The roles of Single

by Lita Diah Rahmawati

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Experimental Research

The roles of Single Nucleotide Polymorphism (SNP) Endoplasmic Reticulum Aminopeptidase 1 (ERAP 1) gene in axial spondyloarthritis Indonesian adults

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ABSTRACT

Background: Axial Spondyloarthritis (AxSpA) is chronic inflammatory arthritis involving the axial joint whose pathogenesis is related to the SNP ERAP1 gene, HLA B27, and cytokine proinflammatory (IL-17A and IL-23).
Objective: Analyzed the role of SNP gene ERAP1 on disease activity and proinflammatory cytokines.
Methods: This study comprised of two phases including a cross-sectional study and an in-vitro experiment in post-test with a control-group design. Participants underwent a PCR investigation searching for HLA-B27. Disease activities were measured by Ankylosing Spondylitis Disease Activity Score-Erythrocyte Sedimentation Rate (ASDAS-ESR) and modified Stokes Ankylosing Spondylitis Spinal Score (mSASSS). Subjects with HLA-B27 positive underwent PCR ERAP1 gene rs27434, genome-sequencing, and analysis. ELISA sandwich method was used to measure ERAP-1, IL-17, and IL-23 levels with lipopolysaccharide and IFN-γ induction. Analysis using independent *t*-test, Mann Whitney, and Pearson correlation test with $p < 0.05$.
Results: The average ASDAS-ESR was 3.33 ± 0.89 and the average mSASSS was 26.53 ± 9.90. In HLA B27 positive group, SNP ERAP1 gene rs 27434 in which alleles A changed to G and A/G with genotypes AA to AG/GG was observed. SNPs of the ERAP1 gene had a correlation on mSASSS ($r = 0.553$; $p < 0.05$) and no correlation on ASDAS-ESR ($r = 0.232$; $p = 0.235$). There were significant differences observed in the SNP ERAP1 gene on ERAP1 and IL-17A levels in subjects with lipopolysaccharide and IFN-γ induction ($p = 0.05$) but no significant difference in IL-23 levels ($p > 0.05$).
Conclusion: The SNP ERAP1 gene affects mSASSS value, ERAP1 levels, and IL-17A levels whereas ASDAS-ESR value and IL-23 level were not associated.

1. Introduction

The role of HLA-B27 in the pathogenesis of Axial Spondyloarthritis (AxSpA) is not clear, in the last decade there has been a role for the Endoplasmic Reticulum Aminopeptidase 1 (ERAP1), which is an aminopeptidase enzyme in the process of cleaving antigen peptides before presentation to Major Histocompatibility Complex (MHC). Single Nucleotide Polymorphism (SNP) in the ERAP 1 gene affects the processing of peptides into the right size for presentation in human leukocyte antigen B27 (HLA B27). Previous studies revealed that allele changes in SNP would affect its activity. Several studies also revealed the SNP of the ERAP1 gene with different results in various populations [1–4]. Previous studies revealed the polymorphism of SNPs of the ERAP1 gene. Thus, the SNPs combination of the ERAP1 gene at certain loci will provide a higher clinical risk in AxSpA [1,2,5].

The role of ERAP1 gene was mentioned in all 3 hypotheses of spondyloarthritis. The Arthritogenic hypothesis reveals the role of ERAP1 gene in cutting and regulating the sequence of antigenic peptides to be presented to HLA-B27. Misfolding the HLA-B27 hypothesis reveals the inappropriate arrangement of peptide as the main culprit of the homodimer free heavy chain (FHC) formation on the cell surface. The last hypothesis, the imperfect peptide cutting process can increase intracellular apoptosis and the endoplasmic reticulum stress (ER Stress) process. These three hypotheses strengthen the involvement of cellular autoinflammatory process in AxSpA. Thus, the role of ERAP1 becomes

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Abbreviations

AS	ankylosing spondylitis
ASAS	assessment of spondyloarthritis international society
ASDAS-ESR	ankylosing spondylitis disease activity score-erythrocyte sedimentation rate
AxSpA	axial spondyloarthritis
BASDAI	bath ankylosing spondylitis disease activity index
BMI	body mass index
DNA	Deoxyribo Nucleic Acid
ELISA	enzyme-linked immunosorbent assay
ERAP1	endoplasmic reticulum aminopeptidase 1
FHC	free heavy chain
HLA B27	human leukocyte antigen B27
IFN-γ	interferon gamma
IL-6	interleukin-6
IL-17A	interleukin-17A
IL-22	interleukin-22
IL-23	interleukin-23
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mSASSS	modified stokes ankylosing spondylitis spinal score
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCR-SSP	polymerase chain reaction sequence specific primer
SNP	single nucleotide polymorphism
SPSS	statistical package for the social sciences
TH17	T helper 17
TNFα	tumor necrosis factor α
UPR	unfolded protein response

important in AxSpA disease activity, because the process of cleaving and regulating antigenic peptides is the basic of the autoinflammatory cascade [6,7].

AxSpA is an auto-inflammatory autoimmune disease with increased inflammatory cytokine pathways. CD8 T cell response to antigenic peptides via MHC class 1 presentation will stimulate innate immune cells to secrete IL-23 which triggers Th17 to produce IL-17A, IFN-γ, IL-22, IL-6, and TNFα. The autoinflammatory processes of AxSpA can be measured by ASDAS-ESR and mSASSS scores. The ASDAS-ESR can be used as a single score with good validity against changes in clinical disease activity. Another assessment of the level of chronic inflammation can be measured by examining radiographic damage through the mSASSS score. Radiographic damage such as erosion, sclerosis, and syndesmophytes in spinal bones are examined through lumbosacral X-ray [8,9]. Therefore, the aimed to analyze the mechanism of the SNP ERAP1 gene against disease activity and inflammatory processes.

2. Material and method

2.1. Participant

Participants in this study were participants diagnosed with AxSpA. Participants' inclusion criteria included AxSpA with the 2010 Assessment of Spondyloarthritis International Society (ASAS) criteria [10,11], aged 20–60 years, and not consuming biologic agents. Participant exclusion criteria included obesity (BMI >30), diabetes mellitus, liver cirrhosis, asthma, and tuberculosis. Participants received an explanation regarding the study objectives was carried out and were required to fill out a consent form if they were willing to become participants.

2.2. Study design

The study was divided into 2 phases and the first phase was a cross-sectional study with HLA-B27 investigation using polymerase chain reaction sequence-specific primer (PCR-SSP), calculating ankylosing spondylitis disease activity score with erythrocyte sedimentation rate (ASDAS-ESR) score, and radiology examination to measure modified stoke ankylosing spondylitis spine score (mSASSS). The number of participants in this study was 28 participants (Fig. 1), which was carried out in the period January to December 2021. PCR gene ERAP1 rs 27434 and sequencing analysis to see the SNP gene ERAP1 were carried out in subjects with HLA-B27 positive. In the second phase, to examine whether there was an association between SNP ERAP-1 gene and proinflammatory cytokines, we conducted a post-test in-vitro experiment on PBMC culture. All subjects were classified into 3 groups such as group 1 = no treatment, group 2 = group with lipopolysaccharide (LPS) induction, and group 3 = group with LPS and interferon-gamma (IFN-γ) induction. LPS is a complex compound of lipids and polysaccharides with covalent bonds in the outer layer of gram-negative bacterial cell membranes [12]. LPS were generated from *Escherichia coli* with a dose of 0.5 μL [13]. IFN-γ human was used in this study with a dose of 10 μL [14]. Then, the concentration of ERAP1, IL-17, and IL-23 in each group was examined by the ELISA sandwich method.

2.3. Ankylosing spondylitis disease activity score with an erythrocyte sedimentation rate (ASDAS-ESR)

ASDAS-ESR is a score used to measure disease activity in ankylosing spondylitis (AS) based on patient-reported assessments of back pain, duration of morning stiffness, peripheral joint pain and/or swelling, general well-being, and serologic markers of inflammation such as ESR [15]. The ASDAS-ESR was declared valid and reliable on the bath ankylosing spondylitis disease activity index (BASDAI) with a value of $r = 0.79$ and $p < 0.001$ [16]. The ASDAR-ESR questionnaire that we used was adopted by the ASDAR-ESR Indonesia version which was adopted based on the study of Machado et al. [9].

2.4. Modified stoke ankylosing spondylitis spine score (mSASSS)

mSASSS was used to describe the degree of chronic inflammation in the form of joint damage observed in the anterior lateral cervical vertebrae and anterior lateral lumbosacral vertebrae [8]. On the score, a total of 24 places from the lower border of the 2nd cervical vertebra to the upper border of the 1st thoracic vertebra, and the lower border of the 1-12th thoracic vertebra to the upper border of the sacral vertebra. The evaluation carried out was to see the presence of joint damage from erosion, sclerosis, flattening of the vertebral body (squaring), the presence of syndesmophyte, and complete bridging. The normal section is given a value of 0. If erosion, sclerosis, or squaring is found, it is given a value of 1, the presence of syndesmophyte is given a value of 2, and the presence of complete bridging is given a value of 3 with a value range of 0–72 [8,17]. Measuring the mSASSS score was carried out based on lumbosacral and cervical radiology with two rheumatologists with inter-observer assessment (Kappa test = 86%) [8,18]. Rheumatologists are specialist doctors with 10 years of experience in their field and have received training in reading the mSASSS score.

2.5. Polymerase chain reaction examination and sequencing analysis

Venous blood samples were collected in 2 tubes which the first tube contains ±5 cc for ESR and PCR SSP examination and the second tube contains ±5 cc for peripheral blood mononuclear cell (PBMC) culture. HLA B27 using PCR (Bio-Rad, Hercules, Calif., USA) assisted by seeing online supplementary methods for cell culturing conditions [19].

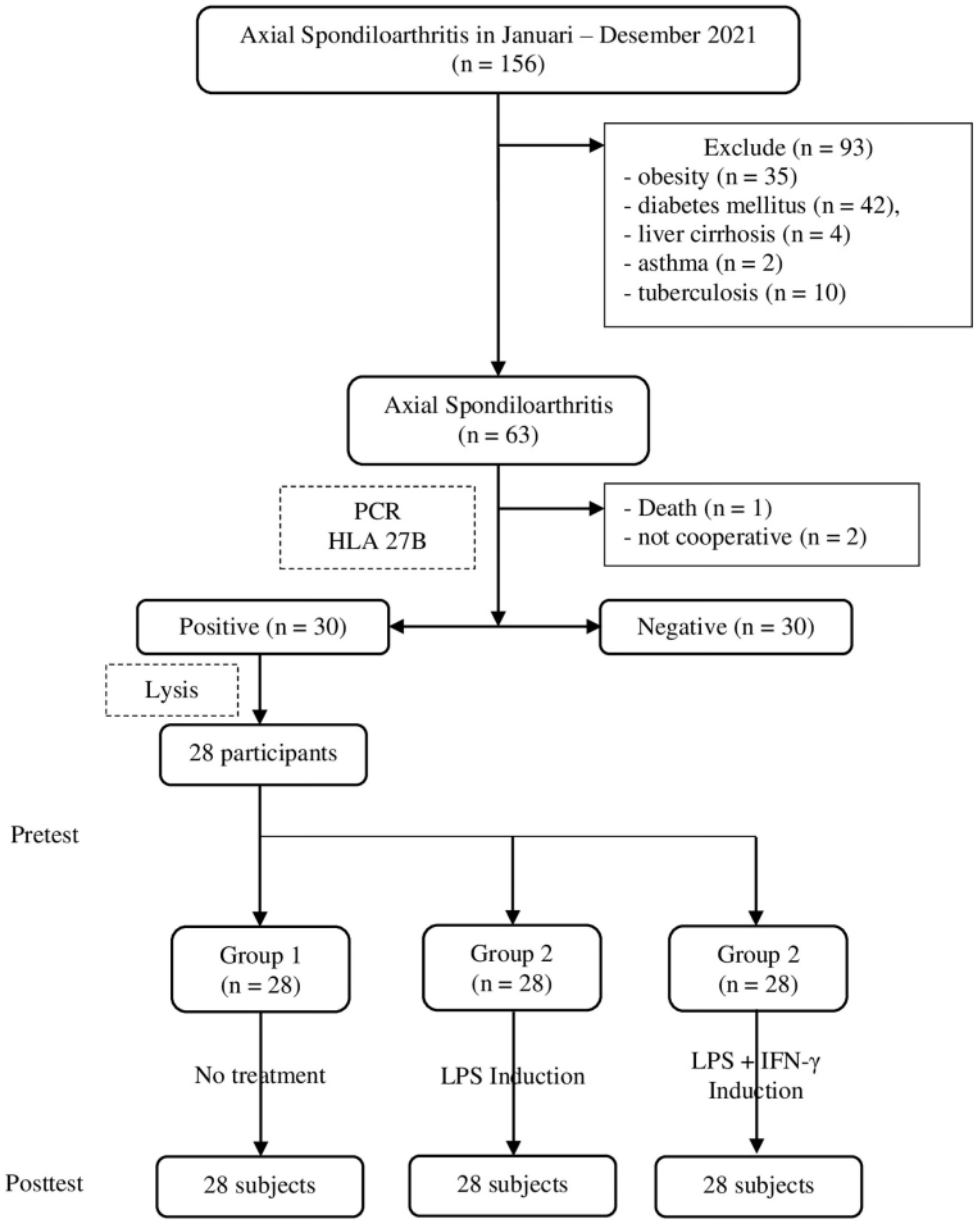

Fig. 1. Requirements of participant.

Meanwhile, ERAP1 refers to Wang et al. procedure in which DNA sequencing analysis of SNP ERAP1 gene rs 27434 using genetic ver.10 system with reference support by PUBMED [6].

2.6. Enzyme-linked immunosorbent assay examination

Calculation of ERAP1, IL-17, and IL-23 using enzyme-linked immunosorbent assay (ELISA) method with single ELISA Humareader

(Humareader, Germany). While the reagent used in IL-17 is the ELISA kit IL-17A E-EL-H0107 (Elabscience, Wuhan, China). Likewise, the IL-23 reagent used the IL-23 ELISA kit E-EL-H010 (Elabscience, Wuhan, China). Meanwhile, the ERAP1 reagent used the ELISA kit by Human ERAP1 EH-14408 (Wuhan Fine Biotech, Wuhan, China).

2.7. Statistical analysis

Statistical analysis using statistical package for the social sciences (SPSS) version 23.0 software (IBM Corp., Armonk, NY, USA). Pearson correlation test was used for the association of ERAP1 SNPs gene on ASDAS-ESR and mSASSS value. An independent *t*-test or Mann Whitney test was used to measure the comparison between ERAP1, IL-17, and IL-23 with induction by LPS and IFN-γ. The statistical test results are declared significant if the *p*-value <0.05.

3. Result

3.1. Characteristic of participant

The mean age of the participants was 35.78 ± 10.38 years with a median of 34.50 (27–44.25) years for participants with positive HLA-B27, the mean age was 35.03 ± 10.17 years and for negative HLA-B27 it was 36.53 ± 10.71 years. Most of the participants have an age range of 20–30 years as many as 20 participants (33.33%) which in HLA-B27 positive there are 12 participants (40%) and in HLA-B27 negative there are 8 participants (26.67%). Most of the male participants were 37 participants (61.67%) of which 25 participants were HLA-B27 positive (83.33%) and HLA-B27 negative were 12 participants (40%). Meanwhile, most of the participants' last education was undergraduate as many as 34 participants (56.67%) of which HLA-B27 positive obtained as many as 18 participants (60%) and HLA-B27 negative obtained as many as 16 participants (53.33%; Table 1).

In the first phase of the study based on PCR SSP, there were two groups of patients with HLA B-27 positive and HLA B-27 negative. HLA B27 positive patients underwent PCR for the SNP gene ERAP1 rs 27434 and was found SNP that allele A wild type becomes mutant type allele A/G (40%) and allele G (40%). While in Allele A wild type was found as much as 20% (Fig. 2).

3.2. Role of SNP gene ERAP1 on disease activity and proinflammatory cytokines

The mean ASDAS-ESR value was 3.33 ± 0.89 and the mean mSASSS value was 26.34 ± 9.91. ASDAS-ESR value is categorized into 2, namely high with 40 participants (66.67%; HLA B27 positive = 53.33% vs HLA negative = 80%) and very high with 20 participants (33.33; HLA B27 positive = 46.67% vs HLA negative = 20%). Meanwhile, the mSASSS value is also categorized into 2, namely the range of values from 3 to 24 with 37 participants (61.67; HLA B27 positive = 73.33% vs HLA negative = 50%) and 24 (38.33; HLA B27 positive = 26.67% vs HLA negative = 50). %). No significant correlation of SNP gene ERAP1 on ASDAS-ESR ($r = 0.232$; $p = 0.235$). Meanwhile, the SNP gene ERAP1 and mSASSS have a significant correlation with $r = 0.533$; $p = 0.004$ (Table 2).

Table 1
Characteristic of participant.

Variable	HLA-B27	
	Positive	Negative
Age	35.03 ± 10.17	36.53 ± 10.71
20–30 years old	12 (40.00)	8 (26.67)
31–40 years old	8 (26.67)	10 (33.33)
41–50 years old	7 (23.33)	9 (30.00)
51–60 years old	3 (10.00)	3 (10.00)
Gender		
Male	25 (83.33)	12 (40.00)
Female	5 (16.67)	18 (60.00)
Education		
Senior high school	12 (40.00)	14 (46.67)
Bachelor	18 (60.00)	16 (53.33)

The comparison of each group based on ERAP1, IL-17A, and IL-23 levels can be seen in Fig. 3. ERAP1 levels in group 1 mutant of 0.10 ± 0.03 ng/mL and non-mutant of 0.13 ± 0.03 ng/mL ($t = 2.114$; $p = 0.044$). ERAP1 levels in group 2 mutant of 0.10 ± 0.02 ng/mL and non-mutant of 0.13 ± 0.03 ng/mL ($t = 2.187$; $p = 0.038$). Meanwhile, ERAP1 levels in group 3 were mutant of 0.09 ± 0.04 ng/mL and 0.12 ± 0.03 ng/mL ($t = 2.098$; $p = 0.046$). IL-17A values in each group were obtained as follows group 1 (mutant = 6.58 ± 1.80 pg/mL vs non-mutant = 8.11 ± 1.86 pg/mL; $t = 2.129$; $p = 0.038$), group 2 (mutant = 8.10 ± 2.23 pg/mL vs non-mutant = 6.50 ± 1.55 pg/mL; $t = 2.124$; $p = 0.043$), and group 3 (mutant = 8.28 ± 2.28 pg/mL vs non-mutant = 6.60 ± 1.64 pg/mL; $t = 2.169$; $p = 0.039$). The value of IL-23 levels showed an insignificant difference in each group which in group 1 obtained IL-23 level of 135.97 ± 297.89 pg/mL (mutant) vs. 135.97 ± 297.89 pg/mL (non-mutant) with $z = 0.697$ and $p = 0.486$; group 2 of 296.73 ± 368.61 pg/mL vs 340.35 ± 298.65 pg/mL with $z = 0.975$ and $p = 0.330$; group 3 of 298.98 ± 421.61 pg/mL vs. 306.65 ± 333.25 pg/mL with $z = 1.161$ and $p = 0.246$ (Table 3).

4. Discussion

Previous studies revealed that SNP ERAP-1 gene rs 27434 consisted of 155 bp located on chromosome 5q15, exon number 6, with the allele A > G/A > T. Its mRNA coding sequence for alanine is located at position 356. Our findings revealed the mutation of alleles A to G and A/G mutation. Therefore, the genotype AA changed into AG and GG genotype. The amino acid produced from this mutation was still alanine. Therefore, it can be classified as a silent mutation. To our knowledge, this is the first study that highlighted this finding. Previous studies reported that the AG genotype was the dominant genotype in the US and Korean populations [6,20,21].

Measuring disease severity is important in AxSPA patients with a clinical score such as ASDAS-ESR or the level of severity with mSASSS score. The ASDAS-ESR in this study had a mean value of 3.33 ± 0.89 classified as high disease activity (68.3% subjects) and very high category (31.7% subjects). The mean mSASSS level of the research subjects was 26.35 ± 9.90. A study of the AS population in Taiwan by Wang et al., divided mSASSS into three categories, <3, 3–24, and >24 and was found more than 50% of subject AS in Taiwan has mSASSS <3. They also concluded that mSASSS greater than 24 was associated with severe prognosis in AS [8,22].

Wang et al. in the Taiwanese population found an association between rs 27044 and rs30187 with mSASSS as the parameter of disease activity. On the other hand, Pearson correlations of the SNP ERAP1 gene with mSASSS in this study were consistent with Wang's findings. Thus, the presence of the SNP ERAP1 gene was correlated with radiographic damage in AxSpA [8,22].

ERAP1 activity and levels influence inflammation through the presentation of MHC to CD8 T cells. SNP ERAP1 gene can change the amino acid structure but does not always affect enzyme activity. This study is the first study that observed that the induction of inflammation with LPS and LPS + IFN-γ on SNP of the ERAP-1 gene was not related to the difference in ERAP1 concentration. Moreover, in this study, it was found that there were no difference in IL-17 used PBMC culture between the without treatment group, with LPS induction treatment and LPS + IFN-γ, so that the SNP of the ERAP1 gene was not associated with IL-17 cytokines. Kenna et al., found an increase of IL-17 cytokines through other pathways, namely a UPR and ER stress, and was not influenced by the ERAP1 gene [3,7].

Consistent with the previous studies, the levels of IL-23 would increase consistently with the increase of IL-17 and IL-22. Therefore, this

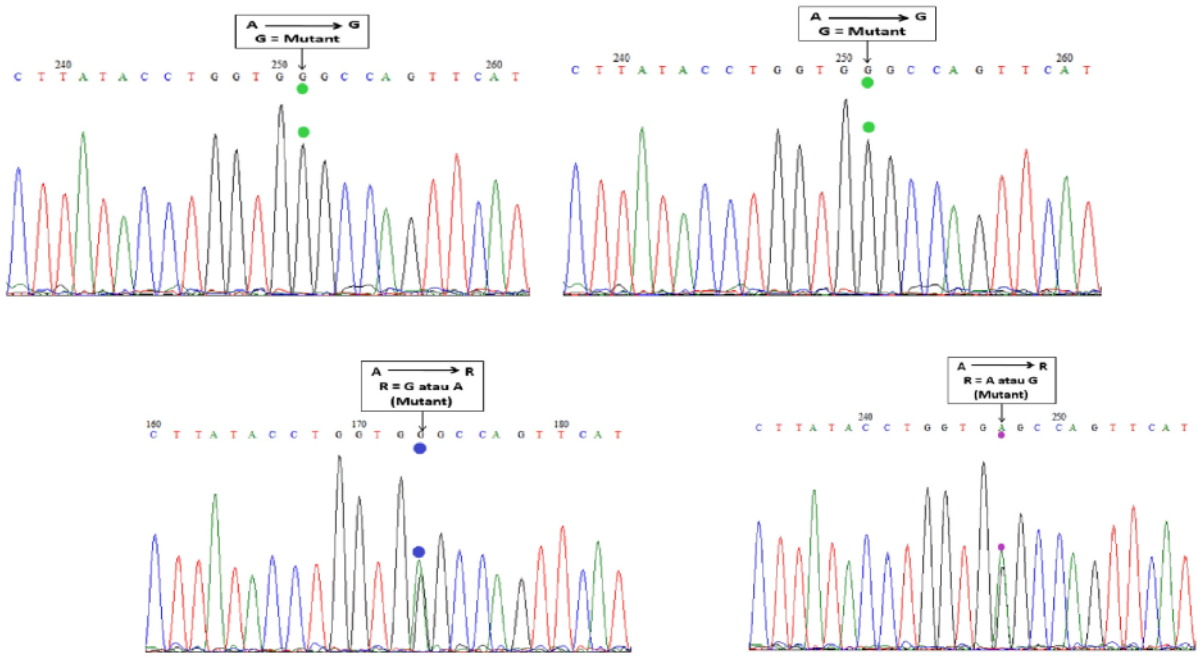

Fig. 2. SNP ERAP1 gene nucleotide Allele A (wild) type, Allele A/G (mutant), and Allele G (mutant).

Table 2
Correlation of SNP ERAP1 on ASDAS-ESR and mSASSS.

Variable	Mean ± SD	*r*	*p*
ASDAS-ESR	3.33 ± 0.89	0.232	0.235
mSASSS	26.34 ± 9.91	0.533	0.004*

Note: ASDAS-ESR = Ankylosing Spondylitis Disease Activity Score-Erythrocyte Sedimentation Rate; mSASSS = modified Stokes Ankylosing Spondylitis Spinal Score; *significant <0.05.

study found no increase in IL-23 levels in PBMC cultures. Haroon et al. stated that ERAP1 plays a role in IL-23R on the cell surface, but this does not affect the levels of inflammatory cytokines such as TNFα, IL-6, and IL-23. SNP ERAP1 gene affects intracellular processes through activation of UPR and ER stress which can increase IL-23 directly without going through the cytokine pathway [23,24].

However, this study has its limitations. Family history of autoimmune was observed only by history taking. Other limitations, such as post-study examination did not use a control group. Finally, this study only observed one SNP, which was rs-27434. Therefore, further studies examining other SNP should be conducted to explore the role of SNP in AxSpA.

5. Conclusion

There was a significant correlation between the SNP ERAP1 gene on mSASSS score and no significant correlation between the SNP ERAP1 gene on ASDAS-ESR. SNP ERAP1 genes were divided into 3 groups such as group 1 = no treatment, group 2 = group with LPS induction, and group 3 = group with LPS and IFN-γ induction. Based on ERAP1 and IL-17A levels, there were significant differences in the three groups. Meanwhile, no significant difference in IL-23 levels to three groups.

Ethical approval

We have conducted an ethical approval base on the Declaration of Helsinki with registration research at the Health Research Ethics Committee in Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (0221/KEPK/VII/2021).

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None.

Author contribution

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Trial registry number

1. Name of the registry: Health Research Ethics Committee in the Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.
2. Unique Identifying number or registration ID: 0221/KEPK/VII/2021.
3. Hyperlink to your specific registration (must be publicly accessible and will be checked):

Guarantor

Aryati is the person in charge of the publication of our manuscript.

Consent

Written informed consent obtained from the patient.

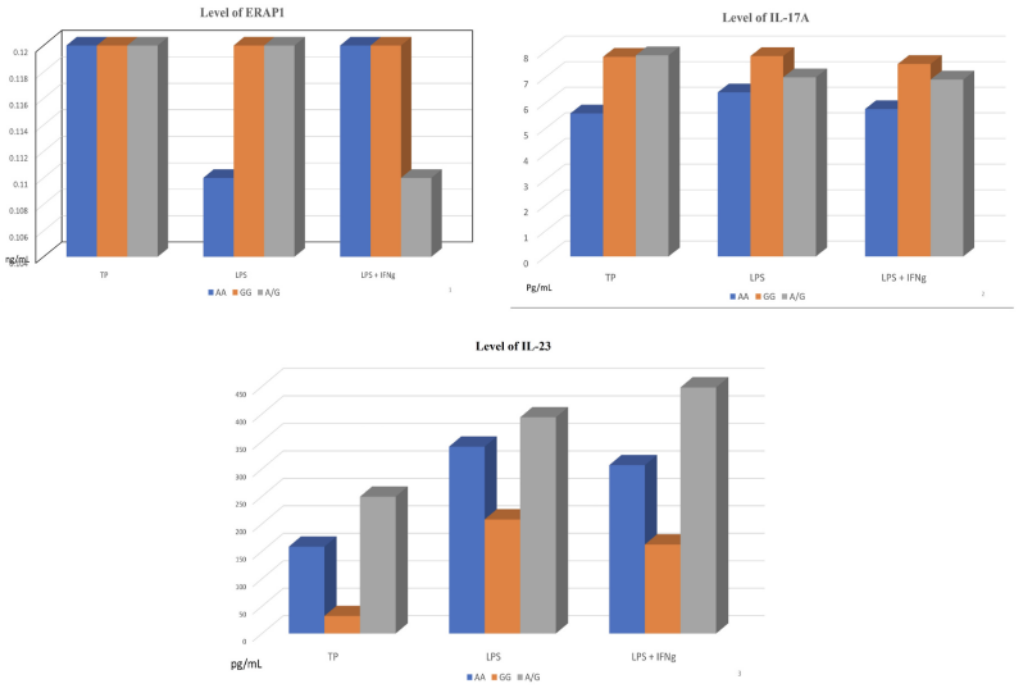

Fig. 3. ERAP1, IL-17, and IL-23 levels pre and post-induction of LPS and LPS + IFN-γ.

Table 3
Comparison of SNP ERAP1 gene based on ERAP1, IL-17A, and IL-23 levels.

Variable	SNP ERAP1 Gene		*p*
	Mutant	Non-Mutant	
ERAP1			
No treatment	0.10 ± 0.03	0.13 ± 0.03	0.044*
LPS	0.10 ± 0.02	0.13 ± 0.03	0.038*
LPS + IFN-γ	0.09 ± 0.04	0.12 ± 0.03	0.046*
IL-17A			
No treatment	6.58 ± 1.80	8.11 ± 1.86	0.038*
LPS	8.10 ± 2.23	6.50 ± 1.55	0.043*
LPS + IFN-γ	8.28 ± 2.28	6.60 ± 1.64	0.039*
IL-23			
No treatment	135.97 ± 297.89	158.29 ± 336.53	0.486
LPS	296.73 ± 368.61	340.35 ± 298.65	0.330
LPS + IFN-γ	298.98 ± 421.61	306.65 ± 333.25	0.246

Note: LPS = Lipopolysaccharide; IFN-γ = interferon gamma; *significant <0.05.

Declaration of competing interest

Lita Diah Rahmawati, Joewono Soeroso, and Aryati declare that they have no conflict of interest.

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