Endothelial Dysfunction and Atherosclerosis in Rheumatoid Arthritis: A Multiparametric Analysis Using Imaging Techniques and Laboratory Markers of Inflammation and Autoimmunity

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ABSTRACT. ***Objective.*** Cardiovascular disease is a leading cause of mortality in rheumatoid arthritis (RA). Endothelial dysfunction often precedes manifest atherosclerosis. We assessed endothelial dysfunction and atherosclerosis in RA in context with laboratory markers.

Methods. Fifty-two patients with RA and 40 matched healthy controls were studied. We assessed common carotid intima-media thickness (ccIMT) and flow- (FMD) and nitroglycerine-mediated vasodilation (NMD). We also assayed numerous immunological and metabolic laboratory markers.

Results. FMD was significantly lower in RA (5.32% ± 4.66%) compared to controls (8.30% ± 3.96%) ($p = 0.001$). NMD was preserved in RA. ccIMT was significantly greater in patients with RA (0.63 ± 0.14 mm) versus controls (0.54 ± 0.15 mm) ($p = 0.012$). In patients with RA, ccIMT correlated with FMD% ($R = –0.318$, $p = 0.022$), age ($R = 0.831$, $p < 0.001$), and anti-dsDNA levels ($R = 0.463$, $p = 0.006$). FMD% correlated with serum interferon-γ (IFN-γ) levels ($R = 0.516$, $p = 0.014$). NMD% correlated inversely with the percentage of Th0 lymphocytes ($R = –0.636$, $p = 0.006$), serum immune complex ($R = –0.692$, $p < 0.001$), and IgM levels ($R = –0.606$, $p = 0.003$). Patients with RA were divided as "low" (< 0.65 mm) versus "high" (> 0.65 mm) ccIMT groups, and into "normal" (> 5%) versus "impaired" (< 5%) FMD% subsets. Low and high ccIMT groups differed significantly in age and serum interleukin 1 (IL-1) and anti-dsDNA levels. RA patients with normal versus impaired FMD% differed significantly in age, disease duration, and serum IFN-γ levels. Lipoprotein(a) [Lp(a)] also correlated with rheumatoid factor (RF) and C-reactive protein (CRP); homocysteine (HCy) correlated with CRP and correlated inversely with folate and vitamin B12 production. Paraoxonase-1 (PON-1) activity correlated with serum tumor necrosis factor-α (TNF-α) and IL-6 levels.

Conclusion. This was a well characterized RA population, where FMD and ccIMT were impaired, indicating early endothelial dysfunction and accelerated atherosclerosis, respectively. RA-related autoimmune-inflammatory mechanisms and metabolic factors including anti-CCP, RF, CRP, circulating immune complexes, IgM, TNF-α, IL-6, Th0/Th1 ratio, HCy, folate, vitamin B12, and PON-1 may all be involved in the development of vascular disease in RA. Although ccIMT and FMD, as well as some laboratory factors, have been assessed by other investigators in RA-associated atherosclerosis, our results regarding the possible involvement of anti-CCP, anti-dsDNA, Lp(a), some cytokines, and PON-1 activity are novel. Early determination of FMD% and ccIMT may be useful to assess RA patients with high cardiovascular risk. (First Release Jan 15 2008; J Rheumatol 2008;35:398–406)

Key Indexing Terms:
RHEUMATOID ARTHRITIS ENDOTHELIAL DYSFUNCTION ATHEROSCLEROSIS
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Rheumatoid arthritis (RA) is a chronic inflammatory disease eventually leading to joint destruction, impaired articular function, and physical disability[1]. Recently, the importance of accelerated atherosclerosis and high risk for cardiovascular disease (CVD) have been acknowledged as leading factors causing reduced life expectancy by 5–10 years in RA[1–9], as well as in other autoimmune diseases[2,3,10-12]. Both RA and atherosclerosis are associated with chronic inflammation[2,4,13], and RA is an independent risk factor for accelerated CVD[1,4,5,14,15]. Numerous recent studies have demonstrated the role of classical, Framingham, and inflammation-associated risk factors in this context[4,14-23].

Regarding traditional risk factors, cigarette smoking may exert a relationship with disease severity[2]. There is no clear evidence that hypertension, diabetes mellitus, dyslipidemia, obesity, and sedentary lifestyle are directly implicated in accelerated atherosclerosis in RA[5,24]. As RA-associated atherosclerosis cannot be solely explained by Framingham risk factors, the inflammatory mechanisms underlying RA may be crucial for early atherosclerosis and CVD development[13,14]. Inflammation-associated risk factors for CVD may include homocysteine (HCy), C-reactive protein (CRP), CD4+/CD28– T cells, some proinflammatory cytokines, chemokines, and proteases[2,4,5,25-28]. Methotrexate (MTX) treatment increases plasma levels of HCy, but concomitant folate supplementation prevented the increase of HCy production and reduced CVD mortality in RA[25].

Increased common carotid intima-media thickness (ccIMT) indicating atherosclerosis has been described in RA[2,3,9,10,19,20,23]. However, there are only a few studies assessing early endothelial dysfunction by measuring flow-mediated vasodilatation (FMD) or nitroglycerine-mediated vasodilatation (NMD) in RA[2,29,30]. FMD and NMD indicate endothelium-dependent and –independent vasodilation, respectively[31,32]. Both impaired[29] and normal[33] FMD were reported in RA.

In a recent review, Giles, *et al*[4] summarized the results of 10 key studies[14-23] assessing risk factors for CVD morbidity and mortality in RA. In most of these studies, age, sex, ethnicity, and traditional risk factors described above, as well as, among RA-related risk factors, disease duration, activity and severity, functional impairment, rheumatoid factor (RF) status, CRP, radiographic indicators, and the shared epitope status and treatment modalities were analyzed[4].

We assessed brachial FMD and NMD as early indicators of endothelial dysfunction, ccIMT, a marker of atherosclerosis, as well as laboratory markers of inflammation, autoimmunity, and accelerated atherosclerosis. Among autoimmune-inflammatory markers, serum cytokine and immunoglobulin levels, anti-cyclic citrullinated peptide (CCP), rheumatoid factor (RF), CRP, peripheral blood lymphocyte subsets, antinuclear antibodies (ANA), anti-dsDNA, complement factors, anticardiolipin (CL) and anti-ß$_2$-glycoprotein I (ß$_2$-GPI) cofactor, and anti-oxidized LDL (oxLDL) production were assessed. Among metabolic markers, a more detailed lipid profile including lipoprotein (a) [Lp(a)], apolipoproteins A (apoA) and B (apoB), serum oxLDL, vitamin B12, HCy and folate levels, paraoxonase-1 (PON-1) activity, and methylene-tetrahydrofolate reductase (MTHFR) gene polymorphism were analyzed. The role of some markers, such as anti-CCP, anti-dsDNA, Lp(a), some cytokines, and PON-1 activity were not assessed in any previous studies[4,14-23].

MATERIALS AND METHODS

Patients. Fifty-two patients with RA (40 women, 77%, and 12 men, 23%; mean age 51 ± 12 yrs, range 23–77 yrs; all Caucasian) and 40 age- and sex-matched healthy control subjects (31 women, 77.5%, and 9 men, 22.5%; mean age 50 ± 10 yrs, range 26–76 yrs; all Caucasian) were included in our study. All patients with RA satisfied the American College of Rheumatology (ACR) criteria for RA[34]. The mean disease duration of RA was 10.5 ± 8.5 years (range 2–34 yrs). We consecutively recruited 52 patients with RA from a pool of 550 patients continuously undergoing followup in our institution. Exclusion criteria included known CVD and cerebrovascular diseases, hypertension (blood pressure > 140/90 mm Hg), diabetes mellitus, cigarette smoking, obesity [body mass index (BMI) ≥ 30 kg/m^2], rheumatoid vasculitis, current infectious disease, or renal failure (serum creatinine ≥ 117 mmol/l). All patients and controls were fasting and had not used alcohol, tobacco, antioxidants, and vasoactive drugs within the past 24 h. At the time of our study, 28 patients were treated with MTX monotherapy (daily dose 10–25 mg), 10 with one of the tumor necrosis factor-α (TNF-α) blockers in combination with MTX, 7 with leflunomide (LEF) monotherapy (daily dose 20 mg), 4 with sulfasalazine (SSZ) monotherapy (daily dose 2000 mg), one with cyclosporin A monotherapy (daily dose 3 mg/kg), one with MTX-LEF, and one with MTX-SSZ combination. No patient received corticosteroids at the time of and at least 3 months prior to the study in order to exclude the atherogenic effects of these compounds. In addition, no patient with RA or control received aspirin, clopidogrel, heparin, or warfarin. The control subjects were recruited from volunteering hospital staff members and visitors in an age- and sex-matched manner. Patients with RA and controls were also normalized for Framingham traditional risk factors for atherosclerosis (Table 1). Informed consent was obtained from each patient and control subject. We obtained local ethical committee approval at the University of Debrecen.

Physical and laboratory examinations. Demographic data including sex, age, and disease duration were recorded at the time of the study. Thorough examinations including assessment of height, weight, blood pressure, and calculation of BMI were performed. After overnight fasting, blood samples were taken from the patients and controls for serum glucose, total choles-

Table 1. Traditional cardiovascular risk factors of patients with RA and controls.

Risk Factors	RA, n = 52	Controls, n = 40	p
Age, yrs	51.2 ± 12.3	50.3 ± 10.1	NS
Systolic blood pressure, mm Hg	131.2 ± 13.0	132.0 ± 12.9	NS
Diastolic blood pressure, mm Hg	82.2 ± 7.0	84.3 ± 6.9	NS
Total cholesterol, mmol/l	5.39 ± 1.19	5.45 ± 0.91	NS
LDL cholesterol, mmol/l	3.16 ± 0.93	3.30 ± 0.82	NS
HDL cholesterol, mmol/l	1.60 ± 0.61	1.65 ± 0.39	NS
Triglyceride, mmol/l	1.55 ± 0.74	1.39 ± 0.78	NS
BMI, kg/m^2	24.2 ± 3.5	24.7 ± 4.8	NS

LDL: low density lipoprotein; HDL: high density lipoprotein; BMI: body mass index; NS: not significant.

terol, low density lipoprotein-cholesterol (LDL-C), high-density lipoprotein (HDL-C), triglyceride, renal and liver function tests, and full blood count. Urinary samples were tested by Uricont-S.

Patients with RA were tested for several other laboratory measures. Among metabolic markers, serum oxLDL concentrations were determined by ELISA developed in our laboratory. Serum Lp(a) was assessed by latex-sensitized immunoturbidimetry (Roche). Plasma HCy was determined by fluorescent polarization immunoassay (FPIA; Abbott). Serum apoA and apoB levels were measured by immunoturbidimetry using Tina-Quant apoA and apoB reagents (Roche) and a Cobas Integra 700 analyzer (Roche). Plasma folate (5-MTHF) concentrations were determined by ion capture assay and serum vitamin B12 levels were measured by microparticular enzyme intrinsic factor assay (both by MEIEA technique, Abbott Diagnostics).

PON-1 activity was determined using paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma) as substrate, measuring the increase in absorbance due to the formation of 4-nitrophenol at 412 nm. One unit of PON-1 activity is defined as 1 nmol of 4-nitrophenol formed per minute under standard assay conditions.

The C677T mutation of the MTHFR gene was assessed by DNA fragmentation using a specific restriction endonuclease enzyme followed by polymerase chain reaction amplification and agarose gel electrophoresis. Patients and controls were genotyped as homozygous for the mutation (TT), heterozygous (CT), or wild-type (CC).

Regarding immunological markers, serum IgM RF and CRP were assessed by quantitative nephelometry (Cobas Mira Plus, Roche), using RF and CRP reagents, respectively (both Dialab, Vienna, Austria). RF levels > 50 IU/ml and high sensitivity CRP levels > 5 mg/l were considered elevated. Anti-CCP autoantibodies were detected in serum samples using the Immunoscan-RA CCP2 ELISA test (Euro Diagnostica, Arnhem, The Netherlands). The assay was performed according to the instructions of the manufacturer. A concentration > 25 IU/ml was considered positive. Serum total immunoglobulin A (IgA), IgG, and IgM levels were assessed by turbidimetry (Dialab). ANA were screened on HEp-2 cells by immunofluorescence. Serum levels of anti-dsDNA autoantibodies were determined by an EIA kit (BioSystems, Barcelona, Spain). Total complement hemolytic activity of the classical pathway was measured by the CH50 assay, adapted on a 96-well microplate, with results expressed in hemolytic units. Complement C3 and C4 serum levels were measured by nephelometry (Behring, Marburg, Germany). Circulating immune complexes (CIC) in the sera were assessed by polyethylene glycol (PEG) precipitation. For detection of anti-ß$_2$-GPI and anti-CL antibodies, commercial ELISA systems were used (Orgentec, Mainz, Germany). Quantification of anti-oxLDL antibodies was performed by a commercial enzyme immunoassay (Immco, Buffalo, NY, USA) according to the manufacturer's instructions. Regarding cytokines, serum TNF-α, IL-1, IL-4, IL-6, IL-10, IFN-γ, and transforming growth factor-ß (TGF-ß) levels were determined by ELISA (R & D Systems). Total lymphocyte counts and lymphocyte subsets including the percentage and absolute numbers of CD3+, CD4+, CD8+, CD19+, and CD56+ T cell subsets were also determined using antigen-specific monoclonal antibodies (all Sigma). The percentage and absolute numbers of Th0 (CD4+/IFN-γ+/IL-4+), Th1 (CD4+/IFN-γ+/IL-4–), Th2 (CD4+/IFN-γ–/IL-4+), Tc0 (CD8+/IFN-γ+/IL-4+), Tc1 (CD8+/IFN-γ+/IL-4–), and Tc2 (CD8+/IFN-γ–/IL-4+) subpopulations were also assessed. Anti-human IFN-γ-FITC and anti-human IL-4-PE antibodies were purchased from Becton-Dickinson.

Brachial FMD and NMD. Brachial FMD and NMD were assessed as described[11,32]. Briefly, ultrasound examination was performed on the right arm using a 10 MHz linear array transducer (HP Sonos 5500 ultrasound system) by a single trained sonographer after 30 min resting in a temperature-controlled room (basal value for FMD). A B-mode longitudinal section was obtained of the brachial artery above the antecubital fossa. In order to assess FMD, reactive hyperemia was induced by release of a pneumatic cuff around the forearm inflated to suprasystolic pressure for 4.5 min. After deflation the maximal flow velocity and arterial diameter was continuously recorded for 90 s. After 15 min of recovery to the baseline diameter (basal value for NMD), 400 µg sublingual nitroglycerine was administered and NMD was assessed. Flow velocities, baseline diameter, FMD, and NMD were electrocardiogram-gated and recorded offline. FMD and NMD values were expressed as percentage change from baseline (resting) value (FMD% and NMD%).

Common carotid IMT (ccIMT). The ccIMT measurements were carried out as described[11,35]. Briefly, a duplex ultrasound system (HP Sonos 5500, 10 MHz linear array transducer) was used to assess the common carotid arteries by a single observer. Longitudinal high-resolution B-mode ultrasound scans were employed over both right and left common carotid arteries and were R-synchronized and recorded. The offline measurements were performed 1 cm proximal to the carotid bulb in the far wall. The ccIMT was defined as the distance between the first and second echogenic lines from the lumen, taking the average of 10 measurements on both sides. ccIMT values were expressed in mm.

Statistical analysis. The descriptive data of normal variables are expressed as the mean ± SD. Statistical analysis was carried out by independent, 2-tailed t-test. Correlations between variables were determined using Pearson correlation analysis for normally distributed values and Spearman correlation analysis as nonparametric test. R values of these correlations were determined and corresponding p values < 0.05 were considered significant.

RESULTS

Characterization of cardiovascular risk factors. As presented in Table 1, the RA patient group and the control group were matched with regard to traditional Framingham risk factors.

Assessment of FMD%, NMD%, and ccIMT. To assess endothelial function, brachial artery FMD was measured by high resolution ultrasonography. FMD in patients with RA

expressed as percentage of the basal value (FMD%) was significantly lower (5.32% ± 4.66%) in comparison to controls (8.30% ± 3.96%) (p = 0.001; Table 2). However, no significant difference was found in NMD% between patients with RA (18.30% ± 15.17%) and controls (17.50% ± 6.96%) (p > 0.1; Table 2).

ccIMT was assessed using a duplex ultrasound system. ccIMT was significantly higher in patients (0.63 ± 0.14 mm) in comparison to controls (0.54 ± 0.15) (p = 0.012; Table 2).

Correlations between FMD%, NMD%, ccIMT, epidemiological and laboratory markers in patients. Within the RA patient population, FMD%, NMD%, and ccIMT values were correlated with each other, as well as with other epidemiological and laboratory indicators described above (Table 3). We found a significant negative correlation between ccIMT and FMD% (R = –0.318, p = 0.022; Figure 1). ccIMT also showed a significant positive correlation with age (R = 0.831, p < 0.001) and serum total cholesterol (R = 0.285, p = 0.041), and a significant inverse correlation with serum IL-1 levels (R = –0.773, p < 0.001; Table 3). Interestingly, there was a significant, positive correlation between ccIMT and anti-dsDNA levels (R = 0.463, p = 0.006), although the absolute value of anti-dsDNA was within the normal range in all patients (Table 3).

FMD% was positively correlated with serum IFN-γ levels (R = 0.516, p = 0.014) and inversely correlated with total leukocyte counts (R = –0.451, p = 0.04; Table 3).

NMD% was inversely correlated with the percentage of Th0 lymphocytes (R = –0.636, p = 0.006), serum CIC (R = –0.692, p < 0.001), and IgM levels (R = –0.606, p = 0.003) (Table 3).

No significant correlations were observed in patients with RA between FMD%, NMD%, or ccIMT values in comparison to sex, drug treatment modalities, or any other laboratory markers (data not shown).

Table 2. ccIMT, FMD%, and NMD% values of patients with RA and controls.

	RA, n = 52	Controls, n = 40	p
ccIMT, mm	0.63 ± 0.14	0.54 ± 0.15	0.012
FMD, %	5.32 ± 4.66	8.30 ± 3.96	0.001
NMD, %	18.30 ± 15.17	17.50 ± 6.96	> 0.05 (NS)

ccIMT: common carotid intima-media thickness; FMD: flow-mediated vasodilation; NMD: nitroglycerine-mediated vasodilation; NS: not significant.

Table 3. Relevant correlations between imaging and laboratory measures in patients with RA (n = 52).

Measure 1	Measure 2	R	p
Imaging data			
ccIMT	FMD%	–0.318	0.022
ccIMT	Age	0.831	< 0.001
ccIMT	Total cholesterol	0.285	0.041
ccIMT	IL-1	–0.773	< 0.001
ccIMT	Anti-dsDNA	0.463	0.006
FMD%	Total lymphocyte count	–0.451	0.040
FMD%	IFN-γ	0.516	0.014
NMD%	Th0%	–0.636	0.006
NMD%	CIC	–0.692	< 0.001
NMD%	IgM	–0.606	0.003
Epidemiological measures			
Age	Triglyceride	0.435	0.018
Age	Lp(a)	0.499	0.007
Disease duration	Total cholesterol	0.506	0.005
Disease duration	CD3+ T cell count	0.334	0.004
Disease duration	CD3+/CD69+ T cell count	0.556	0.003
Disease duration	CD19+ B cell count	–0.618	< 0.01
Laboratory markers			
RF	Lp(a)	0.574	0.001
CRP	Th1 cell count	0.412	0.04
CRP	IL-6	0.522	0.032
CRP	Lp(a)	0.517	0.005
CRP	HCy	0.565	0.002
HCy	Folate	–0.535	0.005
HCy	Vitamin B12	-0.401	0.035
Lp(a)	Th0 count	0.413	0.045
PON-1	TNF-α	0.404	0.030
PON-1	IL-6	0.690	0.002

RF: rheumatoid factor; CRP: C-reactive protein; HCy: homocysteine; Lp(a): lipoprotein (a); PON-1: paraoxonase-1; IL: interleukin; TNF: tumor necrosis factor. Other abbreviations as in Table 2.

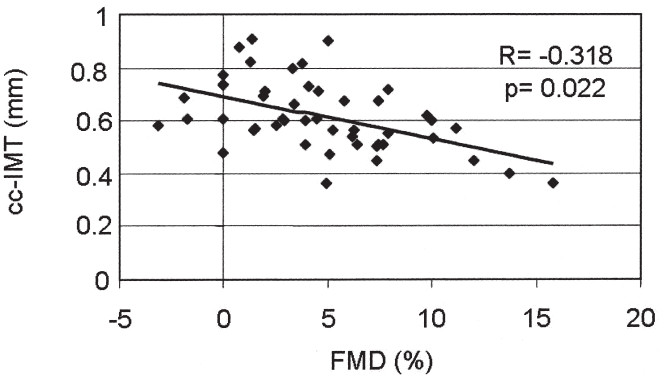

Figure 1. Inverse correlation between flow-mediated vasodilation (FMD)% and common carotid intima-media thickness (ccIMT) in patients with RA. Impaired (lower) FMD% is associated with higher ccIMT, indicating a correlation between endothelial dysfunction and atherosclerosis.

Comparisons of RA patients with low versus high ccIMT and those with impaired versus normal FMD%. As the whole RA patient population was rather heterogenous regarding FMD% and ccIMT, we divided patients with RA into low (< 0.65 mm; n = 26) and high (> 0.65 mm; n = 26) ccIMT groups, and into "normal" (> 5%; n = 27) and "impaired" (< 5%; n = 25) FMD% subsets (Table 4). Regarding ccIMT, the low and high groups differed significantly in age (43.7 ± 8.3 vs 58.2 ± 10.1 yrs; p = 0.001), serum IL-1 (26.8 ± 14.2 vs 6.2 ± 10.9 pg/ml; p = 0.02), anti-dsDNA (8.3 ± 3.9 vs 16.8 ± 12.4 IU/ml; p = 0.011), and IFN-γ levels (21.7 ± 27.3 vs 8.4 ± 11.3 pg/ml; p = 0.04) (Table 4). In addition, anti-CCP may be associated with higher ccIMT (Table 4).

Regarding FMD%, the normal and impaired groups differed significantly in age (45.4 ± 9.8 vs 56.1 ± 11.1 yrs; p = 0.001), disease duration (8.6 ± 5.5 vs 14.4 ± 12.7 yrs; p = 0.042), and serum IFN-γ levels (22.6 ± 28.6 vs 8.8 ± 11 pg/ml; p = 0.037) (Table 4). Again, higher serum anti-CCP concentrations were nonsignificantly associated with impaired compared to normal FMD% (Table 4).

DISCUSSION

CVD and cerebrovascular disease is a leading cause of morbidity and mortality in the general population, as well as in those with autoimmune diseases[1-3,10,11]. Identifying patients with higher risk for vascular disorders allows us to introduce primary prevention or effective pharmacological treatment. Assessing risk for future CVD events includes ultrasensitive CRP measurements, determination of FMD% and NMD%, carotid calcium content, or ccIMT[32,35-37]. A correlation has been reported between FMD% in the brachial artery and in coronary arteries[38]. In addition, impaired endothelium-dependent dilatation in the coronary circulation is associated with higher risk for CVD[39].

Patients with certain autoimmune diseases, such as RA, have been shown to develop accelerated atherosclerosis and need close followup and management[2,3,5,6,10]. Patients with RA have standardized mortality ratios between 0.9 and 3.0[5]. These patients have life expectancy reduced by 5–10 years, and 35%–50% of this excess mortality results from CVD[1,2]. Today CVD is the primary cause of death in RA, and coronary disease developing on the basis of accelerated atherosclerosis represents the main cause of CVD deaths in these patients[1,2,4,5,24]. As most CVD deaths occur in RA patients with high inflammatory activity[1], numerous autoimmune-inflammatory markers involved in RA-associated vascular disease should be investigated.

Table 4. Comparison of demographic and laboratory indicators in RA patients with "high" versus "low" ccIMT and in those with "Impaired" versus "Normal" FMD%.

Factor	ccIMT, mm			FMD, %		
	High (> 0.65 mm), n = 26	Low (< 0.65 mm), n = 26	p	Impaired (< 5%), n = 25	Normal (> 5%), n = 27	p
Age, yrs	58.2 ± 10.1	43.7 ± 8.3	0.001	56.1 ± 11.1	45.4 ± 9.8	0.001
Disease duration, yrs	13.3 ± 12.5	9.7 ± 6.7	NS	14.4 ± 12.7	8.6 ± 5.5	0.04
FMD, %	3.96 ± 1.12	6.67 ± 2.65	0.034	NA	NA	NA
ccIMT, mm	NA	NA	NA	0.66 ± 0.13	0.59 ± 0.15	NS
CRP, mg/l	24.0 ± 45.5	12.1 ± 17.9	NS	23.5 ± 45.9	12.6 ± 17.2	NS
RF, IU/ml	139.5 ± 151.7	111.0 ± 210.7	NS	163.9 ± 225.8	85.6 ± 111.4	NS
Anti-CCP, IU/ml	450.0 ± 532.3	318.8 ± 472.3	NS	482.3 ± 571.6	276.5 ± 396.3	NS
Anti-dsDNA, IU/ml	16.8 ± 12.4	8.3 ± 3.9	0.011	14.0 ± 9.8	10.7 ± 10.3	NS
TNF-α, pg/ml	11.6 ± 35.7	1.4 ± 4.2	NS	8.8 ± 34.4	4.0 ± 6.9	NS
IL-1, pg/ml	6.2 ± 10.9	26.8 ± 34.2	0.02	12.8 ± 24.0	20.2 ± 30.1	NS
IFN-γ, pg/ml	8.4 ± 11.3	21.7 ± 27.3	0.04	8.8 ± 11.0	22.6 ± 28.6	0.037
IL-6, pg/ml	22.8 ± 40.0	25.8 ± 60.0	NS	33.6 ± 60.7	9.4 ± 8.3	NS
IL-4, pg/ml	9.6 ± 20.5	14.6 ± 31.4	NS	11.0 ± 21.8	13.4 ± 31.5	NS
IL-10, pg/ml	30.2 ± 54.6	39.6 ± 66.5	NS	34.8 ± 53.5	34.6 ± 69.3	NS
TGF-ß, pg/ml	7.2 ± 9.0	5.5 ± 6.5	NS	8.2 ± 7.9	3.3 ± 6.1	NS

TGF: transforming growth factor. Other abbreviations as in Table 3.

Numerous studies assessing clinical and laboratory markers of atherosclerosis, as well as imaging techniques including ccIMT measurements, have been performed in RA and other autoimmune diseases[2,3,10,11,35]. Increased ccIMT has been described in RA[2,19,20,23]. A higher annual rate of increase in ccIMT has been demonstrated in RA compared to control subjects[4]. In our study, using a duplex ultrasound system[35], we also found significantly higher ccIMT in patients with RA (0.63 mm) in comparison to healthy subjects (0.54 mm) matched for sex, age, and traditional Framingham risk factors.

Manifest atherosclerosis is preceded by early endothelial damage and dysfunction that lead to vascular damage in RA[2,29,30]. However, there have been only very few reports in this context[29,30,33]. Early endothelial dysfunction may be assessed by FMD and NMD measurements on the brachial arteries[32]. Impaired FMD has been detected in young RA patients with low disease activity[29]. We measured brachial artery FMD and NMD by high resolution ultrasonography[32]. FMD, presented as percentage increase after the application of reactive hyperemia in comparison to baseline, was significantly lower in patients with RA (5.3%) than in healthy subjects (8.3%). No difference was found in NMD presented as percentage increase after the administration of nitroglycerine compared to baseline in patients with RA (18.3%) and controls (17.5%). Thus, RA may be associated with early endothelial dysfunction preceding atherosclerosis, which is indicated by impaired FMD%. However, NMD% is still preserved. Recently, we have made similar observations in systemic sclerosis (SSc), where impaired FMD% is also associated with normal NMD%[11]. In contrast, systemic lupus erythematosus (SLE) has been associated with impairment of both FMD% and NMD%[10,12]. There is a need for early indicators of endothelial dysfunction and vascular damage, better than ccIMT, in order to prevent manifest atherosclerosis in our patients.

Regarding risk factors for endothelial damage and atherosclerosis, our results and several other studies indicate that the pathogenesis of RA and atherosclerosis may overlap closely and may be influenced by numerous immunological factors. Although RA-associated atherosclerosis also involves traditional Framingham risk factors, such as cigarette smoking, hypertension, diabetes, dyslipidemia, or obesity, these do not fully account for the development of vascular damage in RA[2-5]. For example, dyslipidemia may be secondary to chronic inflammation[5]. Therefore RA-related inflammatory events play a key role in this context. In order to exclude the influence of traditional risk factors, we normalized these values, and thus there was no difference between patients with RA and controls regarding these measures (Table 1).

Little information is available regarding the role of the humoral and cellular immunological mechanisms in the development of endothelial dysfunction, assessed by FMD and NMD, and accelerated atherosclerosis, determined by ccIMT, that underlie RA. Impaired FMD in young patients with RA was associated with LDL-C and CRP production[29]. Recently, Giles, *et al*[4] published a metaanalysis of 10 recent studies assessing atherosclerosis and CVD in RA[14-23]. In these studies, mostly epidemiological factors including age, sex and ethnicity, traditional risk factors for CVD, and some disease-associated variables, such as disease duration, activity and severity, as well as RF, CRP, HLA-DR, radiographic progression and means of therapy were assessed in context with RA-associated atherosclerosis. Therefore we went further in investigating immunological and metabolic factors not analyzed before that may be involved in the pathogenesis of vascular damage in RA. Markers such as anti-CCP, HCy, ANA, anti-dsDNA, C3, C4, CH50, serum IgG, IgA, IgM, anti-CL, anti-$ß_2$-GPI, anti-oxLDL, circulating cytokines including TNF-α, IL-1, IL-4, IL-6, IL-10, IFN-γ, and TGF-ß, as well as total lymphocyte counts, lymphocytes, and particularly T cell subpopulations, were assessed in patients with RA. In addition, a detailed metabolic profile including Lp(a), apoA and apoB, oxLDL, vitamin B12, folate levels, PON-1 activity, and MTHFR gene polymorphism were also assessed in these patients. All these markers were correlated with ccIMT, FMD%, and NMD%, as well as with each other within the RA population. In addition, RA patients with high versus low ccIMT and those with impaired versus normal FMD% were compared with each other in context with numerous laboratory markers.

Regarding imaging data, high ccIMT was associated with impaired FMD%. Early endothelial dysfunction indicated by FMD% may precede manifest atherosclerosis indicated by ccIMT. Thus, more pronounced endothelial dysfunction may lead to more accelerated atherosclerosis in RA[2-4]. High ccIMT has also been correlated with age and total cholesterol. Impaired FMD% was also found in higher age. Both age and cholesterol levels are independent factors of atherogenesis[2,5,6]. Other investigators also detected endothelial dysfunction in young patients[29]. In contrast, one study showed normal FMD in adult patients with RA[33]. Disease duration was significantly associated with impaired versus normal FMD%. In addition, there was a tendency for higher ccIMT to be found in RA patients with longer disease duration. Other authors also confirmed the role of age and disease duration in RA-associated atherosclerosis[4,14-23]. Thus, endothelial dysfunction and accelerated atherosclerosis in RA may not only be associated with age, but also with the longer duration of the disease.

Anti-CCP plays a crucial role in the pathogenesis and progression of RA[2,4]. We have not found any previous reports on the possible associations between anti-CCP production and atherosclerosis in RA. In our study, anti-CCP production showed a nonsignificant association with more pronounced atherosclerosis and endothelial dysfunction, as indicated by high ccIMT and impaired FMD%, respectively.

Apart from RA-associated autoantibodies, such as anti-CCP, we found a significant positive correlation between ccIMT and anti-dsDNA. Moreover, when comparing patients with high and low ccIMT, significantly higher anti-dsDNA serum concentrations were found in the high ccIMT group. Anti-dsDNA antibodies can be detected in patients with RA, but the serum anti-dsDNA levels in patients with RA remain within the normal range. However, patients with SLE also experience accelerated atherosclerosis[10]. Further, anti-dsDNA may promote accumulation of cholesterol in macrophages and smooth muscle cells, trigger cytotoxicity, and promote cytokine production[40]. There have been no reports on the possible role of these antibodies in RA-associated atherosclerosis.

Among other immunological factors, immunoglobulins and CIC have also been implicated in RA- and lupus-associated atherosclerosis[4,10]. However, in our study, serum IgM and CIC levels were correlated with impaired NMD. Thus, IgM-containing CIC may also be implicated in endothelial dysfunction. It may also be relevant that IgM and CIC do not seem to be related to FMD%, only to NMD%. Although NMD% seems to be preserved in patients with RA, as well as in those with SSc, our results suggest that immunoglobulins and CIC may be implicated in the mechanism of NMD.

Regarding cytokines, interestingly, we found a strong inverse correlation between ccIMT and IL-1 levels. IL-1 has been implicated in the pathogenesis of atherosclerosis[26]. We have not found any data regarding the exact role of IL-1 in RA-associated atherosclerosis. However, in a study by Dessein, *et al*, IL-1 production was inversely correlated with the production of soluble endothelial adhesion molecules. This study suggests that IL-1 may act differently on endothelial cells compared to TNF-α or IL-6[41] under certain circumstances.

In addition, impaired (low) FMD% has been correlated with low IFN-γ production. IFN-γ, a major regulator of the Th1 response, has also been implicated in the pathogenesis of RA, as well as in that of atherosclerosis[42,43]. However, Kawashima and Miossec[44] suggest that there may be a defective Th1 immune response in RA. When cytokine mRNA expressions in peripheral blood cells were analyzed in relation to serum CRP levels, both IFN-γ and IL-4 mRNA were significantly higher in blood samples with low CRP levels. This study suggests that CRP production and IFN-γ mRNA expression may be inversely regulated in RA[44], which may, in part, explain the inverse relationship between RA-associated vascular disease and IFN-γ production. Yet the exact role of IL-1 and IFN-γ in RA-associated atherogenesis needs to be further elucidated.

It is widely recognized that atherosclerosis, similarly to RA, is associated with a Th0-Th1 response[3,4,13,42,43]. CD4+ T cells are involved in endothelial injury underlying atherosclerosis[2,4,13]. Although the role of T cells in RA was not a major endpoint of our study, our results showing a significant positive correlation of CD3+ T cell counts, activated CD69+ T cell counts, and a significant negative correlation of B cell counts with disease duration suggest the increasing importance of T cells during the progression of RA. Further, there is a correlation between impaired NMD% and the percentage of Th0 cells. In our study, CRP and Lp(a) production were also significantly correlated with Th1 and Th0 cell counts, respectively. Thus, during the development and progression of RA, T cells, predominantly the Th0 and Th1 subsets, may be involved in atherogenesis, which is associated with the production of inflammatory-metabolic factors such as CRP and Lp(a). Both CRP and Lp(a) are independent risk factors for atherosclerosis associated with autoimmune diseases[2,4,10]. In our study, production of CRP and Lp(a) was correlated with each other.

PON-1 has a protective role in vascular diseases by affecting lipid oxidation[45]. Decreased PON-1 activity has been detected in autoimmune diseases such as RA and SLE[46,47]. In our study, PON-1 activity exerted a positive correlation with TNF-α and IL-6 levels. The regulation of PON-1 activity is somewhat controversial. In one study both TNF-α and IL-6 repressed PON expression[48], while others reported that IL-6 may upregulate PON gene expression[49]. Thus, the regulation of PON-1 activity by proinflammatory cytokines in RA needs further confirmation.

There was manifest atherosclerosis indicated by increased ccIMT in our patients with RA compared to matched healthy controls. A significant impairment of FMD in patients with RA compared to controls indicated abnormal endothelium-dependent vasodilatation. At the same time, NMD remained intact. RA-associated atherosclerosis and endothelial dysfunction cannot be solely explained by traditional Framingham risk factors. Apart from age, the duration of RA and other factors related to the pathogenesis of RA including Th0 and Th1 cells and increased production of IgM, CIC, anti-CCP, and possibly anti-dsDNA, Lp(a), and HCy may all be implicated in this process. The suppressed production of IL-4, IL-10, folate, and vitamin B12 may also perpetuate atherosclerosis. The somewhat contradictory roles of IL-1 and IFN-γ in RA-associated endothelial dysfunction or atherosclerosis need further investigation. Although ccIMT and FMD as well as some laboratory factors have previously been assessed by other investigators in RA-associated atherosclerosis, our results regarding the possible involvement of anti-CCP, anti-dsDNA, Lp(a), some cytokines, and PON-1 activity are novel.

Regarding clinical relevance, early endothelial dysfunction precedes manifest atherosclerosis, giving us the opportunity of early detection by FMD. The suppression of metabolic and inflammatory factors involved in atherogenesis may help us to prevent vascular damage. Folate and vitamin B12 supplementation acts by the suppression of HCy production[25]. Statins may not only improve the lipid profile, but may reduce arterial stiffness and may also be mildly anti-

inflammatory[4]. The control of endothelial dysfunction and the subsequent development of atherosclerosis may help us to suppress CVD, a major cause of death in RA.

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