women
BASDAI, improvement	
BASFI, improvement	↑In men
AS DAS, improvement	
ASQoL AS AS-HI	↑In men
EuroQoL	No differences

AS, Ankylosing Spondylitis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; ASDAS, Ankylosing Spondylitis Disease Activity Score; ASQoL, Assessment of Spondyloarthritis Quality of life; ASA-HI, Assessment of Spondyloarthritis international Society Health Index; EQ-5D, EuroQoL-5D; SF-36 HS, SF-36 Health Status.

GENDER AND GENETIC POLYMORPHISMS

Accumulating evidence suggests that gender differences in severity of SpA and treatment response could be due to genetic, immunological and hormonal factors (Brown et al., 1997). Female karyotype includes two X chromosomes, one of which is randomly silenced during embryogenesis. However, about 15% of the genes escape inactivation, leading to overexpression of some X-linked genes in females. The X chromosome encodes several immune-related genes, such as TLR7, TLR8, FOXP3, and IL-2 receptor gamma, whose overexpression may influence the immune response in a sex-dependent manner. For instance, male carriers of TLR9 *rs187084* variant allele have an increased production of IFN-γ and TNFα, and women with *rs5743836∗C* allele have a higher risk of developing SpA (Oliveira-Toré et al., 2019). Moreover, in contrast to the Y chromosome, the X chromosome is highly enriched in miRNAs, and some of them are reported to play a role in immunity or autoimmunity (Ortona et al., 2016). Different variants of the ANKH gene, which encode for a protein involved in osteogenesis and structural damage in axSpA, are expressed in men and women with AS. In women, AS seemed to be associated with genetic markers at the 5′ end of the ANKH gene, whereas in men with genetic markers at the 3′ end of this gene (Tsui et al., 2005). In this latter region, haplotype analysis has shown several SNPs associated with AS, supporting the greater predisposition of the male sex to develop AS. Moreover, different haplotypes combinations were significantly associated with AS in men (including *rs26307* and *rs27356* SNPs) and in women (including *rs28006* and *rs25957)* SNPs) (Ortona et al., 2016). Furthermore, one specific haplotype in TNAP (tissue-non-specific alkaline phosphatase) gene, which interplays with the ANKH gene in ossification, was associated with AS in men but not in women (Tsui et al., 2005). A recent study on AS patients identified an upregulation of IL-17RA gene expression in men compared with women, as well as greater levels of TNFα, IL-18, IL-17A, and peripheral T-helper 17 cells. On the contrary, IL-6 levels were higher in women than in men (Tsui et al., 2007; Gracey et al., 2016). Sex differences in the expression of these genes could explain the higher prevalence and radiographic progression of AS in men compared with women and might be also influenced by sex hormones (Huang et al., 2012; Cutolo and Straub, 2020). Although few studies have investigated sex differences separately, several analyses have assessed sex as a possible predictor in relation to treatment efficacy and drug survival. Recent studies have shown that women tended to discontinue TNFi earlier than men both in axSpA and in PsA (Lubrano et al., 2017; Rusman et al., 2018). This might be partly explained by the lower prevalence of HLA-B27, a longer disease duration, and a greater fat mass in women than men. These factors are associated with a lower TNFi treatment response (Roussou and Sultana, 2011). Moreover, data from registries, such as the DANBIO and BSRBR, suggested that women also suffered from more side effects that have led to the discontinuation of the drugs (Saad et al., 2009; Glintborg et al., 2011). Polymorphisms within the TNF promoter region have been identified to influence clinical efficacy of etanercept in RA, AS, and PsA patients (Pierik et al., 2004; Morales-Lara et al., 2012). TNFR1A variant *rs767455/G36A* in PsA patients has been associated with a better EULAR response at 3 months to infliximab and similar results were obtained in CD patients (Pierik et al., 2004; Morales-Lara et al., 2012). On the contrary, the TNFAIP3 *rs2230926* and *rs610604 variant alleles* seem to be correlated with better TNFi response in PsO patients, while *rs6920220* and *rs610604* are associated with an improvement in the quality of life in PsA patients receiving TNFi (Tejasvi et al., 2012; Ovejero-Benito et al., 2019). One study suggested that TNFi failure can occur in AS patient carriers of variants within macrophage migration inhibitory factor (MIF) gene (*rs755622*), IL-18R gene (rs917997), IL10 (rs1800896), and TNFRSFB1 (rs4355801) (Schiotis et al., 2014). Another study has reported that female gender, elevated basal BASFI index, and being a CHUK gene *rs11591741-GC* genotype carrier are predictors of long-term non-response to TNFi in SpA patients (Polo et al., 2019). Moreover, a lower level of clinical response measured by ASDAS and BASDAI index was observed in females (Rusman et al., 2018). Different miRNAs, such as miR-146a, miR-155, miR-625-3p, and miR-29a were linked to BASDAI, reflecting disease activity in AS with spinal involvement. In addition, miR-146a-5p, miR-125a-5p, and miR-22-3p expression were correlated with the plasma cytokine levels (TNFα, IL-1β, and IL-5), CRP and ESR (Perez-Sanchez et al., 2018). Downregulation of miR-199a-5p has been associated with increased TNFα, IL-17, and IL-23 in AS, suggesting that miRNAs could distinguish SpA phenotypes responsive to inhibitors of TNFα, IL-17, or IL-23 (Berlinberg and Kuhn, 2020). Proinflammatory cytokines, such as IL-17, are decisively involved in all clinical manifestations of SpA, and different concentrations of the cytokines are observed between men and women (Ortona et al., 2016). The use of secukinumab is widely diffused in our clinical practice for SpA management (Chimenti et al., 2019b). Recently the efficacy of secukinumab has been evaluated in patients with SpA, both in PsA and in AS patients. At the multivariate analysis, there was

no gender influence concerning treatment efficacy. However, gender difference was observed according to drug-survival, particularly in the AS population: males had a higher persistence rate than females; this was not demonstrated in PsA patients (Chimenti et al., 2020a).

GENDER AND HORMONES IN AXIAL SpA

Since last century, the role of sex hormones has been hypothesized for the development of immune mediated diseases. Generally, estrogens have roles in both enhancing and inhibiting immune system activity, whereas androgens and progesterone exert suppressive effects (Cutolo and Straub, 2020). It has been reported that sex steroids can influence epigenetic modulation and expression of microRNAs that interfere with immune responses in autoimmune rheumatic diseases (Gracey et al., 2016). However, their role in SpA has been only partially evaluated. Estrogen has mainly anti-inflammatory effects, by inhibiting TNFα production, but contradicting results were presented (Rusman et al., 2018). This concept was previously supported by an old study demonstrating an improvement of arthritis in AS patients treated with oral estrogen and a worsening of disease in patients with low levels of estrogens (Gracey et al., 2016). Conversely, a more recent report showed neither difference in onset nor severity of AS female patients despite differences in serum estrogen concentration (Huang et al., 2012). For instance, estrogen modulates immune-related processes such as T cell differentiation and cytokine production and animal model studies have suggested that estrogen can inhibit Th17 differentiation from naïve T cells and in human 17β-estradiol (E2) levels are lower in patients with active AS than in those with inactive AS (Tyagi et al., 2012). Experimental models, such as E2-treated mice, showed a relationship among cytokines production and sex hormones: a decreased expression of TNF in the joint tissue was demonstrated supporting the inhibition of TNF production by estrogens (Ito et al., 2001). Estrogen-deficient female rats are also reported to have higher serum TNF than estrogen-supplemented animals and high and medium doses of estrogen increased the production of IL-4, IL-10, and TGF-β, whereas estrogen treatment reduced the production of inflammatory cytokines such as IFN-γ, IL-17, and IL-6. The relevant role of IL-17 in SpA pathogenesis and in particular in axial manifestation is well noted in clinical practice. A link between sex hormones and Th17 activation was demonstrated: estrogen inhibits Th17 cell. In ovariectomized DBA/1 mice with collagen-induced arthritis (CIA), E2 treatment reduces the severity of arthritis and results in fewer Th17 cells in the joints compared with controls (Andersson et al., 2015). Jeong et al. (2017) recently aimed to evaluate the effect of estrogen on the disease activity of SpA in a mouse model of SpA. The E2-treated group had significantly suppressed arthritis compared with both the ovariectomized and the sham groups. Furthermore, the expression of Dkk1 was significantly increased in E2-treated mice compared with the ovariectomized and sham groups and the expression of Wnt inhibitors was inhibited by estrogen (Jeong et al., 2017). Considering that blockade of Wnt inhibitors induced the fusion of sacroiliac joints and increased bone formation, estrogens might act in controlling Wnt pathway and used as a therapeutic target (Uderhardt et al., 2010). Unfortunately, in SpA, neither androgens and androgen receptors nor estrogen receptors polymorphisms were evaluated. Shorter CAG repeats of the androgen receptor gene presenting high levels of transactivation activity were demonstrated in a Japanese cohort of AS patients, supporting a role in male AS development (Mori et al., 2000). The role of microbiota has been previously described in SpA and its link with HLA B27 has been reported (Chimenti et al., 2018). Interestingly, women and men tend to have different gut microbiota, suggesting that sex hormones might have an effect on microflora, even if the mechanism still remains unclear (Cutolo and Straub, 2020). Sex-dependent differences in gut microbiota may lead to genetic or epigenetic changes in local gastrointestinal inflammation, systemic immunity, and susceptibility to a range of rheumatic diseases (Rizzetto et al., 2018; Cutolo and Straub, 2020). However, no progress has been made in decoding the unequivocal role of sex steroids in gut microbiota-related effects on SpA patients. Moreover, several studies in humans have highlighted that sex- related differences in the microbiota can occur across the lifespan of an individual, making results questionable (Ding and Schloss, 2014; Jaggar et al., 2019). Other relationships between gender and hormones are associated with levels of circulating adipokines. It is well known that obesity, body composition, and adipokines have an influence on differences in disease activity, progression, and response to treatment, between men and women with SpA (Rusman et al., 2018). Body fat content is higher in women than men and obesity is related to worse disease activity scores in SpA. In details, leptin, which is usually found at higher levels in overweight women and higher in women than men, was associated to spinal radiographic progression. Moreover, women also have higher circulating adiponectin levels, which is an insulin-sensitizing hormone. Positive correlations with inflammatory biomarkers, such as CRP and TNF, have been observed for leptin (Graßmann et al., 2017). Being a woman and being obese, mainly because of the body fat content, are related to a worse response to TNF-α blockers (Ibáñez Vodnizza and van der Horst-Bruinsma, 2020).

CONCLUSION

Based on genetic predisposition related to HLA-B27, SpA was generally considered as a male disease. As a matter of fact, in 1949, the male:female ratio was estimated to be 10:1 for AS patients; yet recent data have established a new 2–3:1 ratio (Kennedy et al., 1993). This epidemiological difference from past to present is due to several considerations: (1) the improvement and the development of new classification criteria aiming at the inclusion of early disease phases as well as the definition of non-radiographic SpA, (2) the better knowledge of clinical manifestations of SpA in the rheumatological field but also in other disciplines, (3) the improvement of radiological techniques for diagnosis and management. However, the highest prevalence of some clinical manifestations in females with respect to male

suggested differences in the pathogenesis of SpA. The relevance of genetic and epigenetic phenomena in SpA pathogenesis was highlightened in this review, as well as the role of sex hormones, supporting the need of a redefinition of clinical and therapeutic targets. bDMARDs dramatically improved the management and quality of life of patients affected by SpA. With the availability of TNFi, research interests were oriented in order to identify potential clinical variables and biomarkers able to define the best responder to these drugs (Rubio Vargas et al., 2016). Different drugs with different mechanisms of action such as Il-17 inhibitors or Jak-inhibitors may have the same effect and efficacy in both men and women.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Effect of *ACTN3* and *VDR* Polymorphisms on Skeletal Muscle Performance in Axial Spondyloarthropathies

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Background: Spondyloarthritis (SpA) are the most common group of chronic inflammatory rheumatic diseases affecting about 1.5% of the adult Caucasian population. Low back pain is the most common symptom. The aetiopathogenesis of SpA is multifactorial, with well-known genetic and environmental contributions. Furthermore, muscle properties might also be involved in the pathophysiological process and these could be modulated by the genetic background. *Alpha-actinin-3* (*ACTN3*) and *Vitamin D receptor* (*VDR*) genes are well-known genes related with muscle performance. Our aim was to analyze four SNPs of these genes and to evaluate their influence in axial SpA (axSpA) susceptibility, phenotype and muscle properties.

Methods: We performed a pilot study based on case-control approach involving 56 participants: 28 axSpA patients and 28 healthy controls matched by age, gender and levels of physical activity. Clinical, epidemiological and muscle characterization data—muscle physical properties (stiffness, tone, and elasticity), strength, mass, and performance, were collected. Two different muscles were considered for analysis, the Multifidus and Gastrocnemius. Four SNPs of *ACTN3* (rs1815739) and *VDR* (rs2228570, rs731236, and rs7975232), were selected, analyzed and correlated with clinical, epidemiological and muscle characterization data.

Results: In total, 51 individuals (27 axSpA patients and 24 matched controls) were eligible for further genetic analysis, 66.7% being male and with a mean age of 36 years. Muscle physical properties, muscle strength and muscle mass were similar in both groups; however, axSpA patients showed a decrease in muscle performance. None of the studied SNPs were associated with disease susceptibility/phenotype, muscle

physical properties, muscle strength or muscle mass. However, *ACTN3* rs1815739 and *VDR* rs2228570 were shown to be associated with muscle performance.

Conclusion: Our results suggest an association between *ACTN3* and *VDR* polymorphisms and muscle performance in axSpA.

Keywords: spondyloarthropathies, muscle, muscle performance, *ACTN3*, *VDR*

INTRODUCTION

Spondyloarthritis (SpA) is one of the most common groups of chronic inflammatory rheumatic diseases, affecting about 1.5% of the Caucasian adult population (Apostolakos et al., 2014; Costantino et al., 2018). SpA is typically characterized by inflammation of the spine and sacroiliac joints accompanied by pain, stiffness and in late stages, by reduced mobility. The disease may also affect the peripheral joints, periarticular structures (enthesitis, dactylitis), and extra-articular systems (acute anterior uveitis, psoriasis, and inflammatory bowel diseases) (Pimentel-Santos et al., 2012; Stolwijk et al., 2016; Costantino et al., 2018).

Entheses play a critical role in SpA ethiopathogeny and in the normal function of the musculoskeletal system. This structure not only allows the transmission of muscle contractile forces into the skeletal attachment site, but also participates in the dissipation of force from tendon into bone (Apostolakos et al., 2014).

The entheses have a unique immune microenvironment that can be stimulated through the combination of several factors (such as genetic predisposition, mechanical stress in the joints, and microbiota immune activation), leading to prostaglandin E2 release and IL-23-IL17 axis activation (Schett et al., 2017). This phenomenon leads to an influx of innate immune cells, promoting chronic inflammation in the entheses, followed by mesenchymal tissues responses and osteogenesis (Benjamin and Mcgonagle, 2001; Schett et al., 2017). In spite of all this information the underlying pathophysiological mechanisms for axial spondyloarthritis (axSpA) susceptibility remain unknown (Asquith et al., 2014; Gill et al., 2015; Van Mechelen and Lories, 2016).

The strong genetic component associated with the presence of HLA-B27 has been validated in different populations (Rosenbaum and Davey, 2011; Rudwaleit et al., 2011; Pimentel-Santos et al., 2013; Costantino et al., 2018). On the other hand, several loci and haplotypes relevant to disease susceptibility were identified through "Genome Wide Association Studies" (GWAS) (Sieper et al., 2009; International Genetics of Ankylosing Spondylitis et al., 2013; Osgood and Knight, 2018). Finally, expression studies also allowed the identification of genes related to inflammation, cartilage, bone and muscle metabolism (Pimentel-Santos et al., 2011). However, these studies can only explain a small portion of disease genetic predisposition and phenotype (Costantino et al., 2018).

In recent years, a link between biomechanical stress and axSpA susceptibility and severity has been raised. In axSpA patients and in animal models of the disease, the occurrence of microtrauma related to physical activities seems to induce inflammation and osteoproliferation within the spine (Mcgonagle et al., 2001; Jacques et al., 2014; Ramiro et al., 2015). Another study demonstrated an increased stiffness of the axial muscles in patients with axSpA (Andonian et al., 2015). Conceptually, microtrauma induced by daily activities (or by the muscle itself), may play an essential role in disease susceptibility/severity. Moreover, many studies support the notion that the gut microbiota plays an important role in axSpA through alterations of intestinal permeability, stimulation of immune responses, and molecular mimicry (Asquith et al., 2014; Gill et al., 2015). Thus, it is reasonable to speculate that general axSpA susceptibility and progression may result from a combination of host genetics, microbiota and micro-trauma. Still, how these factors interact with each other remains largely unknown (Benjamin and Mcgonagle, 2001; Simone et al., 2018). Further insights into genetic factors influencing muscle properties in axSpA patients will help unveil these complex interactions.

Human Alpha- actinin 3 (*ACTN3*) and *Vitamin D receptor* (*VDR*) genes are associated with physical fitness and/or performance and muscular efficiency (Ceglia, 2009; Rejnmark, 2011; Pickering and Kiely, 2017). ACTN3 protein is a fast-twitch-specific isoform uniquely expressed in type-II muscle fibers, having an important role in the generation of contractile forces at high speeds (Pickering and Kiely, 2017). The *VDR* gene exhibits different PCR-RFLP single-nucleotide polymorphisms (SNPs), being *Bsm*I, *Fok*I, *Apa*I, and *Taq*I the most studied, which are thought to be associated with higher VDR activity (Ceglia, 2009; Hamilton, 2010; Rejnmark, 2011) or with protein function, also changing cellular responses to therapies (Hunt et al., 2009).

Thus, the aim of the present study is to characterize the association between *ACTN3* and *VDR* SNPs with axSpA susceptibility or disease characteristics, namely disease activity, functional, and metrological assessments, and in particular, the association with muscle physical properties, muscle strength, mass, and performance.

MATERIALS AND METHODS

Populations Characterization

This pilot study enrolled 56 Caucasian individuals, of which 28 unrelated patients. All patients were previously diagnosed with axSpA and fulfill the Assessment of Spondyloarthritis international Society (ASAS) axSpA classification criteria (Rudwaleit et al., 2009) and the 28 healthy controls were matched by gender, age and level of physical activity. Cases were recruited from a Rheumatology outpatient clinic.

Eligible axSpA participants were recruited according to the following inclusion criteria: (1) axSpA, meeting the ASAS classification criteria, and symptom duration under 10 years;

(2) Age between 18 and 50 years old; (3) Non-steroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids (equivalent to $\leq$ 10 mg of prednisone), in stable doses $\geq$ 4 weeks before screening was allowed; (4) Ability to provide informed consent. The exclusion criteria were: (1) Body mass index (BMI) $\geq$ 35 kg/m^2, (2) Previous exposure to synthetic disease-modifying anti-rheumatic drugs (DMARDs) or biological disease-modifying anti-rheumatic drugs (bDMARDs); (3) Current pregnancy or breastfeeding; (4) Infection that required hospital stay, intravenous antibiotic treatment in the previous 30 days or oral antibiotic treatment within 14 days before screening; (5) Neoplastic disease (except for successfully treated squamous or basal cell carcinoma); (6) Any non-treated conditions (e.g., diabetes mellitus, ischemic heart disease); (7) Intra or peri-articular and tendon sheaths injections within 28 days prior to screening; (8) Rachis ankylosis (with syndesmophytes in all levels from D12 to S1, on lateral spine radiograph).

The current study was submitted and approved by both ethical committees of NOVA Medical School-Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Portugal and Centro Hospitalar Lisboa Ocidental, Hospital de Egas Moniz, EPE, Lisboa, Portugal. The study was conducted in accordance with International Conference on Harmonization good clinical practices and the Declaration of Helsinki. Voluntary written informed consent was obtained from all subjects before starting study procedures.

Clinical Protocol

All 56 participants were submitted to a standardized protocol for extensive epidemiologic and muscle characterization. Physical activity was assessed according to the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003). The axSpA patients were also clinically evaluated (including Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), disease duration, therapy). Additionally, Bath Ankylosing Spondylitis Metrology Index (BASMI) and myofascial characterization were performed by a single investigator (FPS), using different approaches:

(1) Muscle physical properties (stiffness, tone, and elasticity), assessed by a non-invasive device, the MyotonPro®, focusing on axial/torso (multifidus and longissimus dorsi) and on peripheral/lower limbs (gastrocnemius) muscles. The participants were in a prone position and measurements were taken after a 10 min rest.
(2) Muscle strength, measured using the Lafayette Manual Muscle Testing System for torso extension and lower limb extension; global strength evaluated through 5 times Sit-to-Stand test (STS5) (Cruz-Jentoft et al., 2019).
(3) Muscle Mass, assessed by bioimpedance, using direct segmental 8-point multifrequency bioelectric impedance analysis (InBody770, InBody Co., Ltd., Seoul, South Korea).
(4) Muscle performance, assessed by 60 s Sit-to-Stand test (ST60) and Gait speed (Cruz-Jentoft et al., 2019), using a 3D full-body kinematic model (Kinetikos® Coimbra, Portugal).

Due to the absence of already established reference values for some variables related to physical muscular characteristics (stiffness, decrement/inverse of elasticity, tone), we have defined categories (low, intermediate and high) for the whole population group (for details see **Supplementary Material**), without taking into account the differences between genders because of the reduced sample size. Clinical variables were categorized according to the following cut offs in "not active/active disease" (BASDAI $<$ 4, BASDAI $\geq$ 4), "Low/high functional ability" (BASFI $<$ 4, BASFI $\geq$ 4) and low/high reduction in spinal range of motion (BASMI $<$ 3, BASMI $\geq$ 3).

Genotyping

Genomic DNA from all participants was isolated from peripheral blood samples using PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's protocol instructions for blood lysates.

All samples were screened and genotyped for *ACTN3* and *VDR* genes polymorphisms: R557X (rs1815739) and ApaI (rs7975232), FokI (rs2228570), TaqI (rs731236), respectively, that have previously been associated with muscle performance in both men and women.

All SNPs studied (**Table 1**) were previously selected considering the Minor Allele Frequency (MAF) above or equal to 5% for European Caucasian population (HapMap CEU). SNPs under study belong to several parts of the gene: regulatory region, coding region or non-coding region.

The genotyping analysis was performed by quantitative polymerase chain reaction (qPCR) carried out on a 96-well QS5 Real-Time PCR (RT-PCR) System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, United States), following the manufacturer's instructions with the use of the commercially available TaqMan® SNP Genotyping Assays (Applied Biosystems) detailed in **Table 1**. To confirm genotyping and ensure accurate results, inconclusive samples were reanalyzed, and genotyping was repeated in 10–15% of randomly chosen samples, with 100% concordance.

Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences for Windows 22.0 version (SPSS, Inc.). All genotypes were coded accordingly in order to proceed with the statistical analysis. The analysis of Hardy-Weinberg frequencies for all alleles present in patients' populations was carried out using exact probability tests available using the SNPStat software[1] (Sole et al., 2006).

Participants' demographic, clinical, and biomechanical characteristics were described and compared between healthy individual and patients with axSpA, using Chi-Square (χ^2) for discrete data and Wilcoxon-Mann-Whitney-test for non-parametric continuous data.

Since this is not a conclusive final study, but an exploratory one on the role of selected polymorphisms in the *ACTN3* and *VDR* genes, and the data to be obtained should be looked at as

[1]http://bioinfo.iconcologia.net/SNPstats

TABLE 1 | Identification of all genetic variants included in the study.

Gene	dsSNP ID (rs)	SNP	Variant type	A.A residue	Context sequence [VIC/FAM]
ACTN3	rs1815739	**R577X**	Non-sense	R/STOP	CAAGGCAACACTGCCCGAGGCTGAC**[T/C]**GAGAGCGAGGTGCCATCATGGGCAT
VDR	rs2228570	***Fok*I**	Missense	K/R	GGAAGTGCTGGCCGCCATTGCCTCC**[A/G]**TCCCTGTAAGAACAGCAAGCAGGCC
VDR	rs731236	***Taq*I**	Silent	I/I	TGGACAGGCGGTCCTGGATGGCCTC**[A/G]**ATCAGCGCGGCGTCCTGCACCCCAG
VDR	rs7975232	***Apa*I**	Intron	–	AAGGCACAGGAGCTCTCAGCTGGGC**[A/C]**CCTCACTGCTCAATCCCACCACCCC

The bold values refers to the nucleotide change and the correspondence with the correspondent fluorocrome.

proof of concept, the Bonferroni adjustment was deemed as not necessary as it is too conservative.

Logistic regression was used to estimate the risk of each muscle property modification when associated with each genotype: risk estimates were calculated under the codominant model and expressed as crude odds ratios (OR) and corresponding 95% confidence intervals (CI). Association between SNPs and the quantitative variables BASDAI, BASFI, BASMI, stiffness, decrement/inverse of elasticity, tone and strength were tested by linear regression. Results were considered significant when the corresponding two-tailed p-values were < 0.05. The most common homozygous genotype was considered the reference classes for such calculations.

RESULTS

Epidemiological and Clinical Characterization

Even though the study started with 56 enrolled participants, in equal number of controls and patients, due to missing data in some physical measures and in individuals' polymorphisms identification, the total sample population ended up comprising 51 individuals (27 axSpA patients and 24 controls). The axSpA patients, 66.7% male with a mean age of 36 ± 7 years old and a mean of 7.0 ± 0.9 years of disease duration. From the total of patients, 80.8% were HLA-B27 positive, 18.5% had active disease (BASDAI ≥ 4) and 14.8% presented high functional impairment (BASFI > 4). The majority of patients did not exhibit reduction in mobility (88.9% had BASMI < 3). There was no difference between patients and controls in terms of age, gender, level of physical activity and body mass.

Baseline characteristics are shown in detail, in **Table 2**, where all data comparison between patients and controls can be found.

Clinical Data—Muscle Characteristics Analysis

We recorded and analyzed the results obtained from lumbar paravertebral muscle (Multifidus muscle) physical properties, namely: Muscle tonus (M.F); Muscle decrement, i.e., the inverse of elasticity (M.D) and Muscle stiffness (M.S). The regression analysis was performed individually for each characteristic (crude analysis) (**Table 3**).

No significant differences in muscle physical properties, muscle strength (ST5) and muscle mass were identified between patients and controls. However, it seems that patients tend to express higher levels of stiffness in multifidus muscle (trunk) (**Table 3**). Muscle performance, measured by ST60 and gait speed, was significantly reduced in patients, compared to controls ($p < 0.05$).

Genotyping and Individual Susceptibility Analysis

After genotyping analysis, the SNPs distribution was performed and their genetic contribution to disease susceptibility was evaluated (**Table 4**). To perform the correlations, we deemed

TABLE 2 | Study population characterization. Cases group (n = 27) and Healthy control group (n = 24).

Characteristics	Cases, *n* (%)	Mean ± SD	Controls, *n* (%)	Mean ± SD	*p*-value*
Age					
0–29	3 (11.1)		4 (16.7)		
30–39	14 (51.9)	36.8 ± 7.1	10 (41.7)	36.8 ± 7.7	
40–50	10 (37.0)		10 (41.7)		0.728
Gender					
Male	18 (66.7)	–	15 (62.5)	–	
Female	9 (33.3)		9 (37.5)		0.756
Physical Activity level					
Low	22 (81.5)	–	21 (87.5)	–	
High	5 (18.5)		3 (12.5)		0.555
HLA-B27					
Positive	21 (80.8)				
Negative	5 (19.2)	–	–	–	
Not available	1				–
Body height (cm)	–	170.4 ± 7.4	–	171.1 ± 8.8	
Body weight (kg)	–	75.9 ± 12.7	–	71.8 ± 12.9	
BMI					
Low weight	1 (4.2)		1 (4.3)		
Normoponderal	11 (45.8)		13 (56.5)	24.5 ± 3.7	0.410
Overweight	7 (29.2)	26.2 ± 4.3	8 (34.8)		
Obesity	5 (20.8)		1 (4.3)		
Not available	3		1		
BASDAI					
<4	22 (81.5)	3.0 ± 2.0	–	–	–
≥4	5 (18.5)				
BASFI					
≤4	23 (85.2)	2.2 ± 2.7	–	–	–
>4	4 (14.8)				
BASMI					
<3	24 (88.9)	1.1 ± 1.3	–	–	–
≥3	3 (11.1)				

**p-value determined by χ^2-test.*

TABLE 3 | Characterization of multifidus muscle physical properties, strength and mass.

Characteristics	Cases *n* (%)	Controls *n* (%)	*p*-value*	ORcrude (95% CI)
Muscle physical properties				
Muscle tonus#				
Low	6 (22.2)	9 (37.5)		1.000 (reference)
Intermediate	9 (33.3)	10 (41.7)	0.185	1.350 (0.343–5.315)
High	12 (44.4)	5 (20.8)		3.600 (0.829–15.628)
Muscle decrement#				
Low	6 (22.2)	9 (37.5)		1.000 (reference)
Intermediate	12 (44.4)	7 (29.2)	0.406	2.571 (0.640–10.338)
High	9 (33.3)	8 (33.3)		1.687 (0.414–6.878)
Muscle stiffness#				
Low	5 (18.5)	11 (45.8)		1.000 (reference)
Intermediate	12 (44.4)	6 (25.0)	0.099	4.400 (1.041–18.599)†
High	10 (37.0)	7 (29.2)		3.143 (0.751–13.159)
Muscle strength				
ST5				
Normal	25 (92.6)	22 (100.0)	0.192	N.D.
Reduced	2 (7.4)	0 (0.0)		
Muscle mass				
Muscle mass				
Low	1 (4.2)	22 (100.0)		
Normal range	19 (79.2)	0 (0.0)	0.724	N.D.
Above	4 (16.7)			
Muscle performance				
ST60#				
Low	9 (33.3)	0 (0.0)	**0.008**	N.D.
Intermedium	8 (29.6)	7 (31.8)		
High	10 (37.0)	15 (68.2)		
Gait speed				
Good	6 (27.3)	12 (57.1)	**0.047**	1.000 (reference)
Low	16 (72.7)	9 (42.9)		3.556 (0.993–12.733)‡

**p- value determined by χ^2-test; #Swiss reference values were used. ‡borderline effect (P-value = 0.051). BMI (Body mass Index); ST5 (5 Times Sit-to-Stand Test); ST60 (60 s Sit-to-Stand Test). #Categorization parameters. †p-value = 0.044. The bold values refers to values satistical significants.*

TABLE 4 | Genotype distribution between axSpA patients and controls, for *ACTN3* and *VDR* polymorphisms: rs1815739 (R577X); rs2228570 (*Fok*I), rs731236 (*Taq*I), and rs7975232 (*Apa*I).

Genotype	Cases *n* (%)	Controls *n* (%)	*p*-value*	OR[a] (95% CI)
ACTN3 R577X				
C/C	8 (30.8)	6 (25.0)		1.000 (reference)
C/T	15 (57.7)	12 (50.0)	0.463	0.375 (0.066–2.145)
T/T	3 (11.5)	6 (25.0)		0.938 (0.255–3.449)
C/T + T/T	18 (69.2)	18 (75.0)		0.750 (0.216–2.602)
***VDR—Fok*I**				
A/A	4 (15.4)	2 (8.3)		1.000 (reference)
A/G	12 (46.2)	16 (66.7)	0.339	0.375 (0.059–2.397)
G/G	10 (38.5)	6 (25.0)		0.833 (0.115–6.013)
A/G + G/G	22 (84.6)	22 (91.7)		0.500 (0.083–3.017)
***VDR—Taq*I**				
A/A	11 (42.3)	7 (30.4)		1.000 (reference)
A/G	7 (26.9)	9 (39.1)	0.599	0.495 (0.126–1.945)
G/G	8 (30.8)	7 (30.4)		0.727 (0.181–2.914)
A/G + G/G	15 (57.7)	16 (69.6)		0.597 (0.183–1.943)
***VDR—Apa*I**				
C/C	7 (28.0)	3 (12.5)		1.000 (reference)
C/A	9 (36.0)	10 (41.7)	0.400	0.351 (0.070–1.761)
A/A	9 (36.0)	11 (45.8)		0.386 (0.076–1.959)
C/A + A/A	18 (72.0)	21 (87.5)		0.367 (0.083–1.633)

**p-value χ^2-test. Abbreviations: a ORs and 95% CI for specific categories were calculated using logistic regression models; OR, odds ratio; CI, confidence interval.*

it important to verify if the four SNPs were in Hardy-Weinberg Equilibrium (HWE). All the populations followed the Hardy-Weinberg Equilibrium (HWE), except for *Taq*I SNP (p-value = 0.021).

Considering that three SNPs from the same gene (*VDR*) were analyzed, we also evaluated the possibility of establishing an haplotype. However, the allele combination obtained for our populations did not reveal a statistically relevant combination to be correlated with disease susceptibility. After a multiple-SNP analysis, the results showed the existence of *Linkage Disequilibrium* between *Apa*I and *Taq*I SNPs from *VDR* (D' = 0.9382, p-value $\leq$ 0.001).

Another question we wanted to address was related to the potential role of *ACTN3* and *VDR* genes polymorphisms in the etiology of the disease evaluating the risk magnitude. The genotypic frequencies were determined for both groups and for all SNPs under study.

According to the results obtained, the studied SNPs did not seem to be associated with an increased risk of axSpA susceptibility.

In addition, axSpA clinical parameters (BASDAI, BASFI, and BASMI) were also measured in the patient population and the association with the allelic distribution for each SNP was investigated (**Table 5**). *ACTN3* and *VDR* polymorphisms did not markedly influence axSpA disease activity, physical function or severity, as measured by BASDAI, BASFI, and BASMI.

We hypothesize how relevant the genetic background might be to explain muscle properties (physical-stiffness, tone, elasticity; strength; mass) and in particular physical performance (ST60 and Gait Speed), where statistically significant differences between patients and controls were registered. To understand the hypothetical effect of single SNP in these parameters, we applied the logistic regression model adjusted to the presence of the genetic component; the results are presented in the table below (**Table 6**).

Data shown refers only to *ACTN3* and *VDR* Fok1 polymorphisms, since the logistic regression of the other two SNPs did not show statistically significant results.

DISCUSSION

This pilot study, involving 27 young axSpA patients with short disease duration, has not shown any difference in muscle physical properties, global muscle strength or mass compared to controls. However, a reduction in muscle performance assessed by two

TABLE 5 | Clinical characteristics across *ACTN3* and *VDR* genes polymorphisms.

SNPs	BASDAI			BASFI			BASMI			
	Cases *n* (%)		*p*-value*	Cases *n* (%)		*p*-value*	Cases *n* (%)		*p*-value*	
	No active disease	Active disease		Low functional repercussion	High functional repercussion		Mild score	Severe score		Controls *n* (%)
***ACTN3* R577X**										
C/C	6 (28.6)	2 (40.0)		6 (27.3)	2 (50.0)		8 (34.8)	0 (0.0)		6 (25.0)
C/T	12 (57.1)	3 (60.0)	0.700	13 (59.1)	2 (50.0)	0.633	12 (52.2)	3 (100.0)	0.398	12 (50.0)
T/T	3 (14.3)	0 (0.0)		3 (13.6)	0 (0.0)		3 (13.0)	0 (0.0)		6 (25.0)
***VDR*—*Fok*I**										
A/A	3 (14.3)	1 (20.0)		3 (13.6)	1 (25.0)		4 (17.4)	0 (0.0)		2 (8.3)
A/G	9 (42.9)	3 (60.0)	0.534	9 (40.9)	3 (75.0)	0.247	9 (39.1)	3 (100.0)	0.188	16 (66.7)
G/G	9 (42.9)	1 (20.0)		10 (45.5	0 (0.0)		10 (43.5)	0 (0.0)		6 (25.0)
***VDR*—*Taq*I**										
A/A	9 (42.9)	2 (40.0)		11 (50.0)	0 (0.0)		10 (43.5)	1 (33.3)		7 (30.4)
A/G	5 (23.8)	2 (40.0)	0.810	5 (22.7)	2 (50.0)	0.323	6 (26.1)	1 (33.3)	0.886	9 (39.1)
G/G	7 (33.3)	1 (20.0)		6 (27.3)	2 (50.0)		7 (30.4)	1 (33.3)		7 (30.4)
***VDR*—*Apa*I**										
C/C	6 (28.6)	1 (25.0)		7 (31.8)	0 (0.0)		6 (26.1)	1 (50.0)		3 (12.5)
C/A	7 (33.3)	2 (50.0)	0.692	8 (36.4)	1 (33.3)	0.415	9 (39.1)	0 (0.0)	0.528	10 (41.7)
A/A	8 (38.1)	1 (25.0)		7 (31.8)	2 (66.7)		8 (34.8)	1 (50.0)		11 (45.8)

**p-value χ^2-test; BASDAI < 4, "no active disease"; BASDAI ≥ 4 "active disease"; BASFI < 4 "low functional repercussion"; BASFI ≥ 4 "high functional repercussion," and BASMI < 3, low reduction, BASMI ≥ 3, high reduction in movement amplitude.*

different approaches, ST60 and Gait speed, was found. Even using a small participant's sample, the overall results allowed us to generate relevant data on disease susceptibility evidencing low physical performance in spondiloarthritis patients. Thus, we considered interesting to examine if genes associated with muscle performance, such as *ACTN3* and *VDR*, might contribute to explain such differences.

As expected, due to our selection criteria, both groups (SpA patients and controls) are largely similar, regarding age, gender, levels of physical activity. When analyzing disease characteristics, the majority of patients exhibit low disease activity, low functional and low metrological repercussion. Most of our patients are HLA-B27 positive (80.8%), which is in line with the percentages already found for the Portuguese population (Pimentel-Santos et al., 2012). Due to the small size of our study population and low number of female individuals, we did not take into account possible gender differences in any of our analysis.

In this study, we selected several SNPs of well-known genes—*ACTN3* (Ma et al., 2013; Pratt et al., 2019) and *VDR* (Pratt et al., 2019)—related with muscle performance to evaluate their influence in axSpA susceptibility, axSpA phenotype and in muscle properties. In this context, we were interested in studying these genetic variants in the axSpA context looking for the variants related with low muscle performance and simultaneously evaluate any association with muscle physical properties, strength, and lean mass. This would represent an additional method to identify patients that might benefit from a program of physical exercise and to the identification of the best modalities to be used in clinical practice.

In our study, we demonstrate that *ACTN3* and *VDR* are not significantly associated with either susceptibility to axSpA or measures of its activity, disability or severity, as measured by BASDAI, BASFI, and BASMI, respectively. Indeed, no association has been identified in GWAS between these genes and SpA (Australo-Anglo-American Spondyloarthritis et al., 2010; International Genetics of Ankylosing Spondylitis et al., 2013). Nevertheless, some *VDR* polymorphisms have been linked to some musculoskeletal diseases, such as idiopathic scoliosis susceptibility or curve severity, herniation and spinal tissues degeneration and rheumatoid arthritis (Saad et al., 2015; Di Spigna et al., 2016; Vieira et al., 2018; Li et al., 2019).

No association was established between the studied SNPs and muscle physical properties, namely stiffness, tone or elasticity. To the best of our knowledge, this association was never studied. Again, no association for overall strength (ST5) was registered in our cohort. However, in several previous studies, *ACTN3* 577R allele and *VDR* were associated with higher levels of strength. The rs540874 polymorphism of *ACTN3* gene was associated with the muscle function of lower limb (women with the G allele were likely to have higher strength compared with the ones with A allele) but not with the higher limb, in postmenopausal women. Interestingly, in the same study, the improvement of muscle strength after an intervention (exercise and Vitamin D supplementation) was possibly correlated with rs540874, rs618838, and rs2229456 polymorphisms (Xue et al., 2018). A significant association between *VDR* genotype and quadriceps (23% difference) and grip (7% difference) strength was observed in non-obese elderly women (Geusens et al., 1997).

The analysis of these genetic markers regarding lean muscle mass did not show, again, any difference between both groups. It is well-known that genetic factors account for approximately

TABLE 6 | Association of *ACTN3* and *VDR Fok*I polymorphisms and gait speed parameters.

Characteristics	ORcrude (95% CI)	ORadjusted to *ACTN3* (95% CI)	ORadjusted to *VRD*—Fok1 (95% CI)
Gait speed			
Good	1.000 (reference)	1.000 (reference)	1.000 (reference)
Low	3.556 (0.993–12.733)[a]	**3.911 (1.044–14.658)[b]**	**3.785 (1.025–13.982)[c]**

ORs and 95% CI for specific categories were calculated using logistic regression models; OR, odds ratio; CI, confidence interval. [a]p-value = 0.051; [b]p-value = 0.043; [c]p-value = 0.047. The bold values refers to values satistical significants.

50-80% of inter-individual variation in lean body mass, with impacts detected on both "training-naïve" muscle mass and its growth response (Puthucheary et al., 2011). Indeed, these genes have been found to contribute to variation in lean body mass and bone mass density, contributing to understanding the molecular bases of sarcopenia and osteoporosis (Tan et al., 2012; Gonzalez-Mercado et al., 2013; Cho et al., 2017; Scimeca et al., 2018). However, in one study involving older Caucasian men, whole body and thigh non-skeletal lean mass were independent of *ACTN3* R/X polymorphisms (Mccauley et al., 2010). In contrast, VDR expression decreases with age and *VDR* genotype seems to be associated with fat-free mass in elderly men and women (Puthucheary et al., 2011). Differences in populations' characteristics, study methodologies and reduced number of participants (underpowered studies) to detect genes with small effects, potentially lead to discrepancies between results.

This study has shown a clear reduction in axSpA muscle performance without changes in muscle physical properties, strength, and mass. This observation allows us to speculate about a possible muscle dysfunction. As genetics has a strong influence in overall axSpA susceptibility, it is of main interest to investigate a possible genetic base to explain this impairment in muscle performance. Our results indicate that *ACTN3* R577X and *VDR* Fok1 SNPs might influence Gait Speed [OR, 3.911; 95% CI (1.044–14.658) and OR, 3.785; 95% CI (1.025–13.982)]. Several studies have shown an association of these variants on muscle performance. In 2003, Yang and colleagues (Yang et al., 2003) demonstrated a significant association between *ACTN3* genotype and athletic performance. They found that both male and female elite sprint athletes have significantly higher frequencies of the 577R allele compared to controls. Later on, several papers consistently reported a strong association between RR genotype and elite power performance (Paparini et al., 2007; Papadimitriou et al., 2008; Eynon et al., 2009; Chiu et al., 2011; Ma et al., 2013). Similar evidence was documented for *VDR* polymorphisms (Micheli et al., 2011; Puthucheary et al., 2011). In elite Italian soccer players, an interaction of two polymorphisms (*ACE* and *ACTN3*) predicted explosive leg-muscle strength, however, the contribution of genetic factors was only 23.92% (Massidda et al., 2012).

The genotype distribution of all SNPs was in HWE, except for the *Taq*I polymorphism ($p = 0.021$), suggesting the influence of genetic drift due to the small population size. Furthermore, we also evaluated the possibility to establish a specific haplotype for *VDR* gene polymorphisms, however, no haplotype was identified as relevant. Our results allowed us to identify *Linkage Disequilibrium* between *Apa*I and *Taq*I SNPs, indicating that both are segregated together. This corroborates some studies (Hamilton, 2010) and means that both SNPs are always transmitted in block (Cieslinska et al., 2018) (data not shown), even though it was not possible to identify a risk haplotype for our population. Despite the literature revealing that *VDR* SNPs constitute a haplotype, due to their proximity, we could not confirm those reports (Hunt et al., 2009).

To our knowledge, this pilot study is the very first including a genetic susceptibility analysis for muscle properties in the axSpA context. Overall, our results suggest an association between *ACTN3* and *VDR* polymorphisms and muscle performance in the axSpA context. This opens the door to a better stratification of patients regarding exercise programs, but also to identify other candidate genes that might help to characterize the genetic background of the disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the NOVA Medical School | Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Portugal Centro Hospitalar Lisboa Ocidental, Hospital de Egas Moniz, EPE, Lisboa, Portugal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FP-S, JB, and SS mainly developed the conceptualization. IP, HM, FP-S, and SS performed the methodology. SS and FP-S proceeded validation and prepared the visualization. SS and IP did formal analysis. FP-S, SR-M, RP-T, AN, LD, CL, IP, HM, and SS mainly performed the investigation. FP-S collaborated resources acquired in restrict. AN, FP-S, and SS performed the data curation. FP-S, SS, and IP contributed to the writing – original draft preparation. FP-S, SS, SR-M, RP-T, AN, LD, CL, AS, PM, and JB contributed to the writing – review and editing. FP-S, JB, and SS supervised this project. LD contributed to the project administration. FP-S and JB contributed to the funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.688984/full#supplementary-material

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Genetic and Environmental Determinants of T Helper 17 Pathogenicity in Spondyloarthropathies

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In Spondyloarthropathies (SpA), a common group of immune-mediated diseases characterised by excessive inflammation of musculo-skeletal structures and extra-articular organs, T helper 17 (Th17) cells are widely considered the main drivers of the disease. Th17 are able to modulate their genes according to the immune environment: upon differentiation, they can adopt either housekeeping, anti-bacterial gene modules or inflammatory, pathogenic functions, and only the latter would mediate immune diseases, such as SpA. Experimental work aimed at characterising Th17 heterogeneity is largely performed on murine cells, for which the *in vitro* conditions conferring pathogenic potential have been identified and replicated. Interestingly, Th17 recognising different microorganisms are able to acquire specific cytokine signatures. An emerging area of research associates this heterogeneity to the preferential metabolic needs of the cell. In summary, the tissue environment could be determinant for the acquisition of pathogenetic features; this is particularly important at barrier sites, such as the intestine, considered one of the key target organs in SpA, and likely a site of immunological changes that initiate the disease. In this review, we briefly summarise genetic, environmental and metabolic factors that could explain how homeostatic, anti-microbial Th17 could turn into disease-causing cells in Spondyloarthritis.

Keywords: T helper 17 cell, Spondyloarthropathies, ankylosing spondylitis, genetic risk, genome-wide association studies, interleukin 17, pathogenicity

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INTRODUCTION

T helper lymphocytes, characterised by the expression of CD4 on their surface, are the central cell subset of adaptive immunity. They are able to recognise protein antigens belonging to microorganisms thanks to a unique receptor expressed on their surface [T-cell receptor (TCR)] and shape an organised response against them. When a self-antigen is erroneously recognised, or when the activation threshold is altered, CD4+ T cells can cause pathological responses, characterised by uncontrolled inflammation. Together with the recognition of the antigen and the TCR engagement signal, integrated stimuli dictate the transcriptional changes that guide the differentiation of the naïve T cell towards a specialised function. These stimuli include

cytokines, soluble mediators or bacterial products in the microenvironment. Specific intracellular pathways, including Stat (signal transducer and activator of transcription) proteins, regulate this process, which eventually leads to the induction of a dominant transcription factor (TF). The lineage-specific ('master') TF controls the transcriptional programme of the cell, including specific cytokine production and the expression of chemokine receptors that mediate trafficking to the organs: this network helps each T-cell subset to exert specific functions in response to antigens, and in the tissues. The central TFs are as: T-box protein expressed in T cells (T-bet) in Th1, GATA-binding protein 3 in Th2, retinoic acid-related orphan receptor gamma-t (ROR-γt) in T helper 17 (Th17) and Forkhead box P3 in Tregs (Zheng and Flavell, 1997; Szabo et al., 2000; Fontenot et al., 2003; Harrington et al., 2005).

Dysregulated mechanisms in various steps of T-cell commitment, maturation and response to challenges can contribute to pathogenic responses, such as immune-mediated conditions for Th1 and Th17 cells, and allergic responses for Th2 cells. Among immune-mediated diseases driven by Th17 cells are Spondyloarthropathies (SpA), a group of inflammatory arthritides, including Ankylosing Spondylitis (AS) and Psoriatic Arthritis (PsA), characterised by inflammation and structural damage of several musculo-skeletal structures and organs. In these diseases, genetic and immunological alterations suggest a dysregulated 'type 17' response (Simone et al., 2018), that is the arm of cellular immunity characterised by the production of IL-17 and orchestrated by Th17. The focus of this short review will be on Th17 cells, and the genetic and environmental mechanisms that likely determine their pathogenic behaviour thought to cause SpA.

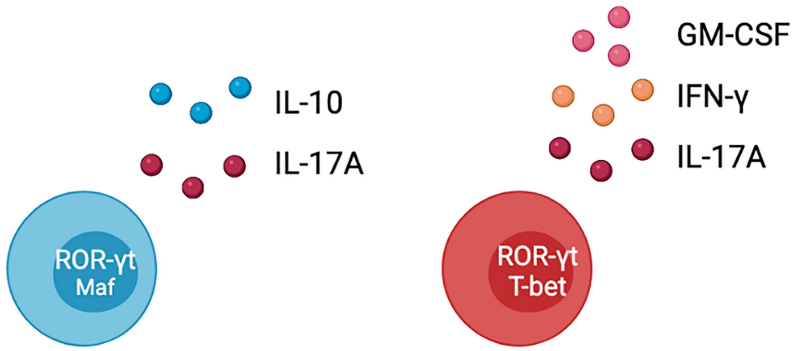
TH17 DIFFERENTIATION

The cytokine environment provides an important contribution to the T naïve fate decision. Although being a disputed matter for years, owing to between-species differences and experimental settings, it is accepted that the process of differentiation towards Th17 requires the presence of IL-6, IL-1β and variable concentrations of TGF-β (Bettelli et al., 2006; Veldhoen et al., 2006; Zhou et al., 2007). The IL-6 receptor, in particular, activates the JAK1/STAT3 pathway to induce the lineage defining TF ROR-γt (Yang et al., 2007). The STAT3/ROR-γt axis is the cornerstone of Th17 differentiation, but more TFs come into play in the process, such as RORa, Ahr, IRF4 and BATF (Brüstle et al., 2007; Schraml et al., 2009), with the last two possibly induced even before ROR-γt, characterising a sort of 'pre-Th17' status (Ciofani et al., 2012). These markers set the initial chromatin accessibility that allows a transcriptional programme, further defined by ROR-γt and decisive for the expression of effector genes (*Il17a* and *Il17f*). As multiple players are involved in the differentiation at different stages, the previously accepted paradigm of T helper differentiation as a linear, irreversible process is not actual. As demonstrated by knock down experiments, inactivation of these regulatory nodes leaves space to transcriptional instability and plasticity: in this scenario, the cytokine milieu could favour, or inhibit, molecular determinants at various stages of the differentiation phase, or even divert the programme towards other T helper lineage. TGF-β is a paradigmatic case: initially identified as essential for murine Th17 differentiation (Mangan et al., 2006), but not for human Th17 cells (Acosta-Rodriguez et al., 2007), and subsequently reinstated as necessary for both human and murine differentiation (Manel et al., 2008; Volpe et al., 2008). In practice, according to a parallel interpretation, two different cytokine cocktails lead to two different Th17 'flavours': TGF-β, together with IL-6, induces 'non-pathogenic' Th17 cells characterised by the co-expression of IL-10 (McGeachy et al., 2007); on the other hand, IL-6 and IL-23 (and no TGF-β) lead to differentiation into 'pathogenic' Th17 cells (Ghoreschi et al., 2010; **Figure 1**). Both subsets would express ROR-γt, but 'pathogenic' Th17 cells, more plastic and polymorphic, have a tendency to transition towards Th1, and consequently the production of IFN-γ. This distinction, popularised by studies that use *in vitro* differentiation assays of murine Th17, likely does not find strict correspondence *in vivo*. In experimental autoimmune encephalomyelitis (EAE), Th17 cells grown in presence of TGF-β are, in fact, also pathogenic (Kebir et al., 2007). It is also difficult to conceive conditions completely devoid of TGF-β, very common in plasma and tissues *in vivo*, and often present (in active or inactive form) in the serum enriching *in vitro* culture media. What might be crucial is the concentration, and the gradient, of TGF-β: high concentrations of TGF-β induce Treg-associated genes while restraining T-bet and other Th1 genes and possibly inhibit Th17 pathogenic responses. The developmental overlap between Th1, Th17 and Treg might in fact be caused by complex cytokine dynamics. At the opposite side of the spectrum, IL-23 has long been considered the main ingredient for pathogenic differentiation and the initiator of the pro-inflammatory module.

TH17 AND SPONDYLOARTHROPATHIES

There is considerable evidence that indicates a central role of Th17 cells in the pathogenesis of SpA. The proportion of Th17 is higher in the blood of AS patients (Shen et al., 2009) and so are the serum concentrations of IL-17A, as reported in a number of studies by different groups (reviewed in Taams et al., 2018). In addition to experimental evidences, current clinical practice offers the empirical demonstration of the importance of 'type 17' lymphocytes in the pathogenesis of SpA: secukinumab (an anti-IL-17A monoclonal antibody) is currently widely used in AS and PsA, while a number of novel agents against IL-17 superfamily cytokines are in the final experimental stages or about to be licenced for clinical use (van der Heijde et al., 2018, 2020).

An important series of evidences originates from genetic studies: Genome-Wide Association Studies (GWAS; International Genetics of Ankylosing Spondylitis Consortium (IGAS) et al., 2013) have convincingly established the association between AS and genetic variants in loci involved in the Th17 pathway, including *TYK2*, *IL6R*, *IL1R1*, *IL1R2* and *IL23R*, whose products are important for the induction and survival of the Th17 response.



	"Non pathogenic" Th17	"Pathogenic" Th17
Genes	*Maf, Ahr, Il10*	*T-bet, Gpr65, Csf2, Toso, Plzp*
Cytokines (other than IL-17A)	IL-9, IL-10, IL-21	GM-CSF, IFN-γ
Surface Markers	CCR4, CCR6, IL6R	CCR4, CCR6, IL6R, CXCR3, IL23R
Differentiation	TGF-β, IL-6	IL-1β, IL-6, IL-23
Micro-organisms	*S. aureus*, SFB (in mouse)	*C. albicans*
Metabolism	oxphosph	glycolysis

FIGURE 1 | List of features of 'non-pathogenic' and 'pathogenic' Th17 cells identified across several studies, most of which performed on murine cells. *In vivo*, this classification should not be considered bimodal, but rather a continuum of states, shaped by the local environment.

In particular, the existence of a genetic variant of the IL-23 receptor gene, possibly causing the altered signalling predisposing to AS, emerged early from one of the first association studies (Wellcome Trust Case Control Consortium et al., 2007). Although one AS-associated SNP (rs11209026, also seen in psoriasis and IBD) causes a missense variation that alters IL-23R signalling, impairing Th17 responses (Di Meglio et al., 2011, 2013), the effect of the other gene variants is unclear. Recently, epigenetic data on chromatin remodelling and transcription factor (TF) binding helped to characterise a second independently AS-associated SNP located in the IL23R-IL12RB2 intergenic region. The observed higher number of IFN-g-secreting cells in the risk allele carriers (Roberts et al., 2016) could in fact explain the functional association with the disease. Although this work could not confirm this alteration is present on Th17 specifically, it provides an example of how allelic variations affect the genetic/epigenetic regulation of inflammatory pathways, thus potentially induce pathogenic functions in Th17 cells. Other genes, whose variants associate with AS, have been carefully studied in the search of an alteration of Th17 responses that can cause AS. *STAT3* and *TYK2*, for example are mediators of Th17 immunity. Their protein products play a central role in IL-6 and IL-23 signalling: they are activated by phosphorylation and indirectly act as transcriptional activators of Th17 differentiation, *via* a chain of other intermediates. Several SNPs within *STAT3* locus have been associated to AS and CD (Danoy et al., 2010), but their impact on Th17 function in these conditions has never been clarified, while different causing SNPs of *STAT3* showed to impact Th17 differentiation in other autoimmune diseases (Tripathi et al., 2017). Another important GWAS hit, involving a gene involved in Th17 responses, is *TYK2*. The AS-associated SNPs (rs12720356) at the *TYK2* locus were found to be associated with increased Th1 frequency and AS disease progression: this, together with the evidence that Tyk2 inhibition is an effective strategy in the experimental model of AS (Gracey et al., 2020), makes the search for functional variants of genes that orchestrate the Th17 response a biologically relevant enterprise, that is also very promising from a translational point of view.

DETERMINANTS OF TH17 PATHOGENICITY

IL-23, a heterodimeric cytokine, is closely related to IL-12, with whom it shares one of the two subunits. Consistent with its kinship to IL-12, it is able to induce both IL-17 and IFN-γ (Aggarwal et al., 2003) in CD4+ cells. More precisely, it is dispensable for Th17 development, but it enhances an accessory transcriptional module, thought to be pathogenic (Volpe et al., 2008). Indeed, IL-23 persistence induces Th17 to acquire a Th1-like phenotype (Lee et al., 2009), and lack of IL-23R on T cells prevents experimental colitis (Ahern et al., 2010). IL-23, together with IL-1, has been associated with Th17 in autoimmune

models (Cua et al., 2003; Langrish et al., 2005; McGeachy et al., 2009). In particular, IL-23 is the key ingredient of pathogenicity in EAE because it induces high levels of T-bet, IL-23R and GM-CSF in the Th17 cell (Lee et al., 2012). Importantly, IL-23R is not constitutively expressed on the naïve T cell, but it appears during the Th17 differentiation secondarily to IL-6 signalling (Chung et al., 2009; Ghoreschi et al., 2010). Through this and other feed forward mechanisms (Kishi et al., 2016; Meyer Zu Horste et al., 2016), the Th17 inflammatory programme is stabilised.

The aforementioned association of genetic variants of the IL23R gene region with AS (International Genetics of Ankylosing Spondylitis Consortium (IGAS) et al., 2013) led to hypothesise that IL-23 was the main driver for pathogenic responses in AS as well. Early experimental data in humans did not confirm this: IL-23 blockade proved ineffective on the axial symptoms in AS (Baeten et al., 2018). Few hypotheses have been proposed to explain this: IL-23 might still be a key player in the initiation phase, losing importance when the disease is already established. An alternative explanation postulates the presence of other IL-23-independent drivers of Th17 pathogenicity. In reality, immune cytokine 'axes' (such as IL-23/17) are almost never linear: IL-23 and IL-17 do participate in the same branch of the immune response but at the same time, have distinct biology and affect different cell subsets in different tissues. Regardless of its induction, we now know that the effector functions of the Th17 pathogenic module consist in IL-17A plus additional inflammatory cytokines, such as IFN-ɣ and GM-CSF, the latter an emerging Th17-associated player of autoimmunity (El-Behi et al., 2011). Recent translational approaches in SpA are focusing on novel cytokine targets, such as GM-CSF (Al-Mossawi et al., 2017; Wade et al., 2019), or upstream pro-inflammatory intracellular signals, such as JAK/STAT (Hammitzsch et al., 2018), a strategy that would simultaneously regulate cell differentiation and inhibit multiple inflammatory cytokines.

Other genetic events leading to unrestrained, disease-causing differentiation are still not clear: *ex vivo* single-cell sequencing led to identify novel pathogenic genes (*Gpr65*, *Toso* and *Plzp*) promoting Th17-mediated CNS inflammation in EAE mice (Gaublomme et al., 2015; Wang et al., 2015), but none of which, as of today, have been validated in humans, with the partial exception of GPR65, an important TF in human GM-CSF + Th17 (Al-Mossawi et al., 2017). The gene, a GWAS hit associated with AS, encodes for a G-protein coupled receptor with an extracellular proton sensing domain, and strongly characterises GM-CSF-producing Th17, where its activation likely concurs to the production of said cytokine in the acidic environment, expanding the inflammatory potential of the Th17 cell.

TH17 RESPONSE TO MICROBIOTA

Not only cytokines, but also different microorganisms have the ability to prime distinct Th17 responses (McGeachy and McSorley, 2012). A study in humans showed, through *in vitro* experiments, that Th17 cells primed with different pathogens are able to acquire specific cytokine signatures (Zielinski et al., 2012). Th17 are inherently plastic having evolved to tailor their response to different pathogens at different anatomical sites: one well-characterised example is provided by a work that described how gut-resident Th17 cells in mice, primed by segmented filamentous bacteria (SFB), exhibit little plasticity and are not involved in tissue inflammation, while Th17 induced by *C. rodentium* have pathogenic potential, and a preferential glycolytic metabolism (Omenetti et al., 2019). Our interpretation is that the combination of host features (e.g., presence of predisposing genetic variants and HLA haplotype) associated with either commensal or pathogenic microbiome would dictate the characteristics of Th17 responses to bacteria and fungi. A recent paper hypothesised that airway Th17 cells in airway inflammation, e.g., during acute allergic bronchopulmonary aspergillosis, could all be cross-reactive to commensal *Candida* (Bacher et al., 2019): it is conceivable that a similar phenomenon of cross-reactivity could be observed in Th17 from SpA patients in response to gut or skin microbes. At the moment, it is not clear whether Th17 cells driving the manifestations of SpA are primed in the barrier organs, such as gut or skin (Gracey et al., 2019). Certainly, specific microbe-induced experimental models, or even gnotobiotic animals, could help replicating different modes of Th17 induction in various disease models and highlight both pathogenic mechanism and natural regulatory responses, such as in the case of cMaf + ROR-ɣt+Tregs, a cell subset specialised in restraining pathogenic bacteria-induced Th17s (Xu et al., 2018).

As anticipated, emerging features of T-cell biology are their inherent instability (loss of expression of transcriptional signature) and plasticity (acquisition of TFs or cytokines typical of other lineages). They are often being seen upon *in vitro* restimulation (Hirota et al., 2011), but also *in vivo*: Th17 cells can acquire Th1-features and transdifferentiate into the so-called Th17.1 cells, a subset that is particularly evident in the inflammatory environment, such as the synovial fluid in the course of arthritis (Nistala et al., 2010). Conversely, Th17 can lose pathogenic features, or even transdifferentiate into Treg-like cells: under pro-inflammatory conditions in the gut, intestinal Th17 cells can differentiate into IL-10-producing Tr1-like cells, important in the resolution of inflammation (Gagliani et al., 2015). This aspect of Th17 biology can provide therapeutic opportunities, through the induction of an anti-inflammatory fate in former pathogenic Th17 cells.

TH17 IN TISSUE HOMEOSTASIS

In the steady state, the natural role of Th17 cells is to maintain tissue homeostasis, by maintaining barrier integrity (Lee et al., 2015) and anti-microbial functions, in particular vs. pathogenic microbes, such as, in humans, *Candida* and *Salmonella* (McGeachy and McSorley, 2012). In mice, colonisation with the commensal SFB (Ivanov et al., 2009) is sufficient to provide Th17-mediated resistance to the intestinal pathogen *Citrobacter rodentium*.

These gut-specific Th17 cells with a homeostatic role can be defined as 'non-pathogenic Th17 cells' being largely responsive to microbial or food-derived antigens and not involved in inflammatory mediated diseases. However, additional environmental cues, such as colonisation by specific microbial species, genetic predispositions and toxins, alter the equilibrium by enforcing effector profiles of autoimmune Th17 cells that can cause pathogenic responses that could then drive disease in the musculo-skeletal structures. The possible driving role of intestinal immunity in the pathogenesis of AS is indirectly confirmed by the susceptibility genes for Crohn's disease (CD), a form of inflammatory bowel disease akin to the colitis observed in SpA, whose list includes a number of Th17-associated genes (*CCR6*, *JAK2*, *TYK2*, *STAT3* and *IL23R*; Anderson et al., 2011).

Finally, it is worth reminding that the IL-17 family includes six cytokines. The role of some of them is poorly understood (McGeachy et al., 2019) but they seem central in intestinal immunity: IL-17D has a homeostatic role in the gut (it maintains ILC3 function; Huang et al., 2021), and conversely, IL-17F could promote an inflammation-promoting microbiota, by interfering with the colonisation of Treg-inducing bacteria (Tang et al., 2018), making it a potentially attractive target for pathologic, non-homeostatic, responses. IL-23 is also upregulated in the gut of AS patients (Ciccia et al., 2009), probably secondary molecular events, such as autophagy (Ciccia et al., 2014), and also relevant in CD (Eken et al., 2014).

DISCUSSION

Experimental data suggesting heterogeneity within the Th17 compartment come largely from mouse studies, where a number of conditioning elements and some molecular networks conferring pathogenic potential have been identified. Whether the majority of these is also relevant in human Th17 is not yet clear. What is increasingly apparent is that Th17 are able to adopt both housekeeping, anti-bacterial gene modules and inflammatory, pathogenic functions, and only the latter would cause immune diseases, such as SpA. The tissue environment is possibly decisive, particularly at barrier sites, where cytokine concentrations or the presence of metabolites can dictate the cell fate. To understand the biology of tissue Th17, careful immune characterisation in steady state and in tissue context will be required. GWAS have discovered associations between genomic loci associated with Th17 function, and the occurrence of AS, providing suggestive hints into pathogenesis (Knight, 2013), but comprehending the functional meaning of the variants is particularly challenging when the genetic association is agnostic of the cell subset and tissue of origin. Since small genetic defects could originate at every phase of cell differentiation or in specific tissues, we believe that combining functional genomics with system immunology will be the next biggest challenge for translational science of immune-mediated diseases.

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All authors listed have made substantial, direct and intellectual contribution to the work and approved it for publication.

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Disruption of c-MYC Binding and Chromosomal Looping Involving Genetic Variants Associated With Ankylosing Spondylitis Upstream of the *RUNX3* Promoter

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Background: Ankylosing Spondylitis (AS) is a common form of inflammatory spinal arthritis with a complex aetiology and high heritability, involving more than 100 genetic associations. These include several AS-associated single nucleotide polymorphisms (SNPs) upstream of *RUNX3,* which encodes the multifunctional RUNT-related transcription factor (TF) 3. The lead associated SNP *rs6600247* ($p = 2.6 \times 10^{-15}$) lies ~13kb upstream of the *RUNX3* promoter adjacent to a c-MYC TF binding-site. The effect of *rs6600247* genotype on DNA binding and chromosome looping were investigated by electrophoretic mobility gel shift assays (EMSA), Western blotting-EMSA (WEMSA) and Chromosome Conformation Capture (3C).

Results: Interrogation of ENCODE published data showed open chromatin in the region overlapping *rs6600247* in primary human $CD14^+$ monocytes, in contrast to the Jurkat T cell line or primary human T-cells. The *rs6600247* AS-risk allele is predicted to specifically disrupt a c-MYC binding-site. Using a 50bp DNA probe spanning *rs6600247* we consistently observed reduced binding to the AS-risk "C" allele of both purified c-MYC protein and nuclear extracts (NE) from monocyte-like U937 cells. WEMSA on U937 NE and purified c-MYC protein confirmed these differences ($n = 3$; $p < 0.05$). 3C experiments demonstrated negligible interaction between the region encompassing *rs6600247* and the RUNX3 promoter. A stronger interaction frequency was demonstrated between the *RUNX3* promoter and the previously characterised AS-associated SNP *rs4648889*.

Conclusion: The lead SNP *rs6600247,* located in an enhancer-like region upstream of the *RUNX3* promoter, modulates c-MYC binding. However, the region encompassing *rs6600247* has rather limited physical interaction with the promoter of *RUNX3*. In contrast a clear chromatin looping event between the region encompassing *rs4648889* and the *RUNX3* promoter was observed. These data provide further

evidence for complexity in the regulatory elements upstream of the *RUNX3* promoter and the involvement of *RUNX3* transcriptional regulation in AS.

Keywords: ankylosing spondylitis, single nucleotide polymorphism (SNP), chromosome conformation capture (3C), RUNX3, c-Myc

INTRODUCTION

Background

Ankylosing Spondylitis (AS) is a form of inflammatory spondyloarthritis predominantly affecting the axial skeleton, which is characterised pathologically by enthesitis (Bridgewood et al., 2020). Extra-skeletal manifestations are also common in AS; these include inflammation of the gut (ranging from low-grade sub-clinical inflammation of the terminal ileum to overt inflammatory bowel disease - IBD), skin (psoriasis), and uveal tract (Stolwijk et al., 2015; Rizzo et al., 2017). AS was one of the first complex diseases in which a specific genetic effect was identified when its strong association with the major histocompatibility complex (MHC) immune response gene HLA-B27 was described nearly 50 years ago (Brewerton et al., 1973) (Schlosstein et al., 1973). However, it is clearly polygenic (Brown et al., 1997); even the MHC association is attributable to several alleles at more than one locus (Cortes et al., 2015) and more than 100 non-MHC genetic associations have now been suggested by genome-wide association studies (Burton et al., 2007; Reveille et al., 2010; Evans et al., 2011; Cortes et al., 2013). Shared genetic susceptibility factors undoubtedly contribute to the excess occurrence of psoriasis, IBD and uveitis not only in individuals with AS but also their relatives (Ellinghaus et al., 2016) (Robinson et al., 2016). One of the strongest non-HLA associations with AS is with the *RUNX3* (Runt-related transcription factor (TF) 3) locus. RUNX3 is involved in T-cell function and plays a key role in the development of $CD8^+$ T-cells (Egawa et al., 2007). It also influences many other cells, including helper T-cells, innate lymphoid, tissue resident, mucosa and gut cells (Ebihara et al., 2015; Behr et al., 2018). We have recently demonstrated that AS-associated non-coding single nucleotide polymorphisms (SNPs) in an enhancer-like region upstream of *RUNX3* affect the binding of different factors: in particular the repressive nucleosome remodelling and deacetylase (NuRD) complex binds preferentially to the risk allele, while conversely interferon regulatory factor (IRF) five to the protective allele (Vecellio et al., 2021). However, the functional effects of these changes on gene transcription are still to be precisely determined. Our earlier observations were made in T-cells, but here we describe some of the functional effects of the lead AS-associated SNP in the vicinity of *RUNX3* (*rs6600247*, $p = 2.6 \times 10^{-15}$ (Cortes et al., 2013) that are more obvious in $CD14^+$ monocyte-like cells than $CD8^+$ T-cells. First, we evaluate the chromatin landscape surrounding *rs6600247* using the ENCODE database (https://genome.ucsc.edu/ENCODE/). Second, we demonstrate differential allelic binding of *rs6600247* to the c-MYC TF. Finally, we investigate the chromosomal architecture and physical interactions between AS-associated sequences in the enhancer-like region upstream of *RUNX3* and its promoter, showing a probable role of chromosome looping in the regulation of *RUNX3*.

METHODS

Genotyping

DNA was extracted using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen Ltd., Manchester, United Kingdom) and genotyped for *rs6600247* using TaqMan SNP assay (custom order by Life Technologies, Paisley, United Kingdom), for the cells (obtained by the buffy coat) used in the functional studies.

In Silico Investigation

We used the UCSC genome browser build hg19 and the Roadmap database [https://genome.ucsc.edu/ENCODE/] to investigate the epigenetic landscape of *rs6600247* upstream of the *RUNX3* promoter, which is strongly associated with AS ($p = 4.2 \times 10^{-15}$) (Cortes et al., 2013). Histone modifications and GeneHancer (a database of human regulatory elements and their inferred target genes) tracks were selected to evaluate regulatory elements and chromosome looping between promoters and enhancer regions (Fishilevich et al., 2017).

Cell Lines, Culture and Primary Human Cell Isolation

Blood samples were obtained from AS patients with ethical approval (COREC **06/Q1606/139)** and informed patient consent. $CD8^+$ T-cells and $CD14^+$ monocytes were isolated from AS patients' peripheral blood mononuclear cells (PBMCs) using a $CD8^+$ T-cell or a $CD14^+$ monocyte isolation kit (Miltenyi, Bisley, Surrey, United Kingdom), respectively. Jurkat, U937, $CD8^+$ and $CD14^+$ cells were resuspended at $1{\times}10^6$/ml in pre-warmed Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum, penicillin/streptomycin and L-glutamine, and rested overnight. Cells were then harvested for experiments.

Electrophoretic Mobility Gel Shift Assay

The impact of *rs6600247*, which lies in a c-MYC binding-site (**Figure 2A**), was assessed by EMSA. We designed DNA probes including either the protective T or the AS-risk variant C to evaluate the disruption of a c-MYC consensus motif. The DNA probes used in EMSAs (50-bp single-stranded biotinylated DNA probe incorporating *rs6600247*) were mixed and annealed at room temperature for 1 h. Probes were then incubated for 20 min with nuclear extracts (NE) obtained either from primary $CD8^+$ T-cells or a monocyte cell line from histiocytic lymphoma (U937) stimulated with phorbol-12-myristate-13-

acetate (PMA). The sequences of the synthetic single-stranded oligonucleotides are listed below:

C* s (sense): 5′-CTCCATGACGCAATTTGGGCTCCGTT ATGAGTCAGCTCAAGTAA-3′; T* s: 5′-CTCCATGACGCA ATTTGGGCTCTGTTATGAGTCAGCTCAAGTAA-3′; C* as (antisense): 5′-TTACTTGAGCTGACTCATAACGGAGCCC AAATTGCGTCATGGAG-3′; T* as: 5′-TTACTTGAGCTG ACTCATAACAGAGCCCAAATTGCGTCATGGAG-3′.

(Underlined base highlights the position of *rs6600247*).

Western Blotting - Electrophoretic Mobility Gel Shift Assay

DNA probes as for EMSA (above) were incubated with nuclear extract obtained from U937, $CD8^+$ T-cells or purified c-MYC human recombinant protein (Abcam, ab169901 Cambridge, United Kingdom) as previously described (Allen et al., 2017) and separated on DNA retardation gels at 100 V on ice. The samples were transferred on to nitrocellulose membranes for Western blotting (WB), then blocked with 5% milk in Tris Buffer Saline +0.1% Tween (TBST) for 1 h at room temperature (RT) before incubating overnight at 4°C with the primary antibody for c-MYC (Santa Cruz Biotechnology sc-40, Dallas, Texas United States). Secondary goat anti-rabbit antibody (1:10,000 dilution) was added (1 h RT) and the membranes washed before Horse Radish peroxidase substrate (Thermo Fisher Scientific, Waltham, Massachusetts, United States) added for imaging. ImageJ (NIH) was used for quantifying WEMSA bands (Schneider et al., 2012).

Chromosome Conformation Capture

Chromosome conformation capture (3C) was performed as previously described (Miele et al., 2006). Briefly, libraries were prepared as follows: 1.5×10^7 of U937 or Jurkat cells were cross-linked with formaldehyde at 1% of the final volume. *Glycine* [0.125M] was used to quench cross-linking and cells were lysed in cold lysis buffer on ice using a Dounce homogenizer (Sigma Aldrich, Gillingham, United Kingdom). Cells were resuspended in specific restriction enzyme buffer (10 μL were kept as undigested control). The remaining samples were digested overnight at 37°C with 500 units of Sac1 (New England Biolabs, Hitchin, United Kingdom). Digestion was stopped by the addition of 10% sodium dodecyl sulfate incubated at 65°C for 30 min. T4 ligase (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to perform ligation for 4 h at 16°C. Proteinase K was added prior to reversal of cross-linking at 65°C overnight. Proteinase K was added to the undigested and digested controls saved earlier. DNA was purified using phenol-chloroform extraction, followed by ethanol precipitation. 3C template was resuspended in 500 ul H_2O, while undigested and digested controls in 50 ul. The quality of the chromatin samples was assessed on agarose gels. Bacterial Artificial Chromosome (BAC) preparations were performed similarly as genomic controls. 3C PCR primers were designed along the same strand and same orientation to accomplish specific amplification across 3C ligation junctions. Full list of primers is available in **Supplementary Table S1** and their genomic position relative to *RUNX3* is shown in **Figure 3**

We interrogated a genomic region upstream the *RUNX3* distal promoter, including few AS-associated SNPs in U937 (monocyte-like) and Jurkat (T-lymphocyte-like) cell lines. The bait was placed at the distal promoter (P2) with amplification primers at the AS-associated SNPs *rs6600247* and *rs4648889* along with three intergenic regions.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from $CD8^+$ and $CD14^+$ cells was isolated with TRIzol (Invitrogen, Paisley, United Kingdom) and reverse transcribed with Superscript III (Invitrogen, Thermo Fisher Scientific, 168 Third Avenue, Waltham, Massachusetts, United States) to synthesise cDNA as previously described (Vecellio et al., 2018). The specific primers were: *RUNX3* sense (s): 5′-ACTCAG CAC CAC AAG CCA CT-3′; *RUNX3* antisense (as): 5′-GTC GGA GAA TGG GTT CAG TT-3′. Quantitative PCR was performed in triplicate and the 2−ΔCt method was used to calculate the expression of *RUNX3* relative to β-actin (ID Assay qHsaCED0036269, Bio-Rad Laboratories, Kidlington, United Kingdom).

Historical Controls and RUNX3 Expression

RUNX3 transcription in AS cases and controls was evaluated from previously published data derived from RNA-seq in PBMCs from 72 AS cases and 62 healthy controls and stratified for *rs6600247* (Li et al., 2017).

RESULTS

Genomic Landscape Interrogation Suggests a Regulatory Role for *rs6600247*

Figure 1A shows the genomic landscape at the *RUNX3* locus, with the lead AS-associated SNP *rs6600247* lying ~13kb upstream of the distal promoter while the regulatory SNP *rs4648889* is physically closer to the promoter. SNP *rs6600247* is situated within a region of open chromatin, defined by a peak for dnase I hypersensitvity (DHS - indicative of regions of open chromatin) (**Figure 1B**), and a peak of H3K4Me1 histone modification. This sequence also binds the transcription factor c-MYC (ENCODE Factorbook (http://www.factorbook.org/human/chipseq/tf/) (**Figure 1B**). Taken together, these data suggest an enhancer-type element surrounding *rs6600247*, so we sought to determine a regulatory role of this SNP. The DHS peak overlapping *rs6600247* is seen specifically in $CD14^+$ monocytes (**Figure 1B**). For this reason, we conducted our functional experiments in U937 cells, a pro-monocytic, human myeloid leukaemia cell line, exhibiting monocyte-like features.

rs6600247 AS-Risk C Allele Alters c-MYC Binding to Deoxyribonucleic Acid

We analysed the DNA sequence at *rs6600247* and found that the SNP lies within a c-MYC consensus binding motif (**Figure 2A**). We hypothesised that binding of c-MYC protein to a DNA sequence containing the risk allele C would be reduced. The

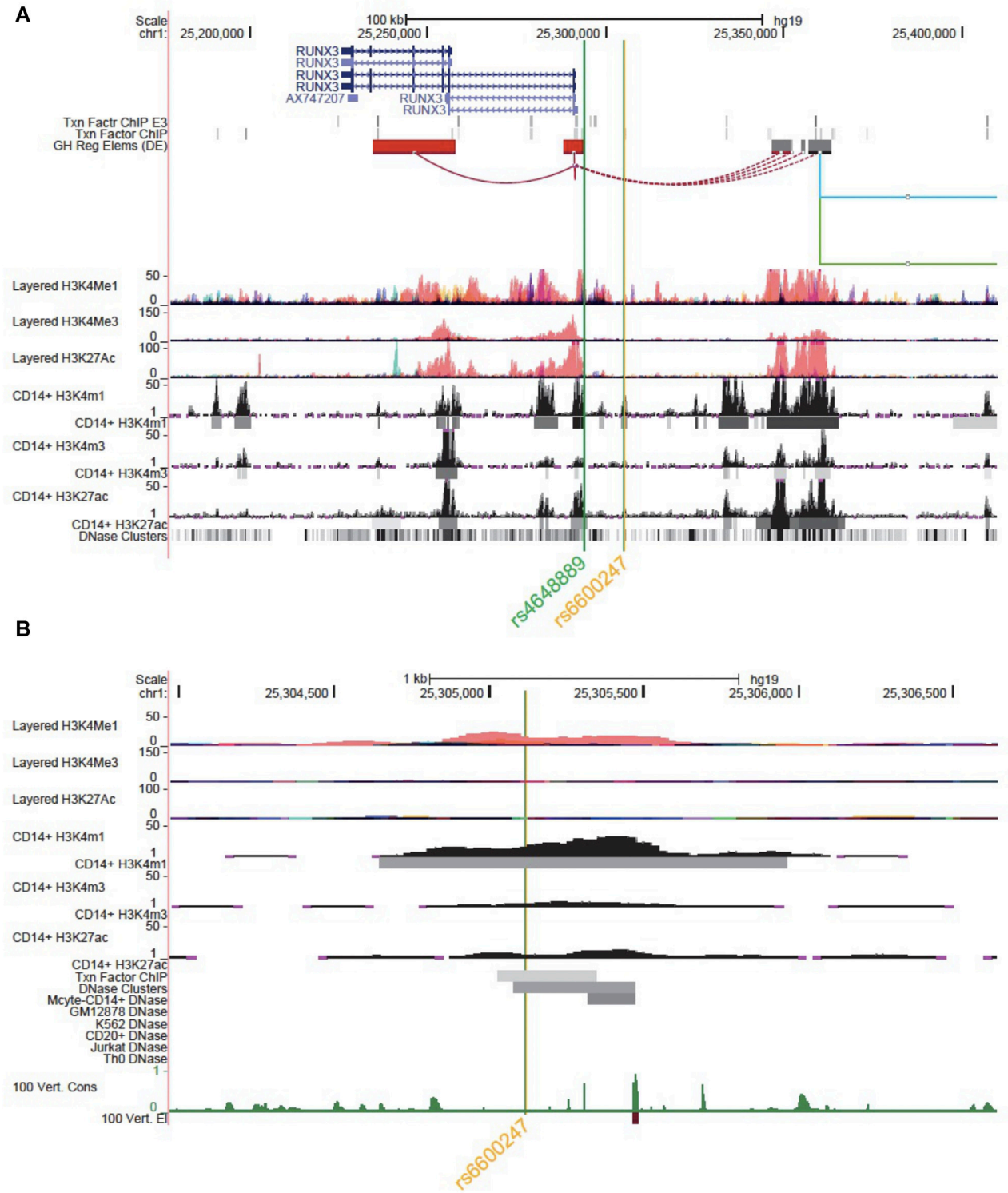

FIGURE 1 | Genomic landscape interrogation suggests a regulatory role for *rs6600247*. UCSC Genome Browser analysis of the *RUNX3* locus at chr1: 25,177,797–25,409,807. The green and orange vertical lines show the location of SNP *rs4648889* and *rs6600247*. *RUNX3* gene location (blue) is from the UCSC genes database. Two tracks show transcription factor binding by ChIP (Txn Facto ChIP E3 and Txn Factor ChIP). GeneHancer track shows human regulatory elements and their inferred target genes. ENCODE layered H3K27ac, H3K4Me1 and H3K4me3 tracks (from 7 cell lines) show enrichment of these marks, indicating promoter and enhancer regions. $CD14^+$ H3K4me1, $CD14^+$ H3K4me3 and $CD14^+$ H3K27ac show enrichment and peaks called specifically in $CD14^+$ monocytes (ENCODE). Dnase clusters show DNase-I hypersensitivity clusters (ENCODE); **(B)** Zoomed Genome Browser view (chr1:25,303,971–25,306,641) upstream of the promoter of *RUNX3* showing a peak for H3K4Me1 enrichment overlapping *rs6600247* (vertical line; Layered tracks and ENCODE histone tracks as in **(A)**. Additional datasets displayed are ENCODE transcription factor Chip-seq peaks and DNaseI HS peaks that directly overlap *rs6600247*, ENCODE dnase I HS for $CD14^+$ monocytes and vertebrate conservation.

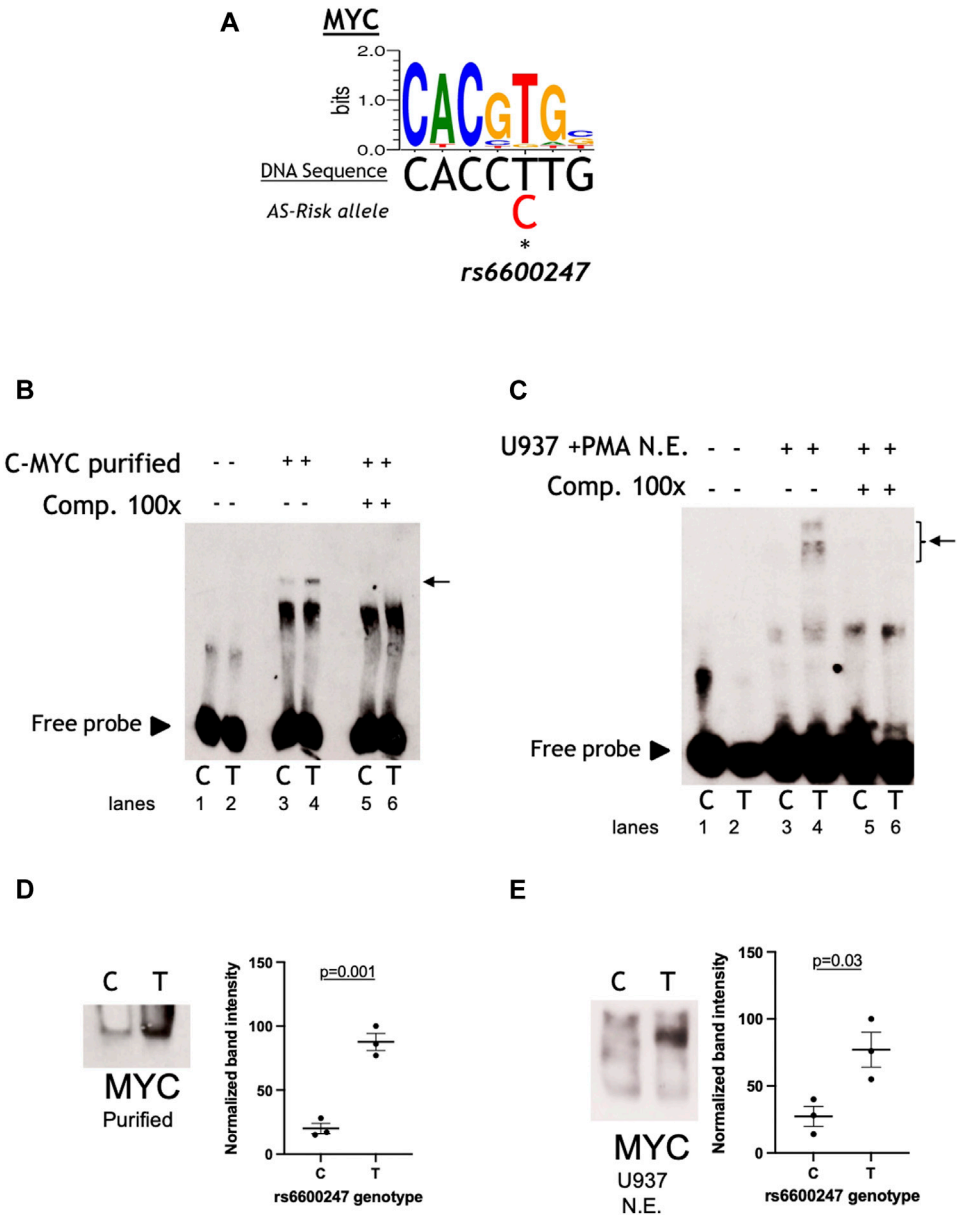

FIGURE 2 | *rs6600247* risk allele affects C-Myc binding. **(A)** C-Myc binding motif analyzed using the MEME program and the location of *rs6600247* risk allele; **(B)** EMSA using c-MYC purified protein with or without specific competitor (Comp 100x), $n = 4$, allele (C or T) of *rs6600247* included in the 50bp biotinylated double-stranded DNA probe is given below the image; horizontal arrow indicates specific protein-DNA complex formation; **(C)** EMSA using nuclear extract (N.E.) from U937 cells stimulated with phorbol 12-myristate 13-acetate (PMA) with or without specific competitor (Comp 100x), $n = 4$; *rs6600247* allele and complex formation indicated as in **(B,D)** WEMSA using C-Myc purified protein and blotted with an antibody against C-Myc; **(E)** WEMSA using U937 nuclear extract and blotted with an antibody against C-Myc. The blot is representative of $n = 3$ experiments. Binding in **(D,E)** was quantified using ImageJ software and is representative of three different experiments, demonstrating that the risk allele for rs6600247 shows fewer binding properties for C-Myc.

results of EMSA assessing the relative c-MYC protein binding to the C or T alleles are shown in **Figure 2**. We first incubated probes with recombinant c-MYC purified protein, and observed a specific DNA/protein complex with both alleles but markedly less to the AS-risk allele C than the protective T allele. (**Figure 2B**; lane 3-4, $n = 3$). We then incubated the same probes with NE from U937 (monocyte-like cells) and observed a major protein/DNA complex binding to the protective T allele, but none with the C allele (**Figure 2C**, lanes 3-4, $n = 3$). In both cases, successful competition with a 100-fold excess of unlabelled probe confirmed the specificity of the complex (**Figure 2B**, lane 5-6 and **Figure 2C** lane 5–6). We next used WEMSA to quantitate the relative binding of c-MYC to each allele of *rs6600247*. Markedly less c-MYC enrichment was seen with the C risk vs T allele using either c-MYC purified protein or U937 NE (**Figures 2D,E**, relative band intensities $p = 0.01$ and $p = 0.05$, respectively,

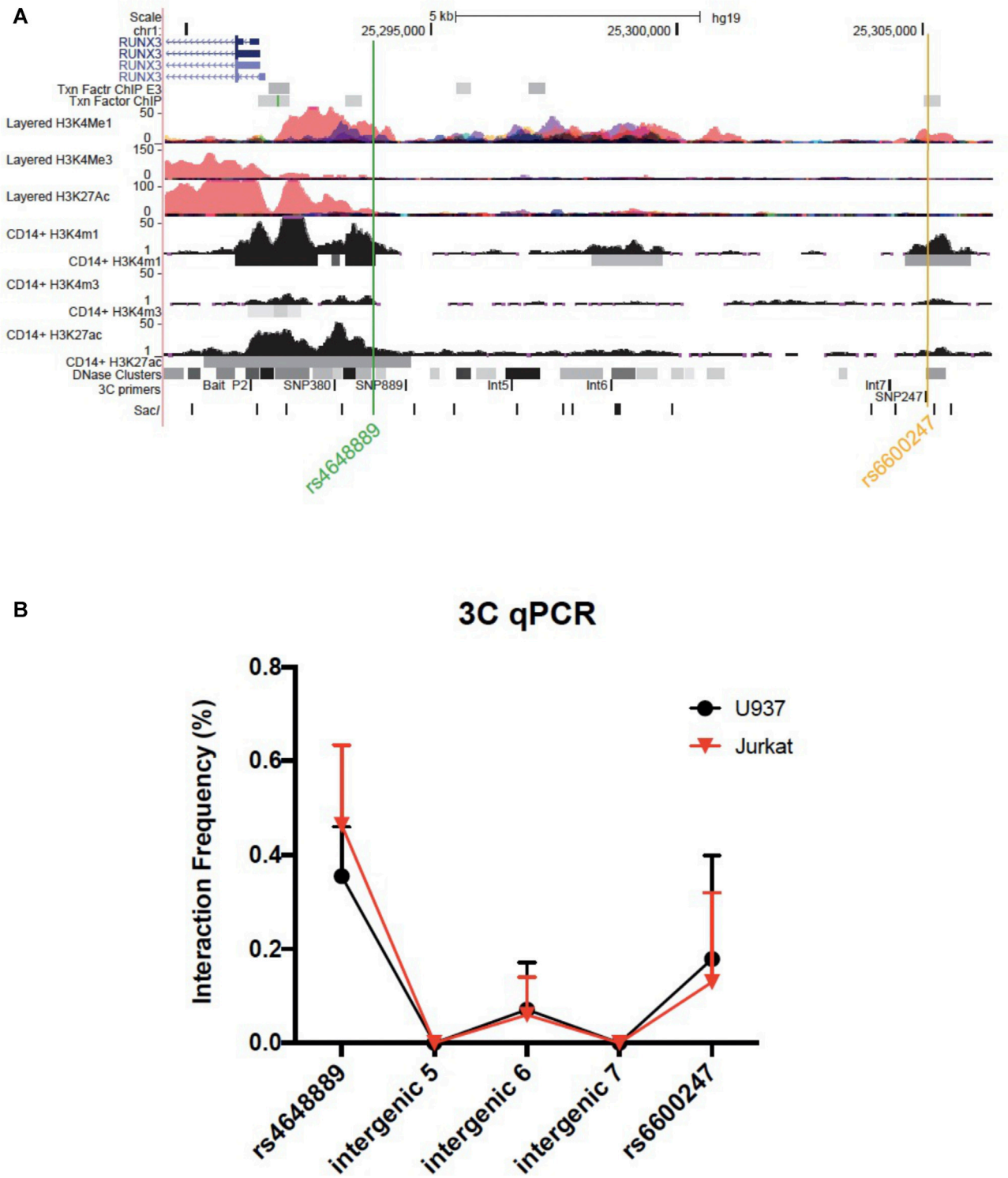

FIGURE 3 | Chromosome looping investigation demonstrates interaction between the *RUNX3* promoter and region encompassing *rs4648889* rather than *rs6600247*. **(A)** Location of the *RUNX3* genomic region chr1:25,289,567–25,306,400. Tracks shown as in **Figure 1**, with the addition of 3C-qPCR primers and SacI enzyme cutting sites. Bait fragment is located at *RUNX3* distal promoter (P2); AS-associated SNPs primers used in these experiments are named as follows: SNP889 (for *rs4648889*), Int5, Int6, Int7 (for intergenic regions 5, 6 and 7) and SNP247 (for *rs6600247*); **(B)** Results of the 3C-qPCR analysis showing Increased relative interaction frequency between *RUNX3* P2 and the region encompassing *rs4648889,* with a modest interaction with the *rs6600247* region. Theses interactions were seen in both U937 (red) and Jurkat (black) cell lines.

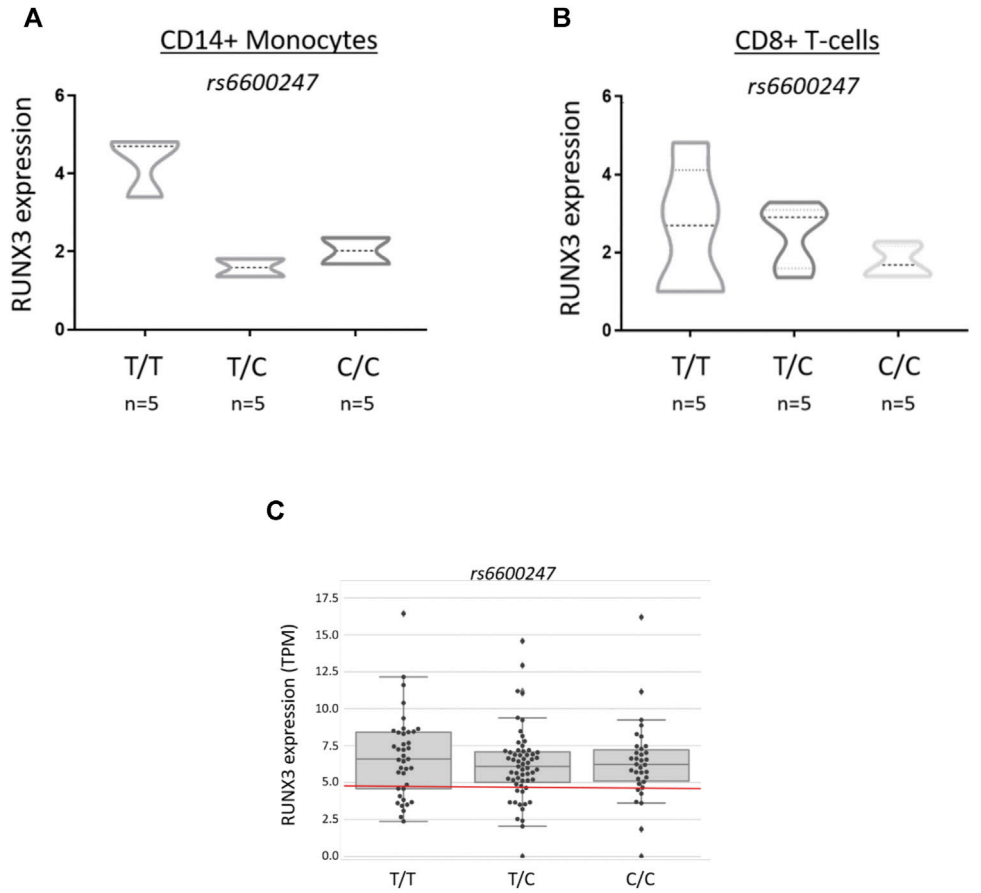

FIGURE 4 | *rs6600247* genotype shows no regulatory effect on RUNX3 expression *RUNX3* expression levels measured by qRT-PCR in freshly isolated **(A)** $CD14^+$ monocytes and **(B)** $CD8^+$ T-cells from 15 AS patients stratified according to *rs6600247* genotype. Statistical analysis performed with Welch's two-sample *t* test; **(C)** Expression of *RUNX3* in an historical RNA-seq dataset (Li et al., 2017) obtained from PBMCs, stratified on *rs6600247*.

two-sample *t* test). We also repeated these experiments using $CD8^+$ T-cells and Jurkat NE, showing no differential binding between the two alleles (**Supplementary Figure S1**).

The *RUNX3* Promoter Interacts With the *rs4648889* Region Rather Than *rs6600247*

We used 3C to test plausible chromosome looping interactions between the AS-associated SNP *rs6600247* and the *RUNX3* distal promoter. **Figure 3A** shows the RUNX3 genomic region interrogated, the location of the primers and Sac1 restriction sites. Baits were designed to capture Sac1 fragments containing *rs6600247*, three intergenic fragments with H3K4me1 enrichment, and additionally with a previously-studied AS-associated SNP *rs4648889*. There was very low interaction frequency between *rs6600247* and the distal *RUNX3* promoter, either in U937 or Jurkat cells. A stronger interaction frequency was observed between the distal promoter and the region encompassing the AS-associated SNP *rs4648889* (**Figure 3B**) confirming its functional role.

rs6600247 Genotype has No Effect on *RUNX3* Expression

Primary $CD14^+$ monocytes and $CD8^+$ T-cells from AS patients were used to evaluate *RUNX3* mRNA expression stratified on *rs6600247* genotype ($n = 5$ each genotype) (**Figures 4A,B**). There was a non-significant trend for lower expression in $CD14^+$ monocytes with the AS-risk CC genotype compared to protective TT and heterozygous TC genotypes (TT vs CC: 4.6 ± 1.8 vs 2.0 ± 0.4; TT vs CT: 4.6 ± 1.8 vs 1.8 ± 0.2; CC vs CT: 2.0 ± 0.4 vs 1.8 ± 0.2, results are expressed as mean ± standard error mean). We also analysed historical RNA-seq data (Li et al., 2017) obtained from AS case PBMCs measuring *RUNX3* mRNA expression, stratified on *rs6600247*: there was no apparent influence from this SNP on *RUNX3* expression (**Figure 4C**).

DISCUSSION

In this study, we have demonstrated that the lead AS-associated SNP in the *RUNX3* region, *rs6600247*, affects the binding of

c-MYC to the region of DNA 13 kb upstream of the *RUNX3* promoter, which lies in a region of open chromatin in $CD14^+$ monocytes. Further, this region showed enrichment for H3K4Me1 modification in the absence of H3K4me3 or H3K27ac enrichment, suggesting a weak or poised enhancer (Gasperini et al., 2020). Although GWAS have identified hundreds of genetic variants associated with AS (Cortes et al., 2013), only a very small portion of these have been investigated to define causal variants. Cell type and stimulation conditions must be taken carefully in consideration in identifying causal SNPs, as both impact on chromatin interaction and gene regulation (Shi et al., 2021).

Recent findings have shown that RUNX3 is highly expressed in monocytes where it has a role in transcriptional repression, metabolic regulation, and in tuning the function of $CD14^+$ monocytes. (Puig-Kröger et al., 2010; Estecha et al., 2012). Expression of RUNX3 has been found also in CD11c + mature intestinal macrophages, suggesting a role for this TF in macrophage maturation (Corbin et al., 2020). Further, both RUNX3 and another TF, ID2 (Inhibitor of DNA binding 2), are required for the differentiation of epidermal Langerhans cells from monocytes (Fainaru et al., 2004).

The interaction between RUNX3 and c-MYC has previously been investigated in T-cell lymphoma (Selvarajan et al., 2017). Double immunofluorescence revealed co-localization of both proteins in the tumour nuclei. In addition, several binding-sites for c-MYC were identified in the RUNX3 enhancer region. Additional evidence for this interaction stems from colorectal cancer studies where upregulation of RUNX3 by Bone Morphogenetic Protein (BMP) reduces c-MYC expression, thereby exerting c-MYC tumour-suppressor activity (Lee et al., 2010). Recently, it has been demonstrated that two super-enhancers located at 59 and 70 kb upstream of the RUNX3 transcription start site are required for both RUNX3 and MYC expression and function (Hosoi et al., 2021). Other studies also indicate a key role of c-MYC in monocyte/macrophage activation, as it is involved in the regulation of different alternative activation genes (Pello et al., 2012).

Our EMSA/WEMSA experiments confirmed c-MYC binding at the *rs6600247* locus, with the AS-risk allele disrupting the binding motif and consequently reducing formation of the c-MYC-DNA complex. Altogether, these observations are consistent with the hypothesis that c-MYC can bind the *RUNX3* promoter and/or regulatory elements upstream of the promoter thereby potentially playing a role in the regulation of *RUNX3*. The processes involved in transcriptional regulation are complex and this finding does not exclude the possibility of other TFs being involved.

The genome is organized in a very dynamic way and TFs mediate chromosome loops to bring enhancers and promoters together (de Wit and de Laat, 2012; Palstra and Grosveld, 2012). 3C and related techniques are the classic approach to demonstrating interactions between target genes and enhancers or enhancer-like regions. Here we demonstrate the presence of chromatin loop between a SNP overlapping a regulatory region and the distal promoter of *RUNX3* using 3C followed by qPCR. This method has been used extensively to demonstrate interactions between various regulatory regions in different cell types and it allows one to quantitate the interaction frequency (Sati and Cavalli, 2017; McCord et al., 2020).

We accept that recent findings highlight the fact that contact frequencies from 3C assays sometimes do not correspond to 3D proximity (Williamson et al., 2014), but taken together with the functional data presented here and other recently published findings (Vecellio et al., 2016; Vecellio et al., 2018; Vecellio et al., 2021) we are confident in our results. However, we are aware that other higher throughput techniques have been developed (eg. 4C, 5C, Hi-C) that might give a more general overview of the regulation of the *RUNX3* locus and the genetic interactions of the SNPs in this region. These will be incorporated into our ongoing genome-wide studies of chromatin interactions and the regulatory effects of AS-associated genetic variants.

Here, we have demonstrated physical interactions between the distal promoter of *RUNX3* and *rs4648889* SNP, which we have previously functionally characterized (Vecellio et al., 2016; Vecellio et al., 2021). Conversely, there was a very low interaction frequency with *rs6600247* that suggests no functional role for this SNP in chromosome looping in the particular context of $CD8^+$ T-cells or monocytes. As previously shown, in a ~15 kb linkage disequilibrium (LD) block upstream the promoter of *RUNX3*, there are 22 *RUNX3* SNPs that are strongly associated with AS ($p \leq 10^{-14}$) (Vecellio et al., 2016). The SNP analysed in this work, *rs6600247* ($p = 1.3 \times 10^{-14}$), is in complete LD with *rs4648889* (~2 kb upstream of the *RUNX3* promoter). Conditional analysis established the primacy of the *rs4648889* association with AS at *RUNX3* (Vecellio et al., 2016), while not excluding additional functional roles for other SNPs in LD with it. The functional experiments described here and in previous publications (Vecellio et al., 2016; Vecellio et al., 2018; Vecellio et al., 2021) represent an approach to identifying more precisely which SNPs in this LD block actually have a functional impact on the *RUNX3* regulatory element and its role in the pathogenesis of AS.

Clearly the presence of an enhancer-promoter loop alone does not ensure activation of a target gene but it provides a platform where transcription factors can bind and regulate gene/s (Espinola et al., 2021; Ing-Simmons et al., 2021). Here, we have confirmed that the genomic regulatory element upstream of the *RUNX3* promoter has potentially important cell-type-specific functional effects. We show that the *rs6600247* AS-risk allele affects c-MYC binding in monocytes, suggesting that c-MYC/RUNX3 modulated pathways could have a role in the pathophysiology of AS. Nevertheless, the region encompassing *rs6600247* has no significant physical interaction with the distal *RUNX3* promoter, thereby confirming that *rs4648889* appears to be the cardinal genetic variant associated with AS at the *RUNX3* locus.

Further studies are required to identify additional higher order chromatin interactions at this locus. For example, HiChIP has been used to delineate promoter-enhancer interactions in keratinocytes and $CD8^+$ T-cell lines exploring psoriasis and psoriatic arthritis disease-associated SNPs and similar methods could be explored in AS (Shi et al., 2020a; Shi et al., 2020b). It is also critical that cell-type and -context specificity are crucial for TF binding and activity, and can also influence chromatin looping data (Nancy et al., 2021). While we have presented here and elsewhere evidence for the involvement of $CD8^+$ T-cells and monocytes in the pathogenesis of AS other cell types must also be considered. These include numerous types

found in bone and cartilage, various other components of the immune system and also cells in the gut where chronic low-grade inflammation is a feature of AS in around two-thirds of cases (Ciccia et al., 2016; Shao et al., 2021). In the future targeted *RUNX3* enhancer element genomic editing strategies could be used to elucidate their effects on RUNX3 (and other gene) expression and downstream cellular signaling.

In conclusion, this work provides new insights into the complex transcriptional regulation of *RUNX3* and the role that AS-associated SNPs may play in this process. We highlight the importance of functional studies in determining which disease associated SNPs are primarily involved in the pathogenesis of such diseases and the importance of interrogating their role in the appropriate cellular context.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by COREC 06/Q1606/139 and OXREC B 07/Q1605/35. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CC, MV, JK and BW conceived and designed the experiments. MV and CC performed the experiments. MV, CC and CD analysed the data. MV, CC, JK and BW drafted the manuscript, and all the authors revised the final version prior submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.741867/full#supplementary-material

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