NF-κB1/IKKε Gene Expression and Clinical Activity in Patients With Rheumatoid Arthritis

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ABSTRACT

Objective: Rheumatoid arthritis (RA) is a systemic and autoimmune disorder whose primary characteristic is the chronic inflammation of joints. The objective of this study was to evaluate whether there was an association between nuclear factor kappa beta1/IKK epsilon (*NF-κB1/IKKε*) gene expression and clinical activity in RA.

Methods: Sixty patients with RA were included in the study: 30 with clinical activity and 30 with clinical remission. *NF-κB1/IKKε* gene expression was performed by real-time quantitative polymerase chain reaction through relative quantification with Taqman probes. A ROC curve for *NF-κB1* and *IKKε* was also constructed.

Results: There were significant differences in *NF-κB1* and *IKKε* gene expression ($P \leq .001$ and $P \leq .029$, respectively) between RA patients with clinical activity and clinical remission. The multivariate lineal general model showed that the use of nonsteroidal anti-inflammatory drugs influenced the *NF-κB1* ($P = .046$) and *IKKε* ($P = .005$) expression. The ROC curves for the event "clinical activity" showed the greater area under the curve for *NF-κB1* (0.827, 95% CI 0.717-0.937), $P \leq .001$.

Conclusion: Although the use of NSAIDs influences the NF-κB1/IKKε pathway, the *IKKε* expression might be a useful laboratorial analysis to evaluate the RA clinical activity.

Keywords: clinical activity; DAS28; *NF-κB1/IKKε*; rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic and autoimmune disorder that primarily affects synovial joints. With a female-to-male ratio of 3:1, its worldwide prevalence is 1%; in Latin America, this is 0.5%, and in Mexico, 0.4%.[1,2] The main symptoms of RA are inflammation and painful joints, morning stiffness of 30-minute duration, fatigue, functional disability, and, in some cases, fever.[3]

There exist various risk factors linked with RA as follows: family history of RA or autoimmunity, obesity, tobacco consumption, social class, exposure to infectious agents, and genetic

Abbreviations:

RA, rheumatoid arthritis; IL, interleukin; TNF-α, tumor necrosis factor alpha; CICMED, Research Committee of the Medical Sciences Research Center; UAEMEX, Autonomous University of the State of Mexico; ACR, American College of Rheumatology; CRP, c-reactive protein; TLR, toll-like receptor

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factors, especially the presence of HLA-DR4 polymorphisms, such as DRB1*04:01, DRB1*04:05, and DRB1*04:04.[4,5]

Some transcription factors are involved in the origin and progression of chronic inflammatory disorders. Among these factors, some family members of the nuclear enabling factor of kappa free light chains of B-cells (NF-κB1) are prominent, in that these genes regulate several biological processes, such as cell growth and survival, the development of tissues and organs, inflammation, and the innate immune response. The latter is related with the expression of cytokines, cytokine receptors, and histocompatibility genes. In contrast, overexpression of the NF-κB1 family members has been associated with multiple ailments, such as autoimmune diseases, chronic inflammation, neurodegenerative processes, metabolic disorders, and cancer.[6]

The *NF-κB1* signaling pathway is important in RA disease progression, in addition to the inflammatory process and synovial cell survival. It was also found that NF-κB1 proteins are capable of stimulating the secretion of inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α), which facilitate T-cell proliferation because

they stimulate the production of IL-2. Likewise, they can suppress the production of type II collagen, an essential compound of cartilage.[7,8] The objective of this study was to evaluate whether there was an association between the *NF-κB1/IKKε* gene expression and the RA clinical activity.

Material and Methods

Experimental Design

A prospective cross-sectional study was conducted at the Rheumatology Service, ISSEMyM Medical Center, Metepec, State of Mexico, Mexico, from November 2013 to June 2014. The study was approved by the Research Committee of the Medical Sciences Research Center (CICMED), Autonomous University of the State of Mexico (UAEMex), and the ISSEMyM Medical Center (code 038/13). The process complied with the ethical principles of the Declaration of Helsinki and of the Official Mexican Norm (NOM-012-SSA3-2012) for medical research in humans,[9] and registered at ClinicalTrials.gov (NCT02689115); written informed consent was obtained from all subjects.

Patients

The key inclusion criteria were patients with RA who met the criteria established by the American College of Rheumatology (ACR).[10] Two groups were conformed based on the Disease Activity Score 28 (DAS28),[11] 1) with clinical activity (DAS28 > 3.6) and 2) with clinical remission (DAS28 < 2.4). Key exclusion criteria included patients with any other inflammatory or autoimmune disease, and patients with infections.

Sample Size

The sample calculation was based on the following formula:

$$n_0 = \frac{2\left(Z_\alpha + Z_\beta\right)^2 S^2}{d^2}$$

Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a 2-sided test, 28 subjects per group were necessary to recognize a statistically significant difference greater than or equal to 3 relative expression units (fold changes). The common standard deviation was assumed to be 4, based on a previous publication[12] in which the same genes were used.

Anthropometric Measurements

Weight (kg) and height (m) were calculated in a mechanical column scale (Seca brand). Patients were classified according to their body mass index (BMI) and weight (kg)/height (m^2) as follows: 1) normal weight (BMI <24.9 kg/m^2), 2) overweight (BMI >24/9 kg/m^2 and <29.9 kg/m^2), and 3) obese (BMI >30 kg/m^2).

Lymphocyte Extraction

Lymphocytes from peripheral blood were extracted using the ACK Lysing Buffer (Life Technologies, Grand Island, NY) kit. Briefly, a venous blood sample (2.5 mL) in an EDTA tube (BD Vacutainer; BD, Franklin Lakes, NJ) was centrifuged at 3500 rpm during 5-8 minutes. The resulting buffy coat was extracted and placed in an Eppendorf tube; 1 mL of ACK Lysing Buffer was added and carefully resuspended. Once again, the suspension was centrifuged at 1900 rcf during 5-8 minutes, and the supernatant was discarded. This latter step was repeated until the leukocyte package (4000-10,000 cells) was completely white (approximately 10-15 mg). Finally, 100 uL of ACK Lysing Buffer was added, and this was frozen at –70ºC for later use.

Gene Expression

From the leukocyte lysate, a messenger RNA (mRNA) extraction was performed using the Magna Pure LC RNA Isolation Kit III (Roche) in the Magna Pure LC 2.0 Instrument. The A260-280-nm absorbance ratio was >1.8 (quality), and total RNA concentration (ng/uL) was calculated by determining absorbance at 260 nm with a NanoPhotometer (Implen GmbH, Germany).

Subsequently, the complementary DNA (cDNA) was synthesized with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science); 100 ng of RNA in a final volume of 15 uL was used per reaction. The quantitative polymerase chain reaction (qPCR) was performed utilizing a 7500 Fast Real-Time PCR System (Applied Biosystems, Applera UK, Cheshire, UK), mixing TaqMan Universal PCR Master Mix and the specific probes (Applied Biosystems) for each gene, including *NF-κB1* (Hs00765730-m1), *IKKε* (Hs01063858-m1), and *18S* (Hs99999901-s1); the last was

Table 1. Conditions for the Quantiative Polymerase Chain Reaction

Component	Volume (1 tube)	10% Error
TaqMan Gene Assay (uL)	1.0	1.1
TaqMan UNIVERSAL (uL)	10.0	11.0
H_2O PCR (uL)	9-sample volume	9-sample volume
Total (uL)	20	22.0

Sample volume = uL of sample to use 100 ng cDNA.

Table 2. General Data of the Patients

Variable	Clinical Activity (n = 30)	Clinical Remission (n = 30)	P
Age (years)	50.0 ± 10.4	54.0 ± 12.8	
BMI (kg/m^2)	26.3 ± 4.3	25.7 ± 4.3	
CRP (mg/L)	15.7 ± 18.0	4.6 ± 6.2	.001
DAS28 (points)	4.3 ± 0.7	1.4 ± 0.5	.001
Glucose (mg/dL)	94.2 ± 16.9	109.2 ± 43.1	
NF-κB1(RE)	3.73 ± 2.67	0.693 ± 0.079	≤.001
IKKε (RE)	0.48 ± 0.36	0.00103 ± 0.0008	.008

Values expressed in Mean ± SD.
BMI, body mass index; CRP, C-reactive protein; DAS28, disease activity score 28; IKKε, activating kinase of NF-kB, NF-κB1, nuclear factor kappa-light-chain-enhancer of activated B cells; RE, relative expression.

used as a control gene. The conditions for the qPCR are depicted in **Table 1**.

According to the CT obtained from the 2 different groups, relative expression was calculated by the fold change in NF KB, and IKK was normalized against the constitutively expressed reference genes, then compared to the untreated controls (calibration sample) as follows: 2 – ΔΔCT, where ΔΔCT = (CT-target – CT-reference) treated-sample – (CT-target – CT-reference) calibrator-sample. *Calibrator -sample* refers to the expression level (1×) of the target gene normalized to the constitutive gene. The calibrator was chosen from the group with clinical activity and given a relative expression value of 1.[13]

Statistical Analysis

The correlation among *NF-κB1*, *IKKε* and DAS28 was performed through the Spearman test. The multivariate lineal general model was used to evaluate the drug effect on the *NF-κB1* and *IKKε* RU and DAS28 punctuation. For the 2 genes, ROC curves with the area under the curve (AUC) and 95% confidence interval (CI) were constructed for the event "clinical activity." Finally we evaluated the best curve model for both genes and the clinical activity. Any *P* value ≤ .05 was considered significant. All tests were performed with the SPSS v 15 software (SPSS, Chicago, IL).

Results

From a total of 680 patients attended in the aforementioned period, 60 patients were included in the study, mean age 52 ± 11.62 years, 30 with clinical activity (28 women and 2 men) and 30 in clinical remission (26 women and 4 men). From the first group, 6 patients had high activity (DAS28 >5.1) and 24 moderate activity (DAS28 <3.2).

Table 2 shows the DAS28 score, anthropometric measurements, biochemical values, and *NF-κB1/IKKε* gene expression, and in **Table 3** the CT values of each sample and gene are displayed. Whilst the anthropometric measurements did not demonstrate significant differences, the converse occurred in the gene expression of both *NF-κB1* (*P* ≤ .001) and *IKKε* (*P* = .008). In contrast, while the *NF-κB1* expression was detected in all cases, *IKKε* expression was confirmed in 16 patients with clinical activity and only in three with clinical remission. The intragroup analysis between moderate and high clinical activity using the Mann-Whitney U test did not show any significant difference for the 2 target genes.

The Kolmogorov test showed a non-Gaussian distribution for the 2 tested genes. Therefore, the Spearman correlation test was performed with the previous 2 genes and the DAS28 scale punctuation, resulting in a significant positive correlation between *NF-κB1* and DAS 28 (r^2 = .495, *P* ≤ .001). Notoriously, 25 patients (83.33%) in the group of clinical activity and 26 (26.66%) in the group of clinical remission were prescribed nonsteroidal anti-inflammatory drugs (NSAIDs). In both groups, the most common combination of drugs was a disease-modifying antirheumatic drug plus an NSAID. The multivariate lineal general model showed that the use of NSAIDs influenced the *NF-κB1* (*P* = .046) and *IKKε* (*P* = .005) relative expression. Moreover, when comparing the expression of both genes, contrasting the use of the drug family, there was a significant difference in *IKKε* when the patients were in NSAIDs treatment (*P* = .003; **Table 4**).

Finally, through the ROC curves, including the missing values, the AUC (95% CI) for *NF-κB1* and *IKKε* were 0.827

Table 3. CP Values of NF-kB1 and IKKε for Each Sample

NF-kB	18s	Δ Cp	ΔΔ Cp	RE	IKKε	18s	Δ Cp	ΔΔ Cp	RE
25.0000	5.5393	19.4607	5.7904	0.0181	26.7779	5.5393	21.2386	3.0647	0.1195
30.7914	15.3075	15.4839	1.8136	0.2845	34.8692	15.3075	19.5617	1.3878	0.3821
32.6788	19.2121	13.4667	−0.2036	1.1516	37.0690	19.2121	17.8569	−0.3170	1.2457
33.4393	20.0963	13.3430	−0.3273	1.2547	40.7389	20.0963	20.6426	2.4687	0.1807
32.8940	19.9053	12.9887	−0.6816	1.6039	35.0393	19.9053	15.1340	−3.0399	8.2243
33.7067	20.9540	12.7527	−0.9176	1.8890	39.5728	20.9540	18.6188	0.4449	0.7346
26.9489	14.3496	12.5993	−1.0710	2.1009	29.5089	14.3496	15.1593	−3.0146	8.0814
27.0737	14.5858	12.4879	−1.1824	2.2695	28.3908	14.5858	13.8050	−4.3689	20.6619
28.9228	16.5027	12.4201	−1.2502	2.3787	36.0770	16.5027	19.5743	1.4004	0.3788
29.0183	16.9648	12.0535	−1.6168	3.0669	35.2065	16.9648	18.2417	0.0678	0.9541
33.2223	21.8552	11.3671	−2.3032	4.9355	34.3395	21.8552	12.4843	undet	undet
32.3036	21.0941	11.2095	−2.4608	5.5052	undet	21.0941	undet	undet	undet
32.7965	21.6030	11.1935	−2.4768	5.5666	undet	21.6030	undet	undet	undet
30.6586	19.4880	11.1706	−2.4997	5.6557	34.5901	19.4880	15.1021	−3.0718	8.4082
35.1412	24.1934	10.9478	−2.7225	6.6002	undet	24.1934	undet	undet	undet
31.9580	21.0205	10.9375	−2.7328	6.6474	undet	21.0205	undet	undet	undet
28.4964	17.7251	10.7713	−2.8990	7.4591	undet	17.7251	undet	undet	undet
28.9882	18.4591	10.5291	−3.1412	8.8226	undet	18.4591	undet	undet	undet
36.0000	26.0000	10.0000	−3.6703	12.7312	undet	26.0000	undet	undet	undet
20.1220	12.9099	7.2121	−6.4582	87.9249	37.0000	12.9099	24.0901	5.9162	0.0166
26.9021	20.2115	6.6906	−6.9797	126.2115	undet	20.2115	undet	undet	undet
31.8488	25.8880	5.9608	−7.7095	209.3104	43.9633	25.8880	18.0753	−0.0986	1.0707
36.2823	31.0425	5.2398	−8.4305	345.0113	undet	31.0425	undet	undet	undet
37.5928	32.5989	4.9939	−8.6764	409.1256	undet	32.5989	undet	undet	undet
36.0468	31.2345	4.8123	−8.8580	464.0061	undet	31.2345	undet	undet	undet
21.8182	17.0752	4.7430	−8.9273	486.8387	undet	17.0752	undet	undet	undet
35.6058	31.2212	4.3846	−9.2857	624.1288	undet	31.2212	undet	undet	undet
30.6817	26.3407	4.3410	−9.3293	643.2787	35.1887	26.3407	8.8480	−9.3259	641.7644
35.2823	33.0425	2.2398	−11.4305	2760.0908	undet	33.0425	undet	undet	undet
33.1743	31.4528	1.7215	−11.9488	3953.1857	undet	31.4528	undet	undet	undet

Cp: crossing point, IKKε: Activating Kinase of NF-kB, NF-κB1: Nuclear factor kappa-light-chain-enhancer of activated B cells, RE: relative expression, undet: undetermined.

Table 4. Difference in the IKKε Expression by Drug Treatment

	Levene Test		t-Test		
	F	Sig	t	Sig (bilateral)	95% CI
Biological	3.356	0.087	0.862	0.402	(−97.7-230.5)
NSAIDs	13586.354	0.000	3.607	0.003	(130.1-505.9)
Steroid	1.308	0.271	0.554	0.588	(−142.9-243.3)

CI, confidence interval; IKKε, activating kinase of NF-kB; NSAIDs, nonsteroidal anti-inflammatory drugs.

(0.717-937), $P \le .001$; and 0.550 (400-700), $P = .506$, respectively (**Figure 1**).

Taking into account the effect of NSAIDs, we proceeded to evaluate the best curve estimation for our dataset, leading to the logarithmic and potency models as those that fit better with our data ($P \le .001$).

It is worth noting that 11 patients from the group of clinical activity suffered from at least 1 episode of infection, while in the group of clinical remission, only 6 had this kind of complication. The type of drugs that the patients were taking is depicted in **Table 5**.

Discussion

It is important to specify that, among the group of patients without clinical activity, the majority recorded no amplification in the *IKKε* transcript, in contrast with patients with active RA, in whom important variations in the relative expression were observed.

Different studies have linked *NF-kB* overexpression with inflammatory and autoimmune processes in diseases such as
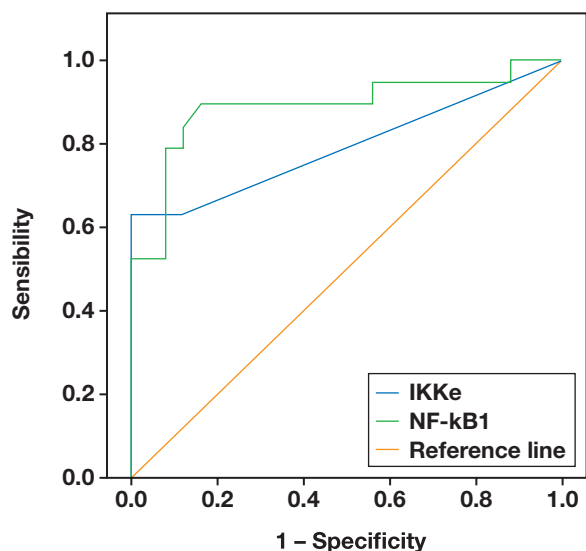
Figure 1

ROC curves for IKKe and NFk-B1.

Table 5. Pharmacological Treatment		
Type of Drug	**Clinical Activity** (n = 30)	**Clinical Remission** (n = 30)
Biologic Drugs	7	8
DMARDs	29	27
NSAIDs	25	26
Steroids	13	5

DMARDs, disease-modifying antirheumatic drugs; NSAIDs, nonsteroidal anti-inflammatory drugs.

RA, systemic lupus erythematosus (SLE), and psoriasis.[14–17] However, there is no clear information on whether there is a link between clinical activity and *NF-κB1* gene expression adjusting the results to the pharmacological treatment.

In this study, we show that there does exist a significant difference between the degree of *NF-κB1* expression of patients with clinical disease activity vs those with clinical remission. Thus, it is possible to suggest that these pathways may be therapeutic targets in RA, in that there is higher expression in patients with clinical activity compared with patients with clinical remission. This especially applies if it is considered as background that the *NF-κB1* family comprises transcription factors that stimulate the synthesis of various inflammatory cytokines involved in RA inception and development, such as IL-1, IL-6, and TNF-α. Therefore, regulating the expression of these transcripts could be a future therapeutic alternative. This is also suggested as a possible inflammatory biomarker, because the clinical activity is directly linked with the presence of the inflammatory process.

In the case of *IKKε*, it is noteworthy that it is a gene triggered by a severe inflammatory stimulus, and that it is expressed predominantly in the cells and tissues of the immune system; it has been described, specifically, that the primary activity of *IKKε* is the chronic activation of synovial inflammation in an arthritic joint;[18] thus, as expected, a significant difference was found between patients without inflammation and patients

with inflammation. Consequently, it is possible to suggest that *IKKε* may be considered in this physiopathology, in that its expression is linked with chronic inflammation and depends on the quite severe inflammatory stimulus, 2 visible aspects in a patient with clinical activity. While further work is required to define in detail the role of *IKKε* in RA, it could be considered an activity indicator, because an important percentage of patients in clinical remission had undetectable levels of *IKKε* expression, contrary to patients with disease activity.

Our results demonstrate a clear difference in the expression of *NF-κB1/IKKε* in peripheral blood mononuclear cells depending on the clinical activity. Whether this finding can be extrapolated to the joint where disease occurs is something to be corroborated, but the molecular mechanism leading to erosions and periarticular and generalized osteoporosis in RA suggest so.[19] Furthermore, the damage to the joints can be modified by the antirheumatic drug group.[20]

During this research, other aspects linked to the RA were also evaluated; this is the case of BMI. The literature reports that obesity is considered at present a low-grade inflammatory pathology whose consequence is the increase of the plasmatic levels of proinflammatory cytokines such as TNF-α and acute-phase reactants, such as the C-reactive protein (CRP).[21,22] Obesity is also considered a risk factor for RA. This has been reported in studies in which it was observed that with regard to the levels of leptin and other types of adipokines that regulate different physiological functions, including the immune response, these levels are elevated in those with obesity. This represents a strong connection with obesity under diverse autoimmune conditions, such as RA, SLE, type 1 diabetes, and Hashimoto thyroiditis.[23,24] Conversely, other authors suggest that being overweight only adds a traumatic load to the joints and that, subsequently, there exists a higher predisposition to suffer osteoarthritis, a mechanical but not a metabolic problem, and the connection with RA is not at all clear.[25,26]

However, in the present study, there was no difference in the degree of *NF-κB1/IKKε* gene expression among patients with normal weight, overweight, and obesity, both for the group with clinical activity and for that with clinical remission. A significant difference was not recorded in the BMI, the weight, or the height of both groups. Therefore, it is suggested that overweight and obesity constitute probable risk factors in the development of the disease because currently, obesity is considered a minor inflammatory process. Notwithstanding, this has no connection with clinical activity. In this regard, the main causes for a clinical improvement are treatment adherence, treatment response, application of attenuated-virus vaccines, and recurrent infections. The latter triggers the immune response and the production of proteins that give rise to inflammatory processes, such as TNF-α, 1 of the cytokines of major relevance in the clinical activity of patients with RA.

In addition, we chose to perform a biochemical analysis on each patient in which the glucose was considered, because some studies mention that patients with RA and active disease exhibit strong resistance to the elevation-induced insulin of TNF-α. Based on this presumption, the patients with moderate-to-high clinical activity would be expected to demonstrate higher levels of glucose than patients with clinical remission; however, the results showed that no differences in the glucose levels of both groups, although it is possible that this is due to patients undergoing treatment for RA.[27–29]

Both groups were analyzed depending on the treatment, without finding any significant difference in BMI, *NF-κB1* and *IKKε*. This was unexpected since the biological treatment is focused on suppressing TNF-α,[30–32] the primary activator in the *NF-κB1* signaling pathway by its canonical or classic pathway. By contrast, *IKKε* belongs to an alternative pathway that is different from the already known pathways and does not depend on TNF-α to be triggered; thus, it should not be affected by the biological treatment.[33] However, the multivariate lineal general model showed that NSAIDs use influences the *NF-κB1* and *IKKε* expression.

Importantly, this pathway appears to have cell type-specific functions, and since many different cell types are involved in the pathogenesis of RA, it is difficult to predict the net overall contribution of the non-canonical NF-κB pathway to synovial inflammation, or clinical indication, and on the other hand, as we propose with this study, this will allow the design of better therapeutic strategies for the management of this disease, including cell type-specific inhibitors

or selective targeting of inhibitors to certain cell types. Some authors propose that NF-κB pathway is a promising new therapeutic target, not only in RA, but also in other immune-mediated inflammatory diseases.[34,35]

Finally, there is a latent possibility of biased results due to the infections in the group with clinical activity. As it is known, NFκB transcription factors are activated upon infection, via triggering of various sensors, like the TLRs (toll-like receptors), which are expressed on cells of the innate immune system, including macrophages, dendritic cells, and mucosal epithelial cells.[36] Surprisingly, information is scarce about RA, infections, and the activation of the NFκB/IKKε module.[37,38]

We are aware that a clear limitation of this study is that the elected genes might be difficult to quantify in an everyday medical practice, but in a second- or third-level hospital with a rheumatology service, they could help to identify patients without clinical improvement. Thus, the major strength of our study is the identification of *IKKε* as clearly positive in RA patients with clinical activity and its negativity in 90% of patients in clinical remission. **LM**

Acknowledgments

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