# Association of Interleukin-12B Polymorphism and Serum Level of Interleukin-12 in a Sample of Egyptian Patients with Rheumatoid Arthritis

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**Rheumatoid arthritis (RA) is one of the common autoimmune diseases, which affected by genetic and environmental factors. IL-12 is important cytokine that play an effective role in the inflammatory reaction of RA. It regulates the balance between Th1 and Th2 cells. Gene polymorphism of cytokines may predispose to susceptibility and severity of RA. To assess the association between single nucleotide polymorphism (SNP) in IL-12B gene (rs3212227 A/C) and serum level of IL-12 with the development and or activity of RA disease in Egyptian population. Sixty RA patients and thirty healthy individuals were studied for IL-12B gene (rs3212227 A/C) polymorphism using PCR-RFLP. Serum level of IL-12 was measured by ELISA. The frequency of genotype AC, CC, AC+CC and C allele were significantly higher in patients compared to control group (*P*<0.02, 0.007, 0.02) respectively. Serum level of IL-12 was significantly higher in patients compared to control (*P*<0.000). Patients who carry AC+CC genotypes had significantly higher DAS28, RF, ACCP and IL-12 compared to AA genotype patients (*P*<0.05, 0.000, 0.000, 0.000) respectively. RA patients who carry AC, CC genotypes had more positive inflammatory markers (RF, ACCP) with *P*<0.000, 0.05 respectively. Significant positive correlation was found between serum IL-12 and number of swollen joints, RF and ACCP. Present findings suggest that IL-12B gene (rs3212227 A/C) may be associated with development and activity of RA and that serum IL-12 can be used as predictor of activity of the disease.**

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune inflammatory diseases. It is complex disorder and affects approximately up to 1 % of the general population worldwide [1]. It could affect multiple tissues and systems, especially joints, characterized by synovitis, progressive erosions, and cartilage destruction leading to significant disability and early mortality [2]. It affects women more than men and the ratio is 3:1. The interaction between genetic susceptibility and environmental factors play an important role in pathogenesis of the disease [3]. Cytokines in the inflammatory cells play an important role in pathogenesis of RA, and polymorphism of cytokine genes may lead to changes in its proteins, that could influence the development or severity of RA [4].

Interlukin-12 (IL-12) is one of the heterodimeric proinflammatory cytokines, which is composed of two disulfide-bonded polypeptide chains of 35 kDa light chain; p35 (encoded by IL-12A gene) and 40 kDa heavy chain; p40 (encoded by IL-12B gene) [5]. It is produced by cells of the innate immune system as well as by B cells and is considered as a connecting point between innate and adaptive immunity [6]. IL-12 is

produced by different antigen presenting cells. It produces a critical role in inducing T helper 1 (Th1) phenotype, thus initiating cell-mediated immune responses, and plays a central role in promoting the differentiation of naive CD4+ T cells into mature interferon-γ (IFN- γ) producing Th1 effector cells. It is also a potent stimulus of natural killer and CD8+ T cells to produce IFN- γ [7]. IL-12 plays an important role in the defense against intracellular microorganisms. In addition, excessive IL-12 production has been found in many autoimmune diseases, including rheumatoid arthritis, type I diabetes mellitus, multiple sclerosis (MS) and Crohn's disease (CD) [8]. IL-12 is expressed by infiltrating macrophages and synovial lining cells in RA [9]. IL-12 could induce the production of IL-1 and tumor necrosis factor alpha (TNF-α) and promote rheumatoid synovial T cells to produce interferon-gamma (IFN-γ) [10]. RA patients have elevated levels of IL-12, in both serum and synovial fluid demonstrating that it is associated with pathogenesis and activity of the disease [11], indicating that the genetic variants related with the RA should be investigated.

IL-12B gene is located on Chromosome 5 [12]. A→C (rs3212227) single nucleotide polymorphism (SNP) located in the 3´ untranslated region (UTR) at position 1188 [13]. The associations between IL-12 gene SNP and autoimmune and inflammatory diseases have been reported recently. IL-12B gene SNP rs3212227A/C is associated significantly with diseases, such as asthma [14], psoriasis [15], type 1 diabetes [16] and RA [17]. Thus, given the important role of IL-12 and IL-12B gene polymorphism in RA and several inflammatory diseases.

The aim of our study is to assess the role of IL-12B gene SNP rs3212227A/C and serum level of IL-12 in development and or activity of RA in Egyptian patients.

## Patients and Methods

### Study subjects

The study included sixty patients with RA, diagnosed as RA according to American College of Rheumatology (ACR) criteria (1987), they were fulfilling the 2010 ACR-EULAR classification criteria for RA [18], who were recruited from outpatient clinic of Physical Medicine, Rheumatology and Rehabilitation Department, Assiut University Hospital between March 2018 to October 2018. Patients less than 18 years or with other autoimmune diseases were excluded from the study. The control group was thirty healthy individuals who showed no clinical or laboratory signs of autoimmune disease selected randomly from blood bank donors, age and sex matched. Patients and control groups had provided written informed consent to be involved in this study. Laboratory analysis and genotyping were done in Clinical Pathology Department, Assiut University Hospital.

### Blood sampling and measurements

Seven ml of blood was collected from each individual and divided into three aliquots; 2 ml of whole blood was collected in sterile EDTA tube and stored at -20°C for further DNA extraction, another 2 ml of whole blood was collected in EDTA tube for ESR determination. The remaining part was collected in serum separator tube, centrifuged at 3500 rpm for 10 min for HsCRP, RF, ACCP and IL-12. HsCRP and RF were done by nephelometry using BN ProSpec analyzer (Siemens Healthineers). ACCP was done by chemiluminescence using Centaur XP analyzer (Siemens Healthineers). IL-12 was measured by ELISA (enzyme-linked immunosorbent assay) according to the manufacturer's instructions (SinoGeneClon Biotech co).

### Genotyping methods

Genomic DNA was extracted from the stored blood, using QIAamp DNA Mini Kit supplied by QIAGEN. The polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method was used to determine the possible SNPs in the IL-12B gene at position +1188A/C. The primer sequences were as follows: forward 5´-CTG ATC CAG GAT GAA AAT TTG-3´, reverse 5´-CCC ATG GCA ACT TGA GAG CTG G-3´ [19].

Amplification reaction was performed using DreamTaq Green PCR Master Mix, Thermo Scientific #K1081. For each 50 µl reaction we used 25 µl of DreamTaq Green PCR Master Mix, 2.0 µM of Forward primer, 2.0 µM of Reverse primer, 10 µl of template DNA and 11 µl of water. The thermal cycling for PCR included an initial denaturing at 95 °C for 15 min, followed by 35 cycles at 94 °C for 45 s, 54 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min.

The PCR product (226 bp) was digested with 1 µl of TaqI restriction enzyme (Thermo Scientific #ER0671, Lot: 00626296), separated in 2.5% agarose gel and visualized with ethidium bromide staining under ultraviolet light. Those samples were used as a positive control for the respective genes. The negative control consisted of PCR-grade water lacking the DNA template. Digestion was visualized and reported manually. A 226-bp full-length PCR product was typed as homozygous AA, samples displaying 71 bp and 155 bp fragments were typed as homozygous CC and samples exhibiting 226 bp, 155 bp and 71 bp were typed as heterozygous AC [19].

### Statistical Analysis

Data were analyzed using the Statistical Package for Social Science (IBM SPSS) version 20. The quantitative data were presented as mean, standard deviations, while qualitative data were presented as number and percentages. The comparison between two independent groups with qualitative data was done by using Chi-square test. The comparison between two independent groups with quantitative data was done by using Independent t-test. Pearson correlation coefficients were used to assess the correlation between two quantitative parameters in the same group. *P*-value was considered significant at $P < 0.05$.

## Results

Demographic, clinical and laboratory data of patients and control groups are demonstrated in table (1) showed the age, sex, disease duration, disease activity score by ESR (DAS28), ESR, RF, ACCP and IL-12, representing high significant elevation of serum IL-12 in patients as compared to control groups.

Table 1. Demographic, clinical and laboratory finding in patients and control groups.

| | | Patients | Control | *P* value |
|---|---|---|---|---|
| Age | Range | 26-73 | 22-55 | - |
| | Mean±SD | 47.08±11.1 | 43.23±6.3 | |
| (male/female) | Male | 2 (3.3%) | 3 (10%) | - |
| | Female | 58(96.7%) | 27(90%) | |
| DD (years) | Range | 1-30 | - | - |
| | Mean±SD | 8.23±5.7 | - | - |
| DAS28 | Range | 1.7-7.9 | - | - |
| | Mean±SD | 4.58±1.4 | - | - |
| ESR | Range | 3-102 | - | - |
| | Mean±SD | 39.8±21.8 | - | - |
| HsCRP | Range | 0.7-14.3 | - | - |
| | Mean±SD | 6.71±3.4 | - | - |
| RF | Range | 6.8-250 | - | - |
| | Mean±SE | 51.01±8.4 | - | - |
| ACCP | Range | 2.2-230 | - | - |
| | Mean±SE | 66.10±9.9 | - | - |
| IL-12 | Range | 54-126 | 13-59.5 | 0.000* |
| | Mean±SD | 77.40±15.5 | 38.43±13.7 | |

DD disease duration, DAS disease activity score, ESR estimated sedimentation rate, HsCRP high sensitive C reactive protein, RF rheumatoid factor, ACCP anti-cyclic citrullinated peptide, IL-12 interleukin 12. *P value was significant if < 0.05.

The genotype distribution of IL-12B gene (+1188 A/C) in patients and control groups are shown in table (2) & Fig 1 and representing significant increase in frequency of the AA, AC and CC genotype in patients when compared to control group. C allele frequency was significantly elevated in patients compared to control group.

Table 2. Distribution of genotypes and alleles frequency of IL-12B gene in patients and control groups.

| IL-12B (+1188A/C) | Patients (N. 60) | Control (N. 30) | *P* value |
|---|---|---|---|
| AA | 26 (43.3%) | 22 (73.4%) | |
| AC | 30 (50%) | 7 (23.3%) | 0.02* |
| CC | 4 (6.7%) | 1 (3.3%) | |
| AC+CC | 34 (56.7%) | 8 (26.7%) | 0.007* |
| A allele | 82 (68.4%) | 51 (85%) | 0.02* |
| C allele | 38 (31.6%) | 9 (15%) | |

* *P* value was significant if < 0.05.

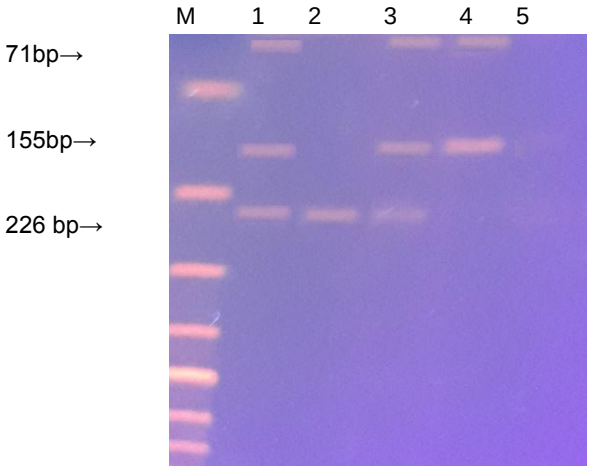


**Figure 1.** Gel electrophoresis of IL-12B genotypes produced by RFLP PCR in RA patients on agarose gel shows the digested fragments by *Taq1* restriction enzyme. The pattern shows DNA marker in position (M), IL-12B AC genotype (with fragment sizes of 226, 155, 71 bp) in position 1 & 3, IL-12B AA genotype (with fragment size 226 bp) in position 2 IL-12B CC genotype (with fragment sizes of 155, 71 bp) in position 4 and negative control in position 5 DNA marker in position (M).

Comparison was made between RA patients having wild genotype (AA) and polymorphic genotypes (AC+CC) regarding clinical and laboratory biomarkers of RA (table 3) showed that there was insignificant difference in age, disease duration, N. of swollen joints, N. of tender joints, ESR and HsCRP, but there was significant difference in DAS28 and very high significant difference ($P$<0.000) in RF, ACCP and IL-12.

Table 3. Comparison of clinical and laboratory data AA genotype and AC+CC genotypes in RA patients

| | AA (N. 26) | AC+CC (N. 34) | *P* value |
|---|---|---|---|
| Age | 45.96±10.1 | 47.94±11.9 | - |
| Sex (M/F) | 2 /24 | 0/34 | - |
| DD | 7.5±4.6 | 8.7±6.4 | - |
| DAS28 | 4.19±1.3 | 4.89±1.5 | 0.05* |
| Swollen joints | 1.23±2.1 | 2.62±3.7 | - |
| Tender joints | 6.69±8.1 | 7.82±6.0 | - |
| ESR | 37.2±22.0 | 41.7±21.8 | - |
| HsCRP | 6.87±3.08 | 6.59±3.68 | - |
| RF | 16.2±2.3 | 77.6±13.07 | 0.000* |
| ACCP | 25.67±9.0 | 97.02±14.2 | 0.000* |
| IL-12 | 65.14±6.6 | 86.77±13.7 | 0.000* |

DD disease duration, DAS disease activity score, ESR estimated sedimentation rate, HsCRP high sensitive C reactive protein, RF rheumatoid factor, ACCP anti-cyclic citrullinated peptide, IL-12 interleukin 12, * *P* value was significant if < 0.05.

The frequency of positive RA laboratory markers were compared between the three different genotypes of IL-12B (+1188 A/C), table (4) (HsCRP positive >3 mg/L, RF positive>12 IU/ml and ACCP positive >5 U/ml) we showed that there was insignificant difference between all genotypes in HsCRP but RF and ACCP were shown significant difference between the three genotypes of IL-12B.

Table 4. Association between IL-12B gene (+1188A/C) polymorphism and laboratory biomarkers in RA patients.

| | | AA | AC | CC | *P* value |
|---|---|---|---|---|---|
| HsCRP | - ve | 4 (15.4%) | 7 (23.3%) | 0 (0%) | - |
| | +ve | 22 (84.6%) | 23 (76.7%) | 4 (100%) | |
| RF | - ve | 17 (65.4%) | 5 (16.7%) | 0 (0%) | 0.000* |
| | +ve | 9 (34.6%) | 25 (83.3%) | 4 (100%) | |
| ACCP | - ve | 7 (26.9%) | 2 (6.7%) | 0 (0%) | 0.05* |
| | +ve | 19 (73.1%) | 28 (93.3%) | 4 (100%) | |

HsCRP high sensitive C reactive protein, RF rheumatoid factor, ACCP anti-cyclic citrullinated peptide
* *P* value was significant if < 0.05.

Correlation between serum level of IL-12 and clinical and laboratory finding of RA patients table (5), Fig (2), (3), (4) showed that significant positive correlation (*P*<0.000) between serum level of IL-12 and N. of swollen joints, RF, ACCP but there was no correlation between serum level of IL-12 and age, DD, DAS28, N. of tender joints, ESR and HsCRP.

Table 5. Correlation between serum IL-12 (pg/ml) and clinical and laboratory findings in RA patients

| | Serum IL-12 (pg/ml) | |
|---|---|---|
| | r | *P* value |
| Age | 0.133 | - |
| DD | 0.027 | - |
| DAS28 | 0.167 | - |
| Swollen joints | 0.334 | 0.009* |
| Tender joints | 0.251 | - |
| ESR | 0.013 | - |
| HsCRP | 0.067 | - |
| RF | 0.634 | 0.000* |
| ACCP | 0.448 | 0.000* |

DD disease duration, DAS disease activity score, ESR estimated sedimentation rate, HsCRP high sensitive C reactive protein, RF rheumatoid factor, ACCP anti-cyclic citrullinated peptide, IL-12 interleukin 12, *P value was significant if < 0.05.

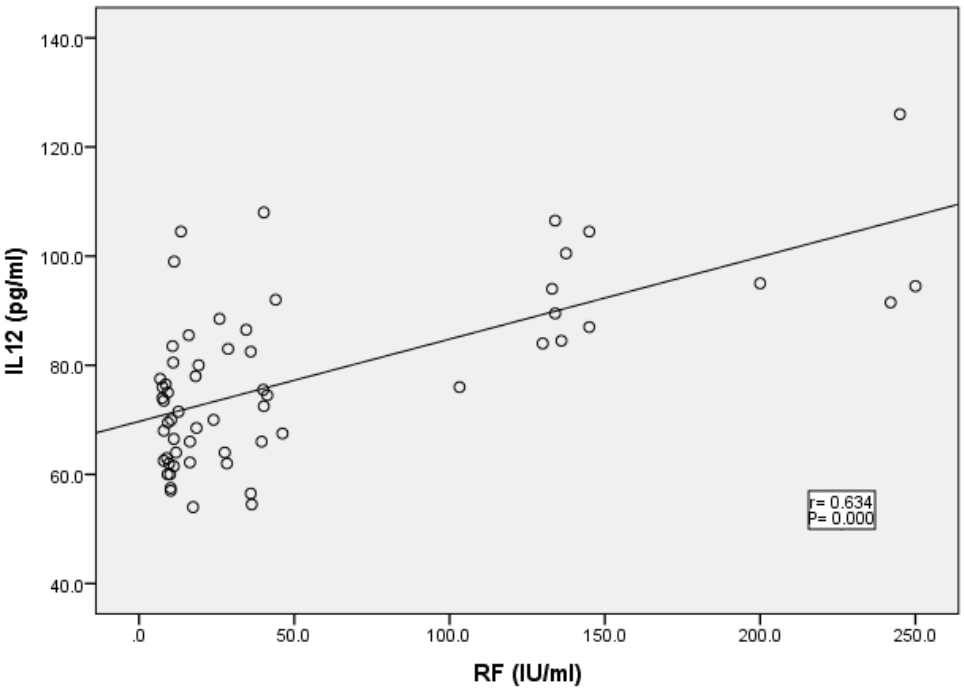


**Figure 2.** Correlation between serum level of IL-12 and RF.

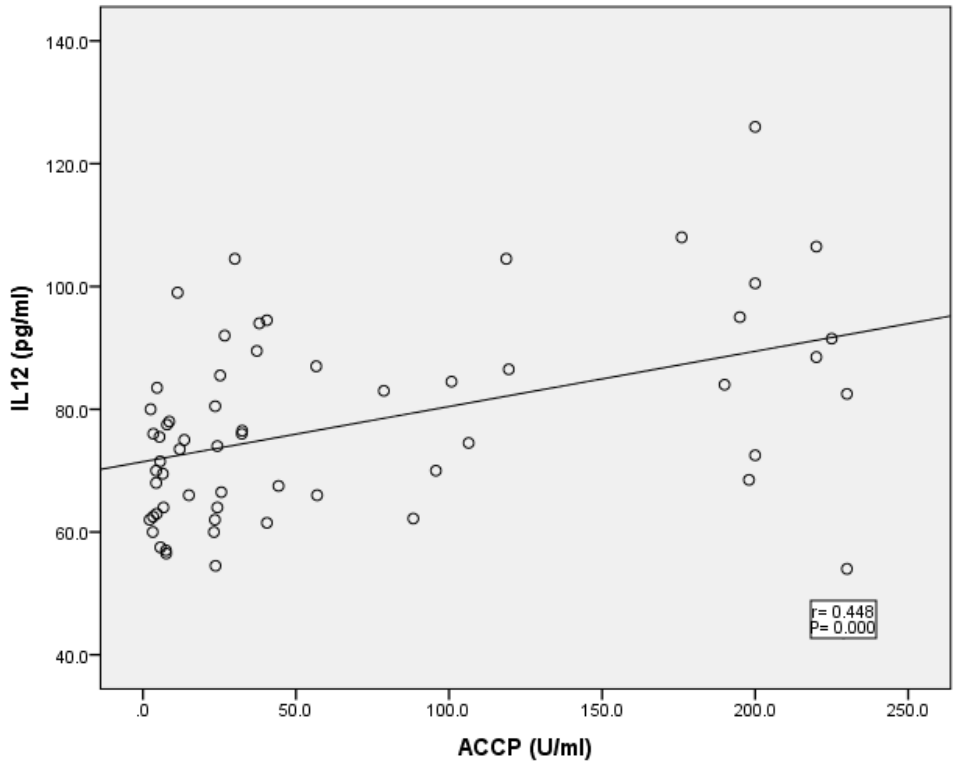


**Figure 3.** Correlation between serum level of IL-12 and ACCP

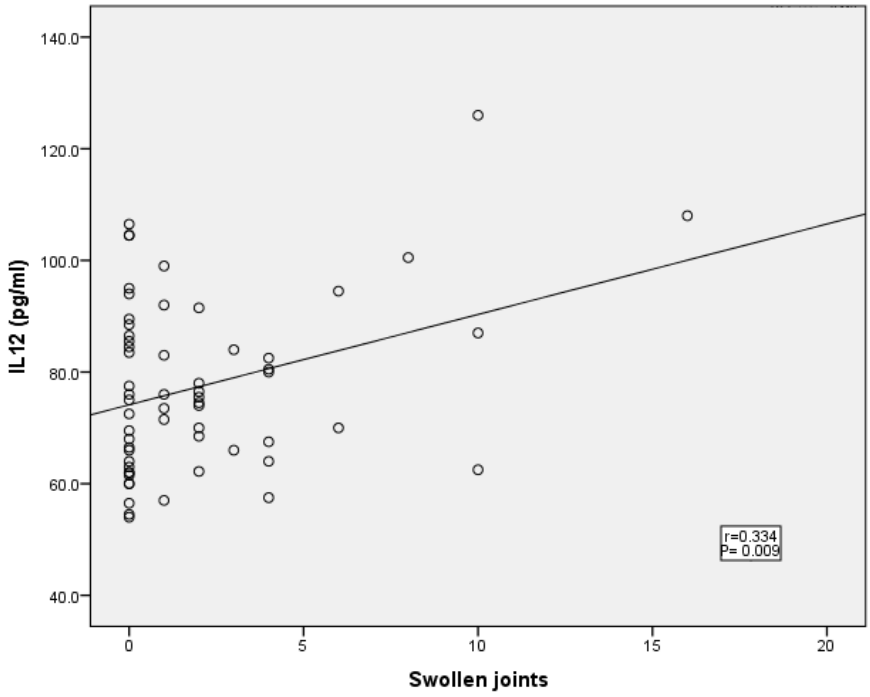


**Figure 4.** Correlation between serum level of IL-12 and N. of swollen joints.

## Discussion

Rheumatoid arthritis (RA) is a complex disease with different phenotypes such as synovitis, progressive erosions, and cartilage destruction [20]. Genetic factors play important roles in the pathogenesis of RA. The total heritability of RA was found to be approximately 66% [21]. Different polymorphisms at the IL-12B locus may affect the course of T cell-mediated immune response and increase the susceptibility of some autoimmune diseases as RA [19].

In our study we found that serum level of IL-12 was significantly increased in patients compared to control group which in agreement with Mokhtar *et al.* [22], Paradowska-Gorycka *et al.* [19] Shen *et al.* [7] that they demonstrated and that serum IL-12 was more important in prediction than TNF-α in active course of the disease.

In our study we found that the frequency of polymorphic genotype (AC+CC) of IL-12B (+1188 A/C) were significantly higher in RA patients compared to control group. C allele was also significantly higher in patients than in control. In agreement with Mokhtar *et al.* [22], Paradowska-Gorycka *et al.* [19], Shen *et al.* [7] and Wang *et al.* [20]. They showed similar results and concluded that IL-12B gene SNP rs3212227 A/C was associated with development of RA and individuals with C allele was significantly at higher risk in developing RA than A allele.

On the other hand, Change *et al.* [23], Alvarez-Rodriguez et al [24] and Angelo et al [25] demonstrated that there was no association between rs3212227 A/C and development of RA. This discrepancies between studies may be explained by heterogenicity of the disease, ethnic variance in the genetic polymorphism between populations and limited sample size.

We found that RA patients with IL-12B (AC+CC) genotypes were significantly higher in DAS28, RF, ACCP and serum level of IL-12 compared to RA patients carry AA genotype in agreement with Mokhtar *et al*. [22] who reported that patients with (AC+CC) were higher in DAS28, ESR, CRP, RF, anti-CCP and serum level of IL-12. Also, Seegers *et al.* [8] found that presence of C allele in IL-12B gene is associated with increase production of serum IL-12.

We found that AC and CC genotypes carriers were significantly more likely to be RF and ACCP positive that in agreement with Shen *et al.* [7] who found that AC and CC genotypes were found more in RF positive patients and Wang *et al.*, who found that CC genotype carriers had more significantly RF positive results and reported that C allele carriers are more likely to develop severe RA and C allele may affect the inflammatory reaction of IL-12 in RA patients.

There was significant positive correlation between serum IL-12 and N. of swollen joints, RF and ACCP but there was no correlation between IL-12 and DD, DAS28, N. tender joints, ESR and HsCRP that was partially in agreement with Mokhtar *et al.* [22] and Kim *et al.* [5] as they found that higher serum IL-12 was positively correlated with swollen joint score, DAS28, ESR, RF and ACCP and also in partial agreement with Paradowska-Gorycka *et al.* [19] who found that higher serum IL-12 was positively correlated with number of swollen and tender joints however no correlation between serum IL-12 and inflammatory markers such as CRP, RF and ACCP. This discrepancy between these studies may arise from racial variances, different sample sizes, and different criteria of the disease (age,

DD, DAS28 and number of tender or swollen joints) and the treatment they received.

We concluded that SNP rs3212227 A/C in IL-12B was associated significantly with increased risk of RA. C allele may increase serum level of IL-12 and that increase activity of the disease in Egyptian population. Serum level of IL-12 can reflect RA disease activity, so IL-12 blockade could be useful for decrease the inflammatory reaction of RA. However, further studies with larger sample size are needed to confirm the conclusion.

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