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Study of Toll -like Receptor 4 and Colony Stimulating Factor 2 Gene Expression for Early Recognition of Axial Spondyloarthritis Changes in Psoriatic cases.

***Sherin S. EL Naidany[1], Wafaa A. Shehata[2], Dalia S. Saif[3], Sally M. El-Hefnawy[1] and Manal A. Ellaithy[1]**

[1]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Menoufia University, Egypt
[2]Dermatology, Andrology and STDs, Faculty of Medicine, Menoufia University, Egypt
[3]Physical Medicine, Rheumatology and Rehabilitation, Faculty of Medicine, Menoufia University, Egypt

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Abstract

Background: About 30% of cases with psoriasis will suffer from psoriatic arthritis (PsA). Heritable element plays a role in PsA as different genes are involved. However, few genes are involved in both psoriasis and PsA. This study aimed to investigate the predictive value of Toll-like receptor (TLR) 4 and colony stimulating factor (CSF) 2 gene expression for early detection of axial spondyloarthritis in psoriatic patients. **Methods:** This study included 200 subjects; 100 psoriatic patients, subdivided into two groups; Group 1: included 66 patients with plaque psoriasis without any articular complaint, and Group 2: included 44 patients with psoriatic arthritis. Group 3 included: 100 age and sex matched healthy controls. Laboratory assessment of TLR4 and CSF2 gene expression by real time polymerase chain reaction technique, and axial joint radiological assessment by Magnetic Resonance Imaging. **Results:** There were significant increase of CSF2 and TLR4 gene expression levels in cases compared with controls ($p < 0.001$) for both. Additionally, a significant rise of CSF2 and TLR4 gene expression levels in cases with psoriatic arthritis compared to cases with psoriatic skin affection only ($U=2.45$, $p= 0.01$, 3.34, $p= 0.001$ respectively. Receiver operating characteristic curve done for earlier detection of sub-clinical changes of axSpA regarding positive MRI results in cases with psoriasis and PsA respectively with P value <0.001. **Conclusion:** TLR4 and CSF2 gene expression have strong predictive value in early detection of axial SpA changes in asymptomatic and non-radiographic psoriatic patients which is equivalent and equal to the MRI predictive value.

Corresponding author: Dr. Manal Abd El-Monem Ellaithy, E-mail: manal.ellaithy@yahoo.com, Tel. 0403273450

INTRODUCTION

Psoriasis is a common recurrent, immune-arbitrated disorder affecting mainly skin and joints of genetically susceptible entities [1]. The disease often starts with non-specific manifestations and the axial involvement, including radiologically-identified sacroiliitis, occurring within the course of psoriasis, there are several biomarker predictors that vary in their specificity [2]. Early diagnosis of axial arthropathy in psoriatic patients is associated with early intervention and management that improves the clinical and radiological outcomes and prevents progressive axonopathy and disabilities [3]. Many studies have indicated that the pathogenesis of psoriatic arthritis (PsA) is caused by an immunological mechanism. Toll like receptor (TLR) activation leads to inflammation in multiple tissues and organs. It is essential in the activation of the innate immune system because it detects lipopolysaccharide from Gram-negative bacteria. TLR expression in the peripheral blood of PsA patients has been documented in some studies as TLR2 and TLR4 expression were found to be higher in PsA patients [4]. Recent studies have shown that formation and maintenance of psoriasis plaques were dependent on TLR-dependent pathways, as TLRs are trans-membrane proteins presents on immune cells that recognize certain sections of molecules both exogenous and endogenous as a portion of the innate and adaptive immune reactions and TLR4 is thought to be an apoptosis triggering factor that interacts with TLR2 in the autoimmune pathway [5]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known as a pleiotropic Th17-related cytokine playing a crucial role in many autoimmune diseases pathogenesis as psoriasis [6]. The pattern of pro-inflammatory cytokine presented in GM-CSF induced dermatitis, with elevated levels of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-12 and reduced IL-10 and transforming growth factor beta (TGF-β), showed high similarities with active psoriasis cytokine pattern. So, GM-CSF considered a potent macrophage polarizing driver and a possible therapeutic target in PsA [7]. Magnetic resonance imaging (MRI) is known to detect initial changes regarding inflammatory processes in symptomatic patients without structural lesions yet, in which osteitis manifested as edema of bone marrow. Additionally, MRI is also specified to identify undiagnosed early clinical stage of the disease in which cases had characteristic features of axial contribution but without systemic alterations [8]. We aimed to explore the predictive value of TLR4 and CSF2 gene expression in initial recognition of axial spondyloarthropathic changes in cases with psoriasis and PsA.

2- Subjects and methods

The current work was accepted by the Local Ethical Research Committee, and an informed permission was attained from all the contributors. A case control study was accompanied on 100 psoriatic patients diagnosed and assessed clinically and 100 sex and age coordinated healthy controls were included. All patients were recruited from the Outpatient Clinic of Rheumatology and Outpatient Clinic of Dermatology and Andrology in the period from April 2018 to July 2020. Cases were categorized into 2 groups, group 1 encompassed: 66 patients with plaque psoriasis without any articular complain, and group 2 included 44 patients with psoriatic arthritis as they diagnosed according to standards for Psoriatic Arthritis (CASPAR) [9]. The psoriatic patients in

both groups were without evidence of changes in X-ray of the spine and the sacroiliac joint (postero-anterior outlook). All patients were subjected to**:** Personal, present and family history taking, general, local dermatological and articular examination of both axial and peripheral joints, and assessing the severity of psoriasis by the application of Psoriasis Area and Severity Index (PASI) score where the body of each case was alienated into four portions: (1) the head (h) (represents 10% of surface area of the skin); (2) upper limbs (u) (20%); (3) trunk (t) (3%); (4) lower limbs (l) (40%). The fraction of the diseased areas was designed and a score was given as the following: 0 (0% influenced part), 1 (< 10% influenced part), 2 (10-29% influenced part), 3 (30-49% influenced part), 4 (50-69% influenced part), 5 (70-89% influenced part) and 6 (90-100% influenced part). The three clinical appearances were evaluated inside every skin portion (A) to estimate severity of the disease: redeness (E), scaling or desquamation (D), and induration (I). The recording of the clinical appearances was graded as the following: 0 (without signs), 1 (mild) 2 (moderate), 3 (severe) and 4 (very severe). At that time, summation of the scores of severity for the clinical manifestations for every part of the skin was determined, then multiplied by the mark assumed to the influenced area and by the heaviness of this part. (0.1 regarding the head; 0.2 for upper limbs; 0.3 for trunk; 0.4 for lower limbs) was done. Finally, estimation of PASI score was done as follows: PASI score = 0.1 (Eh + Ih + Dh) Ah + 0.2 (Eu + Iu + Du) Au + 0.3 (Et + It + Dt) At + 0.4 (El + Il + Dl) Al and 0 to 72 was the range of PASI and PASI lower than 7 is a key for a mild psoriasis; a score from 7–12 denoted a moderate form of psoriasis and a score more than 12 is the score of severe psoriasis [10].

2.1 Exclusion Criteria:

1) Patients with any dermatological disease other than plaque psoriasis.
2) Cases with any of the following diseases such as systemic lupus erythematosus, connective tissue diseases, rheumatoid arthritis, degenerative diseases of joints such as osteoarthritis, metabolic, endocrinal disorders, and any other inflammatory disorders of joints.
3) Cases who were receiving systemic traditional or biological cure for theirs psoriasis.

2.2 Laboratory investigations:

We used disposable syringes to take 3 ml of venous blood samples through venipuncture under perfect aseptic conditions. We used a vacutainer plastic tube containing ethylenediaminetetraacetic acid (EDTA) to transfer blood samples for real-time PCR measurement of TLR4 gene expression. The QIAamp RNA Blood MiniKit was used to purify total RNA from blood (Qiagen, USA). For reverse transcription and complementary DNA (cDNA) creation, Thermo Scientific's RevertAid First Strand cDNA Synthesis Kit was employed. The following two-step reactions were carried out on ice with a net volume of 20 µl: First, 10 µl of template RNA was added to 1 µl of random hexamer and 1 µl nuclease free water to make a total volume of 12 µl, which was then incubated at 65 ºC for 5 min before being placed on ice; second, 4 µl of 5 x reaction buffer, 1 µl ribolock Rnase inhibitor, 2 µl of 10 MM dNTP Mix, and 1 µl of revert-aid. On a 2720 thermal

cycler (Applied Biosystems, Singapore), incubation was done in single cycle at 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 5 minutes. Until the real-time PCR phase, the cDNA was stored at -20 °C.

Quantitative Real-Time PCR (qRT-PCR): The cDNA was used in a SYBR green-based qRT-PCR using the Quanti Tect SYBR Green PCR Kit and ready-made Quanti Tect Primer Assay (Qiagen).

The primers listed below were used to assess TLR4 mRNA levels: F: (5′ - AGCCACGCATTCACAGGG–3), R: (5′ CATGGCTGGGA TCAGAGTCC–3) and granulocyte-macrophage Colony stimulating factor 2: F: (5′-CAGGGCC TGCGGG GCAGCCT-3′), R: (5′-GTCTCACTCCTGGACTGG-3′). Primers for human ß-actin F: (5′-TCCATGACAACTTTGGCATCGTGG-3′) and R: (5′-GTTGCTGTTGAAGTCACA GGAGAC-3′).

A 20 µl mix was made up of 4 µl of the RT product, 10 µl of 2x SYBR Low ROX Master Mix, 4 µl of RNase-free water, and 1 µl of each primer. 45 cycles were performed: 1 minute at 94ºC for denaturation, 1 minute at 55ºC for annealing, and 30 seconds at 72ºC for extension. The data was analyzed using the Applied Biosystems 7500 software version 2.0.1. (Figure 1A, 1B). The mRNA levels were measured using the relative quantification (RQ) approach, which normalizes the target gene to an endogenous reference gene (ß actin) and is relative to a healthy control.

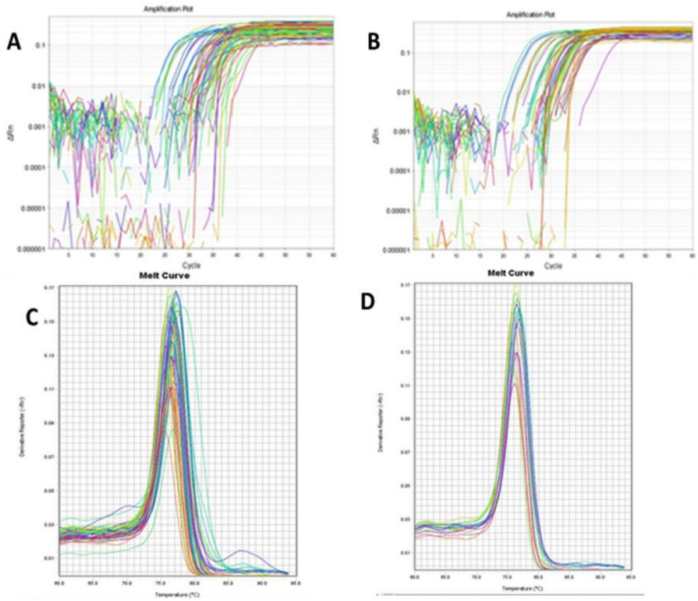

Figure 1: a- Amplification plot of *TL4* gene, b- Amplification plot of *CF2* gene, c- Melting curve for TLR4 gene expression and d- Melting curve for CF2 gene expression

2.4 Radiological investigation

All patients had a non-contrast MRI of the sacroiliac joints and lumbo-sacral spine. It was carried out on a 1.5 T MRI machine (Toshiba Vantage) with a spine and/or body coil. The protocol performed involved oblique coronal with axial T1-weighted picture for detailed anatomy as well as oblique coronal with axial fluid- sensitive T2-weighted fat-saturated/short tau inversion recovery arrangements with a field of view of 16 cm and slice depth of 3-4 mm. The pictures were evaluated for early signs of inflammatory alterations involving bone marrow edema of

the sacroiliac joints and shiny corners of lumbar vertebrae that is regarded a sign of inflammation and those regarded as positive radiological findings. It was regarded significant in degree when it was ≥1 cm in depth as well as visible on at least 2 adjacent MRIs or at 2 detached sites on the same picture of the sacroiliac joint [11].

2.5 Statistical analysis

Data were gathered, presented, and statistically analyzed using an IBM personal computer running the Statistical Package for Social Science (SPSS) version 19 software (SPSS, Inc, Chicago, Illinois, USA) in which the following statistics were used: Descriptive statistics: provided quantitative data in the form of mean (X), standard deviation (SD), range, and qualitative data in the form of numbers (N) and percentages. Analytical statistics are utilized to determine the probable relationship between the components under consideration and the disease under consideration. The following tests of significance were used: The Chi-square test (2nd) was used to investigate the relationship between two qualitative variables. Mann-Whitney: The U test (non-parametric test) is a statistical test used to compare two groups that are not normally distributed and have quantitative variables. The diagnostic performance of our parameters, including sensitivity and specificity at various cut-off points, was evaluated using receiver operating characteristic (ROC) curve analysis. As odds ratios, multiple regression analysis identifies the independent risk variables (ORs). P 0.05 was deemed statistically significant [12]. In the context of logistic regression, the Wald test is used to assess if a certain predictor variable X is significant or not.

Ethics number is 10/2022BIO16-2

3- Results

There were no statistically significant variations in age (p=0.07) or gender (p=0.474) between patients and controls, supporting group matching. There were significant increase of CSF2 and TLR4 gene expression levels in cases compared with controls (p <0.001) for both Table (1).

MRI findings were negative in 10 (29.4%) cases with PsA while findings were positive in 24 (70.6%) cases with PsA. Cases with skin only lesions showed negative MRI finding in 38 (57.5%) cases and there were 28 (42.5%) cases with positive MRI findings with significant difference between both subgroups regarding MRI findings (U=7.13, P=0.008) (Table 2). Indeed, there was significant difference regarding shiny corners of lumbar vertebrae in MRI in cases with PsA compared with cases with psoriatic skin only lesions (P=0.02) Table (2). Furthermore, there were significant increase of CSF2 and TLR4 gene expression levels in cases with PsA when compared to cases with psoriatic skin affection only (U=2.45, p= 0.01, 3.34, p= 0.001 respectively Table (2).

There was significant statistical increase of both of: age, female gender distribution, disease duration, PASI score, CSF2 & TLR4 gene expression levels in MRI positive cases when compared to MRI negative one Table (3).

In order to study whether TLR4 and CSF2 genes expression could be used to identify early axial SpA changes concerning positive MRI findings amongst patients groups, a receiver operating characteristic (ROC) curve was done to identify early sub-clinical axSpA changes concerning positive MRI findings among patients with psoriasis and PsA respectively to be 7.95, 7.6 with a sensitivity of 96.2%, 98.1% specificity 89.6%, 89.6% and accuracy 93%, 94% (area under the curve = 0.937, 0.944) and P value <0.001 Table (4) and (Figure 2a & 2b).

CSF2 and TLR4 gene expression are independent risk factors of initial axSpA changes concerning MRI findings. CSF2 and TLR4 gene expression increases the risk to develop early axial spodyloarthritic changes by 5.33, and 11.97 respectively Table (5).

Table (1): Personal, clinical information, and genetic expression of *CSF2* & *TLR4* of the studied individuals.

Parameter **X± SD or N (%)**	**Cases** **(N =100)**	**Control** **(N =100)**	**Statistical tests.**	**p-value**
Age (years) X ± SD Median Range	 49.40±12.81 51 20 – 68	 46.07±12.59 45 22 - 67	 t-test 1.86	 0.07
Sex Male Female	 45 (45.0) 55 (55.0)	 40 (40.0) 60 (60.0)	X^2 0.51	 0.474
Duration of psoriasis (years) X ± SD Median Range	 6.83±4.35 5 1 – 20	 --	 	 --
PASI score X ± SD Median Range	 13.23±5.84 12.5 1.20 – 27	 --	 	
PSA Peripheral affection LBP	 N = 44 36 (86.4) 8 (13.6)	 --	 	
CSF2 gene expression X ± SD Median Range	 12.05±10.53 8.7 0.91 – 38.0	 0.96±0.49 1.0 0.20 – 1.80	 U 11.03	 **<0.001***
TLR4 gene expression X ± SD Median Range	 11.6±8.67 8.5 1.01 – 34.6	 1.05±0.43 1.0 0.20 –1.80	 U 11.33	 **<0.001***

X^2: Chi square test U: Mann Whitney U test X : Mean SD: Standard deviation IQR: interquartile range
N: Number %: percentage PASI: Psoriasis area and severity index LBP: low back pain TLR4: Toll-like receptor
CSF2: colony stimulating factor 2 * P< 0.05 is the level of significance PsA: Psoriatic arthritis

Table (2): Comparison concerning MRI findings and *CSF2* &*TLR4* gene expression levels in cases with PsA and cases with skin only lesions.

	PsA **N =34** **N (%)**	**PS** **N=66** **N (%)**	**Test of significance**	**P value**
MRI findings Negative Positive	 10 (29.4) 24 (70.6)	 38 (57.5) 28 (42.5)	Z 7.13	 0.008*
Positive MRI findings bone marrow oedema of the SIJ shiny corners of lumbar vertebrae	 18 (52.9%) 6 (17.6%)	 24 (36.4) 4 (6.1)	Z 1.5 2.26	 0.14 0.02*
CSF2 gene expression X± SD Median Range	 14.82±10.62 12.8 0.92 – 38	 10.63±10.28 7.53 0.91 – 38	U 2.45	 0.01*
TLR4 gene expression X ± SD Median Range	 16.33±10.17 14.6 1.1 – 34.6	 9.17±6.66 7.45 1.01 – 33	U 3.34	 0.001*

U: Mann Whitney U test PS: psoriasis patients with skin only lesions PSA: psoriatic arthritis MRI: magnetic resonance imaging X : Mean SD: Standard deviation N: Number %: Percent

Table (3): Association concerning MRI findings and both of personal, clinical information and genetic analysis in patients.

	MRI findings			
	Positive N = 52	Negative N = 48	**Test of significance**	**P value**
Age X ± SD Median Range	 52.38±11.92 54 30 – 68	 46.19±13.08 49 20 – 68	t 2.48	0.02*
Sex Male Female	**N (%)** 18 (34.6) 34 (65.4)	**N (%)** 27 (56.2) 21 (43.8)	U 4.72	0.03*
Disease duration X ± SD Median Range	 7.92±4.59 6.5 2 – 20	 5.65±3.04 5 1 – 20	U 2.73	0.006*
PASI score X ± SD Median Range	 15.45±6.21 14.85 3.3 – 27	 10.83±4.32 10.55 1.2 – 19	U 3.71	<0.001*
CSF2 X ± SD Median Range	 17.82±9.94 13.55 6.5 – 38	 5.81±7.06 4.75 0.91 – 38	U 7.64	<0.001*
TLR4 X ± SD Median Range	 16.96±8.32 14.65 1.5 – 34.6	 5.81±4.16 5.8 1.01 – 22.5	U 7.39	<0.001*

N: Number X: Mean %: Percentage SD: Standard deviation t: student t test
U: Mann Whitney U test * P<0.05 is the level of significance

Table 4: Sensitivity and specificity of TLR 4 to detect early axial SpA changes regarding positive MRI findings among patients groups

	AUC	**P value**	**95% CI**	**Cutoff point**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Accuracy (%)**
TLR4	0.937	<0.001*	0.88 – 99	7.95	96.2%	89.6%	90.9%	95.6%	93%
CSF2	0.944	<0.001*	0.89 – 1.0	7.6	98.1%	89.6%	91.1%	97.7%	94%

AUC: Area under the curve PPV: Positive predictive value NPP: Negative predictive value
* P<0.05 is the level of significance

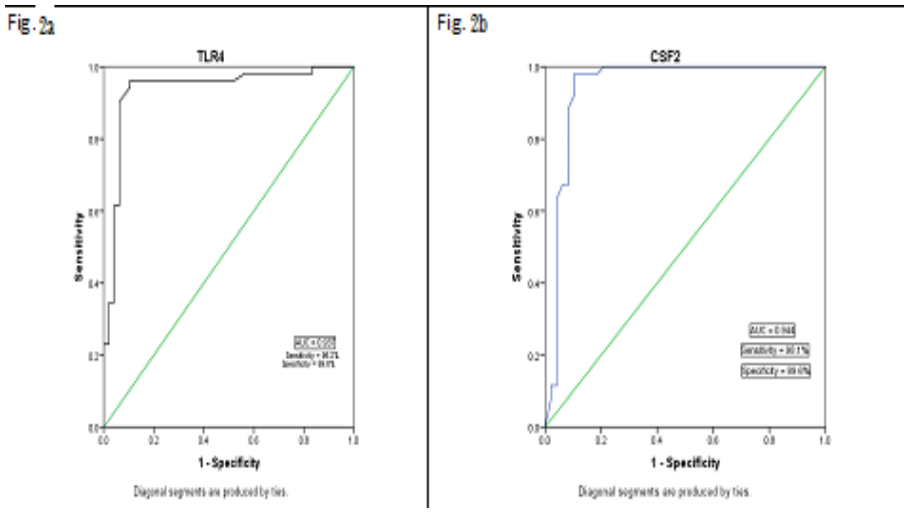

Figure 2: a- ROC curve analysis for sensitivity and specificity of TLR4 gene to detect early axial SpA changes regarding positive MRI findings among patients groups.
b- ROC curve analysis for sensitivity and specificity of CSF2 gene to detect early axial SpA changes regarding positive MRI findings among patients groups.

Table (5): Adjusted odds ratios for the association between variables and the early SPA changes in patients with psoriasis and PsA.

Studied variables	Wald	P-value	Adjusted OR	CI 95 %
Age	0.19	0.66	0.99	0.93 – 1.05
Sex	0.74	0.39	1.85	0.46 – 7.45
Disease duration	3.59	0.06	1.17	0.99 – 1.40
PASI score	3.12	0.08	1.14	0.99 – 1.31
TLR 4	11.97	0.001*	4.83	1.89 – 11.91
CSF 2	5.33	0.02*	3.4	1.59 – 8.02

OR: odds ratio PASI: psoriasis area and severity index PsA: psoriatic arthritis
* P<0.05 is the level of significance

4- Discussion

The present study is the first one that reveals the predictive role of CSF2, and TLR4 genes in the detection of early axial affection in patients with psoriasis and psoriatic arthritis without clinical or radiological axial affection (asymptomatic psoriatic patients and in non-radiographic axSpA (nr-axSpA), in spite of their documented role in the pathogenesis of both diseases. Although our little knowledge about the true mechanisms of both CSF2, and TLR4 genes in the detection of early axial affection, we could explain that many studies including ours showed significant differences in their expression levels among patients of with psoriatic arthritis compared to those with only psoriatic skin lesions, and this attracts our attention that the increased expression level of those genes is responsible for articular affection specially axial type, even in asymptomatic patients and symptomatic ones with normal x-ray findings.

Therefore, from this standpoint and through the results of the present study, we can say that, in cases of elevated TLR4 gene expression levels (above 7.95) and CSF2 (above 7.6), early sub-clinical axSpA alterations should be supposed in nr-axSpA patients and asymptomatic psoriatic patients.

The present study reveals a significant difference between cases and controls and among psoriatic arthritis cases compared to those with only psoriatic skin lesions regarding CSF2 and TLR4 gene expression levels. Many studies reported that TLR4 mRNA level was significantly higher in psoriatic patients in comparison to controls. [13]

Other studies stated that GM-CSF expression was higher in psoriatic patients compared to controls [14].

Gladman et al. [15], reported the stronger heritability for PsA than those with psoriatic skin affection which point to that there could be specific risk loci for these two disease entities and recognition of such loci could possibly enable earlier discovery of PsA and aids in early managing and stoppage of irreversible joint damage.

Indeed, using genome-wide association studies (GWAS) have recognized many genes/genomic loci increasing predisposition for PsA including CSF2 and TLR4 genes which are linked to increased predisposition to axSpA, emphasizing once again the great overlap between these two conditions in terms of their genetic predisposition. As arthritis is the common clinical presentation between the two diseases this pay our attention to their high expression levels in cases of arthritis especially axial arthritis compared to patients with only skin lesions [16].

Mossawi et al. [17], suggested a physiological connection between GM-CSF and the IL-17/IL-23 axis, and as IL17 is greater in PsA patients more than cases with psoriatic skin affection only. According to Saif et al. [18], CSF2 gene present is in a higher rate in PsA patients than cases with psoriatic skin affection only.

Furthermore, Bowes et al. [19], reported that the index single nucleotide polymorphism at long arm of chromosome 5, locus 31, rs715285, maps to an inter-genic site flanked by the genes *CSF2* and *Prolyl 4-Hydroxylase Subunit Alpha 2 (P4HA2)* and this link was studied in the independent cohort of cases with PsA, as well as meta-analysis of PsA cohorts provides undoubted proof of link with predisposition to PsA. By logistic regression, the effect estimates for rs715285 in PsA and psoriasis (odds ratio=1.25 and 0.99, respectively) are expressively different supporting that this genes are a PsA-specific risk site, indicating the highly genetic expression of CSF2 among PsA patients compared to cases with cutaneous psoriatic affection.

Also, Loft et al. [20], reported a higher expression level of TLR4 among patients with PsA compared to those with isolated psoriatic cutaneous lesions.

Our results documented the presence of clinical axPsA in 18% while it was documented by MRI in 57% among patients of both groups. That comes in agreement with Williamson et al. [21]**,** who reported that the sacroiliac joint involvement regarding MRI measures was present in 26/68 (38%), in spite of the presence of clinical sacroiliitis only in 24/68 (35%) patients with PsA**.**

Ibrahim, El-Shazly [22]**,** stated that there were MRI variations in the sacroiliac joint in 6 out of 18 cases having PsA with asymptomatic sacroiliac participation (33.3%).

The current study is the first one reporting a significant correlation between TLR4 and CSF2 gene expression levels with positive MRI results of initial axSpA changes among both groups of cases regarding shiny corners of lumbar vertebrae and bone marrow edema of the sacroiliac joint (SIJ).

This can be explained by the functional link between CSF and the IL-17 documented by Mossawi et al. [17], and as there is a significant relationship between serum IL-17 levels and positive MRI results of initial axSpA changes [23]. So, consequently, CSF2 could be directly correlated with positive MRI findings of early axSpA changes.

The current study documented that both TLR4 and CSF2 genes are independent risk factors of initial axSpA alterations in cases with psoriasis and PsA in spite the presence of many predictors and suggestive indicators for identifying the initial axSpA alterations in cases with psoriasis and PsA as regards clinical, lab and MRI results [24,25]. So, according to the results we got and from our point of view our study proved that TLR4 and CSF2 genes can be considered one of the most important and accurate methods for detecting the initial sub-clinical axSpA alterations in asymptomatic psoriatic cases and those with nr-axSpA, as it is an accurate and objective measures than clinical data and has higher sensitivity, specificity and accuracy parameters compared to other lab parameters as IL17-A,also it has a low cost in comparison to MRI but with an equivalent prognostic importance.

Conclusion

TLR4 and CSF2 gene expression has strong predictive value in early detection of axial spA changes in asymptomatic and non-radiographic

psoriatic patients which is equivalent and equal to the MRI predictive value that could help in the early management and prevention of progressive axial affection in those patients.

All procedures performed in this study were in accordance with ethical standards of national research committee.

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