Acta Biologica Szegediensis

Volume 65(2):271-283, 2021
Acta Biologica Szegediensis
http://abs.bibl.u-szeged.hu/index.php/abs

DOI:10.14232/abs.2021.2.271-283

ARTICLE

Inflammatory and bone biomarkers/composites as a predictive tool for clinical characteristics of rheumatoid arthritis patients

Hameed Hussein Ali[1], Muna Mohammed Yaseen[2], Khalid F. AL-Rawi[1], Shakir F. T. Alaaraji[3], Hussein Kadhem Al-Hakeim[4]*

[1]Department of Chemistry, College of Science, University of Anbar, Iraq
[2]Department of Basic Science, College of Dentistry, University of Anbar, Iraq
[3]Department of Chemistry, College of Education for Pure Science, University of Anbar, Iraq
[4]Department of Chemistry, College of Science, University of Kufa, Iraq

ABSTRACT Rheumatoid arthritis (RA) is related to alterations in different inflammatory and connective tissue biomarkers. The diagnostic values and the factors affecting these biomarkers are conflicting. In the present study, a bone-related composite (B-composite), made from the z-score of stromelysin-1 (MMP3), colony-stimulating factor *2* (CSF2), and osteopontin (OPN), and I-composite, reflecting immune activation, made from the z-score of tumor necrosis factor-*α* (TNFα), interferon-γ (INFγ), and vascular endothelial growth factor-A (VEGF) were examined in RA patients. The biomarkers were measured by ELISA technique in 102 RA patients and 58 age-matched healthy control subjects. Serum MMP3, TNFα, IFNγ, and CSF2 showed significant elevation in RA patients. Multivariate general linear model (GLM) analysis revealed a significant high effect of diagnosis on biomarkers' level (partial $\eta^2 = 0.415$). Duration of disease is significantly associated with VEGF, OPN, and B-composite and negatively correlated with TNFα. B-composite is significantly associated with CRP. A significant fraction of the DAS28 score variance can be explained by the regression on zlnINFγ. The variance in the CRP was explained by zlnOPN and B-composite. More than half of anti-citrullinated protein antibodies (ACPA) variation can be explained by the regression on serum MMP3 and I-composite. The top 3 sensitive predictors for RA disease are INFγ, MMP3, and TNFα. B-composite is associated with the duration of disease and CRP. At the same time, I-composite is negatively associated with the ACPA level. The biomarker composites have potential use as RA disease characteristic biomarkers.

KEY WORDS
diagnosis
inflammation
rheumatoid arthritis
stromelysin-1
osteopontin

ARTICLE INFORMATION
Submitted
28 July 2021
Accepted
26 November 2021
***Corresponding author**
E-mail: headm2010@yahoo.com

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune-inflammatory disorder that affects joints mostly in women and older subjects (Tobón et al. 2010; Agarwal 2011). RA is associated with systemic complications, progressive disability, socioeconomic costs, and even early death (Firestein 2003; Choy 2012). RA patients suffer from chronic inflammation, swollen and painful joints, stiffness following prolonged inactivity, fatigue, burning sensations, and loss of motor control (Alamanos and Drosos 2005; Stack et al. 2014). The etiology of RA remains ambiguous but is attributed to a package of genetic and environmental factors (Vickers 2017). Many parameters were measured and examined for their potential applicability as a factor of diagnosis, prognosis, or RA disease follow-up. Among those parameters, scientists have measured adipokines (Ali et al. 2020), trace elements (Al-Hakeim et al. 2019), various pro- and anti-inflammatory cytokines (Mateen et al. 2016; Wu et al. 2021). Because RA is characterized by inflammation of the synovial membrane that leads to the pulverization and destruction of the joints (Anandarajah 2011), estimation of different inflammation-related cytokines is still an interesting field of study. Due to the increasing production of free radicals in the inflamed joints, inflammation and tissue damage have also been involved in RA's pathogenesis (Mateen et al. 2016; Quiñonez-Flores et al. 2016). Multiple cellular elements, adhesion molecules, soluble mediators, and autoantibodies lead to inflammation of the joints and internal organs and structural changes (McInnes and Schett 2017; Giannini et al. 2020). However, the results of most parameters are not completely decisive and conclusive. Among these measured cytokines in RA is a colony-stimulating factor (CSF2), stromelysin-1 (MMP3), tumor necrosis factor-*α*

(TNFα), osteopontin (OPN), vascular endothelial growth factor-A (VEGF) and interferon-γ (INFγ). TNFα is secreted by monocytes, B cells, and multiple T cell subsets (Zhang et al. 2019). TNFα is bounteously present in RA patients' serum and joint synovium as an essential and significant cytokine upsetting the controlled harmony between pro-inflammatory and anti-inflammatory cytokines (Brennan and McInnes 2008). In one study, TNFα secreted from B-cells, among multiple osteoblasts' inhibitors, can inhibit bone formation in RA's animal model (Sun et al. 2018).

It is known that MMP3 is expressed in the synovium of RA patients (Kanbe et al. 2011; Chen et al. 2014) and represents a potential indicator of early diagnosis and the activity of the disease (Chen et al. 2014; Lerner et al. 2018). During tissue remodeling in RA, MMP3 participates in the breakdown of extracellular matrix proteins (Page-McCaw et al. 2007). Serum levels of MMP3 reflect the activity of RA disease, bone and joint injury and predict drug responsiveness and disease outcome (Vistoli et al. 2013; Lerner et al. 2018; Liu et al. 2018). These findings encouraged some researchers to suggest MMP3 monitoring in the routine assessment to accompany therapeutic modalities in RA management (Lerner et al. 2018).

CSF2 is a cytokine that can rise to high levels in response to immune stimuli (Borriello et al. 2019) to regulate inflammatory responses (Wicks and Roberts 2016). CSF2 is essential for developing inflammatory and arthritic pain in RA models (Cook et al. 2013). It is also increased locally in inflammation sites such as asthmatic patients' lungs and allergic patients' skin (Hamilton 2020). CSF2 has also been detected significantly in the arthritic synovial fluid, where it is expected to destroy bone and joint (Cook et al. 2013).

The binding of vascular endothelial growth factor (VEGF) to its receptor activates a series of signal transduction events that ultimately lead to the secretion of various inflammatory and growth factors that stimulate the proliferation and migration of the endothelial cells to produce new blood vessels (Hicklin and Ellis 2005). VEGF is widely expressed in RA patients' serum and synovial fluid (Lee and Bae 2018; Xu et al. 2019), where it is responsible for vascular growth and blood vessel invasion of the synovial lining membrane in RA (Malemud 2007). VEGF is considered a critical angiogenic factor that causes a synovitis continuous reaction and mainly results in tissue hypoxia (Zimna and Kurpisz 2015). Inflammation in synovium and hypoxia enhances the production of pro-inflammatory cytokines such as IL-1β and TNFα, activating VEGF production (Cho et al. 2006).

The bone matrix contains OPN that connects osteoclasts and hydroxyapatite to bolster bone resorption (Horton et al. 1995). This fact was supported by experiments on OPN-null mice that showed a resistance to the inflammatory joint destruction in collagen-induced arthritis (Yumoto et al. 2002). In a previous study, a manifest elevation of OPN concentration in RA patients' synovial fluid was found, which is suggested to play a significant role in the pathogenesis of RA (Ohshima et al. 2002). Furthermore, plasma OPN was considered an inflammatory bone damage biomarker in RA patients (Iwadate et al. 2014). The presence of the anti-OPN antibodies has been associated with the severity of RA.

In the immune system, IFNγ plays a role in producing cytokine, antigen presentation, cellular differentiation, metabolic pathways, macrophage activation, and cell growth and survival (Lees 2015). The activated T cells and natural killer (NK) cells are the primary cells that secrete IFNγ in plasma (Billiau 1996; Billiau and Matthys 2009), while the dominant source of IFNγ in RA synovium is CD8+ T cells (Zhang et al. 2019). INFγ enhances antigen presentation, mediates antiviral and antibacterial immunity, orchestrate the activation of the innate immune system, regulates Th1/Th2 balance, promotes macrophage activation, and controls cellular proliferation and apoptosis (Billiau 1996; Billiau and Matthys 2009). A previous attempt to use recombinant IFNγ to treat RA without any benefit (Veys et al. 1997). Therefore, the exact effect of these parameters on the RA is not fully understood by studying every parameter alone. In the present study, we built two biomarkers composite from bone biomarkers from MMP3, OPN, and VEGF, reflecting the bone status (B-composite). The second composite (I-composite) was built from TNFα, INFγ, and CSF2, reflecting immune activation. The current study aims to examine the diagnostic role of the above biomarkers and their composites in the RA patients and study the factors affecting their level in the sera of RA patients.

MATERIAL AND METHODS

Participants

The present case-control study was performed in Fallujah General Hospital in Anbar, Iraq, from October 2019 till December 2020. The study included 102 males with RA patients and 58 healthy control subjects. The criteria of the "American College of Rheumatology" and the "European League against Rheumatism" were used for RA diagnosis (Aletaha et al. 2010). Every patient achieved a score higher than six from the results of the four domains: the number and site of the painful joints, positive serologic results (rheumatoid factor (RF), and anti-citrullinated protein antibodies (ACPA)), elevations of inflammatory markers (C-reactive protein (CRP) and/or erythrocyte sedimentation rate (ESR)), and the duration of RA symptoms. The clinical characteristics of the

Table 1. Clinical and demographic characteristics rheumatoid arthritis patients (RA) and healthy controls (HC).

Variables	HC (n = 58)	RA (n = 112)	F/χ^2	df	p
Age (years)	47.128 ± 6.017	48.222 ± 5.941	1.139	1/158	0.289
BMI (kg/m^2)	24.923 ± 2.623	25.917 ± 2.348	2.325	1/158	0.082
DAS28	-	7.406 ± 1.374	-	-	-
Duration of disease (years)	-	9.275 ± 4.788	-	-	-
ACPA (+ve/-ve)	0/58	87/15	59.626	1	<0.001
CRP (+ve/-ve)	0/58	82/20	52.111	1	<0.001
RF (+ve/-ve)	0/58	88/14	61.747	1	<0.001
Urea (mg/dl)	34.258 ± 5.192	35.357 ± 6.028	2.135	1/158	0.272
Creatinine (mg/dl)	0.820 ± 0.201	0.890 ± 0.194	3.164	1/158	0.247
Uric acid (mg/dl)	5.258 ± 1.237	5.891 ± 1.138	3.874	1/158	0.041
T.Protein (g/dl)	7.222 ± 0.949	7.305 ± 0.817	1.127	1/158	0.297
Albumin (g/dl)	4.552 ± 0.610	4.666 ± 0.624	0.817	1/158	0.304
T.Ca (mM)	2.160 ± 0.191	2.168 ± 0.210	0.035	1/158	0.853
I.Ca (mM)	1.254 ± 0.053	1.255 ± 0.058	0.016	1/158	0.901
MMP3 (ng/ml)	16.054 ± 6.557	22.827 ± 9.112	27.305	1/158	<0.001
TNFα (pg/ml)	31.123 ± 8.528	43.471 ± 16.235	14.058	1/158	<0.001
VEGF (pg/ml)	155.258 ± 37.124	159.068 ± 36.848	0.127	1/158	0.685
OPN (ng/ml)	4.777 ± 2.185	5.025 ± 2.681	2.024	1/158	0.122
IFNγ (pg/ml)	58.588 ± 23.582	104.309 ± 31.253	46.951	1/158	<0.001
CSF2 (pg/ml)	66.852 ± 22.136	91.265 ± 38.302	11.027	1/158	0.001
B-Composite	-0.248 ± 1.783	0.238 ± 1.609	1.806	1/158	0.183
I-Composite	0.407 ± 1.847	0.158 ± 1.948	0.380	1/158	0.539

BMI: Body mass index; DAS28: Disease activity score-28; T.Ca: Total calcium; I.Ca: Ionized calcium; CSF2: Colony-stimulating factor; MMP3 (Stromelysin-1): Matrix metalloproteinase-3; TNFα: Tumor necrosis factor-alpha; OPN: Osteopontin; INFγ: Interferon gamma; VEGF: Vascular endothelial growth factor; z: z-score; ln: natural logarithm; B-Composite: Bone cytokines composite = zlnMMP3+zlnVEGF+zlnOPN; I-Composite: Inflammatory cytokines composite = zlnTNFα+zlnINFγ+zlnCSF2.

RA disease and socio-demographic information were recorded from all patients. Disease Activity Score was calculated by using (DAS28-ESR) calculator available online at https://www.mdcalc.com/disease-activity-score-28-rheumatoid-arthritis-esr-das28-esr. The Clinical Disease Activity Index (CDAI) of all patients' RA disease activity was more than 10, indicating moderate to high disease activity. Body mass index (BMI) was calculated by dividing body weight (kilograms) by the squared length of subjects (squared meter). The study was approved by the ethical approval committee (IRB) of the University of Anbar, Iraq (Document number 103/2019), which complies with the "International Guideline for Human Research" standards as mandatory by the Declaration of Helsinki.

A complete medical history evaluated all subjects to exclude any systemic diseases that might influence the measured parameters' results, particularly liver and renal disease, infection, diabetes, cardiovascular events. The smoking subjects were also excluded from the study.

Measurements

After overnight fasting, five millilitres of venous blood were collected from all subjects without a tourniquet and, after complete clotting at 37 °C, centrifuged at 3000 rpm for 15 min. Sera were isolated and stored at -80 °C until analysis. Serum CRP and RF were measured by semi-quantitative kits (Spinreact®, Girona, Spain) based on the latex agglutination principle. A semi-quantitative ACPA test was carried out by kits provided by Hotgen Biotech (Beijing, China). Serum CSF2, INFγ, MMP3, OPN, TNFα, and VEGF were determined by sandwich ELISA assay kits (Mybiosource®, CA, USA). Serum albumin, total protein, calcium, urea, creatinine, and uric acid were measured spectrophotometrically using ready-to-use kits (Spinreact®, Girona, Spain). Ionized calcium (I.Ca) in serum was calculated from the following equation "$I.Ca = 0.813 \times T.Ca^{0.5} - 0.006 \times Albumin^{0.75} + 0.079$" (Mateu-de Antonio 2016), which give the best approximate result. The sensitivities of the ELISA kits were: CSF2 < 1 pg/ml, INFγ < 4 pg/ml, MMP3 < 0.068 ng/ml, OPN < 0.1 pg/ml, TNFα < 1 pg/ml, and VEGF < 1 pg/ml. The intra-assay coefficients of variance of all kits were less than 10%.

Table 2. Multivariate GLM analysis examining the differences in biomarkers between rheumatoid arthritis patients and normal controls.

Tests	Dependent variables	Explanatory variables	F	df	p	Partial η^2
Multivariate	All 8 biomarkers	Diagnosis	14.112	8/149	<0.001	0.415
		Sex	1.124	8/149	0.352	0.102
		BMI	1.224	8/149	0.307	0.911
		Age	1.248	8/149	0.411	0.090
Between-subject effects	T.Ca	Diagnosis	1.714	1	0.194	0.021
	I.Ca	Diagnosis	1.695	1	0.197	0.020
	MMP3	Diagnosis	13.173	1	<0.001	0.141
	TNFγ	Diagnosis	1.687	1	0.207	0.023
	VEGF	Diagnosis	0.988	1	0.294	0.032
	OPN	Diagnosis	0.214	1	0.516	0.017
	IFNγ	Diagnosis	23.777	1	<0.001	0.227
	CSF2	Diagnosis	2.945	1	0.078	0.049

All results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as an explanatory variable while adjusting for extraneous variables. Diagnosis: RA versus healthy controls; BMI: Body mass index; DAS28: Disease Activity Score-28; T.Ca: Total calcium; I.Ca: Ionized calcium; CSF2: Colony-stimulating factor; MMP3 (Stromelysin-1): Matrix metalloproteinase-3; TNFα: Tumor necrosis factor-alpha; OPN: Osteopontin; INFγ: Interferon-gamma; VEGF: Vascular endothelial growth factor.

Statistical analysis

The distribution of all biomarkers results was found normal, as examined by the Kolmogorov-Smirnov test. Therefore, all results were presented as mean ± standard deviation. Chi-square test (χ^2-test) was used to estimate the associations between categorical variables, and analysis of variance (ANOVA) test was used for checking the differences in scale variables between groups. Correlations among biomarkers and between biomarkers and clinical and demographic parameters were assessed using Pearson's product-moment correlation analysis. Associations between RA diagnosis and the measured biomarkers were examined using the multivariate general linear model (GLM) analysis while directing for age and BMI as confounding variables. Accordingly, tests for between-subject effects were performed to explain the associations between diagnosis and every biomarker. The effect size of the analysis was expressed as partial eta-squared (η^2). Various z-unit weighted composite scores were calculated based on the levels of the biomarkers. Firstly, we computed the sum of z values of three normalized inflammatory biomarkers values (zlnTNFα) + zlnINFγ + zlnCSF2) reflecting immune activation (I-composite). Secondly, we computed the sum of z values of three normalized bone-related biomarkers (zlnMMP3) + zlnVEGF + zlnOPN) reflecting bone tissue status (B-composite).

The multiple regression analysis was employed to assess the most significant biomarkers that predict some measured biomarkers. Receiver Operating Curve (ROC) was used to study the measured biomarkers' diagnostic ability for RA. We estimated the best cut-off value of the parameters that produce the best sensitivity and specificity. A two-tailed test was used, and a p-value <0.05 would be considered as a statistical significance. The IBM SPSS package for windows-10, version 25, was used for performing all analyses.

RESULTS

Demographic, clinical and blood parameters characteristics

The demographic data of RA patients in comparison with the healthy controls are shown in Table 1. No significant differences in BMI and age between the groups. Table 1 also shows no significant difference in the levels of urea, creatinine, total protein, albumin, T.Ca and I.Ca between groups. Serum uric acid showed a slight significant increase (p = 0.041) in the RA group compared with the control group. Serum MMP3, TNFα, IFNγ, and CSF2 were significantly increased in RA patients than in the control group. While the serum level of VEGF and OPN showed no significant difference between groups. The weighted composites of bone tissue (B-composite) and inflammatory state (I-composite) showed no significant difference in the RA group than the control group. Co-varying for the drug state did not alter the results.

GLM analysis

The multivariate GLM analysis results, in Table 2, did not show any significant effect of age (F = 1.248, df = 8/149, p = 0.411), and BMI (F = 1.244, df = 8/149, p = 0.307) on the eight biomarkers. The presence of RA (diagnosis) has a highly significant effect (F = 14.112, df = 8/149, p<0.001) on the levels of the biomarkers with a high effect size

Table 3. Correlation matrix of the measured biomarkers with the demographic and among other biomarkers in all the study subjects.

Parameter	zlnMMP3	zlnTNFα	zlnVEGF	zlnOPN	zlnINFγ	zlnCSF2	B-Composite	I-Composite
Age	-0.094	-0.152	-0.055	-0.168	-0.151	-0.156	-0.191	0.256*
BMI	-0.110	-0.025	0.048	-0.003	-0.148	-0.089	-0.035	-0.146
T. Ca	-0.261*	-0.053	-0.121	-0.129	-0.027	-0.150	-0.308**	-0.131
I. Ca	-0.210*	-0.100	-0.150	-0.141	-0.082	-0.151	-0.304**	-0.187
S. Uric acid	-0.122	-0.029	0.085	-0.024	-0.061	-0.098	-0.030	-0.106
zlnMMP3	1	-0.061	-0.166	0.093	0.174	0.124	0.531**	0.134
zlnTNFα	-0.061	1	0.013	-0.002	0.075	-0.075	-0.028	0.541**
zlnVEGF	-0.166	0.013	1	-0.078	-0.052	-0.103	0.504**	-0.082
zlnOPN	0.093	-0.002	-0.078	1	0.100	0.154	0.606**	0.144
zlnINFγ	0.174	0.075	-0.052	0.100	1	0.111	0.128	0.649**
zlnCSF2	0.124	-0.075	-0.103	0.154	0.111	1	0.098	0.604**
B-Composite	0.531**	-0.028	0.504**	0.606**	0.128	0.098	1	0.112
I-Composite	0.134	0.541**	-0.082	0.144	0.649**	0.604**	0.112	1

*: Significant correlation (p<0.01); **: Significant correlation (p<0.001); BMI: Body mass index; DAS28: Disease Activity Score-28; T.Ca: Total calcium; I.Ca: Ionized calcium; CSF2: Colony-stimulating factor; MMP3 (Stromelysin-1): Matrix metalloproteinase-3; TNFα: Tumor necrosis factor-alpha; OPN: Osteopontin; INFγ: Interferon gamma; VEGF: Vascular endothelial growth factor; z: z-score; ln: natural logarithm; B-Composite: Bone cytokines composite expressed as zlnMMP3+zlnVEGF+zlnOPN; I-Composite: Inflammatory cytokines composite expressed as zlnTNFα+zlnINFγ+zlnCSF2.

(partial $\eta^2 = 0.415$). Tests for between-subject showed that the diagnosis has the major effects on the levels of IFNγ ($F = 23.777$, df = 1, $p<0.001$, partial $\eta^2 = 0.227$), followed by MMP3 ($F = 13.173$, df = 1, $p < 0.001$, partial $\eta^2 = 0.141$). Other biomarkers parameters showed lower effects by diagnosis with low-size effects.

Intercorrelation matrix

Table 3. shows the intercorrelation matrix of the measured parameters in 170 subjects. There were significant negative associations between the zlnMMP3 and T.Ca ($r = -0.261$, $p<0.01$) and I.Ca ($r = -0.210$, $p<0.05$) across the entire study group. I-composite is significantly correlated with age ($r = 0.256$, $p<0.01$).

In the RA patients group only, the zlnMMP3 was correlated with ACPA ($r = 0.379$, $p<0.01$), while negatively associated with T.Ca ($r = -0.311$, $p<0.01$) and I.Ca ($r = -0.318$, $p<0.01$). Duration of disease is significantly associated with zlnVEGF ($r = 0.325$, $p<0.01$), zlnOPN ($r = 0.383$, $p<0.001$), and B-composite ($r = -0.433$, $p<0.001$). While zlnTNFα is negatively and significantly associated with the duration of disease ($r = -0.303$, $p<0.01$), and zlnTNFα ($r = -0.281$, $p<0.05$). CRP is significantly associated with B-composite ($r = -0.375$, $p<0.01$) and zlnVEGF ($r = -0.357$,

Table 4. Results of multiple regression analysis with the routinely measured parameters in rheumatoid arthritis patients in addition to DAS28 and the duration of disease as dependent variables.

Regression	Explanatory variables	β	t	p	F_{model}	p	R^2
#1. DAS28	**Model**				5.235	0.012	0.127
	zlnINFγ	0.313	-2.208	0.015			
#2. Duration of disease	**Model**				7.323	<0.001	0.259
	zlnMMP3	-0.234	-3.0021	<0.001			
	Sex	0.244	2.877	<0.001			
#3. CRP	**Model**				5.912	0.006	0.218
	zlnOPN	-0.106	2.168	0.027			
#4. RF	**Model**				6.432	0.001	0.311
	GM-CSF	1.848	2.025	<0.001			
#5. ACPA	**Model**				7.927	<0.001	0.501
	zlnMMP3	0.281	3.077	0.002			

DAS28: Disease Activity Score-28; CSF2: Colony-stimulating factor; MMP3 (Stromelysin-1): Matrix metalloproteinase-3; OPN: Osteopontin; INFγ: Interferon gamma; CRP: C-reactive protein; RF: Rheumatoid factor; ACPA: anti-citrullinated protein antibodies; z: z-score (standard score); ln: natural logarithm.

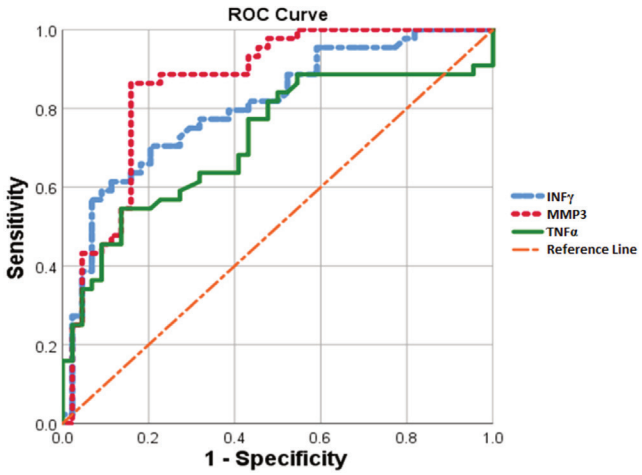

Figure 1. ROC analysis curve for the biomarkers (IFNγ, MMP3, and TNFα) that have the highest area under the curve as predictors for rheumatoid arthritis compared with the reference line.

p<0.01). DAS28 is associated with the zlnINFγ. ACPA is significantly and negatively associated with I-composite (r = -0.336, p<0.01).

Table 4. presents the results of different automatic multiple regression analyses with the routine measured parameters (ACPA, CRP, and RF) in addition to DAS28 and the duration of disease as dependent variables. Regression #1 explained that 12.7% of the variance in the DAS28 score on zlnINFγ. Regression #2 shows that the regression can explicate 25.9% of the variance in disease duration on B-composite (positively associated) and zlnMMP3 (inversely associated). Regression #3 shows that 21.8% of the CRP variance was explicated by zlnOPN (inversely associated) and B-composite (positively associated). Regression #4 shows that 31.1% of the RF variance was explained by zlnCSF2 (positively associated). More than half (50.1%) of the variation in ACPA level can be explained by the regression on serum calcium, MMP3, and I-composite in Regression #5.

Effects of background variables

We also examined the effects of drugs taken on the serum levels of the measured parameters in RA patients by using univariate-GLM analysis. The analysis shows no significant effects of taking sulfasalazine (F = 2.981, df = 1/100, p=0.071) and methotrexate (F = 2.110, df = 1/100, p=0.971) on the serum level of the eight measured biomarkers. There was a slight significant effect of prednisolone on TNFα (p = 0.032; partial η^2 = 0.048) and INFγ (p = 0.047, partial η^2 = 0.066), naproxen on CSF2 (p = 0.039, partial η^2 = 0.072), and tofacitinib on TNFα (p = 0.0292 partial η^2 = 0.061). The other administered drugs have no remarkable effect. The drug administration's overall effects on the measured parameters were only minimum (partial η^2< 0.059).

Predictive value of the measured biomarkers

Table 5. shows the ROC parameters of the measured biomarkers for the diagnosis of rheumatoid arthritis. Only the biomarkers that showed a significant efficacy was cited in Table 5. Figure 1. shows only the top 3 sensitive biomarkers (p<0.001) for RA's diagnosis in a suspected subject. The serum concentration of 62.7 pg/ml represents the cut-off value that when the suspected subjects have higher serum INFγ than it, may refer to the presence of RA with sensitivity = 86.4% and specificity = 84.1%. The cut-off value of MMP3 was 17.7 ng/ml with sensitivity = 70.5% and specificity = 79.5%. While TNFα have sensitivity = 65.6% and specificity = 68.2% at cut-off value of 36.2 pg/ml. While the other biomarkers (CSF2, OPN, and VEGF) showed lower sensitivities and specificities than the top 3 biomarkers.

DISCUSSION

The initial stage in ensuring the quality of the research was to enroll subjects who were not suffering from renal issues with normal urea and creatinine levels as seen in Table 1. The most important finding of the current study is the increased serum MMP3, TNFα, IFNγ, and CSF2 in RA patients compared with the HC group. The elevation in these biomarkers values suggested a more inflammatory state in RA group. Tissue damage is thought to be controlled by cytokines (Alstergren and Kopp 2006). TNFα is commonly acknowledged as a pro-inflammatory cytokine involved in RA pathophysiology (Vervoordeldonk and Tak 2002; Yamanaka 2015; Guo et al. 2019). The outcome of other previous research showed higher levels of TNFα in RA patients (Thilagar et al. 2018). As a result of elevated TNFα, osteoclast precursor cells are stimulated, causing bone resorption (Bartold et al. 2005). TNFα triggers an infection or inflammatory immunological response. However, it promotes an increase in osteoclast precursors and osteoclast development, causing bone resorption (Cessak et al. 2014). A comprehensive evaluation confirmed the advantages of anti-TNF medication for RA patients' rheumatic joints (Zamri and de Vries 2020).

The rise in IFNγ levels in the patients' group is attributable to the inflammatory character of RA, as several cell types, including T-cells, B-cells, NK cells, and monocytes/macrophages, produce IFN (Lees 2015). CD8+ T lymphocytes in the RA synovium are the primary source of IFNγ. T cells, on the other hand, are required for the generation of tissue-degrading enzymes and proinflammatory cytokines, and CD4 T cells are an important regulator of

Table 5. Receiver operating characteristic-area under curve analysis of the measured biomarkers for the diagnosis of rheumatoid arthritis.

Variable	Cut-off concentration	Sensitivity %	Specificity %	Youden's J statistics	AUC	95% CI of AUC	p-value
IFNγ	62.7 pg/ml	86.4	84.1	0.67	0.863	0.784-0.942	<0.001
MMP3	17.7 ng/ml	70.5	79.5	0.46	0.804	0.713-0.896	<0.001
TNFα	36.2 pg/ml	65.6	68.2	0.40	0.718	0.609-0.828	<0.001
CSF2	69.2 pg/ml	53.6	52.4	0.31	0.668	0.556-0.780	0.017
OPN	4.65 ng/ml	44.4	47.4	0.27	0.624	0.505-0.743	0.038
VEGF	167.2 pg/ml	42.3	49.8	0.24	0.480	0.357-0.602	0.042

AUC: area under curve; CI: Confidence interval; CSF2: Colony-stimulating factor; MMP3 (Stromelysin-1): Matrix metalloproteinase-3; TNFα: Tumor necrosis factor-alpha; OPN: Osteopontin; INFγ: Interferon-gamma; VEGF: Vascular endothelial growth factor.

these processes (Klimiuk et al. 1999, Petrasca et al. 2020). IFNγ signaling can regulate many genes (Rusinova et al. 2013) that may affect the inflammatory process and the destruction or degeneration of synovial membranes. The presence of IFNγ receptors is associated with RA symptoms, and an increase in IFNγ-induced inflammation is a sign of therapy success and RA remission (Page et al. 2010; Lee et al. 2017).

MMP3 levels were found to be elevated in our RA patients' group, which was previously reported (Czeczuga and Zajkowska 2008; Mamehara et al. 2010; Ma et al. 2015; Tuncer et al. 2019). However, one study showed no significant difference in MMP3 in RA and control groups (Abd-Allah et al. 2012). When it comes to bone damage detection, the serum level of MMP3 is a good predictor, and suppressing MMP3 levels may be an essential treatment approach for individuals with early RA (Fiedorczyk et al. 2006; Houseman et al. 2012; Tokai et al. 2018). According to a recent research, serum MMP3 levels are better at predicting clinical remission of RA disease than CRP levels (Hattori et al. 2019). It is possible that serum MMP3 will serve as a biomarker for histological synovitis and the diagnosis of RA (Ma et al. 2014), joint erosions in the early stages of the disease, and monitoring disease activity (Tuncer et al. 2019).

As seen previously, RA patients have a higher serum level of CSF2 than the control group (Fiehn et al. 1992; Nakamura et al. 2000). CSF2 is expressed in the synovial membrane and is higher in the synovial fluid of patients with RA (Field and Clinton 1993; Bell et al. 1995). CSF2 receptors are also upregulated by RA subjects in synovial tissue and circulating mononuclear cells (Field and Clinton 1993; Berenbaum et al. 1994). Populations of synovial tissue macrophages are associated with articular damage, and a drop in macrophage numbers is a responsive biomarker for treatment response in RA patients (Haringman et al. 2005). Since CSF2 plays a central role in macrophages' differentiation, activation, and survival, inhibiting CSF2 activity may affect the function of macrophages and provide RA with clinical benefit. In RA pathogenesis, CSF2 contributes to the differentiation and pathogenicity of Th17 cells (Wijbrandts et al. 2007; Codarri et al. 2011; El-Behi et al. 2011).

According to the results of Table 2, multivariate GLM analysis showed no significant effects of the cofounders (age and BMI) on the levels of the measured biomarkers. The biomarkers' levels are significantly affected only by RA disease in the subject with a big effect size (partial $\eta^2 = 0.415$). Tests for between-subject effects revealed that 22.7% of the variance in the IFNγ level. At the same time, 14.1% of the variance in the MMP3 level was due to the RA disease. This analysis excluded any effect of the cofounders on the levels of the measured parameters. A multivariate general linear model (GLM) analysis was used to delineate the relationships between diagnosis and biomarkers while controlling for confounding variables (age and BMI). As a result, to establish the relationship between diagnosis and each biomarker, we conducted an inter-subject effect analysis. This analysis revealed the importance of activating monocytes/macrophages in RA because IFNγ activates monocytes/macrophages activated in RA and contributes to the progression and advancement of the disease (Udalova et al. 2016). Furthermore, IFNγ deficiency or defects in the IFNγ-receptors may have an important effect on the activity and the serum level of IFNγ in RA disease (Lee et al. 2017; Sharma et al. 2018).

MMP3 enzyme can degrade collagen molecules, laminin, proteoglycans, elastin, and fibronectin (Verma and Hansch 2007), which are components of the connective tissues' matrix joints, bones, tendons, and their reduction causes destruction in the joints and exaggerate the RA state. The increase in MMp3 from the synovial fibroblasts or B cells is well-known as a producer of MMP3 (Tetlow et al. 1993). Since MMP3 has a major role in connective tissue remodeling, it may affect their calcium contents and even serum calcium level, as seen in Table 3. However, previously, serum MMP3 is correlated with systemic inflammation and not an independent joint damage marker (So et al. 1999). In previous work, plasma OPN levels were strongly associated with MMP3

levels, and after treatment, the responders' plasma OPN levels decreased dramatically (Iwadate et al. 2014). Also, B-composite showed a significant negative correlation with serum calcium. As B-composite represents the bone biomarkers, it is important to notice that bone biomarkers' elevation, as a composite, is more indicative than each biomarker alone. Previous work showed that circulating pro-inflammatory molecules are inversely correlated with the calcium status parameters and serum calcium level (Poddar et al. 2016). The increase in the inflammatory composite (I-composite) with age is due to each component's cumulative effects. Therefore, I-composite is better than each parameter separately as a tool for studying inflammatory effects. The TNFα level presented a positive correlation with age (Milan-Mattos et al. 2019). Because age is considered a major risk factor for many chronic inflammatory diseases (Larbi et al. 2008; Ferrucci and Fabbri 2018), immune aging contributes to many autoimmune diseases.

The results of Table 4. indicated the direct effect of INFγ on the severity of disease expressed as DAS28. This result indicated the importance of pro-inflammatory biomarkers INFγ on disease progression (Meyer et al. 2010). Duration of the disease depends on the magnitude of the MMP3 and B-composite. As the disease extends, the bone composite increases with time. In one study, MMP3 level was not correlated to the age, disease duration, and the DAS28 scores. However, MMP3 is significantly correlated to CRP and ESR, which are markers of inflammation (Sun et al. 2014). MMP3 was associated with age (Kodama et al. 2018). Furthermore, CRP was explained by B-composite. These results indicated the dependence of the level of bone-related cytokines on the inflammation state biomarkers. However, other researchers showed that raised serum biomarkers might not act as a risk factor for low bone mineral density (Sponholtz et al. 2014). A significant fraction of the RF level can be explained by CSF2. The same explanations can be used, i.e. the inflammation state in RA is the major cause of the biomarker's changes. ACPA level is affected by serum calcium, MMP3, and I-composite levels, as seen in Regression #5 of Table 4. Previously, ACPA had the highest predictive value for RA development (van de Stadt et al. 2011; van Heemst et al. 2015). However, the concomitant existence of RF could increase the risk of RA production. (Rantapää-Dahlqvist et al. 2003; Kokkonen et al. 2011). A significant correlation between ACPA-positivity status and arthritis progression has also been confirmed in multiple patients that have subsequently developed RA. (van Gaalen et al. 2004; Rakieh et al. 2015).

Figure 1. and Table 5. showed a good predictive value of the serum INFγ, MMP3, and TNFα for the presence of RA in a suspected subject. MMP3 is a biomarker for connective tissues, while INF and TNF are inflammatory parameters. Thus, RA is a result of a cascade of inflammatory processes in the joints and bone tissues. However, the diagnostic cut-off value is rather high. MMP3 expression is a good indicator for disease activity in patients with RA (Ma et al. 2015). MMP3 levels elevated as the stage and type of RA progressed and eventually reduced after effective therapy (Uemura et al. 2015). MMP3 levels in the serum were favorably linked with serum CRP or RF levels or with joint destruction (Li et al. 2013). However, a statistically significant correlation was observed between MMP3 and CRP and ESR (So et al. 1999; Hattori et al. 2019). Elevation of serum MMP3 in RA patients is a sign of inflammation (Mamehara et al. 2010) and serves as an early marker of developing joint destruction and a good prognostic indicator of active rheumatoid arthritis illness (Ma et al. 2014; Ma et al. 2015; Galil et al. 2016). Joint injury may be detected with the use of a blood test for serum MMP3, which is linked to systemic inflammation (Mamehara et al. 2010).

Different immune cells, such as neutrophils, release pro-inflammatory indicators, such as prostaglandins, cytokines, and reactive oxygen intermediates, which contribute to synovitis (Cascao et al. 2010). Mast cells in the synovium also release large quantities of cytokines, chemokines, proteases, and vasoactive amines, all of which are harmful to the joint tissues (Nigrovic and Lee 2007; Hueber et al. 2010). ACPA has been widely accepted as a biomarker for RA activity and types (Schuerwegh et al. 2010). ACPA has been widely used to diagnose RA with specificity (Hill et al. 2008). ACPA endorsed inflammatory cytokines production in RA patients and accumulated at the citrulline site, causing bone damage (Umeda et al. 2017).

CONCLUSION

The top 3 sensitive predictors for RA are INFγ, MMP3, followed by TNFα. A significant part of CRP variance was explained by zlnOPN and B-composite, while CSF2 can explain a significant RF variance. Duration of the disease can be explained and B-composite (positively associated). About half (50.1%) of the variation in ACPA level can be explained by the regression on serum calcium, MMP3, and I-composite. B-composite is associated with the duration of disease and CRP. I-composite is negatively associated with the ACPA level. The overall results indicated the importance of composite from the biomarkers with common properties as a new biomarker for estimating disease characteristics.

Concerning the limitations of the study, the first drawback is the small scale of the research sample. Test

experiments with a larger sample size will be needed to ensure sufficient generalization of the analysis' results. The second limitation is that the study recruited only male subjects, and we excluded women to eliminate the effect of the estrogens on the measured parameters. The third limitation is the relatively significant inter-assay CV% of the ELISA kits, which were less than 10% for all kits.

ACKNOWLEDGMENT

The authors acknowledge Fallujah General Hospital's staff in Anbar Governorate, Iraq to help with sample collection and the biochemical measurements.

REFERENCES

Abd-Allah SH, Shalaby SM, Pasha HF, El-Shal AS, Abou El-Saoud AM (2012) Variation of matrix metalloproteinase 1 and 3 haplotypes and their serum levels in patients with rheumatoid arthritis and osteoarthritis. Gen Test Mol Biomark 16(1):15-20.

Agarwal SK (2011) Biologic agents in rheumatoid arthritis: an update for managed care professionals. J Manag Care Pharm 17(9 Suppl B):S14-18.

Al-Hakeim HK, Moustafa SR, Jasem KM (2019) Serum cesium, rhenium, and rubidium in rheumatoid arthritis patients. Biol Trace Elem Res 189(2):379-386.

Alamanos Y, Drosos AA (2005) Epidemiology of adult rheumatoid arthritis. Autoimmun Rev 4(3):130-136.

Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD (2010) 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 62(9):2569-2581.

Ali DM, Al-Fadhel SZ, Al-Ghuraibawi NHA, Al-Hakeim HK (2020) Serum chemerin and visfatin levels and their ratio as possible diagnostic parameters of rheumatoid arthritis. Reumatologia 58(2):67-75.

Alstergren P, Kopp S (2006) Insufficient endogenous control of tumor necrosis factor-alpha contributes to temporomandibular joint pain and tissue destruction in rheumatoid arthritis. J Rheumatol 33(9):1734-1739.

Anandarajah AP (2011) Clinical aspects of rheumatoid arthritis: highlights from the 2010 ACR conference. Int J Clin Rheumatol 6(3):267.

Bartold PM, Marshall RI, Haynes DR (2005) Periodontitis and rheumatoid arthritis: a review. J Periodontol 76(11 Suppl):2066-2074.

Bell AL, Magill MK, McKane WR, Kirk F, Irvine AE (1995) Measurement of colony-stimulating factors in synovial fluid: potential clinical value. Rheumatol Int 14(5):177-182.

Berenbaum F, Rajzbaum G, Amor B, Toubert A (1994) Evidence for GM-CSF receptor expression in synovial tissue. An analysis by semi-quantitative polymerase chain reaction on rheumatoid arthritis and osteoarthritis synovial biopsies. Eur Cytokine Netw 5(1):43-46.

Billiau A (1996) Interferon-gamma: biology and role in pathogenesis. Adv Immunol 62:61-130.

Billiau A, Matthys P (2009) Interferon-gamma: a historical perspective. Cytokine Growth Fact Rev 20(2):97-113.

Borriello F, Galdiero MR, Varricchi G, Loffredo S, Spadaro G, Marone G (2019) Innate immune modulation by GM-CSF and IL-3 in health and disease. Int J Mol Sci 20(4):834.

Brennan FM, McInnes IB (2008) Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest 118(11):3537-3545.

Cascao R, Rosário H, Souto-Carneiro M, Fonseca J (2010) Neutrophils in rheumatoid arthritis: more than simple final effectors. Autoimmun Rev 9(8):531-535.

Cessak G, Kuzawińska O, Burda A, Lis K, Wojnar M, Mirowska-Guzel D, Bałkowiec-Iskra E (2014) TNF inhibitors - Mechanisms of action, approved and off-label indications. Pharmacol Rep 66(5):836-844.

Chen JJ, Huang JF, Du WX, Tong PJ (2014) Expression and significance of MMP3 in synovium of knee joint at different stage in osteoarthritis patients. Asian Pac J Trop Dis 7(4):297-300.

Cho ML, Jung YO, Moon YM, Min SY, Yoon CH, Lee SH, Park SH, Cho CS, Jue DM, Kim HY (2006) Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblasts via AP-1-dependent pathways. Immunol Lett 103(2):159-166.

Choy E (2012) Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology (Oxford) 51(Suppl-5):v3-11.

Codarri L, Gyülvészi G, Tosevski V, Hesske L, Fontana A, Magnenat L, Suter T, Becher B (2011) RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. Nat Immunol 12(6):560-567.

Cook AD, Pobjoy J, Sarros S, Steidl S, Durr M, Lacey DC, Hamilton JA (2013) Granulocyte-macrophage colony-stimulating factor is a key mediator in inflammatory and arthritic pain. Ann Rheum Dis 72(2):265-270.

Czeczuga A, Zajkowska J (2008) [Usefulness of examinations of serum levels of matrix metalloproteinases 1, MMP-3, MMP-9, tissue inhibitor of metalloproteinases 1, hyaluronic acid and antibodies against cyclic citrullinated peptide in Lyme arthritis, rheumatoid arthritis and patients with arthritic complaints]. Przegl Epidemiol

62 (Suppl 1):20-29.
El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, Zhang GX, Dittel BN, A Rostami (2011) The encephalitogenicity of TH 17 cells is dependent on IL-1-and IL-23-induced production of the cytokine GM-CSF. Nat Immunol 12(6):568-575.
Fiedorczyk M, Klimiuk PA, Sierakowski S, Gindzienska-Sieskiewicz E, Chwiecko J (2006) Serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with early rheumatoid arthritis. J Rheumatol 33(8):1523-1529.
Fiehn C, Wermann M, Pezzutto A, Hüfner M, Heilig B (1992) [Plasma GM-CSF concentrations in rheumatoid arthritis, systemic lupus erythematosus and spondyloarthropathy]. Z Rheumatol 51(3):121-126.
Field M, Clinton L (1993) Expression of GM-CSF receptor In rheumatold arthritis. Lancet 342(8881):1244.
Firestein GS (2003) Evolving concepts of rheumatoid arthritis. Nature 423(6937):356-361.
Galil SM, El-Shafey AM, Hagrass HA, Fawzy F, Sammak AE (2016) Baseline serum level of matrix metalloproteinase-3 as a biomarker of progressive joint damage in rheumatoid arthritis patients. Int J Rheum Dis 19(4): 377-384.
Giannini D, Antonucci M, Petrelli F, Bilia S, Alunno A, Puxeddu I (2020) One year in review 2020: pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol 38:387-397.
Guo X, Wang S, Godwood A, Close D, Ryan PC, Roskos LK, White WI (2019) Pharmacodynamic biomarkers and differential effects of TNF- and GM-CSF-targeting biologics in rheumatoid arthritis. Int J Rheum Dis 22(4):646-653.
Hamilton JA (2020) GM-CSF in inflammation. J Exp Med 217(1).
Haringman JJ, Gerlag DM, Zwinderman AH, Smeets TJ, Kraan MC, Baeten D, McInnes IB, Bresnihan B, Tak PP (2005) Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. Ann Rheum Dis 64(6):834-838.
Hattori Y, Kida D, Kaneko A (2019) Normal serum matrix metalloproteinase-3 levels can be used to predict clinical remission and normal physical function in patients with rheumatoid arthritis. Clin Rheumatol 38(1):181-187.
Hicklin DJ, Ellis LM (2005) Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 23(5):1011-1027.
Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, Lee DM, Hueber W, Robinson WH, Cairns E (2008) Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. J Exp Med 205(4):967-979.
Horton MA, Nesbit MA, Helfrich MH (1995) Interaction of osteopontin with osteoclast integrins. Ann N Y Acad Sci 760:190-200.
Houseman M, Potter C, Marshall N, Lakey R, Cawston T, Griffiths I, Young-Min S, Isaacs JD (2012) Baseline serum MMP-3 levels in patients with rheumatoid arthritis are still independently predictive of radiographic progression in a longitudinal observational cohort at 8 years follow up. Arthritis Res Ther 14(1):R30.
Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, Leipe J, Melendez AJ, McInnes IB (2010) Cutting edge: mast cells express IL-17A in rheumatoid arthritis synovium. J Immunol 184(7):3336-3340.
Iwadate H, Kobayashi H, Kanno T, Asano T, Saito R, Sato S, Suzuki E, Watanabe H, Ohira H (2014) Plasma osteopontin is correlated with bone resorption markers in rheumatoid arthritis patients. Int J Rheum Dis 17(1):50-56.
Kanbe K, Chiba J, Nakamura A (2011) Decrease of CD68 and MMP-3 expression in synovium by treatment of adalimumab for rheumatoid arthritis. Int J Rheum Dis 14(3):261-266.
Klimiuk PA, Yang H, Goronzy JJ, Weyand CM (1999) Production of cytokines and metalloproteinases in rheumatoid synovitis is T cell dependent. Clin Immunol 90(1):65-78.
Kodama R, Muraki S, Iidaka T, Oka H, Teraguchi M, Kagotani R, Asai Y, Hashizume H, Yoshida M, Kawaguchi H, Nakamura K, Akune T, Tanaka S, Yoshimura N (2018) Serum levels of matrix metalloproteinase-3 and autoantibodies related to rheumatoid arthritis in the general Japanese population and their association with osteoporosis and osteoarthritis: the ROAD study. J Bone Miner Metab 36(2):246-253.
Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadell G, Ronnelid J, Rantapaa-Dahlqvist S (2011) Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis. Arthritis Res Ther 13(1):R13.
Lee SH, Kwon JY, Kim SY, Jung K, Cho ML (2017) Interferon-gamma regulates inflammatory cell death by targeting necroptosis in experimental autoimmune arthritis. Sci Rep 7(1):10133.
Lee YH, Bae S-C (2018) Correlation between circulating VEGF levels and disease activity in rheumatoid arthritis: a meta-analysis. Zeitsch Rheumatol 77(3):240-248.
Lees JR (2015) Interferon gamma in autoimmunity: A complicated player on a complex stage. Cytokine 74(1):18-26.
Lerner A, Neidhofer S, Reuter S, Matthias T (2018) MMP3 is a reliable marker for disease activity, radiological monitoring, disease outcome predictability, and therapeutic response in rheumatoid arthritis. Best Pract Res Clin Rheumatol 32(4):550-562.
Li L, Cai B, Liao J, Yang B, Huang Z, Wang L (2013) [Clinical value of serum matrix metalloproteinase-3 in evaluating joint destruction and therapeutic effect in rheumatoid arthritis patients]. Chinese J Cell Mol Immunol 29(9): 966-969.

Liu MK, Wang LC, Hu FL (2018) [Value of serum matrix metalloproteinase 3 in the assessment of early rheumatoid arthritis]. Beijing Da Xue Xue Bao Yi Xue Ban 50(6): 981-985.

Ma J, Wang X, Mo Y, Chen L, Zheng D, Wei X, Dai L (2015) [Value of serum matrix metalloproteinase-3 in the assessment of active disease in patients with rheumatoid arthritis]. Zhonghua Yi Xue Za Zhi 95(47):3823-3828.

Ma JD, Zhou JJ, Zheng DH, Chen LF, Mo YQ, Wei XN, Yang LJ, Dai L (2014) Serum matrix metalloproteinase-3 as a noninvasive biomarker of histological synovitis for diagnosis of rheumatoid arthritis. Mediators Inflamm 2014:179284.

Ma M, Liu H, Qu X, Wang J (2015) Matrix metalloproteinase-3 gene polymorphism and its mRNA expression in rheumatoid arthritis. Genet Mol Res 14(4):15652-15659.

Malemud CJ (2007) Growth hormone, VEGF, FGF: Involvement in rheumatoid arthritis. Clin Chim Acta 375(1):10-19.

Mamehara A, Sugimoto T, Sugiyama D, Morinobu S, Tsuji G, Kawano S, Morinobu A, Kumagai S (2010) Serum matrix metalloproteinase-3 as predictor of joint destruction in rheumatoid arthritis, treated with non-biological disease modifying anti-rheumatic drugs. Kobe J Med Sci 56(3):E98-107.

Mateen S, Moin S, Khan AQ, Zafar A, Fatima N (2016) Increased Reactive Oxygen Species Formation and Oxidative Stress in Rheumatoid Arthritis. PLoS One 11(4):e0152925.

Mateen S, Zafar A, Moin S, Khan AQ, Zubair S (2016) Understanding the role of cytokines in the pathogenesis of rheumatoid arthritis. Clin Chim Acta 455:161-171.

Mateu-de Antonio J (2016) New predictive equations for serum ionized calcium in hospitalized patients. Med Princ Pract 25(3):219-226.

McInnes IB, Schett G (2017) Pathogenetic insights from the treatment of rheumatoid arthritis. Lancet 389(10086):2328-2337.

Meyer PW, Hodkinson B, Ally M, Musenge E, Wadee AA, Fickl H, Tikly M, Anderson R (2010) Circulating cytokine profiles and their relationships with autoantibodies, acute phase reactants, and disease activity in patients with rheumatoid arthritis. Mediators Inflamm 2010:158514.

Milan-Mattos JC, Anibal FF, Perseguini NM, Minatel V, Rehder-Santos P, Castro CA, Vasilceac FA, Mattiello SM, Faccioli LH, Catai AM (2019) Effects of natural aging and gender on pro-inflammatory markers. Braz J Med Biol Res 52(9):e8392.

Nakamura H, Ueki Y, Sakito S, Matsumoto K, Yano M, Miyake S, Tominaga T, Tominaga M, Eguchi K (2000) High serum and synovial fluid granulocyte colony stimulating factor (G-CSF) concentrations in patients with rheumatoid arthritis. Clin Exp Rheumatol 18(6):713-718.

Nigrovic PA, Lee DM (2007) Synovial mast cells: role in acute and chronic arthritis. Immunol Rev 217(1):19-37.

Ohshima S, Yamaguchi N, Nishioka K, Mima T, Ishii T, Umeshita-Sasai M, Kobayashi H, Shimizu M, Katada Y, Wakitani S, Murata N, Nomura S, Matsuno H, Katayama R, Kon S, Inobe M, Uede T, Kawase I, Saeki Y (2002) Enhanced local production of osteopontin in rheumatoid joints. J Rheumatol 29(10):2061-2067.

Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8(3):221-233.

Page CE, Smale S, Carty SM, Amos N, Lauder SN, Goodfellow RM, Richards PJ, Jones SA, Topley N, Williams AS (2010) Interferon-γ inhibits interleukin-1β-induced matrix metalloproteinase production by synovial fibroblasts and protects articular cartilage in early arthritis. Arthritis Res Ther 12(2):R49.

Petrasca A, Phelan JJ, Ansboro S, Veale DJ, Fearon U, Fletcher JM (2020) Targeting bioenergetics prevents CD4 T cell-mediated activation of synovial fibroblasts in rheumatoid arthritis. Rheumatology (Oxford) 59(10):2816-2828.

Poddar A, Behera DD, Ray S (2016) Serum alkaline phosphatase activity & serum calcium levels: an assessment tool for disease activity in rheumatoid arthritis. IJBAMR 5(4):1324.

Quiñonez-Flores CM, González-Chávez SA, Del Río Nájera D, Pacheco-Tena C (2016) Oxidative stress relevance in the pathogenesis of the rheumatoid arthritis: a systematic review. BioMed Res Int 2016:6097417.

Rakieh C, Nam JL, Hunt L, Hensor EM, Das S, Bissell LA, Villeneuve E, McGonagle D, Hodgson R, Grainger A, Wakefield R J, Conaghan PG, Emery P (2015) Predicting the development of clinical arthritis in anti-CCP positive individuals with non-specific musculoskeletal symptoms: a prospective observational cohort study. Ann Rheum Dis 74(9):1659-1666.

Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ (2003) Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 48(10):2741-2749.

Rusinova I, Forster S, Yu S, Kannan A, Masse M, Cumming H, Chapman R, Hertzog PJ (2013) Interferome v2.0: an updated database of annotated interferon-regulated genes. Nucleic Acids Res 41(Database issue):D1040-1046.

Schuerwegh AJ, Ioan-Facsinay A, Dorjee AL, Roos J, Bajema IM, van der Voort EI, Huizinga TW, Toes RE (2010) Evidence for a functional role of IgE anticitrullinated protein antibodies in rheumatoid arthritis. Proc Natl Acad Sci USA 107(6):2586-2591.

Sharma V, Pope BJ, Bolland M, Reynolds R, Sun D, Bridges SL, Raman C (2018). Enhanced interferon gamma re-

sponse contributes to disease remission in rheumatoid arthritis. J Immunol 200(Suppl 1):45.18.
So A, Chamot AM, Peclat V, Gerster JC (1999) Serum MMP-3 in rheumatoid arthritis: correlation with systemic inflammation but not with erosive status. Rheumatology (Oxford) 38(5):407-410.
Sponholtz TR, Zhang X, Fontes JD, Meigs JB, Cupples LA, Kiel DP, Hannan MT, McLean RR (2014) Association between inflammatory biomarkers and bone mineral density in a community-based cohort of men and women. Arthritis Care Res 66(8):1233-1240.
Stack RJ, van Tuyl LD, Sloots M, van de Stadt LA, Hoogland W, Maat BMallen CD, Tiwana R, Raza K, van Schaardenburg D (2014) Symptom complexes in patients with seropositive arthralgia and in patients newly diagnosed with rheumatoid arthritis: a qualitative exploration of symptom development. Rheumatol 53:1646-1653.
Sun S, Bay-Jensen A-C, Karsdal MA, Siebuhr AS, Zheng Q, Maksymowych WP, Christiansen TG, Henriksen K (2014) The active form of MMP-3 is a marker of synovial inflammation and cartilage turnover in inflammatory joint diseases. BMC Musculoskelet Disord 15:93.
Sun W, Meednu N, Rosenberg A, Rangel-Moreno J, Wang V, Glanzman J, Owen T, Zhou X, Zhang H, Boyce BF, Anolik JH, Xing L (2018) B cells inhibit bone formation in rheumatoid arthritis by suppressing osteoblast differentiation. Nat Commun 9(1):5127.
Tetlow LC, Lees M, Ogata Y, Nagase H, Woolley DE (1993) Differential expression of gelatinase B (MMP-9) and stromelysin-1 (MMP-3) by rheumatoid synovial cells in vitro and in vivo. Rheumatol Int 13(2):53-59.
Thilagar S, Theyagarajan R, Sudhakar U, Suresh S, Saketharaman P, Ahamed N (2018) Comparison of serum tumor necrosis factor-α levels in rheumatoid arthritis individuals with and without chronic periodontitis: A biochemical study. J Indian Soc Periodontol 22(2):116-121.
Tobón GJ, Youinou P, Saraux A (2010) The environment, geo-epidemiology, and autoimmune disease: rheumatoid arthritis. Autoimmun Rev 9(5):A288-A292.
Tokai N, Yoshida S, Kotani T, Yoshikawa A, Kimura Y, Fujiki Y, Matsumura Y, Takeuchi T, Makino S, Arawaka S (2018) Serum matrix metalloproteinase 3 levels are associated with an effect of iguratimod as add-on therapy to biological DMARDs in patients with rheumatoid arthritis. PLoS One 13(8):e0202601.
Tuncer T, Kaya A, Gulkesen A, Kal GA, Kaman D, Akgol G (2019) Matrix metalloproteinase-3 levels in relation to disease activity and radiological progression in rheumatoid arthritis. Adv Clin Exp Med 28(5):665-670.
Udalova IA, Mantovani A, Feldmann M (2016) Macrophage heterogeneity in the context of rheumatoid arthritis. Nat Rev Rheumatol 12(8):472-485.
Uemura Y, Hayashi H, Takahashi T, Saitho T, Umeda R, Ichise Y, Sendo S, Tsuji G, Kumagai S (2015) [MMP-3 as a biomarker of disease activity of rheumatoid arthritis]. Rinsho Byori 63(12):1357-1364.
Umeda N, Matsumoto I, Sumida T (2017) The pathogenic role of ACPA in rheumatoid arthritis. Japan J Clin Immunol 40(6):391-395.
van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, van Schaardenburg D (2011) Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis Rheum 63(11):3226-3233.
van Gaalen FA, Linn-Rasker SP, van Venrooij WJ, de Jong BA, Breedveld FC, Verweij CL, Toes RE, Huizinga TW (2004) Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. Arthritis Rheum 50(3):709-715.
van Heemst J, Trouw LA, Nogueira L, van Steenbergen HW, van der Helm-van Mil AH, Allaart CF, Serre G, Holmdahl R, Huizinga TW, Toes RE, van der Woude D (2015) An investigation of the added value of an ACPA multiplex assay in an early rheumatoid arthritis setting. Arthritis Res Ther 17:276.
Verma RP, Hansch C (2007) Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. Bioorg Med Chem 15(6):2223-2268.
Vervoordeldonk MJ, Tak PP (2002) Cytokines in rheumatoid arthritis. Curr Rheumatol Rep 4(3):208-217.
Veys EM, Menkes CJ, Emery P (1997) A randomized, double-blind study comparing twenty-four-week treatment with recombinant interferon-gamma versus placebo in the treatment of rheumatoid arthritis. Arthritis Rheum 40(1):62-68.
Vickers NJ (2017) Animal communication: when i'm calling you, will you answer too? Curr Biol 27(14):R713-R715.
Vistoli G, De Maddis D, Cipak A, Zarkovic N, Carini M, Aldini G (2013) Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. Free Radic Res 47(Suppl 1):3-27.
Wicks IP, Roberts AW (2016) Targeting GM-CSF in inflammatory diseases. Nat Rev Rheumatol 12(1):37-48.
Wijbrandts CA, Vergunst CE, Haringman JJ, Gerlag DM, Smeets TJ, Tak PP (2007) Absence of changes in the number of synovial sublining macrophages after ineffective treatment for rheumatoid arthritis: Implications for use of synovial sublining macrophages as a biomarker. Arthritis Rheum 56(11):3869-3871.
Wu J, Li Q, Deng J, Zhao JJ, Yu QH (2021) Association between IL-33 and other inflammatory factors in patients with rheumatoid arthritis and in fibroblast-like synoviocytes in vitro. Exp Ther Med 21(2):161.
Xu J, Feng Z, Chen S, Zhu J, Wu X, Chen X, Li J (2019) Taxol

alleviates collagen-induced arthritis in mice by inhibiting the formation of microvessels. Clin Rheumatol 38(1):19-27.

Yamanaka H (2015) TNF as a target of inflammation in rheumatoid arthritis. Endocr Metab Immune Disord Drug Targets 15(2):129-134.

Yumoto K, Ishijima M, Rittling SR, Tsuji K, Tsuchiya Y, Kon S, Nifuji A, Uede T, Denhardt DT, Noda M (2002) Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. Proc Natl Acad Sci USA 99(7):4556-4561.

Zamri F, de Vries TJ (2020) Use of TNF inhibitors in rheumatoid arthritis and implications for the periodontal status: for the benefit of both? Front Immunol 11:591365.

Zhang F, Wei K, Slowikowski K, Fonseka CY, Rao DA, Kelly S, Goodman SM, Tabechian D, Hughes L B, Salomon-Escoto K, Watts GFM, Jonsson AH, Rangel-Moreno J, Meednu N, Rozo C, Apruzzese W, Eisenhaure TM, Lieb DJ, Boyle DL, Mandelin AM, 2nd, A Accelerating Medicines Partnership Rheumatoid, C Systemic Lupus Erythematosus, Boyce BF, DiCarlo E, Gravallese EM, Gregersen PK, Moreland L, Firestein GS, Hacohen N, Nusbaum C, Lederer JA, Perlman H, Pitzalis C, Filer A, Holers V M, Bykerk V P, Donlin LT, Anolik JH, Brenner MB, Raychaudhuri S (2019) Defining inflammatory cell states in rheumatoid arthritis joint synovial tissues by integrating single-cell transcriptomics and mass cytometry. Nat Immunol 20(7):928-942.

Zimna A, Kurpisz M (2015) Hypoxia-Inducible Factor-1 in physiological and pathophysiological angiogenesis: applications and therapies. Biomed Res Int 2015:549412.