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Original article

Methylene tetrahydrofolate reductase, transforming growth factor-β1 and lymphotoxin-α genes polymorphisms and susceptibility to rheumatoid arthritis

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ABSTRACT

Background: Rheumatoid arthritis is a widely prevalent autoimmune disorder with suggested genetic predisposition.
Objectives: The aim of this study is to detect the pattern of genetic polymorphism of methylene tetrahydrofolate reductase (MTHFR C677 T and A1298 C), transforming growth factor-β1 (TGF-β1 T869 C) and lymphotoxin-α (LT-α A252G) in patients having rheumatoid arthritis and correlate these patterns to disease activity and serum levels of tumor necrosis factor-alpha (TNF-α), B-Cell Activating Factor (BAFF), and osteopontin.
Methods: A total of 194 subjects, 90 controls and 104 patients with rheumatoid arthritis were genotyped for MTHFR C677 T and A1298 C, TGF-β1 T869 C and LT-α A252G polymorphisms using a methodology based on PCR-RFLP. Also serum levels of TNF-α, osteopontin and BAFF were measured by ELISA kits.
Results: The CT genotype and T allele of MTHFR C677 T and GG genotype and G allele of LT-α A252G are associated with the risk of RA and with higher levels of the pro-inflammatory cytokine, TNF-α in patients with rheumatoid arthritis.
Conclusion: Our findings suggest that there is association between MTHFR C677 T and LT-α A252G genes polymorphisms and increased risk of RA in this sample of Egyptian population.

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Polimorfismos dos genes metilenotetrahidrofolato redutase, fator de crescimento transformador β1 e linfotoxina-α e susceptibilidade à artrite reumatoide

Palavras-chave:
Artrite reumatoide
Polimorfismo genético
MTHFR C677 T e A1298 C
TGF-β1 T869 C
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RESUMO

Antecedentes: A artrite reumatoide é uma doença autoimune amplamente prevalente com sugerida predisposição genética.
Objetivos: Detectar o padrão de polimorfismo dos genes metilenotetrahidrofolato redutase (MTHFR C677 T e A1298 C), fator de crescimento transformador β1 (TGF-β1 T869 C) e linfotoxina-α (LT-α A252G) em pacientes com artrite reumatoide e correlacionar esses padrões com a atividade da doença e os níveis séricos de fator de necrose tumoral alfa (TNF-α), fator ativador de linfócitos B (BAFF) e osteopontina.
Métodos: Foram genotipados 194 indivíduos – 90 controles e 104 com artrite reumatoide – à procura de polimorfismos dos genes MTHFR C677 T e A1298 C, TGF-β1 T869 C e LT-α A252G com uma metodologia baseada na PCR-RFLP. Mensuraram-se também os níveis séricos de TNF-α, osteopontina e BAFF com kits de Elisa.
Resultados: O genótipo CT e o alelo T do MTHFR C677 T e o genótipo GG e alelo G do LT-α A252G estão associados ao risco de AR e a níveis mais elevados da citocina pró-inflamatória TNF-α em pacientes com artrite reumatoide.
Conclusão: Os achados do presente estudo sugerem que há associação entre os polimorfismos dos genes MTHFR C677 T e LT-α A252G e um risco aumentado de AR nessa amostra da população egípcia.

Introduction

Rheumatoid arthritis (RA) is a widely prevalent autoimmune disorder which affects ~1% of the populations in developed countries with women more frequently affected than men (~3:1).[1] In this disease, the joints are the main target of attack with great tendency to joint destruction and consequently impairment of all aspects of life quality.[2] The exact causes of the disease are unknown, but environmental factors and genetic predisposition are involved. Although these factors are not sufficient for development of disease, yet they may have a role in the heterogeneity of the clinical picture, the response to treatment and they can be target for therapeutic agents. Polymorphic forms of methylene tetrahydrofolate reductase (MTHFR) gene and some cytokine genes have been studied as possible markers of susceptibility, severity, and/or protection in RA.[3]

C677 T (Ala 222 Val) and A1298 C (Glu 429 Ala) are two common genetic polymorphisms of MTHFR gene. They are both associated with decrease in the activity of the enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) with lower degree in A1298 C compared to C677 T polymorphism.[4] This enzyme is responsible for the synthesis of 5-methyltetrahydrofolate, required for pyrimidine and purine synthesis and regeneration of methionine from homocysteine.[5] Decreased activity of this enzyme results in high levels of homocysteine which is commonly found in patients with rheumatoid arthritis (RA), and is partially responsible for the high rate of cardiovascular complications in these subjects.[6]

Transforming growth factor-β1 (TGF-β1) is a growth factor that regulates cellular proliferation, wound healing, and angiogenesis in a cell-specific manner.[7] It is present abundantly in rheumatoid joints and it has anti-inflammatory function because overexpression of this gene reduced arthritis in an animal model.[8] Individuals with TGF-β1 polymorphisms in the coding and regulatory regions have a greater propensity to develop immune system disorders. TGF-β1 (T869 C) polymorphism affects the serum levels of TGF-β1 and is used as marker for increased disease risk in several diseases.[9]

Cytokines play an important role in pathogenesis of RA and its associated inflammatory processes and articular destruction. This occurs mainly through deviation of balance toward higher levels of pro-inflammatory cytokines on the expense of anti-inflammatory cytokines. Thus, the concentration of members of pro-inflammatory TNF-α superfamily have been directly correlated with disease pathology.[10] TNF-α is a potent pro-inflammatory cytokine produced mainly by macrophages. It is considered one of the main inflammatory mediators of joint inflammation and destruction in RA by inducing other inflammatory cytokines and stimulating expression of adhesion molecules by fibroblasts.[11] Lymphotoxin-α (LT-α), another member of the TNF superfamily previously known as tumor necrosis factor-β (TNF-β) induces cell apoptosis and inflammatory responses upon binding to TNF receptor type 1 and 2 respectively.[12] A single nucleotide polymorphism (A252G) was detected within the first intron of LT-α gene and it was reported to increase expression of LT-α plasma level.[13] The two alleles resulting from this single nucleotide polymorphism are designated LT-α (10.5 kb) and LT-α (5.5 kb). The LT-α (5.5 kb) allele is associated with higher plasma levels of LT-α, lymphoid malignancies and a worse outcome of autoimmune diseases.[14]

Another member of TNF family is B cell activating factor (BAFF) which can bind B cells stimulating their proliferation and promoting their survival and consequently helping in

regulation of both innate and adaptive immune responses. Deregulation of BAFF has been observed in patients with autoimmune diseases such as rheumatoid arthritis.[15] Osteopontin (OPN) is also a pro-inflammatory cytokine which has immunoregulatory effects in autoimmune diseases and could be involved in the pathogenesis of RA.[16]

The aim of this study is to detect the pattern of genetic polymorphism of MTHFR types (C677 T) and (A1298 C), TGF-β1 (T869 C) and LT-α (A252G) in rheumatoid arthritis Egyptian patients and if there is any correlation of those patterns with disease activity and serum levels of TNF-α,BAFF and osteopontin.

Subjects e methods

Subjects selection

This is a cross sectional observational comparative study that was conducted in Kasr Al Aini rheumatology unit, Faculty of Medicine, Cairo University from May to December 2013. The study was conducted according to the principles of the Helsinki Declaration and was approved by the local ethics committee of the Faculty of Medicine of Cairo University.

Informed written consent was obtained from all subjects who participated in this study after explaining the aim and nature of the study. One hundred and four patients with rheumatoid arthritis (16 males and 88 females) and ninety healthy control subjects of matched age and sex were included in the study. The patients were on regular treatment with non-steroidal anti-inflammatory drugs (NSAID) ($n = 42$), methotrexate ($n = 88$), Prednisone ($n = 24$), Leflunomide ($n = 32$) and Hydroxychloroquine ($n = 56$). Patients were treated by different combinations of the previous drugs. Thorough history taking and physical examination were done for all patients and Disease activity score in 28 joints (DAS-28) and Health Assessment Questionnaire total (HAQ) total were calculated.

Laboratory assays

Serum BAFF, OPN and TNF-α were measured by ELISA kits obtained from R&D Systems, Minneapolis, MN, for the former two and from AviBion, Helsinki Finland for TNF-α according to the manufacturer's protocol.

Molecular analysis

DNA extraction

Genomic DNA was extracted from peripheral blood using a QIA amp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Genotyping of MTHFR A1298 C[17]

One set of forward "5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3″ and reverse "5′-CAC TTT GTG ACC ATT CCG GTT TG-3″ primers were used for amplification of a fragment of 241 base pairs and then the amplified fragment was digested with MboII enzyme. The PCR profile was: initial denaturation at 95 °C for 5 min, followed by 35 cycles each of 30 s at 94 °C, 51 °C and 72 °C, then for 10 min at 72 °C. The AA genotype produces two bands of 211 and 30 bp, the CC genotype produces a single band of 241 bp (uncut), and AC genotypes produces three bands of 241, 211 and 30 bp.

Genotyping of MTHFR C677 T[18]

One set of forward "5′-CAT CCC TAT TGG CAG GTT AC-3″ and reverse "5′-GAC GGT GCG GTG AGA GTG-3″ primers were used for amplification of a fragment of 198 base pairs, and then the amplified fragments were digested with HinfI enzyme. The PCR profile was: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s each at 94 °C, 61 °C and 72 °C and a final elongation at 72 °C for 5 min. The wild CC genotype was identified by only a 198 bp fragment, the (TT) genotype by the 175/23 bp fragments and heterozygotes (CT) genotype by both the 198, 175 and 23 bp fragments.

Genotyping of TGF-β1 T869 C genotypes[19]

One set of forward primers 5′-TTCCCTCGAGGCCCTCCTA-3′ and reverse 5′-GCCGCAGCTTGGACAGGATC-3′ primers were used for amplification of 294 bp fragments of the TGF-β1 gene. PCR products were then digested by MspA1I enzyme. The PCR profile was: denaturation at 96 °C for 10 min, followed by 35 cycles of 75 s each at 96 °C, 62 °C and 72 °C, and a final extension at 72 °C for five minutes. The T allele resulted in 4 fragments of 161, 67, 40, and 26 bp. The C allele resulted in fragments of 149, 67, 40, 26, and 12 bp.

Genotyping of LT-α (A252G)[20]

A 782 bp fragment of the intron 1 (+252A/G) of the LT-α gene was PCR-amplified with the primer set: (forward) "5′-AGAGGGGTGGATGCTTGGGTTC-3″ and (reverse) "5′-CCGTGCTTCGTGCTTTGGACTA-3″. PCR products were digested with NcoI restriction enzyme. The A allele gives a single fragment of 782-bp (not digested). The G allele is digested into 586-bp and 196-bp bands. The PCR profile was: incubation for 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 52 °C, and 1 min at 72 °C, with a final extension of 72 °C for 7 min.

Statistical analysis

Statistical Package of Social Science Software program (SPSS), version 19 was used to analyze data. Data were summarized using frequency and percentage for qualitative data or mean and standard deviation for quantitative ones. Chi-squared analysis was used to test for deviation of genotype distribution from Hardy-Weinberg. Comparison of patients and healthy control groups were performed using Chi square test or Fisher's exact test for qualitative data and Student *t* test and ANOVA test for quantitative ones. Bivariate regression analysis was used to detect association of different genetic polymorphic forms with the disease. Receiver Operating Characteristics (ROC) curve analysis was conducted to explore the discriminant ability of osteopontin and TNF for RA patients. *p* values less than 0.05 were considered statistically significant.

Table 1 – Patients' characteristics.

Parameters	Min	Max	Mean ± SD
Age (years)	22.0	70.0	42.7 ± 12.1
Disease duration (month)	6	216	66.7 ± 52.5
Morning stiffness (min)	3.0	120.0	29.9 ± 38.2
Number of swollen joints	0.0	18.0	3.3 ± 5.5
Number of tender joints	0.0	20.0	5.0 ± 5.9
ESR	5.0	125.0	36.4 ± 36.0
DAS 28	1.4	7.7	3.9 ± 1.6

Results

Patients' characteristics including age, disease duration, clinical manifestations, disease activity and ESR are shown in Table 1.

Comparison between serum level of Osteopontin, BAFF and TNF-α (expressed as mean ± SD) in patients and control groups showed that serum levels of OPN and TNF-α were significantly higher in cases compared to controls ($p < 0.001$). Serum BAFF level showed no statistical difference between patients and control groups ($p = 0.6$) (Table 2).

On comparing the ability of TNF and Osteopontin in discriminating control from rheumatoid arthritis patients the ROC curve showed that TNF-α has a higher discriminating ability than OPN with sensitivity (94.2%) and specificity (84.4%) at cut off value 15.2 pg/ml. On the other hand, OPN has sensitivity (71.2%) and specificity (53.3%) at cut off value 5.7 ng/ml as calculated by ROC curve (Figure 1).

The results of this study showed that the genotype frequencies of all studied cases were in Hardy-Weinberg equilibrium for MTHFR C677 T & A1298 C and TGF-β1 T869 C. As for LT-α A252G, the genotype frequencies were in Hardy-Weinberg equilibrium only in control group.

On studying the association between rheumatoid arthritis and different polymorphic forms of MTHFR C677 T, MTHFR A1298 C, TGF-β1 T869 C and LT-α A252G (Table 3). The results of this study showed significant association between rheumatoid arthritis and CT genotype, T allele of MTHRF C677 T and the GG genotype and G allele of LT-α A252G. On the other hand, the AC genotype of MTHFR A1298 C showed a protective effect from the disease when compared to the wild genotype AA. Moreover, no significant association has been detected between different polymorphic forms of TGF-β1 T869 C polymorphism and rheumatoid arthritis (Table 3).

HAQ total showed no association with different polymorphic forms of MTHFR C677 T polymorphism ($\chi^2 = 5.5$, $p = 0.06$), MTHFR A1298 C ($\chi^2 = 2.26$, $p = 0.32$), TGF-β1 T869 C, ($\chi^2 = 1.31$, $p = 0.52$) or LT-α ($\chi^2 = 1.1$, $p = 0.57$). Also, disease activity as shown by DAS 28 was not associated with polymorphic forms of all examined genes; for MTHFR C677 T ($\chi^2 = 0.4$, $p = 0.81$), for MTHFR A1298 C ($\chi^2 = 0.69$. $p = 0.7$), for TGF-β1 T869 C ($\chi^2 = 4.27$, $p = 0.12$) and for LT-α ($\chi^2 = 4.54$, $p = 0.1$).

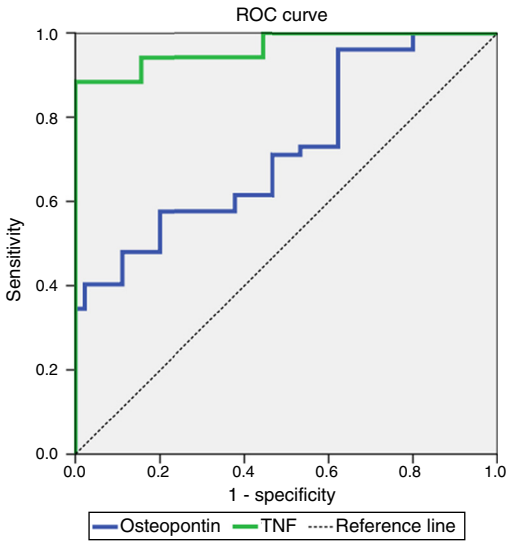

Figure 1 – ROC curve showing ability of TNF and osteopontin in discriminating control from rheumatoid arthritis patients.

We found that serum TNF-α level differs significantly in the different polymorphic forms of all tested genes except MTHFR A1298 C as shown in Table 4. Post Hoc test showed that serum TNF-α is significantly higher in CT genotype than in CC genotype in MTHFR C677 T, $p = 0.009$, in CT genotype than that in TT genotype in TGF-β1 T869 C, $p = 0.005$, in GG genotype when compared to that in AA genotype, $p = 0.008$, and AG genotype, $p = 0.005$, in LT-α A252G. There is no significant difference in OPN and BAFF serum levels in the different genotypes of examined genes (Table 4).

Discussion

Although the etiology of RA remains unclear, susceptibility factors that include environmental and genetic factors are evident. The genetic factors constitute about 50% of these factors.[3] The pro-inflammatory cytokines amplify the inflammatory process and destruction in rheumatoid joints. Tumor necrosis factor, osteopontin and BAFF are among these cytokines.[3]

Table 2 – Comparison between serum level of osteopontin, BAFF and TNF-α (expressed as mean ± standard deviation) in patients and control groups.

	Cases ($n = 104$) Mean ± SD	Controls ($n = 90$) Mean ± SD	p value
Osteopontin (ng/ml)	12.9 ± 10.6	5.9 ± 2.5	<0.001
BAFF (pg/ml)	474.5 ± 151.1	489.7 ± 145.6	0.6
TNF-α (pg/ml)	36.0 ± 14.3	8.9 ± 6.3	<0.001

Table 3 – Association between rheumatoid arthritis and different polymorphic forms of MTHFR C677 T, MTHFR A1298 C, TGF-β1 T869 C and LT-α A252G.

	Genotype and allele	Patients		Control		*p*	OR (95% CI)
		n	%	*n*	%		
	CC	46	44.2	64	71.1		
	CT	50	48.1	22	24.4	0.011	3.16 (1.30–7.69)
MTHRF C677 T	TT	8	7.7	4	4.4	0.260	2.78 (0.47–16.5)
	C	142	68.3	159	83.3		
	T	66	31.7	30	16.7	0.028	2.18 (1.09–4.38)
	AA	46	44.2	20	22.2		
	AC	34	32.7	56	62.2	0.006	0.26 (0.10–0.69)
MTHFR A1298 C	CC	24	23.1	14	15.6	0.629	0.75 (0.23–2.45)
	A	126	60.6	96	53.3		
	C	82	39.4	84	46.7	0.383	0.74 (0.42–1.32)
	TT	18	17.3	20	22.2		
	CT	68	65.4	36	40.0	0.173	2.10 (0.72– 6.10)
TGF-β1 T869 C	CC	18	17.3	34	37.8	0.390	0.59 (0.18–1.97)
	C	104	50	104	58		
	T	104	50	76	42	0.313	0.73 (0.41–1.29)
	AA	6	5.8	16	28.9		
	AG	42	40.4	48	17.8	0.252	2.33 (0.55–9.95)
LT-α A252G	GG	56	53.8	26	53.3	0.021	5.74 (1.31–25.26)
	A	54	26	80	44		
	G	154	74	100	56	0.007	2.28 (1.25–4.18)

In this study, serum levels of TNF-α are significantly higher in patient compared to control group. The results regarding TNF-α are coincided with results of previous studies such as Gheita et al.,[21] and Ismail et al.,[22] who explained these results by its vital and central role in the etiology and pathogenesis of RA, hence TNF inhibitors were the first of the biological disease modifying anti-rheumatic drugs (DMARDs) to be approved for the treatment of RA and now they are part of the routine treatment of patients with this disease.[23] However, Ebrahimi et al.,[24] reported non-significant increase in serum TNF-α levels in patients when compared to control group.

Also, our results showed significant increase in serum OPN levels in patients with RA than in healthy control group which are in accordance with results of Ji et al.,[25] Chen et al.[26] who explained these results by the cardinal role of OPN and its receptors in pathogenesis of RA. So, several experimental studies aiming to use OPN as therapeutic target are being performed and the outcomes of these results are promising.[27]

On the contrary, the serum BAFF showed no significant difference between both groups which is coming with previous results of Eldin et al.[28] However, Mahdy et al.,[29] and Moura et al.,[30] reported significant increase in serum BAFF levels among RA patients especially patients with higher disease activity and shorter disease duration. This discrepancy in results may be attributed to different disease activity and duration in the studied group.

The CT genotype and T allele of MTHFR C677 T are associated with RA, higher serum level of TNF-α, but they have no significant effect on OPN and BAFF serum levels. On the other hand, the different MTHFR A1298 C polymorphic forms were not associated with RA, or serum levels of TNF-α, OPN or BAFF. The AC genotype of MTHFR A1298 C showed protective effect from the disease. This may be due to higher

Table 4 – Comparison between serum levels of osteopontin, TNF-α and BAFF in different genotypes in all studied cases (*n* = 194).

	Serum osteopontin (ng/ml)			Serum TNF (pg/ml)		Serum BAFF (pg/ml)	
	Geno-type	Mean ± SD	*p*	Mean ± SD	*p*	Mean ± SD	*p*
	CC	8.32 ± 7.75		19.20 ± 15.076		505.16 ± 149.65	
MTHFR C677 T	CT	11.24 ± 9.92	0.248	28.93 ± 19.817	0.031	444.67 ± 133.17	0.162
	TT	11.62 ± 7.91		25.62 ± 16.080		486.83 ± 197.19	
	AA	12.27 ± 11.30		23.61 ± 16.05		486.49 ± 136.9793	
MTHFR A1298 C	AC	7.79 ± 6.00	0.076	20.58 ± 18.17	0.233	480.98 ± 157.43	0.233
	CC	9.293 ± 8.00		28.75 ± 17.85		474.47 ± 151.22	
	CC	6.79 ± 5.99		22.17 ± 11.99		518.42 ± 164.63	
TGF-β1 T869 C	CT	10.54 ± 8.66	0.151	27.09 ± 19.39	0.018	482.25 ± 139.42	0.137
	TT	10.91 ± 11.07		14.04 ± 15.32		429.32 ± 139.09	
	AA	14.99 ± 16.98		14.43 ± 3.62		525.27 ± 174.13	
LT-α A252G	AG	9.44 ± 7.86	0.075	19.35 ± 15.45	0.004	469.87 ± 135.90	0.542
	GG	8.35 ± 5.53		29.82 ± 19.66		482.71 ± 155.02	

effect of MTHFR C677 T on MTHFR enzyme resulting in hyper-homocysteinaemia and subsequent cascade of cytokine activation. Results of previous studies were heterogeneous; some showed no association between risk of RA and different polymorphic forms of MTHFR C677 T and A1298 C.[31,32] Others reported association of T allele, but not any of the MTHFR C677 T polymorphic forms with RA.[3] Rubini et al.[33] reported association of CC genotype of MTHFR A1298 C but not any of MTHFR C677 T polymorphic forms with susceptibility to RA in Italian population. This heterogeneity in results may be attributed to racial variations in allele and genotype frequencies as reported by Hughes et al.,[32] who found significant increase in T allele in MTHFR C677 T and C allele of MTHFR A1298 C (independent on disease status) in Caucasians when compared to African Americans. Also, a significant interaction between MTHFR polymorphisms and nutrient/environmental factors (i.e. folate status, age, smoking and alcohol intake) was reported.[4] Transforming growth factor-β1 (TGF-β1) is an anti-inflammatory cytokine associated with disease remission, and in addition has the potential to be pro-inflammatory cytokine. Polymorphisms of TGF-β1 have been reported to be associated with variations in the serum levels of TGF-β1 and with several diseases.[34] In this study, the TGF-β1 (T869 C) genotypes were not associated with RA. However, the CT genotype was associated with significant increase in serum TNF when compared to TT genotype. This is coming with results of the two meta-analysis studies done by Zhang et al.[35] and Chang et al.[36] which showed no clear association of TGF-β1 T869 C polymorphism and T allele with RA on a worldwide population but association was suggested only in the people of Asian descent. The results of Hussein et al.[37] was different as they found association of TGF-β1 T allele with susceptibility to RA in Egyptian patients. Our results also showed association of the G allele and the GG genotype of LT-α A252G with RA and higher TNF-α serum levels. These results are in accordance with that of Karray et al.[38] and Al-Rayes et al.,[39] which reported association between GG and AA genotypes with RA, while GA genotype was refractory.

From the above we can conclude that CT genotype of MTHFR C677 T, AC genotype of MTHFR A1298 C and GG genotype of LT-α A252G are associated with increased risk of RA in Egyptian patients.

To our knowledge, this is the first study to evaluate simultaneously the association of MTHFR C677 T and A1298 C, TGF-β1 T869 C and LT-α A252G polymorphisms with RA and with pro-inflammatory cytokines TNF, BAFF and OPN in Egyptian patients.

Some limitations were facing this study as it included a very broad group of patients regarding disease duration, disease activity, patients taking different drugs and no mention was made about rheumatoid factor and Anti-CCP positivity. The cytokines measurements may vary widely with these factors and also with other methodology details, such as time between the sample is collected and processed, DAS-28, drugs and its' doses when the samples were collected.

Conflicts of interest

The authors declare no conflicts of interest.

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