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Genetically determined high activities of the TNF-alpha, IL23/IL17, and NFkB pathways were associated with increased risk of ankylosing spondylitis

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Abstract

Background: Ankylosing spondylitis (AS) results from the combined effects of susceptibility genes and environmental factors. Polymorphisms in genes regulating inflammation may explain part of the heritability of AS.

Methods: Using a candidate gene approach in this case-control study, 51 mainly functional single nucleotide polymorphisms (SNPs) in genes regulating inflammation were assessed in 709 patients with AS and 795 controls. Data on the patients with AS were obtained from the DANBIO registry where patients from all of Denmark are monitored in routine care during treatment with conventional and biologic disease modifying anti-rheumatic drugs (bDMARDs). The results were analyzed using logistic regression (adjusted for age and sex).

Results: Nine polymorphisms were associated with risk of AS ($p < 0.05$). The polymorphisms were in genes regulating a: the TNF-α pathway ($\text{TNF} -308 \ G > A$ (rs1800629), and $-238 \ G > A$ (rs361525); $\text{TNFRSF1A} -609 \ G > T$ (rs4149570), and $\text{PTPN22} -609 \ G > T$ (rs4149570); $\text{TNFRSF1A} -609 \ G > T$ (rs4149570)); b: the IL23/IL17 pathway ($\text{IL23R} G > A$ (rs11209026), and $\text{IL18} -137 \ G > C$ (rs187238)); or c: the NFkB pathway ($\text{TLR1} 743 \ T > C$ (rs4833095), $\text{TLR4} T > C$ (rs1554973), and $\text{LY96} -1625 \ C > G$ (rs11465996)). After Bonferroni correction the homozygous variant genotype of $\text{TLR1} 743 \ T > C$ (rs4833095) (odds ratios (OR): 2.59, 95% confidence interval (CI): 1.48–4.51, $p = 0.04$), and $\text{TNFRSF1A} -609 \ G > T$ (rs4149570) (OR: 1.79, 95% CI: 1.31–2.41, $p = 0.01$) were associated with increased risk of AS and the combined homozygous and heterozygous variant genotypes of $\text{TNF} -308 \ G > A$ (rs1800629) (OR: 0.56, 95% CI: 0.44–0.72, $p = 0.0002$) were associated with reduced risk of AS.

Conclusion: We replicated associations between AS and the polymorphisms in $\text{TNF}$ (rs1800629), $\text{TNFRSF1A}$ (rs4149570), and $\text{IL23R}$ (rs11209026). Furthermore, we identified novel risk loci in $\text{TNF}$ (rs361525), $\text{IL18}$ (rs187238), $\text{TLR1}$ (rs4833095), $\text{TLR4}$ (rs1554973), and $\text{LY96}$ (rs11465996) that need validation in independent cohorts. The results suggest that genetically determined high activity of the TNF-α, IL23/IL17, and NFkB pathways increase risk of AS.

Keywords: Ankylosing spondylitis, Single nucleotide polymorphism, SNP, Case-control study
Background
Ankylosing spondylitis (AS) is a type of spondyloarthritis in which hallmark clinical features are inflammation at entheses and subchondral bone of the pelvic and spinal joints with subsequent abnormal new bone formation at these sites. Ultimately, this leads to ossification of entheses and joints resulting in loss of joint mobility. The incidence varies between 0.1 and 1.8% with the highest incidence in Scandinavia. Onset is typically in young adults with a male predominance. Medications used include non-steroid anti-inflammatory drugs (NSAIDs), and biological disease-modifying anti-rheumatic drugs (bDMARDs), i.e. tumor necrosis factor-α inhibitors (anti-TNF) and more recently also an interleukin(IL)-17A inhibitor (secukinumab) [1].

The cause of AS is unknown but is believed to involve a combination of genetic and environmental factors [2]. The heritability is polygenic and estimated to exceed 90%, with the HLA-B27 allele as the major contributor accounting for approximately 25% of the heritability of AS [2]. The IL-17/IL-23 pathway and the TNF-α pathway are central in the pathogenesis of AS and alterations in these pathways have been shown in mouse models to affect development and severity of enthesitis [3, 4].

TNF-α can be activated by Pathogen-Associated Molecular Patterns (PAMPs) such as bacterial or viral DNA, flagellin, or lipopolysaccharide (LPS), through the NFkB pathway. PAMPs can be recognized by Toll-like receptors (TLRs) thereby initiating a kinase cascade which phosphorylates and degrades the NFkB inhibitor IkBα [5]. This releases NFkB which is transported from the cytosol to the nucleus where it initiates expression of pro- and anti-inflammatory cytokines including TNF-α and IL-17 (http://www.bu.edu/nf-kb/gene-resources/target-genes/). The TNF-α and NFkB pathway are intertwined and TNF-α can feedback stimulate NFkB by binding to TNF receptors (TNFR1 or TNFR2), resulting in a kinase cascade similar to, but distinct from, the pathway induced by TLRs [5].

The IL23/IL17 pathway can also stimulate TNF-α activity. The pro-inflammatory cytokine IL-17 enhances the production of other pro-inflammatory cytokines including TNF-α, and the secretion IL-17 itself can be enhanced by IL-23 [6].

PAMPs can also be recognized by intracellular Nod-like receptors (NLRs). In turn, NLRs can activate pro-inflammatory cytokines including IL-18 [7]. IL-18 is involved in the IL23/IL17 pathway and can enhance the production of IL-17 [8].

The aim of this study was to assess whether functional single nucleotide polymorphisms (SNPs) in genes involved in the TNF-α, IL23/IL17, NFκB, and other pro- and anti-inflammatory pathways were associated with risk of AS.

Methods
Patients and samples

The DANBIO registry includes prospectively collected clinical data on patients with inflammatory joint diseases including smoking status, disease characteristics e.g. HLA-B27 status, disease activity, treatment, and treatment outcomes. Patients from all of Denmark are monitored in routine care during treatment with conventional and biologic disease modifying anti-rheumatic drugs (bDMARDs) [9].

Screening for tuberculosis before initiation of treatment with biological drugs is routinely performed in Denmark. Left over blood clots (after whole blood analysis for Mycobacterium tuberculosis) were collected from all patients screened for tuberculosis at Statens Serum Institut (Copenhagen, Denmark) from 01.09.2009 to 31.01.2013; the Department of Respiratory Diseases B and the Department of Clinical Microbiology, Aarhus University Hospital (Aarhus, Denmark) from 01.01.2011 to 31.01.2014; the Department of Clinical Biochemistry, Herlev and Gentofte Hospital (Hellerup, Denmark) from 01.03.2012 to 31.01.2014; the Department of Biochemistry, Hospital of Lillebaelt (Vejle, Denmark); and the Department of Biochemistry, Hospital of Slagelse (Slagelse, Denmark) from 01.01.2014 to 31.01.2014. Furthermore, from 01.01.2013 to 31.12.2013 blood samples were collected from all patients with AS treated with or without anti-TNF drugs at the Department of Rheumatology, Frederiksberg Hospital (Frederiksberg, Denmark).

By linking the unique personal identification number of Danish citizens (CPR-number) from each blood sample with the clinical data from DANBIO, 709 patients with AS (ICD-10: M45.9) were identified. The control group consisted of 795 healthy blood donors recruited from Viborg, Denmark.

Genotyping

Fifty-one SNPs in genes involved in the TNF-α, IL23/IL17, NFκB, and other pro- and anti-inflammatory pathways were assessed. A list of all SNPs studied and genotype distribution is presented in Table 1 and SNPs associated with AS are summarized in Table 2.

DNA extraction (Maxwell 16 LEV Blood DNA Kit; Promega, Madison, WI, USA) was performed as described by Bank et al. [10]. For the healthy controls, DNA was extracted from EDTA-stabilized peripheral blood by either PureGene (Qiagen, Hilden, Germany) or Wizard Genomic (Promega, Madison, Wisconsin, USA) DNA purification kit according to the manufacturers’ instructions [11–17]. Competitive Allele-Specific Polymerase chain reaction (KASP™), an end-point PCR technology, was used by LGC Genomics for genotyping (LGC Genomics, Hoddesdon, United Kingdom) (http://www.lgcgenomics.com/).
Table 1. Odds ratios (OR) and 95% confidence interval (95CI) for genotypes studied among healthy controls and patients with ankylosing spondylitis (AS)

| Gene | rs-number | Healthy controls | AS | Unadjusted | Adjusted, age & sex | Adjusted, age, sex & smoking |
|------|-----------|-----------------|----|------------|---------------------|-----------------------------|
|      |           | OR (95 CI)      | p  | OR (95 CI) | p                   | OR (95 CI) | p |
| TLR1 | rs4833095  |                 |    |            |                     |               |
| TT   | 485       | 1.07 (0.86–1.33) | 0.57 | 1.03 (0.82–1.29) | 0.83 | 1.05 (0.78–1.42) | 0.73 |
| TC   | 261       | 2.51 (1.45–4.34) | 0.00095 | 2.59 (1.48–4.51) | 0.00081 | 2.86 (1.44–5.68) | 0.0026 |
| CC   | 20        | 1.17 (0.95–1.44) | 0.15 | 1.14 (0.91–1.41) | 0.25 | 1.18 (0.89–1.58) | 0.26 |
| TLR2 | rs3804099  |                 |    |            |                     |               |
| TT   | 241       | 1.10 (0.87–1.40) | 0.42 | 1.07 (0.84–1.37) | 0.58 | 1.02 (0.73–1.42) | 0.90 |
| TC   | 393       | 1.21 (0.89–1.63) | 0.22 | 1.24 (0.91–1.68) | 0.17 | 1.30 (0.87–1.96) | 0.20 |
| CC   | 144       | 1.13 (0.90–1.41) | 0.29 | 1.11 (0.89–1.40) | 0.36 | 1.10 (0.80–1.50) | 0.57 |
| TLR2 | rs11938228 |                 |    |            |                     |               |
| CC   | 327       | 0.89 (0.71–1.10) | 0.27 | 0.86 (0.69–1.07) | 0.17 | 0.80 (0.60–1.08) | 0.15 |
| AA   | 76        | 0.95 (0.66–1.36) | 0.76 | 0.92 (0.63–1.33) | 0.66 | 1.03 (0.62–1.69) | 0.92 |
| CA   | 368       | 0.90 (0.73–1.10) | 0.30 | 0.87 (0.70–1.07) | 0.19 | 0.84 (0.63–1.11) | 0.22 |
| TLR2 | rs4696480  |                 |    |            |                     |               |
| AA   | 199       | 0.93 (0.72–1.19) | 0.55 | 0.89 (0.69–1.15) | 0.38 | 0.94 (0.60–1.18) | 0.31 |
| AT   | 417       | 1.21 (0.90–1.63) | 0.20 | 1.16 (0.86–1.58) | 0.33 | 1.18 (0.78–1.78) | 0.44 |
| TT   | 155       | 1.00 (0.79–1.27) | 0.97 | 0.97 (0.76–1.23) | 0.78 | 0.92 (0.67–1.27) | 0.62 |
| TLR4 | rs5030728  |                 |    |            |                     |               |
| GG   | 359       | 1.03 (0.83–1.28) | 0.80 | 1.01 (0.81–1.27) | 0.91 | 0.93 (0.69–1.25) | 0.62 |
| GA   | 323       | 1.00 (0.70–1.43) | 1.00 | 0.98 (0.68–1.42) | 0.93 | 0.87 (0.53–1.42) | 0.57 |
| AA   | 78        | 1.02 (0.83–1.26) | 0.83 | 1.01 (0.82–1.25) | 0.94 | 0.91 (0.69–1.21) | 0.53 |
| TLR4 | rs1554973  |                 |    |            |                     |               |
| TT   | 440       | 1.07 (0.86–1.33) | 0.55 | 1.06 (0.85–1.32) | 0.62 | 0.98 (0.73–1.32) | 0.90 |
| TC   | 272       | 0.59 (0.38–0.92) | 0.02 | 0.55 (0.34–0.86) | 0.01 | 0.68 (0.38–1.23) | 0.20 |
| CC   | 62        | 0.98 (0.80–1.21) | 0.85 | 0.96 (0.78–1.19) | 0.72 | 0.93 (0.70–1.24) | 0.63 |
| TLR4 | rs12377632 |                 |    |            |                     |               |
| TT   | 306       | 1.01 (0.81–1.26) | 0.96 | 1.05 (0.84–1.32) | 0.66 | 1.07 (0.78–1.46) | 0.67 |
| TC   | 358       | 1.06 (0.77–1.47) | 0.71 | 1.11 (0.80–1.55) | 0.52 | 1.41 (0.92–2.17) | 0.12 |
| CC   | 102       | 1.02 (0.83–1.26) | 0.86 | 1.06 (0.86–1.32) | 0.58 | 1.14 (0.85–1.53) | 0.37 |
| TLR5 | rs5744168  |                 |    |            |                     |               |
| CC   | 672       | 1.17 (0.95–1.44) | 0.15 | 1.14 (0.91–1.41) | 0.25 | 1.18 (0.89–1.58) | 0.26 |
Table 1  Odds ratios (OR) and 95% confidence interval (95CI) for genotypes studied among healthy controls and patients with ankylosing spondylitis (AS) (Continued)

| Gene  | rs-number | Healthy controls | AS | Unadjusted OR (95 CI) | p | Adjusted, age & sex OR (95CI) | p | Adjusted, age & sex & smoking OR (95 CI) | p |
|-------|-----------|------------------|----|-----------------------|---|-------------------------------|---|------------------------------------------|---|
| CT    | 94        | 89               |    | 1.05 (0.77–1.43)      | 0.75 | 1.05 (0.77–1.45) | 0.74 | 0.89 (0.58–1.37) | 0.60 |
| TT    | 5         | 2                |    | 0.44 (0.09–2.30)      | 0.33 | 0.45 (0.08–2.43) | 0.35 | 0.04 (0.00–3.54) | 0.16 |
| CT or TT | 99      | 91               |    | 1.02 (0.75–1.39)      | 0.89 | 1.02 (0.75–1.40) | 0.88 | 0.84 (0.55–1.29) | 0.43 |
| TLR5  | rs5744174 |                  |    |                       |     |                               |    |                                          |    |
| TT    | 215       | 216              |    |                       |     |                               |    |                                          |    |
| TC    | 399       | 337              |    | 0.84 (0.66–1.07)      | 0.15 | 0.85 (0.67–1.09) | 0.20 | 0.82 (0.60–1.14) | 0.24 |
| CC    | 144       | 138              |    | 0.95 (0.71–1.29)      | 0.76 | 1.02 (0.75–1.39) | 0.91 | 0.87 (0.57–1.32) | 0.51 |
| TC or CC | 543  | 475              |    | 0.87 (0.69–1.09)      | 0.23 | 0.90 (0.71–1.13) | 0.36 | 0.84 (0.62–1.14) | 0.26 |
| TLR9  | rs187084  |                  |    |                       |     |                               |    |                                          |    |
| TT    | 262       | 237              |    |                       |     |                               |    |                                          |    |
| TC    | 366       | 335              |    | 1.01 (0.80–1.27)      | 0.92 | 1.03 (0.82–1.31) | 0.78 | 1.09 (0.79–1.50) | 0.60 |
| CC    | 142       | 120              |    | 0.93 (0.69–1.26)      | 0.66 | 0.91 (0.67–1.24) | 0.56 | 1.07 (0.71–1.61) | 0.76 |
| TC or CC | 508  | 455              |    | 0.99 (0.80–1.23)      | 0.93 | 1.00 (0.80–1.25) | 0.98 | 1.08 (0.80–1.46) | 0.60 |
| TLR9  | rs352139  |                  |    |                       |     |                               |    |                                          |    |
| GG    | 255       | 211              |    |                       |     |                               |    |                                          |    |
| GA    | 347       | 324              |    | 1.13 (0.89–1.43)      | 0.32 | 1.08 (0.85–1.38) | 0.52 | 1.01 (0.73–1.40) | 0.93 |
| AA    | 167       | 139              |    | 1.01 (0.75–1.34)      | 0.97 | 0.96 (0.71–1.30) | 0.79 | 0.80 (0.53–1.20) | 0.27 |
| GA or AA | 514  | 463              |    | 1.09 (0.87–1.36)      | 0.45 | 1.04 (0.83–1.31) | 0.72 | 0.94 (0.69–1.27) | 0.68 |
| LY96  | rs11465996|                  |    |                       |     |                               |    |                                          |    |
| CC    | 344       | 341              |    |                       |     |                               |    |                                          |    |
| CG    | 337       | 298              |    | 0.89 (0.72–1.11)      | 0.30 | 0.91 (0.73–1.14) | 0.42 | 0.89 (0.66–1.20) | 0.45 |
| GG    | 81        | 53               |    | 0.66 (0.45–0.96)      | 0.03 | 0.68 (0.46–1.00) | 0.0498 | 0.65 (0.39–1.10) | 0.11 |
| CG or GG | 418  | 351              |    | 0.85 (0.69–1.04)      | 0.11 | 0.87 (0.70–1.07) | 0.18 | 0.84 (0.63–1.12) | 0.24 |
| CD14  | Rs2569190 |                  |    |                       |     |                               |    |                                          |    |
| GG    | 236       | 194              |    |                       |     |                               |    |                                          |    |
| GA    | 360       | 339              |    | 1.15 (0.90–1.46)      | 0.27 | 1.18 (0.92–1.51) | 0.19 | 1.27 (0.91–1.78) | 0.16 |
| AA    | 170       | 157              |    | 1.12 (0.84–1.50)      | 0.43 | 1.20 (0.89–1.61) | 0.24 | 1.46 (0.98–2.19) | 0.06 |
| GA or AA | 530  | 496              |    | 1.14 (0.91–1.43)      | 0.26 | 1.18 (0.94–1.50) | 0.15 | 1.32 (0.96–1.82) | 0.08 |
| TIRAP | rs8177374 |                  |    |                       |     |                               |    |                                          |    |
| CC    | 556       | 521              |    |                       |     |                               |    |                                          |    |
| CT    | 185       | 159              |    | 0.92 (0.72–1.17)      | 0.49 | 0.99 (0.77–1.27) | 0.94 | 1.38 (0.99–1.91) | 0.06 |
| TT    | 21        | 15               |    | 0.76 (0.39–1.49)      | 0.43 | 0.76 (0.38–1.53) | 0.45 | 1.31 (0.55–3.12) | 0.55 |
| CT or TT | 206  | 174              |    | 0.90 (0.71–1.14)      | 0.39 | 0.97 (0.76–1.23) | 0.81 | 1.38 (1.00–1.89) | 0.047 |
| SUMO4 | rs237025  |                  |    |                       |     |                               |    |                                          |    |
| TT    | 215       | 195              |    |                       |     |                               |    |                                          |    |
| TC    | 362       | 358              |    | 1.09 (0.86–1.39)      | 0.48 | 1.08 (0.84–1.38) | 0.55 | 1.04 (0.75–1.44) | 0.80 |
| CC    | 195       | 136              |    | 0.77 (0.57–1.03)      | 0.08 | 0.75 (0.55–1.01) | 0.06 | 0.55 (0.36–0.84) | 0.01 |
| TC or CC | 557  | 494              |    | 0.98 (0.78–1.23)      | 0.85 | 0.96 (0.76–1.22) | 0.75 | 0.87 (0.64–1.19) | 0.38 |
Table 1 Odds ratios (OR) and 95% confidence interval (95CI) for genotypes studied among healthy controls and patients with ankylosing spondylitis (AS) (Continued)

| Gene rs-number | Healthy controls | AS | Unadjusted OR (95 CI) | p | Adjusted, age & sex OR (95 CI) | p | Adjusted, age, sex & smoking OR (95 CI) | p |
|----------------|------------------|----|-----------------------|---|-------------------------------|---|----------------------------------------|---|
| NFKBIA rs696   |                  |    |                       |   |                               |   |                                        |   |
| GG             | 298              | 259| 1.06 (0.85–1.32)      | 0.63| 1.06 (0.84–1.33)              | 0.64| 1.02 (0.75–1.39)                     | 0.88|
| GA             | 366              | 336| 1.03 (0.74–1.43)      | 0.88| 0.97 (0.69–1.36)              | 0.86| 1.07 (0.67–1.69)                     | 0.78|
| AA             | 101              | 90 | 1.05 (0.85–1.30)      | 0.65| 1.04 (0.84–1.29)              | 0.73| 1.03 (0.77–1.38)                     | 0.84|
| NFKB1 rs28362491|                 |    |                       |   |                               |   |                                        |   |
| Ins/Ins        | 269              | 258| 0.88 (0.70–1.10)      | 0.25| 0.89 (0.70–1.12)              | 0.31| 0.74 (0.54–1.01)                     | 0.06|
| −/−            | 122              | 100| 0.85 (0.62–1.17)      | 0.33| 0.82 (0.59–1.13)              | 0.22| 0.78 (0.51–1.19)                     | 0.25|
| Ins/− or −/-   | 498              | 416| 0.87 (0.70–1.08)      | 0.21| 0.87 (0.70–1.08)              | 0.21| 0.75 (0.56–1.01)                     | 0.06|
| TNF rs1800629  |                  |    |                       |   |                               |   |                                        |   |
| GG             | 527              | 549| 0.56 (0.43–0.71)      | 0.0000032| 0.58 (0.45–0.75)              | 0.000029| 0.63 (0.45–0.89)                     | 0.01|
| GA             | 223              | 129| 0.35 (0.16–0.75)      | 0.01| 0.39 (0.18–0.85)              | 0.02| 0.19 (0.04–0.79)                     | 0.02|
| AA             | 25               | 9 | 1.00 (1.00–1.00)      | 0.00 | 1.00 (1.00–1.00)              | 1.00| 1.00 (1.00–1.00)                     | 1.00|
| T NF rs361525  |                  |    |                       |   |                               |   |                                        |   |
| GG             | 708              | 669| 0.85 (0.70–1.03)      | 0.21| 0.87 (0.70–1.08)              | 0.21| 0.75 (0.56–1.01)                     | 0.06|
| GA             | 60               | 30 | 0.53 (0.34–0.83)      | 0.01| 0.52 (0.32–0.82)              | 0.0049| 0.61 (0.33–1.12)                     | 0.11|
| AA             | 3                | 0 | 1.00 (1.00–1.00)      | 0.00 | 1.00 (1.00–1.00)              | 1.00| 1.00 (1.00–1.00)                     | 1.00|
| TNFRSF1A rs4149570|              |    |                       |   |                               |   |                                        |   |
| GG             | 307              | 217| 1.35 (1.07–1.70)      | 0.01| 1.33 (1.05–1.68)              | 0.02| 1.46 (1.06–2.00)                     | 0.02|
| GT             | 355              | 339| 1.71 (1.26–2.33)      | 0.000060| 1.79 (1.31–2.46)              | 0.00027| 2.26 (1.48–3.47)                     | 0.00017|
| TT             | 109              | 132| 1.44 (1.16–1.78)      | 0.0010| 1.44 (1.15–1.80)              | 0.0013| 1.64 (1.21–2.22)                     | 0.0014|
| TNFAIP3 rs6927172|              |    |                       |   |                               |   |                                        |   |
| GG             | 473              | 415| 1.06 (0.85–1.32)      | 0.61| 1.06 (0.85–1.33)              | 0.61| 1.03 (0.76–1.39)                     | 0.85|
| CT             | 297              | 299| 0.70 (0.42–1.19)      | 0.20| 0.70 (0.41–1.19)              | 0.18| 0.51 (0.23–1.10)                     | 0.09|
| TT             | 86               | 53 | 1.02 (0.83–1.26)      | 0.83| 1.00 (0.81–1.23)              | 0.97| 1.14 (0.86–1.52)                     | 0.35|
| TGFB1 rs1800469|                  |    |                       |   |                               |   |                                        |   |
| CC             | 383              | 344| 1.12 (0.90–1.39)      | 0.30| 1.08 (0.87–1.35)              | 0.48| 1.28 (0.95–1.71)                     | 0.11|
| CT             | 297              | 299| 0.69 (0.47–1.00)      | 0.047| 0.69 (0.47–1.02)              | 0.06| 0.69 (0.40–1.17)                     | 0.17|
| TT             | 86               | 53 | 1.01 (0.82–1.26)      | 0.91| 1.00 (0.81–1.23)              | 0.97| 1.14 (0.86–1.52)                     | 0.35|
| PTPN22 rs2476601|              |    |                       |   |                               |   |                                        |   |
| GG             | 588              | 557|                       |   |                               |   |                                        |   |
Table 1: Odds ratios (OR) and 95% confidence interval (95CI) for genotypes studied among healthy controls and patients with ankylosing spondylitis (AS) (Continued)

| Gene name | rs-number | Healthy controls OR (95 CI) | Unadjusted | Adjusted, age & sex | Adjusted, age, sex & smoking |
|-----------|-----------|-----------------------------|------------|---------------------|-----------------------------|
| GA        | 166       | 0.78 (0.60–1.01) 0.06       | 0.77 (0.59–1.00) 0.05 | 0.75 (0.52–1.09) 0.13 |
| AA        | 11        | 0.58 (0.21–1.57) 0.28       | 0.57 (0.20–1.58) 0.28 | 0.83 (0.21–3.28) 0.80 |
| GA or AA  | 177       | 0.76 (0.59–0.99) 0.04       | 0.76 (0.58–0.98) 0.04 | 0.76 (0.53–1.09) 0.13 |
| PPARC     | rs1801282 |                             |            |                     |                             |
| CC        | 548       | 0.87 (0.68–1.10) 0.23       | 0.85 (0.66–1.08) 0.18 | 0.87 (0.63–1.21) 0.42 |
| CG        | 207       | 1.15 (0.55–2.40) 0.71       | 1.33 (0.62–2.83) 0.46 | 1.54 (0.60–3.98) 0.37 |
| GG        | 14        | 0.88 (0.70–1.11) 0.29       | 0.88 (0.69–1.11) 0.27 | 0.91 (0.67–1.26) 0.58 |
| IL1B      | rs4848306 |                             |            |                     |                             |
| GG        | 246       | 1.08 (0.85–1.36) 0.52       | 1.09 (0.86–1.39) 0.48 | 1.16 (0.84–1.60) 0.38 |
| AA        | 151       | 0.95 (0.70–1.28) 0.72       | 0.96 (0.71–1.31) 0.81 | 0.88 (0.57–1.34) 0.55 |
| GA or AA  | 524       | 1.04 (0.83–1.30) 0.72       | 1.06 (0.84–1.33) 0.64 | 1.08 (0.79–1.46) 0.64 |
| IL1B      | rs1143623 |                             |            |                     |                             |
| GG        | 401       | 0.97 (0.78–1.20) 0.76       | 0.98 (0.79–1.22) 0.87 | 1.07 (0.80–1.44) 0.66 |
| CC        | 55        | 1.04 (0.69–1.56) 0.85       | 1.12 (0.74–1.69) 0.59 | 0.87 (0.48–1.57) 0.64 |
| GC or CC  | 371       | 0.98 (0.80–1.20) 0.83       | 1.00 (0.81–1.24) 0.98 | 1.04 (0.78–1.38) 0.79 |
| IL1B      | rs1143627 |                             |            |                     |                             |
| GG        | 340       | 1.00 (0.81–1.25) 0.98       | 1.00 (0.79–1.25) 0.97 | 1.05 (0.78–1.42) 0.75 |
| CC        | 97        | 0.99 (0.71–1.37) 0.94       | 1.01 (0.72–1.41) 0.95 | 0.85 (0.53–1.36) 0.50 |
| GC or CC  | 436       | 1.00 (0.81–1.23) 1.00       | 1.00 (0.81–1.24) 1.00 | 1.00 (0.76–1.34) 0.97 |
| IL1RN     | rs4251961 |                             |            |                     |                             |
| TT        | 298       | 1.09 (0.87–1.36) 0.47       | 1.04 (0.83–1.32) 0.71 | 1.22 (0.89–1.67) 0.21 |
| CC        | 112       | 1.13 (0.83–1.55) 0.44       | 1.05 (0.76–1.46) 0.76 | 1.41 (0.92–2.17) 0.12 |
| TC or CC  | 472       | 1.10 (0.89–1.36) 0.40       | 1.05 (0.84–1.30) 0.68 | 1.26 (0.94–1.71) 0.12 |
| IL4R      | rs1805010 |                             |            |                     |                             |
| AA        | 209       | 0.80 (0.63–1.02) 0.08       | 0.79 (0.62–1.02) 0.07 | 0.73 (0.52–1.02) 0.07 |
| AG        | 410       | 0.88 (0.65–1.19) 0.41       | 0.91 (0.67–1.24) 0.55 | 0.87 (0.58–1.33) 0.53 |
| GG        | 157       | 0.83 (0.66–1.04) 0.10       | 0.83 (0.65–1.05) 0.12 | 0.77 (0.56–1.06) 0.11 |
| AG or GG  | 567       |                             |            |                     |                             |
| IL6       | rs10499563|                             |            |                     |                             |
| TT        | 476       | 0.94 (0.76–1.17) 0.60       | 0.94 (0.75–1.18) 0.60 | 0.77 (0.57–1.05) 0.10 |
| TC        | 259       | 0.81 (0.48–1.36) 0.42       | 0.72 (0.42–1.25) 0.24 | 0.80 (0.39–1.63) 0.53 |
| CC        | 35        | 0.93 (0.75–1.14) 0.48       | 0.92 (0.74–1.14) 0.43 | 0.77 (0.57–1.04) 0.09 |
| Gene   | rs-number | Healthy controls | AS | Unadjusted OR (95 CI) | p      | Adjusted, age & sex OR (95 CI) | p      | Adjusted, age, sex & smoking OR (95 CI) | p      |
|--------|-----------|------------------|----|----------------------|--------|-------------------------------|--------|----------------------------------------|--------|
| IL6R   | rs4537545 |                  |    |                      |        |                               |        |                                        |        |
| CC     | 289       | 247              |    | 1.03 (0.82–1.29)     | 0.82   | 1.05 (0.83–1.32)              | 0.71   | 1.07 (0.79–1.47)                      | 0.65   |
| CT     | 369       | 324              |    | 1.13 (0.83–1.54)     | 0.44   | 1.18 (0.86–1.63)              | 0.30   | 1.17 (0.76–1.79)                      | 0.48   |
| TT     | 117       | 113              |    | 1.05 (0.85–1.30)     | 0.64   | 1.08 (0.86–1.34)              | 0.51   | 1.09 (0.81–1.47)                      | 0.55   |
| CT or TT | 486   | 437              |    | 1.05 (0.85–1.30)     | 0.64   | 1.08 (0.86–1.34)              | 0.51   | 1.09 (0.81–1.47)                      | 0.55   |
| IL10   | rs1800872 |                  |    |                      |        |                               |        |                                        |        |
| CC     | 482       | 408              |    | 1.03 (0.83–1.29)     | 0.79   | 1.01 (0.80–1.27)              | 0.94   | 0.93 (0.68–1.26)                      | 0.63   |
| CA     | 258       | 225              |    | 1.42 (0.89–2.26)     | 0.14   | 1.35 (0.83–2.18)              | 0.22   | 1.47 (0.79–2.73)                      | 0.22   |
| AA     | 35        | 42               |    | 1.08 (0.87–1.33)     | 0.50   | 1.05 (0.84–1.30)              | 0.67   | 0.99 (0.74–1.33)                      | 0.95   |
| CA or AA | 293  | 267              |    | 1.08 (0.87–1.33)     | 0.50   | 1.05 (0.84–1.30)              | 0.67   | 0.99 (0.74–1.33)                      | 0.95   |
| IL10   | rs3024505 |                  |    |                      |        |                               |        |                                        |        |
| CC     | 518       | 467              |    | 1.00 (0.80–1.26)     | 0.97   | 1.01 (0.80–1.28)              | 0.95   | 1.19 (0.87–1.61)                      | 0.28   |
| TT     | 22        | 24               |    | 1.21 (0.67–2.19)     | 0.53   | 1.32 (0.72–2.42)              | 0.37   | 1.80 (0.79–4.12)                      | 0.16   |
| CT or TT | 243  | 224              |    | 1.02 (0.82–1.27)     | 0.84   | 1.04 (0.83–1.30)              | 0.76   | 1.23 (0.92–1.66)                      | 0.17   |
| IL12B  | rs3212217 |                  |    |                      |        |                               |        |                                        |        |
| GG     | 499       | 460              |    | 0.92 (0.74–1.16)     | 0.49   | 0.95 (0.75–1.19)              | 0.64   | 0.94 (0.69–1.29)                      | 0.72   |
| GC     | 235       | 200              |    | 0.91 (0.50–1.65)     | 0.76   | 0.94 (0.51–1.72)              | 0.84   | 0.57 (0.23–1.41)                      | 0.22   |
| CC     | 25        | 21               |    | 0.92 (0.74–1.15)     | 0.47   | 0.95 (0.76–1.19)              | 0.63   | 0.91 (0.67–1.23)                      | 0.53   |
| GC or CC | 260  | 221              |    | 0.92 (0.74–1.15)     | 0.47   | 0.95 (0.76–1.19)              | 0.63   | 0.91 (0.67–1.23)                      | 0.53   |
| IL12B  | rs6887695 |                  |    |                      |        |                               |        |                                        |        |
| GG     | 385       | 324              |    | 1.22 (0.98–1.52)     | 0.07   | 1.24 (0.99–1.55)              | 0.06   | 1.31 (0.97–1.77)                      | 0.07   |
| GC     | 293       | 301              |    | 1.16 (0.81–1.66)     | 0.43   | 1.16 (0.80–1.69)              | 0.43   | 0.98 (0.59–1.61)                      | 0.94   |
| CC     | 72        | 70               |    | 1.21 (0.98–1.49)     | 0.07   | 1.22 (0.99–1.51)              | 0.06   | 1.24 (0.93–1.64)                      | 0.14   |
| GC or CC | 365  | 371              |    | 1.21 (0.98–1.49)     | 0.07   | 1.22 (0.99–1.51)              | 0.06   | 1.24 (0.93–1.64)                      | 0.14   |
| IL12B1 | rs401502  |                  |    |                      |        |                               |        |                                        |        |
| CC     | 360       | 304              |    | 1.22 (0.98–1.51)     | 0.08   | 1.21 (0.96–1.51)              | 0.10   | 1.19 (0.88–1.61)                      | 0.26   |
| CG     | 303       | 311              |    | 0.95 (0.67–1.35)     | 0.79   | 0.97 (0.68–1.39)              | 0.87   | 1.18 (0.74–1.88)                      | 0.48   |
| GG     | 87        | 70               |    | 1.16 (0.94–1.42)     | 0.17   | 1.15 (0.93–1.43)              | 0.19   | 1.19 (0.89–1.58)                      | 0.24   |
| IL17A  | rs2275913 |                  |    |                      |        |                               |        |                                        |        |
| GG     | 340       | 307              |    | 0.99 (0.80–1.24)     | 0.94   | 0.98 (0.79–1.23)              | 0.89   | 0.90 (0.67–1.22)                      | 0.51   |
| GA     | 336       | 301              |    | 0.98 (0.70–1.36)     | 0.90   | 1.00 (0.71–1.40)              | 0.98   | 1.00 (0.63–1.57)                      | 0.99   |
| AA     | 95        | 84               |    | 0.99 (0.80–1.22)     | 0.92   | 0.99 (0.80–1.22)              | 0.89   | 0.92 (0.69–1.22)                      | 0.57   |
| IL18   | rs187238  |                  |    |                      |        |                               |        |                                        |        |
| GG     | 387       | 380              |    |                      |        |                               |        |                                        |        |
Table 1 Odds ratios (OR) and 95% confidence interval (95CI) for genotypes studied among healthy controls and patients with ankylosing spondylitis (AS) (Continued)

| Gene | rs-number | Healthy controls | AS | Unadjusted | | Adjusted, age & sex | | Adjusted, age, sex & smoking |
|------|-----------|-----------------|----|------------|---|------------------|---|---------------------|
|      |           | OR (95 CI) | p  | OR (95 CI) | p  | OR (95 CI) | p  |
| GC   | 312       | 259         | 0.85 (0.68–1.05) | 0.13 | 0.83 (0.66–1.03) | 0.09 | 0.74 (0.55–1.00) | 0.049 |
| CC   | 64        | 41          | 0.65 (0.43–0.99) | 0.04 | 0.69 (0.45–1.06) | 0.09 | 0.58 (0.32–1.04) | 0.07 |
| GC or CC | 376     | 300         | 0.81 (0.66–1.00) | 0.0499 | 0.80 (0.65–0.99) | 0.04 | 0.71 (0.53–0.95) | 0.02 |
| IL18 rs1946518 |       |              |     |             |     |             |     |
| GG   | 282       | 259         | 0.99 (0.79–1.24) | 0.91 | 0.96 (0.76–1.12) | 0.71 | 0.89 (0.65–1.21) | 0.45 |
| GT   | 363       | 329         | 0.93 (0.68–1.29) | 0.68 | 0.95 (0.68–1.31) | 0.74 | 0.80 (0.51–1.24) | 0.32 |
| TT   | 113       | 97          | 0.97 (0.79–1.21) | 0.81 | 0.96 (0.77–1.19) | 0.68 | 0.86 (0.64–1.16) | 0.32 |
| IL23R rs11209026 |       |              |     |             |     |             |     |
| GG   | 680       | 646         | 0.59 (0.41–0.85) | 0.0045 | 0.63 (0.43–0.91) | 0.02 | 0.64 (0.38–1.05) | 0.08 |
| GA   | 89        | 50          | 1.00 (1.00–1.00) | 1.00 | 1.00 (1.00–1.00) | 1.00 | 1.00 (1.00–1.00) | 1.00 |
| AA   | 5         | 1           | 0.57 (0.40–0.82) | 0.0021 | 0.60 (0.42–0.87) | 0.01 | 0.63 (0.38–1.03) | 0.06 |
| IFNG rs2430561 |       |              |     |             |     |             |     |
| TT   | 199       | 181         | 1.02 (0.80–1.30) | 0.88 | 1.01 (0.79–1.30) | 0.92 | 1.08 (0.77–1.52) | 0.65 |
| TA   | 398       | 369         | 0.95 (0.70–1.29) | 0.74 | 0.97 (0.71–1.32) | 0.85 | 1.09 (0.72–1.64) | 0.68 |
| AA   | 161       | 139         | 1.00 (0.79–1.26) | 0.99 | 1.00 (0.79–1.27) | 0.99 | 1.08 (0.79–1.50) | 0.62 |
| IFNGR1 rs2234711 |       |              |     |             |     |             |     |
| TT   | 290       | 232         | 1.20 (0.96–1.51) | 0.11 | 1.20 (0.95–1.51) | 0.12 | 1.15 (0.84–1.57) | 0.40 |
| TC   | 361       | 348         | 1.13 (0.83–1.55) | 0.43 | 1.09 (0.79–1.50) | 0.60 | 1.11 (0.72–1.70) | 0.65 |
| CC   | 119       | 108         | 1.19 (0.96–1.47) | 0.12 | 1.17 (0.94–1.46) | 0.16 | 1.14 (0.84–1.53) | 0.40 |
| IFNGR2 rs8126756 |       |              |     |             |     |             |     |
| TT   | 553       | 522         | 0.82 (0.63–1.06) | 0.13 | 0.83 (0.64–1.09) | 0.18 | 0.86 (0.60–1.24) | 0.42 |
| TC   | 168       | 130         | 0.71 (0.54–1.48) | 0.36 | 0.69 (0.32–1.49) | 0.35 | 0.53 (0.18–1.54) | 0.24 |
| CC   | 18        | 12          | 1.00 (0.78–1.29) | 0.98 | 1.00 (0.77–1.30) | 0.99 | 1.01 (0.71–1.42) | 0.97 |
| IFNGR2 rs17882748 |      |              |     |             |     |             |     |
| CC   | 199       | 173         | 1.31 (1.07–1.61) | 0.08 | 1.31 (1.07–1.61) | 0.08 | 1.16 (0.86–1.57) | 0.48 |
| CT   | 391       | 341         | 1.09 (0.85–1.38) | 0.48 | 1.09 (0.85–1.38) | 0.48 | 1.05 (0.76–1.45) | 0.76 |
| TBX21 rs17250932 |       |              |     |             |     |             |     |
| TT   | 526       | 497         | 0.87 (0.69–1.08) | 0.21 | 0.90 (0.72–1.14) | 0.39 | 0.78 (0.56–1.07) | 0.12 |
Power calculation
The Genetic Power Calculator was utilized for power analysis of discrete traits (http://zzz.bwh.harvard.edu/gpc/cc2.html). The lowest minor allele frequency (MAF) of the studied SNPs was 0.10. The ‘high-risk allele frequency’ was set to 0.10, the ‘prevalence’ was set to 0.0018 [18], D-prime was set to 1, type I error rate was set to 0.05 and number of cases and control-case ratio was 795:709. This cohort study had more than 80% chance of detecting a dominant effect with an odds ratio (OR) of 1.4 for AS.

Statistical analysis
Logistic regression was used to compare genotype distributions among patients with AS versus healthy controls. Crude odds ratio, odds ratio adjusted for age and sex, and odds ratio adjusted for age, sex, and smoking status were assessed (Table 1). A chi-square test was

| Gene | rs-number | Healthy controls | AS | Unadjusted OR (95CI) | p | Adjusted, age & sex OR (95CI) | p | Adjusted, age, sex & smoking OR (95CI) | p |
|------|-----------|-----------------|----|---------------------|---|-----------------------------|---|-----------------------------|---|
| NLRP1 | rs2670660 |                 |    |                     |   |                             |   |                             |   |
| AA   |           | 222             | 202| 0.92 (0.73–1.18)    | 0.52| 0.96 (0.75–1.23)            | 0.73| 1.12 (0.80–1.56)            | 0.52|
| AG   |           | 390             | 328| 1.10 (0.82–1.47)    | 0.53| 1.11 (0.82–1.49)            | 0.51| 1.12 (0.75–1.67)            | 0.59|
| GG   |           | 154             | 154| 0.97 (0.78–1.22)    | 0.82| 1.00 (0.79–1.26)            | 0.98| 1.11 (0.81–1.52)            | 0.50|
| AG or GG | 544 | 482             |    | 0.99 (0.79–1.22)    | 0.82| 1.00 (0.79–1.26)            | 0.98| 1.11 (0.81–1.52)            | 0.50|
| NLRP1 | rs878329 |                 |    |                     |   |                             |   |                             |   |
| GG   |           | 217             | 206| 0.89 (0.70–1.13)    | 0.34| 0.89 (0.69–1.14)            | 0.35| 0.99 (0.71–1.38)            | 0.93|
| GC   |           | 394             | 333| 1.05 (0.79–1.41)    | 0.73| 1.05 (0.78–1.41)            | 0.75| 1.03 (0.69–1.54)            | 0.90|
| CC   |           | 155             | 155| 0.94 (0.75–1.17)    | 0.57| 0.93 (0.74–1.18)            | 0.56| 1.00 (0.73–1.36)            | 0.98|
| GC or CC | 549 | 488             |    | 0.97 (0.78–1.22)    | 0.82| 1.00 (0.79–1.26)            | 0.98| 1.11 (0.81–1.52)            | 0.50|
| NLRP3 | rs10754558 |                 |    |                     |   |                             |   |                             |   |
| CC   |           | 294             | 248| 1.08 (0.86–1.36)    | 0.50| 1.06 (0.84–1.34)            | 0.61| 1.10 (0.81–1.51)            | 0.54|
| CG   |           | 355             | 324| 1.24 (0.91–1.69)    | 0.18| 1.25 (0.91–1.71)            | 0.17| 1.11 (0.71–1.72)            | 0.65|
| GG   |           | 111             | 116| 1.12 (0.90–1.39)    | 0.30| 1.11 (0.89–1.38)            | 0.36| 1.11 (0.82–1.49)            | 0.51|
| GC or GG | 466 | 440             |    | 1.18 (0.96–1.45)    | 0.12| 1.23 (0.99–1.52)            | 0.06| 1.24 (0.94–1.65)            | 0.13|
| NLRP3 | rs4612666 |                 |    |                     |   |                             |   |                             |   |
| CC   |           | 435             | 360| 1.20 (0.96–1.49)    | 0.11| 1.23 (0.99–1.54)            | 0.07| 1.28 (0.95–1.72)            | 0.10|
| CT   |           | 280             | 277| 1.09 (0.72–1.66)    | 0.67| 1.19 (0.78–1.82)            | 0.41| 1.07 (0.59–1.94)            | 0.82|
| TT   |           | 53              | 48 | 1.18 (0.96–1.45)    | 0.12| 1.23 (0.99–1.52)            | 0.06| 1.24 (0.94–1.65)            | 0.13|
| CARDS | rs2043211 |                 |    |                     |   |                             |   |                             |   |
| AA   |           | 321             | 298| 1.00 (0.80–1.24)    | 0.97| 0.98 (0.79–1.23)            | 0.89| 0.90 (0.67–1.22)            | 0.50|
| AT   |           | 342             | 316| 0.89 (0.64–1.25)    | 0.52| 0.89 (0.63–1.26)            | 0.50| 0.91 (0.57–1.44)            | 0.68|
| TT   |           | 94              | 78 | 0.97 (0.79–1.20)    | 0.80| 0.96 (0.78–1.19)            | 0.72| 0.90 (0.67–1.19)            | 0.45|
| AT or TT | 436 | 394             |    | 1.01 (0.82–1.25)    | 0.90| 0.98 (0.79–1.21)            | 0.86| 0.86 (0.64–1.14)            | 0.29|

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| Gene   | Rs-number | Pathway            | Model   | OR (95% CI)       | P-value / Bonferroni | Effect of minor-allele                                                                 | Biological interpretation                                                                                                                                                                                                 |
|--------|-----------|--------------------|---------|-------------------|----------------------|----------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| TLR1   | rs4833095 | Pathogen recognition | CC vs TT | 2.59 (1.48–4.51) | 0.00081 / 0.04        | 743C increase TLR1 level in PBMC [56]                                                   | Increased TLR1 level was associated with increased risk of AS. This could indicate that a genetically determined high activity of the NFκB pathway, and thus high TNF-α and IL-17 activity, was associated with increased risk of AS.                                     |
| TLR4   | rs1554973 | Pathogen recognition | CC vs TT | 0.55 (0.34–0.86) | 0.010 / 0.51          | Unknown [67]                                                                            | Increased MD-2 and TNF-α level was associated with a reduced risk of AS. In contrast to the other results this indicates that genetically determined high TNF-driven inflammatory response was associated with reduced risk of AS.                                     |
| LY96   | rs11465996| Pathogen recognition | GG vs CC | 0.68 (0.46–1.00) | 0.049 / 1.00          | -1625G increase MD-2 and TNF-α levels in human U937 cells and whole blood leukocytes [57] | Increased MD-2 and TNF-α level was associated with a reduced risk of AS. In contrast to the other results this indicates that genetically determined high TNF-driven inflammatory response was associated with reduced risk of AS.                                     |
| TNF    | rs1800629 | Cytokines          | GA or AA vs GG | 0.56 (0.44–0.72) | 0.000047 / 0.00024     | -308A increase expression in jurkat cells [65], reduce mRNA level in PBMC and serum [48] or no association was found [49] | Reduced TNF-α mRNA level was associated with reduced risk of AS. This could indicate that genetically determined high TNF-driven inflammatory response was associated with increased risk of AS.                                     |
| TNF    | rs361525  | Cytokines          | GA or AA vs GG | 0.49 (0.31–0.78) | 0.0024 / 0.12          | -238A reduce expression in PBMC [49]                                                   | Reduced TNF-α expression was associated with reduced risk of AS. This indicates that genetically determined high TNF-driven inflammatory response was associated with increased risk of AS.                                     |
| TNFRSF1A | rs4149570 | Cytokines          | GT or TT vs GG | 1.44 (1.15–1.80) | 0.0013 / 0.0066b      | -609 T increase expression in PBMC [50]                                                | Increased TNF-α receptor 1 expression was associated with increased risk of AS. This indicates that genetically determined high TNF-driven inflammatory response was associated with increased risk of AS.                                     |
| PTPN22 | rs2476601 | Immune response    | GA or AA vs GG | 0.76 (0.58–0.98) | 0.037 / 1.00          | 1858A reduce TNF-α level in serum [51]                                                 | Reduced TNF-α level was associated with reduced risk of AS. This indicates that genetically determined high TNF-driven inflammatory response was associated with increased risk of AS.                                     |
| IL18   | rs187238  | Cytokines          | GC or CC vs GG | 0.80 (0.65–0.99) | 0.044 / 1.00          | -137C reduce IL-18 level in serum [53] and expression in PBMC [54]                      | Reduced IL-18 expression, and thus reduced IL-17 and TNF-α activity, was associated with reduced risk of AS. This indicates that a genetically determined high activity of the IL23/IL17 pathway was associated with increased risk of AS.                                     |
| IL23R  | rs11209026| Cytokines          | GA or AA vs GG | 0.60 (0.42–0.87) | 0.0071 / 0.36          | rs11209026A reduce IL-17 level in PBMC [52]                                             | Reduced IL-17 level was associated with reduced risk of AS. This indicates that a genetically determined high activity of the IL23/IL17 pathway was associated with increased risk of AS.                                     |

OR Odds ratio
95% CI 95% confidence interval
PBMC peripheral blood mononuclear cell

**The Bonferroni calculations were based on the 51 SNPs assessed in this study**

**The TNFRSF1A (rs4149570) TT vs GG: OR = 1.79, 95% CI: 1.31–2.41, p = 0.00027, Bonferroni = 0.014**
used to test for deviation from Hardy-Weinberg equilibrium in the healthy controls and for haplotype analysis (Tables 3, 4, 5 and 6).

Statistical analyses were performed using STATA version 15 (StataCorp LP, College Station, TX, USA).

Results

Study population
Among the patients with AS the median age was 32 years (SD: 11.5) and 68% (483/709) were males. The healthy controls had a median age of 43 years (SD: 11.5) and 52% (411/384) were males. Among the patients 37% (118/323), 23% (73/323), and 41% (132/323) and among the controls 26% (207/788), 24% (189/788), and 50% (392/788) were current smokers, former smokers and never smokers, respectively. HLA-B27 staus was available for 498 patients of which 83% (411/498) were positive. Sixty percent (427/709) of the patients were treated with anti-TNF.

The genotype distributions among the healthy controls deviated from Hardy-Weinberg equilibrium for TLR1 (743 T > C (rs4833095)) (p = 0.03), TLR2 (−16,934 A > T (rs4696480)) (p = 0.02), TLR4 (rs1554973 T > C) (p = 0.03), TLR9 (1174 G > A (rs352139)) (p = 0.02) and TGFB1 (−509 C > T (rs1800469)) (p = 0.02). After correction for multiple testing, all SNPs studied were in Hardy-Weinberg equilibrium.

Polymorphisms associated with susceptibility of AS
In the age and sex adjusted analysis, the homozygous variant genotype of TLR1 743 T > C (rs4833095) (OR: 2.59, Table 3)

Table 3

| Haplotype combinations | Haplotypes rs4696480 A > T | rs11938228 C > A | rs3804099 T>C | N_AS (%) | N_Control (%) | OR a (95% CI) | P-value |
|------------------------|----------------------------|-----------------|--------------|-----------|---------------|--------------|---------|
| 11                     | T:T                       | AA              | T:T          | 69 (11)   | 76 (10)       | 1.00         | –       |
| 22                     | A:A                       | CC              | CC           | 72 (11)   | 74 (10)       | 1.07         | 0.68–1.70 | 0.82    |
| 33                     | A:A                       | CC              | T:T          | 28 (4)    | 34 (5)        | 0.91         | 0.50–1.65 | 0.76    |
| 44                     | T:T                       | CC              | CC           | 14 (2)    | 10 (1)        | 1.52         | 0.64–3.70 | 0.38    |
| 12                     | T:A                       | CA              | CT           | 158 (24)  | 197 (27)      | 0.88         | 0.60–1.30 | 0.55    |
| 13                     | T:A                       | CA              | T:T          | 76 (12)   | 103 (14)      | 0.81         | 0.52–1.26 | 0.37    |
| 14                     | T:T                       | CA              | CT           | 59 (9)    | 49 (7)        | 1.33         | 0.80–2.19 | 0.31    |
| 23                     | A:A                       | CC              | CT           | 77 (12)   | 89 (12)       | 0.95         | 0.61–1.49 | 0.91    |
| 24                     | T:A                       | CC              | CC           | 52 (8)    | 55 (8)        | 1.04         | 0.63–1.72 | 0.90    |
| 34                     | T:T                       | CC              | CT           | 51 (8)    | 44 (6)        | 1.28         | 0.76–2.14 | 0.43    |

OR Odds ratio

*OR was calculated for each haplotype combination by using the haplotype 11 as reference group

The biological effect of the three polymorphisms in TLR2 was unknown

The variant allele of rs3804099T T > C has been shown to decrease TNF-α, IL-1β & IL-6 level [68]

Statistical analyses were performed using STATA version 15 (StataCorp LP, College Station, TX, USA).

Polymorphisms associated with susceptibility of AS
In the age and sex adjusted analysis, the homozygous variant genotype of TLR4 743 T > C (rs4833095) (OR: 2.59, Table 4)

Table 4

| Haplotype combinations | Haplotypes rs12377632 T > C | rs1554973 T > C | rs5030728 G > A | N_AS (%) | N_Control (%) | OR a (95% CI) | P-value |
|------------------------|-----------------------------|-----------------|-----------------|-----------|---------------|--------------|---------|
| 11                     | C:C                         | T:T             | G:G             | 95 (14)   | 101 (14)      | 1.00         | –       |
| 22                     | T:T                         | T:T             | A:A             | 69 (10)   | 74 (10)       | 0.99         | 0.64–1.53 | 1.00    |
| 33                     | T:T                         | C:C             | G:G             | 29 (4)    | 57 (8)        | 0.54         | 0.32–0.92 | 0.03    |
| 44                     | T:T                         | T:T             | G:G             | 3 (0)     | 5 (1)         | 0.64         | 0.15–2.74 | 0.72    |
| 12                     | T:C                         | T:T             | G:A             | 154 (23)  | 188 (25)      | 0.87         | 0.61–1.24 | 0.47    |
| 13                     | T:C                         | T:C             | G:G             | 126 (19)  | 129 (17)      | 1.04         | 0.72–1.51 | 0.85    |
| 14                     | T:C                         | T:T             | G:G             | 30 (5)    | 32 (4)        | 1.00         | 0.56–1.77 | 1.00    |
| 12                     | T:T                         | C:C             | G:A             | 99 (15)   | 106 (14)      | 0.99         | 0.67–1.47 | 1.00    |
| 24                     | T:T                         | T:T             | G:A             | 31 (5)    | 24 (3)        | 1.37         | 0.75–2.51 | 0.36    |
| 34                     | T:T                         | T:T             | G:G             | 28 (4)    | 26 (4)        | 1.14         | 0.63–2.09 | 0.76    |

OR Odds ratio

*OR was calculated for each haplotype combination by using the haplotype 11 as reference group
95% CI: 1.48–4.51, \( p = 0.0008 \) and the combined homozygous and the heterozygous variant genotypes of \( \text{TNFRSF1A} -609 \ G > T \) (rs4149570) (OR: 1.44, 95% CI: 1.15–1.80, \( p = 0.001 \)) were associated with increased risk of AS. The homozygous variant genotype of \( \text{TLR4} \ T>C \) (rs1554973) (OR: 0.55, 95% CI: 0.34–0.86, \( p = 0.01 \)) and \( \text{LY96} -1625 \ C > G \) (rs11465996) (OR: 0.68, 95% CI: 0.46–1.00, \( p = 0.05 \)), and the combined homozygous and the heterozygous variant genotypes of \( \text{TNF} -308 \ G > A \) (rs1800629) (OR: 0.56, 95% CI: 0.44–0.72, \( p = 0.000005 \)), \( \text{TNF} -238 \ G > A \) (rs361525) (OR: 0.49, 95% CI: 0.31–0.78, \( p = 0.002 \)), \( \text{PTPN22} 1858 \ G > A \) (rs2476601) (OR: 0.76, 95% CI: 0.58–0.98, \( p = 0.04 \)), \( \text{IL18} -137 \ G > C \) (rs187238) (OR: 0.80, 95% CI: 0.65–0.99, \( p = 0.04 \)), and \( \text{IL23R} G>A \) (rs11209026) (OR: 0.60, 95% CI: 0.42–0.87, \( p = 0.01 \)) were associated with reduced risk of AS (Table 1).

After Bonferroni correction for multiple testing the homozygous variant genotype of \( \text{TLR1} 743 \ T > C \) (rs4848306) (OR: 2.59, 95% CI: 1.48–4.51, \( p = 0.04 \)) and \( \text{TNFRSF1A} -609 \ G > T \) (rs4149570) (OR: 1.79, 95% CI: 1.31–2.41, \( p = 0.01 \)) were associated with increased risk of AS and the combined homozygous and the heterozygous variant genotypes of \( \text{TNF} -308 \ G > A \) (rs1800629) (OR: 0.56, 95% CI: 0.44–0.72, \( p = 0.0002 \)) were associated with reduced risk of AS (Table 2).

SNPs associated with AS and the biological effect of the SNPs are summarized in Table 2.

### Table 5

| Haplotype combinations | N<sub>AS</sub> (%) | N<sub>Control</sub> (%) | OR<sup>a</sup> (95% CI) | P-value |
|------------------------|------------------|---------------------|-------------------|--------|
| rs4848306-3737G>A [69, 70] | | | | |
| rs1143623-1464G>C [69, 71] | | | | |
| rs1143627-31T>C [69, 71, 72] | | | | |
| 11 A:A G:G T:T 125 (18) 148 (20) 1.00 – – | | | | |
| 22 G:G C:C C:C 52 (8) 54 (7) 1.14 0.73–1.79 0.65 | | | | |
| 33 G:G C:C C:C 32 (5) 41 (5) 0.92 0.55–1.55 0.79 | | | | |
| 44 G:G G:G T:T 5 (1) 3 (0) 1.97 0.46–8.42 0.48 | | | | |
| 12 A:G G:C T:C 163 (24) 185 (24) 1.04 0.76–1.43 0.81 | | | | |
| 13 A:G G:C T:C 141 (20) 147 (19) 1.14 0.82–1.58 0.50 | | | | |
| 14 A:G G:C T:C 44 (6) 38 (5) 1.37 0.84–2.25 0.26 | | | | |
| 23 G:G C:G C:T 84 (12) 92 (12) 1.08 0.74–1.58 0.70 | | | | |
| 24 G:G C:G C:T 28 (4) 34 (4) 0.98 0.56–1.70 1.00 | | | | |
| 34 G:G G:G T:C 14 (2) 16 (2) 1.04 0.49–2.21 1.00 | | | | |

**OR** Odds ratio

The variant allele of −3737 G>A [69], −1464 G>C [70] and −31 T>C [71, 72] have been shown to decrease IL-1β level [69–72]

*aOR was calculated for each haplotype combination by using the haplotype 11 as reference group.

### Table 6

| Haplotype combinations | N<sub>AS</sub> (%) | N<sub>Control</sub> (%) | OR<sup>a</sup> (95% CI) | P-value |
|------------------------|------------------|---------------------|-------------------|--------|
| rs361525 G>A<sup>b</sup> | | | | |
| rs1800629 G>A<sup>c</sup> | | | | |
| 11 G:G G:G 523 (76) 469 (61) 1.00 – – | | | | |
| 22 G:G A:A 9 (1) 25 (3) 0.32 (0.15–0.70) 0.005 | | | | |
| 12 G:G G:A 125 (18) 210 (28) 0.53 (0.41–0.69) < 0.0001 | | | | |
| 13 G:A G:G 26 (4) 47 (6) 0.50 (0.30–0.81) 0.007 | | | | |
| 14 G:A G:A 4 (1) 12 (2) 0.30 (0.10–0.93) 0.05 | | | | |

**OR** Odds ratio

*aOR was calculated for each haplotype combination by using the haplotype 11 as reference group.

*bThe variant allele of TNF -238A rs361525A G > A has been shown to reduce expression of TNF-α [49]

*cThe variant allele of TNF -308A rs1800629 G > A has been shown to reduce mRNA level [48]
Discussion

In this case-control study, polymorphisms in α: the TNF-α (TNF (rs1800629 and rs361525), TNFRSF1A (rs4149570), and PTPN22 (rs2476601)), b: the IL23/IL17 (IL23R (rs11209026), and IL18 (rs187238)), or c: the NFkB (TLR1 (rs4833095), TLR4 (rs1554973), and LY96 (rs11465996)) pathways were associated with risk of AS.

The found associations for TNF (rs1800629) [19–22], TNFRSF1A (rs4149570) [23], and IL23R (rs11209026) [24–33] are in agreement with other case-control studies. Furthermore, Zhao et al. found that the variant allele of NLRP3 (rs4612666) was associated with increased risk of AS in Chinese patients [23]. In our study we found a trend for associations of the variant allele of NLRP3 (rs4612666) with increased risk of AS (p = 0.06). However, our results are in contrast to a meta-analysis of the PTPN22 (rs2476601) polymorphism that did not find an association with AS [34]. Finally, we identified novel risk loci in TNF (rs361525), IL18 (rs187238), TLR1 (rs4833095), TLR4 (rs1554973), and LY96 (rs11465996) that need validation in independent cohorts.

Most of the SNPs assessed in our study have known biological effects thus allowing a biological interpretation of the observed associations based on increased or reduced gene activity as summarized in Table 2 [35–47]. The associations observed for the TNF (rs1800629 and rs361525) polymorphisms suggest that reduced TNF-α mRNA level and expression of TNF-α was associated with reduced risk of AS [48, 49]. This is supported by our haplotype analysis which also suggests that the variant alleles of TNF rs1800629 and rs361525 were associated with reduced risk of AS. Likewise, the associations observed for the TNFRSF1A (rs4149570) polymorphism indicates that increased expression of the TNF-α receptor 1 was associated with increased risk of AS [50]. Furthermore, the associations observed for the PTPN22 (rs2476601) polymorphism suggests that reduced TNF-α serum level was associated with reduced risk of AS [51]. Taken together, this suggests that genetically determined high activity of the TNF-α pathway was associated with increased risk of AS.

IL-17 is known to induce the production of many cytokines including TNF-α [6]. IL-18 is a pro-inflammatory cytokine known to enhance the production of IL-17, TNF-α, and IL-1β [8]. In this study, the association observed for the IL23R (rs11209026) polymorphism suggests that reduced IL-17 serum level, and thus reduced TNF-α activity, was associated with reduced risk of AS [52]. Furthermore, the associations observed for the IL18 (rs187238) polymorphism indicates that reduced IL-18 expression, and thus reduced IL-17 and TNF-α activity, was associated with reduced risk of AS [53, 54]. The associations found in the IL23R (rs11209026) and the IL18 (rs187238) polymorphisms thus suggest that a genetically determined high activity of the IL23/IL17 pathway was associated with increased risk of AS. The two SNPs furthermore support that genetically determined high activity of the TNF-α pathway was associated with increased risk of AS. The observed associations between the polymorphisms in IL23R and IL18 and risk of AS are in line with previous studies pointing out the IL23/IL17 pathway as central to the pathophysiology of AS [3, 4, 55].

This study also suggests that the NFkB pathway may be involved in the etiology of AS. The associations observed for the TLR1 (rs4833095) polymorphism suggests that increased TLR1 level was associated with increased risk of AS [56]. High level of TLR1 may lead to increased NFkB activation and thus increased TNF-α and IL-17 activity, which is in line with the other results. However, in contrast to the other results, the associations observed for the IL18 (rs11465996) polymorphism suggests that increased MD-2 (IL18) and TNF-α level was associated with a reduced risk of AS [57]. Finally, the TLR4 (rs1554973) polymorphism was associated with reduced risk of AS which was supported by the haplotype results (Table 4). The biological effect of the TLR4 (rs1554973) polymorphism is unknown, however, the result supports the notion that the NFkB pathway may be involved in the etiology of AS.

Both TNF-α [58] and interleukin-17 inhibitors [59] have been shown to reduce inflammation and improve symptoms in patients with AS [60]. Furthermore, increased levels of TNF-α, IL-17, IL-23, IL-1β, and IL-6 have been found in sera and synovial fluid from AS patients [61–64]. The genetic associations between AS and the polymorphisms in TLR1, TLR4, LY96, TNF, TNFRSF1A, IL18, and IL23R found in this study, could potentially – in part – explain this altered cytokine milieu present in AS patients.

There are aspects of this study which should be interpreted with care. Conflicting results have been reported for the TNF (rs1800629) polymorphism [48, 49, 65]. Furthermore, the TNF polymorphisms, as well as the HLA-B27 locus, are located on chromosome 6, and there is a risk that even a minor linkage disequilibrium could have confounded our results [2]. TLR1 (rs4833095), TLR2 (rs4696480), TLR4 (rs1154973), TLR9 (rs352139), and TGFB1 (rs1800469) were not in Hardy-Weinberg equilibrium among the healthy controls. Due to the number of polymorphisms analyzed this is probably a type II error. The polymorphisms do not deviate from Hardy-Weinberg equilibrium when corrected for multiple testing. We cannot exclude that some of our positive findings may be due to chance due to the obtained p-values and the number of statistical tests performed. When the results were corrected for multiple testing only the variant allele of TLR1 (rs4833095) and TNFRSF1A (rs4149570) were associated with increased risk of AS and the
variant allele of TNF (rs1800629) was associated with reduced risk of AS.

A major strength of this study was that the cohort was rather large including 709 patients with AS and 795 healthy controls and the associations that we report were biologically plausible. Also, the validity of the diagnosis is expected to be high, since the patients were identified via a clinical database that the rheumatologist use for prospective monitoring of patients as part of routine care [66].

Conclusions

In conclusion, we replicated associations between AS and the polymorphism TNF (rs1800629), TNFRSF1A (rs4149570), and IL23R (rs11209026). Furthermore, we identified novel risk loci in TNF (rs361525), IL18 (rs187238), TLR1 (rs4833095), TLR4 (rs1554973), and IL16 (rs11465996) that need validation in independent cohorts. The results suggest that genetically determined high activity of the TNF-α, IL23/IL17, and NFκB pathways increase the risk of AS.

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In memory of Niels Henrik Heegaard:

Co-author Niels H.H. Heegaard, Professor, MD, DMsc, DNaSc, died unexpectedly on September 26, 2017, at age 57. As director of the Department of Autoimmunology and Biomarkers, Statens Serum Institut, Copenhagen, Dr. Heegaard advanced research in autoimmunology and neurodegenerative disease. He had an extensive international research network and published more than 200 papers in scientific journals, focusing on biomarkers such as autoantibodies, microRNA, and microparticle proteins. He was a patient and unpretentious collaborator who always sought to highlight the work of other collaborators and co-workers. Dr. Heegaard was characterized by humor, kindness, and optimism. He is survived by his wife and 2 children.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JS, SB, UV, PSA, SBS, HL, NHH and VA designed the research study and PSA, ABB, MRA, IB, RBQ, HJH, BG and MLH collected the materials. JS and SB analysed the data and wrote the first draft. UV, PSA, SBS, HL, NHH and VA critically revised the manuscript. All authors agreed to be accountable for all aspects of the work and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Regional Ethics Committees of Central (M20100153) and Southern (5-2012/0113) Denmark and the Danish Data Protection Agency of Central (RM-J. 2010–41–4719) and Southern (RSD: 2008–58-0353) Denmark. For blood samples collected after routine TB screening, the Ethics Committees gave exemption from informed consent requirements because samples were taken as part of routine care and data were not identifiable. Written informed consent was obtained from patients donating blood samples at Frederiksberg Hospital as this involved collecting additional samples from patients.

Consent for publication

Not applicable.

Competing interests

VA receives compensation as a consultant and for being member of an advisory board for MSD and Janssen. BG has received research funding from Abbvie, Biogen, Pfizer. The other authors declare no conflicts of interest.

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