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Serum Biomarkers of Bone and Immune Function for Diagnosis of Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovial membrane that leads to the destruction of the joints. Measurements of cytokines have been carried out in many previous works with no decisive result. In the present study, three bone biomarkers (osteopontin, vascular-endothelial growth factor-A (VEGF), and Stromelysin-1 (MMP3)), and three inflammatory biomarkers (colony-stimulating factor (GM-CSF), interferon-γ, and tumor necrosis factor-alpha (TNFα)) are assayed and examined in RA by using artificial neural-network analysis and regression analysis. The study enrolled 112 patients with RA and 58 healthy controls. The biomarkers were measured by the enzyme-linked immune sorbent assay (ELISA) technique. The neural-network analysis showed that the top 3 sensitive predictors for RA are MMP3, TNFα, and osteopontin, followed by VEGF, GM-CSF, and interferon-γ. A significant part of the variance in the disease activity scale (DAS28), rheumatoid factor (RF), C-reactive protein (CRP), and anti-citrullinated protein antibodies (ACPA)) could be explained by interferon-γ, GM-CSF, osteopontin, and MMP3, respectively. The neural network and logistic regression findings showed that RA could predict MMP3, TNFα, and osteopontin with a good area under the curve of 0.938. In conclusion, the neural network analysis showed that MMP3, TNFα, and osteopontin are diagnostic biomarkers for RA disease and correlated with many disease-related characteristics.

Keyword: Rheumatoid arthritis; inflammation; diagnosis;stromelysin-1; and Osteopontin

1. Introduction

Rheumatoid arthritis (RA) is a disease that usually affects females and older subjects characterized by joint pain that worsens over time due to an autoimmune-inflammatory process [1, 2]. The etiology of RA is still unclear, but a package of genetic and environmental considerations is attributed [3]. Numerous parameters were assessed for their possible utility as a predictor in the diagnosis, prognosis, or follow-up of RA disorder. Among these biomarkers, pro- and anti-inflammatory cytokines [4, 5], trace elements [6], and adipokines [7] have been examined in RA disease. Because RA is characterized by inflammation of the synovial membrane that leads to the pulverization and destruction of the joints [8], many cellular elements, adhesion molecules, soluble mediators, and autoantibodies have various effects on the inflammation of the joints and internal organs and structural changes [9, 10]. Estimation of different inflammation-related cytokines is still an interesting field of study because the results of most parameters are not completely decisive and conclusive. Among these measured cytokines in RA are a colony-

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stimulating factor(GM-CSF), *i*nterferon-γ (INFγ), osteopontin (OPN), tumor necrosis factor-alpha (TNFα), and vascular endothelial growth factor-A (VEGF), in addition to an important enzyme stromelysin-1 (MMP3), The bone matrix contains osteopontin, which connects osteoclasts and hydroxyapatite to promote bone resorption [11]. Experiments on osteopontin-null mice revealed a resilience to the inflammatory joint damage seen in collagen-induced arthritis [12]. Previous research has revealed a significant increase in osteopontin content in the synovial fluid of RA patients, where it is believed to play an influential role in the pathogenesis of RA [13]. Furthermore, in RA patients, plasma osteopontin was considered an inflammatory bone injury biomarker [14]. TNFα is abundantly present in the serum and the joint synovium of RA patients as an essential and significant cytokine upsetting the controlled harmony between anti-inflammatory and proinflammatory cytokines [15] and may act as a drug target for the treatment of RA [16].

MMP3 is reported to be found in the synovium of RA patients [17], and it may act as a biomarker for the diagnosis and progression of the disease [17, 18]. MMP3 is involved in the degradation of the proteins' extracellular matrix during tissue remodeling in RA [19]. MMP3 levels in the blood indicate RA disease activity, bone and joint damage, and medication susceptibility and disease outcome [18, 20]. These results prompted several researchers to recommend MMP3 testing as part of a standardized evaluation to go along with RA treatment options [18].

GM-CSF is an inflammatory cytokine that can reach elevated levels in response to immune stimulation [21] and is involved in regulating inflammatory responses [22]. In RA models, GM-CSF is needed for the development of inflammatory and arthritic pain [23]. Local inflammatory areas, such as asthmatic patients' lungs and allergic patients' skin, are often elevated [24]. GM-CSF has been found in large amounts in the synovial fluid of patients with RA, where it is presumed to be implicated in bone and joint deterioration [25].The attachment of vascular endothelial growth factor (VEGF) to its receptor initiates a cascade of signal transduction events that result in the secretion of various inflammatory and growth factors that promote endothelial proliferation and migration, thus promoting the formation of new blood vessels [26]. VEGF is highly distributed in the serum and synovial fluid of RA patients [27], where it promotes vascular development and blood vessel penetration of the synovial lining membrane in RA [28]. In the immune system, IFNγ is secreted mainly from the natural killer (NK) cells and the activated T cells [29], while the dominant source of IFNγ in RA synovium is CD8+ T cells [30]. The biomarkers mentioned above were statistically examined in the current study by using artificial neural network analysis in addition to the binary and multivariate logistic analysis to test the ability to diagnose RA disease.

2. Experimental

2.1 Participants

The current case-control study was carried out from September 2019 to December 2019 at the Fallujah General Hospital in Anbar Governorate in Iraq. The research was authorized by the University of Anbar's ethical approval committee (IRB) (Document number 103/2019), and it fulfills the "International Guideline for Human Research" guidelines established by the Helsinki Declaration. All subjects were informed about the research and agreed to participate in the study and to donate the blood samples. The research enrolled 112 males who had been diagnosed with RA and 58 age-matched healthy control subjects. The RA patients were diagnosed according to the European League Against Rheumatism and the American College of Rheumatology guidelines [31]. The inclusion criteria include that each patient had a score greater than six based on the following four domains: amount and location of painful joints, favorable serologic findings (rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)), elevations of inflammatory markers (C-reactive protein (CRP) and/or erythrocyte sedimentation rate (ESR)), and length of RA symptoms. Disease Activity Score with ESR (DAS28-ESR) was calculated using the online 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 RA patients was greater than 10, suggesting moderate to severe disease activity. Body mass index (BMI) was calculated by dividing subjects' body weight (kilograms) by their height squared (squared meter). Exclusion criteria were recorded through the medical profile to rule out any existing systemic disorders that

may affect the effects of the calculated parameters, especially liver and renal disease, infection, diabetes, and cardiovascular incidents. Subjects that smoked were rolled out from the study.

2.2 Measurements

Five millilitres of venous blood were extracted without a tourniquet from all fasting subjects and centrifuged at 3000rpm for 15 minutes after full clotting at 37°C. Sera were collected and stored at -80°C until they were analyzed. Based on the latex agglutination concept, serum CRP and RF were assessed using semi-quantitative kits provided by the Spinreact® Co., Girona, Spain. Kits supplied by Hotgen Biotech Co., Ltd., Beijing, China, were used to conduct a semi-quantitative ACPA examination. Sandwich ELISA assay kits provided by Mybiosource®, Inc., CA, USA, were used to assess serum GM-CSF, INFγ, MMP3, osteopontin, TNFα, and VEGF. Spinreact®, Girona, Spain, provided ready-to-use kits to test serum urea and creatinine spectrophotometrically. The procedures were carried out exactly as the manufacturers instructed without any changes. GM-CSF <1pg/ml, INFγ <4 pg/ml, MMP3<0.068ng/ml, osteopontin <0.1pg/ml, TNFα <1pg/ml, and VEGF <1pg/ml were the sensitivities of the ELISA kits. All of the kits' intra-assay coefficients of variance were less than 10%**.**

2.3 Statistical analysis

The Kolmogorov-Smirnov test showed that all biomarker results were normally distributed. As a consequence, all of the data are interpreted as mean ± standard deviation. The Chi-square test (χ^2 test) was used to estimate the relationships between categorical variables, and the measurement of variation (ANOVA) test was used to equate scale variables between categories. Pearson's product-moment association analysis was used to determine correlations among biomarkers and between biomarkers and clinical and demographic parameters. The correlation between dichotomous variables (CRP, RF, and ACPA) with the continuous variables (biomarkers) was examined by point-biserial correlation analysis. The "multivariate general linear model" (GLM) study was used to look for links between RA diagnosis and the assessed biomarkers regarding age and BMI as confounding variables. As a result, the between-subject effects test was employed to determine the impact of the RA on each biomarker. The partial eta-squared (η^2) effect size was used in the study. Based on the levels of the biomarkers, various z-unit weighted scores were determined. The most important biomarkers that predict certainly observed biomarkers were evaluated using multiple regression analysis. We have used multilayer perceptron (MLP) Neural Network processing to evaluate the predictability of the existence of RA in a subject using input variables and biomarkers. We used an artificial feedforward model of two hidden layers. The stopping criteria were one successive move with no further decrease in the error expression. The research sample was split into three categories: training (50%), testing (20%), and holdout (30%). The relative error, area under the ROC curve and the value of the explanatory variables (displayed in an importance chart) were all calculated. The tests are two-tailed, with a statistical significance level of 0.05. The IBM SPSS package for windows-10, version 25, was used for performing all analyses.

3. Results

3.1 Demographic, clinical and biomarkers characteristics

Table 1 compares the demographic details of RA patients to those of healthy controls. No statistically significant variations in BMI or age between the groups. Table 1 also indicates that there are no major differences in urea and creatinine levels between groups. Serum MMP3, TNFα, IFNγ, and GM-CSF levels were substantially elevated in RA patients compared to the control group. However, there was no significant variation in serum levels of VEGF and osteopontin between groups.

3.2 GLM analysis

Table 2 shows the effects of the multivariate GLM study, which showed that age (Partial η^2=0.096, p=0.443) and BMI (Partial η^2=0.109, p=0.341) had no substantial impact on the six biomarkers (MMP3, TNFα, VEGF, Osteopontin, IFNγ, and GM-CSF). The involvement of RA (diagnosis) has a highly significant effect (p<0.001) on biomarker levels, with a large effect size (partial η^2=0.445). Between-subject tests revealed that the diagnosis had the greatest impact on the serum IFNγ levels (partial η^2=0.219, p<0.001), followed by MMP3 (partial η^2=0.135, p=0.001). Other biomarkers had an insignificant effect by the presence of RA and had small effects, as seen in Table 2.

3.3 Intercorrelation matrix

The most notable correlations were significant negative interactions between zLnMMP3

and ACPA (r = 0.379, p < 0.01). The duration of illness is related to zLnVEGF (r = 0.325, p < 0.01) and zLnOPN (r = 0.383, p < 0.001). Although zLnTNFα is negatively associated with the disease duration (r = -0.303, p < 0.01), and zLnTNFα (r = -0.281, p < 0.05). CRP has a negative correlation with zLnVEGF (r = -0.357, p < 0.01). DAS28 is connected to the zLnINFγ (r = 0.297, p < 0.05).

3.4 Multiple regression analysis

Table 3 shows the outcomes of various automated multiple regression tests using the routinely calculated parameters (ACPA, CRP, and RF) and DAS28 and disease duration as dependent variables. Regression #1 demonstrated 10.4% of the difference in the DAS28 score on zLnINF. Regression #2 demonstrates that the regression will explain 26.3 % of the variation in the disease period on zLnMMP3 (inversely associated). Regression #3 reveals that zLnOPN explained 22.3 % of the CRP variation (inversely associated). Regression #4 reveals that zLnGM-CSF explains 32.5 % of the RF variation (positively associated). More than half (51.9%) of the difference in ACPA level can be described by the regression on serum MMP3 in Regression #5.

3.5 Results of binary logistic regression analysis

The results of binary logistic regression tests with RA as a dependent variable (and healthy controls as the reference group) are seen in Table 4. The regression discriminates RA patients from healthy controls, found that four input variables, namely GM-CSF, MMP-3, osteopontin, and TNFα, substantially discriminated both study groups (χ^2=82.814, df=6, p<0.001) (all positively associated). The Nagelkerke effect size was 0.813, and the classification precision was 89.8%, with a sensitivity of 90.9% and a precision of 88.6%.

3.6 Effects of background variables.

The univariate-GLM analysis has been used to assess the impact of medications on the serum levels of the assessed parameters in RA patients. The study found no significant effects of sulfasalazine (F=3.102, df=1/110, p=0.088) or methotrexate (F=2.417, df=1/110, p=0.126) on the blood levels of the eight biomarkers tested. Prednisolone had a marginally significant impact on TNF (p=0.021; partial η^2=0.057) and INF (p=0.042, partial η^2=0.049), naproxen had a marginally significant effect on GM-CSF (p=0.045, partial η^2=0.044), and tofacitinib had a marginally significant effect on TNF (p=0.029, partial η^2=0.052). The other medications prescribed have a little discernible impact. The cumulative effects of medication administration on the assessed parameters were minor (partial η^2 =0.057).

3.7 Results of neural networks

The results of the neural network analysis are mentioned in Table 5. The final neural network was trained with eight units, two in the hidden layer 1 and two in the hidden layer 2, with hyperbolic tangent as the activation function in the hidden layers, identity as the activation function in the output layer, and the sum of squares as the error term. The sum of squares in the testing set (1.400) was significantly less than that in the training set (4.163), and the relative error was also significantly less (11.1 % versus 14.0 %, respectively), indicating that the neural network model learned to generalize from the trend. The holdout set's relative error was 14.8 %. The area under the curve of the receiver operating characteristic (AUC ROC) curve was 0.938, with an 86.7 % sensitivity and 83.3 % specificity. The importance and relative importance of the input variables are depicted in Figure 1. MMP3, TNFα, and osteopontin were the top three determinants of the model's predictive power, followed by VEGF, GM-CSF, and INFγ.

4.Discussion

The first step to ensure the work's quality is to recruit patients who have no kidney problems. All patients had normal urea and creatinine, as seen in Table 1. These results are important to exclude any excretion of small-molecular weight proteins by diseased kidneys. The major findings of the current study are the elevations in serum MMP3, TNFα, IFNγ, and GM-CSF in RA patients compared with the control group. Higher levels of these parameters indicated an overall state of the inflammatory response in RA patients. Several studies have found anomalies in biomarkers that represent systemic inflammation throughout the preclinical RA period, such as circulating cytokines and chemokines [32, 33]. It is assumed that cytokines control the counterbalance between tissue development and

destruction, contributing to tissue destruction [34]. The involvement of proinflammatory cytokines, including TNFα, is widely accepted in RA's pathogenesis [35]. The outcome of other previous researches showed higher levels of TNFα in RA patients [36]. Increased TNF levels cause RA as well as endothelial dysfunction by raising vascular oxidized low-density lipoprotein content, decreasing NO bioavailability, and lowering cGMP levels [37]. The increased TNFαinduces bone resorption by assigning to the receptor activator of a certain signaling pathway, leading to stimulating osteoclast precursor cells [38]. TNFα sets a natural immune response to an infection or inflammation. However, it causes increases in the number of osteoclast precursors and osteoclast formation at high levels, resulting in inducing bone resorption [39]. All these results are enforced by a systematic review that revealed the benefits of anti-TNFα treatment for RA patients' rheumatic joints [40]. Although it is unclear whether specific inflammatory pathways precede the development of autoimmunity, it is reasonable to assume that there is some degree of local or systemic inflammation associated with the early development of autoimmunity in RA, and that both inflammatory and autoimmune processes then expand over time until clinically apparent arthritis develops [41].

Since many cell types, including T-cells, B-cells, NK cells, and monocytes/macrophages secrete INFγ, the increase in IFNγ level in the patients' group is due inflammatory nature of RA [42]. In RA synovium, CD8+ T cells are the main source of IFNγ[43]. However, the production of tissue-degrading enzymes and proinflammatory cytokines are T cell-dependent and that CD4 T cells are key regulatory cells in these processes [44]. Expression of IFNγ receptors correlates with RA illness, and rise in response to IFNγ is representative of treatment response and remission of RA [45].

The increase in MMP3 in our RA patients' group was reported previously [46-48]. One research, however, found no substantial difference in MMP3 levels between the RA and control groups [49]. The serum level of MMP3 is a valuable predictor for detecting bone injury, and suppression of MMP3 levels can be an important therapeutic strategy for early RA patients [50]. In a recent study, serum MMP3 level has a larger accuracy than CRP level for predicting clinical remission [51]. Serum MMP3 can be a potential marker for histological synovitis and diagnosis of RA [52], joint erosions in the early phases of the disease, and keeping track of the disease's progress [48].

The study's second main conclusion is that a neural network algorithm was able to externally confirm the clinical diagnosis of RA against control with an AUC ROC curve of 0.938, with the most important discriminatory factors being MMP3, TNFα, and osteopontin. Simultaneously, logistic regression revealed that GM-CSF levels (OR=165.43) had additional effects. As previously demonstrated, RA patients had a higher level of GM-CSF in their blood than the control group [53, 54]. GM-CSF is widely produced in the synovial membrane and increases in RA's synovial fluid [55]. GM-CSF receptors are often upregulated in synovial tissue and circulating mononuclear cells of RA patients [56]. Synovial tissue macrophage populations are correlated with articular injury, and a decline in macrophage numbers is a sensitive biomarker of treatment response in RA patients [57].

According to the findings of Table 2, multivariate GLM analysis revealed that the cofounders (age and BMI) had no substantial impact on the levels of the assessed biomarkers. The levels of biomarkers are substantially impaired only by RA in the subject, with a large impact size (partial η^2=0.445). Tests for between-subject effects showed that the presence of RA in a subject might explain 21.9 % of the variation in the IFNγ level. Simultaneously, 13.5 % of the difference in the MMP3 concentration was attributed to RA. The multivariate GLM study was used to establish correlations between biomarkers and diagnosis when adjusting for confounding variables (age and BMI). This research established the critical role of monocytes/macrophages in RA, as the increase in IFNγ activates the monocytes/macrophages that in turn secreted proinflammatory cytokines that exacerbate the RA disease's development and progression [58].

Additionally, IFN*γ* dysfunction or deficiencies in IFN-receptors can significantly impact IFN*γ* function and serum levels in RA [45, 59]. MMP3 output is increased by synovial fibroblasts or B cells, well-known as MMP3 producers[60].Previously published research established a close correlation between plasma osteopontin levels and MMP3 levels. Plasma osteopontin levels in responders declined significantly after therapy[61].

The findings of Table 3 showed that INF had a direct impact on disease severity, as measured by

DAS28. This finding underscores the critical function of proinflammatory biomarkers such as INFγ in disease progression [62]. The duration of the RA diagnosis is dependent on the severity of the MMP3. MMP3 levels were not associated with age, disease length, or DAS-28 scores in one research. Serum MMP3 is strongly associated with the levels of two inflammatory indicators, CRP and ESR [63]. These findings revealed a correlation between the levels of bone-related cytokines and biomarkers of inflammation. Other researchers, however, demonstrated that elevated serum biomarkers could not be a risk factor for reduced bone mineral density [64]. GM-CSF accounts for a substantial portion of the RF level. The same reasons apply: the inflammatory state associated with RA is the primary source of biomarker changes. ACPA levels are influenced by serum MMP3 levels, as seen in Table 3's Regression #5. Previously, ACPA was found to have the best predictive importance for the production of RA [65]. However, the presence of RF concurrently can increase the risk of RA development [66]. A strong association between ACPA positivity and arthritis development has also been established in several patients who later experienced RA [67].

There is a good predictive value of the serum INFγ, MMP3, and TNFα for the presence of RA in a suspected subject. Two of these parameters (INFγ and TNFα) are inflammatory biomarkers, while MMP3 is a connective tissue biomarker. However, the cut-off value for diagnosis is relatively high. MMP3 level is useful as a biomarker for disease activity in RA patients [68]. MMP3 levels increased with advanced stage and RA class and gradually decreased after successful treatment [69]. Serum MMP3 level was positively correlated with serum CRP or RF levels or joint injury [70]. Furthermore, the correlation of MMP3 with CRP and ESR was statistically significant [51]. Elevation of serum MMP3 in RA patients indicates inflammation [46] and acts as an early predictor of progressive joint damage and a strong prognostic marker of RA disease activity [47]. Serum MMP3 is associated with systemic inflammation and can be a valuable marker for detecting joint damage [46]. Inflammation is responsible for raising the number of proinflammatory biomarkers in different immune cells, such as neutrophils' contribution to synovitis by synthesizing prostaglandins, cytokines and reactive oxygen intermediates [71, 72]. Synovial mast cells also produce high amounts of chemokines, proteases, cytokines, and vasoactive amines [73]. InRA patients, ACPA endorsed inflammatory cytokines production and accumulated at the citrulline site, causing bone damage [74]. These findings were enforced by the fact that a significant fraction of ACPA was correlated with the RA [75]. The first analysis of the current analysis is the limited size of the test sample. To ensure adequate generalization of the study findings, a greater sample size would be needed. The second drawback is that the analysis only included male participants and omitted women to minimize the influence of estrogens on the measured parameters. The third drawback is the relatively high inter-assay coefficient of variance percentage (CV%), less than 10% for all kits.

5.Conclusion

There was a significant elevation in the serum levels of MMP-3, IFNγ, TNFα, and GM-CSF levels in RA patients compared to the controls. The neural network analysis showed that the top 3 sensitive predictors for RA are MMP3, TNFα, and osteopontin. A significant part of the variance in DAS28, RF, CRP, and ACPA could be explained by INFγ, GM-CSF, osteopontin, and MMP3, respectively.

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Conflict of interest

The authors have no conflict of interest.

Author's contributions

All authors have contributed equally in the study design, writing, and editing of the manuscript.

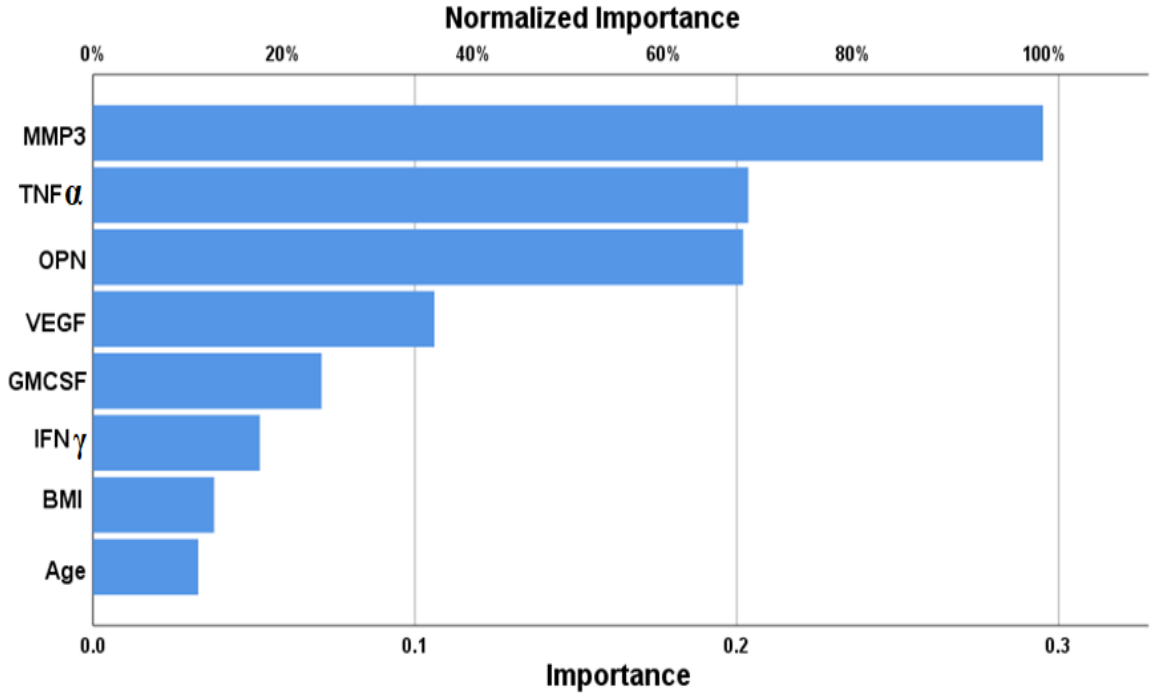

Figure 1. Results of neural network analysis showing the (normalized) importance of all input variables predicting rheumatoid arthritis entered as output variable. BMI: Body mass index,GMCSF*:* Colony-stimulating factor, MMP3: Matrix metalloproteinase-3, TNFα: *T*umour necrosis factor-alpha, INFγ: Interferon-gamma, and VEGF: Vascular endothelial growth factor.

Table 1. Demographic and clinical data in healthy controls (HC) and patients with rheumatoid arthritis (RA).

Variables		HC (n=58)	RA (n=112)	F/χ^2	p
Age	years	48.409±5.358	49.705±6.052	1.130	0.291
BMI	kg/m^2	24.883±2.899	26.050±2.024	2.510	0.071
DAS28		-	7.377±1.236	-	-
Duration of Disease	years	-	9.502±5.005	-	-
ACPA	(+/-)	0/58	94/18	58.113	<0.001
CRP	(+/-)	0/58	88/24	50.236	<0.001
RF	(+/-)	0/58	96/16	60.342	<0.001
Urea	mg/dl	37.386±6.391	38.591±7.53	2.823	0.171
Creatinine	mg/dl	0.820±0.201	0.890±0.194	3.164	0.247
MMP3	ng/ml	13.541±7.603	24.419±10.090	32.623	<0.001
TNFα	pg/ml	33.078±8.631	43.047±16.044	13.169	<0.001
VEGF	pg/ml	167.043±56.782	161.173±56.794	0.235	0.629
Osteopontin	ng/ml	4.642±2.636	5.519±2.443	2.622	0.109
IFNγpg/ml		56.284±40.871	120.759±50.691	43.139	<0.001
GM-CSF	pg/ml	70.227±36.249	97.771±45.169	9.952	0.002

BMI: Body mass index, DAS28: Disease Activity Score-28, GM-CSF*:* Colony-stimulating factor, MMP3: Matrix metalloproteinase-3, TNFα: Tumour necrosis factor-alpha, INFγ: Interferon-gamma, VEGF: Vascular endothelial growth factor, CRP: C-reactive protein, RF: Rheumatoid factor, ACPA: anti-citrullinated protein antibodies z: z-score, Ln: natural logarithm.

Table 2. Results of multivariate GLM analysis examining the differences in biomarkers between rheumatoid and normal controls.

Tests	Dependent variables	Explanatory variables	p	Partial η^2
Multivariate	All Biomarkers (MMP3, TNFα, VEGF, Osteopontin, IFNγ, and GM-CSF)	Diagnosis	<0.001	0.445
		BMI	0.341	0.109
		Age	0.443	0.096
	MMP3	Diagnosis	0.001	0.13
	TNFα	Diagnosis	0.22	0.018
	VEGF	Diagnosis	0.344	0.011
	Osteopontin	Diagnosis	0.67	0.002
	IFNγ	Diagnosis	<0.001	0.219
	GM-CSF	Diagnosis	0.133	0.027

Diagnosis: RA versus healthy controls, BMI: Body mass index, GM-CSF*:* Colony-stimulating factor, MMP3: Matrix metalloproteinase-3, TNFα: Tumour necrosis factor-alpha, INFγ: Interferon-gamma, VEGF: Vascular endothelial growth factor.

Table 3.Results of multiple regression analysis with the routinely measured parameters in RA 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**				4.877	0.033	0.104
	zLnINFγ	0.410	-2.208	0.033			
#2. Duration of disease	**Model**				7.323	0.002	0.263
	zLnMMP3	-1.576	1/168	0.046			
#3. CRP	**Model**				5.884	0.006	0.223
	zLnOPN	-0.106	1/168	0.044			
#4. RF	**Model**				6.432	0.001	0.325
	GM-CSF	1.848	1/168	0.002			
#5. ACPA	**Model**				8.168	<0.001	0.519
	zLnMMP3	0.118	3.128	0.003			

DAS28: Disease Activity Score-28, GM-CSF*:* 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.

Table 4. The binary logistic regression analysis results with RA results as dependent variable (and control as the reference group) and biomarkers as explanatory variables.

Dependent Variables	Explanatory variables*	B (SE)	p	OR	95% CI
RA versus Controls	MMP-3	2.561(0.740)	0.001	12.955	3.039-55.226
	TNFα	1.575(0.649)	0.015	4.832	1.355-17.233
	VEGF	0.293(0.558)	0.600	1.340	0.449-3.997
	Osteopontin	1.600(0.541)	0.003	4.952	1.714-14.305
	IFNγ	0.619(0.497)	0.213	1.857	0.701-4.924
	GM-CSF	5.109(1.820)	0.005	165.430	4.668-586.327

(*): Standardized values, OR: Odd ratio, SE: standard error, and CI: confidence interval, GM-CSF*:* Colony-stimulating factor, MMP3 (Stromelysin-1): Matrix metalloproteinase-3, TNFα: Tumour necrosis factor-alpha INFγ: Interferon-gamma, and VEGF: Vascular endothelial growth factor.

Table 5. Results of neural networks with rheumatoid arthritis (RA) versus controls as output variables and biomarkers as input variables.

	Models	RA vs. Control
Input Layer	No. of units	8
	Rescaling method	Normalized
Hidden layers	No. of hidden layers	2
	No. of units in hidden layer 1	2
	No. of units in hidden layer 2	2
	Activation Function	Hyperbolic tangent
Output layer	Dependent variables	RA vs. Control
	Number of units	2
	Activation function	Identity
	Error function	Sum of squares
Training	Sum of the squares error term	4.163
	% incorrect or relative error	14.0%
	Prediction (sensitivity, specificity)	85.7%, 86.4%
Testing	Sum of Squares error	1.400
	%incorrect or relative error	11.1%
	Prediction (sensitivity, specificity)	100.0%, 80.0%
	AUC ROC	0.938
Holdout	%incorrect or relative error	14.8%
	Prediction (sensitivity, specificity)	86.7%, 83.3%

AUC ROC: area under the curve of receiver operating curve.

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