Rapid immunoprofiling of cytokines, chemokines and growth factors in patients with active rheumatoid arthritis using Luminex Multiple Analyte Profiling technology for precision medicine

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Abstract

Objective

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease of unknown aetiology, characterised by symmetric erosive synovitis, leading inevitably to the destruction of cartilage and bone as well as bursa and tendon sheaths of joints. The aim of this study was to decipher the differential expression of cytokines, chemokines and growth factors in the plasma of RA patients with active disease, using magnetic bead-based Luminex Multiple Analyte Profiling (xMAP) technology, for precision medicine.

Methods

We obtained plasma samples from RA patients (n=25) from the Rheumatology Clinic at the King Abdulaziz University Hospital (KAUH), Jeddah, Kingdom of Saudi Arabia (KSA) after written informed consent for their inclusion in this study. Besides, we have used the plasma samples from inflammatory osteoarthritis (OA) patients (n=10) and healthy volunteers (n=10) for comparison analyses. Plasma samples were examined using the Human Cytokine Magnetic 30-plex panel (Novex®), Invitrogen, USA) and analysed by MAGPIX® instrument (Luminex Corporation, USA).

Results

Though several pro-inflammatory cytokines, chemokines and growth factors present in the 30plex magnetic bead panel were not significantly ($p>0.05$) increased in the plasma of RA patients, the levels of plasma Th1 associated proinflammatory cytokines TNFα, and IL-6 and Th2 associated cytokines such as IL-4, IL-5 and IL-13 were significantly ($p<0.05$) upregulated compared to OA and normal controls. The proinflammatory IL-12 as well as anti-inflammatory IL-10 and IL-1RA were significantly ($p<0.05$) upregulated in the plasma of RA patients compared to normal controls. Also, the chemokines such as IP-10, RANTES and IL-8 as well as growth factors such as EGF, and VEGF were significantly ($p<0.05$) increased in RA.

Conclusion

The MAGPIX data showed that the cytokines, chemokines and growth factors were differentially regulated systemically in patients with active RA compared to OA and normal controls. Hence, the Luminex xMAP technology-based multiplex immunoassays offer clues to formulate effective therapeutic strategies for RA patients with active disease irrespective of their treatment regimen and duration of treatment and, thus, an indispensable tool in precision medicine.

Key words

rheumatoid arthritis, autoimmunity, osteoarthritis, cytokines, chemokines, growth factors, Luminex, precision medicine

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease exhibited by synovial inflammation, joint destruction coupled with a range of comorbidities affecting the bone, brain, lungs, and underlying vasculature (1). RA progressively leading to permanent disability and severely impacts the socio-economic status of these patients (1-3). The incidence of RA in the general population is 1–2% and it may arise at any age (4). There is a female predisposition of about 3:1 with an array of complex clinical characteristics with significant variation or intensity in joint and extra-articular manifestations amongst patients (2-6). Various genetic and environmental factors play a crucial role in the aetiopathogenesis of RA, chiefly by increasing the biosynthesis of proinflammatory cytokines compared to anti-inflammatory cytokines both systemically in the blood and the synovial membranes of the joints (2, 7). The synovitis in RA is characterised by influx and proliferation of neutrophils, monocytes, T and B-lymphocytes causing tissue damage and the production of cytokines, chemokines and growth factors in the joints, subsequently, causing systemic inflammation (1). Studies have established that the levels of pro inflammatory mediators outweigh the anti inflammatory mediators in the RA synovial membrane and augment the destruction of adjacent cartilages and bone erosion (1-3, 8).

For decades, the conventional disease-modifying anti-rheumatic drugs (DMARDs) such as azathioprine, hydroxychloroquine, methotrexate (MTX), and sulphasalazine are the main tools for the treatment of RA (1-3, 9). Ironically, the exact mechanisms of action of conventional DMARDs remain obscure and essentially they are not targeted therapies and do not target the immune cells and the disease specific pathways. Nevertheless, studies have shown that DMARDs are effective in the treatment of RA in most patients (1-3, 5, 8, 9).

Though RA patients are being treated with both conventional and biologic disease-modifying drugs to attain sustained or complete remission, still many patients exhibit either partial or full-blown disease activity during the treatment period. In the present study, we examined using a 30-plex human panel of cytokines (Th1, Th2, Th17), chemokines, and growth factors in plasma in order to diagnostically interpret the precise role of proinflammatory and anti-inflammatory mediators in RA patients with active disease, using Luminex Multiple Analyte Profiling (xMAP) Technology.

Materials and methods

Sample collection and processing

Peripheral blood samples were obtained from healthy volunteers (n=10), Osteoarthritis (n=10) and RA patients (n=25) who met the diagnostic criteria of 2010 ACR/EULAR (5). Patient's samples were obtained from Rheumatology Clinic of the King Abdulaziz University Hospital (KAUH), Jeddah, KSA, after obtaining the written informed consent. Plasma samples were obtained by the centrifugation of blood at 3,000 g for 10 min and stored immediately at -80°C until further use.

Patient characteristics

The normal control, OA, and RA groups were age and sex matched, the healthy volunteers (10 females) with a mean age of 43.3 years, (10 females) the OA patients with a mean age of 48.9 years, while the RA patients (25 Females) with the mean age of 44.6 years. The clinical details of RA patients as follows: The mean duration of disease was 9 years (range 1–25 years); all the RA patients were seropositive for IgM rheumatoid factor (RF). The disease activity of RA patients was calculated using the Disease Activity Score in 28 joints (DAS28) and all of them were in acute disease activity and receiving treatment. Patient's characteristics and clinical details such as the disease duration and treatment regimen for each patient were presented in Table I.

Multiple analyte profiling (xMAP) in normal, OA, and RA patients

Plasma samples isolated from normal controls, OA, and RA patients were analysed for an array of cytokines, chemokines, cytokine receptor and growth factors using the Human Cytokine Magnetic 30-Plex Panel (Novex®), Invitrogen, USA) according

to the manufacturer's instruction. Briefly, the magnetic beads coupled with antibodies for 30 different analytes were added and washed twice with 1X wash buffer. The standard was prepared by mixing 16plex and 14plex solutions provided by the manufacturer and a serial dilution protocol was followed to prepare a range of standard solutions (1:3 serial dilution). Both the standards and the serum samples (1:2 dilution) were prepared and added to the washed beads at the designated wells in the Mylar plates and incubated in an orbital shaker at 500 rpm for 2 h. In between incubations with different antibodies, the plate was washed twice with wash buffer. The plate was then incubated with secondary antibodies for 1h and streptavidin-RPE coupled detection antibodies for 30 min. The plate was finally washed thrice and resuspended in wash buffer and analysed using MAGPIX® instrument (Luminex Corporation, USA).

Statistical analyses

The raw data obtained for individual analytes was analysed by the Luminex xPONENT® multiplex assay analysis software (Luminex Corporation, USA) to calculate the absolute concentration in normal control, OA, and RA groups. Additionally, the concentration of each analyte calculated was further analysed using GraphPad Prism v. 7 (GraphPad Software, USA) to compute the statistical significance using Student's unpaired *t*-test (two-tailed) and one-way analysis of variance (ANOVA). The *p*-values ≤0.05 were considered to be statistically significant.

Table I. RA Patient characteristics and clinical information.

S. no	Disease type	Sex	Age	Disease duration (Years)	Disease activity (DA)	Medication
1	RA	Female	17	4	Active	Methotrexate
2	RA	Female	55	25	Active	Methotrexate
3	RA	Female	54	12	Active	Methotrexate
4	RA	Female	37	2	Active	Corticosteroids, Hydroxychloroquinone
5	RA	Female	60	15	Active	Methotrexate
6	RA	Female	37	5	Active	Methotrexate
7	RA	Female	35	6	Active	Methotrexate, folic acid
8	RA	Female	54	10	Active	Methotrexate, Eternacept
9	RA	Female	31	4	Active	Methotrexate
10	RA	Female	37	5	Active	Methotrexate
11	RA	Female	35	4	Active	Methotrexate, folic acid
12	RA	Female	35	6	Active	Methotrexate
13	RA	Female	65	5	Active	Methotrexate
14	RA	Female	31	6	Active	Folic acid, Methotrexate
15	RA	Female	65	7	Active	Methotrexate
16	RA	Female	47	7	Active	Hydroxychloroquinone, Leflunomide, Etarnacept, folic acid
17	RA	Female	40	11	Active	Corticosteroids, Hydroxychloroquinone
18	RA	Female	51	8	Active	Corticosteroids
19	RA	Female	63	25	Active	Methotrexate
20	RA	Female	54	11	Active	Methotrexate, Etarnacept
21	RA	Female	48	20	Active	Methotrexate, folic acid
22	RA	Female	53	11	Active	Corticosteroids, Hydroxychloroquinone
23	RA	Female	38	1	Active	Methotrexate, folic acid, Hydroxychloroquinone
24	RA	Female	27	13	Active	Corticosteroids
25	RA	Female	45	2	Active	Hydroxychloroquinone

Results

In the present study, we have examined the systemic levels of proinflammatory and anti-inflammatory mediators in RA patients with active disease (Table I) using a magnetic 30plex panel (Table II) based on Luminex xMAP technology. We have observed a significant increase in Th1 associated cytokines such as TNF- α ($p<0.0069$) and IL-6 ($p<0.0481$) in RA compared to normal control. No significant changes ($p>0.05$) were observed in IL-1β, IFN-α, and IFN-γ in RA compared to OA and normal control groups (Fig.1). On the other hand, all the Th2 associated cytokines present in the 30plex panel like IL-4 ($p<0.0151$), IL-5 ($p<0.0174$) and IL-13 ($p<0.0085$) were significantly increased in RA compared to normal control (Fig. 2). Unlike IL-13 ($p<0.0646$), both IL-4 ($p<0.0338$) and IL-5 ($p<0.019$) were significantly different in RA compared to OA.

The plasma levels of chemokines such as IL-8 ($p<0.00212$), IP-10 ($p<0.0244$) and RANTES ($p<0.0342$) were significantly elevated in RA patients with active disease compared to normal controls. Besides, the IL-8 ($p<0.0248$) was significantly different in RA when compared to OA. There was no significant difference observed in the levels of IP-10 and RANTES between RA and OA groups. In addition, there were no significant changes ($p>0.05$) observed in the levels of MIP-1a, MIP-1b, MIG, MCP-1 and Eotaxin in RA compared to OA and normal control groups (Fig. 3). Amongst the growth factors investigated, only EGF ($p<0.0005$) and VEGF ($p<0.0388$) levels were significantly elevated in RA patients with active disease compared normal control. On the other hand, the levels of GM-CSF, G-CSF, FGF and HGF were not significantly different ($p>0.05$) compared to both normal control and OA (Fig. 4).

The anti-inflammatory mediators such as IL-1RA ($p<0.0349$) and IL-10 ($p<0.0154$) were also significantly elevated in RA patients compared to normal controls (Fig. 5). Though IL-10 was significantly different ($p<0.0265$) between OA and normal control, the IL-1RA ($p<0.5983$) was not different between these groups. Besides, the level of IL-12 in RA ($p<0.0049$) and OA ($p<0.0036$) was significantly decreased compared to normal controls. However, there were no significant differences observed in the levels of IL-2, IL-2R, IL-15 and IL-17 in RA compared to OA and normal control groups (Fig. 6).

Discussion

RA is a systemic inflammatory disease with poorly defined aetiology (3). An

Table II. Statistical significance between normal control, OA and RA groups based on student's unpaired *t*-test (two-tailed) and one-way analysis of variance (ANOVA).

S. no	Analyte	Normal control *vs*. OA (*p*-value)	*p*-value Summary	Normal control *vs*. RA (*p*-value)	*p*-value Summary	OA vs RA (*p*-value)	*p*-value Summary	ANOVA (*p*-value)
1	Tumour necrosis factor alpha (TNF-α)	0.0079	**	0.0069	**	0.1182	NS	0.0078
2	Interleukin-6 (IL-6)	0.0106	*	0.0481	*	0.0775	NS	0.0321
3	Interleukin-1 beta (IL-1β)	0.3306	NS	0.1911	NS	0.2605	NS	0.2293
4	Interferon alpha (IFN-α)	0.1713	NS	0.3747	NS	0.0858	NS	0.1491
5	Interferon gamma (IFN-γ)	0.6129	NS	0.3207	NS	0.6252	NS	0.5805
6	Interleukin-4(IL-4)	0.0337	*	0.0151	*	0.0338	*	0.0066
7	Interleukin-5 (IL-5)	0.8765	NS	0.0174	*	0.019	*	0.0049
8	Interleukin-13 (IL-13)	0.1885	NS	0.0085	**	0.0646	NS	0.0083
9	Interleukin-8 (IL-8) or CXCL8	0.9285	NS	0.0212	*	0.0248	*	0.0099
10	Interferon γ-inducible protein-10 (IP-10) or C-X-C-motif chemokine ligand 10 (CXCL10)	0.0022	**	0.0244	*	0.4393	NS	0.0427
11	Chemokine (C-C motif) ligand 5 (CCL5) or RANTES (regulated on activation, normal T cell expressed and secreted)	0.0011	**	0.0342	*	0.0921	NS	0.027
12	Eotaxin or C-C motif chemokine Ligand 11 (CCL11)	0.1271	NS	0.1299	NS	0.8796	NS	0.2711
13	Monokine induced by gamma interferon (MIG) or C-X-C motif chemokine ligand 9 (CXCL9)	0.1315	NS	0.3204	NS	0.3913	NS	0.4224
14	Monocyte chemotactic protein-1 (MCP-1) or CCL2	0.5164	NS	0.8073	NS	0.4329	NS	0.6384
15	Macrophage inflammatory protein alpha (MIP-1α) or CCL3	0.9346	NS	0.6509	NS	0.7322	NS	0.8751
16	Macrophage inflammatory protein-1 beta (MIP-1β) or CCL4	0.1428	NS	0.9029	NS	0.8982	NS	0.4222
17	Granulocyte Macrophage -Colony Stimulating factor (GM-CSF) or colony stimulating factor-2 (CSF-2)	0.1319	NS	0.1149	NS	0.2949	NS	0.1681
18	Granulocyte-colony stimulating factor or G-CSF or CSF3	0.3306	NS	0.0979	NS	0.1088	NS	0.0735
19	Hepatocyte growth factor (HGF) or Scatter Factor (SF)	0.2516	NS	0.4624	NS	0.701	NS	0.6049
20	Fibroblast growth factor (FGF)	ND	NS	0.3801	NS	0.3801	NS	0.4634
21	Epidermal growth factor (EGF)	0.1688	NS	0.0005	***	0.0622	NS	0.0019
22	Vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF)	0.1091	NS	0.0388	*	0.4305	NS	0.1362
23	Interleukin 1-receptor antagonist (IL-1RA)	0.5983	NS	0.0349	*	0.1364	NS	0.061
24	Interleukin-10 (IL-10)	0.0265	*	0.0154	*	0.2865	NS	0.0254
25	Interleukin-2 (IL-2)	0.3306	NS	0.3106	NS	0.3578	NS	0.3928
26	Interleukin-2 receptor (IL-2R)	0.0841	NS	0.7224	NS	0.2229	NS	0.3989
27	Interleukin-15 (IL-15)	0.4267	NS	0.5814	NS	0.5059	NS	0.6878
28	Interleukin-17 (IL-17)	ND	NS	0.5631	NS	0.5631	NS	0.7157
29	Interleukin-7 (IL-7)	0.5554	NS	0.2999	NS	0.7605	NS	0.6163
30	Interleukin-12 (IL-12)	0.0036	**	0.0049	**	0.2724	NS	0.0084

array of genetic and environmental factors have found to play an indispensible role in the aetiopathogenesis of RA, chiefly by increasing the biosynthesis of proinflammatory cytokines compared to anti-inflammatory cytokines in the synovial membranes of the joints (10, 11). An array of cytokine regulated molecular pathways is pivotal for the pathogenesis of RA (11). The successful blockade or inhibition of cytokines such as TNF-α, IL-1β and IL-6 are important for the drug free remission of RA (7, 12).

IL-1RA is commercially produced as Anakinra (brand name Kineret), is a FDA approved interleukin-1 receptor antagonist for treating moderate to severe RA that has been insensitive to conventional DMARDs (13-17). Here, the IL-1RA levels were increased and the IL-1β levels were not significantly higher in RA patients. This reduction could be attributed to the increased IL-1RA levels in the plasma of RA patients. On the contrary, the increased levels of other Th1 associated cytokines; TNF-α and IL-6 in RA patients in our study could contribute to the disease activity even through these patients are continuously treated with MTX and other treatment modalities.

A recent study has shown that IL-10 plays an important regulatory function in the NLRP3-inflammasome mediated joint destruction in arthritis (18). IL-10 levels are found to be increased in the synovial fluids of RA patients and has been associated with the regulation of bone resorption by reducing osteoclastogenesis (19, 20). Here, we observed that, the levels of IL-10, similar to IL-

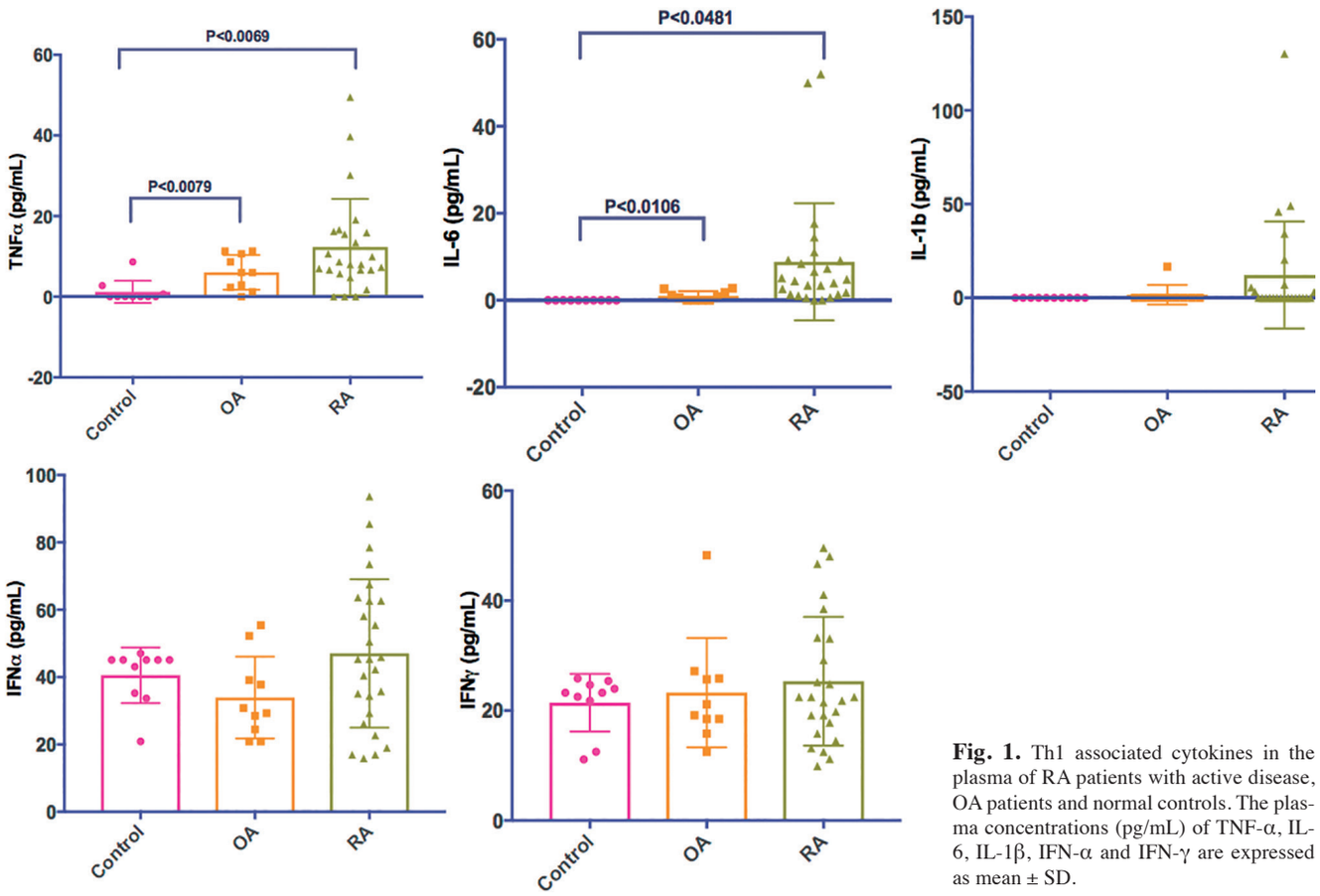

Fig. 1. Th1 associated cytokines in the plasma of RA patients with active disease, OA patients and normal controls. The plasma concentrations (pg/mL) of TNF-α, IL-6, IL-1β, IFN-α and IFN-γ are expressed as mean ± SD.

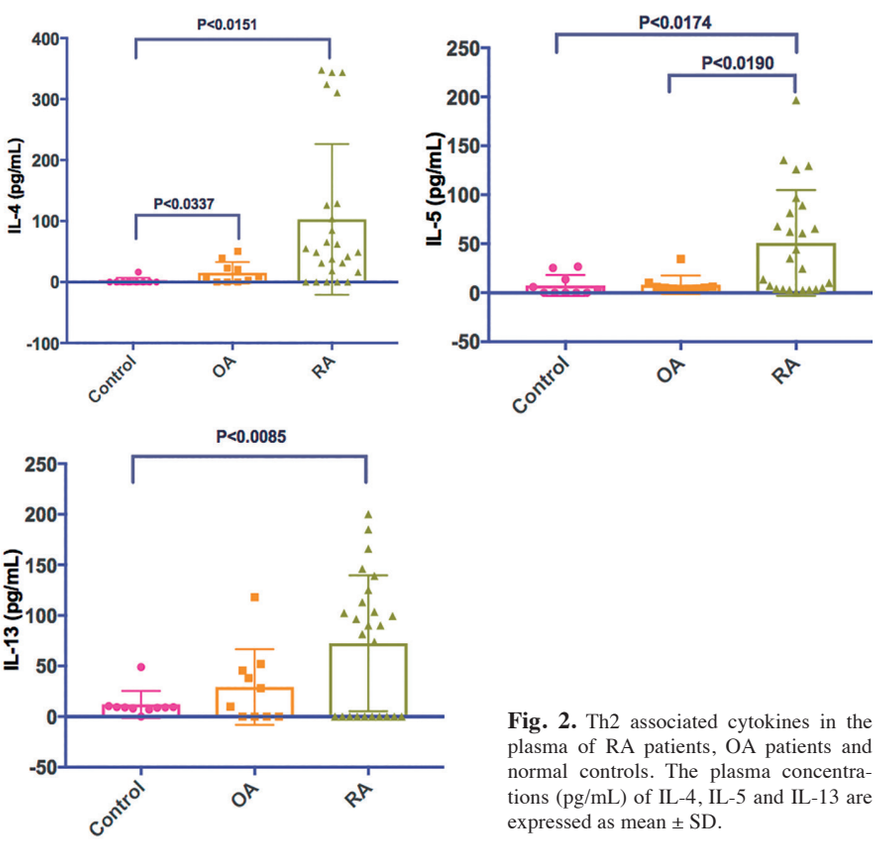

Fig. 2. Th2 associated cytokines in the plasma of RA patients, OA patients and normal controls. The plasma concentrations (pg/mL) of IL-4, IL-5 and IL-13 are expressed as mean ± SD.

1RA, were increased in RA patients treated with MTX and other treatment modalities. IL-10 inhibits the key transcription factor, NF-κB and diminishes the expression of an array of proinflammatory mediators linked with the progression of RA. In animal models of inflammatory arthritis, IL-10 knockout mice showed a RA like phenotype by exhibiting an aggravated joint inflammation (18-20). IL-10 is protective, and an immunomodulatory cytokine, by inhibiting the synthesis of proinflammatory cytokines including IL-1β and IL-33, affecting both innate and adaptive immune responses (18-20). In the present study, the reduction in IL-1β may be attributed to the systemic increase in IL-10 in RA patients with active disease. Additionally, the systemic increase in the Th2 associated cytokines; IL-4, IL-5 and IL-13 in the plasma of RA patients in our study corroborates with a previous study using the synovial fluids derived from RA patients (21). It indicates that the Th2 associated cytokines are upregulated in

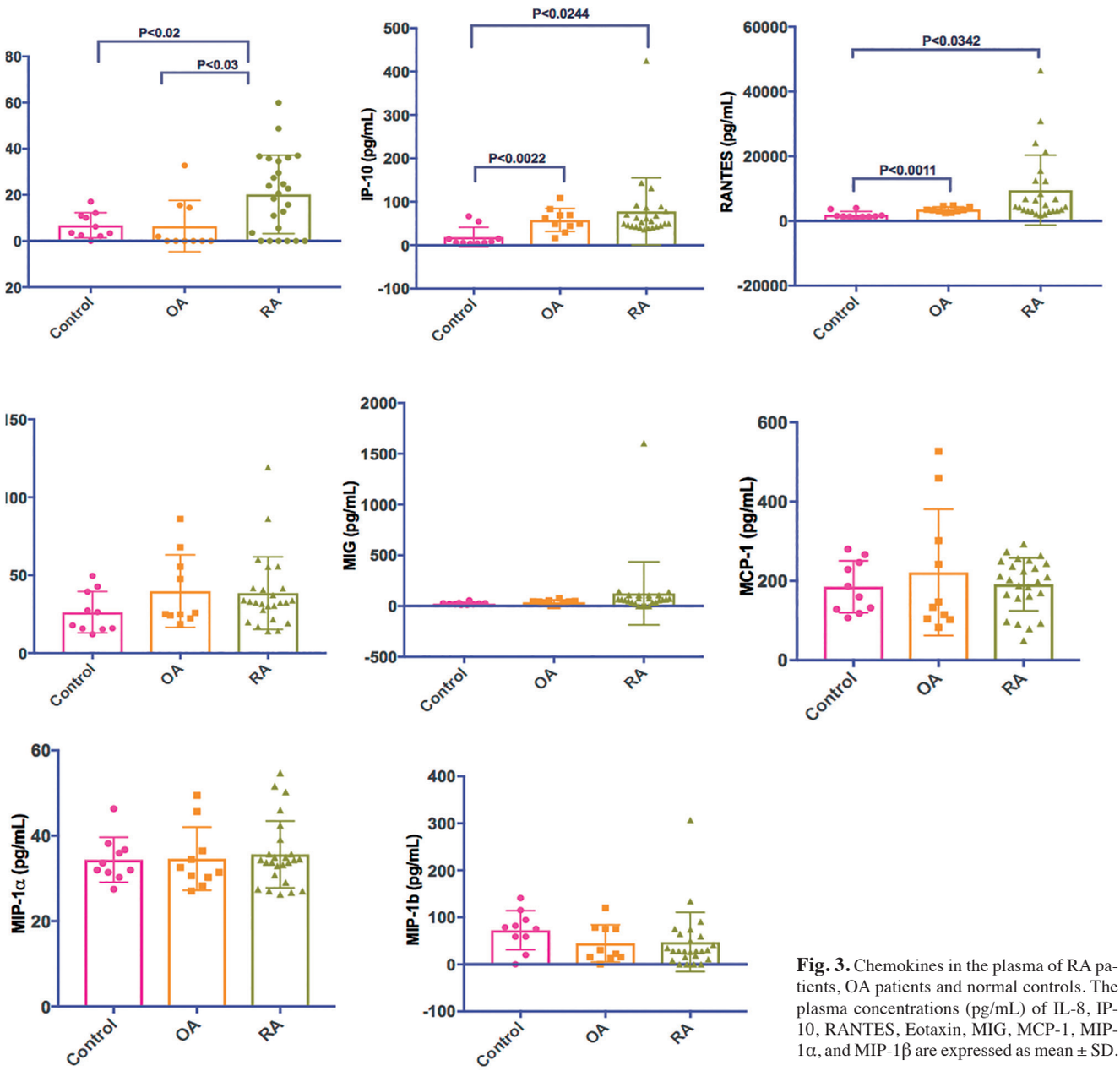

Fig. 3. Chemokines in the plasma of RA patients, OA patients and normal controls. The plasma concentrations (pg/mL) of IL-8, IP-10, RANTES, Eotaxin, MIG, MCP-1, MIP-1α, and MIP-1β are expressed as mean ± SD.

both plasma and synovial fluids of RA patients playing an anti-inflammatory role (22, 23) and, more importantly, their elevation may be ascribed to MTX treatment (24).

Studies indicate that chemokines such as IL-8, IP-10 and RANTES as well as IL-12 are essential for the progression of RA (25-27). The elevation of these chemokines and cytokines in the plasma of RA patients in our study shows that the MTX treatment may not be adequate to successfully control these pro-inflammatory chemokines and cytokines that augment the disease leading to relapse or active disease phenotype. Besides, the increase in the pro-angiogenic growth factors such as VEGF and EGF levels in the plasma may contribute to the higher disease activity in these patients (28, 29).

In conclusion, the rapid Immunoprofiling of an array of cytokines, chemokines and growth factors in the plasma of RA patients with active disease provides clues to interpret the precise nature of systemic inflammation and helps the clinicians to devise better or alternate therapeutic modalities such as the biologic targeted therapies to achieve tolerance and complete or sustained drug free remission for a long period of time.

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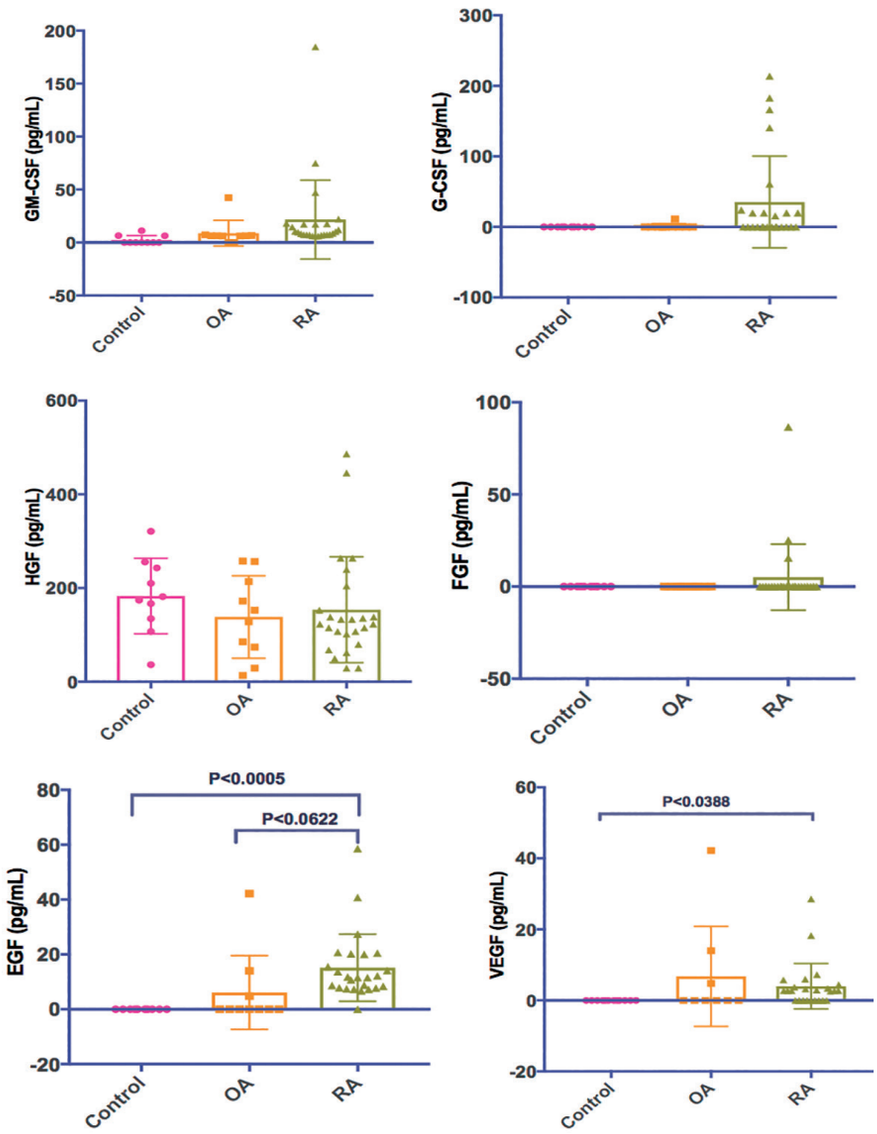

Fig. 4. Growth factors in the plasma of RA patients, OA patients, and normal controls.
The plasma concentrations (pg/mL) of GM-CSF, G-CSF, HGF, FGF, EGF and VEGF are expressed as mean ± SD.

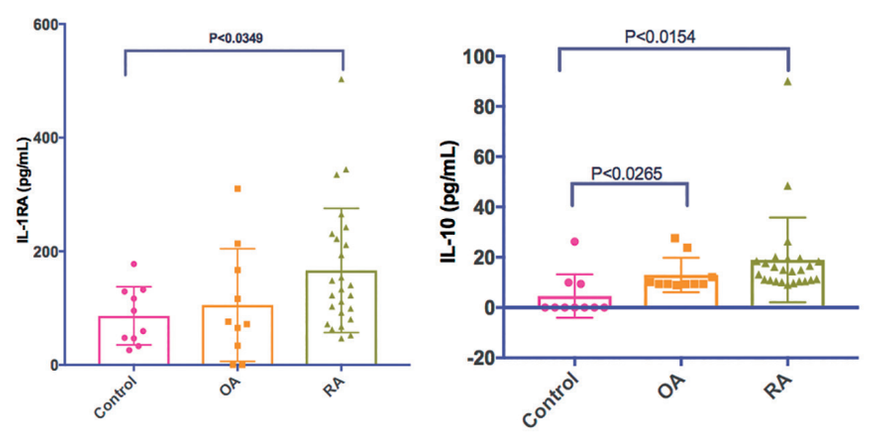

Fig. 5. The anti-inflammatory mediators in the plasma of RA patients, OA patients, and normal controls. The plasma concentrations (pg/mL) of IL-1RA and IL-10 are expressed as mean ± SD.

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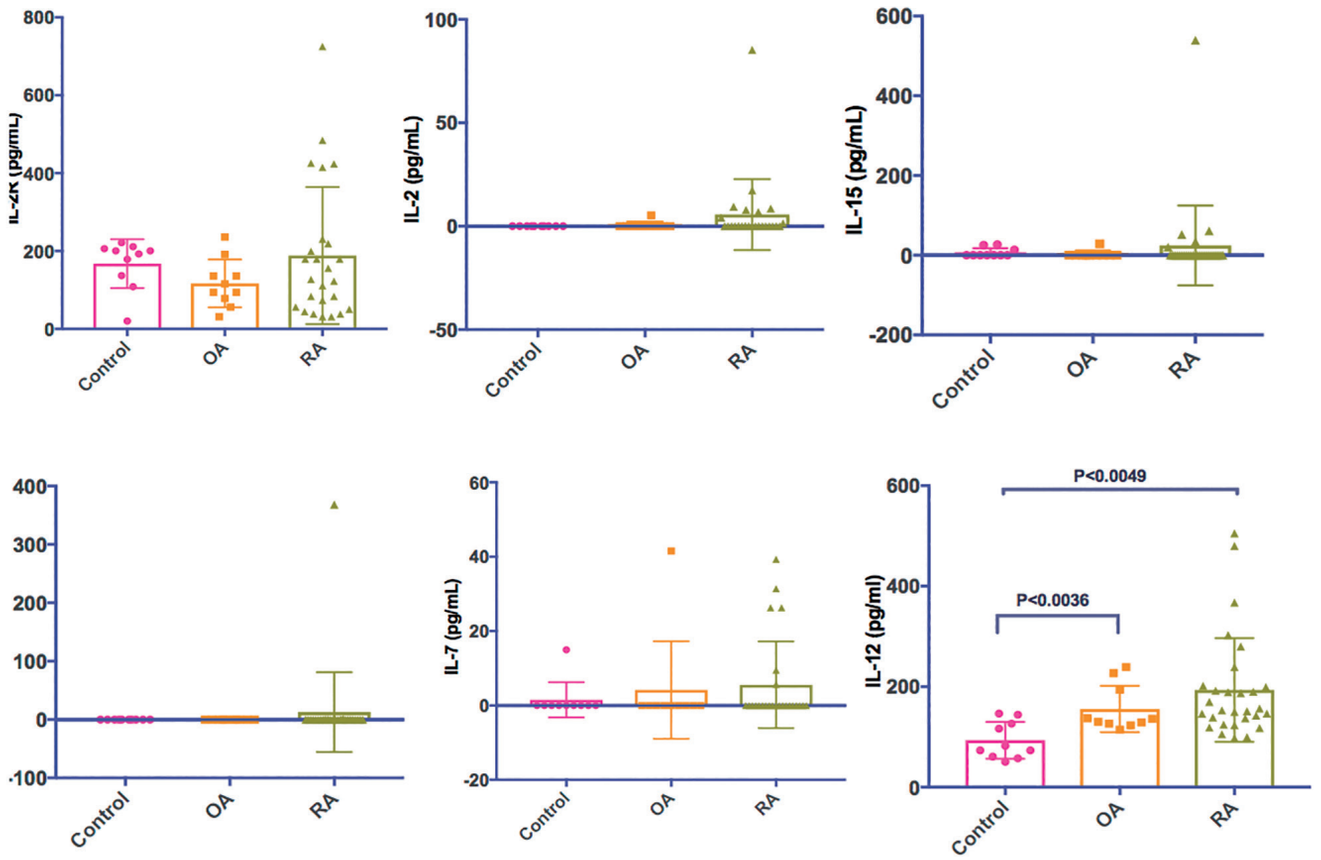

Fig. 6. The proinflammatory mediators in the plasma of RA patients, OA patients, and normal controls. The plasma concentrations (pg/mL) of IL-2R, IL-2, IL-15, IL-17, IL-7, and IL-12 are expressed as mean ± SD.

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