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Soluble Mediators and Clinical Features Discern Risk of Transitioning to Classified Disease in Relatives of Systemic Lupus Erythematosus Patients

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Abstract

Objective—Systemic lupus erythematosus (SLE) and other autoimmune diseases cause significant morbidity. Identifying populations at risk of developing SLE is essential to curtail irreversible inflammatory damage. The objective of this study was to identify factors associated with transition to classified disease that inform SLE risk.

Methods—Previously identified lupus patient blood relatives with < 4 American College of Rheumatology SLE classification criteria at baseline (n=409) were enrolled in this follow-up study. Participants provided detailed family, demographic, and clinical information, including the SLE-specific portion of the Connective Tissue Disease Screening Questionnaire (SLE-CSQ). Plasma samples were tested for the presence of lupus-associated autoantibodies and 52 soluble mediators. Generalized estimating equations (GEE) were applied to identify factors anticipating disease transition.

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Results—Forty-five relatives (11%) transitioned to classified SLE during follow-up (mean time=6.4 years). Relatives who transitioned displayed more lupus-associated autoantibody specificities and higher SLE-CSQ scores ($p<0.0001$) at baseline than non-transitioned relatives. Importantly, they also had elevated baseline plasma levels of inflammatory mediators, including B-lymphocyte stimulator (BLyS), stem cell factor (SCF), and interferon-associated chemokines ($p\leq0.02$), with concurrent decreases in levels of regulatory mediators, tumor growth factor (TGF)-β and interleukin (IL)-10 ($p\leq0.03$). GEE revealed that baseline SLE-CSQ or ACR scores and plasma levels of SCF and TGF-β ($p\leq0.03$), but not autoantibodies, were significant and independent predictors of SLE transition.

Conclusions—Altered levels of soluble mediators anticipate transition to classified disease in lupus relatives. Thus, immune perturbations precede SLE classification and can help identify high-risk relatives for rheumatology referral and potential enrollment in prevention trials.

Keywords

SLE; relatives; risk factors; cytokines; chemokines; BLyS

Autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus (SLE), are increasingly prevalent (1, 2), with irreversible morbidity and early mortality due to immune dysfunction, chronic inflammation, and end-organ damage (3). Ongoing studies revealing the benefits of early intervention for patients at high risk of type 1 diabetes (2) and rheumatoid arthritis (4) suggest that early intervention could also be particularly beneficial in SLE, where irreversible organ damage is often present by the time patients are diagnosed and treated (5). Identifying preclinical factors that herald disease transition is essential. Although healthy relatives of SLE patients have an increased risk of developing SLE compared to the general population (6), the vast majority will never transition to classified disease (7, 8).

Accumulation of lupus-associated autoantibodies prefaces SLE classification (9); however, autoantibody specificities alone appear to be insufficient to identify relatives at highest risk of developing lupus. Indeed, as many as 37% of unaffected relatives (10-12) and up to 14% of unrelated individuals (13) are antinuclear antibody (ANA) positive, yet remain healthy, suggesting that other forms of immune dysregulation coincide with autoantibody production to precipitate SLE transition. Interferon (IFN) pathways are associated with autoantibodies against DNA/RNA-binding proteins (6) and SLE (14). Indeed, IFN-induced, downstream mediators are also increased in the periphery of SLE patients, including chemokines MCP-1, MCP-3, and MIP-1β (15), as well as B-lymphocyte stimulator (BLyS), a tumor necrosis factor receptor (TNFR) superfamily member (16). BLyS contributes to altered B-lymphocyte activation and autoantibody production (15) and is a current therapeutic target in SLE (16). Stem cell factor (SCF), associated with hematopoiesis, T-cell differentiation, and chemokine release (17, 18), is also elevated in SLE patients before clinical flare (15). Other immunoregulatory mechanisms, including levels of circulating IL-10 and TGF-β, may also be altered in SLE (15).

Although extensive work has been performed in SLE patients with established disease, little is known about inflammatory pathway dysregulation in the pre-classification time period,

where the absence of confounding immunomodulatory therapy and organ damage would help identify targets for pathway-directed therapy. We therefore assembled a unique cohort of previously unaffected blood relatives of SLE patients to investigate demographic, familial, clinical, and biological factors that distinguish relatives who transitioned to classified SLE in this follow-up cohort versus relatives who did not transition.

PATIENTS AND METHODS

Study Population/Plasma Samples

Experiments were performed in accordance with the Helsinki Declaration and approved by the Oklahoma Medical Research Foundation (OMRF) and Medical University of South Carolina (MUSC) Institutional Review Boards. All participants provided written informed consent prior to study enrollment. Unaffected blood relatives (meeting <4 cumulative ACR criteria for SLE) (19), previously enrolled in the Lupus Family Registry and Repository (LFRR) (20) or Systemic Lupus Erythematosus in Gullah Health (SLEIGH) (21) cohort (between 1992 and 2011), were recruited to participate in a follow-up study (between March 2010 and May 2012) to identify individuals who transitioned to classified SLE (meeting ≥4 cumulative ACR criteria via medical record review) (19, 22). Upon enrollment in the parent cohort (baseline) and in this study (follow-up), participants provided serum and plasma samples, along with demographic and clinical information. Samples were stored at −20°C and assays performed on freshly thawed samples. Participants completed the SLE-specific portion of the connective tissue disease screening questionnaire (SLE-CSQ) at baseline and follow-up and were scored using the SLE-CSQ algorithm (23). All relatives who transitioned to classified SLE at follow-up were compared to all non-transitioned relatives. In addition, each transitioned relative was matched by race, gender, and age (± 5 years) to one ANA positive and one ANA negative (by indirect immunofluorescence [IIF]) non-transitioned relative from unique families for case-control analysis to identify factors elucidating transition to classified SLE (Table 1). See Supplementary material for additional details.

Detection of SLE-associated Autoantibodies and Soluble Mediators

Serum samples were screened for ANAs and SLE-associated autoantibodies in the OMRF College of American Pathologists certified Clinical Immunology Laboratory as previously described (12). Briefly, ANAs (HEp-2 cells) and anti-dsDNA (*Crithidia luciliae*) were measured using IIF (Inova Diagnostics); positive ANA was defined at titer of ≥1:120 and positive anti-dsDNA at titer of ≥1:30. Anticardiolipin (aCL) antibodies were measured by enzyme linked immunosorbent (ELISA) assay; positive aCL was defined at titer of >20 IgG or >20 IgM units. Plasma samples were assessed for autoantibody specificities, including SLE-associated specificities toward dsDNA, chromatin, Ro/SSA, La/SSB, Sm, SmRNP complex, and RNP by xMAP BioPlex 2200 (Bio-Rad Technologies, Hercules, CA) (12). Plasma levels of BLyS (R&D Systems, Minneapolis, MN) and APRIL (eBioscience/ Affymetrix, San Diego, CA) were determined by ELISA, per the manufacturer protocol. An additional fifty analytes, including innate and adaptive cytokines, chemokines, and soluble TNF superfamily members (Supplementary Table 1), were assessed by xMAP multiplex

assays (eBioscience/Affymetrix, Santa Clara, CA) on BioPlex200 (Bio-Rad Technologies, Hercules, CA) (15). See Supplementary material for additional details.

Statistical Analyses

Relatives who transitioned to classified SLE were compared to non-transitioned relatives at baseline (pre-transition) and follow-up (post-transition). Chi-square or Fisher's exact test were used, as appropriate, to determine differences in gender, race, and familial relationship, and with Bonferroni adjustment to determine differences in the presence of ACR criteria and lupus-associated autoantibody specificities. Age differences were assessed by unpaired *t*-test with Welch's correction. Number of ACR criteria (ACR scores), SLE-CSQ scores, ANA titers, number of autoantibody specificities, and plasma soluble mediator levels were compared by Mann-Whitney test. Correlations between plasma soluble mediator levels and SLE-CSQ or ACR score were determined by Spearman rank correlation. Generalized estimating equations (GEE), adjusting for correlation within families, were used to assess whether univariately associated demographic, familial, clinical, and serologic factors at baseline could forecast relatives who transitioned to classified SLE and those who remained unaffected at follow-up (24). Unless noted, analyses were performed using GraphPad Prism 6.02 (GraphPad Software, San Diego, CA). GEE analyses were carried out in SAS version 9.3 (Cary, NC). See Supplementary material for additional details.

RESULTS

Identification of Relatives Who Transitioned to Classified SLE at Follow-up

We recruited previously identified, unaffected (meeting <4 cumulative ACR classification criteria for SLE) blood relatives of patients with medical record-confirmed SLE (20, 21) to participate in this follow-up study (n=3645; mean time to follow-up=8.0 years). Of the 409 previously unaffected relatives who agreed to participate (mean time to follow-up=6.4 years), the majority (n=364, 89%) did not reach disease classification at follow-up, while 45 (11%) transitioned to classified SLE (19). There were no differences in age at baseline, nor time to follow-up, between relatives who did and did not transition to classified SLE (non-transitioned; Table 1). There was also no difference in time to follow-up between relatives who transitioned to classified SLE (6.4 ± 3.6 years) and ANA positive relatives who did not transition (6.0 ± 3.7 years, $p=0.5339$). Relatives who transitioned were demographically similar to all enrolled participants, as the majority of relatives who transitioned to SLE were of European American (EA) descent (36 EA, 5 African American and 4 American Indian). The percent of relatives who transitioned was 11.6% among EA, compared to 11.8% for non-EA relatives.

Although relatives of lupus patients are at increased risk of developing SLE (25), families with >1 SLE patient at baseline (multiplex) were not enriched for relatives who subsequently transitioned to classified disease ($p=0.7462$; Supplementary Table 2). Both first degree relatives (FDRs; parent, child, or sibling) and non-FDR blood relatives of SLE patients had transitioned to classified SLE at follow-up, whether from simplex or multiplex families (Table 1).

Increased Baseline SLE Clinical Features in Relatives Who Transition to Classified SLE

Transitioned relatives displayed higher numbers of both medical-record confirmed ACR criteria (ACR score, $p<0.0001$, 4.8 ± 0.8 [transitioned] vs. 1.2 ± 0. 9 [non-transitioned]) and self-reported SLE-CSQ (23) scores ($p<0.0001$, 6.1 ± 3.0 vs. 2.1 ± 2.2) compared to non-transitioned relatives (Supplementary Figure 1). At baseline (pre-transition), the majority of lupus relatives met only 0-1 ACR criteria (n=294, 72%), and the average baseline ACR score was higher for relatives who transitioned to classified SLE than for non-transitioned relatives ($p<0.0001$, 2.3 ± 0.7 vs. 0.8 ± 0.8, Supplementary Figure 1A). In addition to ACR criteria, baseline SLE-CSQ (23) scores were significantly higher in relatives who transitioned to SLE ($p<0.0001$, 5.9 ± 2.7 vs. 2.2 ± 2.2, Supplementary Figure 1B). Compared to the ANA positive (≥1:120 titer by IIF) subset of relatives who did not transition, relatives who transitioned still displayed higher baseline ACR scores ($p<0.0001$, 2.3 ± 0.7 [Transitioned] vs. 1.4 ± 0.6 [ANA positive]) and SLE-CSQ scores ($p<0.0001$, 5.9 ± 2.7 [Transitioned] vs. 2.6 ± 2.4 [ANA positive]). Thus, average ACR and SLE-CSQ scores were higher at baseline for relatives who transitioned to SLE at follow-up than for non-transitioned relatives.

ACR scores reflect a combination of currently observed and previously documented criteria, including clinical criteria, serum ANA positivity (≥1:120 titer by IIF), and immunologic criteria (antibody reactivity to dsDNA, Sm, or cardiolipin) (12). Thus, ACR score differences could be due to clinical, ANA, and/or immunologic distinctions between relatives who later transitioned to classified SLE and those who had not transitioned in this follow-up cohort (Table 2). Relatives who transitioned to SLE, as well as ANA positive and ANA negative relatives who did not transition, met clinical and immunologic ACR criteria for SLE at both baseline and follow-up, including mucocutaneous criteria, arthritis, and anti-cardiolipin autoantibodies (Table 2). However, relatives who transitioned to classified SLE were more likely to meet one clinical criterion at baseline, with a higher prevalence of malar rash, photosensitivity, arthritis, and serositis than non-transitioned relatives, irrespective of ANA status ($p<0.0001$, Table 2). At follow-up, only those relatives who transitioned to classified SLE met criteria for discoid rash (n=7, 16%), serositis (n=20, 44%), or renal disease (n=5, 11%, Table 2).

In all relatives, regardless of subsequent SLE classification status, ANA positivity (≥1:120 titer by IIF) was common at baseline (52% of the total cohort; 89% of the subset that transitioned to SLE and 49% of the non-transitioned subset), and the frequency of ANA positivity was higher at follow-up (70%; 96% transitioned and 67% non-transitioned, Table 2). However, relatives who transitioned to SLE had higher ANA titers (Supplementary Table 3, $p\leq0.0007$) and more lupus-specific autoantibody specificities against DNA and RNA-binding proteins at both baseline and follow up (Supplementary Table 3, $p<0.0001$), with the greatest number in non-European-American relatives who transitioned (baseline: $p=0.0194$, 0.63 ± 0.90 [EA] vs. 1.67 ± 1.32 [Non-EA], follow up: $p=0.0077$, 0.56 ± 0.88 [EA] vs. 1.67 ± 1.32 [Non-EA]). Of the tested autoantibody specificities, anti-Ro/SSA was significantly higher at both baseline (pre-classification) and follow-up (post-classification; $p=0.0004$, after Bonferroni correction), in relatives who transitioned to classified SLE (27% at baseline) compared to relatives who did not transition (7.7% at baseline, Supplementary

Table 3). Relatives who transitioned to classified SLE were also more likely to be positive for anti-nRNP at baseline (*p=0.0020*, 13% transitioned vs. 2.2% non-transitioned).

Altered Plasma Soluble Mediators in Relatives Who Transition to SLE

Altered immune mediators are linked to SLE pathogenesis (15) and may be altered prior to disease classification (26). Utilizing a nested, case-control, approach, we assessed plasma levels of 52 soluble mediators from multiple immune pathways (Supplementary Table 1) in the 45 relatives who transitioned to classified SLE vs. non-transitioned relatives matched by race, gender, and age ± 5 years (n=90, 45 ANA positive and 45 ANA negative non-transitioned relatives, Table 1). As before (Supplementary Figure 1), relatives who transitioned to classified SLE had significantly higher baseline ACR (*p<0.0001*, 2.3 ± 0.7 [transitioned] vs. 0.8 ± 0.8 [matched non-transitioned] and SLE-CSQ (*p<0.0001*, 5.9 ± 2.7 [transitioned] vs. 2.0 ± 1.9 [matched non-transitioned]) scores compared to matched, non-transitioned relatives. However, there was not a significant difference in SLE-CSQ scores between matched ANA positive and ANA negative non-transitioned relatives (*p=0.0669*, 2.3 ± 2.0 [ANA positive] vs. 1.6 ± 1.7 [ANA negative]).

Consistent with their putative contributions to SLE pathogenesis, baseline (pre-transition) levels of a number of soluble mediators correlated with evidence of SLE at follow-up (Figure 1). Baseline plasma levels of BLyS (*p=0.0028*), SCF (*p<0.0001*), MCP-1 (*p=0.0107*), and MCP-3 (*p=0.0003*) positively correlated with cumulative follow-up ACR scores (Figure 1A). In parallel, baseline BLyS (*p=0.0151*), SCF (*p<0.0001*), and MCP-3 (*p=0.0011*) levels also positively correlated with follow-up SLE-CSQ scores (Figure 1B). Further, baseline levels of BLyS (*p=0.0156*, Spearman r = 0.208), SCF (*p<0.0001*, Spearman r = 0.345), and MCP-3 *(p=0.0004*, Spearman r = 0.300) levels significantly correlated with baseline ACR scores. Baseline levels of BLyS (*p=0.0006*, Spearman r = 0.291), SCF (*p=0.0003*, Spearman r = 0.306), and MCP-3 (*p=0.0007*, Spearman r = 0.288) also significantly correlated with baseline SLE-CSQ scores, prior to disease transition. Conversely, levels of the regulatory mediator TGF-β at baseline (Figure 1A, *p=0.0241*) and follow-up (Supplementary Figure 2A, *p=0.0054*) negatively correlated with cumulative follow-up ACR scores.

Baseline soluble mediators identified relatives who transitioned to classified SLE. Relatives who transitioned had higher baseline plasma levels of BLyS (Figure 1C) and SCF (Figure 1D) compared to relatives who remained unaffected, including ANA positive (BLyS *p=0.0229* and SCF *p=0.0004*) and ANA negative subsets (BLyS *p=0.0003* and SCF *p<0.0001*). Relatives who transitioned to SLE and matched, ANA positive, non-transitioned relatives had similar baseline plasma levels of the IFN-driven chemokines MCP-1 (*p<0.0001*, Figure 1E) and MCP-3 (*p<0.0001*, Figure 1F) that were significantly higher than matched, ANA negative, non-transitioned relatives. Compared to relatives who did not transition, those who transitioned had significantly reduced levels of the regulatory mediators IL-10 (*p=0.0284* vs. ANA negative non-transitioned relatives, Figure 1G) and TGF-β (*p=0.0082* median ANA positive and *p=0.0121* vs. ANA negative non-transitioned relatives, Figure 1H).

Follow-up levels of multiple inflammatory mediators continued to correlate with ACR (Supplementary Figure 2A) and SLE-CSQ (Supplementary Figure 2B) scores, after transition to classified disease. Conversely, the regulatory mediators IL-10 ($p=0.0039$) and TGF-β ($p=0.0054$) negatively correlated with cumulative ACR scores (Supplementary Figure 2A). Follow-up plasma levels of BLyS, SCF, MCP-1, MCP-3, IL-10, and TGF-β continued to be altered in relatives who transitioned to SLE compared to matched relatives who remained unaffected (Supplementary Figure 2C-H). In addition, a number of mediators at follow-up were altered between lupus relatives and matched unrelated healthy controls (Ctl) with no medical or family history of SLE (Supplementary Figure 2). Relatives who transitioned had significantly higher levels of BLyS ($p<0.0001$), MCP-1 ($p<0.0001$), MCP-3 ($p=0.05$), and IL-10 ($p=0.0002$) than Ctls (Supplementary Figure 2C and 2E-G, respectively). ANA negative and positive relatives who did not transition also had higher plasma levels of BLyS ($p\leq0.01$), MCP-1 ($p\leq0.003$), IL-10 ($p\leq0.0002$), and TGF-β ($p\leq0.01$) than Ctls (Supplementary Figure 2C, 2E, and 2G-H, respectively).

Baseline SCF and TGF-β Forecast Transition to SLE in Relatives Independent of Clinical Measures

We ascertained several factors that anticipated transition to classified disease in previously unaffected relatives of SLE patients. GEE analysis, adjusting for familial correlation, was performed to determine whether a multivariable model including univariate-associated demographic and relationship variables, SLE-CSQ scores, ACR classification criteria, autoantibody status, and/or select soluble mediators at baseline could forecast the risk of transition to SLE for unaffected relatives (Tables 3-4). All models were adjusted for age, gender, and race to verify effective demographic matching of transitioned and non-transitioned relatives. MCP-1, MCP-3, and BLyS did not reach significance alone or in combination and were excluded from the final models.

Relationship to confirmed SLE patients (blood relative, parent, child, or sibling) did not determine who would transition to classified SLE (Tables 3-4, Model 1). However, increased baseline levels of SCF and decreased baseline levels of TGF-β associated with transitioning to SLE (Tables 3-4, Model 2). Increased SLE-CSQ scores (Table 3, Model 3), as well as number of baseline ACR criteria (Table 4, Model 3), significantly associated with transitioning to SLE. In addition, altered SCF and TGF-β levels reached significance independently of SLE-CSQ (Table 3, Model 4) and ACR scores (Table 4, Model 4). These associations were attenuated only slightly by adjustment for SLE-CSQ (Table 3, Model 4) and ACR (Table 4, Model 4) scores, indicating that immune dysregulation alone may identify relatives at high risk of developing SLE. Although relatives who transitioned to classified SLE had more autoantibody specificities than non-transitioned relatives (Supplementary Table 3), neither ANA positivity (Tables 3-4, Model 5) nor number (adjusted OR = 1.74 [0.79-3.85], $p=0.1726$) of DNA and RNA-binding autoantibody specificities informed risk of SLE transition.

Overall, the best model that identified relatives who would subsequently transition to SLE combined soluble mediator information with clinical criteria derived either from SLE-CSQ scores (Table 3, Model 4, AUC = 0.92 [0.88-0.97] and 0.81 [0.66-0.95] for test [n=158] and

validation [n=77] datasets, respectively) or medical record ACR scores (Table 4, Model 4, AUC = 0.93 [0.88-0.98] and 0.89 [0.80-0.97] for test [n=158] and validation [n=77] datasets, respectively). Significantly more lupus relatives who transitioned to SLE at follow-up were positive for SCF (cutoff = 486.1 pg/ml by ROC curve/Youden index analysis) and negative for TGF-β (cutoff = 62.77 pg/ml by ROC curve/Youden index analysis) at baseline compared to matched ANA positive and ANA negative relatives who remained unaffected ($p<0.0001$ for SCF and $p=0.0028$ for TGF-β by χ^2). However, neither SCF positivity nor TGF-β negativity associated with any particular ACR criterion in lupus relatives who did or did not transition to SLE. Rather, baseline levels of these mediators positively (SCF) or negatively (TGF-β) correlated with overall ACR and SLE-CSQ scores at follow-up (Figure 1A-B).

Based on a pre-test probability of transitioning to classified SLE of 0.11 (11% of the cohort transitioned to classified SLE at follow-up), combining self-reported SLE-CSQ data and soluble mediator data at baseline increased the post-test probability to 0.41 (Table 3, Model 4, averaging the test and validation sets), while combining physician-confirmed ACR criteria and soluble mediator data at baseline increased the post-test probability to 0.50 (Table 4, Model 4). We additionally assessed baseline differences in SCF and TGF-β levels among relatives who transitioned to SLE with a baseline ACR score of 1-2 (ANA positivity and/or meeting immunological criteria, n=25) vs. a baseline ACR score of 3 (also meeting clinical criteria, n=20). Levels of SCF and TGF-β were not different between these groups (Supplementary Figure 3A-B). No significant differences were noted in either SCF or TGF-β levels based on history of prednisone or hydroxychloroquine use (Supplementary Figure 3C-F). For those relatives who did not transition to classified SLE (pre-test probability = 0.89), the post-test probability of remaining unaffected based on baseline SLE-CSQ scores and soluble mediators is 0.99 (Table 3, Model 4) and 0.98 if based on baseline ACR scores and soluble mediators (Table 4, Model 4).

DISCUSSION

Early intervention may ameliorate some autoimmune diseases, but this is currently not possible for lupus because those at highest risk of SLE development cannot be reliably identified. As a step toward developing monitoring and early intervention strategies to limit the accrual of SLE-induced organ damage (3), this study provides critical new information to help identify lupus relatives at the highest risk of transition to SLE. Further, it enables identification of those relatives who are less likely to develop SLE and may not require the same level of clinical monitoring. A strength of the current study is that we were able to re-enroll lupus relatives positioned across the spectrum of SLE pre-classification at baseline, ranging from meeting no criteria to exhibiting ANA positivity with clinical features. During the relatively short follow-up period of this study (mean = 6.4 ± 3.9 years), blood relatives were identified who transitioned to classified disease. Although some who transitioned were ANA positive with clinical features at baseline (pre-transition), a number of relatives who transitioned to SLE exhibited no clinical features at baseline. Yet, the vast majority of lupus relatives did not transition to classified SLE despite many exhibiting ANA positivity and/or clinical features at baseline, with 68% exhibiting no change in ACR criteria between baseline and follow-up evaluations (ACR score 0-3 at baseline and follow-up).

Although ANA positivity was more frequent and the number of autoantibody specificities greater in relatives who transitioned, neither factor independently identified future SLE classification in multivariable models. Thus, ANA positivity alone does not reliably denote future disease transition, as 85% of ANA positive relatives at follow-up did not develop SLE during the period of observation. Rather, increased SCF and decreased TGF-β levels, independent of ACR and CSQ scores, identified individuals who would transition to SLE (or remain unaffected, with decreased SCF and increased TGF-β levels) in multivariable models. Measurement of these select soluble mediators could help identify individuals in need of rheumatology referral, closer monitoring, or early intervention. Moreover, our findings support the emerging paradigm that SLE pathogenesis involves both enhanced pro-inflammatory pathways and insufficient compensatory regulatory pathways (27, 28).

Several inflammatory mediators were elevated at baseline in relatives who subsequently developed SLE. In particular, baseline plasma SCF levels were highest in relatives who transitioned and significantly predicted SLE. Together with our previous results showing that increased SCF levels immediately precede disease flare in patients with active SLE (15), these results suggest that SCF may promote SLE pathogenesis. Although typically known for its role in hematopoiesis, SCF has also been shown to drive IL-6 production and influence Th2 and Th17 pathways in several inflammatory conditions by interacting with the receptor c-kit (18). Such mechanisms may drive SLE pathogenesis by inducing the secretion of MCP chemokines (17). Indeed, the chemokines MCP-1 and MCP-3 and their downstream mediator BLyS (29) showed similar patterns of significantly increased plasma levels at baseline and follow-up in relatives who transitioned to SLE. Though considered a promising SLE therapeutic target (16), BLyS did not contribute independently to any of our models. Thus, upstream inflammatory factors, rather than downstream mediators like BLyS, may be primary independent factors in early pathogenesis (15, 17, 18, 29, 30).

Along with enhanced inflammatory pathways, SLE patients showed signs of inadequate regulatory mechanisms compared to healthy, ANA positive individuals, suggesting that a failure of active regulation contributes to SLE pathogenesis in patient relatives (31-33). Indeed, TGF-β levels were lowest in relatives who transitioned to SLE, differentiating them from unaffected relatives. Baseline IL-10 levels were also reduced in relatives who transitioned. TGF-β and IL-10 are required for the development and propagation of T-regulatory cells (33), which may have altered numbers and/or functions in SLE (31). The effectiveness of regulatory pathways in SLE patients may be further reduced by resistance of T-effector cells to T-regulatory cells (32). Conversely, compensatory T-regulator functions may help unaffected relatives avoid SLE (34), as the highest levels of TGF-β at baseline and follow-up are in those relatives who did not transition to classified SLE, irrespective of ANA status. Future studies could address these possibilities.

Eleven percent of previously unaffected lupus relatives transitioned to classified SLE in this follow-up cohort (n=409), highlighting the likelihood of identifying at-risk relatives for early intervention or clinical trial enrollment. Even in this primarily European-American cohort with limited numbers of individuals meeting renal and neurological classification criteria, utilizing the multivariable model incorporating clinical (self-reported SLE-CSQ or physician-confirmed ACR criteria) and serologic features increases the baseline risk of

transitioning to SLE to 42% for those relatives who demonstrate clinical criteria, increased SCF, and decreased TGF-β. Such individuals may benefit from clinical trials to prevent or delay SLE classification. Those relatives who are found to be autoantibody positive yet exhibit elevated levels of regulatory mediators may be identified as having a decreased risk of transitioning to classified disease. Utilizing the multivariable model incorporating clinical and serologic features increases the negative predictive value to ≥98% for those relatives who demonstrate few clinical criteria, decreased SCF, and increased TGF-β. Such a population would have the potential to reveal novel mechanisms of incomplete breaks in tolerance that can be harnessed and applied to high-risk individuals to delay or prevent disease transition. This is particularly tantalizing given that we see immune profile differences between lupus relatives who have not transitioned compared to matched unrelated, healthy controls with no family history of SLE, with increases in both inflammatory and regulatory mediators in lupus relatives. The increased inflammatory profile in lupus relatives may be due to the presence of heritable risk factors (35), offset by enhanced regulatory mechanisms that have been detected in the current study, and by others (34, 36).

While we were able to re-enroll just over 400 lupus relatives and confirm the presence of ACR classification criteria in the medical record, this study is limited by the presence of a single follow-up point and the collection of clinical data prior to the publication of proposed SLICC classification criteria (37). Prospective, longitudinal monitoring of lupus relatives over a long period of time (>10 years), where serial collection of clinical data and biologic specimens can occur, would improve our ability to pinpoint immune dysregulation associated with the natural progression to classified SLE (38, 39). Such studies could potentially provide insight into the relationship between genetic, epigenetic, and environmental risk factors (40) and immune dysregulation that leads to the accrual of autoantibody specificities and development of clinical disease.

Currently, ACR criteria and serology, particularly soluble mediator levels, may be used to evaluate unaffected relatives to help identify individuals at the highest risk of developing SLE. This evaluation requires a trained rheumatologist and may miss more subtle signs and symptoms that result in a clinician identifying a patient as having “potential SLE” (41). Screening families of lupus patients with the SLE-CSQ and serology may substantially facilitate identifying relatives who are at increased risk of disease compared to relatives who do not require enhanced monitoring or treatment with potentially toxic medications.

Such information may assist when counseling family members about future disease risk and allow for the identification of relatives for inclusion in prospective prevention trials. Given the humanistic and economic burden of SLE (42, 43), addressing immune dysregulation prior to disease classification may prove beneficial (44). Although a therapeutic challenge (45), this study reveals inflammatory and regulatory mechanisms that may be applied to the development of novel SLE therapies (46). In addition, early intervention with hydroxychloroquine has been shown to reduce organ damage (47), decrease the accumulation of lupus-associated autoantibodies, and delay the transition to classified SLE (48). Such an approach may allow for decreased rate of damage and a reduced need for

multiple and/or immunosuppressant treatments that perpetuate morbidity and increased healthcare costs (49) in lupus relatives at high risk of transitioning to classified SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

Biomarker	Spearman r	95% CI	p value
SLE-CSQ	0.613	0.492 to 0.711	***<0.0001***
SCF	0.393	0.236 to 0.531	***<0.0001***
MCP-3	0.304	0.138 to 0.454	***0.0003***
BLyS	0.256	0.086 to 0.3411	***0.0028***
MCP-1	0.230	0.059 to 0.389	***0.0072***
MIP-1β	0.219	0.047 tp 0.379	***0.0107***
TGF-β	-0.194	-0.356 to -0.021	***0.0241***
IL-10	-0.128	-0.295 to 0.047	*0.1385*
TNFRII	0.134	-0.041 to 0.300	*0.1225*

B

Biomarker	Spearman r	95% CI	p value
ACR Score	0.421	0.266 to 0.554	***< 0.0001***
SCF	0.356	0.194 to 0.499	***< 0.0001***
TNFRII	0.284	0.116 to 0.436	***0.0009***
MCP-3	0.277	0.108 to 0.430	***0.0011***
MIP-1β	0.230	0.059 to 0.389	***0.0072***
BLyS	0.209	0.036 to 0.369	***0.0151***
MCP-1	0.110	-0.065 to 0.279	*0.1102*
TGF-β	-0.091	-0.261 to 0.084	*0.2941*
IL-10	-0.064	-0.235 tp 0.111	*0.4601*

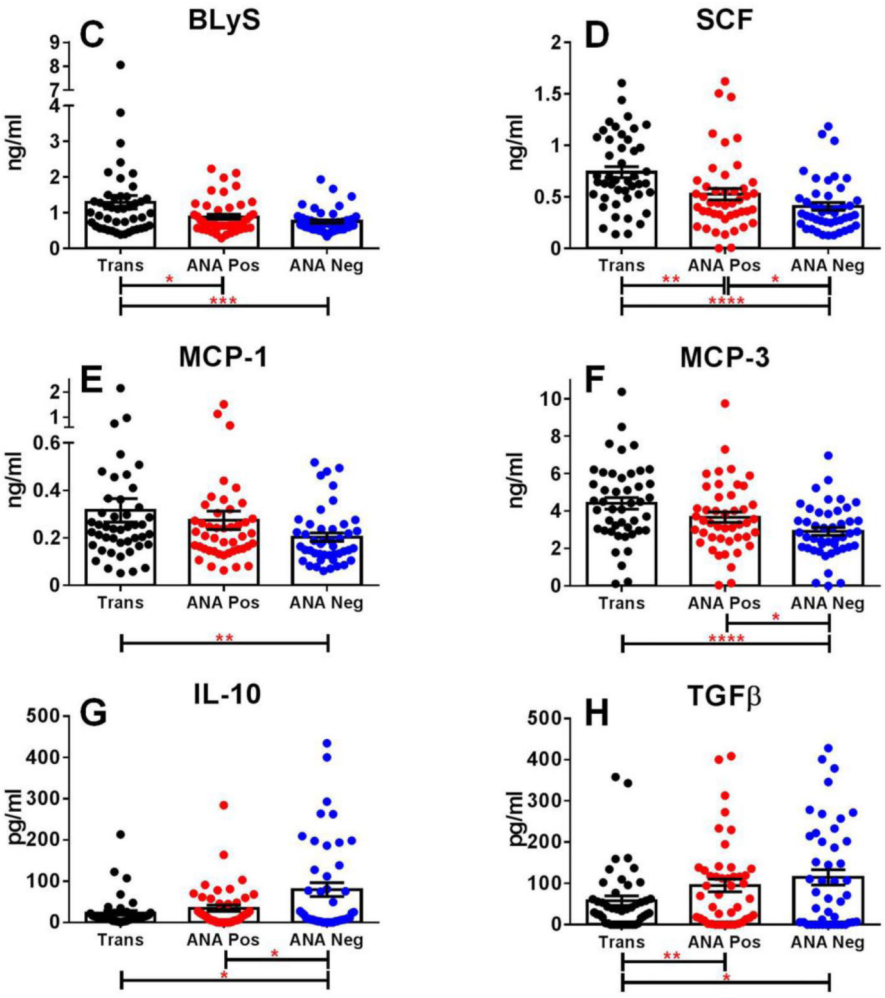

Figure 1.

Altered soluble mediators of inflammation at baseline in relatives who transition to classified SLE at follow-up. Spearman correlation of baseline SLE-CSQ scores and plasma soluble mediator levels vs. ACR scores at follow-up are presented (**A**). Spearman correlation of ACR scores and plasma soluble mediators vs SLE-CSQ scores at follow-up are presented (**B**). Plasma levels of BLyS (**C**), SCF (**D**), MCP-1 (**E**), MCP-3 (**F**), IL-10 (**G**), and TGF-β (**H**) were measured at baseline in 45 lupus relatives who transitioned to classified SLE at follow-up (Trans) vs. age (±5 years), race, gender, and time of sample procurement matched

unaffected relatives who were ANA positive (ANA Pos) or ANA negative (ANA Neg) by IIF. Mean ± SEM. ****$p<0.0001$; ***$p<0.001$; **$p<0.01$; *$p<0.05$ by Kruskal-Wallis with Dunn's multiple comparison.

Table 1

Study Participant Demographics

			Non-transitioned Matched to Transitioned to SLE[a]	
	Non-transitioned	**Transitioned to SLE**	**ANA Positive**	**ANA Negative**
	(n = 364)	**(n = 45)**	**(n = 45)**	**(n = 45)**
Gender[b]				
Female (%)	304 (84%)	40 (89%)	40 (89%)	40 (89%)
Baseline Age (SD)[c]	47.3 (15.9)	47.2 (12.8)	47.9 (13.7)	48.0 (17.0)
Follow-up Age (SD)[c]	53.8 (15.5)	53.4 (12.6)	54.0 (13.2)	55.3 (16.9)
Timespan (SD)[c]	6.5 (3.9)	6.4 (3.6)	6.1 (3.5)	7.3 (3.5)
Race (n, %)[b]				
EA	270 (74.2 %)	36 (80.0%)	36 (80.0%)	36 (80.0%)
AA	52 (14.3%)	5 (11.1%)	5 (11.1%)	5 (11.1%)
AI	15 (4.1%)	4 (8.9%)	4 (8.9%)	4 (8.9%)
Asian	14 (3.8%)		--	--
Hispanic	11 (3.0%)		--	--
PI	2 (0.6%)		--	--
Relationship status (n, %)[d]				
Parent of SLE patient	167 (45.9%) **	10 (22.2%)	24 (53.3%) **	23 (51.1%) **
Child of SLE patient	30 (8.2%) **	10 (22.2%)	3 (6.7%)	4 (8.9%)
Sibling of SLE patient	255 (70.0%) *	24 (53.3%)	37 (82.2%) **	27 (60.0%)
Non-FDR of SLE Patient[e]	115 (31.6%) *	23 (51.1%)	5 (11.1%) ****	14 (31.1%)

[a] ANA Positive and ANA negative (determined by IIF, titer ≥ 120) Non-transitioned relatives matched to Transitioned to SLE group by race, gender, and age (± 5 years)

[b] *ns* Fisher's exact test vs. Transitioned to SLE

[c] *ns* by unpaired t-test with Welch's correction compared to Transitioned to SLE

[d] First degree relative (FDR) = sibling, child, or parent of SLE patient (from singlex or multiplex families)

[e] Non-FDR=aunt/uncle, niece/nephew, first cousin, grandparents, grandchildren, and other distant relatives (from simplex or multiplex families)

* $p<0.05$;

** $p<0.01$;

**** $p<0.0001$ Fisher's exact test vs. Transitioned to SLE

Race: European-American (EA), African-American (AA), American Indian (AI), Pacific Islander (PI)

Table 2

ACR Criteria in Non-transitioned Relatives vs. Relatives Who Transition to SLE

FU ACR Score	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**					
Baseline ACR													
0	84	47	12	1									
1		98	42	5	5		1	1					
2			56	8	8	9	1						
3				11	7	8	4	1					
	Non-transitioned (n = 364)				**Transitioned to SLE (n = 45)**				**Non-Transitioned (n= 364)**		**Transitioned (n= 45)**		
ACR Criteria (Baseline)									**n**	**%**	**n**	**%**	***p-value*[a]**
Malar Rash			2	1	2	3	1		3	1%	6	13%	***<0.0001***
Discoid Rash									0	--	0	--	--
Photosensitivity			1	7	5	4	1	1	8	2%	11	24%	***<0.0001***
Oral Ulcers			1			1			1	0.3%	1	2%	*0.2082*
Arthritis			3	6	7	6	2		9	2.5%	15	33%	***<0.0001***
Serositis					3		1		0	--	4	9%	***0.0001***
Renal Disease							1		0	--	1	2%	*0.1103*
Neurologic						1			0	--	1	2%	*0.1100*
Hematologic			1	5	1	2	1		6	1.6%	4	9%	*0.0164*
Immunologic		21	71	13	7	10	2	1	105	29%	20	44%	*0.0394*
ANA Positivity		77	75	22	17	15	6	2	174	48%	40	89%	***<0.0001***
ACR Criteria (Follow-up)									**n**	**%**	**n**	**%**	***p-value*[b]**
Malar Rash			3	3	11	7	6	2	6	1.6%	26	58%	***<0.0001***
Discoid Rash					1	5		1	0	--	7	16%	***<0.0001***
Photosensitivity			1	9	10	9	4	2	10	2.7%	25	56%	***<0.0001***
Oral Ulcers			1	2	9	7	3	1	3	1%	20	44%	***<0.0001***
Arthritis			5	14	13	14	5	2	19	5.2%	34	76%	***<0.0001***
Serositis					6	7	5	2	0	--	20	44%	***<0.0001***
Renal Disease					1	2	2		0	--	5	11%	***<0.0001***
Neurologic			2			3	1	1	2	0.6%	5	11%	***0.0002***

FU ACR Score	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**					
Hematologic			1	6	2	3	2		7	1.9%	7	16%	***0.0002***
Immunologic		30	101	17	8	12	2	1	148	41%	23	51%	*0.2014*
ANA Positivity		115	106	24	19	16	6	2	245	67%	43	96%	***<0.0001***

[a] Fisher's exact test;Bonferroni-adjusted *p-value significance* for multiple comparisons is *0.0050*

[b] Fisher's exact test; Bonferroni-adjusted *p-value* significance for multiple comparisons is *0.0045*

FU= Follow-up; N/A=Not applicable (ANA positive and ANA negative defined by ANA status)

Table 3

Baseline SCF and TGF-β, independent of SLE-CSQ scores, differentiate transition to SLE[a]

	Model 1		Model 2		Model 3		Model 4		Model 5	
Parameter (at baseline)	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**
Age	1.01 (0.97,1.04)	*0.7099*	1.01 (0.97,1.05)	*0.6389*	1.01 (0.97,1.06)	*0.5230*	1.01 (0.97,1.06)	*0.5961*	1.01 (0.96,1.06)	*0.6360*
Gender	0.61 (0.17,2.19)	*0.4500*	0.47 (0.11,2.10)	*0.3241*	0.42 (0.08,2.10)	*0.2899*	0.30 (0.05,1.72)	*0.1764*	0.27 (0.05,1.52)	*0.1377*
Race										
EA	1		1		1		1		1	
AA	0.60 (0.12,2.91)	*0.5243*	0.74 (0.13,4.08)	*0.7255*	0.73 (0.13,4.28)	*0.7296*	0.84 (0.13,5.45)	*0.8528*	0.73 (0.11,4.90)	*0.7416*
Other	1.68 (0.46,6.17)	*0.4381*	1.00 (0.21,4.76)	*0.9985*	4.17 (0.88,19.80)	*0.0727*	2.28 (0.39,13.33)	*0.3612*	2.39 (0.41,14.08)	*0.3353*
Relationship to SLE patient										
Blood Relative	1		1		1		1		1	
Parent of	0.90 (0.09,18.83)	*0.9240*	0.88 (0.07,10.53)	*0.9195*	4.19 (0.36,149.37)	*0.2548*	5.98 (0.39,91.44)	*0.1989*	7.30 (0.44,120.37)	*0.1644*
Child of	2.92 (0.65,13.10)	*0.1621*	4.56 (0.79,26.47)	*0.0910*	1.79 (0.33,9.72)	*0.4988*	3.37 (0.45,25.30)	*0.2383*	3.73 (0.49,28.62)	*0.2055*
Sibling of	1.19 (0.43,3.28)	*0.5317*	1.51 (0.46,4.91)	*0.4956*	1.44 (0.45,4.63)	*0.5367*	2.53 (0.60,10.75)	*0.2078*	2.66 (0.62,111.49)	*0.1891*
SLE-CSQ Score	--	--	--	--	1.64 (1.35, 1.98)	***<0.0001***	1.62 (1.29, 2.02)	***<0.0001***	1.61 (1.28, 2.02)	***<0.0001***
ANA Positivity[b]	--	--	--	--	--	--	--	--	1.78 (0.49, 6.47)	*0.3831*
SCF[d]	--	--	3.96 (2.19,57.16)	***<0.0001***	--	--	3.78 (1.94,7.35)	***<0.0001***	3.62 (1.84,7.12)	***0.0002***
TGF-β[d]	--	--	0.20 (0.08,0.52)	***0.0010***	--	--	0.27 (0.10,0.69)	***0.0067***	0.25 (0.10,0.67)	***0.0058***
Test Group (n=158)										
ROC (AUC [95% CI])	0.60 (0.47,0.72)		0.84 (0.76,0.92)		0.86 (0.80,0.92)		0.92 (0.88,0.97)		0.93 (0.89,0.97)	
Sensitivity	0.35		0.86		0.93		0.97		0.97	
Specificity	0.85		0.72		0.70		0.81		0.81	
LR+[e]	2.33		3.07		3.10		5.11		5.11	
LR–[f]	0.76		0.19		0.10		0.04		0.04	
PPV[g]	0.22		0.28		0.28		0.39		0.39	
NPV[h]	0.91		0.98		0.99		1.00		1.00	
Validation Group (n=77)										

	Model 1		Model 2		Model 3		Model 4		Model 5	
Parameter (at baseline)	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**
ROC (AUC [95% CI])	0.57 (0.39,0.75)		0.73 (0.56,0.88)		0.77 (0.64,0.91)		0.81 (0.66,0.95)		0.80 (0.65,0.95)	
Sensitivity	0.50		0.69		0.63		0.75		0.75	
Specificity	0.71		0.79		0.89		0.87		0.87	
LR+[e]	1.72		3.29		5.73		5.77		5.77	
LR−[f]	0.70		0.39		0.42		0.29		0.29	
PPV[g]	0.18		0.29		0.41		0.42		0.42	
NPV[h]	0.92		0.95		0.95		0.97		0.97	

[a] Compared to race, gender, and age (± 5 years) matched unaffected relatives

[b] ANA status determined by IIF, as described in *Methods*

[c] P-values determined by Wald Chi-square

[d] OR determined per standard deviation (SD) increase. SCF and TGF-β SD = 329.2 and 147.2, respectively.

[e] Positive Likelihood Ratio (LR+) based on cohort SLE transition prevalence/pre-test probability = 0.11

[f] Negative Likelihood Ratio (LR−) based on cohort SLE transition prevalence/pre-test probability = 0.11

[g] Positive Predictive Value (PPV) based on cohort SLE transition prevalence/pre-test probability = 0.11

[h] Negative Predictive Value (NPV) based on cohort SLE transition prevalence/pre-test probability = 0.11

Table 4

Baseline SCF and TGF-β, independent of ACR scores, differentiate transition to SLE[a]

	Model 1		Model 2		Model 3		Model 4		Model 5	
Parameter (at baseline)	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**
Age	1.01 (0.97,1.04)	*0.7099*	1.01 (0.97,1.05)	*0.6389*	1.00 (0.96,1.05)	*0.8822*	1.00 (0.95,1.05)	*0.9988*	1.00 (0.96,1.05)	*0.9356*
Gender	0.61 (0.17,2.19)	*0.4500*	0.47 (0.11,2.10)	*0.3241*	0.37 (0.07,1.97)	*0.2450*	0.39 (0.06,2.38)	*0.3074*	0.47 (0.07,3.07)	*0.4268*
Race										
EA	1		1		1		1		1	
AA	0.60 (0.12,2.91)	*0.5243*	0.74 (0.13,4.08)	*0.7255*	0.50 (0.08,3.29)	*0.4679*	0.47 (0.06,3.43)	*0.4533*	0.55 (0.07,4.09)	*0.5552*
Other	1.68 (0.46,6.17)	*0.4381*	1.00 (0.21,4.76)	*0.9985*	1.10 (0.18,6.65)	*0.9218*	.57 (0.07,4.57)	*0.5948*	0.47 (0.05,4.50)	*0.5149*
Relationship to SLE patient										
Blood Relative	1		1		1		1		1	
Parent of	0.90 (0.09,18.83)	*0.9240*	0.88 (0.07,10.53)	*0.9195*	3.79 (0.24,60.23)	*0.3452*	4.44 (0..23,85.97)	*0.3241*	3.68 (0.21,63.35)	*0.3693*
Child of	2.92 (0.65,13.10)	*0.1621*	4.56 (0.79,26.47)	*0.0910*	5.57 (0.83,37.16)	*0.0763*	6.94 (0.83,58.32)	*0.0744*	5.87 (0.70,49.52)	*0.3693*
Sibling of	1.19 (0.43,3.28)	*0.5317*	1.51 (0.46,4.91)	*0.4956*	1.34 (0.38,4.72)	*0.6467*	1.61 (0.38,6.85)	*0.5210*	1.45 (0.33,6.41)	*0.6256*
ACR Score			--	--	7.40 (3.54, 15.45)	***<0.0001***	5.96 (2.69, 13.19)	***<0.0001***	6.62 (2.98, 14.72)	***<0.0001***
ANA Positivity[b]	--	--	--	--	--	--	--	--	0.40 (0.09, 1.81)	*0.2324*
SCF[d]	--	--	3.96 (2.19,57.16)	***<0.0001***	--	--	2.69 (1.42,5.10)	***0.0024***	2.81 (1.46,5.38)	***0.0019***
TGF-β[d]	--	--	0.20 (0.08,0.52)	***0.0010***	--	--	0.29 (0.11,0.79)	***0.0156***	0.30 (0.11,0.83)	***0.0203***
Test Group (n=158)										
ROC (AUC [95% CI])	0.60 (0.47,0.72)		0.84 (0.76,0.92)		0.90 (0.84,0.96)		0.93 (0.88,0.98)		0.93 (0.87,0.98)	
Sensitivity	0.35		0.86		0.93		0.86		0.90	
Specificity	0.85		0.72		0.70		0.90		0.87	
LR+[e]	2.33		3.07		3.10		8.60		6.92	
LR−[f]	0.76		0.19		0.10		0.16		0.11	
PPV[g]	0.22		0.28		0.28		0.51		0.46	
NPV[h]	0.91		0.98		0.99		0.98		0.99	
Validation Group (n=77)										

	Model 1		Model 2		Model 3		Model 4		Model 5	
Parameter (at baseline)	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**	**OR (95% CI)**	***P-value*[c]**
ROC (AUC [95% CI])	0.57 (0.39,0.75)		0.73 (0.56,0.88)		0.87 (0.79,0.96)		0.89 (0.80,0.97)		0.89 (0.81,0.98)	
Sensitivity	0.50		0.69		0.63		0.81		0.81	
Specificity	0.71		0.79		0.89		0.89		0.92	
LR+[e]	1.72		3.29		5.73		7.36		10.13	
LR–[f]	0.70		0.39		0.42		0.21		0.21	
PPV[g]	0.18		0.29		0.41		0.48		0.56	
NPV[h]	0.92		0.95		0.95		0.97		0.97	

[a] Compared to race, gender, and age (± 5 years) matched unaffected relatives

[b] ANA status determined by IIF, as described in *Methods*

[c] P-values determined by Wald Chi-square

[d] OR determined per standard deviation (SD) increase. SCF and TGF-β SD = 329.2 and 147.2, respectively.

[e] Positive Likelihood Ratio (LR+) based on cohort SLE transition prevalence/pre-test probability = 0.11

[f] Negative Likelihood Ratio (LR−) based on cohort SLE transition prevalence/pre-test probability = 0.11

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