Antinuclear antibodies in the general population: positive association with inflammatory and vascular biomarkers but not traditional cardiovascular risk factors

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Abstract

Objective

Patients with clinically evident autoimmune disease are at increased risk for premature cardiovascular disease (CVD). Markers of serological autoimmunity such as anti-nuclear antibodies (ANA) are found in approximately 25% of the general population. Yet, the vast majority will not develop clinical autoimmune disease. Serological autoimmunity is a risk factor for CVD death in individuals without autoimmune disease; however, the mechanisms mediating this excess CVD risk have not been elucidated.

Methods

We examined associations of ANA with traditional cardiovascular risk factors, inflammatory mediators, and vascular biomarkers in the Dallas Heart Study – a large, representative multiethnic population-based cohort. Plasma ANA were measured by enzyme linked immunosorbent assay in 3,488 Dallas Heart Study participants aged 30 to 65 years who do not have known rheumatologic disease. Associations of ANA with demographic characteristics, cardiovascular risk factors, and biomarkers were assessed using univariable and multivariable linear regression.

Results

Factors independently associated with higher ANA include female sex, African-American race/ethnicity, soluble intracellular adhesion molecule-1, soluble CD40 ligand, chemokine CXCL-2, and Cystatin C ($p<0.05$ for each). ANA was not associated with traditional cardiovascular risk factors, high sensitivity C-reactive protein, coronary artery calcium scores, or aortic wall thickness.

Conclusion

ANA are associated with inflammatory mediators and biomarkers of vascular activation, but not with traditional cardiovascular risk factors in a multiethnic population-based cohort. These findings suggest that the cardiovascular risk associated with ANA may involve pathways distinct from traditional risk factors and include dysregulation of endothelial cells and the immune system.

Key words

anti-nuclear antibodies, cardiovascular disease, autoimmunity, Dallas Heart Study

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Reprints will not be available from the author.

Received on October 25, 2017; accepted in revised form on March 5, 2018.

UT Southwestern IRB numbers:
STU 072010-186 and STU 072010-162

Funding: The Dallas Heart Study was funded by the Donald W. Reynolds Foundation and was partially supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under award no. UL1TR001105.
E.B. Solow received research support from the American Heart Association, National Center for Advancing Translational Sciences of the NIH, and Bristol-Myers Squibb;
J.A. de Lemos has received grant support and consulting income from Roche Diagnostics and Abbott Diagnostics, and consulting income from Roche Diagnostics, Abbott Diagnostics, Siemen's Health Care Diagnostics, and Ortho Clinical Diagnostics.
D.R. Karp received support from National Institutes of Health P50 AR055503;
W. Vongpatanasin receives support from UT Southwestern O'Brien Kidney Center P30 DK079328 and R01HL113738;
B. Skaug and C. Ayers have no relevant financial relationships to disclose.

Introduction

Individuals with systemic autoimmune disease are at greater risk for developing cardiovascular disease (CVD) than expected based on traditional CVD risk factors (1, 2). These individuals most commonly experience thomboembolic events such as myocardial infarction and stroke (1). Those with higher titres of autoantibodies may be at particularly increased risk (3). Moreover, CVD events may precede the clinical presentation of autoimmunity (4, 5). The association of clinical autoimmune disease with CVD is presumed to reflect the action of inflammatory mediators on the endothelium and the cellular component of atherosclerotic plaque (6-8). It is well established that there is a continuum of autoimmunity that begins with the appearance of asymptomatic autoantibodies and subsequent up-regulation of inflammatory chemokines and cytokines before the onset of clinically evident autoimmune disease (9). Antinuclear autoantibodies (ANA), which reflect the presence of autoantibodies to self-antigens (10), are common and have been reported in up to 25% of the general population free from clinical autoimmune disease (11). Only a small proportion of individuals who are ANA positive will eventually progress to clinical autoimmune disease, thus pretest probability for autoimmune disease matters when requesting ANA (12). For example, systemic lupus erythematosus (SLE) has prevalence estimates ranging from 0.1% to 0.15% (13). Previously thought of as "benign autoimmunity", ANA may serve as a marker of cardiovascular risk in the general population. We have reported that ANA are associated with all-cause mortality, cardiovascular death, and atherosclerotic vascular disease events in participants from the Dallas Heart Study (DHS), a representative multiethnic population-based cohort of young and middle-aged individuals (14). Moreover, the associations persisted after exclusion of participants with high ANA, suggesting that even minimally detectable levels of ANA present in the population portend a heightened risk of death. To investigate potential mechanistic underpinnings of the associations between ANA and CVD events, we performed comprehensive analyses within the DHS cohort for associations of ANA with traditional CVD risk factors, biomarkers of inflammation and endothelial cell activity, and imaging markers of atherosclerotic vascular disease.

Materials and methods

The Dallas Heart Study (DHS), a multiethnic population-based probability sample of Dallas County residents ages 30–65 years, was established in 2000, and previously described in detail (11, 15). All participants in the DHS provided written informed consent, and the UT Southwestern Medical Center Institutional Review Board approved the study (IRB STU 072010-186 and STU 072010-162). The DHS data collection included an in-home visit (n=6,101) to collect medical history, blood pressure and anthropometric measurements, followed by a second in-home visit (n=3,557) to collect fasting blood and urine specimens, and then a final visit to UT Southwestern Medical Center (n=2,971) where detailed cardiovascular phenotyping by electron-beam computed tomography (EBCT), and cardiac and aortic magnetic resonance imaging (MRI) were performed. ANA were measured from plasma banked at the second visit in 3,488 participants. We excluded 57 participants who both self-reported a history of an autoimmune disease (rheumatoid arthritis, SLE, or inflammatory bowel disease) and were taking an immunosuppressive medication (11).

Blood samples collected at the second visit were collected into EDTA tubes, refrigerated at 4°C for 4 hours or less, centrifuged, and the plasma removed and stored at -70°C. ANA were measured from these stored plasma samples using Quanta Lite™ enzyme linked immunosorbent assay (ELISA) (Inova Diagnositics, San Diego, CA) and are reported as ELISA units (EU), as previously described (11). ANA of greater than 1 EU are considered detectable and greater than 20 EU are considered "positive" for potential rheumatic disease based on a comparator healthy control subset described by the Inova ELISA. The biomarkers soluble vascular cell

adhesion molecule (sVCAM-1), intracellular adhesion molecule (sICAM-1), soluble endothelial cell-selective adhesion molecule (sESAM), matrix metalloproteinase-9 (MMP-9), soluble CD40 ligand (sCD40L), chemokine (C-X-C motif) ligand-1 (CXCL-1), CXCL-2, CXCL-10, D-dimer, interleukin-18 (IL-18), monocyte chemoattractant protein-1 (MCP-1), Cystatin C, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), and high sensitivity C-reactive protein (hsCRP) were measured from these samples as previously reported (16-23).

Race/Ethnicity was self-reported. Comorbidity definitions for smoking, hypertension, diabetes, hypercholesterolaemia, and metabolic syndrome may be found in the supplementary material. Glomerular filtration rate (eGFR) was estimated using the Modification of Diet in Renal Disease equation. Detailed methods for MRI and EBCT in the DHS have been previously reported (16). Briefly, EBCT was performed in duplicate on a single scanner (Imatron Inc, San Bruno, CA) to assess coronary artery calcium, with results averaged and reported as Agatston units. Cardiac and aortic MRI was performed using a 1.5-Tesla system (Intera, Philips Medical Systems, Best, the Netherlands).

Continuous variables are reported as median and interquartile range and categorical variables as proportions. Comparisons of ANA between dichotomous groups were performed using the Wilcoxon Rank-Sum test due to the right skewing of ANA. Demographic variables, biomarkers, and cardiovascular risk factors were compared using Spearman partial correlation coefficients, with adjustment for race and sex. Backwards stepwise multivariable linear regression was used to identify variables independently associated with ANA. Candidate variables with a $p<0.1$ from Tables I and II were tested in the regression model, with the exception of sVCAM-1, which was excluded due to multicollinearity with sICAM-1. Variables retained in the final model were required to have a p-value <0.05. All p-values are 2-sided and $p<0.05$ was considered statistically significant. Gender and race/ethnicity interactions with biomarkers were tested by including multiplicative interaction terms with individual biomarkers that were associated significantly with ANA. Statistical analyses were performed using SAS v. 9.2 (SAS Institute, Cary, NC).

Table I. Clinical characteristics and cardiovascular risk factors of DHS participants at enrolment and distribution of ANA (n=3488).

	DHS Cohort (%)	ANA Median (IQR)	p-value
Demographics			
Overall population		12.8 (8.5, 20.7)	
Age			
30-40y	33	12.9 (8.6, 21.2)	
40-50y	32	12.8 (8.5, 19.8)	
50-60y	24	12.8 (8.8, 20.9)	
>60y	7	13.8 (8.8, 23.3)	0.2
Gender			
Male	44.3	11.9 (8.27, 19.2)	
Female	55.7	13.6 (9.0, 22.2)	<0.0001
Ethnicity/Race			
Black	51	13.9 (9.4, 22.4)	
White	29	11.7 (7.7, 18.9)	
Hispanic	17	12.2 (8.2, 19.7)	
Other	2	11.1 (8.2, 17.2)	<0.0001
Smoking			
Current	29	11.8 (7.9, 18.3)	
Former/Never	71	13.2 (8.8, 21.8)	<0.0001
Cardiovascular risk factors			
Hypertension			
Yes	34	13.1 (9.0, 21.7)	
No	66	12.7 (8.3, 20.3)	0.02
Diabetes			
Yes	12	13.0 (8.7, 21.4)	
No	88	12.8 (8.5, 20.6)	0.9
Hypercholesterolaemia			
Yes	13	12.5 (8.2, 19.7)	
No	87	12.9 (8.6, 20.9)	0.1
Metabolic syndrome			
Yes	34	12.8 (8.5, 20.7)	
No	66	12.9 (8.5, 20.7)	0.9

Values are presented as % or median and interquartile range (IQR). ANA: antinuclear antibody measured by ELISA. p-value for trend <0.05 significant when comparing ANA.

Results

ANA were measured in 3,488 participants. Those with higher ANA were more likely to be female and African-American, have hypertension, and be nonsmokers, shown in Table I. As previously reported, we did not find differences in ANA based on other cardiovascular risk factors including age, diabetes, hypercholesterolaemia and metabolic syndrome (14).

We examined the association of ANA with baseline variables and biomarkers of endothelial activation and inflammation by Spearman correlation, following sex and race adjustments. ANA were associated with soluble cell surface adhesion molecules sVCAM-1 ($\varrho=0.12$, $p=0.04$) and sICAM-1 ($\varrho=0.16$, $p=0.004$). ANA were positively associated with the inflammatory chemokine CXCL-2 ($\varrho=0.16$, $p=0.005$), Cystatin C ($\varrho=0.16$, $p=0.005$), and soluble CD40 ligand ($\varrho=0.13$, $p=0.02$). ANA were inversely associated with HOMA-IR ($\varrho=-0.12$, $p=0.02$) and total cholesterol ($\varrho=-0.15$, $p=0.009$). ANA did not significantly associate with hsCRP, body mass index, eGFR, D-dimer, coronary artery calcium score, or aortic wall thickness (Table II).

In multivariable linear regression analyses, female sex, African-American race/ethnicity, and biomarkers reflecting inflammation and endothelial dysfunction such as CXCL-2, Cystatin C, sCD40 ligand, and sICAM-1 were positively and independently associated with ANA. In contrast, smoking, HOMA-IR, and hypercholesterolaemia were inversely associated with ANA

Table II. Spearman correlations of ANA with continuous baseline variables, biomarkers and vascular imaging studies.

	Rho	*p*-value
Age, years	-0.01	0.9
Body Mass Index, kg/m^2	-0.08	0.2
Total Cholesterol, mg/dl	-0.15	0.009
Low density lipoprotein, mg/dl	-0.11	0.06
High density lipoprotein, mg/dl	-0.01	0.9
Triglycerides, mg/dl	-0.10	0.08
eGFR, mL/min/1.73 m^2	-0.02	0.8
HOMA-IR	-0.14	0.02
sVCAM-1, ng/mL	0.12	0.04
sICAM-1, ng/mL	0.16	0.004
Soluble CD40 ligand, ng/mL	0.13	0.02
CXCL-2, ng/mL	0.16	0.005
Cystatin C, mg/L	0.16	0.005
MMP-9, ug/l	-0.13	0.02
hsCRP, mg/L	-0.03	0.6
sESAM, ng/ml	0.02	0.7
MMP-9, ug/l	-0.13	0.02
CXCL-1, ng/ml	0.03	0.6
CXCL-10, ng/ml	0.03	0.6
D-dimer, ug/ml	0.03	0.6
IL-18, pg/ml	0.07	0.2
MCP-1, pg/ml	-0.002	0.9
Aortic wall thickness, mm	-0.02	0.7
Coronary artery calcium, %	0.01	0.6

Values are Spearman partial correlations after sex and race adjustments. eGFR: glomerular filtration rate by MDRD equation, HOMA-IR: Homeostasis Model Assessment of Insulin Resistance Index, sVCAM-1: Soluble vascular cell adhesion molecule-1, sICAM-1: Soluble intracellular adhesion molecule-1, CXCL: chemokine (C-X-C motif) ligand, hsCRP: high sensitivity C-reactive protein, sESAM: soluble endothelial cell-selective adhesion molecule, MMP: matrix metalloproteinase, IL: interleukin, MCP-1: monocyte chemoattractant protein-1; Aortic wall thickness and coronary artery calcium scores are measured by MRI and CT respectively. ANA: antinuclear antibody measured by ELISA.

Table III. Multivariable associations of participant characteristics and biomarkers with ANA.

Outcome variable	$\beta_{\log(ANA)*}$	*p*-value
Female	0.19 ± 0.04	<0.0001
African-American race	0.18 ± 0.04	<0.0001
Smoking	-0.13 ± 0.04	0.002
Hypercholesterolaemia	-0.15 ± 0.05	0.006
HOMA-IR	-0.01 ± 0.004	0.0005
sICAM-1, ng/mL	0.10 ± 0.03	0.005
CXCL-2, ng/mL	0.45 ± 0.09	<0.0001
Cystatin C, mg/L	0.37 ± 0.06	<0.0001
sCD40 Ligand, ng/mL	0.05 ± 0.02	0.03

Multivariable linear regression using log(ANA). The β coefficient of logANA ± standard error are reported for each outcome variable. HOMA-IR: Homeostasis Model Assessment of Insulin Resistance Index, sICAM-1: soluble intercellular adhesion molecule 1, CXCL: chemokine (C-X-C motif) ligand. ANA: antinuclear antibody measured by ELISA.

(*p*<0.01 for each, Table III). No significant sex by biomarker or race/ethnicity by biomarker interactions were seen (data not shown).

Discussion

The major finding of this study is that ANA are associated with elevated levels of endothelial adhesion markers and biomarkers of immune up-regulation in the general population. Although the magnitude of the associations observed was modest, they may provide a mechanistic clue regarding the association of ANA elevation with cardiovascular outcomes.

Autoimmune diseases are associated with a substantially increased risk of premature cardiovascular morbidity and mortality (1, 2). This risk appears to be mediated only in part by traditional risk factors (24). Activation of the innate and adaptive immune system likely contributes to cardiac risk, though the exact role of these processes is not yet known (3, 6). SLE is rare, with prevalence estimates ranging from 0.1% to 0.15% (13). On the contrary, low-level autoimmunity, as measured by ANA, is common and is an independent risk factor for cardiovascular complications irrespective of clinical autoimmune disease (14, 25-27).

We did not observe associations between ANA and measures of subclinical atherosclerosis, including coronary artery calcium and aortic wall thickness. It is noteworthy that ANA were either inversely or not associated with several traditional CVD risk factors. These findings suggest that ANA-associated cardiovascular death may result from processes distinct from progressive coronary artery disease that is caused by traditional risk factors. A study using cardiac magnetic resonance noted disparate patterns of gadolinium uptake into the coronary vessel wall in individuals with SLE compared with those with known coronary artery disease. Vessels in patients with SLE demonstrated diffuse gadolinium uptake, while those with coronary artery disease displayed a patchy, irregular pattern. However, both groups had significantly higher gadolinium uptake than control subjects (28). This finding suggests that individuals with systemic autoimmune disease may develop vascular injury by different mechanisms than traditional risk factors. Those with antiphospholipid antibody syndrome (APS), characterised by autoantibodies and thrombosis, experience an increased risk of premature cardiovascular death (8, 29). In APS models, autoantibodies induce endothelial cell vascular activation and leukocyte adhesion with thrombus formation via dysregulation in the nitric oxide pathway (8). It is also possible that low level autoimmunity is associated with a higher propensity for an index cardiovascular event being fatal. Rheumatoid arthritis patients are notably at risk for silent myocardial infarction and sudden death early in the course of their

disease (4). Given that the DHS is an observational cohort, we cannot assess whether the associations of ANA with biomarkers of inflammation and immune activation reflect causal pathways leading to future cardiovascular events. We hypothesise that the presence of ANA heralds changes in the immune system that may directly or indirectly affect vascular integrity. By what means low-level immune autoreactivity in the general population may contribute to mortality will require further study.

In our study, ANA were correlated with biomarkers of endothelial activation (sICAM-1, sVCAM-1) and chemotaxis (CXCL-2), and B-cell regulation (CD40L), but not with broad spectrum inflammatory markers such as hsCRP. This may be due to the fact that IL-6, the main regulator of CRP expression, is not increased in ANA positive individuals, unlike other inflammatory cytokines (30). Cell adhesion molecules (CAM) are augmented in areas of plaque formation, and serum levels are elevated during a cardiac event, though their ability to predict cardiovascular death is mixed (31, 32). The association of ANA with sICAM-1 and sVCAM-1 is intriguing given that *in vitro* studies have demonstrated that autoantibodies isolated from SLE patients directly induce endothelial cell activation, as measured by the expression of ICAM-1 and VCAM-1 (33). ICAM and VCAM have been associated with subclinical atherosclerosis in SLE independent of the Framingham risk score (34).

CD40L is an important regulator of B cell activity and antibody production (35). CD40/CD40L interactions are also present in developing atherosclerotic lesions, and elevated levels of sCD40L are associated with an increased risk of recurrent ischemic events in patients with acute coronary syndromes (36-38). Platelets expressing CD40L bind to endothelial cells inducing cell adhesion molecule expression (39), and when CD40L binds to its receptor on macrophages, tissue factor is produced (40). These factors play an important role in mediating inflammation and thrombosis in the vasculature. Evidence of this was apparent in a clinical trial in patients with SLE; blocking CD40L successfully reduced autoantibody production, however, the trial was terminated early due to excess thromboembolic events (41). Subsequent work suggests CD40/CD40L interactions are complex and re-engineering of the drug preparation as well as the drug target may eliminate thrombosis risk (42, 43).

ANA were positively associated with CXCL-2 and Cystatin C. The chemokine CXCL-2, also known as macrophage inflammatory protein-2, is secreted by activated monocytes in response to ischaemia. CXCL-2 promotes endothelial cell migration and leukocyte chemotaxis that may enhance vascular injury (44-46). Cystatin C, a cysteine protease inhibitor that is ubiquitously expressed, is an important biomarker of renal function. Cystatin C has been shown to be predictive of CVD and concentric left ventricular hypertrophy in the general population (23). Furthermore, Cystatin C was associated with venous thromboembolism in patients with normal renal function (47). We found no associations of ANA with MCP-1, CXCL-1, and CXCL-10, which is similar (except for CXCL-10) to the findings of Slight-Webb *et al.* (30). This could be due to our definitions of ANA positive individuals, with their group using an assay capturing more specific nuclear antigens.

ANA were inversely associated with smoking, hypercholesterolaemia, and HOMA-IR. HOMA-IR levels serve as a risk factor for developing metabolic syndrome, and patients with SLE are at increased risk for developing metabolic syndrome driven in part by corticosteroid use, disease activity including lupus nephritis, and ethnicity (48-50). Slight-Webb *et al.* found those with SLE and as well as those with only ANA positivity had lower levels of the metabolic syndrome markers leptin and resistin (30). We did not find studies showing ANA titres were associated with metabolic syndrome in SLE. Smoking is strong predictor for damage in SLE, however, smoking may also impact antibody production in SLE and first degree relatives though mechanisms are not clear (51-54). Given the cross-sectional nature of these variables, longitudinal studies will be needed to further elucidate their impact on antibody production.

Several limitations of the study are noteworthy. We cannot account for variability in ANA levels over time as we measured ANA at the baseline time point only. The use of ELISA to measure ANA is not as common in clinical practice as HEp2-cell indirect immunofluorescence, however ELISA for ANA measurement has been validated and may actually under-report positive ANA compared to indirect immunofluorescence in some cases (55). In a previous study from the DHS, ANA ELISA units were highly correlated with indirect immunofluorescence titres (ϱ=0.8, p=0.02). ANA titres by indirect immunofluorescence of 1:20 and 1:40 corresponded to ELISA levels of 10 and 20 EU, respectively (11). Exclusion of participants with autoimmune disease was based on self-report and a review of their prescribed medications, rather than formal evaluation by a rheumatologist.

In aggregate, our findings suggest that a low level of immune autoreactivity, as measured by ANA, may be associated with endothelial cell activity and immune up-regulation in the absence of clinically evident autoimmune disease or traditional CVD risk factors in an ethnically diverse cohort. The associations described here may indicate a propensity for thrombosis mediated by dysregulated endothelial cell/platelet interactions, which could serve as a potential mechanism for further study in understanding the association of ANA with cardiovascular events and mortality. Characterisation of the interactions amongst ANA, inflammatory and immune mediators, and the vascular endothelium could yield novel insights into CVD pathogenesis.

ANA associates with markers of inflammation and vascular endothelial activity in a multiethnic, population-based cohort. In contrast, ANA does not associate with traditional cardiovascular risk factors or imaging markers of atherosclerosis in this cohort. These findings suggest a unique mechanistic underpinning for the association of ANA with CVD.

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