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# TO IDENTIFY THE EXPRESSION OF INTRACELLULAR TOLL-LIKE RECEPTORS (TLRS) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF ALOPECIA AREATA.

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**Abstract**

**Background** Alopecia areata (AA) is a hair loss of scalp with out scarring and other hair bearing area. In AA immune system invade hair follicles The innate immunity and Toll-like receptors (TLRs) can express significant role in this autoimmune disorder. According to available data the role of expression of TLRs studied in multiple cutaneous disorders; however, some were investigated in relation to pathogenesis of AA

**Methodology** This retrospective cross-sectional study is to investigate the role of TLR-3, TLR-7, TLR-8, and TLR-9 in the AA patients. 40 (AA) patients were recruited from the Institute of Skin Disease Sindh and 10 healthy controls after getting an informed consent. Venous whole blood was used to extract the total RNA, and by using a real time quantitative PCR (RT-qPCR) expression of four TLRs with *GAPDH* (a house-keeping gene) was determined. The ANOVA statistical test was applied to analyze the expression values.

**Results** The expressions of all the four TLRs in AA were considerably significant than in the controls. The average relative expression of TLR-3, TLR-7, TLR-8, and TLR-9 was 54.40, 111.69, 34.45, and 112.51 respectively in all the AA patients. By comparing between male and female AA patients, the expression of all the four TLRs was non-significantly higher in male AA patients ($p<0.5$). TLRs expression was observed highest in the age group 21-30 yrs.

**Conclusion** This study identifies the significance of TLRs expression in AA patients indicating the fundamental role in autoimmunity, which will further provide a new insight in the therapeutic strategies of AA and other linked cutaneous diseases. Furthermore, studies are required to understand the targeted TLRs expression in introducing new modulated therapies for management of AA patients.

**Keyword:** Alopecia areata, autoimmune, gene expression, hair follicles, RT-qPCR, toll like receptors, SALT-score

## 1 Introduction

Alopecia areata (AA) is a condition, which includes hairloss without scarring. Onset of AA is uncertain, which can be extremely stressful for the patients (1). It appears with discrete areas of hair loss to generalized the scalp or body involvement. AA is reported with extensive hair loss along with relapse and remission of disease. (2). AA affect on the quality of life of patients, and it has the potential to cause psychiatric illnesses (such as anxiety and sadness) (3).

There are three types of AA, including alopecia totalis (AT), which affects the entire scalp; patchy-type AA (AAP), which is frequently limited to small regions on the scalp or in the beard area; and alopecia universalis (AU), which affects the entire body surface area (4). Numerous hypotheses exist about the pathophysiology of illness of AA. It involves a number of variables, including unknown etiology, autoimmune diseases and genetics. According to different studies, the interplay between AA familial stimuli and environmental factors determines the onset and severity of AA (1, 5). In AA white blood cells attacks hair follicles(HF) in hair bulge as a result of autoimmune response with accumulation of lymphocytes which further shrinks and decreases the new hair growth (5). HF mainly involve in pathogenesis of AA where immune reaction take place specially in the bulge of hair cycle and hair bulb in anagen phase is mostly prevented (6). The main way for achieving this is via suppressing the synthesis of MHC class I molecules in anagen hair bulbs, which may sequester autoantigens and shield them from CD8+ T cell presentation (7). In addition, lower portion of the hair bulb is infiltrated by T helper (Th) cells, cytolytic T cells, natural killer cells, and plasmacytoid dendritic cells during the anagen, or growth phase (8, 9). Furthermore, the secretion of regional immunosuppressive metabolites which include transforming growth factor-1 (TGF-1), interleukin-10 (IL-10), indoleamine-2,3-dioxygenase (IDO), melanocyte-stimulating hormone (MSH), and vasoactive intestinal peptide (VIP), may aid in the creation of a regional immune-inhibitory milieu (7). However, the increasing IFN- secretion in HF may facilitate anagen HF-associated antigens and loss of HF-IP, upregulating the MHC I and MHC II molecules, and chemokines (such as IL-15, IL-2, and CXCLs), and leading to the initiation of AA.

Innate immune response immediately recognize and denatures the microorganisms. (10). This immune response is responsible for identification and recognition of cytokines, produced by pathogens. TLRs have extracellular, ligand-binding domains that are similar in structure, but they are able to identify a variety of ligands. The primary domain gains a leucine-rich repeat motif-based horseshoe frame. It is ideal for two extracellular domains to form an 'm'-shaped dimer sandwich upon ligand binding, bringing the transmembrane and cytoplasmic domains of the ligand molecule close together and activating the downstream cascade. Ligands lead to the dimerization of receptors having similar characteristics. The interactions between ligands and extracellular domains of TLRs differ greatly from those of TLR paralogs (11-13).

Animals use pattern-recognition receptors to perceive whether disease-causing entities are present in the body (14). Pattern recognition receptors are transmembrane proteins known as TLRs. The expression of TLRs on antigen-presenting cells, for instance macrophages, starts signaling and supports both innate and adaptive immunity. It exhibits both molecular expression and the activation of inflammatory cytokines. It works by enhancing immunity to microbes through immunoadjuvant actions. Additionally, TLR agonists are important in immunological disorders like allergies. Understanding the basic mechanism of TLRs is crucial for developing new therapeutic approaches for autoimmune diseases and allergies (15).

These are crucial for the immune response to the offending substances and the identification of infections in humans. TLRs are type 1 transmembrane proteins with an LRR-rich extracellular domain and a conserved area known as the Toll/IL(TIR) domain in the cytoplasm. Extracellular domain has a massive horseshoe-shaped structure, according to crystallography research. They are primarily

found in immune-related tissues such the spleen, peripheral blood leukocytes, lungs, and digestive system. Depending on where the tissues and cell types are located, they may seem differently. Although TLR-3, TLR-7, and TLR-9 are located in the endosomal compartment, the majority of these receptors are found on the plasma membrane. Deoxy ribonucleotides, ribonucleotides, flagellin, and imidazoquinolines were used to identify the presence of these TLR receptors on endosomal compartments (14, 16).

The functions of the Toll-like receptor genes may mediate dermatologic symptoms of autoimmune responses such as AA. However, there are few studies reporting the role of TLRs in AA. In this study, we recruited AA patients belonging to urban as well as rural areas of the Sindh province of Pakistan. The expression of the TLRs (TLR3, TLR7, TLR8, TLR9) was assessed by using the real time quantitative PCR. The expression of TLRs was compared between the AA patients and controls. This study identifies the significance of TLRs in AA patients.

## 2 Methodology

### 2.1 Study Design

In this retrospective investigation, we chose individuals with AA who faced an episode of attacks of the disease, and the patients had active disease. The patients were recruited in the Institute of Skin Disease Sindh outpatient clinic. The study comprised both male and female participants of all ages. The study was conducted as per the guidelines of the Alopecia Areata Foundation Clinical Assessment. The Severity of Alopecia Tool (SALT) score was used to classify the AA patients and determine the severity of the condition. The patients with SALT ≥ 25% were designated as severe AA, whereas those with SALT <25% were considered as moderate AA patients.in exclusion criteria, those patients will not be included if they are on any medications either topical or systemic for three months. No history of smoking or any chronic disease such HTN, DM or other chronic disease. Children suffering from acute illness were excluded. Trichoscopy was done to confirm the clinical progression of disease.

### 2.2 Samples collection

About 10 mL of peripheral venous blood samples were collected in EDTA tubes from the AA patients and the healthy controls. The Histopaque-1077 media was used in gradient centrifugation to rapidly recover the viable lymphocytes and other mononuclear cells.

### 2.3 RNA Isolation from the whole blood

By using the EZ-10, spin column total RNA micro prep super kit (Bio Basic, Canada) as per the manufacturer's instructions, total RNA was extracted from whole blood. Briefly, 500µL sample's whole blood and 1µl water mix were mixed in a tube. The tube was inverted several times to mix the contents sufficiently, and then it was spin at 5,000 rpm for 1 minute. The supernatant was discarded, and the pellet was dissolved in 500 µL of RLT solution provided in the kit. The tube was inverted for 30 seconds to homogenize the lysate. Then equal volume (1 mL) of 70% ethanol was added to the lysate following well mixing with a pipette. An EZ spin column was placed in a 2.0 mL collection tube, and centrifuged at 8000 rpm for 30 seconds. Then the whole ethanol-lysate mixture was loaded into the spin column following centrifugation at 8000 rpm for 1 minute. The flow-through was discarded and 500µL of the RW solution was added onto the spin column, and then it was centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and 500 µL of the PRE solution was added onto the spin column, and again it was centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and the column was air dried for 1 minute. Then the EZ spin column was placed in a clean RNase-free 1.5 mL micro tube. About 50µL, RNase-free water was added onto the column following incubation at 50°C for 2 minutes. After incubation, the column was centrifuged at 10000 rpm for 1 minute to collect the total RNA in the 1.5 mL tube. The isolated RNA was stored at -70°C.

The workflow of total RNA isolation summarized in Fig. 1. The quantity and quality of the isolated RNA was estimated using a nanodrop instrument (Thermo Fischer Scientific, USA).

### 2.4 Synthesis of the first strand cDNA, and qPCR

The first strand cDNA was synthesized from the isolated RNA by using the TruScript™ First Strand cDNA Synthesis kit (Norgen Biotek, Canada) according to the protocol provided by the manufacturer. To create the first strand cDNA, 1µg of the total RNA was used. For setting the reaction, 10 µL 2x reaction mix, 2 µL TruScript™ enzyme mix, 1 µg total RNA, nuclease-free water (x µL) were used to make a total volume of 20 µL. A thermal cycler used to provide different temperatures to the reaction mixture for the first strand cDNA synthesis as per the program indicated in Table 1.

The expressions of four TLR-3, TLR-7, TLR-8, and TLR-9 along with an endogenous control GAPDH were quantified using real-time polymerase chain reaction (qPCR). Gene specific primers were used to amplify the targeted TLRs using the Green-2-Go qPCR Master mix (Bio Basic, Canada). This master mix contains all the components to perform a qPCR reaction, and includes a hot-start Taq polymerase enzyme, 2X mix of dNTPs, MgCl2, a fluorescent detection dye, a reference (passive) dye, and the requisite buffer to control the pH of reaction. The nucleotide sequence of the primers given in Table 2. For the qPCR, 20 µL reaction was prepared as 10 µL master mix, 0.6 µL each of forward and reverse primers, cDNA x µL (500 ng), and nuclease free water x µL to make the final volume of 20 µL. For performing the qPCR, initial denaturation (and enzyme activation) was carried out at 95 °C for 5 min, then 40 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min were carried out.

The fluorescence data collected at the annealing step. The threshold cycle (Ct) values for all TLRs were recorded, and the level of mRNA of our target gene was normalized to the level of GAPDH to find the ΔCt values, as Δ Ct (sample) = Ct (gene of interest) − Ct (GAPDH). Then the ΔCt values of the patients and controls were assessed as Δ Ct (patient) – Δ Ct (healthy control) to evaluate the ΔΔCt values, and two-fold change in expression analyzed by using the formula 2-ΔΔCt. (17).

### 2.5 Statistical analysis

To analyze Δ Ct values of each TLRs, average of Ct values of the TLRs were determined. The correlation between quantitative measures was investigated by using the Spearman correlation test. A two-tailed T-test and ANOVA (where applicable) were used to evaluate and compare the TLRs expression in the females and males. At p 0.05, the significance level was established. Statistical v.12 or Microsoft excel 2016 software were used to conduct the statistical analysis, where applicable.

## 3 Results

### 3.1 Cohort Description

The AA cohort comprised of 20 males and females each, with an average age of 17.325 years (SD 11.17), and median age of 13 years (IQR 8 - 24.25). The average age of females was 16.9 years (± 11.67), and median 11 years (IQR 7.75 – 22.75), and average age of males was 17.75 years (± 10.93), and median age was 17 years (IQR 8 – 24.75). Although average ages of the females and male participants were comparable (16.9 v.s. 17.75 years), yet a boxplot indicated higher median and variation in the ages of male participants (Fig. 2).

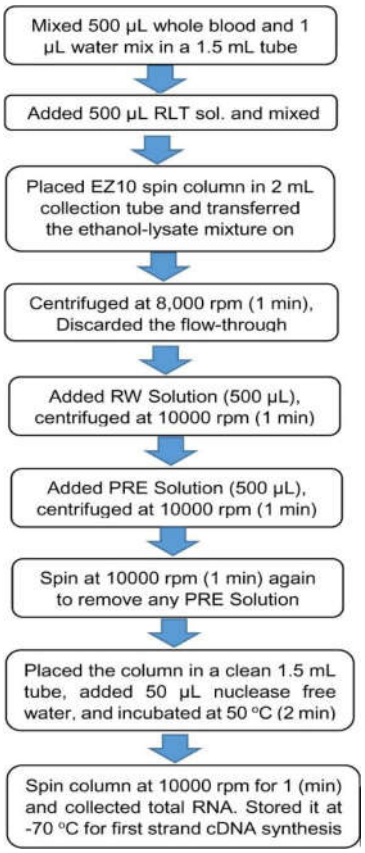


**Fig. 1:** Workflow for the isolation of total RNA from whole blood, using the Bio Basic SP-10 mini spin kit.

### 3.2 Expression level of TLRs

The qPCR technique used to evaluate the expression of the four TLRs (TLR-3, TLR-7, TLR-8, and TLR-9) in AA patients and healthy control subjects. The expressions of these TLRs in the AA patients and Controls compared to determine the differences (Fig. 3). This comparison highlighted proportional higher expression of all the TLRs in AA patients compared with that in the control subjects. The average expression of TLR-3, TLR-7, TLR-8, and TLR-9 was 15.32, 33.93, 10.77, and 33.10 elevated in the AA patients respectively compared with control subjects. The expression of TLR evaluated in both the males and females, and in different age groups, as described below:

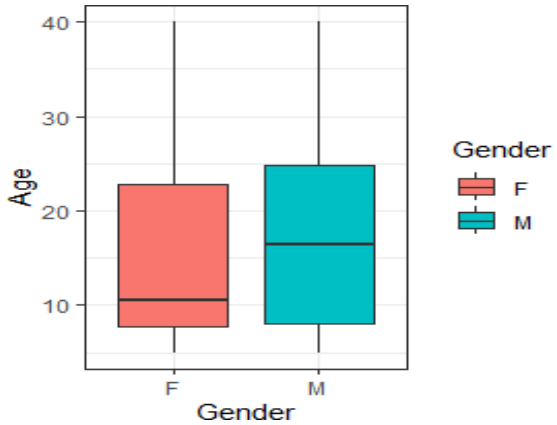


**Fig. 2:** Boxplots showing the distribution of ages of females (F) and males (M) of the AA participants of this study. Here, heights of the boxes represent variation in ages (years), and the central lines in the boxes represent median ages of both the genders.

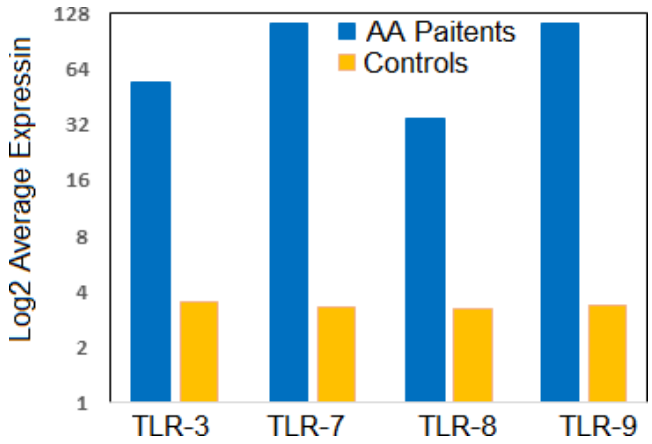


**Fig. 3:** TLR-3, TLR-7, TLR-8, TLR-9 expression in the AA and Control subjects of this study. The average expression of all the four TLRs was observed higher in the AA patients.

### 3.3 Expression of TLR-3

The expression of TLR-3 found quite variable in the study cohort. Overall, the average relative expression of TLR-3 was 54.40 (SD 199.80).

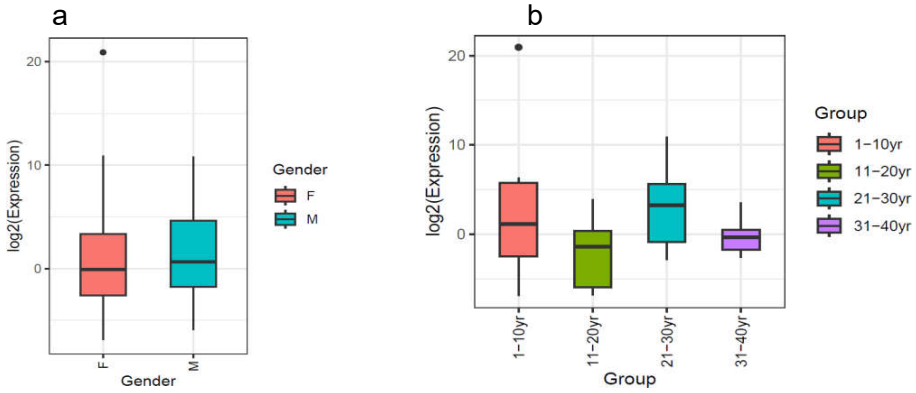


**Fig. 4:** Boxplots showing the distribution of expression of TLR-3. a. The TLR-3 expression in females (F) and males (M) of AA patients. b. The TLR-3 expression in four different age groups of the patients.

The higher standard deviation value represented higher variation in TLR-3 expression. The median TLR-3 expression was 1.73 (IQR 0.61-14.87). Next, TLR-3 expression in males and females participants evaluated, and the participants of different age groups (1-10 years, 11-20 years, 21-30 years, and 31-40 years) to determine the point of variation in the gene expression. The average TLR-3 expression in females was 93.09 (SD 279.53), and median expression 1.73 (IQR 0.16 - 6.66) (Fig. 4A). Likewise, the average TLR-3 expression in males was 15.72 (SD 25.66), and median expression 2.02 (IQR 0.75 - 16.02) (Fig. 3A). Statistically, there was non-significant difference ($P>0.05$), using two-tailed T-test, in the TLR-3 expression in males and females. Finally, the analysis of TLR-3 expression in all age groups of AA participants showed that AA patients aged 21–30 had increased expression of TLR-3, followed by those aged 1–10, 11–20, and 31–40 years. (Fig. 4B).

### 3.4 Expression of TLR-7

The expression of TLR-7 observed as relatively higher compared with that of TLR-3 in the AA study cohort. The average relative expression of TLR-3 was 111.69 (SD 415.93) in all the 40 patients. The standard deviation was higher than the average value, which represented greater variation in TLR-7

expression. The median TLR-7 expression was 1.17 (IQR 0.26-12.54). The TLR-3 expression also evaluated in male and female AA patients. This analysis highlighted that the average TLR-7 expression was 116.45 (SD 436.89) in the females, and median expression was 0.94 (IQR 0.16 - 10.06) (Fig. 4A). Likewise, the average TLR-7 expression in males was 106.92 (SD 405.19), and median expression 1.60 (IQR 0.29 - 27.67) (Fig. 5A). The large differences in the average and median values indicated that some outliers, which resulted in higher, average values of TLR-7 expression in the AA patients. A two-tailed T-test indicated that the difference in TLR-7 expression in males and females AA was non-significant (P>0.05). The assessment of the expression of TLR-7 in different age groups of AA patients indicated that the TLR-7 expression was the highest in AA patients aged 21-30 years, followed by 1-10 years, 31-40 years, and 11-20 years (Fig. 5B).

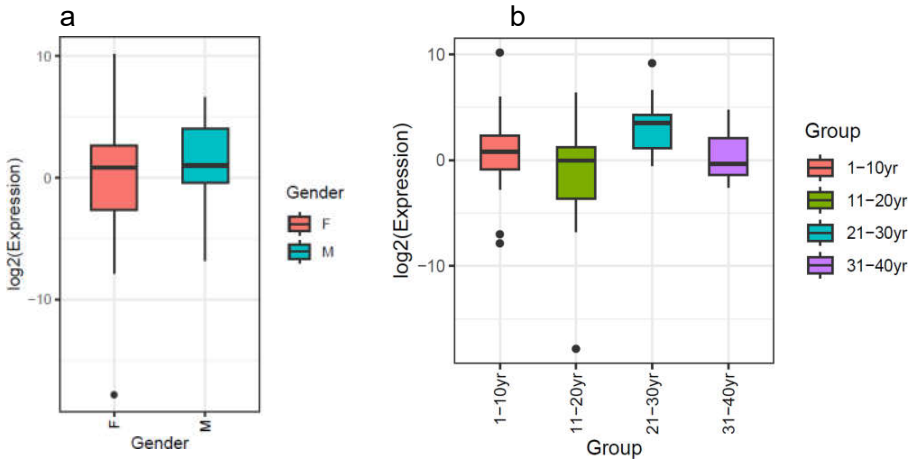


**Fig. 5:** Boxplots showing the distribution of expression of TLR-7 in AA patients. **a.** The TLR-7 expression was slightly higher in males than in females. **b.** Age wise, the TLR-7 expression was the highest in the AA patients of 21-30 years of age.

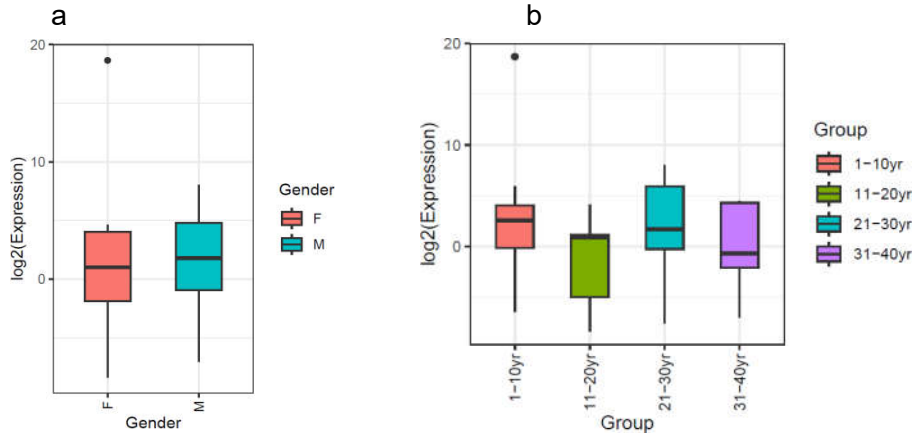


**Fig. 6:** Boxplots showing the distribution of expression of TLR-8 in AA patients. A. The TLR-8 expression was slightly higher in males than in females. B. Age wise, the median TLR-8 expression was the highest in the AA patients of 1-10 years of age.

### 3.5 Expression of TLR-8

The cumulative expression of TLR-8 was comparatively lower than those of TLR-3, and TLR-7 gene. The average relative expression of TLR-8 was 34.45 (SD 84.81), and median expression was 2.18 (IQR 0.31-17.26). This difference in the average and median values of TLR-8 expression indicated a moderate dispersion in the expression data. Gender wise, in females, the average TLR-8 expression

was 26.68 (SD 89.99), and median was 2.01 (IQR 0.28-16.56). In males, the average TLR-8 expression was 42.23 (SD 80.86), and median expression 3.48 (IQR 0.55-30.09) (Fig. 6A). Statistically, a two-tailed T-test indicated non-significant difference (P>0.05) in the TLR-8 expression in males and females. Age wise, the median TLR-8 expression was highest in the AA patients of 1-10 years old, followed by AA patients of 21-30 years, 11-20 years, and 31-40 years (Fig. 6B).

### 3.6 Expression of TLR-9

Across all the AA patients, the average relative expression of the TLR-9 gene was significantly higher than that of the TLR-8 gene. The average relative expression of TLR-9 was 112.51 (SD 443.60), and median expression was 1.89 (IQR 0.28-18.54). The difference in the average and median values of TLR-9 expression was quite higher, indicating a higher dispersion in the expression data. Gender wise, the average TLR-9 expression was 12.59 (SD 25.81), and median was 1.23 (IQR 0.48-5.44) in the female AA patients. In the male AA patients, average TLR-9 expression was 212.52 (SD 618.17), and median expression 2.23 (IQR 0.28-26.14) (Fig. 7A). Further, there was non-significant difference (P>0.05) in the TLR-9 expression in males and females by using a two-tailed T-test. In different age categories of AA patients, the median TLR-9 expression was highest in the AA patients of 21-30 years old, followed by AA patients of 31-40 years, 1-10 years, and 11-20 years (Fig. 7B).

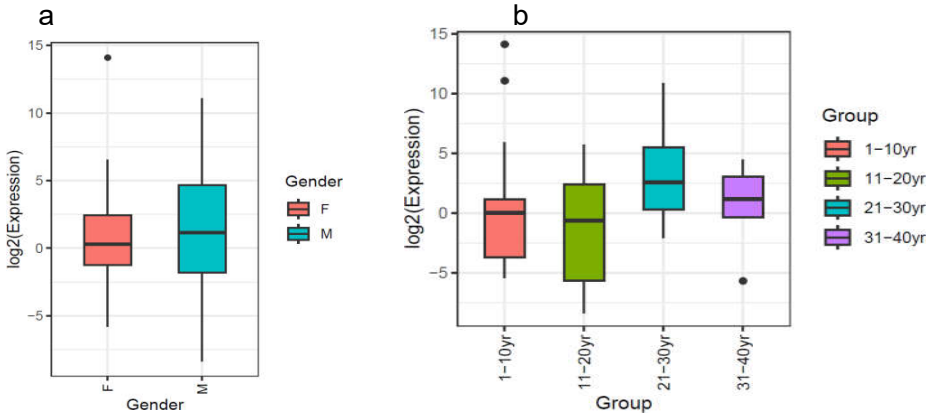


**Fig. 7:** Boxplots showing the distribution of expression of TLR-9 in AA patients. A. The expression of TLR-9 was slightly higher in males than in females. B. Age wise, the median TLR-9 expression was highest in the AA patients of 21-30 years of age.

### 3.7 Comparison of expressions of four TLR genes

The cumulative expression of the four TLR genes (TLR-3, TLR-7, TLR-8, and TLR-9) evaluated to compare the expression level of these TLRs (Fig. 7). Notable, the median expression of all the four TLRs was comparable (1.73, 1.17, 2.18, 1.89 for TLR-3, TLR-7, TLR-8, and TLR-9 respectively). However, the difference in the average expression of TLRs was notably larger (54.40, 111.69, 34.45, and 112.51 for TLR-3, TLR-7, TLR-8, and TLR-9 respectively) (Fig. 8).

## 4 Discussion

The pattern-recognition receptors also called as toll-like receptors (TLRs) detect different infections and trigger both innate and adaptive immune responses. Currently, the expression of TLR in the circulatory hematological cells implicated in different cutaneous diseases including the alopecia areata (AA), and systemic lupus erythematosus (SLE) (18, 19). Alzolibani et al (2016) reported that the expression of certain TLRs was significantly increased in the peripheral blood-mononuclear cells (PBMCs) of AA patients belonging to Arab ancestry (19). This study to address the real-time quantitative PCR to assess the expression of four TLRs (TLR-3, TLR-7, TLR-8, and TLR-9) in the peripheral blood of alopecia areata patients. The study provides the molecular role involved in the pathogenesis of AA.

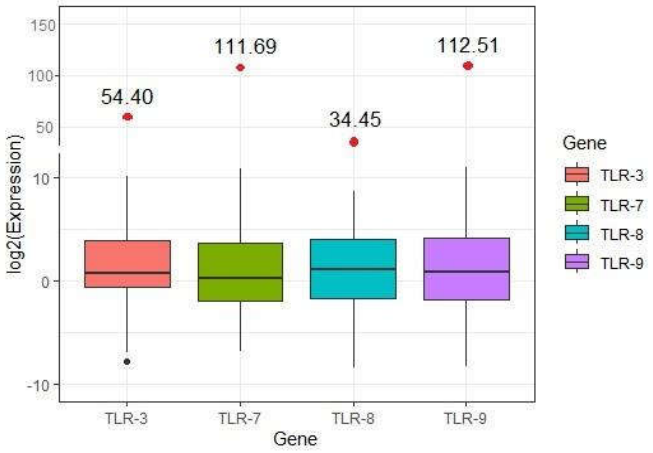


**Fig. 8:** Boxplots showing the distribution of expression of four TLRs in AA patients. The central horizontal lines in middle of the boxes represent the median expression values. The red dots (and the adjacent numbers) represent the average expression values.

The nucleic acids work on invading pathogens which are captured by TLRs. These TLRs are expressed intracellularly in endosomal compartments (20). The TLR-3 can recognize polyinosinic-polycytidylic acid, a synthetic counterpart of double-stranded RNA produced by viruses. TLR3 stimulates transcription factors to express type I interferon and inflammatory cytokines once it binds (21). TLR-7 and TLR-9 on the cell endosomal membranes detected the virus-derived RNA and DNA and other expressed antiviral gene including the interferons. (22). Human TLR-8 detects viral ssRNA and imidazoquinoline compounds (23). The expression of TLRs link makes a bridge between the innate and adaptive immunity. Multiple autoimmune disorders occur as a result of these inappropriate activations (24). It has been discovered that peripheral blood cells' activation of TLR signaling cascades controls the expression of a variety of cytokines. TLRs and cytokines are implicated in underlying disease process of AA, it is conceivable that abnormal regulation of TLRs expression following incorrect TLR activation may actively contribute to AA pathogenesis. In this study, the results indicated that the expression of TLR-3, TLR-7, TLR-8, and TLR-9 significantly higher in the circulatory leukocytes in AA than in the healthy controls of matching age, gender. The findings of the study is in accordance with the previous reports where increased expression of various TLRs identified in the peripheral blood and the follicle cells (19, 25). The increased levels of TLRs found in the blood of the patients indicate that TLRs are crucial to the pathophysiology of the AA, which will be helpful in new-targeted therapy. It is likely that after the exposure with some environmental pathogens, these Toll like receptors are activated leading to an auto-immune response which in result affect the hair follicles and ultimately causing the disease of hair-loss. This is determine by previous studies in which the TLR-9 mRNA expression was found increased in the follicle bulbs of AA patients (25).

It is observed that increased TLR-3, TLR-7, TLR-8, and TLR-9 expression in AA. Although it does not fully explain the complex AA etiology, the TLR receptors are one plausible category of molecules for further exploration for their potential involvement in occurrence of AA. In future, new studies are essential to investigate the targeted TLRs expression in AA for new modulated therapies in management of AA patients.

**Table 1:** Thermal cycler program to carry out the first strand cDNA synthesis

| Temperature | Time |
|---|---|
| 25 °C | 5 minutes |
| 50 °C | 60 minutes |
| 70 °C | 15 minutes |
| 4 °C | Hold |

**Table 2:** the primers used to quantify the expression of 5 genes using real time qPCR.

| Gene | Primer | Accession number & Sequence |
|---|---|---|
| TLR-3 | Forward | NM_003265.2<br>5′-TTTGCGAAGAGGAATGTTTAAATCT-3′ |
| | Reverse | 5′-CACCTATCCGTTCTTTCTGAACTG-3′ |
| TLR-7 | Forward | NM_016562.3<br>5′-TCCTTGTGCGCCGTGTAAA-3′ |
| | Reverse | 5′-GTCAGCGCATCAAAAGCATTT-3′ |
| TLR-8 | Forward | NM_016610.3<br>5′-GCTACGGCAGCGGATCTGT-3′ |
| | Reverse | 5′- CCTTCTGCCTTCGGGTTGT-3 |
| TLR-9 | Forward | NM_017442.3<br>5′-GGCAAAGTGGGCGAGATG-3′ |
| | Reverse | 5′-GCTCTGCGTTTTGTCGAAGAC-3 |
| GAPDH | Forward | NM_002046.5<br>5′-TCGACAGTCAGCCGCATCTTCTTT-3′ |
| | Reverse | 5′-ACCAAATCCGTTGACTCCGACCTT-3′ |

## References


1. Brzezińska-Wcisło L, Bergler-Czop B, Wcisło-Dziadecka D, Lis-Święty A. New aspects of the treatment of alopecia areata. Advances in Dermatology and Allergology. 2014;31(4):262-5.
2. Pratt CH, King LE, Messenger AG, Christiano AM, Sundberg JP. Alopecia areata. Nature Reviews Disease Primers. 2017;3(1):17011.
3. Toussi A, Barton VR, Le ST, Agbai ON, Kiuru M. Psychosocial and psychiatric comorbidities and health-related quality of life in alopecia areata: a systematic review. Journal of the American Academy of Dermatology. 2021;85(1):162-75.
4. Jabbari A, Cerise JE, Chen JC, Mackay-Wiggan J, Duvic M, Price V, et al. Molecular signatures define alopecia areata subtypes and transcriptional biomarkers. EBioMedicine. 2016;7:240-7.
5. Islam N, Leung PS, Huntley AC, Gershwin ME. The autoimmune basis of alopecia areata: a comprehensive review. Autoimmunity Reviews. 2015;14(2):81-9.
6. Rajabi F, Drake L, Senna M, Rezaei N. Alopecia areata: a review of disease pathogenesis. British Journal of Dermatology. 2018;179(5):1033-48.
7. Bertolini M, McElwee K, Gilhar A, Bulfone-Paus S, Paus R. Hair follicle immune privilege and its collapse in alopecia areata. Experimental Dermatology. 2020;29(8):703-25.
8. Simakou T, Butcher JP, Reid S, Henriquez FL. Alopecia areata: A multifactorial autoimmune condition. Journal of Autoimmunity. 2019;98:74-85.
9. Xing L, Dai Z, Jabbari A, Cerise JE, Higgins CA, Gong W, et al. Alopecia areata is driven by cytotoxic T lymphocytes and is reversed by JAK inhibition. Nature Medicine. 2014;20(9):1043-9.
10. Koenderman L, Buurman W, Daha MR. The innate immune response. Immunology letters. 2014;162(2):95-102.
11. Botos I, Segal DM, Davies DR. The structural biology of Toll-like receptors. Structure. 2011;19(4):447-59.
12. Asami J, Shimizu T. Structural and functional understanding of the toll-like receptors. Protein Science. 2021;30(4):761-72.
13. Behzadi P, García-Perdomo HA, Karpiński TM. Toll-like receptors: general molecular and structural biology. Journal of Immunology Research. 2021;2021:1-21.
14. Li D, Wu M. Pattern recognition receptors in health and diseases. Signal Transduction and Targeted Therapy. 2021;6(1):291.

15. Kaisho T, Akira S. Toll-like receptor function and signaling. The Journal of allergy and clinical immunology. 2006;117(5):979-87; quiz 88.
16. Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. Immunology letters. 2003;85(2):85-95.
17. Gohar A, Shakeel M, Atkinson RL, Haleem DJ. Potential mechanisms of improvement in body weight, metabolic profile, and liver metabolism by honey in rats on a high fat diet. PharmaNutrition. 2020;14:100227.
18. Toubi E, Vadasz Z. Innate immune-responses and their role in driving autoimmunity. Autoimmun Rev. 2019;18(3):306-11.
19. Alzolibani AA, Rasheed Z, Bin Saif G, Al-Dhubaibi MS, Al Robaee AA. Altered expression of intracellular Toll-like receptors in peripheral blood mononuclear cells from patients with alopecia areata. BBA clinical. 2016;5:134-42.
20. Krieg AM, Vollmer J. Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. Immunological reviews. 2007;220:251-69.
21. Zainol MIB, Kawasaki T, Monwan W, Murase M, Sueyoshi T, Kawai T. Innate immune responses through Toll-like receptor 3 require human-antigen-R-mediated Atp6v0d2 mRNA stabilization. Scientific Reports. 2019;9(1):20406.
22. Wagner H. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Current opinion in microbiology. 2002;5(1):62-9.
23. Sun Y, Reddy P. CH 18 - Intracellular sensors of immunity and allogeneic hematopoietic stem cell transplantation. In: Socié G, Blazar BR, editors. Immune Biology of Allogeneic Hematopoietic Stem Cell Transplantation. San Diego: Academic Press; 2013. p. 425-47.
24. Alexoudi I, Kapsimali V, Vaiopoulos A, Kanakis M, Vaiopoulos G. Toll-like receptors pathways implication in common autoimmune diseases and therapeutic perspectives. Giornale italiano di dermatologia e venereologia: organo ufficiale, Societa italiana di dermatologia e sifilografia. 2015;150(2):255-60.
25. Kang H, Wu W-Y, Yu M, Shapiro J, McElwee KJ. Increased expression of TLR7 and TLR9 in alopecia areata. Experimental Dermatology. 2020;29(3):254-8.