Cholinergic Anti-Inflammatory Pathway Activity and High Mobility Group Box-1 (HMGB1) Serum Levels in Patients with Rheumatoid Arthritis

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High Mobility Group Box-1 (HMGB1) is a cytokine implicated in the pathogenesis of rheumatoid arthritis (RA) and other inflammatory diseases. The cholinergic anti-inflammatory pathway, a vagus nerve-dependent mechanism, inhibits HMGB1 release in experimental disease models. Here, we examine the relationship between vagus nerve activity and HMGB1 in patients with RA. We compared RR interval variability, an index of cardiac vagal modulation, HMGB1 and hsCRP serum levels, and disease activity scores in thirteen RA patients and eleven age- and sex-matched controls. In RA patients, serum levels of HMGB1 and hsCRP were elevated as compared with controls (HMGB1 = 71 ng/mL (45-99) vs. 18 ng/mL (0-40), $P < 0.0001$; hsCRP = 14.5 mg/L (0.7-59) vs. 1 mg/L (0.4-2.9), $P < 0.001$). RR interval variability in RA patients was significantly decreased as compared with controls (HF = 38 msec^2 (14-80) vs. 288 msec^2 (38-364), $P < 0.0001$; rMSSD = 20.9 ± 9.79 msec, 52.6 ± 35.3 msec, $P < 0.01$). HMGB1 levels and RR interval variability were significantly related (rho = -0.49, $P < 0.01$). HMGB1 serum levels significantly correlated with disease activity scores (DAS-28) in patients with RA ($P = 0.004$). The study design does not enable a determination of causality, but the results are consistent with the hypothesis that decreased cholinergic anti-inflammatory pathway activity is associated with increased HMGB1 levels in patients with RA.

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INTRODUCTION

Rheumatoid arthritis (RA), a chronic autoimmune destructive polyarthropathy, has a worldwide prevalence of 0.5-1%, and an incidence of three cases per 10,000 persons annually (1-3). Affected individuals have significantly diminished quality of life and a three to ten year decrease in life expectancy (4). Over-expression of cytokines mediates tissue injury and drugs that decrease the activity of TNF or IL-1 have improved the lives of RA patients significantly. High mobility group box-1 (HMGB1), a recently identified proinflammatory cytokine, has been implicated in the pathogenesis of arthritis (5-10). HMGB1 expression is increased in the synovium of patients with RA and in experimental animal models of collagen and adjuvant-induced arthritis (6). Anti-HMGB1 antibodies confer significant protection against the development of experimental arthritis in animals, but little is known about mechanisms that regulate HMGB1 release in this disease (5-7).

The cholinergic anti-inflammatory pathway is a neural mechanism that inhibits the expression of HMGB1 and other cytokines (11-16). Signals transmitted via the vagus nerve, the principal nerve of the parasympathetic nervous system, significantly attenuate the release of HMGB1 and other cytokines in animal models of inflammation (11-16). Here we considered whether decreased activity in the cholinergic anti-inflammatory pathway might occur in patients with RA. The results indicate that depressed levels of vagus nerve activity correlate with elevated levels of HMGB1.

MATERIALS AND METHODS

Patients

This prospective observational study protocol was approved by the General Clinical Research Advisory Committee (GAC) and the North Shore-LIJ Institutional Review Board. Patients were recruited from the North American Rheumatoid Arthritis Consortium (NARAC) and by affiliated Rheumatology clinic referrals.

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Informed consent was explained and subsequently obtained in all subjects. The total number of subjects enrolled was twenty-four (RA patients = 13, healthy volunteers = 11). Patients had an established diagnosis of RA according to the ACR criteria (17). None of the RA patients had a history of smoking. Two RA patients had hypertension controlled with medication. No other cardiovascular risk factors were identified in the RA subjects. Healthy volunteers were age- and sex-matched and were free of cardiovascular risk factors.

Table 1. Patient Demographics

	RA	Controls	P value
M/F (n)	4/9	5/6	ns
Age (years)	52 (32–82)	38 (26–73)	0.05
Disease Duration (years)	13	–	–
Rheumatoid-factor positive (%)	11(85%)	–	–
C-reactive protein (mg/L)	14.5 (0.2–5.9)	1 (0.4–2.9)	P<0.01
Disease activity score-28 (mean ± SD)	4.5 ± 0.94	–	–
co-Morbidities:			
HTN	0	1	ns
Hypothyroidism	1	1	ns
SLE	1	0	ns
Medications:			
Corticosteroids	7	0	0.006
NSAIDS	2	0	ns
β-Blockers	2	0	ns
Calcium Channel Antagonists	0	1	ns
DMARDs	10	0	0.002
TNF receptor Blockers	7	0	0.005

[a]Data expressed as median values. Range is given in parentheses. DAS-28 score is expressed as mean ± SD.

Disease Activity

Disease activity was measured with the 28 joint disease activity score (DAS 28), a validated composite score which in our study incorporated three variables including tender and swollen joint counts and high-sensitivity CRP (i.e. DAS 28 – 3(CRP) = (0.56*SQRT(TJC28) + 0.28*SQRT(SJC28) + 0.36*ln (CRP + 1))* 1.10 + 1.15) (18). A DAS-score ≤ 2.6 indicates remission; a score between 2.6 and 3.2 indicates low disease activity; a score between 3.2 and 5.1 indicates intermediate activity; a score ≥ 5.1 is considered high disease activity (18). Joint tenderness and swelling were assessed by a single physician to limit interrater variability as a potential confounding variable. Patient and control characteristics and clinical data are summarized in Table 1.

RR Interval Variability Measurements

Subjects were instructed not to consume food or caffeine prior to arrival at the North Shore-LIJ General Clinical Research Center (GCRC) between 0700-1000. RR interval variability was measured in each subject by placing three ECG electrodes on the anterior chest wall and a respiratory sensor around the circumference of the abdomen. After a five min rest in a dimly lit room, RR interval variability was recorded for a twenty min interval. Subjects were awake in the seated position in a reclining chair, and although the depth and rate of breathing was not controlled, the observed respiratory rates were in the high frequency range (ten to twenty breaths per minute). Ectopic beats and artifacts on the ECG's were edited by averaging, splitting, or adding consecutive beats. The total amount of editing for each patient was less than one percent of the recording duration.

RR interval variability measurements were computed using CardioPro 2.0 software (Thought Technology Ltd., Canada). Fast-Fourier analysis was used to generate frequency domain measures. For each five min epoch, HF power (0.15-0.40 Hz), LF power (0.04-0.15 Hz), the LF/HF ratio and rMSSD were computed. This analysis adhered to the standards developed by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (19). The low frequency to high frequency ratio (LF/HF), a measure of sympathovagal balance, and rMSSD, a reflection of vagal nerve activity, were calculated. Absolute values of frequency domain analysis were calculated from normalized data (i.e. HF = HFN × [Total Power-VLF]/100, LF = LFN × [Total Power-VLF]/100). Means of all 4 epochs were calculated for each subject.

HMGB1 Measurements

Whole blood was collected in non-heparinized tubes and allowed to clot at room temperature (22-24°C) for 2 h. Tubes were centrifuged at 2000*g* for ten min and supernatant collected. Serum was aliquoted into microfuge tubes and stored at -80°C. HMGB1 levels were measured by Western immunoblotting analysis. In brief, sera samples were thawed on ice and microfuged for ten min at 9700*g*. The supernatant (100-200 μL) was ultrafiltered with Microcon YM-100 filters (Millpore, Billerica, MA, USA). Filtrate was transferred to a new tube, mixed with 5X Laemmli Sample Buffer (BioRad, Hercules, CA, USA), heated at 95°C for five min and spun down at 8160*g* for five min in tabletop microfuge. 12.5 μL of heated sample (10 μL serum per lane) was fractionated through 10-20% Tris-HCL acrylamide gels (BioRad) and transferred to preactivated polyvinyline fluoride (PVDF) membrane (Bio-Rad). PVDF membrane was pre-activated 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% non-fat dry milk in wash buffer). The membrane was probed overnight at 4°C

with purified IgG from anti-HMGB1 antiserum (2 μg/mL in 1% non-fat dry milk in wash buffer). The membrane was washed two times at ten min intervals in wash buffer and incubated with peroxidase conjugated anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ, USA, 1:7500 dilution) for 1 h. The membrane was washed three times at fifteen min intervals in wash buffer followed by five min in 1× PBS. After rinsing the membrane in distilled water, the signals were visualized using 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. PVDF membrane was then exposed to Hyperfilm (Amersham Biosciences). Autoradiograph films were scanned and densitometric analyses performed using Quantity One software (BioRad) and Microsoft Excel. The levels of HMGB1 were determined by reference to standard curves generated with purified HMGB1.

CRP Analysis

High sensitivity CRP serum analysis was performed using a Hitachi 917 Analyzer (Roche Diagnostics, Indianapolis, IN, USA). The standard reference range for hsCRP is 0.0 - 3.0 mg/L.

Role of the Funding Source

The funding source had no role in the study design, data collection, data analysis, data interpretation, or writing of the study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit the manuscript for publication.

Statistical Methods

All values are expressed as median (range), unless otherwise indicated. A P value < 0.05 was considered to be statistically significant. Comparisons between two groups were assessed for continuous variables with the Wilcoxon test. Differences among three groups were analyzed by analysis of variance (ANOVA) using the Kruskal-Wallis test. Spearman's rank correlation was used to determine correlations between continuous variables. Fisher's Exact test was used for comparing categorical variables between the two groups. The statistical analysis was performed using statistical software SAS version 9.1.4 (SAS Campus Drive, Cary, NC, USA). Covariant analysis was performed using respiratory rates for the established effect that respiration has on HF power (21).

Table 2. Patient medications and co-morbidities

Gender	Age	ACR Criteria	Duration of RA (years)	Smoking	RA Tx
M/F=4/9	Avg = 52.7 Median=52				
F	54	Y	4	n	Sulfasalazine
F	63	Y	5	n	TNF-alpha blocker
F	47	Y	10	n	TNF-alpha blocker Methotrexate Prednisone
F	55	Y	6	n	TNF-alpha blocker Methotrexate Folic Acid
F	52	Y	17	n	TNF-alpha blocker Methotrexate Folic Acid Hydroxychloroquine NSAIDS
F	38	Y	3	n	Hydroxychloroquine
M	51	Y	16	n	TNF-alpha blocker Prednisone
F	58	Y	10	n	Hydroxychloroquine Prednisone
M	46	Y	16	n	TNF-alpha blocker Methotrexate Folic Acid Prednisone
F	38	Y	10	n	Methotrexate Prednisone NSAIDs
F	32	Y	5	n	Sulfasalazine Methotrexate Folic Acid Prednisone
M	70	Y	31	n	TNF-alpha blocker Methotrexate Folic Acid Prednisone
M	82	Y	3	n	NSAIDs

RESULTS

There were no statistically significant differences in age, gender, or the diagnosis of hypertension in the RA patients as compared with healthy controls (Table 1). All concurrent medications and co-morbidities of RA patients are listed in Table 2.

HMGB1, CRP, and Vagus Nerve Activity

HMGB1 levels were significantly increased in RA patients as compared with the healthy control group (RA =

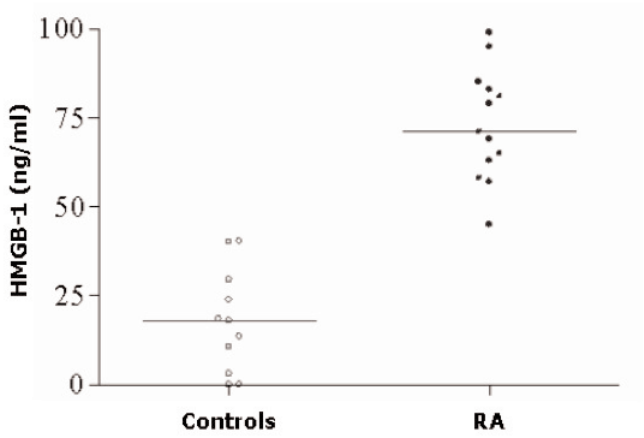

Figure 1. Detection of HMGB1 in patients with Rheumatoid Arthritis. Rheumatoid arthritis serum HMGB1 = 71 ng/mL (45-99), control serum HMGB1 = 18 ng/mL (0-40); $P < 0.0001$.

71 ng/mL [45-99] *vs*. controls = 18 ng/mL [0-40], $P < 0.0001$) (Figure 1). High sensitivity CRP values also were significantly elevated in the RA patients as compared with control subjects (RA = 14.5 mg/L [0.2-59] vs. controls = 1 mg/L [0.4-2.9], $P < 0.001$). As described elsewhere, a substantial body of evidence has demonstrated that RR interval variability from the electrocardiogram provides a noninvasive index of cardiac autonomic modulation. Variability in the spectrally-defined high frequency (HF) range (0.15-0.50 Hz) has been linked to cardiac parasympathetic regulation, as shown by studies employing vagal stimulation, pharmacological blockade, and vagotomy (22,23). RR interval oscillations at lower frequencies (LF) (0.04-0.15 Hz) reflect mixed parasympathetic and sympathetic contributions, with the magnitude of the sympathetic contribution varying as a function of several factors including posture and activity (23-25). We observed that the absolute value of HF power was significantly lower in RA patients as compared with controls (38 $msec^2$ [14-80] *vs*. 288 [38-364], $P < 0.0001$) indicating that vagus nerve activity is decreased in RA (Figure 2a). rMSSD, another parameter to measure vagus nerve activity, was also attenuated in RA subjects as compared with healthy volunteers (20.9 ± 9.79 msec *vs*. 52.6 ± 35.3 msec, $P < 0.01$). Resting heart rate was elevated in RA patients as compared with controls, but this difference was not statistically significant (HR 77 beats/min [60-115] *vs*. 68 beats/min [44-83], $P = 0.09$). Respiratory rate in the RA group was significantly increased as compared with healthy controls (15 breaths/min [12-17] *vs*. 12 breaths/min [9-15], $P < 0.001$) (Figures 2b, 2c). Covariant analysis corrected for any respiratory rate influences that may have occurred on HF power and rMSSD. Together these findings indicate that vagus nerve activity is significantly reduced in patients with RA.

Correlation of Vagus Nerve Activity, HMGB1 Levels, and Disease Activity

Serum HMGB1 levels were significantly correlated to HF power (rho = –0.49, $P < 0.01$). Causality cannot be determined in this study and it is theoretically possible that advanced inflammation and subsequent damage cause the observed decrease in vagus nerve activity. If decreased vagus nerve activity occurs as a result of damage, however, then vagus nerve activity should be less in patients with severe disease as compared with mild disease. On the contrary, we observed that vagus nerve activity is significantly decreased even in patients with mild disease (Figure 3a). This observation is consistent with the hypothesis that decreased vagus nerve activity might facilitate increased cytokine levels and worsen disease. A covariance analysis was performed to correct for respiratory influences on HF power. There remained a statistical significant correlation between HF power *vs*. HMGB1 ($P < 0.01$) and HF power *vs*. DAS-28 ($P < 0.01$). Including healthy controls in the analysis using the DAS-28 formula, the score correlated significantly to HMGB1 serum levels (rho = 0.83, $P < 0.0001$) (Figure 3b). Within the RA subjects alone however, the DAS-28 did not correlate to HMGB1 levels.

DISCUSSION

Recent evidence indicates an essential role of the vagus nerve in suppressing cytokine expression during inflammation (11-16). Cytokine expression in human macrophages is attenuated through the interaction of the principle vagus nerve neurotransmitter, acetylcholine, and the α7nAChR subunit located on cytokine expressing cells (12,13). Stimulating the vagus nerve either by electrical or pharmacological methods improves survival in animal models of inflammation (14). Administration of cholinergic agonists inhibited HMGB1 release and improved survival from infection in vivo by inhibiting NF-κB signaling through an α7nAChR dependent mechanism (15). We and others have suggested previously that decreased activity in the cholinergic anti-inflammatory pathway may facilitate excessive cytokine expression in the development and progression of inflammatory diseases.

RR interval variability is a noninvasive tool that measures autonomic regulation of the heart. In this study, patients with RA had significantly lower cardiac vagal modulation compared with healthy controls. Evrengul and colleagues reported that HF power was decreased in RA patients as compared with healthy controls, and they suggested that lower vagal nerve activity may contribute to the increased incidence of sudden death observed in RA (26). Loothernoo et al. studied subjects with RA and SLE and observed autonomic nervous system

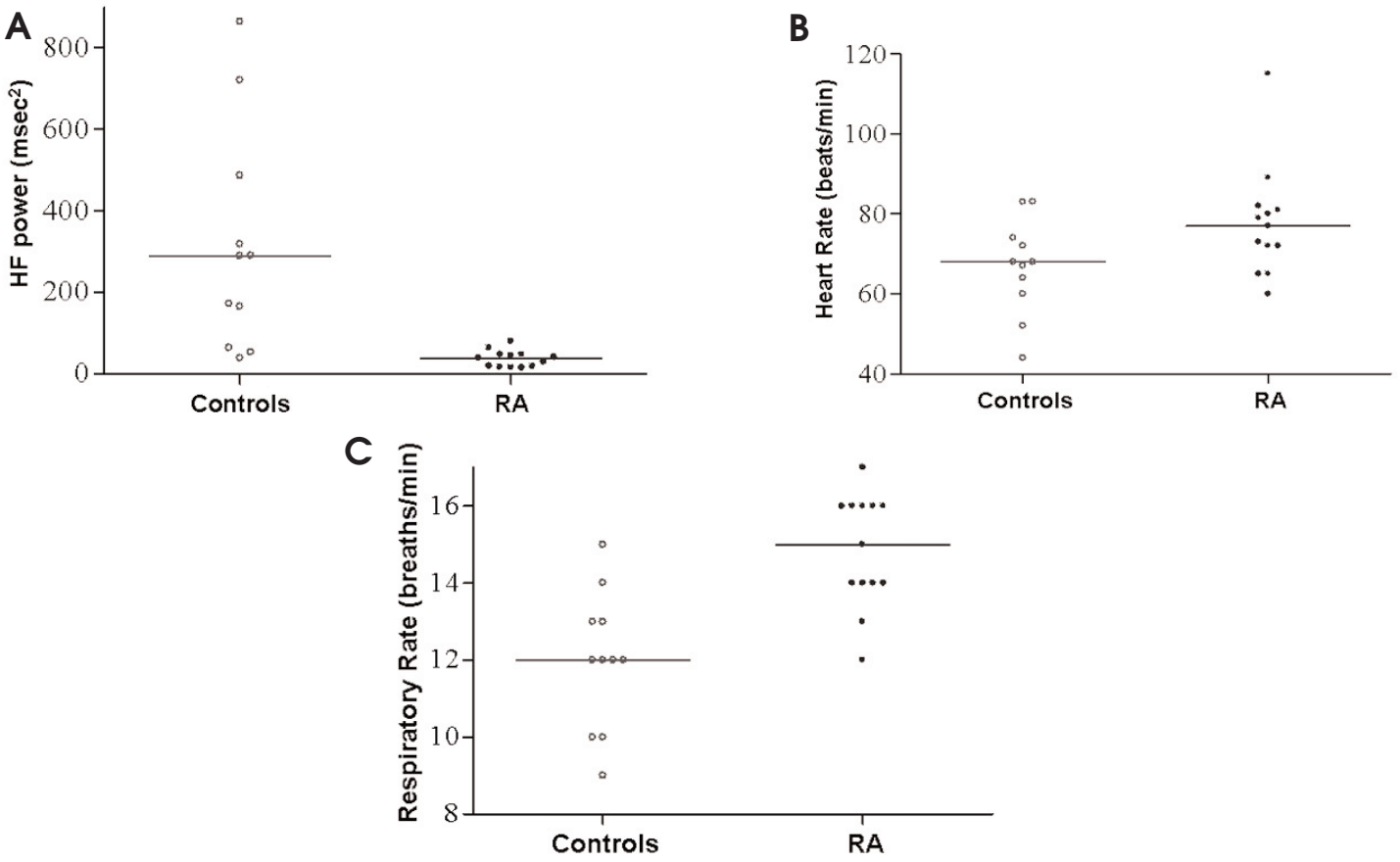

Figure 2. A) Decreased vagus nerve activity in Rheumatoid Arthritis patients. HF power, a measurement of vagus nerve activity, was measured in patients with RA and healthy control subjects. RA patients had significantly decreased vagus nerve activity as compared with control subjects (RA; HF = 38 msec^2 (14-80), controls; HF = 288 msec^2 (38-364), $P < 0.0001$); B) Elevated heart rate in Rheumatoid Arthritis patients. Heart rates were measured from electrocardiograms in RA patients and control subjects (RA; Heart Rate = 77 beats/min (60-115), Controls; Heart Rate = 68 beats/min (44-83), $P = 0.09$); C) Elevated respiratory rates in Rheumatoid Arthritis patients. Respiratory rates were measured using an abdominal sensor. RA patients; Respiratory Rate = 15 breaths/min (12-17), controls; Respiratory Rate = 12 breaths/min (9-15), ($P < 0.001$).

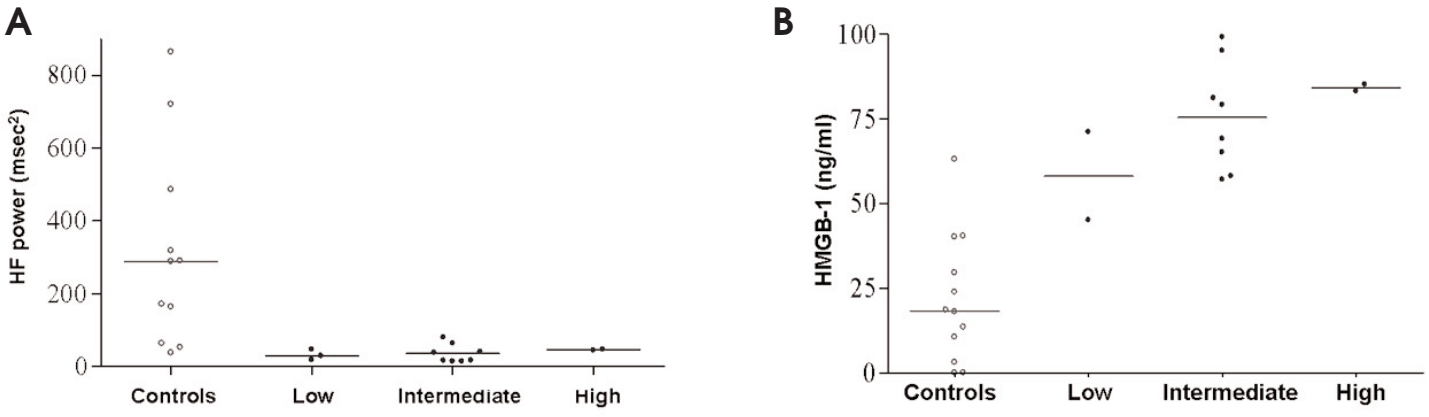

Figure 3. A) Disease activity and vagus nerve activity. Disease activity was measured using DAS-28. Note that disease activity is independent of HF power. Kruskal-Wallis ANOVA = 16.72, $P = 0.0008$; B) Relationship between HMGB1 serum levels and disease activity. HMGB1 serum levels were compared with disease activity scores (DAS-28). Below 3.2 represents low activity, a score of 3.2-5.1 represents intermediate activity, and > 5.1 represents high disease activity. Kruskal-Wallis ANOVA = 13.14 $P = 0.004$.

dysfunction in response to sustained hand grip and valsalva maneuvers (27). Our findings here are in agreement with previous observations that HF is decreased in patients independent of disease severity. Diminished vagal tone was significantly correlated with levels of HMGB1. To our knowledge this is the first study to demonstrate a link between insufficient activity in the cholinergic anti-inflammatory pathway, and levels of an inflammatory marker (HMGB1) in human inflammatory disease. We did not observe a significant correlation between HMGB1 levels and disease activity in this group of RA patients only, but this may be due to a restriction in the range of the measure of disease activity. Consistent with this possibility, when healthy volunteers were included in the analysis a significant correlation between HMGB1 and DAS-28 emerged, see Figure 3b.

The presence of subclinical cardiovascular disease in RA has been described previously and may have contributed partly to the lower RR variability observed in this study (28,29). It is interesting to consider whether subclinical cardiovascular disease is the result of increased inflammation from decreased vagus nerve activity.

Although the present study design does not enable a determination of causality, in the future it should be possible to increase vagus nerve activity via biofeedback, vagus nerve stimulation, or other measures and determine whether these maneuvers can decrease HMGB1 levels and improve the disease.

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REFERENCES

1. Kvien TK. (2004) Epidemiology and burden of illness of rheumatoid arthritis. *Pharmacoeconomics*.22:1-12.
2. Kobelt G, Lindgren P, Lindroth Y, Jacobson L, Eberhardt K. (2005) Modelling the effect of function and disease activity on costs and quality of life in rheumatoid arthritis. *Rheumatology*. 44:1169-75.
3. Kvien TK, Uhlig T, Odegard S, Heiberg MS. (2006) Epidemiological aspects of rheumatoid arthritis; the sex ratio. *Ann. N. Y. Acad. Sci.* 1069:212-22.
4. Mutru O, Laakso M, Isomaki H, Koota K. (1985) Ten year mortality and causes of death in patients with rheumatoid arthritis. *Br. Med. J.* 290:1797-9.
5. Kokkola R et al. (2003) Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum.* 48:2052-8.
6. Taniguchi N et al. (2003) High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum*. 48:971-81.
7. Pullerits R et al. (2003) High mobility group box chromosomal protein 1, a DNA binding cytokine, induces arthritis. *Arthritis Rheum*. 48:1693-700.
8. Andersson U, Tracey KJ. (2004) HMGB1 as a mediator of necrosis-induced inflammation and a therapeutic target in arthritis. *Rheum. Dis. Clin. North Am*. 30:627-37.
9. Lotze MT, Tracey KJ. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol*. 5:331-42.
10. Wang H et al. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 285:248-51.
11. Tracey KJ. (2002) The inflammatory reflex. *Nature*. 420:853-9.
12. Borovikova LV et al. (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*. 405:458-62.
13. Wang H et al. (2003) Nicotinic acetylcholine receptor α7 receptor is an essential regulator of inflammation. *Nature*. 421:384-8.
14. Bernik TR et al. (2002) Pharmacological stimulation of the cholinergic anti-inflammatory pathway. *J. Exp. Med*. 195:781-8.
15. Wang H et al. (2004) Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat. Med*. 10:1216-21.
16. Pavlov VA, Wang H, Czura CJ, Friedman SG, Tracey KJ. (2003) The cholinergic anti-inflammatory pathway: a missing link in neuromodulation. *Mol. Med*. 9:125-34.
17. Arnett FC et al. (1988) The American rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*. 31:315-24.
18. Fransen J, Stucki G, van Riel P. (2003) Rheumatoid arthritis measures. *Arthritis Rheum*. 49:S214-24.
19. (1996) Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *Circulation*. 93:1043-65.
20. Song HS, Lehrer PM. (2003) The effects of specific respiratory rates on heart rate and heart rate variability. *Appl. Psychophysiol. Biofeedback*. 28:13-23.
21. Akselrod S et al. (1981) Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. *Science*. 213:220-2.
22. Pomeranz B et al. (1985) Assessment of autonomic function in humans by heart rate spectral analysis. *Am. J. Physiol*. 248:151-3.
23. Saul JP et al. (1991) Transfer function analysis of the circulation: unique insights into cardiovascular regulation. *Am. J. Physiol*. 261:1231-45.
24. Taylor JA, Carr DL, Myers CW, Eckberg DL. (1998) Mechanisms underlying very-low-frequency RR-interval oscillations in humans. *Circulation* 98:547-55.
25. Evrengul H et al. (2004) Heart rate variability in patients with rheumatoid arthritis. *Rheumatol. Int*. 24:198-202.
26. Louthrenoo W, Ruttanaumpawan P, Aramrattana A, Sukitawut W. (1999) Cardiovascular autonomic nervous system dysfunction in patients with rheumatoid arthritis and systemic lupus erythematosus. *QJM*. 92:97-102.
27. Gonzalez-Juanatey C et al. (2003) Increased prevalence of severe subclinical atherosclerotic findings in long-term treated rheumatoid arthritis patients without clinically evident atherosclerotic disease. *Medicine*. 82:407-13.
28. Park YB et al. (2002) Atherosclerosis in rheumatoid arthritis: morphologic evidence obtained by carotid ultrasound. *Arthritis Rheum*. 46:1714-9.