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Whole blood cytokine attenuation by cholinergic agonists *ex vivo* and relationship to vagus nerve activity in rheumatoid arthritis

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Abstract

Objective—The central nervous system regulates innate immunity in part via the cholinergic anti-inflammatory pathway, a neural circuit that transmits signals in the vagus nerve that suppress pro-inflammatory cytokine production by an α -7 nicotinic acetylcholine receptors (α 7nAChR) dependent mechanism. Vagus nerve activity is significantly suppressed in patients with autoimmune diseases, including rheumatoid arthritis (RA). It has been suggested that stimulating the cholinergic anti-inflammatory pathway may be beneficial to patients, but it remains theoretically possible that chronic deficiencies in this pathway will render these approaches ineffective.

Methods—Here we addressed the hypothesis that inflammatory cells from RA patients can respond to cholinergic agonists with reduced cytokine production in the setting of reduced vagus nerve activity.

Results—Measurement of RR interval variability (heart rate variability, HRV), in RA patients ($n = 13$) and healthy controls ($n = 10$) revealed that vagus nerve activity was significantly depressed in patients. Whole blood cultures stimulated by exposure to endotoxin produced significantly less tumour necrosis factor in samples from RA patients as compared to healthy controls. Addition of cholinergic agonists (nicotine and GTS-21) to the stimulated whole blood cultures however significantly suppressed cytokine production to a similar extent in patients and healthy controls.

Conclusion—These findings suggest that it is possible to pharmacologically target the α 7nAChR dependent control of cytokine release in RA patients with suppressed vagus nerve activity. As

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Conflict of interest statement

K.J. Tracey is an inventor on patents related to vagus nerve stimulation and cholinergic agonists as anti-inflammatory agents.

$\alpha 7$ nAChR agonists ameliorate the clinical course of collagen induced arthritis in animals, it may be possible in the future to explore whether $\alpha 7$ nAChR agonists can improve clinical activity in RA patients.

Keywords

autonomic dysfunction; cholinergic agonists; LPS; monocyte cytokine attenuation; RA

Introduction

The central nervous system, via inflammatory reflexes, modulates the innate immune response by suppressing the release of cytokines. One well studied mechanism is a neural circuit in which signals transmitted via the vagus nerve control cytokine release by cell receptor signal transduction requiring the $\alpha 7$ nAChR subunit. The motor arc of this reflex, termed the 'cholinergic anti-inflammatory pathway' reduces pro-inflammatory cytokine production and ameliorates disease in many inflammatory disease models [1–3]. Stimulation of the efferent vagus nerve specifically attenuates tumour necrosis factor (TNF) and other cytokines via a mechanism that is dependent on $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChR) [4,5]. The release of pro-inflammatory cytokines, such as TNF and high mobility group box-1 protein (HMGB1), from endotoxin-stimulated macrophages is significantly suppressed by acetylcholine or nicotine [5]. GTS-21 (also known as DMBX-anabaseine), a selective $\alpha 7$ nAChR agonist, has recently been found to dose-dependently inhibit serum TNF and HMGB1 in mice with lethal endotoxemia and sepsis, giving evidence that cholinergic modalities that activate $\alpha 7$ nAChR may be useful as anti-inflammatory agents [6].

Previous results from clinical studies suggest that decreased vagus nerve activity occurs in subjects with acute inflammatory conditions [7,8]. Vagus nerve activity can be measured in humans by recording RR interval variability (RRV) from each QRS complex in an electrocardiogram (ECG). RRV data are subjected to Power spectral analysis to give a noninvasive measurement of cardiac autonomic modulation known as heart rate variability (HRV). Variability in the spectrally defined high frequency (HF) range (0.15–0.50 Hz) has been linked to cardiac parasympathetic regulation, reflecting vagus nerve tone. Oscillations at the lower frequencies (LF) range (0.04–0.15 Hz) reflect mixed parasympathetic and sympathetic contributions and the low frequency to high frequency ratio (LF/HF) is considered a measurement of sympathovagal balance [9,10]. Reduced HRV is also a strong independent predictor of mortality in the elderly, and predicts the rate of the progression of focal coronary atherosclerosis [11,12]. Heart rate variability dysfunction is inversely related to C-reactive protein (CRP) levels and white blood cell counts in middle-aged and elderly subjects with no apparent heart disease, suggesting that the autonomic imbalance might interact with an inflammatory process leading to atherosclerosis [13].

Chronic inflammatory diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), are associated with autonomic dysfunction and reduced vagus nerve activity [14–16]. In a recent animal model of mice with collagen-induced arthritis clinical arthritis was exacerbated by vagotomy and ameliorated by cholinergic agonists [17]. In RA patients $\alpha 7$ nAChR is expressed in the inflamed synovia [18]. Here, in a prospective observational human study we hypothesize that the addition of cholinergic agonists to whole blood cultures stimulated with endotoxin can attenuate cytokine release, even in RA patients with significantly reduced vagus nerve activity.

Materials and methods

Study subjects

The study population consisted of two groups, RA patients ($n = 13$) and healthy volunteers ($n = 10$). Rheumatoid arthritis patients fulfilled the American College of Rheumatology criteria for RA. The RA subjects were recruited through the North American Rheumatoid Arthritis Consortium at North Shore-LIJ health system, Manhasset, NY, USA. Healthy controls were recruited through the North Shore-LIJ General Clinical Research Center. None of the subjects smoked or had diabetes. Two of the RA subjects and one of the healthy controls used anti-hypertensive medication. A summary of age, gender, RA disease duration, and concurrent medication is found in Table 1. Informed consent was obtained from all subjects by one of the co-investigators. The protocol was approved by the General Clinical Research Center Approval Committee and the Institutional Review Board at the North Shore LIJ Health System.

EKG/HRV analysis—Subjects were assessed during morning hours at the North Shore-LIJ General Clinical Research Center after an overnight fast. RRV measurements were taken whilst each subject was in a semi-recumbent position, in a quiet examining room. ECG monitoring was obtained using ECG sensors provided by Thought Technology Unigel electrodes which were placed on the anterior chest wall. After a 5 min rest subjects were asked to sit comfortably for a 20-min duration when each subject's RRV was recorded. Heart rate variability measurements were averaged over four 5-min epoch recordings. Cardiopro 2.0 software (Thought Technology Ltd, Plattsburg, NY, USA) was used for HRV data collection. All recordings were visually edited. Nonstationary signals (artefacts or ectopic beats) were removed by averaging, splitting, or adding signals. The total amount of editing for each subject was <1% of the recording duration. Data generated from the session included frequency domain parameters including very low frequency (VLF), LF, HF, and the LF/HF-ratio, as well as the time domain parameters. However, for the purposes of this study, statistical calculation was focused only on the frequency domain parameters LF, HF and LF/HF. Other parameters included respiration and heart rate.

Serum collection—Subsequent to each subject's HRV measurements, approximately 10–15 mL and 5–10 mL of whole blood were collected into two heparinized tubes and one nonheparinized tube, respectively. The nonheparinized tube was left to clot for 2 h at room temperature (approximately 25 °C) and subsequently centrifuged [5–10 min, 828 g (2000 rpm in Sorvall RT7 Plus centrifuge; Thermoscientific, Ashville, NC, USA)]. About 500 μ L aliquots of harvested serum were frozen at –20 °C for future analysis.

Whole blood assay

Heparinized tubes of blood were immediately incubated at 37 °C with 5% CO₂ until aliquoted in experimental setup (incubation did not exceed 1 h). Endotoxin [lipopolysaccharide (LPS), *Escherichia coli* 0111:B4, Sigma cat. No. L4130] was re-suspended to 5mg mL^{–1}, sonicated for 30 min, vortexed well and diluted with 1× phosphate-buffered saline (PBS) to create a working 1 mg mL^{–1} stock. This stock was serially diluted with 1× PBS to final concentrations of 1, 10 and 100 ng mL^{–1}, in 500 μ L blood aliquots. Microfuge tubes aliquoted with blood and endotoxin were gently pulsed on a vortexer and incubated in a test tube rack on a rocking platform at 37 °C with CO₂. After 4 h incubation plasma was collected by centrifugation [5 min, 2000 g (5000 rpm in Microfuge 5415C; Brinkmann, Westbury, NY, USA)] and frozen at –20 °C for future analysis. All samples were performed in duplicate.

Cholinergic agonist treatment—*Ex vivo* whole blood samples were stimulated with the final LPS concentrations of 1, 10 and 100 ng mL^{–1} as described above in the presence of 100 μ mol L^{–1} GTS-21. Alternatively nicotine was added at a final concentration of 100 μ mol

L^{-1} along with 100 ng mL^{-1} LPS. Endotoxin and cholinergic agonist treatments were effectively simultaneous. Incubation time and conditions, as well as subsequent centrifugation, harvest, and storage were performed as indicated above. Each sample was performed in duplicate. Nicotine was obtained from Sigma-Aldrich (St Louis, MO, USA). GTS-21 was provided by Yousef Al-Abed who synthesized, isolated and standardized it. Yousef Al-Abed is a medicinal chemist and investigator at the Feinstein Institute for Medical Research.

Cytokine analysis—Tumour necrosis factor levels in plasma and serum samples were analyzed by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

High sensitivity CRP serum analysis was performed using Hitachi 917 Analyzer (Roche Diagnostics, Indianapolis, IN, USA) at the core laboratory at North Shore LIJ Health System. The reference value for hs-CRP is $0.0\text{--}3.0\text{ mg L}^{-1}$.

HMGB1 serum determination—Whole blood was collected in nonheparinized tubes and allowed to clot at room temperature ($22\text{--}24\text{ }^{\circ}\text{C}$) for 2 h. The clotted blood samples in the tubes were centrifuged at 2500 rpm (2000 g) for 10 min and supernatant collected. Serum was aliquoted into microfuge tubes and stored at $-80\text{ }^{\circ}\text{C}$. HMGB1 levels were measured by Western immunoblotting analysis. In brief, serum samples were thawed on ice and microfuged for 10 min at 11 000 rpm (9700 g). The supernatant ($100\text{--}200\text{ }\mu\text{L}$) was ultrafiltered with Microcon YM-100 filters (Millipore, Billerica, MA, USA). Filtrate was transferred to a new tube, mixed with $5\times$ Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA), heated at $95\text{ }^{\circ}\text{C}$ for 5 min and spun down at 10 000 rpm (8160 g) for 5 min in tabletop microfuge. About $12.5\text{ }\mu\text{L}$ of heated sample ($10\text{ }\mu\text{L}$ serum per lane) was fractionated through 10–20% Tris–HCl acrylamide gels (Bio-Rad) and transferred to preactivated Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Polyvinylidene Difluoride membrane was preactivated with methanol, rinsed and equilibrated in Tris/Glycine/20% Methanol transfer buffer. After transfer, the membrane was rinsed briefly with wash buffer (0.2% Tween 20 in PBS) and incubated for 1 h in blocking buffer (5% nonfat dry milk in wash buffer). The membrane was probed overnight at $4\text{ }^{\circ}\text{C}$ with purified IgG from anti-HMGB1 antiserum ($2\text{ }\mu\text{g mL}^{-1}$ in 1% nonfat dry milk in wash buffer). The membrane was washed two times at 10-min intervals in wash buffer and incubated with peroxidase conjugated anti-rabbit secondary antibody (1: 7500 dilution; Amersham Biosciences, Piscataway, NJ, USA) for 1 h. The membrane was washed three times at 15-min intervals in wash buffer followed by 5 min in $1\times$ PBS. After rinsing the membrane in distilled water, the signals were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences). Equal volumes of ECL A and B were mixed and added to the PVDF membrane and incubated for 1 min. Excess reagent was removed by blotting the edge of the PVDF membrane on a paper towel. Polyvinylidene Difluoride membrane was then exposed to Hyperfilm (Amersham Biosciences). Autoradiograph films were scanned and densitometric analyses performed using Quantity One software (Bio-Rad) and Microsoft Excel. The levels of HMGB1 were determined by reference to standard curves generated with purified HMGB1.

Statistical methods

All subjects came for 1–8 visits for patients with RA and 1–5 visits for controls. The respiration rate, HF, LF, LF/HF-ratio, basal TNF, TNF-levels after whole blood assay, (exposure to LPS, nicotine response, GTS-21 response) were measured on all visits. There were no statistical significant differences in variables at repeated visits at various time points in individual subjects. Data in figures and tables are expressed as mean of all multiple measurements of each subject. C-reactive protein and HMGB1 were measured on the first visit only. Continuous variables were expressed as median (10–90 percentiles) and nominal variables were expressed

as percentage. Group differences for continuous variable were determined by nonparametric Wilcoxon two sample rank sum test or Kruskal–Wallis test as appropriate. Nominal variables were compared by chi-square test.

For any measurements that were below detectable levels of 7.81 pg mL^{-1} for TNF, the value of 1 pg mL^{-1} was used. Cholinergic responsiveness focusing on nicotine responsiveness was defined as TNF levels after whole blood assay exposure to LPS 100 ng mL^{-1} divided by TNF levels after whole blood assay exposure to LPS 100 ng mL^{-1} + nicotine. Cholinergic responsiveness focusing on GTS-21 responsiveness was defined as the ratio of the TNF levels after LPS exposure with 1, 10 and 100 ng mL^{-1} divided for LPS + GTS-21.

To explore an association between cholinergic responsiveness and each of HF, a mixed models approach to analysis of covariance (ancova) was used, where cholinergic responsiveness was modelled as a function of group and HF, age, gender, and respiration rate were included as covariates in analyses including HF. Differences were considered significant with a *P*-value of <0.05 . All statistical analyses were performed using the sas 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

The first phase of the study aimed to assess patient characteristics, disease activity and inflammatory markers. With the exception of age ($P = 0.002$), there were no significant differences between RA patients and healthy controls with regards to gender or hypertension. These data and other patient characteristics such as RA disease duration, disease activity score (DAS 28-CRP a clinical score based upon the presence of number of swollen, tender joints and CRP levels) and medications are summarized in Table 1. C-reactive protein and HMGB1 levels were significantly elevated in RA patients compared to controls (Table 2).

Measurement of vagus nerve activity parameters based on HRV (HF, LF, LF/HF) revealed that HF was significantly reduced in RA as compared with healthy controls (Table 2). Respiration rate was also significantly elevated in RA subjects but heart rate did not differ significantly between groups (though the mean heart rate was higher in RA patients). Respiration rate was included as a covariate as respiration can influence HRV but failed to change the significant difference for either HF or LF between groups [19]. The ratio LF/HF was affected by including respiration as a covariate, but the observed differences remained statistically significant. Together, these physiological results are consistent with the previously described findings that autonomic dysfunction and reduced vagus nerve activity occurs in RA.

We next addressed the hypothesis that inflammatory cells are capable of responding to cholinergic agonists in the setting of decreased vagus nerve activity in RA patients. Baseline serum TNF levels were slightly elevated in several RA patients, and generally nondetectable in controls, but these differences were not significant (Table 3). Total inducible TNF production in whole blood cultures stimulated by increasing exposure to endotoxin was significantly lower in RA patients as compared to controls (Table 3 and Fig. 1). The reduction in inducible TNF levels remained significantly greater after exposure to nicotine and GTS-21 in the controls compared with RA patients (Table 3 and Fig. 1). The total magnitude of suppressed inducible TNF production by addition of cholinergic agonists was however comparable when looking at the data as a ratio and did not differ significantly between the RA patients and control groups (Table 3). These results suggest that TNF producing cells in whole blood organ culture can be targeted by cholinergic agonists to suppress cytokine release even in a patient group with low inducible TNF capacity.

We observed no association between cholinergic responsiveness (nicotine or GTS-21) and HF, suggesting that cholinergic responsiveness to $\alpha 7\text{nAChR}$ agonists is independent of underlying

autonomic dysfunction. Age, gender and respiration rate were included as covariates in the model but was not significant. There was no association between cholinergic responsiveness and HMGB1 levels.

Discussion

Rheumatoid arthritis is a systemic chronic inflammatory disease primarily affecting the joints. TNF has been implicated in the pathogenesis of disease progression in RA, and inhibiting TNF activity significantly reduces disease manifestations [20]. Vagus nerve stimulation by physiological and pharmacological modalities has recently been shown to attenuate HMGB1 and TNF levels in animal models of inflammation, including collagen induced arthritis [2,4–6]. Patients with RA have decreased vagus nerve activity as compared to healthy individuals, raising the possibility that RA patients may be incapable of responding to cholinergic agonist-based therapy to control TNF production [14,15]. Accordingly, here we examined whole blood culture responses to LPS and the subsequent effect of cholinergic agonists on total TNF production in RA patients with reduced vagus nerve activity compared with healthy controls. Extensive experience with this model indicates that the cells producing TNF are CD14+ monocytes [21] that are responsive to $\alpha 7$ nAChR mediated signaling [22,23].

Previous studies of untreated RA and SLE patients compared with healthy controls revealed that basal levels of IL-1ra were higher and IL-6 were lower whereas TNF levels were similar. When Peripheral Blood Mononuclear Cells (PBMC) from patients were challenged with LPS, cells from RA patients produced significantly lower amounts of IL-1ra as compared to controls [24]. Rossol *et al.* found that TNF production by PBMC *in vitro* was significantly lower in treated and untreated patients with RA as compared with controls after stimulating monocytes using glutaraldehyde fixed T-cells [25]. Popa *et al.* recently examined cytokine production from stimulated whole blood cultures in RA patients treated with the TNF blocking agents adalimumab or etanercept. Stimulation with Salmonella LPS resulted in a significantly lower production of IL-1beta, TNF and a trend towards lower IL-6 and IFN-gamma production in RA patients compared to healthy controls. Therapy for at least 3 months with either of the agents did not significantly alter cytokine production capacity, with the exception of a lower IFN-gamma and IL-8 production in patients treated with adalimumab [26]. Here we also analyzed TNF levels in different groups of RA patients based on the treatment with or without TNF-blockers but found no significant difference between groups. Taken together these findings suggest that the decreased innate response to endotoxin stimulation is associated with the RA disease and not the TNF-blocking treatment. However, in line with previous studies the patients on steroids did express significantly less TNF ($P = 0.01$) after LPS induction [27–29]. Our observations that RA monocytes *ex vivo* respond to cholinergic agonists support the hypothesis that the inflammatory cells in RA still can respond to cholinergic stimulation despite having been exposed to a milieu of decreased vagus nerve activity *in vivo*. In this study we did not record total white blood cell counts or leucocyte differentials which could potentially differ between RA patients and controls and should be addressed in future studies. It is however likely that the relative influence of leucocytes is small as monocytes have been shown to be the major source of TNF in the LPS model [21]. Further limitations of this study are the small sample size and that the control population was significantly younger than the RA patients. The latter could have some importance when considering the HRV data, since HRV is known to decrease with age [30]. Also gender could be a significant factor as women have a higher vagus nerve tone than men [31]. However, the main finding of this study that inflammatory cells from RA patients do respond to cholinergic agonists *ex vivo* in a similar fashion compared to healthy controls seems to be independent of vagus nerve activity in this study, as HF, which was significantly decreased in RA patients did not correlate with cholinergic responsiveness even when controlling for factors such as age, gender and respiration rate. Methods to utilize the cholinergic anti-inflammatory pathway by cholinergic modalities in

chronic inflammatory diseases such as RA could be of considerable interest and may be explored as an anti-inflammatory strategy in prospective clinical studies.

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Fig. 1. The stimulation with lipopolysaccharide (LPS) (vehicle) at a concentration of 1, 10 and 100 ng mL⁻¹ and LPS + GTS-21 in patients and controls in whole blood. Data are expressed as mean and standard error of mean. Rheumatoid arthritis (RA) patients (□); Controls (●).

Table 1

Characteristics of the rheumatoid arthritis patients and healthy controls

	RA, <i>n</i> = 13	Controls, <i>n</i> = 10	<i>P</i> -value
Sex (female, %)	69	54	ns
Age (years)*	52 (34–77)	32 (26–71)	0.05
RA duration (years)	13.2 (3–31)	–	n.a
Rheumatoid-factor positive (%)	11/13	–	n.a
Disease activity score (DAS-28 CRP)	3.9 (2.3–5.9)	–	n.a
Medications			
Corticosteroids	7	0	n.a
NSAID	2	0	n.a
B-blockers	2	0	ns
Calcium channel antagonist	0	1	ns
DMARDS	10/13	0	n.a
TNF receptor blocker	7	0	n.a

CRP, C-reactive protein; DMARDS, disease modifying anti-rheumatic drugs; ns, not significant; n.a, not applicable; TNF, tumour necrosis factor; RA, rheumatoid arthritis; NSAID, nonsteroidal anti-inflammatory drug.

* Expressed as median (10–90 percentiles).

Table 2

Inflammatory markers, respiration rate, heart rate, heart rate variability: high frequency power (HF), low frequency power (LF) and low frequency/high frequency ratio (LF/HF) in rheumatoid arthritis patients ($n = 13$) and healthy controls ($n = 10$)

	RA	Controls	P-value
CRP	14.5 (0.4–54.6)	1.0 (0.4–2.9)	0.007
HMGB1	71 (49.8–97.4)	19 (0–40)	<0.0001
Respiration rate	15 (12–16)	12 (9–15)	0.002
Heart rate	77 (45–104)	71 (45–89)	ns
HF	44 (14–122)	288 (19–1288)	0.009
LF	141 (6–251)	158 (88–2073)	0.09
LF/HF	1.6 (0.3–8.2)	1.3 (0.4–12.7)	ns

CRP, C-reactive protein; HF, high frequency power; LF, low frequency power; RA, rheumatoid arthritis; ns, not significant.

Data expressed as median (10–90 percentiles).

Table 3

Basal tumour necrosis factor (TNF), stimulated TNF (after whole blood assay exposure to LPS), effect on TNF after nicotine, effect on TNF after GTS-21. Cholinergic responsiveness (nicotine) defined as a ratio of TNF levels after whole blood assay exposure to lipopolysaccharide (LPS) 100 ng mL⁻¹ divided by TNF levels after whole blood assay exposure to LPS 100 ng mL⁻¹ + nicotine. Cholinergic responsiveness (GTS-21) defined as the ratio of TNF levels after LPS exposure with 1, 10 and 100 ng mL⁻¹ divided for LPS + GTS-21

	RA	Controls	P-value
Basal TNF* (pg mL ⁻¹)	18 (1–209)	1 (1–172)	0.32
TNF levels after exposure to LPS (1 ng mL ⁻¹)*	1383 (112–4420)	2315 (1–6125)	0.26
TNF levels after exposure to LPS (10 ng mL ⁻¹)*	3203 (389–9381)	7024 (2092–12 814)	0.0006
TNF levels after exposure to LPS (100 ng mL ⁻¹)*	4181 (781–15 814)	10 208 (4864–17 879)	0.0001
TNF levels after nicotine response*	3305 (831–11 523)	8227 (3989–20 511)	0.0001
TNF levels after LPS exposure(1 ng mL ⁻¹) and GTS response*	858 (1–2841)	687 (1–3827)	0.96
TNF levels after LPS exposure(10 ng mL ⁻¹) and GTS response*	1403 (208–5092)	3834 (1167–7507)	0.0001
TNF levels after LPS exposure(100 ng mL ⁻¹) and GTS response*	2217 (477–8232)	5000 (2917–12 995)	0.0001
Cholinergic responsiveness			
Nicotine responsiveness*	1.24 (0.88–1.49)	1.33 (0.85–1.54)	0.57
LPS exposure (1 ng mL ⁻¹) GTS-21 responsiveness*	1.84 (0.93–4.51)	1.73 (1.06–5.02)	0.96
LPS exposure (10 ng mL ⁻¹) GTS-21 responsiveness*	1.92 (1.13–2.95)	1.73 (1.24–2.55)	0.39
LPS exposure (100 ng mL ⁻¹) GTS-21 responsiveness*	1.85 (1.17–2.52)	1.74 (1.22–3.06)	0.86

LPS, lipopolysaccharide; TNF, tumournecrosis factor; RA, rheumatoid arthritis.

* Expressed as median (10–90 percentiles).