UNDERSTANDING THE CONCEPT OF PRE-CLINICAL AUTOIMMUNITY

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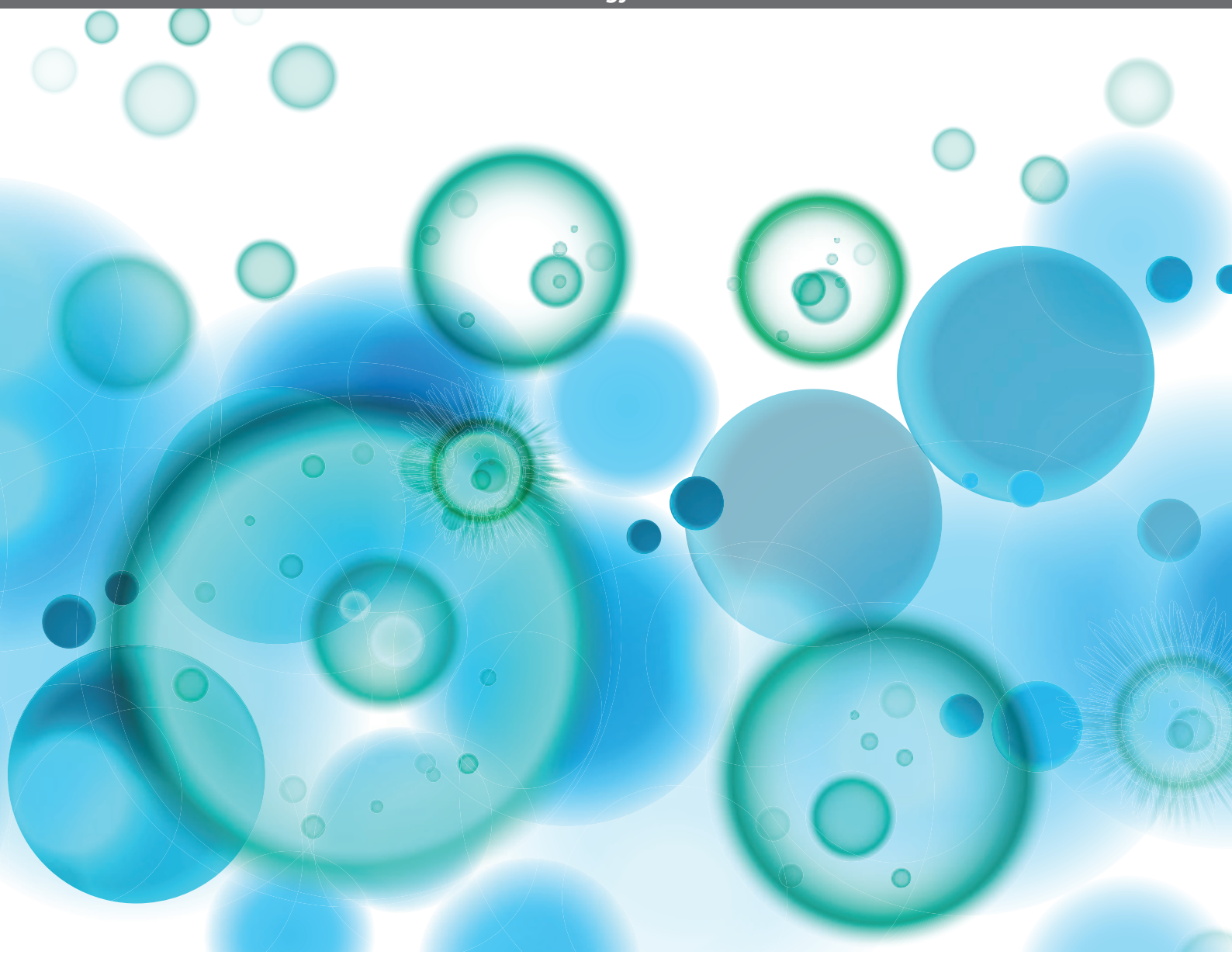

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UNDERSTANDING THE CONCEPT OF PRE-CLINICAL AUTOIMMUNITY

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Editorial: Understanding the concept of pre-clinical autoimmunity

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Editorial on the Research Topic
Understanding the concept of pre-clinical autoimmunity

The concept of autoimmune disease covers at least 80 different conditions. Each of these diseases is relatively rare, but together they have been estimated to occur in 7.6-9.4 percent of the US population (1). Autoimmune diseases occur most often in females, typically during childbearing years, and contribute substantially to morbidity and mortality in this age group (2). Over the last two decades, a combination of translational, clinical, and epidemiological research has led to the concept in Figure 1. One of the central tenants of immunology is tolerance to self, with central and peripheral immunologic mechanisms designed to prevent the occurrence of self-reactive T or B cells. Thus, the "normal" immune system is envisioned as one without demonstrable high affinity IgG autoantibodies or activated self-reactive T cells. However, some types of asymptomatic autoimmunity are relatively common. For example, anti-nuclear antibodies are found in at least 15% of asymptomatic individuals (3), including young children (4). The boundary between autoantibody-negative and autoantibody-positive (Transition 1) is clear-cut, as it is defined with standardized laboratory testing. What is less clear is the importance, if any, of the presence of laboratory defined autoimmunity in the absence of signs or symptoms of immune-mediated pathology in an individual patient.

In retrospective cohorts, asymptomatic autoimmunity precedes clinical disease by up to a decade, suggesting a prognostic role for autoantibodies. Given the low prevalence of disease in an unselected population, the predictive value of most autoantibodies alone is relatively weak but can allow the identification of at-risk individuals for mechanistic studies and prevention trials. The addition of other laboratory testing such as measurement of serum cytokines and chemokines, or the addition of environmental or

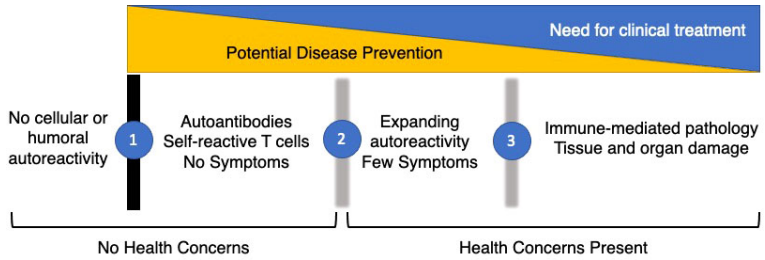

FIGURE 1
Phases of Autoimmunity. The majority of the healthy population has no evidence of cellular or humoral autoimmunity. However, a substantial fraction develops autoantibodies or self-reactive T cells while remaining asymptomatic (1). These individuals typically do not seek medical care unless it is to explain serological findings. After a period of years, characterized by expanding serological autoimmunity and up-regulation of inflammatory cytokines and chemokines, early symptoms develop in a sub-set of people with asymptomatic autoimmunity (2). With the accumulation of sufficient clinical signs and symptoms, patients are classified with definite autoimmune conditions (3). If possible, the prevention of autoimmunity will take place in the earliest phases before there are significant health concerns present. Later clinical treatments will address organ damage and dysfunction but are less likely to halt disease progression over time.

genetic risk factors to focus biomarker testing increases the ability to make meaningful predictions in people with asymptomatic autoimmunity. Transition 2 occurs in a subset of people with asymptomatic autoimmunity when they begin to develop early signs or symptoms of an organ-specific or systemic autoimmune condition. This might be arthralgia in the absence of synovitis in the case of rheumatoid arthritis, or a skin rash without other clinical features of systemic lupus erythematosus. This transition is less clear cut, as laboratory features such as neutropenia can have other causes and the presence of joint inflammation depends on whether it is assessed by physical examination or by imaging. Transition 3 occurs at the point when the individual is felt to have the autoimmune disease in question and meets either clinical diagnostic or classification criteria. This, too, is a subject to ambiguity. Criteria exist to classify individuals for entry into clinical research studies and are often proxies for diagnostic criteria. Nonetheless, the boundary between early and established disease is artificial and it remains to be determined whether treatments developed for established disease will slow or prevent progression to established disease.

This Research Topic of *Frontiers in Immunology* addresses the important questions regarding the development of asymptomatic autoimmunity and the progression from few clinical symptoms to well-defined autoimmune disease. It consists of fourteen articles and includes both reviews and original research. Most of the articles deal with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), reflecting the large body of research in these areas. The inclusion of articles focused on the precursor states to multiple sclerosis (MS), systemic sclerosis, and celiac disease illustrates the common features of pre-clinical autoimmunity.

Several reviews look at the epidemiology of pre-clinical autoimmunity and the methodology needed to study it. Kowalski et al., examined the natural history of RA through retrospective population-based and administrative datasets, prospective case-control or cohort studies, studies of first-degree relatives of RA patients, biomarker-driven studies and studies that focus on patients with early symptoms. Together, these studies describe distinct phases of RA that exist prior to definite classification and illustrate the need to focus on these phases to design effective clinical trials for disease prevention.

Choi and Costenbader describe similar studies in SLE that have documented the genetic, epidemiological, and lifestyle risks for developing disease and the stepwise timeline of disease progression from autoantibody positively to the presence of soluble mediators to early disease and finally to full disease classification. Notably, they document the fact that in the Nurses Health Studies, healthy lifestyle habits – diet, regular exercise, smoking avoidance, moderate alcohol use, and healthy weight each led to a 19% decrease in the risk of SLE. Together these modifiable lifestyle factors contribute 50% of the population attributable risk. The authors discuss studies to prevent SLE in people at risk using hydroxychloroquine (5) and vitamin D or omega 3 fatty acids (6).

Calderon and Pope performed a scoping review of SLE and systemic sclerosis to identify homogeneous groups of individuals in each disease that typify the pathophysiology in each disease. In systemic sclerosis, there is dysregulated immune signaling followed by vasculopathy and fibrogenesis. In SLE the dysregulated signaling precedes the development of autoantibody production. Curtiss et al., describe the progression from autoimmunity with a restricted set of clinical signs – cutaneous lupus erythematosus – to SLE. While the pace varied in each study they reviewed, the progression from CLE to SLE occurred in 42% of patients, suggesting this group be targeted for intervention.

The original research in this collection ranges from the very earliest phases of pre-clinical autoimmunity to screening strategies of populations at risk. Gupta et al. evaluated a cohort of clinically healthy individuals with positive

antinuclear antibodies (ANA) and performed detailed immunophenotyping on their peripheral blood compared to people with early or established disease. The ANA+ individuals had more activated T and B cells than ANA- controls, and had more Tfh and Tph cells, consistent with an active cellular immune response driving the production of autoantibodies. In general, Th2 and to a lesser extent, Th17 responses predominated. In the ANA+ individuals with no symptoms, a greater Treg response was seen than in people with early or established disease, suggesting effective control mechanisms are preventing progression to clinically apparent disease. This concept was echoed by Munroe et al., who extended their previous studies of first-degree relatives of lupus patients, using the self-administered SLE Connective Tissue Screening Questionnaire and measurement of soluble mediators to characterize relatives that progress to SLE and those that do not. The unaffected relatives had higher levels of inflammatory soluble mediators, but those who did not transition to SLE also had higher levels regulatory cytokines IL-10 and TGF-β.

In an examination of healthy individuals recruited from community health fairs, Bergstedt et al., determined that the 29% who had antibodies to citrullinated protein antigens at baseline developed RA over a mean of 2 yr. The rate of progression to RA was significantly influenced by the presence of both IgM and IgA isotypes of rheumatoid factor and HLA alleles known to confer RA risk. They conclude that these clinically available serological markers could be used to assess risk for RA in the general population.

Two contributions addressed the pre-clinical phase of MS. Rival et al. reviewed the biomarkers available for the radiologically isolated syndrome – those individuals with MRI findings but no clinical evidence of demyelinating disease. 50% of these individuals develop MS over 10 years. They discuss the ability of cytokines including IL-8, neurofilament light chains from injured neurons and specific micro RNA species predict this transition. In a single center study by Levraut et al. a care pathway that uses CSF kappa free light chains is shown to classify individuals who develop MS versus other inflammatory and non-inflammatory neurological diseases with 76% sensitivity and 91% specificity while elevated CSF CD25 and IL-6 would rule out the condition.

Lastly, Falahee and Raza discussed the qualitative and quantitative studies that examine the perspectives of patients on screening and prevention strategies for autoimmune diseases. There is a clear interplay between the perception of disease risk and risks arising from a potential intervention. Given the uncertainty in the effectiveness of therapies to prevent RA, SLE and other autoimmune diseases people identified as having pre-clinical disease have a certain reluctance to take medications. As much as epidemiological and translational research needs to be done to elucidate the causes and course of pre-clinical autoimmunity, there is work that needs to be done in parallel to understand the perceptions and concerns of patients and their families.

In conclusion, this Research Topic of *Frontiers in Immunology* provides us with important information on the timely topic of pre-clinical autoimmunity, describing the research to date and possible care pathways to prevent the morbidity and mortality of these conditions.

Author contributions

This editorial was drafted by DK then reviewed and revised by VH, NO and DO. All authors approve of the version published and agree to be accountable for all aspects of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Circulating miRNAs as Potential Biomarkers for Celiac Disease Development

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Background & Aims: Celiac disease (CeD), an immune-mediated disease with enteropathy triggered by gluten, affects ~1% of the general European population. Currently, there are no biomarkers to predict CeD development. MicroRNAs (miRNAs) are short RNAs involved in post-transcriptional gene regulation, and certain disease- and stage-specific miRNA profiles have been found previously. We aimed to investigate whether circulating miRNAs can predict the development of CeD.

Methods: Using next-generation miRNA-sequencing, we determined miRNAs in >200 serum samples from 53 participants of the PreventCD study, of whom 33 developed CeD during follow-up. Following study inclusion at 3 months of age, samples were drawn at predefined ages, diagnosis (first anti-transglutaminase antibody (TGA) positivity or

diagnostic biopsy) and after the start of a gluten-free diet (GFD). This allowed identification of circulating miRNAs that are deregulated before TGA positivity. For validation of the biomarkers for CeD and GFD response, two additional cohorts were included in subsequent meta-analyses. Additionally, miRNAs were measured in duodenal biopsies in a case-control cohort.

Results: 53 circulating miRNAs were increased (27) or decreased (26) in CeD *versus* controls. We assessed specific trends in these individual miRNAs in the PreventCD cohort by grouping the pre-diagnostic samples of the CeD patients (all had negative TGA) by how close to seroconversion (first sample positive TGA) the samples were taken. 8/53 miRNAs differed significantly between controls and samples taken <1 year before TGA positivity: miR-21-3p, miR-374a-5p, 144-3p, miR-500a-3p, miR-486-3p let-7d-3p, let-7e-5p and miR-3605-3p. 6/26 downregulated miRNAs reconstituted upon GFD, including miR-150-5p/-3p, whereas no upregulated miRNAs were downregulated upon GFD. 15/53 biomarker candidates also differed between CeD biopsies and controls, with a concordant direction, indicating that these circulating miRNAs might originate from the intestine.

Conclusions: We identified 53 circulating miRNAs that are potential early biomarkers for CeD, of which several can be detected more than a year before TGA positivity and some start to normalize upon GFD.

Keywords: small RNA sequencing, pre-diagnostic marker, pre-clinical marker, autoimmunity, celiac disease

INTRODUCTION

In celiac disease (CeD), genetically susceptible individuals develop a small intestinal immune response to gluten, a group of storage proteins present in food items containing wheat, rye or barley (1). Partially degraded gluten proteins pass the small-intestinal epithelial barrier and are deamidated by the enzyme transglutaminase 2 (TG2). Specific deamidated gluten peptides bind strongly to HLA-DQ2 or -DQ8, resulting in activation of gluten-specific CD4+ T cells, which then initiate an immune response by secreting cytokines that activate CD8+ T cells (2, 3). The activated CD8+ T cells that migrate to the epithelial layer (called intra-epithelial lymphocytes) and are then "licensed to kill" epithelial barrier cells, resulting in villous atrophy (4). Simultaneously, B cells interact with activated gluten-specific CD4+ T cells and secrete disease-specific autoantibodies against TG2 (TGA), of which the detection is the current mainstay of CeD diagnosis (3). The only current treatment for CeD is a strict lifelong gluten-free diet (GFD).

Epidemiological studies based on screening for TGA seroprevalence suggest that approximately 1-2% of the Caucasian population has CeD, but that at least half of the individuals with CeD remain undiagnosed (4, 5). The age of CeD diagnosis ranges from the first encounter with gluten in the first year, too late in life. Moreover, CeD is characterized by a wide array of symptoms varying from gastrointestinal symptoms (abdominal pain, bloating, chronic diarrhea, constipation) and/or extra-intestinal symptoms (e.g. iron-deficiency anemia, fatigue, poor growth in children, weight loss), and many persons with CeD have no signs and symptoms at all. Altogether, these features make it difficult to diagnose CeD (2, 6–8). Untreated CeD may aggravate symptoms (e.g. weight loss, failure to thrive in children, moodiness and loss of energy) and CeD-associated complications (e.g. osteoporosis) that decrease quality of life (9–12). The importance of early diagnosis for avoiding symptoms and complications underlines the need for tools that can detect CeD as early as possible, ideally before disease onset and accompanying symptoms.

Historically, the 'gold standard' for diagnosing CeD was the histopathological detection of villous atrophy and increased numbers of intra-epithelial lymphocytes in duodenal biopsies collected by upper endoscopy. However, these lesions are not specific for CeD. In the last few decades, increased TGA and anti-endomysium autoantibody concentrations in serum have been added to the diagnostic work-up and have been used for screening of persons at risk for CeD (2, 3, 13). The major drawback of these antibody-based tests is that they cannot be used as predictive markers of disease development because in the majority of patients these antibodies are found elevated when intestinal mucosal lesions are already present (3, 14–16). For early detection of CeD, preferably before the onset of intestinal damage, it would be valuable to identify novel biomarkers for CeD development. Ideally, these biomarkers would be blood-based, detectable at an early stage of CeD onset and able to monitor GFD adherence.

Circulating microRNAs (miRNAs) represent such biomarker candidates. These small non-coding RNAs (19-24 nucleotides) appear to be stable in the extracellular environment in different biofluids, including blood, and specific circulating miRNAs have been shown to be detectable in blood in a disease- or even disease

stage-specific fashion (17–21). In previous studies applying array-based approaches, CeD-specific miRNA profile changes were observed in small intestinal biopsies of CeD patients (22–24). Some of the deregulated miRNAs were also later detected in the circulation of CeD patients at the time of diagnosis (25).

We applied a next-generation miRNA-sequencing approach to profile extracellular/circulating miRNAs. The advantage of the next generation sequencing approach is that it is not limited by an array-design nor dependent on PCR-primer sets, thus allowing for holistic screening of the entire miRNA repertoire catalogued in the current version of miRbase (26). To find biomarkers, we used three different studies, including the longitudinal prospective CeD birth cohort, PreventCD (15). Participants of PreventCD are at high risk of developing CeD because they carry the HLA-risk alleles and have at least one 1st degree family member diagnosed with CeD. They were enrolled at birth and were followed up to 12 years of age. The availability of longitudinal samples from birth for both participants who did develop CeD and those who did not, enabled us to search for CeD biomarkers that arise before celiac-specific autoantibodies (TGA) are increased in serum.

Altogether, we detected 53 miRNAs in circulation that are potential early biomarkers for CeD. Changes in several of these miRNAs were detectable in blood more than two years before CeD diagnosis by TGA antibody detection and small bowel biopsies, and six of them began to normalize once the participant started treatment with a GFD. We therefore propose that these miRNAs represent novel biomarker candidates for early detection of CeD.

MATERIAL AND METHODS

Sample Collection

Serum samples of the PreventCD cohort collected in the context of a prospective, multicenter study were used to generate the explorative dataset. In short, infants at high risk of developing CeD were included after birth and followed up prospectively (15, 27). Circulating microRNA (here defined as all extracellular miRNAs present in the circulation, which includes exosomic miRNAs and miRNAs potentially present in other extracellular vesicles or in protein-miRNA aggregates) profiles were generated from 250 serial serum samples obtained from 53 participants of whom 33 developed CeD during the course of the study (**Table 1** shows the number of samples included in the final analyses after the quality control; **Supplementary Table S4** shows the number of samples excluded in the quality control). The remaining 20 individuals who did not develop CeD within the timeframe of the PreventCD study provided the control samples. Samples were drawn at 4, 6, 9, 12, 18 or 24 months of age, at time of CeD diagnosis (taken at first positive TGA sample or at the diagnostic biopsy). The samples included in this "Diagnosis" group, were taken on average 1.71 months after seroconversion (first positive TGA sample). Additional samples were included after start of a GFD (median: 7.4 months after start of the GFD, range: 2.3–40 months). Serum TGA levels were determined at each timepoint by the Celikey™ Varelisa ELISA or ELIA assays, where positivity was assigned to results above 6 U/ml or 7 U/ml, respectively.

Additionally, samples were derived from an independent case-control cohort consisting of patients included in the University Medical Hospital of Milano-Bicocca, Monza, Italy (**Table 1** shows the number of samples included in the final analyses after the quality control; **Supplementary Table S4** shows the number of samples excluded in the quality control) (discussed as the 'Milano-Bicocca cohort'). In this cohort, plasma samples were collected from 33 pediatric CeD patients at time of diagnosis and from 10 of these patients 2 years after start of the GFD. Control plasma samples were obtained from 10 pediatric patients in whom CeD was excluded by histopathological examination of small-intestinal biopsies. For all Milano-Bicocca subjects (both CeD patients and controls), we also had biopsy-derived RNA taken at the time of plasma collection (time of diagnosis). Additional clinical characteristics of participants of the PreventCD and the Milano-Bicocca cohorts are presented in the **Supplementary Materials** and **Supplementary Tables S1**–**3**.

We also aimed to investigate the effect of GFD on circulating miRNA profiles. For this analysis, we used the GFD samples available from the PreventCD and the Milano-Bicocca cohorts but also included samples from a healthy adult cohort of 12 healthy adults without self-reported intestinal or immune-mediated disease background (28, 29) who voluntarily followed a 4-week GFD (**Supplementary Table S4** shows the number of

TABLE 1 | Overview of samples.

Controls					CeD Patients						Healthy Volunteers	
Non-CeD	High-risk CeD				Before Diagnosis				At Diagnosis	On GFD	On GFD	Off GFD
	M4	M6-9	M12	M18-24	**M4**	M6-9	M12	M18-24				
	13	20	17	18	19	22	23	24	21	29		
9*									33*	10		
											12	12

*This overview shows how many circulating microRNA samples were included in the final analyses. M4-M24: months of age. In the CeD patients of the PreventCD cohort, the first sample showing positive IgA anti-transglutaminase antibodies (at seroconversion) or samples close to the diagnostic biopsy were grouped in the "At diagnosis" group. All samples of PreventCD CeD patients taken prior to seroconversion, with negative IgA anti-transglutaminase antibodies, were grouped in the "Before Diagnosis group". *In the Milano-Bicocca cohort intestinal microRNA profiles were generated from duodenal biopsies from 10 controls (all control samples in the biopsy group passed quality control) and 33 patients at diagnosis.*

PreventCD cohort.
Milano-Bicocca cohort.
Healthy volunteer GFD cohort.

samples excluded in the quality control). Circulating miRNA profiles were generated from two plasma samples per individual: one taken during the GFD (4 weeks after start of the GFD) and one taken when eating a regular, gluten-containing diet (either before start of the GFD or after a 2-week wash-out period following the GFD intervention). The study protocol for the GFD cohort was described in detail in Baranska et al. and Bonder et al. (28, 29).

All the protocols of the three studies included in this project were approved by the medical ethics committees of the participating centers and conducted according to the Declaration of Helsinki (15, 27, 29, 30).

Sample Pre-Processing

Samples were collected for the PreventCD study using BD Vacutainer® SST II Advance (number 367957). Samples were centrifuged for 10 minutes at 3000 RPM after which serum was collected and stored at -80°C. For the healthy volunteer GFD cohort, samples were collected using BD Vacutainer® K2E (EDTA) tubes (number 367525). Samples were centrifuged for 10 min at 1300 RPM after which plasma was collected and stored at -80°C.

For the Milano-Bicocca cohort, samples were collected using glass BD Vacutainer® K3EDTA tubes. After collection the tubes were immediately inverted several times to prevent clotting. The samples were maintained at 4°C and processed within 30 min (meaning the time necessary to come back from the hospital). Separation was obtained by centrifugation at 1500 rcf for 15 min in a refrigerated centrifuge and the upper two thirds of the volume was collected to prevent cell contamination. Hemolyzed samples were not collected. Plasma samples were stored at -80°C and shipped to the Netherlands on dry ice.

Previous studies have shown that extracellular microRNA profiles extracted from serum and plasma microRNA are highly correlated (31). However, to avoid bias related to sample type, we did not pool samples from plasma and serum, instead performing separate analyses in the separate cohorts.

RNA Isolation

Serum or plasma samples (50-250 µl) were centrifuged at 1.000xg for 5 min at 4°C to pellet cellular debris. RNA was isolated from the supernatant using the mirVana PARIS kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. To increase RNA purity and yield, the acid-chloroform extraction step and RNA elution step were repeated (32). Subsequently, total RNA was precipitated by adding 0.1 volume of 3M Sodium acetate (pH 5.2), 3 volumes 100% molecular-grade ethanol and glycoblue (Ambion). After vortexing, samples were stored at -80°C for at least 1 hr. Samples were then centrifuged at maximum speed for 30 min at 4°C in an Eppendorf centrifuge. Supernatant was discarded and pellets were washed with 70% molecular-grade ethanol, upon which the samples were centrifuged again for 10 min at 4°C. The supernatant was then removed, and the pellet was dried in a vacuum desiccator for 5 min max. The RNA pellet was subsequently re-dissolved in 5 µl RNAse-free water. RNA was isolated from small-intestinal biopsy material with the miRVana kit (Ambion), and small RNA-libraries were generated from 500 ng isolated RNA.

Small-RNA Library Preparation and Sequencing

Small-RNA libraries were generated as described in the TruSeq Small RNA Sample Prep Kit manual (Illumina, San Diego, CA, USA), performing 15 cycles in the amplification step. In the purification step after cDNA synthesis, glycoblue (Ambion) was used. The cDNA concentration was measured using the LabChip GX (Caliper). Twenty libraries were pooled equimolarly per lane and sequenced on an Illumina HiSeq2500.

Alignment of miRNA Reads and Quality Control

Raw sequencing reads were trimmed and aligned to the most up-to-date version of the reference database, miRBase 22 (26), using a stand-alone version of sRNAbench (version 1.5 - 6/2018). Default settings were applied, with the exception that the number of mismatches allowed between reference database and reads was set from 1 to 0. We used a cut-off of minimally 100 uniquely aligned miRNAs with >1 read counts and >1,000 read counts aligned to miRNAs in total. Samples that met these criteria were subjected to further Quality Control (QC) steps that are explained in more detail in the **Supplementary Methods**: Quality control of the miRNA profiles.

Differential Expression Analyses

All statistical analyses were performed in "R" (version 3.5.1). The R-package compareGroups (version 4.0.0) was applied to assess differences in clinical baseline characteristics between cases and controls, including the Shapiro-Wilks test to decide between normally or non-normally distributed variables. Differential expression analysis was performed using the DESeq2 package (version 1.22.2). For further details, including covariates that were taken into account, see **Supplementary Tables S5**–**S7**. P-values for the differential expression analyses and meta-analyses were adjusted for multiple testing using the Benjamini-Hochberg correction for False Discovery Rate (FDR) (33). MiRNAs were considered significantly differentially expressed at an FDR-corrected P-value < 0.1. The R-package Pheatmap (version 1.0.12) was used to create heatmaps to visualize the log2foldchanges of the differential expression analyses. All other figures were generated using the R-package ggplot2 (version 3.1.0). In the figures that display regularized log-normalized miRNA counts, the counts were corrected for batch and age.

Identification of Circulating miRNAs That Are Early Biomarker Candidates for CeD

To identify circulating miRNAs associated with CeD development, we performed three independent analyses using the PreventCD cohort and the Milano-Bicocca cohort (see **Figure 2**, part 1 *Finding biomarkers for CeD development* and **Table 1**). The results of these three separate analyses were combined to identify which miRNAs showed the most consistent trends over all three analyses. Before this meta-

analyses, the Cochrane's Q test was performed. For all miRNAs that did not show significant heterogeneity (Cochrane's Q P-value >0.05), a fixed-effects meta-analysis was performed using the inverse-variance method to pool the log2fold changes and their standard errors of different comparisons (meta package, version 4.9-5).

Next, after identifying the miRNAs that show characteristic global trends for CeD development, we zoomed in further to examine more specific trends. To get insight into whether the miRNA levels change depending on how close an individual is to seroconversion, we grouped the pre-diagnostic, TGA negative, samples of the PreventCD patients based on how long before seroconversion they were taken (more than 2 years (>2 years), between 2 and 1 year before diagnosis (2>x>1 years), less than 1 year before diagnosis (<1 year)) and compared these to controls (corrected for sex, age and batch). Samples taken at 4 months of age, i.e. before introduction of gluten, were excluded from this analysis.

A potential source of the circulating miRNAs that are biomarker candidates for CeD is the tissue that is affected in CeD – the small intestine. To investigate whether the circulating miRNAs reflect the intestinal miRNA environment in CeD, we performed a differential expression analysis using the miRNA profile of intestinal biopsies of CeD patients *versus* the profile of control biopsies (patients and control biopsies obtained from Milano-Bicocca cohort participants) and compared these results with the circulating miRNA profile.

Identification of GFD-Associated miRNAs

To identify miRNAs that change in response to GFD, three different analyses were performed and subsequently combined in a meta-analysis (see comparisons A-C in **Figure 2** – part 2 *Finding miRNAs that change upon gluten-free diet*; **Table 1**). We applied the same statistical methods for the meta-analysis as described above.

Pathway Analyses

Pathway analyses were performed with the online tool DIANA-miRPath v3.0 database (34). This tool produces a list of genes based on available databases that contain miRNA-gene pairs and performs pathway enrichment analyses using genes that are predicted to be targeted by the set of miRNAs. The standard settings were used, using the KEGG pathways, and only enrichments with FDR <0.05 were considered significant.

RESULTS

Cohort Characteristics

We used three cohorts to identify whether miRNAs in circulation could be indicative of CeD (at diagnosis and in timepoints prior to TGA positivity) or change upon initiation of GFD. The clinical parameters of the three cohorts (PreventCD, the Milano-Bicocca cohort and a GFD intervention cohort) are summarized in **Tables S3A–1C**, and more detailed participant information for the PreventCD and Milano-Bicocca cohorts is described in the "**Supplementary Methods**: Additional participant characteristics of the PreventCD and Milano-Bicocca cohort".

In the PreventCD cohort, the duration of follow up did not differ between high-risk participants who did develop CeD during the study and those who did not develop CeD (P=0.38) (see **Table S3A**). The CeD cases carried the DQ2.5/DQ2.5 or the DQ2.5/DQ2.2 HLA haplotype significantly more often compared to participants who did not develop CeD, consistent with what was observed in the full cohort (15). **Figure 1** shows the levels of TGA of the patients in PreventCD divided by age group, at time of diagnosis and after start of the GFD. For the participants that developed CeD, the diagnostic samples were defined throughout the manuscript as the samples at seroconversion (first sample with positive TGA antibodies) or at diagnostic biopsy, and all the negative TGA samples were designated pre-diagnostic timepoint samples. One of the control individuals displayed transiently elevated TGA levels at 3 years of age, but did not develop CeD in follow up (age 9.5). In most patients, TGA levels normalized after start of the GFD (**Figure 1**). More detailed information on the PreventCD participants is provided in the "**Supplementary Methods**:

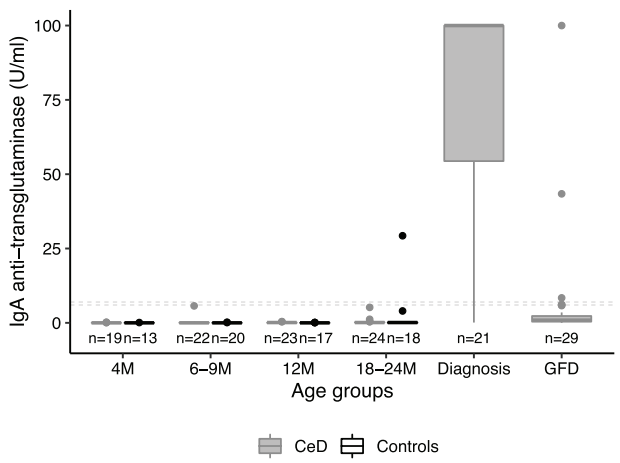

FIGURE 1 | IgA anti-transglutaminase levels peak at diagnosis in the patient group only. IgA anti-transglutaminase levels (TGA) in serum samples of PreventCD participants displayed by age of sampling (CeD=individuals who developed CeD; Ctr=age-matched samples of individuals who did not develop CD; M=Months). For the individuals that developed CeD, we also show serology at diagnosis and after initiating a gluten-free diet (GFD). Samples of individuals in the CeD group that were taken at timepoint of first positive TGA (seroconversion) or at the time of the diagnostic biopsy, were grouped in the diagnosis group (age median: 24, range: 13 - 64 months). One control individual showed positive TGA (29 U/L), but this individual did not have or develop CeD in the follow up (see **Supplemental Methods** for more information). This sample with a positive TGA in the control taken at 3 years of age was grouped with the M18-M24 age group for visualization and analysis purposes. Dashed lines indicate the cutoffs used to assign positivity, depending on the two types of tests used (see *Methods*). Boxplots were generated using the default parameters in the R package ggplot2 (median, second and third quartiles shown by the hinges, individual datapoints are displayed outside the whiskers beyond 1.5 * interquartile range).

Additional participant characteristics of the PreventCD and Milano-Bicocca cohort".

Additionally, samples were collected from an independent Milano-Bicocca cross-sectional cohort consisting of pediatric controls, pediatric CeD patients at time of diagnosis and from 10 of these patients 2 years after start of the GFD (see **Supplementary Methods** for more information about the included participants). In the Milano-Bicocca cohort, no differences were observed in age or sex between non-CeD controls and cases at time of CeD diagnosis and after start of the GFD (the results are displayed in **Table S3B**). TGA levels normalized in the majority of patients for whom we also had samples after start of the GFD (**Table S3B**).

The GFD intervention cohort consisted of adults without self-reported intestinal or immune-mediated diseases who voluntarily followed a 4-week GFD. Unfortunately, anti-transglutaminase antibody measurements were not available for this cohort. In the GFD intervention study no differences were observed with regards to the food-related phenotypes measured (mean energy, protein, carb, fat content per day) or with regards to plasma cytokines, when these individuals were on their normal diet *vs* when on GFD (the results are displayed in **Table S3C**) (29).

Quality Control

After extracting miRNA, library preparation and sequencing, we performed rigorous quality control (QC) to ensure that only high-quality samples were included in our analysis (see "**Supplementary Methods**: Quality control of the miRNA profiles" and **Supplementary Figures S1–4** for an overview of the QC workflow, **Supplementary Table S4** for an overview of the samples excluded during the QC). In total, 206 samples of the PreventCD study (82% of the sequenced total; 53 individuals), 52 samples of the Milano-Bicocca cohort (98%; 42 individuals) and 24 samples of the GFD intervention study (100%; 12 individuals) were included for further analysis (an overview of the samples excluded during QC is provided in **Supplementary Table S4**). All 43 miRNA libraries generated from the small-intestinal biopsy RNA available for the Milano-Bicocca cohort passed QC. The reason for the difference in library preparation efficiency between circulating RNA samples and biopsy-derived RNA samples may be that RNA yield from circulation is low and cannot be detected prior to sequencing of the miRNA libraries when starting with the available serum volumes (50-250 µl). The biopsy library preparations were started with a standard 500 ng RNA. High-quality samples were subsequently used for differential expression analysis.

Circulating miRNAs as Potential Early Biomarkers For CeD Development

To find circulating miRNAs that could function as biomarkers for distinct stages of CeD development, we performed a systematic comparison in three independent cohorts and the results were subsequently summarized in a meta-analysis (see **Figure 2** – part 1 *Finding biomarkers for CeD development* and **Table 1**). The first comparison was performed to identify circulating miRNAs that are predictive markers for CeD development (**Figure 2** part 1, comparison A). Pre-diagnostic samples of children who developed CeD, taken prior to detection of elevated TGA levels, were compared to samples from high-risk controls (the results of this comparison are displayed in **Supplementary Table S5**). The country of sample collection (Netherlands *vs* others) had a limited effect on the differences between CeD and controls: after adding country as a confounder to the statistical analyses, the fold changes between pre-diagnostic samples of CeD and controls were highly correlated to the fold changes without country in the model (R=0.94, P $<2.2*10^{-16}$). Because only one of the control individuals was HLA-DQ2.5 homozygous, we only checked within the patient group whether HLA type had an effect on the miRNA profile (HLA-DQ2.5 homozygous *vs* other HLA). Of the miRNAs significantly different between the pre-diagnostic and control samples, none were significantly different between the HLA groups (FDR>0.3).

Next, in a second comparison, to identify biomarkers at time of diagnosis, we compared the circulating miRNA profile in the PreventCD cohort between diagnostic samples (taken at seroconversion or at diagnostic biopsy) and samples taken at 4 months of age (**Figure 2**, part 1, comparison B; the results of this comparison are displayed in **Supplementary Table S6**). In this comparison, the 4 months samples were used as the baseline because the entire PreventCD cohort is considered free of CeD at this age since gluten has not yet been introduced into their diet. Finally, we used a pediatric case-control cohort (Milano-Bicocca cohort) to find miRNAs that differ between controls and CeD at time of diagnosis (**Figure 2** part 1, comparison C; the results of this comparison are displayed in **Supplementary Table S7**).

To identify which miRNAs had the most consistent trends over these three comparisons (**Figure 2**, part 1, A-C), we combined the results in a meta-analysis. By considering the effect size (including direction of effect) in the meta-analysis, our results are less dependent on the sample size. This approach identified 53 significant miRNAs that were consistently associated with CeD development (the results of the meta-analysis that combines the results of the three separate comparisons are shown in **Supplementary Table S8**). Of the 53 miRNAs, 26 showed decreased levels in CeD and 27 showed increased levels. The trends for these 53 miRNAs in the three separate analyses (**Figure 2**, part 1, A-C) are displayed in **Figure 3**, including the beta of the meta-analysis that represents the pooled direction across the three comparisons.

To assess the contribution of the Milano-Bicocca cohort, we also performed an additional meta-analysis with only the comparisons performed in the PreventCD cohort (**Figure 2**, part 1, comparisons A-B), yielding 41 significant microRNAs. Of the 53 biomarkers significant in the meta-analysis of comparisons A-C (**Figure 2**, part 1), 29 were also significant in the meta-analysis of comparisons A-B (**Supplementary Table S12**). Moreover, there was a high concordance between the direction of effect between the 53 microRNAs significant in the meta-analyses of arms A-C and that of arms A-B (Pearson's correlation coefficient of 0.96 (P<2.2*10-16). These results indicate that the addition of the

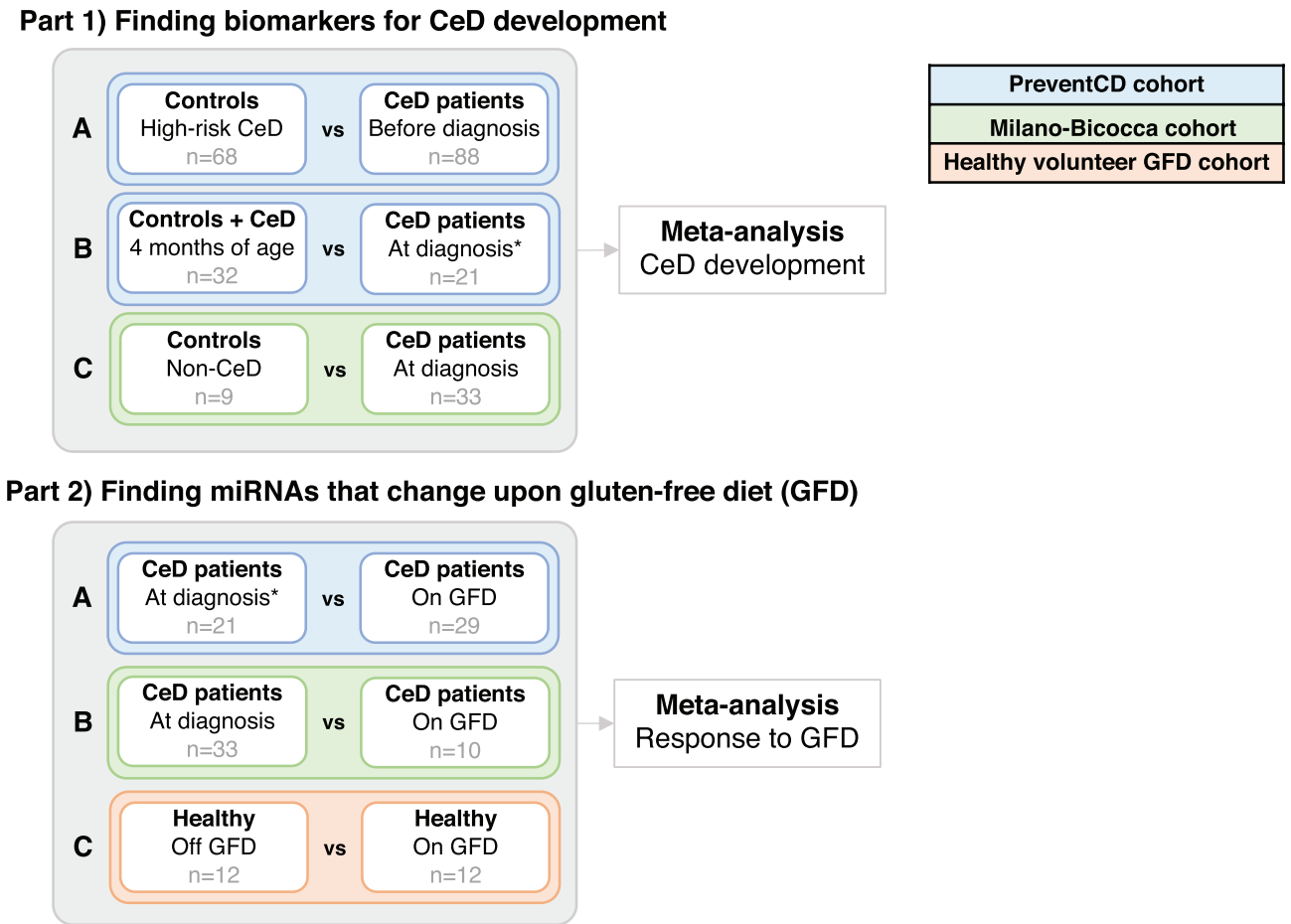

FIGURE 2 | Analyses in the separate cohorts that were performed before combining the results of the differential expression in two meta-analyses. The goals here were to: in part 1) find miRNAs that are potential biomarkers for CeD development and part 2) find miRNAs that change upon the gluten-free diet (GFD). Corresponding sample sizes are shown in grey. *In the PreventCD cohort, the samples "at diagnosis" include samples at seroconversion (first positive IgA anti-transglutaminase (TGA) levels) and samples taken close to the diagnostic biopsy. All samples in the "before diagnosis" groups had negative TGA levels.

Milano-Bicocca cohort (arm C) adds power to the meta-analysis. Therefore, throughout the manuscript the meta-analysis including arms A-C is used to prioritize the biomarker candidates for CeD development.

We then zoomed in on specific trends in the prioritized 53 biomarker candidates in the PreventCD cohort, by grouping the pre-diagnostic samples of the CeD patients (all had negative TGA) by how close to seroconversion the samples were taken (<1 year, 1-2 years and >2 years before seroconversion) (the results of these comparisons are displayed in **Supplementary Table S8** and **Supplementary Figures S5, 6**). Eight of the 53 prioritized miRNAs that were identified in the meta-analysis (miR-21-3p, miR-374a-5p, 144-3p, miR-500a-3p, miR-486-3p let-7d-3p, let-7e-5p and miR-3605-3p) are significantly different between the samples taken closest to seroconversion (<1 year) and control samples (the fold changes and adjusted P-values of these comparisons for these eight microRNAs are shown in **Table 2**; the results for all 53 miRNAs are shown in **Supplementary Table S8**). For some of these eight miRNAs, including miR-500a-3p and miR-3605-3p, the levels in pre-diagnostic samples increasingly diverge from controls coming up to seroconversion and diagnosis, and then show a normalizing trend after start of a GFD (see **Table 2** and **Figure 4**). For two of these eight miRNAs, miR-21-3p (shown in **Figure 5**) and let-7d-3p, we detected a significant difference between pre-diagnostic samples and controls more than 2 years before seroconversion and subsequent diagnosis (**Figure 4**).

To assess the potential influence of age on miRNA levels in controls, we compared the samples taken at 4 and 24 months in controls (**Supplemental Table S11**). This revealed 11 microRNAs that overlapped in the same direction with the comparison M4 *versus* diagnosis (**Figure 2** part 1 comparison B). Only two of these microRNAs (miR-29c and miR-224) were among the 53 biomarker candidates that were prioritized in the final meta-analysis. These results indicate that by combining different comparisons in the meta-analysis, we could filter out most microRNAs for the which the main driver is age-related changes.

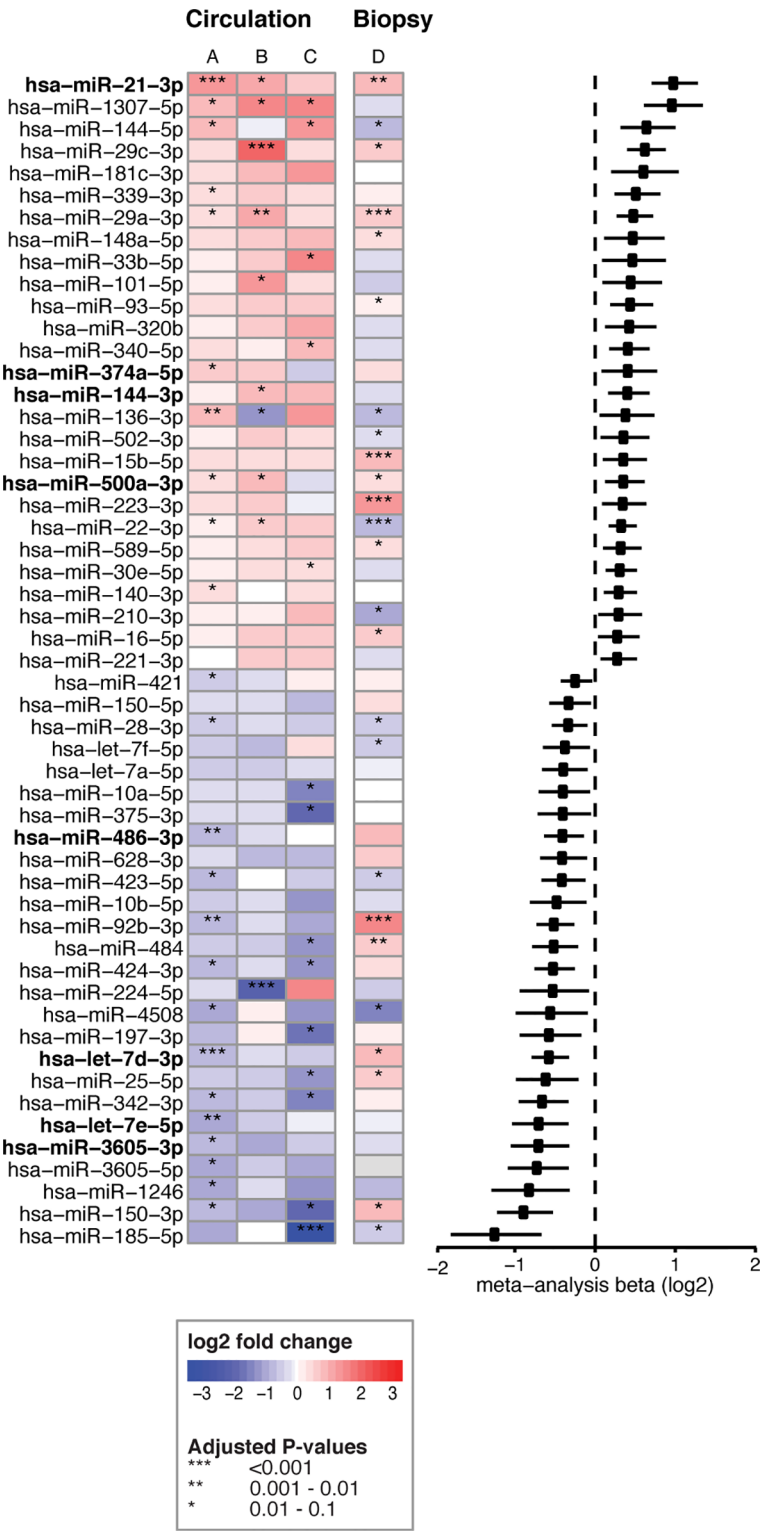

FIGURE 3 | 53 circulating miRNA biomarker candidates for CeD development. Log2fold changes are depicted for three separate differential expression (DE) analyses **(A–C)** of 53 microRNAs that were significant in the meta-analysis combining these analyses. **(A)** PreventCD: pre-diagnostic samples of CeD patients (IgA anti-transglutaminase (TGA) negative) *versus* controls. **(B)** PreventCD: CeD at diagnosis (at seroconversion (TGA positivity) or at diagnostic biopsy) *versus* samples at 4 months of age (before gluten consumption). **(C)** Milano-Bicocca: CeD at diagnosis *versus* controls. **(D)** Milano-Bicocca: CeD at diagnosis *versus* controls in intestinal biopsy samples. Right panel shows a forest plot for the meta-analysis (beta and 95% confidence interval). miRNAs that are detectable < 12 months before diagnosis are indicated in bold.

TABLE 2 | Of the 53 circulating miRNA biomarker candidates for CeD development identified in the meta-analysis (**Figure 2**), these eight miRNAs were significantly different in samples taken <12 months before diagnosis.

	Meta-analysis				>24 M *vs* Controls		12-24 M *vs* Controls		<12 M *vs* Controls		Biopsies (CeD *vs* Controls)	
	beta	se	P	P_{adj}	log2(FC)	P_{adj}	log2(FC)	P_{adj}	log2(FC)	P_{adj}	log2(FC)	P_{adj}
hsa-miR-21-3p	0.99	0.15	1.5E-11	3.9E-09	1.40	4.1E-03	1.25	1.1E-03	1.31	3.5E-04	0.81	4.4E-03
hsa-miR-374a-5p	0.43	0.18	1.6E-02	7.8E-02	0.69	3.3E-01	0.70	3.1E-01	1.10	2.3E-02	0.46	1.6E-01
hsa-miR-144-3p	0.42	0.13	1.4E-03	1.3E-02	-0.15	8.7E-01	0.38	5.3E-01	0.77	3.6E-02	-0.17	7.2E-01
hsa-miR-500a-3p	0.37	0.13	3.3E-03	2.5E-02	0.30	6.4E-01	0.47	3.3E-01	0.96	2.7E-03	0.32	3.0E-02
hsa-miR-486-3p	-0.39	0.13	2.2E-03	1.9E-02	-0.27	6.4E-01	-0.39	3.9E-01	-0.64	7.0E-02	0.74	1.7E-01
hsa-let-7d-3p	-0.56	0.12	3.0E-06	1.3E-04	-0.65	1.0E-01	-0.90	2.8E-03	-0.94	4.8E-04	0.72	8.5E-02
hsa-let-7e-5p	-0.68	0.18	1.4E-04	2.8E-03	-0.27	7.9E-01	-0.89	1.3E-01	-1.53	4.8E-04	-0.01	9.9E-01
hsa-miR-3605-3p	-0.69	0.19	2.3E-04	3.4E-03	-0.82	2.1E-01	-1.08	3.7E-02	-1.09	2.7E-02	-0.20	7.6E-01

Some can even be detected more than 2 years before the first detection of IgA anti-transglutaminase antibodies (seroconversion), >24 M vs Controls. The first set of columns show the results of the meta-analysis. The next three sets of columns show the comparisons in the PreventCD cohort between the samples taken >24 months, 12-24 or <12 months before seroconversion versus control samples [corrected for sex, age and batch and after exclusion of samples taken before introduction of gluten (Month 4)]. The last set of columns shows the comparison between CeD and controls in the small intestinal biopsies (Milano-Bicocca cohort), corrected for age and sex. FC, Fold Change; se, standard error of the beta; Padj, P-value adjusted for multiple testing; Colors, A positive beta or log2(FC) (displayed in green) indicates that the miRNA level is higher in patients who developed CeD; Red, lower in patients who developed CeD; Yellow, Padj<0.1.

Overall, we identified 53 miRNAs that could indicate if a person will develop CeD before the TGA elevation that accompanies intestinal mucosal damage. We hypothesized that the affected tissue in CeD, the small intestine, is a potential source of the 53 CeD-associated circulating miRNAs. Indeed, for the 53 circulating biomarker candidates for CeD, 15 miRNAs are differentially expressed in intestinal biopsies from CeD patients compared to controls, with a concordant direction between circulating and intestinal biopsy-derived miRNAs. The results of the comparison between CeD and controls in the biopsy material are shown in **Figure 3** and **Supplementary Table S8** for the 53 miRNAs that were identified in meta-analysis A. Two of the eight miRNAs that show an early pre-diagnostic increase in circulation, miR-21-3p (displayed in **Figures 4**, **5**) and miR-500a-3p (displayed in **Figures 4** and **Supplementary S6**), are also significantly increased in CeD biopsies (for the results of the comparison in biopsies see **Table 2**). To check if there was a statistically significant enrichment for upregulated miRNAs in CeD biopsies within the miRNAs that are upregulated in circulation, we used a hypergeometric test considering all miRNAs detected by miRNA-seq in both the biopsies and in plasma samples in the Milano-Bicocca cohort. We found a significant enrichment for these miRNAs (P= 5.1 x 10-6), indicating that there is a higher concordance between the differentially expressed miRNAs in circulation and biopsies beyond what would be expected by chance.

Circulating Biomarkers in Relation to the Initiation of a Gluten Free Diet

Next, to assess if miRNAs can be used to assess the impact of a GFD, we performed separate comparisons of miRNA profiles of participants on a GFD (**Figure 2** – part 2). These included comparisons in the PreventCD cohort (CeD) (**Figure 2** part 2 comparison A, no miRNAs were significantly differentially expressed), the Milano-Bicocca cohort (CeD) (**Figure 2** part 2 comparison B; significantly differentially expressed miRNAs in this comparison are shown in **Supplementary Table S9**) and the healthy volunteer cohort (**Figure 2** part 2 comparison C; significantly differentially expressed microRNAs in this comparison are shown in **Supplementary Table S10**) and then subsequently combined these results in a meta-analysis. To discern dietary induced microRNA changes from changes due to healing processes in CeD, we have also investigated a cohort of healthy volunteers that were subjected to GFD. In total, 15 circulating miRNAs were significantly associated with the GFD (the results of the meta-analysis are summarized in **Figure 6**). Of the 53 CeD-associated miRNAs, six miRNAs that had decreased levels in circulation at time of diagnosis were significantly increased in response to the GFD: miR-150-5p, miR-150-3p, miR-1246, miR-342-3p, miR-375-3p and let-7a-5p. **Figure 7** shows miR-150-5p, one example of these CeD-associated miRNAs that start to normalize upon GFD. Circulating miR-150-5p increased upon GFD in all 10 individuals for whom we had paired data at diagnosis and after start of the GFD in the Milano-Bicocca cohort. Thus, we were able to identify several miRNAs that can delineate the start of GFD in CeD patients and control individuals.

Pathway Analyses

We used the DIANA-miRPath v3.0 tool to predict the pathways in which the prioritized circulating miRNAs might play a role. The pathway analysis was performed for the 53 biomarker candidates for CeD development (**Supplementary Figures S7A, B**). The enriched pathways for the miRNAs that were increased in active CeD participants (**Supplementary Figure S7A**) largely overlapped with the pathways found for the miRNAs that decreased upon active CeD (**Supplementary Figure S7B**), as well as the pathways found for the eight miRNAs (**Supplementary Figure S7C**) that increasingly diverge from controls up to diagnosis (shown in **Figure 4**). Top significant pathways include, for example, cell-cycle regulation (hippo signaling pathway, cell-cycle), TGF-beta signaling, fatty-acid metabolism, extracellular matrix interactions and adherence junctions (barrier function). However, because of this overlap, it is difficult to speculate on a functional role for the profiles associated with CeD.

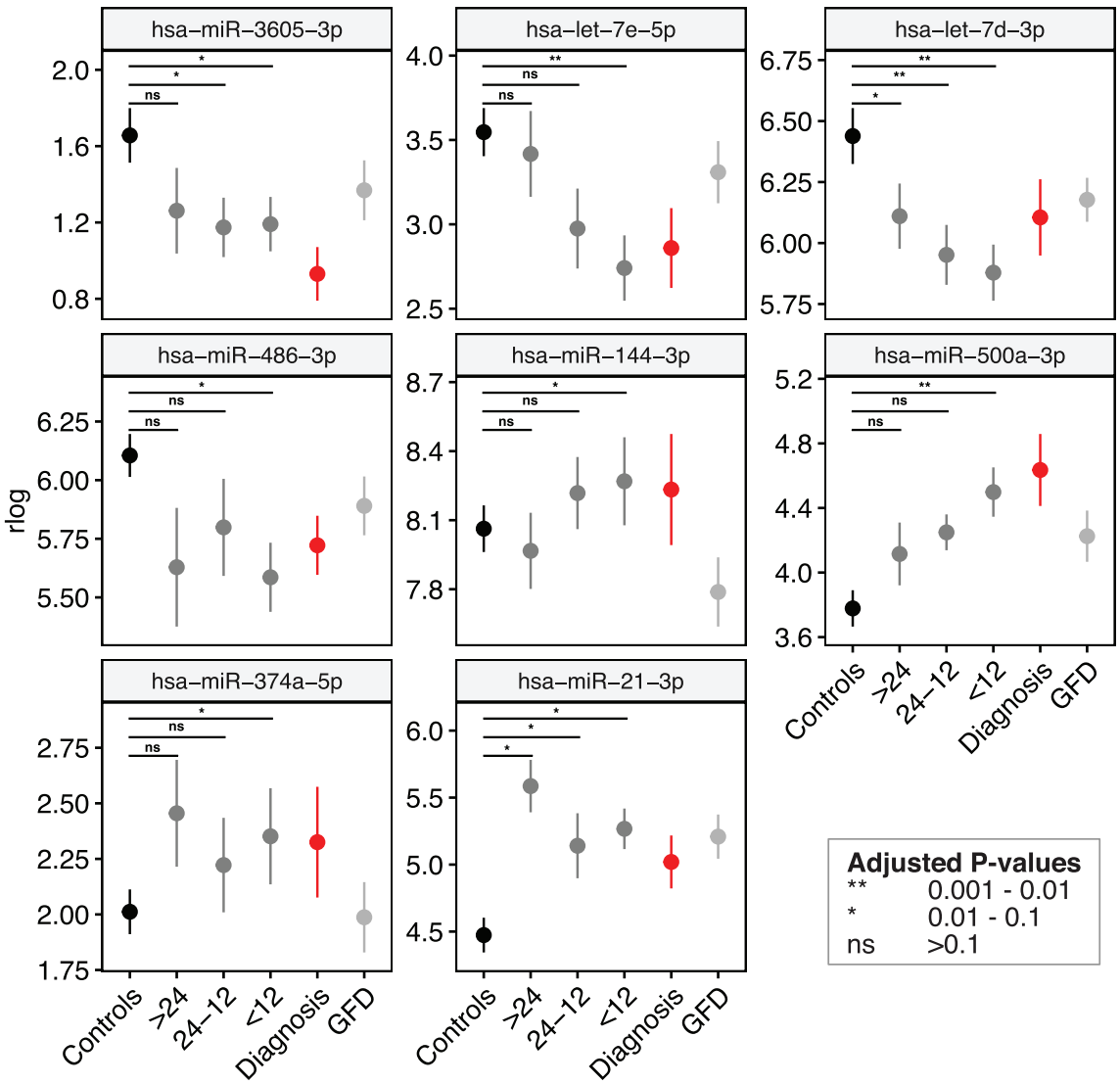

FIGURE 4 | Several miRNA biomarkers for CeD change months to years before detection of CeD serology. The levels of eight out of the 53 microRNAs listed in differ from controls < 12 months before seroconversion (first IgA anti-transglutaminase positivity). Shown are mean values ± standard error of the regularized log-normalized miRNA counts, corrected for batch and age. Black: controls; Dark-grey: pre-diagnostic samples of CeD patients grouped by months till seroconversion (all samples had negative IgA anti-transglutaminase levels); Red: samples at diagnosis (samples at seroconversion or at time of biopsy); grey: CeD patients after start of the GFD.

DISCUSSION

Currently, there are no biomarkers available that can predict the development of CeD before the detection of increased TGA in serum, that is in most cases already accompanied with intestinal mucosal damage. We therefore set out to find novel, non-invasive biomarkers for CeD. For our study, we used three cohorts, including a unique prospective cohort (PreventCD). To our knowledge, our study is the first to apply next generation sequencing to identify miRNAs in circulation in CeD patient samples. By combining the cohorts in a meta-analysis, we identified 53 significant miRNAs that represent potential miRNA biomarker candidates for the development of CeD. Remarkably, eight of these 53 CeD-associated miRNAs could be detected in circulation at an early stage, in some cases more than 2 years before TGA levels were detected above the upper limit of normal. Moreover, we also found six downregulated miRNAs in CeD, including miR-150-3p and miR-150-5p, showed an increased upon a GFD. These miRNA markers are therefore potential markers for CeD, and may be useful for monitoring dietary adherence after start of the GFD. Thus, we have identified a panel of potential miRNA biomarkers that may indicate onset of CeD long before traditional diagnosis of CeD with TGA above the upper limit of normal.

The 53 biomarkers candidates include some miRNAs that have previously been linked to CeD but also some that are being

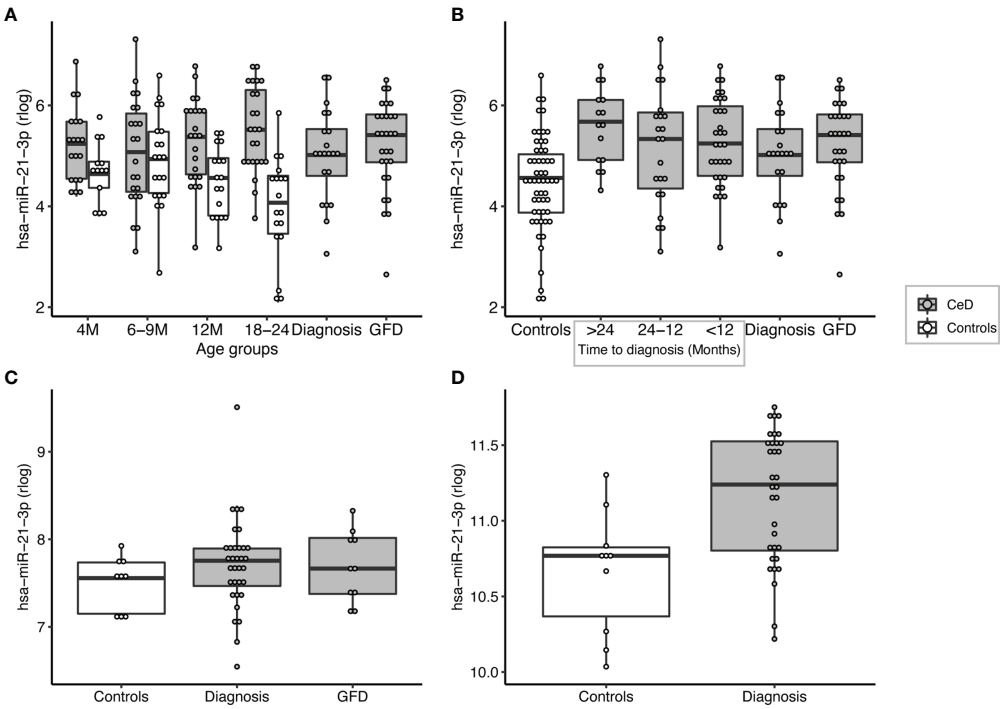

FIGURE 5 | miR-21-3p can be detected at high levels in pre-diagnostic samples of patients but not in age-matched controls and is significantly upregulated in the small intestinal biopsies of CeD patients. **(A)** PreventCD cohort: grouped by age of sampling (M=Months). **(B)** PreventCD cohort: pre-diagnostic (IgA anti-transglutaminase negative) samples of CeD patients are grouped by time till seroconversion: more than 24 months before seroconversion (>24), between 24-12 months before seroconversion (24-12), less than 12 months before seroconversion (<12), or at diagnosis (taken at seroconversion or at time of biopsy) and 6 months after starting GFD. Controls: all samples of the PreventCD controls. **(C)** Circulating miR-21-3p in the Milano-Bicocca cohort (circulation). **(D)** miR-21-3p expression in small-intestinal biopsies in the Milano-Bicocca cohort.

associated with CeD for the first time. For example, Buoli Comani et al. reported that both miR-21-3p and miR-21-5p are highly upregulated in the small intestine of CeD patients and that this elevation was reflected in the circulation (25). This finding was then confirmed by two independent qPCR based studies in which circulating miRNAs were measured (35, 36). Our study, however, is the first to describe that increased levels of miR-21-3p can be detected more than 2 years before the peak in TGA antibodies and the diagnosis of CeD.

Of most interest are the eight miRNAs that were detectable in circulation at a much earlier stage than TGA (in some cases years earlier): miR-21-3p, miR-374a-5p, miR-144-3p, miR-500a-3p, miR-486-3p let-7d-3p, let-7e-5p and miR-3605-3p. For some of these miRNAs, e.g. miR-500a-3p and miR-3605-3p, the difference between pre-diagnostic samples and controls increased depending on how close the samples were taken to the first detection of TGA. In addition, levels of several miRNAs, e.g. miR-500a-3p, normalized after start of a GFD in the PreventCD cohort, although the normalizing effect was not significant. In contrast, miR-21-3p did not (start to) normalize after start of the GFD in the PreventCD cohort. Previously, Bascuñán et al. also reported that miR-21 levels in circulation did not return to normal levels after start of the GFD (37). The observations that miR-21 levels are already elevated more than two years before detection of positive TGA and diagnosis raises the question whether this miRNA is correlated with the development of CeD or rather reflects intrinsic differences between CeD and controls that are independent of the (intestinal) inflammation and intestinal damage. Additionally, the lack of a quick response of these miRNA levels to a GFD might indicate that these miRNAs are not changing because of inflammation/mucosal damage. However, it should be noted that the mucosal healing could take longer than the 6 months after start of the GFD studied in the PreventCD cohort, and adherence to GFD might also influence the response to GFD.

Thus, we observed that that some miRNAs change towards diagnosis (e.g. miR-500a-3p), suggesting that these markers could reflect the pathogenesis of CeD, including immune cell activation, barrier function and mucosal damage. It would be interesting to combine measurements of these miRNAs with other read-outs to detect immune-cell or intestinal function. Other miRNAs, such as miR-21-3p, might represent inherent differences between those individuals who will develop CeD and those who will not, suggesting that this miRNA reflects intrinsic differences between CeD and controls. These intrinsic differences might be linked with factors such as genetic differences and/or immune and intestinal barrier function. Both the biomarkers that reflect the active disease process and the biomarkers that

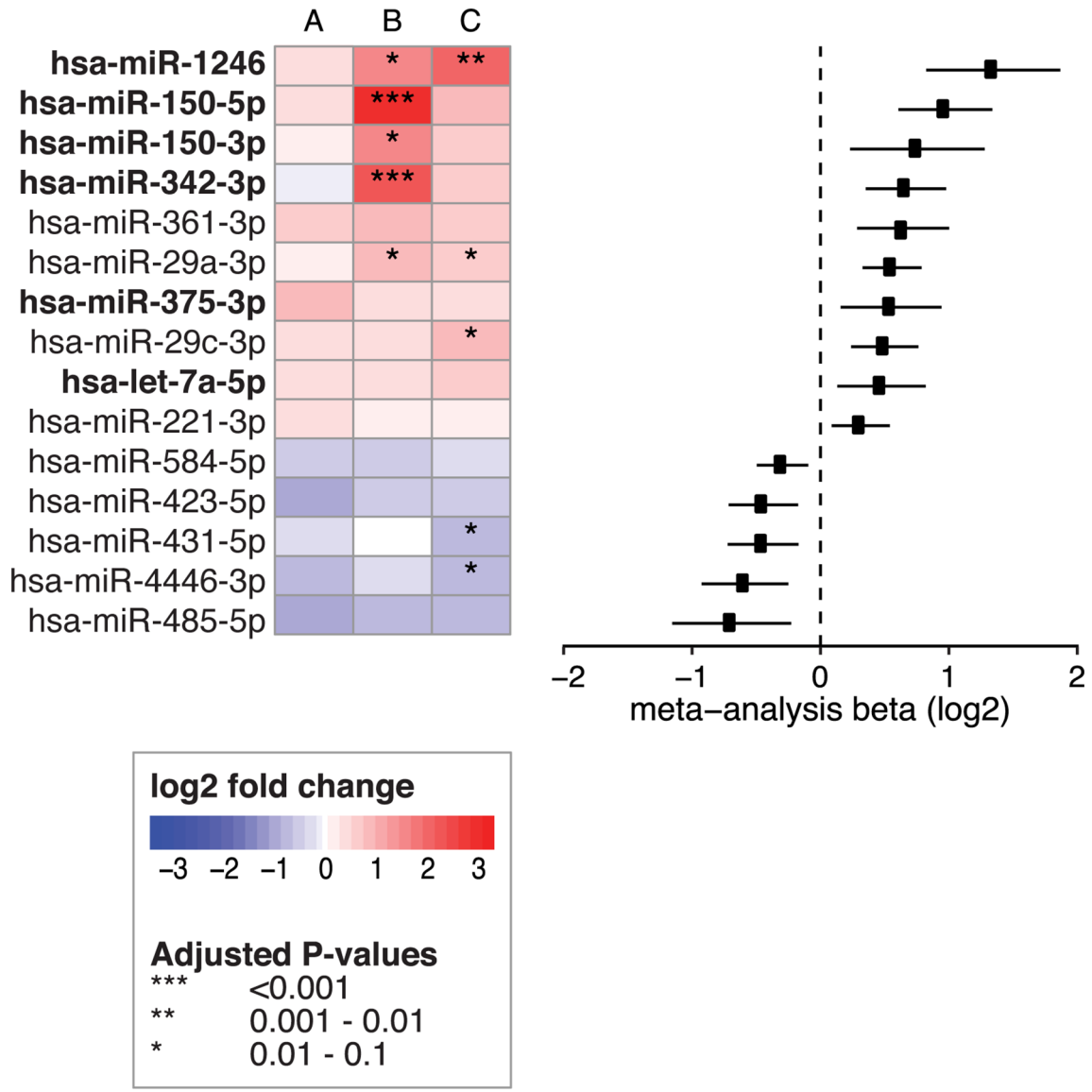

FIGURE 6 | Fifteen circulating miRNAs change after start of the GFD. Left panel shows the 15 circulating miRNAs that were significant in the meta-analysis when combining the following comparisons: **(A)** PreventCD: GFD *vs* CeD at diagnosis (taken at seroconversion or at time of biopsy) **(B)** Milano-Bicocca: GFD *vs* CeD at diagnosis and **(C)** GFD volunteers: GFD *vs* gluten containing diet. Right panel shows forest plot for the meta-analysis (beta and 95% confidence interval). Bold text indicates miRNAs that are also among the 53 CeD biomarker candidates and show a normalizing trend upon GFD.

reflect intrinsic risk factors for development of CeD could be valuable in predicting which individuals are at highest risk of developing CeD.

The tissue and cell type of origin for the 53 extracellular circulating microRNAs that we find to be associated with CeD has yet to be uncovered. We did find that 15 of the 53 miRNAs were differentially expressed in active CeD intestinal biopsies, with a concordant direction between circulation and intestinal biopsies. These included the biomarker candidates mentioned above, miR-21-3p and miR-500a, and an increase of miR-21 and miR-500 in CeD biopsies has also been reported by other independent studies (22, 25). Increased miR-21-3p expression in affected gut mucosa has also been described in inflammatory bowel disease (IBD), as has increased expression of the other strand of miR-21 (miR-21-5p) (38, 39). A possible role of miR-21 in intestinal inflammation is also provided by the observation that, in dextran sulphate sodium mouse models, an experimental model for colitis, inflammation is alleviated in miR-21 knock-out mice (40).

This raises the possibility that the 53 miRNAs identified in this study are associated to intestinal inflammation but not specific for CeD. To our knowledge, miR-21-3p in circulation

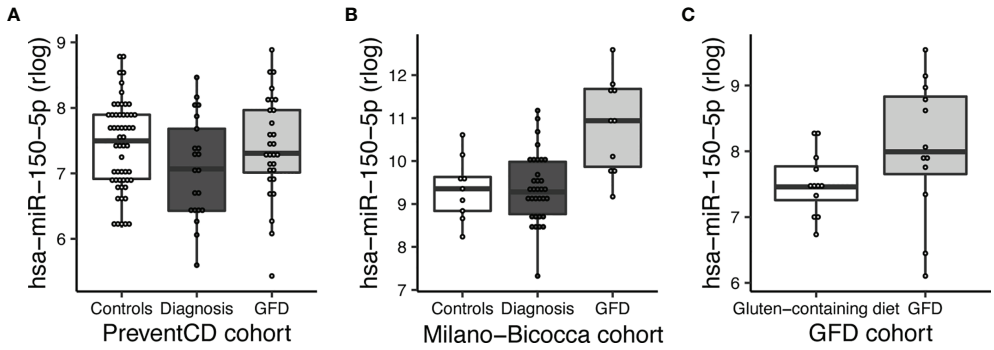

FIGURE 7 | MiR-150-5p is significantly decreased in CeD and reverses after start of a GFD. **(A)** PreventCD: high-risk controls and CeD patients at time of diagnosis (taken at seroconversion or at time of biopsy) and CeD patients after start of a GFD. **(B)** Milano-Bicocca cohort: controls at time of diagnosis (CeD) and at GFD. **(C)** GFD volunteers: on gluten-containing diet or on GFD.

has not been linked to IBD, but the miR-21-5p form is increased in pediatric Crohn's disease (21). If we also compare the other 53 potential CeD markers with two previous array-based studies in IBD, several microRNAs (miR-16, miR-93 and miR-30e) are elevated in serum of IBD compared to controls (21, 41). However, other microRNAs are elevated in IBD but decreased in the serum of CeD patients, including miR-185, miR-484, miR-25 and members of the let-7 family (21, 41). Therefore, the specificity of this panel of potential biomarkers should be tested, including testing in other intestinal enteropathies and autoimmune diseases.

MiRNAs can function as useful biomarkers but may also have distinct roles in CeD pathophysiology through fine-tuning of gene expression levels. It would be interesting to investigate whether the cell types that play a key role in CeD pathophysiology, e.g. intestinal epithelial cells, gluten-specific T cells or intra-epithelial lymphocytes, selectively secrete or take up miRNAs after the cells are stimulated with compounds that mimic the pathogenic conditions in CeD. Examples of previous efforts to identify the source of CeD-associated miRNAs include those of Bascuñán et al., who showed that miR-21 expression is higher in circulating immune cells (peripheral blood mononuclear cells (PBMC)) isolated from active CeD patients than in PBMC from controls. The levels of miR-21-3p did not increase after stimulation with gliadin and/or interferon-γ. These results indicate that miR-21-3p is expressed by immune cells and, according to reference dataset in peripheral blood, has the highest expression in monocytes, CD4+ and CD8+ T-cells (42).

Predicting miRNA function remains difficult. The functions of individual miRNAs are diverse, as one miRNA can target up to hundreds of genes and one gene can have binding sites for multiple miRNAs (43). This makes it difficult to interpret our pathway analysis results, where we saw overlap between miRNAs increased and decreased in CeD. However, we did find non-immune pathways that have been linked to CeD pathophysiology, such as barrier function (adherence junctions) and fatty acid metabolism, and immune pathways like TGF-beta signaling (44–50). We therefore present the pathway analyses to encourage hypothesis-generation about the potential functions of the circulating miRNA profile associated with CeD but acknowledge that further evidence is needed to confirm that these miRNAs influence these biological pathways.

In summary, we show that circulating miRNAs are promising blood-based biomarker candidates to detect pediatric CeD at an earlier stage than the currently available serological tests. Tests could be designed for these miRNAs that can be more easily implemented in clinics than the next-generation sequencing approach used in this study. However, future independent studies are first needed to confirm whether single or combinations of prioritized miRNAs indeed have value in earlier recognition of CeD in high-risk cohorts. The markers that we found to be associated with the GFD should also be confirmed and compared with other potential markers for gluten intake (such as gluten immunogenic peptides) (51). We did not perform sensitivity/specificity analysis of individual single markers in the current study because testing such statistical prediction models in a cross-validation approach requires a larger sample size, or alternatively needs to be assessed in independent studies. These studies would ideally also test other potential biomarkers for CeD, such as T cell receptor bias, that might also provide specificity and sensitivity, although it is still unclear if these will also be predictive of CeD prior to TGA conversion. It might also be beneficial to measure serum miRNAs in individuals who have positive TGA but no villous atrophy (potential CeD) to see whether the miRNA profile is different between individuals who will develop CeD and those who will not. Moreover, the specificity of the miRNAs to CeD as compared to other immune-mediated diseases, especially those of the gastrointestinal tract, should also be investigated. Finally, future studies should further study factors that could potentially influence circulating miRNA levels, including age (pediatric *vs* controls), genetics (e.g. the role of HLA and regional differences). Nonetheless, our findings hopefully pave the way toward preventative strategies in miRNA-positive individuals in the future, which might minimize the onset of active inflammation, decrease villous atrophy and prevent CeD-associated complications in the future (52).

DATA AVAILABILITY STATEMENT

The raw data generated for this paper cannot be shared because this possibility was not covered by the Institutional Review Boards agreement when we initiated the study. However, the miRNA count data are available as **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the participating centers in the different centers that participated in the three different studies (details were published previously). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualization and study design: All authors. Sample collection: DB, CM, MR, EM-O, RS, RA, RT, IK-S, GC, HS, and SK. Sample processing: RCA, RM, and AS. Data analysis and visualization: IT and RCA. Data analysis supervision: VK, YL, IJ, and SW. Writing — original draft preparation: IT, IJ, and SW. Writing — review and editing: RCA, RM, AS, JD, DB, CM, MR, EM-O, RS, RA, RT, IK-S, GC, HS, SK, AZ, VK, YL, MV, RW, MM, and CW. Supervision: SW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.734763/full#supplementary-material

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A Systematic Review of the Progression of Cutaneous Lupus to Systemic Lupus Erythematosus

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Lupus erythematosus is an autoimmune disease that may manifest in a variety of organs and tissues including the skin, kidney, brain, heart and lung. Many patients present with cutaneous lupus, where disease is often limited to the skin, but are at risk for developing systemic lupus. The objective of our present study is to perform a systematic review of studies that investigated patient cohorts and populations for the occurrence of cutaneous lupus progressing to systemic lupus. Inclusion criteria required that studies present longitudinal data of patients with limited cutaneous lupus erythematosus who were followed for development of systemic lupus erythematosus. Studies were excluded if patients had concurrent diagnosis of SLE, or if they failed to present longitudinal data. Medline and Embase were searched for English language studies using the Ovid platform. A total of 25 adult studies were identified, as well as 8 pediatric studies. The rate of cutaneous to systemic lupus progression ranged between 0% to 42% in the adult studies and 0% to 31% in the pediatric groups. The variability in these rates were due to differences in patient populations, study design, criteria used to diagnose systemic lupus, and follow-up time. Common risk factors associated with systemic lupus erythematosus development including having positive anti-nuclear antibodies, hematologic abnormalities, and higher number of lupus classification criteria at baseline. This study emphasizes the importance for providers to routinely monitor for systemic lupus in patients with cutaneous lupus.

Keywords: cutaneous lupus erythematosus (CLE), systemic lupus erythematosus, systematic review, autoimmunity, progression

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INTRODUCTION

Cutaneous lupus erythematosus (CLE) is an autoimmune skin disease with a wide range of clinical presentations. Several subtypes exist including acute cutaneous lupus (ACLE), subacute cutaneous lupus (SCLE), and chronic cutaneous lupus (CCLE), with the most common CCLE subtype being discoid lupus erythematosus (DLE). As early as 1872, Moritz Kaposi identified a characteristic subset of patients with DLE and found that while they may present with limited cutaneous disease, some may progress to systemic involvement (1). Systemic involvement can range from mild in severity, affecting only a single organ system, to potentially severe systemic involvement, affecting multiple organ systems.

Since then, several classification criteria, including the American Rheumatism Association (ARA) criteria, American College of Rheumatology (ACR) criteria, Systemic Lupus International Collaborating Clinics (SLICC) criteria, and the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) criteria, have been developed to help clinicians monitor for the progression of CLE to systemic lupus erythematosus (SLE) (2–5). Clinically, the risk of patients with isolated CLE developing SLE is an area of interest to both the dermatologist and rheumatologist, and CLE patients. Current screening recommendations suggest monitoring patients for various lab abnormalities and clinical symptoms included in the lupus classification criteria sets, including the development of hematological abnormalities, autoantibodies including anti-nuclear antibodies (ANA) and double-stranded DNA (dsDNA) antibodies, and signs of joint, kidney or neurologic involvement (6). Current standard of care involves checking CLE patients for systemic disease on presentation as well as interval assessments for the development of SLE (6, 7).

The phenomenon of CLE developing to SLE has been studied in a variety of settings and populations, with the rate of progression ranging from zero to over thirty percent (8–10). Notably, methodologies amongst studies have often differed with respect to the studied population, definitional criteria of SLE, length of follow up, and study design. Prior reviews aimed at summarizing these studies have been limited to narrative reviews, narrow timeframe, or confined to a single subtype of CLE (11, 12). In order to better summarize these data, we performed a systematic reviews of all studies that have investigated patient cohorts and populations for the occurrence of CLE progressing to SLE. The information gleaned from this systematic review will help equip providers with counseling these patients about their prognosis and direct the management of these patients to track disease progression.

METHODS

This systematic review was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (13). The objective was to identify studies of patients with skin limited cutaneous lupus and the rates of development of systemic lupus to better examine how studies evaluate and characterize this transition. The primary outcome of interest was the proportion of patients with CLE who developed SLE. Inclusion criteria were that studies identified cohorts of patients with CLE without SLE initially. Studies were excluded if patients had concurrent presentation of CLE and SLE, or did not present longitudinal data (either retrospective or prospective) for the development of SLE.

English language literature was searched using the MEDLINE and Embase databases. Databases were searched from inception until the date of the search using the Ovid platform. Databases were searched for articles with keywords, titles, abstracts including cutaneous lupus or its subtypes (i.e. discoid lupus, lupus panniculitus, lupus profundus, bullous lupus, subacute cutaneous lupus, lupus tumidus) and systemic lupus. Two separate reviewers (P.C. and A.W.) independently appraised all studies meeting inclusion and exclusion criteria. Disagreements were discussed and consensus reached involving a third reviewer (B.F.C.) whenever appropriate. Full text articles were then screened for inclusion in the present study and reference lists of primary studies were searched for additional studies meeting inclusion criteria.

RESULTS

After removing duplicates in the OVID platform, a total of 2,842 titles and abstracts were screened for articles potentially meeting inclusion criteria. Of these, 85 full-text articles were selected for in-depth review with a total of 33 articles relevant articles identified meeting our inclusion criteria. This included 25 articles of adult CLE patients, and 8 pediatric CLE studies, which will be summarized in the following sections. A complete PRISMA flow chart is included in **Supplementary Figure 1** (13).

Adult CLE

Studies looking at adult CLE patients reported a broad range of CLE to SLE progression. The rate of CLE to SLE progression ranged from 0 to 42 percent of CLE patients developing SLE (**Table 1**). The number of patients with CLE only and therefore eligible to progress varied widely amongst studies, ranging from small cohorts of only 5 patients to large, database studies of over 20,000 patients (18, 24, 30). DLE was the most commonly studied CLE subtype amongst all studies examined (20/25). SCLE was the second most commonly represented subtype (10/25). Notably, one study found that patients with SCLE had higher rates of progression than those with DLE (9). Most studies analyzed CLE patients from multiple subtypes. While several studies did report on various CLE subtypes other than DLE (e.g. lupus erythematosus panniculitis, lupus erythematosus tumidus), this accounted for a relatively small proportion of the overall data studied.

Studies used several different metrics to define SLE. Most studies (7/25) used the 1982 ACR SLE criteria (18, 22, 23, 26, 35–37). Four studies pre-dated the development of the 1982 ACR criteria and used ARA criteria (25, 27, 28, 33). Two studies used the 2012 SLICC classification criteria (21, 31). None have employed the 2019 EULAR/ACR criteria. One study used more than one classification criteria set to compare rates of CLE to SLE progression. From a cohort of 93 patients with CLE, our group reported 10.8% developing SLE under the SLICC criteria and 16.1% under the ACR criteria, highlighting potential differences between criteria sets (17). Five adult studies used diagnostic codes for large data sets (9, 16, 24, 30, 32). Six studies did not specify a defined criteria set/methodology (14, 15, 19, 20, 29, 34).

The length of follow up was variable among studies. For instance, 11 out of 25 studies only reported a range of years from which records were reviewed instead of average follow-up time

TABLE 1 | Summary of results from adult cohort studies.

Author	Year	Total CLE Patients (n)	CLE to SLE n, (%)	Time to Progression	SLE Diagnostic Method
Aitmehdi et al. (14)	2021	14	1 (17)	NA	NA
Al-Saif et al. (15)	2012	56	6 (11.8)	10.5 months (mean)	NA
Baek et al. (16)	2020	27	27 (4.3)	1.53 years (mean)	ICD-10
Black et al. (17)	2021	93	10 (10.8) by SLICC, 15 (16.1) by ACR	7.8 years (SLICC, mean)	SLICC and ACR
Braunstein et al. (18)	2013	5	1 (20)	NA	ACR
Callen et al. (19)	1982	56	4 (6.5)	NA	NA
Casarrubias et al. (20)	2019	8	2 (25)	NA	NA
Chanprapaph et al. (21)	2021	42	4 (9.5)	5.6 months (median)	SLICC
Drenkard et al. (22)	2019	190	9 (5.3) at one year and 16 (12.3) at three years	NA	ACR
Durosaro et al. (23)	2009	156	19 (12.2)	8.2 years (mean)	ACR
Gronhagen et al. (9)	2011	828	107 (12.9)	NA	ICD-10
Hall et al. (24)	2017	20,878	4,715 (11)	12.8 months (mean)	ICD-9
Healy et al. (25)	1995	58	3 (5.2)		ARA
Kindle et al. (26)	2016	9	0 (0)	NA	ACR
Leibowitch et al. (27)	1981	42	4 (9.5)	NA	ARA
Millard et al. (28)	1979	92	6 (6.5)	NA	ARA
Ng et al. (29)	2002	10	1 (10)	NA	NA
Petersen et al. (30)	2018	1674	199 (11.9)	2.05 years (median)	ICD-10
Preti et al. (31)	2019	12	5 (42)	NA	SLICC
Rees et al. (32)	2015	1002	145 (14)	NA	Read Codes
Schiodt et al. (33)	1984	56	5 (8.9)	NA	ARA
Scott et al. (34)	1959	274	14 (5)	NA	NA
Wieczorek et al. (35)	2014	77	13 (17)	8.03 years (mean)	ACR
Wu et al. (36)	2018	25	6 (24)	NA	ACR
Xie et al. (37)	2020	17	5 (29.4)	NA	ACR

ACR, American College of Rheumatology; ARA, American Rheumatism Association; CLE, cutaneous lupus erythematosus; ICD-9, International Classification of Diseases, ninth revision; ICD-10, International Classification of Diseases, tenth revision; NA, not applicable; SLE, systemic lupus erythematosus; SLICC, Systemic Lupus International Collaborating Clinics.

(9, 15, 20–23, 27, 30–33). Some studies chose to report a range of years from which records were obtained and a minimum length of follow up of 6 months (16, 17, 37). Other studies chose to report median or mean length of time to follow up, ranging from a median of 40 to 48 months or a mean of 16.7 months to 5.75 years (14, 19, 26, 29). In addition, some studies reported variable rates that were dependent on length of follow up. For instance, Gronhagen et al. reported that when follow up data for one year was analyzed, 9.7% of CLE patients developed SLE; when sufficient follow up data was available for 3 years, this shifted to 16.7% (9).

Heterogeneous data on risk factors for CLE to SLE progression and time to progression were available from a minority of studies. From the adult studies, the most common patient and clinical risk factors associated with SLE development included positive ANA (5/25), hematologic abnormalities (2/25), and number of classification criteria met at baseline (2/25) (15, 17, 21, 25, 28, 35). Studies often differed on significant risk factors. Al-Saif et al. reported that CLE patients who progressed to SLE had more sunlight exposure, were ANA positive, and had a positive dsDNA antibody. They also found that progression of disease was significantly correlated with an earlier age of onset (p=0.044). Our group identified baseline risk factors for disease progression under the SLICC criteria including positive ANA (p=0.02), SLICC immunologic criteria (p=0.002), and SLICC total criteria (p=0.007) (17). Other studies identified baseline risk factors including non-scarring alopecia and high initial ANA titer ≥1:320 (21), hematologic abnormalities and positive ANA (28), and mucocutaneous criteria, positive ANA, total number of ACR criteria, and generalized DLE (35). Time to progression was reported inconsistently among studies and ranged anywhere from a mean of 5.6 months to a median of 8.2 years for adult cohorts (21, 23). One study reported significantly different median time to progression for subtypes of CLE including 3.04 years for DLE, 1.65 years for SCLE, and 1.04 years for localized CLE (p=0.018) (30).

Pediatric CLE

Eight studies looking at CLE to SLE progression amongst pediatric cohorts were found. Similar to the adult cohort studies, there was also a broad range of progression rates among pediatric populations, ranging from 0 to 31 percent of patients developing SLE (**Table 2**). However, the cohort size of patients with CLE and therefore eligible to progress to SLE was notably smaller than that of adult cohort studies, ranging from 10 to 276 total patients (41, 43). Similar to adult studies, DLE was the most commonly analyzed subtype representing over 60% of pediatric studies. Two studies examined a mixed cohort of multiple subtypes (8, 40). One small cohort study was dedicated to lupus erythematosus profundus (43).

In terms of criteria sets for SLE diagnosis, pediatric studies most commonly used the ACR criteria to define SLE progression (3/8 studies) (8, 38, 42). Ezeh et al. reported rates of progression for both ACR (20%) and SLICC (25%) criteria in the same cohort of patients (41). The remainder of pediatric studies did not specify a specific classification or diagnostic criteria used to determine the progression of CLE to SLE in their patient cohorts (10, 39, 40, 43). Like adult studies, follow-up length for pediatric cohorts was variably reported, with studies reporting a median follow up time ranging between 1 and 11 years (8, 10).

TABLE 2 | Summary of results from pediatric cohort studies.

Author	Year	Total CLE Patients (n)	CLE to SLE (n, %)	Time to Progression	SLE Diagnostic Method
Arkin et al. (38)	2015	34	9 (26)	NA	ACR
Cherif et al. (39)	2003	16	0 (0)	NA	NA
Dickey et al. (40)	2013	38	1 (2.6)	NA	NA
Ezeh et al. (41)	2019	276	55 (20) by ACR and 69 (25) by SLICC	NA	ACR and SLICC
George et al. (10)	1993	16	5 (31)	NA	NA
Lee et al. (8)	2019	11	0 (0)	NA	ACR
Moises Alfaro et al. (42)	2003	27	7 (26)	NA	ACR
Tinoco-Fragoso et al. (43)	2016	10	0 (0)	NA	NA

ACR, American College of Rheumatology; CLE, cutaneous lupus erythematosus; NA, not applicable; SLE, systemic lupus erythematosus; SLICC, Systemic Lupus International Collaborating Clinics.

Only three studies commented on risk factors for progression. Risk factors included: higher age at diagnosis of DLE and positive autoantibodies, positive serologies and higher-titer ANA, and positive family history for rheumatic disease ($p<0.05$) (38, 41, 42). Only one study, Arkin et al., reported data on time to progression and noted that pediatric patients were at greatest risk for CLE to SLE progression within the first year after CLE diagnosis (38). However, they note that their study was limited to a follow-up duration of 5 years.

DISCUSSION

This systematic review encompassed a broad range of studies, reporting on both adult and pediatric CLE groups. In adults, all but one study showed a proportion of CLE patients ultimately developing SLE. While a minority of CLE patients will go on to develop SLE, this proportion is sizeable enough to highlight the need for CLE patients to have ongoing monitoring for the development of SLE. Interestingly, data was somewhat more bimodal in the pediatric studies, with several studies reporting that no CLE patients progressing to SLE, but other studies reporting higher risk of 20%-30%. This discrepancy in reported risks may reflect study level characteristics or varying patient populations. The relatively limited number of pediatric studies highlights the need for more data to better characterize the risk of developing SLE within the pediatric population.

Studies used a variety of different metrics to define SLE. Larger population studies used diagnostic codes to identify patients with SLE. While this may be less rigorous on a patient level basis, it does allow for examining a significantly broader segment of the population and provide greater context of this phenomenon. For smaller studies, specific SLE classification criteria, including the ARA, ACR, and SLICC criteria, were employed for each patient and their disease course. Studies that examined multiple diagnostic criteria both supported the risk of transition to SLE. The similarly reported rates within studies that employed multiple SLE diagnostic criteria suggests that this distinction may not account greatly for the discrepancies in progression rates between studies. For example, Ezeh et al. reported on both SLICC and ACR criteria, yielding 20% progression under ACR criteria and 25% under SLICC criteria (41). Conversely, Black et al. reported 10.8% development from CLE to SLE using SLICC criteria and 16.1% with ACR criteria (17). The small variation in rates were thought to be, in part due to application of photosensitivity as a diagnostic criteria in ACR but not SLICC.

A variety of risk factors have been proposed to influence the risk of development of SLE, which was more commonly studied in adult CLE patients than pediatric CLE patients. Disease severity, CLE subtype, autoantibodies (anti-dsDNA and anti-Smith), arthritis, and high titers of ANAs have been reported to be more commonly found in CLE patients progressing to SLE than those who have not (11, 44). In our review of prior studies, the most common risk factor reported was a positive ANA (15, 17, 21, 28, 35, 41). Other common risk factors included hematologic abnormalities, age at CLE onset, lupus specific antibodies like dsDNA, and mucocutaneous criteria (15, 21, 25, 28, 35, 38, 41). Disparities in risk factor reporting can be attributed to differences in study design, population, and methods of reporting SLE diagnosis. Future larger-scale studies with uniform SLE diagnosis reporting are needed to further confirm risk factors that portend higher chance for systemic progression in CLE patients. In addition, most CLE patients who ultimately progressed to SLE in the studies examined by this review rarely met criteria that would signify involvement of major organ systems (e.g. renal, neuro), highlighting the overall mild severity of systemic involvement seen in CLE patients who progress to SLE (17, 21, 35).

It has been hypothesized that antimalarial treatment with may slow or prevent the progression of systemic disease (45). To address this hypothesis, there is an ongoing multi-center randomized controlled trial looking at whether hydroxychloroquine can halt progression of lupus in at-risk individuals such as those with CLE (46). Given that lupus medications may slow development to SLE, the rate of progression may be higher in untreated CLE individuals. While none of the reported studies looked at effects of therapies on progression, we hypothesize that because most patients in these studies were under treatment, reported rates of progression from CLE to SLE may be conservative.

In conclusion, this study summarized findings from adult and pediatric CLE patient groups showing ranges of progression to SLE. Prior studies showing up to 42% of CLE patients progressing to SLE highlight the importance for monitoring CLE patients for the development of systemic disease clinically at routine intervals. We recommend that providers perform complete review of systems to identify any new systemic symptoms such as small joint pains, and thorough skin exams to check for worsening skin disease and presence of oral ulcers lasting more than two weeks. Laboratory tests including ANAs

and complete blood counts can be also ordered, with positive ANA titers being followed up with additional autoantibody tests including dsDNA and extractable nuclear antibody tests (6). Importantly, larger multi-center studies using standard and uniform reporting of SLE diagnosis and heterogeneous populations are necessary to better estimate rates of and identify risk factors for development of SLE in CLE patients.

AUTHOR CONTRIBUTIONS

PC, AW, and BC contributed to conception and design of the study. PC and AW contributed to the acquisition and analysis of the data. PC and AW drafted the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.866319/full#supplementary-material

Supplementary Figure 1 | PRISMA Flow Diagram for literature search. Diagram shows searching and selection strategy at each stage of search.

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Kappa Free Light Chains, Soluble Interleukin-2 Receptor, and Interleukin-6 Help Explore Patients Presenting With Brain White Matter Hyperintensities

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Introduction: Many patients are referred to multiple sclerosis (MS) tertiary centers to manage brain white matter hyperintensities (WMH). Multiple diagnoses can match in such situations, and we lack proper tools to diagnose complex cases.

Objective: This study aimed to prospectively analyze and correlate with the final diagnosis, cerebrospinal fluid (CSF) interleukin (IL)-1β, soluble IL-2 receptor (CD25), IL-6, IL-10, and kappa free light chains (KFLC) concentrations in patients presenting with brain WMH.

Methods: All patients over 18 years addressed to our MS tertiary center for the diagnostic workup of brain WMH were included from June 1, 2020, to June 1, 2021. Patients were separated into three groups—MS and related disorder (MSARD), other inflammatory neurological disorder (OIND), and non-inflammatory neurological disorder (NIND) groups—according to clinical presentation, MRI characteristics, and biological workup.

Results: A total of 176 patients (129 women, mean age 45.8 ± 14.7 years) were included. The diagnosis was MSARD ($n = 88$), OIND ($n = 35$), and NIND ($n = 53$). Median CSF KFLC index and KFLC intrathecal fraction (IF) were higher in MSARD than in the OIND and NIND groups; $p < 0.001$ for all comparisons. CSF CD25 and IL-6 concentrations were higher in the OIND group than in both the MSARD and NIND groups; $p < 0.001$ for all comparisons. KFLC index could rule in MSARD when compared to NIND (sensitivity, 0.76; specificity, 0.91) or OIND (sensitivity, 0.73; specificity, 0.76). These results were similar to those with oligoclonal bands (sensitivity, 0.59; specificity, 0.98 compared to NIND; sensitivity, 0.59; specificity, 0.88 compared to OIND). In contrast, elevated CSF CD25 and IL-6 could rule out MSARD when compared to OIND (sensitivity, 0.58 and 0.88; specificity, 0.95 and 0.74, respectively).

Discussion: Our results show that, as OCBs, KFLC biomarkers are helpful tools to rule in MSARD, whereas elevated CSF CD25 and IL-6 rule out MSARD. Interestingly, CSF IL-6 concentration could help identify neuromyelitis optica spectrum disorder, myelin oligodendrocyte glycoprotein antibody-associated disease, and central nervous system (CNS) vasculitis. These results need to be confirmed within more extensive and multicentric studies. Still, they sustain that KFLC, CSF CD25, and CSF IL-6 could be reliable biomarkers in brain WMH diagnostic workup for differentiating MSARD from other brain inflammatory MS mimickers.

Keywords: white matter hyperintensities, multiple sclerosis, biomarker, IL-6, sIL-2R, kappa free light chains

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating central nervous system (CNS) disease. It presents as relapsing clinical demyelinating events or a progressive worsening neurological deficit disease with suggestive white matter hyperintensities on the brain or spinal cord MRI T2-weighted images. Clinical research has focused on diagnosing MS as early as possible to prevent relapse and disability by initiating disease-modifying treatments. In this condition, many patients may have an early demyelinating disease diagnosis: i) after a single demyelinating event (1, 2) or ii) before any clinical event (3, 4). In early-MS patients, biology may have an essential role in identifying an intrathecal B-cell activation by the detection of cerebrospinal fluid (CSF) oligoclonal bands (OCBs) on isoelectric focusing, which can replace dissemination in time in patients presenting with a typical first demyelinating event (1, 2). Unfortunately, misdiagnosis may occur in such situations (5, 6), while many other neurological diseases may mimic early MS (6, 7).

The immunopathology of MS is complex and implicates a large number of cells. CD4+ Th1 and Th17 cells are thought to promote while CD4+ Th2 and Treg cells are thought to downregulate inflammation in MS (8–10). B cells are also crucial effector cells in MS (11). In contrast, i) B cell-depletive therapies are effective in relapsing MS (12, 13), and ii) intrathecal immunoglobulin synthesis is part of the MS diagnostic criteria (1). However, we lack a reliable biomarker that could help separate MS from other inflammatory-mimicking diseases to avoid misdiagnosis.

During the last decade, many biomarkers have been explored. Kappa free light chains (KFLC), low-weighted immunoglobulin compounds, are a reliable biomarker in MS (14–16). This activated B-cell biomarker has the advantage, compared to OCBs, to quantify intrathecal B-cell activity by an automatized procedure. However, prospective data on the effectiveness of KFLC biomarkers are poor (14). Cytokines are low-molecular-weight proteins secreted by many cells, implicated in many immune functions, such as chemotaxis, activation, or repression of the immune cells. In autoimmune CNS diseases, cytokine measurement may reflect a unique immunopathological profile and help etiological diagnosis. It has been shown that CSF interleukin (IL)-6 is increased in neuromyelitis optica spectrum disorders (NMOSD) or in myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD) compared to MS (17). Soluble IL-2 receptor (s-IL2R), also called CD25, is increased in many CNS granulomatosis, such as neurosarcoidosis or in infectious meningitis (18), and CSF IL-10 is now part of the diagnostic workup in CNS lymphoma (19). However, our knowledge about cytokine expression in such diseases comes from retrospective cohorts. Based on these data, our MS tertiary center included OCBs, KFLC, and CSF IL-1β, sIL-2R, IL-6, and IL-10 concentration measurement in the routine diagnostic workup of patients presenting with white matter hyperintensities suggestive of MS.

Therefore, in this study, we prospectively evaluated the expression of KFLC biomarkers and CSF concentration of IL-1β, sIL-2R (CD25), IL-6, and IL-10, and we correlated each biomarker measurement with diagnosis in patients referred to our MS center for suspected MS.

METHODS

Patients

All patients referred to our MS tertiary center in the University Hospital of Nice, France, were eligible for the study from June 1, 2020, to June 1, 2021. Patients were included if they i) were at least 18 years old and ii) had brain white matter hyperintensities on MRI T2-weighted images. According to routine care, all patients underwent the same diagnostic workup with a blood and CSF analysis and 3-T brain MRI.

At the end of the diagnostic workup, patients were separated into three groups according to their diagnosis. First, patients were divided as having an inflammatory or a non-inflammatory CNS disorder according to clinical presentation, MRI (topography, number, size, and gadolinium enhancement of the lesions), and biology (identification of blood or CSF red flags for MS). All non-inflammatory diagnoses were pooled together as a control group named non-inflammatory neurological disorder (NIND). Patients identified as having an inflammatory CNS disorder were separated into two groups. Patients who fulfilled the 2017 McDonald criteria for MS and clinically isolated syndrome (CIS) (1), or the 2009 criteria for radiologically isolated syndrome (RIS) (3, 20), were pooled together into the MS and related disorder (MSARD) group.

The other inflammatory patients were pooled together as having another inflammatory disease: the other inflammatory neurological disease (OIND) group.

A non-opposition to research was obtained for each patient according to French law. Our institutional review board approved the study design, and the study was registered on Clinical Trial (NCT05056740) as the CyBIRD (Cytokine and Brain Inflammatory Related Disorders) Study.

Kappa Free Light Chains and Cytokine Measurement

Blood and CSF were collected the same day for all patients. Fluids were sent within 2 h after collection into the Immunology Laboratory of Nice's University Hospital.

Detection of OCBs was performed by isoelectric focusing and subsequent immunoglobulin using IgG-specific antibody staining. OCB patterns were evaluated by experienced biologists and classified as negative or positive. A cutoff ≥2 CSF-restricted bands was used to define OCB positivity. CSF KFLC was measured on fresh samples using the turbidimetric analyzer Optilite® (The Binding Site, Birmingham, UK) with the serum-free light chain immunoassay Freelite® (The Binding Site, Birmingham, UK). Serum and CSF albumin were also measured with the same turbidimetric analyzer and permitted to calculate KFLC index and KFLC intrathecal fraction (KFLC IF) as follows:

(i) KFLC index = KFLC quotient/albumin quotient with:

$$KFLCquotient = CSFKFLC/serumKFLC$$

$$Albuminquotient = CSFalbumin/serumalbumin$$

(ii) KLC IF was determined with Reiber's formula (21):

$$KFLCIF(\%) = (KFLC_{loc}/CSFKFLC) \times 100 \ with$$

$$KFLC_{loc} = (KFLCquotient/KFLCquotient(\mathrm{lim})) \times serum\ KFLC\ with$$

$$KFLCquotient(\mathrm{lim}) = 3.27\left(albuminquotient^2 + 33\right)^{0.5} - 8.2\left(\times 10^{-3}\right)$$

The turbidimetric analyzer's lower detection limit (LDL) for KFLC was 0.33 mg/L. For patients with CSF KFLC concentration lower than the LDL, an empirical value of KFLC = LDL/2 = 0.16 mg/L was assigned.

For cytokine measurement, CSF was directly centrifuged and kept frozen at −80°C until there were enough stored samples to perform analysis (16-well cartridges). CSF was thawed once, just before cytokine analysis. CSF IL-1β, sIL-2R (or CD25), IL-6, and IL-10 concentration were determined using a mixture of 25 µl of CSF and buffer, in a custom-designed cartridge Ella (ProteinSimple, Santa Clara, CA, USA) for the detection of IL-1β, sIL-2R, IL-6, and IL-10, according to the manufacturer's instructions. The LDL of ELISA kits for cytokine measurement was 0.32 pg/ml for IL-1β, 6.56 pg/ml for sIL-2R, 0.5 pg/ml for IL-6, and 1.16 pg/ml for IL-10. As for CSF KFLC measurement, when the CSF cytokine concentration was under the LDL, an empirical CSF cytokine value of LDL/2 was assigned.

Statistical Analysis

Statistical analysis was performed using the online application EasyMedStat (version 3.14; www.easymedstat.com).

Data were presented as means with their SD for continuous values and counts and percentages for categorical variables for descriptive statistics. The data's normality and heteroscedasticity were assessed using the Shapiro–Wilk and Levene's tests. Constant values were compared using the Mann–Whitney U test. When more than two groups needed to be compared, the Kruskal–Wallis test was performed with a *post hoc* Conover's multiple comparison test. Categorical variables were compared using the chi-square test. Receiver operating characteristic (ROC) curves were used to assess the ability of each biomarker to predict MSARD diagnosis and to calculate the area under the curve (AUC). DeLong's test was performed to make pairwise comparisons of the predictive biomarkers according to MSARD diagnosis. The test implementation follows "Fast Implementation of DeLong's Algorithm for Comparing the Areas Under Correlated Receiver Operating Characteristic Curves, by Xu Sun and Weichao Xu." An optimal threshold that best discriminates MSARD from control populations was then determined with Youden's index. Based on the defined threshold values, patients were classified as positive or negative for each biomarker as a binary result. Sensitivity, specificity, and positive and negative predictive values were then calculated for each biomarker. All comparisons were two-tailed. To identify the impact of demographic and clinical features on each biomarker concentration, CSF KFLC, IL-6, and CD25 were included in a multivariate linear regression model. According to the three identified groups, the explanatory variables were age, gender, disease duration, immune-modifying drug use at sampling, and final diagnosis. Data were checked for multicollinearity with the Belsley–Kuh–Welsch test. Patients with missing data were excluded from the analysis. The differences were considered significant when the p-value was <0.05.

RESULTS

Study Cohort

In the study period, two hundred seventeen patients have been referred to our center for brain white matter T2 hyperintensities. Forty-one patients were excluded because of subnormal brain MRI or age <18 years. One hundred seventy-six patients were included in the study. After the diagnostic workup, patients were separated into the following groups: 88 patients (50%) in the MSARD group, 35 (20%) in the OIND group, and 53 (30%) in the NIND group (flowchart available in the **Supplementary Material**, **Figure S1**). MSARD patients were younger than OIND ($p = 0.002$) and NIND patients ($p = 0.001$). All MSARD patients, except for RIS, experienced a clinical demyelinating event, while 63% and 0% in the OIND and NIND groups, respectively, experienced the same ($p < 0.001$ for both comparisons). All groups were comparable for immune treatment exposure at sampling. MSARD patients had lower CSF protein level ($p < 0.001$), CSF white blood cell count ($p < 0.001$), and albumin quotient ($p < 0.001$) than had OIND patients but had a higher level of CSF immunoglobulin G (IgG) and positive OCB status ($p = 0.005$ and $p < 0.001$, respectively). All characteristics are shown in **Table 1**.

Quantification of Kappa Free Light Chains Biomarkers

Median values of CSF KFLC (**Figure 1A**), KFLC index (**Figure 1B**), and KFLC IF (**Figure 1C**) were higher in the MSARD group (2.59 (IQR 9.18) mg/L; 37.80 (IQR 132.07); 95.06% (IQR 22.09%), respectively) than in the NIND group (0.16 (IQR 0.06) mg/L, $p < 0.001$ for CSF KFLC; 2.38 (IQR 1.82), $p < 0.001$ for KFLC index; 10.11% (IQR 38.10%), $p < 0.001$ for KFLC IF) and the OIND group (0.43 (IQR 1.03) mg/L, $p = 0.001$ for CSF KFLC; 4.53 (IQR 7.35), $p < 0.001$ for KFLC index; 60.21% (IQR 66.98%), $p < 0.001$ for KFLC IF).

In the MSARD group, median values of CSF KFLC (figure in the **Supplementary Material**, **Figure S2A**), KFLC index (**Figure S2B**), and KFLC IF (**Figure S2C**) were lower in CIS (0.63 (IQR 1.64) mg/L, 14.2 (IQR 22.2), and 75.1% (IQR 87.19%), respectively) than in MS patients (3.8 (IQR 10.1) mg/L, 69.1 (IQR 161.23), and 96.6% (IQR 11.1%), respectively; $p < 0.001$ for all comparisons). There was no difference of KFLC biomarkers values between MS and RIS patients (3.4 (IQR 11.2) mg/L, 48.5 (IQR 213.6), and 94.2% (IQR 73.9%), for CSF KFLC ($p = 0.403$), KFLC index ($p = 0.377$), and KFLC IF ($p = 0.320$), respectively).

Quantification of Cerebrospinal Fluid IL-1β, CD25, IL-6, and IL-10

CSF concentration of IL-1β was often under the LDL of the analyzer (68% of the all cohort). Therefore, median values of CSF IL-1β (**Figure 2A**) were similar between groups: 0.16 (IQR 0.20) pg/ml in the MSARD group, 0.16 (IQR 0.17) pg/ml in the NIND group, and 0.16 (IQR 0.32) pg/ml in the OIND group. Median values of CSF CD25 (**Figure 2B**) were higher in the OIND group (45.9 (IQR 65.75) pg/ml) compared to the MSARD group (19.35 (IQR 12.12) pg/ml, $p < 0.001$), and the NIND group (15.7 (IQR 8.60) pg/ml, $p < 0.001$). Similar to CSF CD25, median values of CSF IL-6 (**Figure 2C**) were higher in the OIND group (13.6 (IQR 48.90) pg/ml) compared to the MSARD group (2.99 (IQR 1.67) pg/ml, $p < 0.001$), and the NIND group (2.68 (IQR 2.07) pg/ml, $p < 0.001$). CSF IL-10 concentration was under the LDL in most of the MSARD patients (67%) and the NIND patients (91%). Median values of CSF IL-10 (**Figure 2D**) were higher in the OIND group (1.40 (IQR 3.99) pg/ml) compared to the MSARD group (0.58 (IQR 0.69) pg/ml, $p < 0.001$) and the NIND group (0.58 (IQR 0.1) pg/ml, $p = 0.002$).

In the MSARD group, median values of CSF CD25 (**Figure S3A**) were higher in MS (20.5 (IQR 16.3) pg/ml) than in CIS patients (14.6 (IQR 11.4) pg/ml), $p = 0.023$. CSF CD25 median values were similar between MS and RIS patients (20.3 (IQR 7.9) pg/ml), $p = 0.836$. Median values of CSF IL-6 (**Figure S3B**) and IL-10 (**Figure S3C**) were similar between MS (3.1 (IQR 1.6) and 0.58 (IQR 0.8) pg/ml, respectively), RIS (2.5 (IQR 1.7) and 0.58 (IQR 0.2) pg/ml, respectively), and CIS patients (2.9 (IQR 2.1) and 0.58 (IQR 0.1) pg/ml, respectively), $p > 0.1$ for all comparisons.

TABLE 1 | Baseline demographic and clinical data.

	MSARD group *n* = 88	OIND group *n* = 35	*p*-Value MSARD vs. OIND	NIND group *n* = 53	*p*-Value MSARD vs. NIND
Median age, [IQR]	41.6 ± 13.0	50.7 ± 17.0	0.002	49.5 ± 13.5	0.001
Female gender, *n* (%)	67 (76)	19 (54)	0.028	43 (81)	0.535
Type of disease, *n* (%)	MS, 58 (66) CIS, 22 (25) RIS, 8 (9)	NMOSD/MOGAD, 9 (26) CNS vasculitis, 9 (26) CNS lymphoma, 3 (9) Immune encephalitis, 2 (6) CNS infection, 2 (6) Neurosarcoidosis, 2 (6) Other, 8 (23)	–	Migraine, 13 (25) SCVD, 18 (34) Stroke, 3 (6) Ischemic ON, 1 (2) Myelopathy, 3 (6) Cerebellar atrophy, 2 (4) Mechanical, 6 (11) Other, 7 (13)	–
Clinical event, *n* (%)	80 (91)	22 (63).	<0.001	0* (0)	<0.001
Optic neuritis, *n* (%)	7 (8)	4 (11)	–	0 (0)	
Myelitis, *n* (%)	41 (47)	8 (23)	–	0 (0)	
Brainstem/cerebellar, *n* (%)	20 (23)	4 (11)	–	0 (0)	
Other, *n* (%)	12 (13	6 (16)	–	0 (0)	
Autoimmune medical history, *n* (%)	14 (16)	6 (17)	1	16 (30)	0.056
Immune-modifying drug at sampling, *n* (%)	8 (9)	5 (15)	0.349	6 (11)	0.773
Gadolinium enhancement on baseline MRI, *n* (%)	28 (33)	21 (60)	0.008	2 (4)	<0.001
Median disease duration (months), [IQR]	5.3 [1.3; 35.5]	1.3 [0.3; 2.9]	<0.001	12.3 [3.8; 19.5]	0.108
Median CSF protein concentration (g/L), [IQR]	0.33 [0.27; 0.40]	0.45 [0.31; 0.94]	<0.001	0.33 [0.26; 0.48]	0.667
Median CSF WBC count (/µl), [IQR]	2 [0; 5]	2 [0; 25]	<0.001	0 [0; 1]	0.015
Median albumin quotient (%), [IQR]	0.44 [0.33; 0.58]	0.71 [0.51; 1.45]	<0.001	0.47 [0.36; 0.66]	0.308
Median IgG index, [IQR]	0.75 [0.61; 0.99]	0.60 [0.50; 0.71]	0.005	0.56 [0.50; 0.61]	<0.001
Median serum KFLC (mg/L), [IQR]	13.8 [11.7; 16.2]	15.0 [11.5; 19.6]	0.017	13.7 [11.2; 16.8]	0.258
Positive OCBs status, *n* (%)	52 (60)	4 (11)	<0.001	1 (2)	<0.001

CIS, clinically isolated syndrome; CNS, the central nervous system; CSF, cerebrospinal fluid; IQR, interquartile range; KFLC, kappa free light chains; MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease; MS, multiple sclerosis; MSARD, multiple sclerosis and related disorder; NIND, non-inflammatory neurological disorder; NMOSD, neuromyelitis optica spectrum disorder; OCBs, oligoclonal bands; OIND, other inflammatory neurological disorder; ON, optic neuritis; RIS, radiologically isolated syndrome; SCVD, small cerebral vessel disease; WBC, white blood cell.

**Clinical event non-evocative of demyelinating events (optic neuritis presented as an acute and non-painful event, myelopathies presented as progressive motor weakness of lower limbs).*

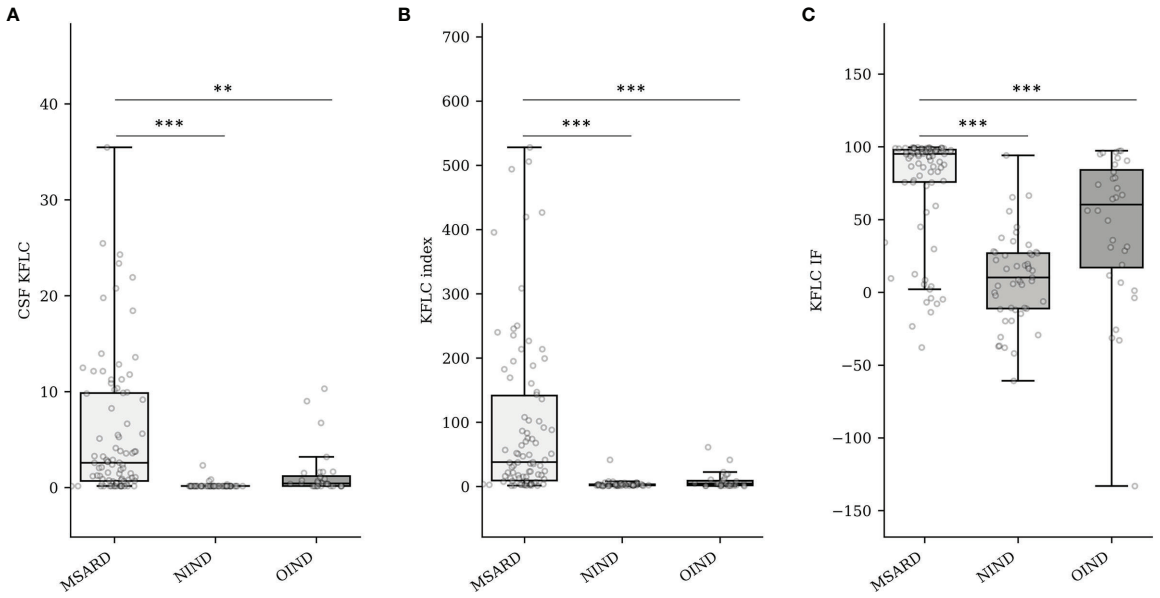

FIGURE 1 | Quantification of CSF KFLC (mg/L) **(A)**, KFLC index **(B)**, and KFLC IF (%) into groups **(C)**. MSARD, multiple sclerosis and related disorder (n = 88); NIND, non inflammatory neurological disorder (n = 53); OIND, other inflammatory neurological disorder (n = 35). ** determined a p-value < 0.01. *** determined a p-value < 0.001. CSF, cerebrospinal fluid; KFLC, kappa free light chains; IF, intrathecal fraction.

Biomarker Diagnostic Performances

We analyzed the ability of the KFLC index, KFLC IF, CSF CD25, CSF IL-6, and CSF IL-10 to diagnose MSARD i) against a non-inflammatory-mimicking disease by the comparison of the MSARD and NIND groups and ii) against another inflammatory-mimicking disease by the comparison of the MSARD and OIND groups. CSF IL-1β was not analyzed because of its low CSF concentration in most patients.

Kappa Free Light Chains Biomarkers Performed Better Than Cerebrospinal Fluid CD25, IL-6, and IL-10 in Separating Multiple Sclerosis and Related Disorder From Non-Inflammatory Neurological Disorder

KFLC index and KFLC IF had similar, and good, overall performances (AUC, 0.900 [0.849; 0.952] and 0.887 [0.830; 0.943], respectively) to diagnose MSARD compared to NIND. However, CSF CD25, CSF IL-6, and CSF IL-10 had lower performances (AUC, 0.596 [0.501; 0.690], 0.569 [0.467; 0.671], and 0.627 [0.565; 0.688], respectively) than both KFLC biomarkers (p < 0.001 for all comparisons). The thresholds that best separated MSARD from NIND were 8.4 for KFLC index, 73.1% for KFLC IF, 21.5 pg/ml for CSF CD25, 2.0 pg/ml for CSF IL-6, and 1.2 pg/ml for CSF IL-10. All data are shown in **Table 2**, and the ROC curves are available in the **Supplementary Material** (**Figure S4A**).

These cutoffs, KFLC index, KFLC IF, CSF CD25, IL-6, and IL-10 were changed into binary variables, and patients were categorized as positive or negative for each biomarker. As shown in **Table 3**, both OCBs and the KFLC index had good overall performances for MSARD diagnosis as compared to NIND. OCBs were more specific than the KFLC index (0.98 vs. 0.91, respectively) but less sensitive (0.59 vs. 0.76, respectively). However, the KFLC index diagnostic accuracy seemed to be higher than OCBs' (0.82 vs. 0.74). Interestingly, the combination of an elevated KFLC index and CSF IL-6 had the same specificity for MSARD diagnosis than OCBs (specificity of 0.96 vs. 0.98, respectively) with a higher sensitivity (0.69 vs. 0.59, respectively) and higher diagnostic accuracy (0.79 vs. 0.74).

Cerebrospinal Fluid IL-6, CD25, and Kappa Free Light Chains Index Showed Good Performances in Diagnosing Multiple Sclerosis and Related Disorder Compared to Other Inflammatory Neurological Disorder

When comparing MSARD to OIND, KFLC index showed better diagnostic performances than KFLC IF (AUC, 0.823 [0.746; 0.900], and 0.745 [0.652; 0.838] respectively, p = 0.008). In contrast with the comparison with the NIND group, in this situation, CSF CD25 and CSF IL-6 showed good diagnostic performances (AUC, 0.770 [0.656; 0.885], and 0.874 [0.798; 0.950], respectively), statistically similar to the KFLC index (p = 0.358, and p = 0.436, respectively). However, diagnostic performances of CSF IL-10 (AUC, 0.680 [0.566; 0.794]) were lower than those of both KFLC index (p = 0.02) and IL-6 (p < 0.001). The thresholds that best separated MSARD from OIND were 13.1 for KFLC index, 82.8% for KFLC IF, 41.5 pg/ml for CSF CD25, 4.1 pg/ml for CSF IL-6, and 2.4 pg/ml for CSF IL-10.

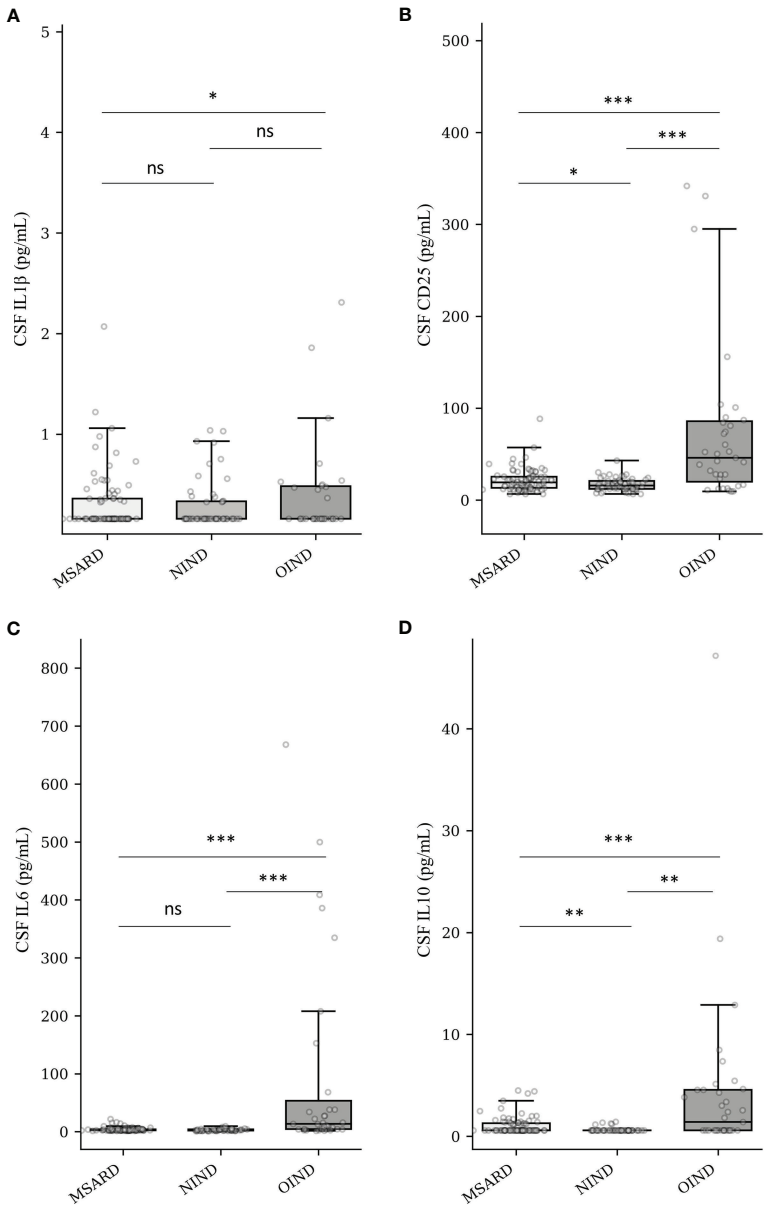

FIGURE 2 | Quantification of CSF IL-1β **(A)**, CD25 (sIL-2R) **(B)**, IL-6 **(C)**, and IL-10 **(D)** into groups. MSARD, multiple sclerosis and related disorders (n = 88); NIND, non-inflammatory neurological disorders (n = 53); OIND, other inflammatory neurological disorders (n = 35); ns, non-significant ($p > 0.05$). *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. CSF, cerebrospinal fluid.

All data are shown in **Table 2**, and the ROC curves are available in the **Supplementary Material** (**Figure S4B**).

As shown in **Table 4**, CSF IL-6 could separate both groups with better sensitivity and the same specificity than OCBs and a better specificity for the same sensitivity than the KFLC index (sensitivity of 0.74, 0.59, and 0.73 and specificity of 0.88, 0.88, and 0.76 for CSF IL-6, OCBs, and KFLC index, respectively). The better specific combination for MSARD diagnosis in such a situation was the association of low CSF IL-6 and CD25 (sensitivity 0.72, specificity 0.94).

Kappa Free Light Chains Index, Cerebrospinal Fluid CD25, and Cerebrospinal Fluid IL-6 Diagnostic Performances Needed to Be Studied in Homogenized Other Inflammatory Neurological Disorder Populations

As shown in **Figure 3A**, elevated KFLC index strongly suggests MS diagnosis independently of the compared OIND subgroups (median of 69.1, 5.49, 1.46, and 4.05 for MS, NMOSD/MOGAD, CNS vasculitis, and OIND diagnoses, respectively; $p < 0.001$ for all comparisons). The comparison between MS and CNS infection

TABLE 2 | Diagnostic performances of the different biomarkers for MSARD diagnosis compared to both control populations.

	MSARD vs. NIND *n* = 141			MSARD vs. OIND *n* = 123		
	AUC (%)	95% CI	Optimal threshold	AUC (%)	95% CI	Optimal threshold
KFLC index	90.0[a]	[84.9; 95.2]	8.4	82.3	[74.6; 90.0]	13.1
KFLC IF	88.7[a]	[83.0; 94.3]	73.1	74.5[c]	[65.2; 83.8]	82.8
CSF CD25	59.6[b]	[50.1; 69.0]	21.5	77.0[d]	[65.6; 88.5]	41.5
CSF IL-6	56.9[b]	[46.7; 67.1]	2.0	87.4[e]	[79.8; 95.0]	4.1
CSF IL-10	62.7[b]	[56.5; 68.8]	1.2	68.0	[56.6; 79.4]	2.4

CSF, cerebrospinal fluid; KFLC, kappa free light chains; MSARD, multiple sclerosis and related disorder; NIND, non-inflammatory neurological disorder; OIND, other inflammatory neurological disorder; AUC, area under the curve.

[a]*KFLC index and KFLC IF AUC are not statistically different (p = 0.404).*

[b]*AUCs of CSF CD25, IL-6, and IL-10 were all lower than both KFLC biomarkers (p < 0.001 for all 6 comparisons). There was no AUC difference between CSF CD25 and CSF IL-6 (p = 0.717), CSF CD25 and CSF IL-10 (p = 0.523), and CSF IL-6 and CSF IL-10 (p = 0.314).*

[c]*KFLC index and KFLC IF AUC are statistically different (p = 0.008).*

[d]*CSF CD25 AUC is not statistically different than KFLC index AUC (p = 0.358), CSF IL-6 AUC (p = 0.119), or CSF IL-10 AUC (p = 0.149).*

[e]*CSF IL-6 AUC is not statistically different than KFLC index AUC (p = 0.436) and CSF CD25 AUC (p = 0.119) and is higher than CSF IL-10 AUC (p = 0.007).*

did not seem valid, while only two patients presented with a CNS infection in our cohort. The CSF CD25 concentration (**Figure 3B**) did not seem to be effective to separate MS from NMOSD/MOGAD (median CSF CD25 of 20.5 vs. 27.6 pg/ml for MS and NMOSD/MOGAD, respectively, $p = 0.755$). Nevertheless, CSF CD25 could separate MS from CNS vasculitis (median CSF CD25 of 81.0 pg/ml, $p < 0.001$) or other types of OIND (median CSF CD25 of 51.5 pg/ml, $p = 0.012$). Finally, CSF IL-6 (**Figure 3C**) seemed to be a good biomarker to distinguish MS from NMOSD/MOGAD (median CSF IL-6 of 3.1 vs. 27.0 pg/ml for MS and NMOSD/MOGAD, respectively, $p < 0.001$) and from CNS vasculitis (median CSF IL-6 for vasculitis of 27.7 pg/ml, $p < 0.001$). However, median CSF IL-6 concentrations were not different between MS and the other OIND ($p = 0.392$).

Cerebrospinal Fluid Kappa Free Light Chains, CD25, and IL-6 Concentrations Were Not Influenced by Age, Gender, Disease Duration, and Immune-Modifying Drug Use at Sampling

Based on the linear regression multivariate analysis model, CSF KFLC, CSF CD25, and CSF IL-6 concentrations were not influenced by age ($p = 0.423$, 0.508, and 0.891, respectively), gender ($p = 0.840$, 0.564, and 0.072, respectively), immune-modifying drug use at sampling ($p = 0.906$, 0.530, and 0.215, respectively), or disease duration ($p = 0.0931$, 0.163, and 0.126, respectively). The only factor associated with elevated CSF KFLC was MSARD diagnosis ($p < 0.001$ when compared to NIND group, and $p = 0.001$ when compared to the OIND group as reference), and the only one associated with elevated CSF CD25 and IL-6 was OIND diagnosis ($p = 0.018$ for CD25 when compared to MSARD as a reference, and $p = 0.003$ for IL-6 when compared to MSARD as reference). All data are shown in **Table 5**.

DISCUSSION

Our study evaluates prospectively multiple CSF biomarkers in patients presenting for a diagnostic workup of brain white matter hyperintensities suggestive of MS. Our results suggest that activated B-cell biomarkers (OCBs or KFLC index/IF) may strongly recommend MSARD diagnosis regardless of the chosen control population. KFLC index has the advantage of being more sensitive than OCBs but suffered from less specificity. These results are consistent with previous retrospective (15, 16) and prospective (14, 22, 23) studies. We found that CIS patients may present with lower KFLC biomarkers values than MS and RIS patients. It may be explained that the 2017 McDonald criteria were applied for MS diagnosis. In doing so, all CIS patients presenting with radiological dissemination in space and positive OCBs were diagnosed as having MS. Therefore, in our cohort, most of the CIS patients presented with low intrathecal B-cell activity (negative OCBs).

We found that CSF CD25 and CSF IL-6 concentrations were lower in MSARD than in OIND. However, these biomarkers cannot rule in MSARD, while NIND patients also express low CSF CD25 and IL-6 concentrations. Nevertheless, high CSF CD25 and IL-6 could be helpful in rolling out MSARD diagnosis, while it would favor another MS-mimicking inflammatory CNS disease. Of note, elevated CSF CD25 presents the highest positive predictive value for OIND diagnosis, more than low KFLC index or negative OCBs. However, CSF CD25 lacks diagnostic performance in separating MSARD from NMOSD and MOGAD, whereas IL-6 seems to be an effective tool in such situations. This is why we think that CSF CD25 and CSF IL-6 should both be used in practice. Moreover, CSF KFLC, CD25, and IL6 concentrations were not influenced by age, gender, disease duration, or immune treatment used during sampling. This point is important, while diagnostic biomarkers need to be efficient at any time of the diagnostic workup.

Our results are consistent with previously published data, showing that a high KFLC index or KFLC IF is associated with MS diagnosis (14–16, 22, 24). KFLC has the advantage, compared to OCBs, in quantifying CSF B-cell activity. This is an important point to consider, while it has been shown, on pathological brain analysis, that MS patients present higher amounts of activated B cells than other inflammatory CNS disorders (25). However, many different KFLC index cutoff values were published to assess

TABLE 3 | Diagnostic performance of the different biomarkers comparing MSARD to NIND (*n* = 141).

	TP (*n*)	FP (*n*)	TN (*n*)	FN (*n*)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Positive OCBs	52	1	52	36	59.1 [48.1; 69.5]	98.1 [89.9; 99.9]	98.1 [88.1; 99.7]	59.1 [52.8; 65.1]	73.8 [65.7; 80.8]
KFLCi > 8.4	67	5	48	21	76.1 [65.9; 84.6]	90.6 [79.3; 96.9]	93.1 [85.2; 96.9]	69.6 [60.9; 77.0]	81.6 [74.2; 87.6]
CD25 > 21.5	37	13	40	51	42.0 [31.6; 53.0]	75.5 [61.7; 86.2]	74.0 [62.6; 82.9]	44.0 [38.3; 49.8]	54.6 [46.0; 63.0]
IL-6 > 2.0	79	37	16	9	89.8 [81.5; 95.2]	30.2 [18.3; 44.3]	68.1 [63.8; 72.1]	64.0 [45.8; 78.9]	67.4 [59.0; 75.0]
CD25 > 21.5 and IL-6 > 2.0	31	8	45	57	35.2 [25.3; 46.1]	84.9 [72.4; 93.3]	79.5 [65.8; 88.6]	44.1 [39.5; 48.9]	53.9 [45.3; 62.3]
KFLCi > 8.4 and CD25 > 21.5	29	0	53	59	33.0 [23.3; 43.8]	100.0 [93.3; 100.0]	100.0 [–]	47.3 [43.7; 51.0]	58.2 [49.6; 66.4]
KFLCi > 8.4 and IL-6 > 2.0	60	2	51	28	68.2 [57.4; 77.7]	96.2 [87.0; 99.5]	96.8 [88.4; 99.2]	64.6 [57.2; 71.3]	78.7 [71.0; 85.2]
KFLCi > 8.4 and CD25 > 21.5 and IL-6 > 2.0	25	0	53	63	28.4 [19.3; 39.0]	100.0 [93.3; 100.0]	100.0 [–]	45.7 [42.4; 49.0]	55.3 [46.7; 63.7]

FN, false negative; FP, false positive; KFLCi, kappa free light chains index; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive; MSARD, multiple sclerosis and related disorder; NIND, non-inflammatory neurological disorder.

TABLE 4 | Diagnostic performance of the different biomarkers comparing MSARD to OIND (*n* = 123).

	TP (*n*)	FP (*n*)	TN (*n*)	FN (*n*)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Positive OCBs	52	4	29	36	59.1 [48.1; 69.5]	87.9 [71.8; 96.6]	92.9 [83.6; 97.1]	44.6 [37.8; 51.6]	66.9 [57.8; 75.2]
KFLCi > 13.1	64	8	25	24	72.7 [62.2; 81.7]	75.8 [57.7; 88.9]	88.9 [81.2; 93.7]	51.0 [41.3; 60.7]	73.6 [64.8; 81.2]
CD25 < 41.5	84	14	19	4	95.5 [88.8; 98.7]	57.6 [39.2; 74.5]	85.7 [80.1; 90.0]	82.6 [63.6; 92.8]	85.1 [77.5; 90.9]
IL-6 < 4.1	65	4	29	23	73.9 [63.4; 82.7]	87.9 [71.8; 96.6]	94.2 [86.5; 97.6]	55.8 [46.5; 64.7]	77.7 [69.2; 84.8]
CD25 < 41.5 and IL-6 < 4.1	63	2	31	25	71.6 [61.0; 80.7]	93.9 [79.8; 99.3]	96.9 [89.1; 99.2]	55.4 [46.8; 63.6]	77.7 [69.2; 84.8]
KFLCi > 13.1 and CD25 < 41.5	61	3	30	27	69.3 [58.6; 78.7]	90.9 [75.7; 98.1]	95.3 [87.3; 98.4]	52.6 [44.4; 60.8]	75.2 [66.5; 82.6]
KFLCi > 13.1 and IL-6 < 4.1	48	2	31	40	54.5 [43.6; 65.2]	93.9 [79.8; 99.3]	96.0 [86.1; 98.9]	43.7 [37.8; 49.7]	65.3 [56.1; 73.7]
KFLCi > 13.1 and CD25 < 41.5 and IL-6 < 4.1	46	0	33	42	52.3 [41.4; 63.0]	100.0 [89.4; 100.0]	100.0 [-]	44.0 [38.7; 49.4]	65.3 [56.1; 73.7]

FN, false negative; FP, false positive; KFLCi, kappa free light chains index; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive; MSARD, multiple sclerosis and related disorder; OIND, other inflammatory neurological disorder.

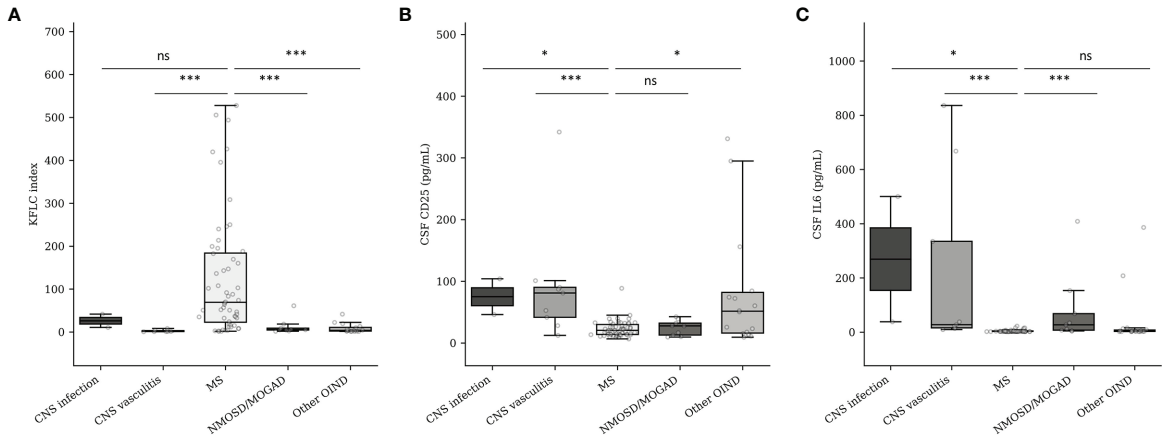

FIGURE 3 | KFLC index **(A)**, CSF CD25 **(B)**, and CSF IL-6 **(C)** expression in MS and OIND subgroups. CNS, central nervous system; MS, multiple sclerosis; MOGAD, myelin oligodendrocyte antibody-associated disorder; NMOSD, neuromyelitis optica spectrum disorder; OIND, other inflammatory neurological disorder. Number of patients according to the different subgroups: CNS infection (n = 2), CNS vasculitis (n = 9), MS (n = 58), NMOSD/MOGAD (n = 9), OIND (n = 15). ns, non-significant ($p > 0.05$). *$p < 0.05$. ***$p < 0.001$.

TABLE 5 | Identification of clinical and demographic data influencing CSF KFLC, CSF CD25, and CSF IL-6 concentrations by linear regression multivariate analysis.

	CSF KFLC n = 174		**CSF CD25 n = 174**		**CSF IL-6 n = 174**	
	β coefficient [IQR]	***p*-Value**	**β coefficient [IQR]**	***p*-Value**	**β coefficient [IQR]**	***p*-Value**
Age	0.024	0.423	0.316	0.508	–0.071	0.891
Risk for each 1 year increase	[–0.035; 0.083]		[–0.625; 1.260]		[–1.100; 0.953]	
Gender	0.24	0.840	–7.76	0.564	40.53	0.072
Reference: women	[–2.07; 2.54]		[–34.23; 18.72]		[–3.61; 84.66]	
Disease duration	–0.04	0.093	–0.20	0.163	–0.42	0.126
Risk for each 1 month increase	[–0.08; 0.01]		[–0.48; 0.08]		[–0.97; 0.12]	
Immune drug ongoing at sampling	0.11	0.906	11.52	0.530	45.32	0.215
Reference: yes	[–1.71; 1.92]		[–24.59; 47.63]		[–26.6; 117.23]	
Diagnosis						
Reference: MSARD						
NIND group	–5.53	<0.001	–7.24	0.146	–0.823	0.896
	[–7.53; –3.54]		[–17.04; 2.55]		[–13.23; 11.59]	
OIND group	–4.82	0.001	60.55	0.018	93.56	0.003
	[–7.66; –1.98]		[10.58; 110.52]		[33.29; 153.83]	

CSF, cerebrospinal fluid; KFLC, kappa free light chains; MSARD, multiple sclerosis and related disorder; NIND, non-inflammatory neurological disorder; OIND, other inflammatory neurological disorder.

intrathecal immunoglobulin synthesis (i.e., KFLC index cutoff range from less than 3 to more than 10) (26, 27). This discrepancy could be explained by the heterogeneity of the different control populations, while many inflammatory CNS disorders may have an intrathecal B-cell activity. Therefore, as suggested by our study, cutoff values of KFLC biomarkers should be different depending on the suspected underlying MS-mimicking disorder, to avoid misdiagnosis.

Our findings agree with other retrospective studies that found an increased concentration of CSF IL-6 in NMOSD (17, 28, 29) and MOGAD (17, 30) compared to MS. Added to our results, these findings suggest that CSF IL-6 measurement may impact early diagnosis, while cytokine measurement is easy and fast to perform as compared to aquaporin-4 or MOG antibody, and may guide early therapeutic action, in suspected NMOSD/MOGAD patients. Moreover, tocilizumab, an IL-6 receptor blockade therapy, has shown promising efficacy in NMOSD (31–33) and has been reported to be effective in relapsing steroid-dependent MOGAD (31, 34), reinforcing the impact of the IL-6 pathway in these diseases. In contrast, CSF CD25 could not separate MSARD from NMOSD and MOGAD, as it has already been suggested in two previously published retrospective studies (17, 35). However, because of its high positive predictive value for OIND diagnosis, elevated CSF CD25 should be used as a non-specific MSARD red flag. Even if CSF CD25 is not associated with a specific disease in our heterogeneous cohort, it could be an exciting tool in neurosarcoidosis (18), bacterial meningitis (18), or CNS lymphoma (18, 35, 36).

Nonetheless, in clinical practice, a spinal tap is not performed in all suspected MS patients, while MS diagnostic

criteria are based on the presence of a typical demyelinating event and MRI presentation (1). However, CSF analysis is often performed, while identifying OCBs is a key point to ensure MS diagnosis and avoid misdiagnosis. Moreover, our results show that extensive CSF analysis could help etiological diagnosis in many complicated cases. Importantly, none of the NIND patients in our cohort experienced a typical clinical demyelinating event, reinforcing the importance of clinical presentation in fulfilling MS criteria and identifying red flags for MS diagnosis. According to these findings and the current recommendations for MS diagnosis (1, 37, 38), we provide an MS practical diagnostic algorithm for patients presenting with brain white matter hyperintensities suggestive of MS (**Figure 4**).

Our study suffers from several limits. First, being a monocentric study, our results need to be confirmed by others, even if these results are consistent with multiple retrospective data. Second, our cohort's small size and heterogeneity, particularly in the OIND group, do not permit us to conclude on the effectiveness of these biomarkers for the different MS-mimicking diseases. However, it allows figuring out which biomarker may help in rolling in or rolling out MSARD. Third, it would have been interesting to measure serum IL-1β, CD25, IL-6, and IL-10 to calculate cytokine indexes, but our routine diagnostic workup of white matter hyperintensities does not include these analyses. Nevertheless, this study is pragmatic, evaluating these biomarkers prospectively in daily practice for the diagnostic workup of suspected MS. We think that these results will increase the etiological diagnostic accuracy of such patients.

CONCLUSION

In patients presenting for a diagnostic workup of MRI white matter hyperintensities, elevated CSF activated B-cell biomarkers such as KFLC index or KFLC IF strongly suggest MSARD. In contrast, elevated CSF IL-6 and CD25 suggest another

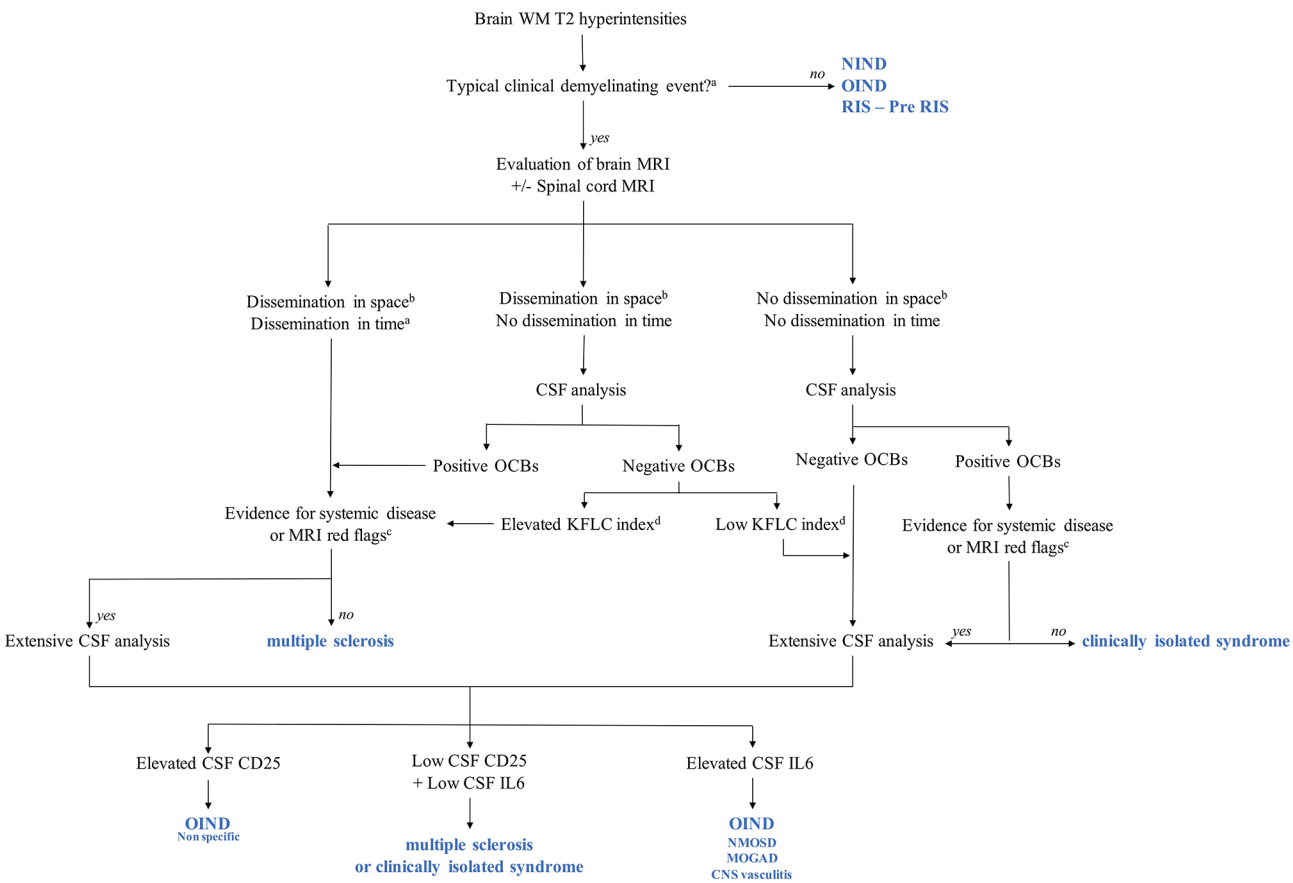

FIGURE 4 | Multiple sclerosis (MS) diagnostic algorithms including KFLC index, CSF CD25, and CSF IL-6. MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease; NIND, non-inflammatory neurological disorder; NMOSD, neuromyelitis optica spectrum disorder; OIND, other inflammatory neurological disorder; Pre-RIS, radiologically isolated syndrome with one or two specific dissemination in space criteria; RIS, radiologically isolated syndrome; WM: white matter. [a]According to reference (1). [b]According to reference (37). [c]According to reference (38). [d]In our study, the KFLC index cutoff was 13.1. This cutoff is specific to our cohort and should not be used in daily practice, while each MS tertiary center should determine its threshold values. KFLC, kappa free light chains; CSF, cerebrospinal fluid.

inflammatory-mimicking disease. These findings need to be confirmed in other prospective cohort studies within larger samples.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML and CL-F designed the study, collected the data, performed the data analysis, and drafted the manuscript. CL, LM, MC, and SB helped in designing the study, data collection, preparation of the manuscript, and review for intellectual content. VB and BS-P performed the biomarker analysis and helped in reviewing the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.864133/full#supplementary-material

Supplementary Figure 1 | Flow chart. CNS, central nervous system; MS, multiple sclerosis; MSARD, multiple sclerosis and related disorder; OIND, other inflammatory neurological disorder; WMH, white matter hyperintensities.

Supplementary Figure 2 | Distribution of KFLC biomarkers into RIS, CIS, and MS subgroups. CIS, clinically isolated syndrome (*n*=22); MS, multiple sclerosis (*n*=58); RIS, radiologically isolated syndrome (*n*=8); ns, non significant ***p < 0.001.

Supplementary Figure 3 | Distribution of CSF CD25, IL6, and IL10 into RIS, CIS, and MS subgroups. CIS, clinically isolated syndrome (*n*=22); MS, multiple sclerosis (*n*=58); RIS, radiologically isolated syndrome (*n*=8). ns, non significant *p < 0.05.

Supplementary Figure 4 | ROC curves of KFLC index, KFLC IF, CSF CD25, IL6, and IL10.[(**A**) ROC curve that separate MSARD from NIND (*n*=141)]. Panel **(B)** ROC curve that separate MSARD from OIND (*n*=123).

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Biological Markers in Early Multiple Sclerosis: the Paved Way for Radiologically Isolated Syndrome

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Radiologically Isolated Syndrome (RIS) is characterized by MRI-typical brain lesions fulfilling the 2009 Okuda criteria, detected in patients without clinical conditions suggestive of MS. Half of all RIS patients convert to MS within 10 years. The individual course of the disease, however, is highly variable with 12% of RIS converting directly to progressive MS. Demographic and imaging markers have been associated with the risk of clinical MS in RIS: male sex, younger age, infra-tentorial, and spinal cord lesions on the index scan and gadolinium-enhancing lesions on index or follow-up scans. Although not considered as a distinct MS phenotype, RIS certainly shares common pathological features with early active and progressive MS. In this review, we specifically focus on biological markers that may help refine the risk stratification of clinical MS and disability for early treatment. Intrathecal B-cell activation with cerebrospinal fluid (CSF) oligoclonal bands, elevated kappa free light chains, and cytokine production is specific to MS, whereas neurofilament light chain (NfL) levels reflect disease activity associated with neuroaxonal injury. Specific microRNA profiles have been identified in RIS converters in both CSF and blood. CSF levels of chitinases and glial acidic fibrillary protein (GFAP) reflecting astrogliosis might help predict the evolution of RIS to progressive MS. Innovative genomic, proteomic, and metabolomic approaches have provided several new candidate biomarkers to be explored in RIS. Leveraging data from randomized controlled trials and large prospective RIS cohorts with extended follow-up to identify, as early as possible, biomarkers for predicting greater disease severity would be invaluable for counseling patients, managing treatment, and monitoring.

Keywords: multiple sclerosis (MS), radiologically isolated syndrome (RIS), prognosis, biomarkers, personalized medicine, Kappa free-light chain index (kFLC index), glial fibrillary acidic protein (GFAP), neurofilament-light chain (NfL)

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1 INTRODUCTION

In 2013, the classical definitions of MS clinical courses were modified to take disease activity and disease progression into account (1). Additionally, a clinically isolated syndrome (CIS), the first attack of typical clinical MS symptoms, was defined as early-stage MS, later becoming relapsing–remitting multiple sclerosis (RRMS) if subsequently clinically active and fulfilling the current MS

diagnostic criteria (1). Various signs and symptoms (namely, fatigue, pain, bowel and bladder dysfunction, sleep disturbances, and cognitive impairment) and increased healthcare usage may occur in the latent period between the start of neuropathological lesions and CIS, defining the concept of a prodromal phase of MS (2). However, profiles associating multiple biological and clinical features suggestive of MS should be carefully defined to reach appropriate diagnostic specificity before they can be used as markers to screen for MS in populations at risk, such as the offspring of MS patients. In the absence of clinical conditions suggestive of MS, only MRI lesions that fulfill the 2005 dissemination in space criteria (the so-called Okuda criteria) have shown enough specificity for the risk of clinical conversion during follow-up and therefore reached a consensus for the definition of radiologically isolated syndrome (RIS) (3, 4). With this definition, one-third of RIS patients experience their first clinical event, typical of RRMS, after 5 years, while another third show new brain lesions on follow-up scans (5). A long-term retrospective multinational study showed that more than 50% of RIS subjects converted to MS within 10 years, with 11.7% meeting the criteria for primary progressive MS (PPMS) (6).

Predicting the evolution of RIS is of utmost importance for adapting follow-up and therapeutic strategies for effective, personalized care. In large cohorts, male sex and younger age have been identified as baseline predictors of clinical conversion (5–8). Validated MRI prognostic biomarkers are infra-tentorial (IT) and spinal cord (SC) lesions on the index scan and the presence of gadolinium-enhanced (Gd+) lesions on index or follow-up scans (5–8). Recently, studies have shown that the presence of white matter lesions with a central vein sign (CVS) or a paramagnetic rim sign in RIS patients is associated with the presence of SC lesions, suggesting their potential for predicting RIS evolution (9, 10). Optic nerve demyelination identified by visual evoked potentials (VEP), thinning of the peripapillary retinal nerve fiber layer (pRNFL) and the common ganglion cell and inner plexiform layer (GCIP) at baseline and during follow-up on optical coherence tomography (OCT) has also been correlated with a higher risk of clinical conversion (8, 11).

Although RIS is not considered a distinct MS phenotype due to the absence of MS symptoms (12), it certainly shares common pathological features with CIS and early progressive MS, encompassing several biological characteristics and markers, forming a set of putative biological markers for the prognosis of RIS (13–15). Except for oligoclonal bands (OCBs) from cerebrospinal fluid (CSF), for 40 years now, have been considered as a biomarker for MS (12), biological markers for early MS remain largely unexplored in RIS. There is a need to identify biomarkers for early MS that may help refine the risk stratification for clinical MS and disability for early treatment. Exploring the pathophysiological pathways for MS involving risk factors for MS, immune system dysfunction, neuroaxonal injury and degeneration, and glial activation in RIS might improve our understanding of this complex disease (16). Additionally, biomarkers for RIS might reveal early pathological features of MS that were unidentified in

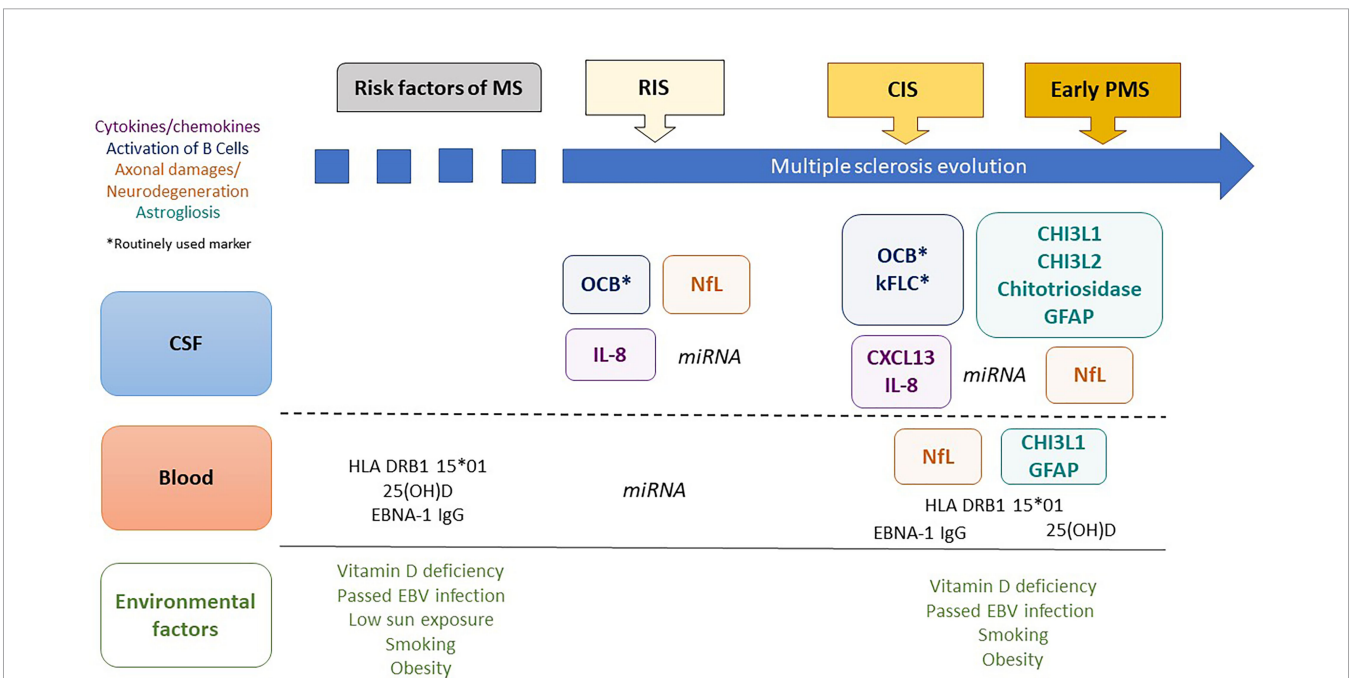

FIGURE 1 | Biological markers predictive of clinical evolution in early multiple sclerosis. MS, multiple sclerosis; CSF, cerebrospinal fluid; RIS, radiologically isolated syndrome; CIS, clinically isolated syndrome; PMS, progressive MS; 25(OH)D, 25-hydroxy vitamin D; EBNA1-IgG, Epstein–Barr Virus-encoded nuclear antigen 1 specific immunoglobulin G; OCBs, oligoclonal bands; CHI3L1, chitinase 3-like protein 1; CHI3L2, chitinase 3-like protein 2; GFAP, glial fibrillary acidic protein; NFL, neurofilament-light chain; kFLC, kappa Free Light Chains; miRNA, microRNA.

the later stages and may constitute future therapeutic targets to slow the disease in its pre-symptomatic phase. In this review, we focus on published biological markers predictive of disease activity and progression at the earliest stages of MS, as depicted in **Figure 1**, and discuss their potential interest in RIS subjects.

2 INFLUENCE OF RISK FACTORS FOR MS ON THE EVOLUTION OF RIS

In the relatives of MS patients, the risk of MS is much greater and correlates with the degree of kinship, origin, and sex, partly due to several genetic risk factors for MS, especially human leukocyte antigen (HLA) genes (16). Accordingly, there is a higher incidence of RIS in healthy relatives of patients with MS compared to people with healthy relatives (17). HLA-DRB1∗1501 is the main allele responsible for the genetic risk of MS in patients with European ancestry (18). It has also been associated with the risk of clinical events in CIS patients (19), but not in RIS patients (20). Although they are not routinely determined in MS and RIS, analysis of genetic variants associated with MS might still have a minor interest in clinical care.

Low sun exposure, poor vitamin D intake, and low 25-hydroxy vitamin D (25(OH)D) levels in serum, smoking, obesity, and a history of Epstein–Barr virus (EBV) infection are all environmental risk factors for MS (21). Immunoglobulins against EBV-encoded nuclear antigens (EBNA-1,2,3,4,6-IgG) are associated with the risk of developing MS (22). Most of these have also been linked to disease severity (25(OH)D, EBNA1-IgG, obesity, and smoking) (23, 24). Smoking, especially in healthy relatives of patients with MS, is associated with the presence of white matter (WM) signal abnormalities, whereas obesity is related to the presence of ≥9 WM signal abnormalities and fulfillment of the Swanton criteria (17). Lower 25(OH)D levels were associated with the risk of clinical events in a large cohort of CIS patients in univariate analysis, but EBNA1-IgG and smoking status as defined by cotinine levels (>14 ng/ml) were not (25). In a small RIS cohort, there was no difference in 25(OH)D levels in the serum of converters or non-converters (20). The predictive value of 25(OH)D deficiency should be investigated further, as the relatively minor clinical impact of vitamin D therapy in MS may be enhanced if started before disease onset (26, 27).

3 PROGNOSTIC BIOMARKER CANDIDATES FOR RIS

3.1 CSF B Cell Lineage and Biomarkers

3.1.1 CSF B Cells

B cells are a key component of acute and chronic inflammatory activity in MS (28), with specific activated clones promoting cytokine production, antigen presentation, differentiation into plasma cells, T cell activation, and CNS invasion by immune cells (29). Inflammatory aggregates of B cells in the subarachnoid spaces were associated with a worse evolution of the disease (30). In analyzing different B-cell subsets (transitional, mature naive, marginal zone, switched memory B cells, IgM-only, IgD-only B cells, and plasmablasts), Guerrier et al. observed that double-negative IgD2/CD272 B cells increased in CIS patients (31). Analysis of the different subsets of T and B cells in RIS could bring new insights into the mechanisms of MS and serve as biomarkers.

3.1.2 Immunoglobulin G and M Intrathecal Synthesis

Clonally expanded B and plasma cells in the CNS locally produce clonal IgGs, leading to CSF restricted oligoclonal bands (OCBs). The presence of OCBs was the first established biological marker for the diagnosis of MS (29) and predicts CIS conversion to clinically definite MS (29). Moreover, RRMS or CIS patients with intrathecal IgG synthesis had a higher risk of and shorter time-to-EDSS worsening over a 4-year follow-up period (32).

In RIS, the presence of OCBs is predictive of clinical conversion in adults (33) and children (34, 35) (**Table 1**), although the presence of OCBs is not correlated with the conversion time in adults (33). Conversely, the IgG index has not shown an independent prognostic value (8, 20). In large cohorts, abnormal CSF, defined as the presence of ≥2 OCBs and/or an IgG index >0.7, revealed a relevant predictive value for disease activity (5, 6, 8). It was also an independent predictor of clinical conversion at 10 years in a multivariate analysis compared to MRI and epidemiological data (6) but not in shorter term studies (5) (**Table 1**). Interestingly, OCBs have been accurately detected in tears and could be used as a minimally-invasive diagnostic tool for RIS if further confirmed in independent cohorts (38).

Intrathecal synthesis of IgM has been associated with higher disease activity and shorter progression toward disability compared with abnormal CSF in RRMS patients and an active inflammatory disease phenotype in PPMS patients, but its prognostic value has not been studied for RIS (39, 40).

3.1.3 Kappa-Free Light Chains

Kappa free light chains (kFLC) measured by nephelometry (41) reflect the quantitative intrathecal immunoglobulin synthesis with better accuracy than OCBs and IgG index for MS diagnosis (42) and for predicting clinical conversion in CIS (43), suggesting that it could represent a good candidate biomarker for RIS prognosis. However, studies evaluating small numbers of pooled RIS and CIS patients provide divergent results, and sound investigations of kFLC in RIS are needed (44, 45).

3.1.4 B Cell Cytokines and Chemokines

CXCL13 is a pro-inflammatory chemokine involved mainly in the migration of B cells, a critical stage in the pathology of MS (46). CXCL13 levels assessed in CSF by ELISA have been associated with the conversion of CIS to MS, a higher relapse rate and accumulation of disability (47–49). In only one study of a few RIS patients (n = 4), the CXCL13 index in RIS showed no difference from healthy controls or other stages of MS (50).

In the study by Guerrier, an imbalance in the cytokine production by circulating B cells, especially the alteration of

TABLE 1 | Prognostic value of oligoclonal bands and/or IgG index in cerebrospinal fluid in patients with radiologically isolated syndrome.

	Study	Patient characteristics			End-point	Statistical test	Univariate analysis	Multivariate analysis
		N (W%)	Age* (y)	Follow- up* (y)				
Abnormal CSF	Lebrun et al. (8)	70 (75.7)	35.6	5.2	attack	Log-rank test	n.s.	**p = 0.02** [#]
	Lebrun et al. (6)	415 (86.5)	37.2	*6.7*	attack or progression	Cox proportional hazards models	HR 2.15 [1.40–3.31] **P <0.001**	HR 1.74 [1.07–2.85] **p = 0.027**
	Okuda et al. (5)	451 (78.4)	37.2	4.4–*2.8*	attack or progression	Cox proportional hazards models	HR 1.78 [1.11–2.87] **p =0.017**	ns
	Thouvenot et al. (36)	71 (76.1)	38.0	*1.3*	attack	Cox proportional hazards models	HR 2.9 [0.83–10.2] p = 0.097	HR 2.22 [0.57–8.59] p = 0.249
	Lebrun et al. (7)	354 (74.6)	38.6	3.8	attack or progression	Cox proportional hazards models	HR 1.26 [0.51–3.09] p = 0.61	–
Oligoclonal Bands	Matute-Blanch et al. (33)	75 (73.3)	36.6	2.8	attack	Cox proportional hazards models	HR 10.31 [1.37–76.61] **p = 0.024**	HR 14.70 [1.80–120.15] **p = 0.012**
	Makhani et al. (34)	38 (71.1)	*15.4*	4.8–*2.5*	attack	Cox proportional hazards models	not shown	HR 10.9 [1.4–86.2] **p = 0.020**
	Makhani et al. (35)	61 (68.9)	*15.0*	4.2–*2.4*	attack	Cox proportional hazards models	HR 4.1 [1.1–14.4] **p = 0.03**	HR 3.0 [1.1–8.5] **p = 0.04**
	Lebrun et al. (8)	70 (75.7)	35.6	5.2	attack	Fisher's exact test	p = 0.69	NA
	Rossi et al. (37)	18 (50)	29.7	2	attack	Multivariate logistic regression model	not shown	OR 4.45 [0.12–154.07] p = 0.400
	Munoz et al. (20)	15 (73.3)	*38*	*6.5*	attack or progression	Fisher's exact test	p = 0.200	NA
IgG index	Lebrun et al. (8)	70 (75.7)	35.6	5.2	attack	Fisher's exact test	p = 0.26	NA
	Munoz et al. (20)	15 (73.3)	*38*	*6.5*	attack or progression	Mann–Whitney U test	p = 0.127	NA

*Abnormal CSF was defined as IgG index positive (>0.7) and/or the presence of OCBs (≥2). All adult patients fulfilled Okuda's criteria, children RIS-Ped criteria. *Mean or Median value in years. [#]significant only among patients with ≥9 T2 lesions on MRI. P-values <0.005 are in bold [95% confidence interval]. N, total number of patients included; W%, percentage of women; HR, hazard ratio; OR, odds ratio; n.s., not significant.*

IL-10 production with a high IL-6/IL-10-producing B-cell ratio, was associated with clinical conversion and its delay in a mixed cohort of CIS and RIS patients (31). Concentrations of B cell-related factors, notably CD27, FCRL2, CXCL10, and CXCL13, increase in MS CSF, especially in the early stages of the disease (51). Further studies must confirm B-cell phenotyping as a valuable prognostic biomarker.

3.2 Other Inflammatory Biomarkers

3.2.1 Soluble CD27

A soluble form of CD27 (sCD27) is released by activated T cells and co-stimulates B and T cell activation and proliferation in autoimmune diseases like MS (52–54). High sCD27 levels in the CSF of CIS patients have been associated with a 5.5 times higher annual relapse rate (53) and the CSF sCD27/T-cell ratio increases in progressive MS (55). However, serum sCD27 levels do not discriminate between MS patients and healthy individuals (54).

3.2.2 Interleukin-8

Interleukin-8 (IL-8) is a pro-inflammatory chemokine produced by astrocytes and microglia in response to active intrathecal inflammation (56). It activates monocytes and neutrophils (37) and binds to oligodendrocytes and hypertrophic astrocytes in MS (57). Elevated CSF IL-8 levels are predictive of MS conversion following a CIS (37). In a small group of 18 RIS patients, a high level of CSF IL-8 was an independent predictor of clinical conversion (37), making IL-8 a candidate for RIS prognosis to be further validated.

3.2.3 Interleukin 17A

Studies on experimental autoimmune encephalomyelitis, an animal model of MS, highlighted the role of Th17 lymphocytes, characterized by interleukin 17A (IL-17A) secretion, as strong inducers of pro-inflammatory responses (58). In a large cohort of 1,327 MS spectrum patients (RIS-CIS-RRMS), IL-17A levels were higher than in healthy controls

in CSF but not in serum (59). Serum and CSF IL-17A did not discriminate between MS subtypes and did not demonstrate any prognostic value in 35 RIS patients (59).

3.3 Markers of Neuroaxonal Damage and Glial Activation

3.3.1 Neurofilaments

Neurofilaments encompass a family of 5 intermediate filaments (heavy, medium, light chains (NfL), a-internexin, and peripherin) involved in axonal growth and stability as well as mitochondrial and synaptic functions in central and peripheral neurons (60). Neurofilaments can be released into the interstitial fluid from injured neurons, either due to the loss of neuronal membrane integrity or to active secretion related to axonal damage or neurodegeneration. According to other brain protein clearance, degraded neurofilaments may be absorbed from interstitial fluid into lymphatic vessels or directly absorbed by the blood vessels *via* perivascular drainage along the basement membranes of capillaries (61). Different levels of blood–brain barrier leakage induced by inflammation probably modify the kinetics of the neurofilament-light chain, circulating between the brain and blood compartments and its final blood concentration (60). NfL in CSF (cNfL) has been associated with clinical activity in CIS patients (62). cNFL can tell RIS apart from RRMS and PPMS, but not from early-stage CIS or healthy controls (63). Among 75 RIS patients, high cNfL measured by ELISA (Uman-Diagnostics; Umeå, Sweden) has been associated with an increased risk of conversion to CIS or to RRMS (CIS was based on the 2010 McDonald criteria in this study) (33).

Recently, ultrasensitive technologies such as the single molecule array (Simoa™) and the microfluidic platform (Simple Plex™ Ella) have been developed, allowing for the accurate determination of NfL levels in serum (sNfL) and highly correlated cNfL levels (64, 65). Using Simoa™, sNfL levels have been associated with disease activity, treatment response, and long-term outcomes at different stages of MS (66, 67) and identified as an independent predictor of relapse in newly-diagnosed MS and CIS patients (68, 69). The prognostic value of sNfL has not been investigated in RIS subjects. However, in a large epidemiological study among US military personnel, it was significantly higher among people who developed MS within 6 years (70). sNfL might provide a potentially less invasive option for assessing RIS prognosis when a lumbar puncture cannot be performed.

3.3.2 Glial Fibrillary Acidic Protein

Glial Fibrillary Acidic Protein (GFAP) measurement has recently been implemented with NfL in multiplex kits (2-PLEX B and 4-PLEX A) by Quanterix®, making it possible to investigate astrocytic activation along with neuroaxonal damage in serum samples. GFAP is one of the major intermediate filament proteins expressed in astrocytes. CSF GFAP levels correlate with different subtypes of MS, reflecting different degrees of damage to astrocytes and may represent a useful marker of disease progression (71). CSF and serum GFAP (sGFAP) levels are correlated with MS patients (72). sGFAP has been associated with a higher Expanded Disability Status Scale (EDSS) score, older age, longer disease duration, progressive disease course, and MRI pathology (73, 74). The positive correlation between sGFAP and the clinical severity of the disease may highlight a particular role of astrocytes in progressive MS and mark the potential of sGFAP as a marker of disease severity (73). In RIS, the prognostic value of sGFAP as a minimally invasive biomarker of conversion to PPMS should be evaluated.

3.3.3 Chitinase 3-Like protein 1

Chitinase 3-like protein 1 (CHI3L1, also known as YKL-40) is a protein of the chitin family mainly released in the CNS by activated astrocytes (75), microglia, and macrophages (76) in response to acute and chronic inflammation. It has been described as inhibiting oxidant-induced injury, increasing Th2 immunity, and regulating apoptosis (77). CSF CHI3L1 levels (cCHI3L1) measured by ELISA predict conversion from CIS to clinically definite MS and development of disability (75, 78). Indeed, cCHI3L1may reflect non-lymphocytic low-grade inflammation leading to active neurodegeneration (79), explaining its association with neurological disability quantified by EDSS in PPMS (80). However, all studies consistently show the absence of prognostic value of cCHI3L1 in RIS (20, 33, 36), suggesting that astrocytic and microglial activation is too scarce at the pre-symptomatic stage of MS. However, chitotriosidase and chitinase 3-like protein 2 (CHI3L2), two other members of the chitin family with similar properties, also need to be evaluated (75, 81, 82).

Although at a much lower concentration than in the CSF, ELISA made it possible to quantify serum CHI3L1 (sCHI3L1) levels, which are also associated with the risk of conversion to RRMS in CIS patients (75). Additionally, sCHI3L1 is higher in PMS patients than in RRMS patients and correlates with disability as determined by EDSS in PMS patients (83). However, the prognostic value of sCHI3L1 for the conversion to CIS or to PMS in RIS patients has not been assessed.

Altogether, NfL, likely associated with acute neuroaxonal injury, might have an interesting predictive value in the early stages of MS for disease activity, whereas GFAP and sCHI3L1 seem rather to be associated with glial activation, and could be of interest for predicting conversions to progressive MS. Their association in a CSF or serum "glia score" (GFAP*CHI3L1/NfL) better discriminates RRMS vs. PPMS than each biomarker alone, CSF being more accurate than serum (AUC 0.80 vs. 0.68, respectively) (83).

3.4 Innovative Genomic, Proteomic, and Metabolomic Approaches

3.4.1 MicroRNA

MicroRNA (miRNA) is an extremely stable class of non-coding single-stranded RNA with post-transcriptional regulatory functions (84) that can be detected in peripheral blood or CSF. Some serum and CSF miRNA profiles have been associated with MS (84, 85), while others predict clinical evolution in CIS patients (86). In 15 RIS patients, miRNA specific profiles in CSF (miR-144-3p, miR-448, and miR-653-3p) and in plasma (miR-142-3p, miR-338-3p, miR-363-3p, miR-374b-5p, miR-424-5p, and miR-483-3p) have been associated with the risk of conversion after 5 years of follow-up (20) and require further validation.

3.4.2 Mass Cytometry

Mass cytometry (CyTOF) can help decipher immune cell phenotypes. In CSF from early MS patients, a B-cell population expressing CD49d, CD69, CD27, CXCR3, and HLA-DR could be a strong candidate for an MS-specific cell type (51). In the blood of CIS patients, an increased proportion of both a T-bet-expressing B cell subset and a CD206+ classical monocyte subset has been identified, especially in very active MS patients (disease activity after 6 months of disease modifying therapy or two or more relapses within one year with residual disability and radiological activity) (87).

These approaches provide new insights into the pathophysiology of MS and allow the identification of immunological biomarkers of early MS. Further studies will be required to determine the exact role of new candidate biomarkers and validate their diagnostic and prognostic value in RIS patients.

3.4.3 Proteomics and Metabolomics

In the past few years, technical breakthroughs have made it possible to screen for many molecules as candidate biomarkers through unbiased -omic approaches. SOMAscan™ has identified specific protein profiles in the CSF extracellular vesicles of RRMS patients (88). The Olink inflammation panel has identified CCL11 and CCL20 as plasma biomarkers associated with MS progression and severity (89).

Metabolomics can identify the disturbed pathways involved in signaling and energy supply, providing potential signature profiles for MS diagnosis, stages, and assessment of drug responses, especially involving the alpha-linoleic acid pathway, nucleotide metabolism, amino acid metabolism, tricarboxylic acid cycle, D-ornithine, and D-arginine pathways (90).

The multi-omics-based algorithm based on protein profiling by SOMAScan™ and nuclear magnetic resonance metabolite measures has outperformed the current individual biomarkers for predicting the risk of conversion to clinically definite MS in CIS patients (91), although a reproducible MS-specific metabolome-based signature remains to be identified. Applied to RIS, these approaches could bring new insights into the molecular pathways promoting the disease and more accurately predict individual prognoses.

4 DISCUSSION

Prognostic values of several biological factors have been tested in RIS owing to their interest in different subtypes of MS, especially in CIS and early progressive MS.

First, the most studied biomarker in MS and validated MS diagnostic criteria, OCBs, remains the most relevant prognostic biomarker for RIS. Physiologically linked to OCBs and with greater accuracy in other phases of the disease, kFLC might be a good candidate prognostic biomarker for RIS.

Secondly, although unavailable in routine clinical care, data concerning NfL, IL-8, and miRNA profiles in CSF have encouraged us to explore their potential as biomarkers for RIS prognosis (**Figure 1**). Additionally, CHI3L1 and GFAP, reflecting glial activation, need to be explored in CSF as possible biomarkers for early PPMS and disability progression.

Finally, no peripheral biological markers have so far been identified as providing additional prognostic value, except for the miRNA profile. CHI3L1, GFAP, and NfL, accurately measurable in blood, might also constitute potential peripheral biomarkers of disease activity and progression.

Along with candidate biomarkers from current knowledge of early MS and -omics approaches, therapeutic response biomarkers may arise from ongoing randomized controlled trials (RCTs) in RIS subjects [TERIS, NCT03122652 (92) and ARISE, NCT02739542 (93)]. Leveraging samples and data from RIS patients in RCTs and large prospective cohorts with extended follow-up will be necessary to validate these candidate biomarkers for RIS, which predict greater disease severity. Moreover, identifying biological biomarkers obtained from blood samples—far less invasive than a lumbar puncture—should be a priority for future studies.

AUTHOR CONTRIBUTIONS

MR wrote the first draft of the manuscript, wrote sections of the manuscript, and contributed to manuscript revision, read, and approved the submitted version. MG contributed to manuscript revision, read, and approved the submitted version. ET contributed to the conception and design of the study, wrote sections of the manuscript, contributed to manuscript revision, read, and approved the submitted version. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Perspectives of at-Risk Individuals on Preventive Intervention for Rheumatoid Arthritis: A Mini Review

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There has been intense research focus on the biological mechanisms underlying the transition from health to disease for rheumatoid arthritis (RA) over recent years, and it is now well established that a state of autoimmunity precedes the development of symptoms for a large proportion of patients. This has led to an increased interest in the identification of at-risk groups and the potential for preventive intervention. The ability of several immunomodulatory agents to delay or prevent RA is under investigation and novel cellular therapies are in development. Preventive approaches are also being assessed in other chronic autoimmune diseases. For example, an anti-CD3 antibody has recently been shown to delay progression to type 1 diabetes in non-diabetic relatives of patients identified as being at high risk. The identification and treatment of individuals as being at risk of a disease where there is a degree of uncertainty around the potential for benefit is socially and ethically challenging. Recently reported difficulties in recruitment to RA prevention trials have underlined the importance of understanding the perspectives of at-risk individuals to identify barriers and facilitators that need to be addressed in order for preventive strategies to be acceptable. Understanding of their preferences for benefits and risks of preventive interventions can inform efficient intervention prioritization, prevention trial design and the development of informational resources for those at risk. In this review we summarize current knowledge of preferences for RA prevention and make recommendations for further research needed to ensure efficient development of preventive therapies and clinical implementation.

Keywords: rheumatoid arthritis, prediction, prevention, at-risk groups, perceptions, preferences, choice - behaviour

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory disease that causes painful swelling of the joints, fatigue, depression, and extra-articular manifestations including accelerated cardiovascular disease. There is currently no cure, and long-term treatment is usually required to prevent joint erosion and loss of function (1). Although the introduction of biologic and targeted synthetic disease modifying anti-rheumatic drugs (b/ts DMARDs) has revolutionized management of RA, approximately 10-15% of patients do not respond to multiple sequential therapies (2). Risks of treatments for RA include infection and lung, liver and haematological toxicity. In addition to the disease burden experienced by patients, RA presents a significant socioeconomic burden (3, 4). There is thus a clear rationale for the development of a cure and/or preventive interventions for this condition.

It is established that early treatment of RA is associated with improved outcomes (5). This has led to increased focus on the earliest stages of disease development, including pre-clinical phases (6). Understanding of the biological mechanisms operating at articular and extra-articular sites in at-risk individuals has evolved rapidly (7), and algorithms to predict the development of clinical arthritis in at-risk populations have become increasingly sophisticated (8). Recognition of groups at risk of RA presents possibilities for preventive intervention. Such intervention could prevent or delay the onset of clinical arthritis, and also reduce the complex symptom burden often experienced before diagnosis (9). Intervention at this stage could also reduce RA severity if it were to subsequently develop.

The European Alliance of Associations for Rheumatology (EULAR) has provided recommendations for terminology to identify distinct at-risk phases (based on genetic and environmental risk factors, RA-related autoantibodies and symptoms) (10). Key target groups for preventive approaches may have one or more of the following: (a) genetic risk factors (e.g. risk is increased approximately fourfold in first-degree relatives [FDRs) (11)]; (b) environmental risk factors [e.g. smoking (12)]; (c) systemic autoimmunity associated with RA (typically indicated by rheumatoid factor and/or anti–citrullinated protein/peptide antibodies); (d) symptoms suggestive of underlying inflammation but without clinically apparent synovitis [clinically suspect arthralgias (CSAs) (13)]; or (e) early arthritis that does not fulfil RA classification criteria. Different approaches are likely to be appropriate at each phase. Primary prevention of seropositive RA would involve intervention to prevent development of systemic autoimmunity, while secondary prevention of seropositive RA would involve prevention of RA development in individuals with pre-existing systemic autoimmunity (6).

EULAR guidance for trials and observational studies in individuals at-risk of RA, based on expert consensus and evidence from systematic reviews (14, 15), is now available and the scene is set for progress towards a new paradigm of prevention, rather than treatment of RA (16). Evaluation of candidate preventive therapies for RA is a nascent research area, though early findings are promising. Whilst intramuscular glucocorticoid did not delay arthritis development in seropositive arthralgia patients (17), it prevented 10% of patients with early inflammatory polyarthritis from progressing to RA and delayed DMARD prescription (18). B-cell depletion with a single infusion of rituximab delayed, but did not prevent RA onset in individuals with seropositive arthralgia and either imaging synovitis or evidence of an acute phase response (19). The effects of time-limited courses of other immunomodulatory therapies, including abatacept (20) and hydroxychloroquine (21), on RA development are currently being assessed in other at-risk groups, including asymptomatic FDRs (21). Preventive treatments are also under investigation in other chronic autoimmune conditions. For example, an anti-CD3 antibody delayed progression to type 1 diabetes in non-diabetic relatives of patients identified as being at high risk based on the presence of diabetes-related autoantibodies and other risk factors (22).

Although trials of lifestyle interventions to prevent RA are currently lacking, Vitamin D supplementation for five years has been shown to reduce risk of autoimmune diseases (23). Omega 3 fatty acids have been inversely associated with the presence of RA-related autoantibodies (24, 25), though a prospective cohort study did not find an association between fish intake with RA development (26). There is a robust rationale for studies of smoking cessation to reduce risk of RA (12, 27, 28), and other interventions such as periodontal treatment and weight control have preventive potential (29, 30).

Whilst prevention of diseases such as RA has considerable potential to improve outcomes and reduce societal costs, the identification of individuals as being at risk, and the use of preventive treatment where there is a degree of uncertainty around disease development and progression, is ethically challenging (31, 32). Those at risk may face complex decisions around accepting predictive assessments and risks associated with immunomodulatory interventions in exchange for uncertain benefit. A recent trial of 40mg atorvastatin daily for three years to prevent arthritis development in seropositive arthralgia patients was terminated prematurely due to unwillingness to participate (33). A related qualitative study exploring barriers to trial participation highlighted perceptions that the need for treatment was low and outweighed by concerns about treatment risks and the burden of trial participation (34).

Understanding the perceptions and preferences of those at risk for preventive approaches is therefore essential to inform the development of balanced, tailored informational resources for those considering trial participation, and to support efficient clinical translation. There is increasing recognition of the value of information about patient preferences for decision-making by the pharmaceutical industry, regulatory agencies, and health technology assessors (35–37). Systematically collected data on patient preferences can support efficient, patient-focused medicine development, including target product profile development, endpoint selection, benefit-risk assessment, and regulatory approval (38, 39). The integration of patient preference information into drug development is more likely to result in treatments that are acceptable to patients. This is especially important in the context of disease prevention, where uptake and adherence to medications can be low (40, 41). Therefore, the objective of this article is to provide a

narrative review of what is known about the perceptions and preferences of at-risk populations (EULAR at-risk stages a-d) and other key stakeholders for predictive and preventive strategies for RA, and identify opportunities for further investigation. The search strategy used to identify relevant literature is summarized in **Supplementary Material**.

EXPLORATORY QUALITATIVE STUDIES

A summary of published qualitative investigations exploring perceptions of predictive testing and/or preventive interventions for RA can be found in **Table 1**. Perceptions of predictive approaches have been studied in those with CSA (43, 48), asymptomatic individuals who have tested positive for RA-related autoantibodies (48), FDRs (44), the general public (49), and RA patients (who may be involved in providing access and/or information to FDRs) (45). Participants across these studies recognized the value of disease risk information in terms of increased self-awareness and also the potential for early or preventive treatment (15). However, several studies noted concerns around the uncertainty associated with disease development and potential for psychological distress (44, 45, 48). Mosor et al. (2020) reported that these concerns were particularly salient for participants with joint symptoms (48). However in another study, FDRs who received personalized risk education reported greater levels of reassurance than those who received standard RA risk information (50).

In a focus group study of CSA patients, participants had negative views of the utility of numerical information about risk (43). Interview studies with FDRs (44) and patients (45) suggested that positive views of predictive testing for RA were associated with the misperception that such tests could rule in/out RA. Negative viewpoints were associated with an understanding of the probabilistic nature of risk information (45). In focus groups, members of the general public reflected misperceptions about the severity of RA that had been found in previous studies, and held beliefs that risk assessment was more appropriate for diseases that were perceived to be more serious (49). Lack of public awareness about the negative personal impact of RA was highlighted by RA patients as a potential barrier to predictive strategies (45). Several studies emphasized unmet needs for information about RA and risk factors for RA (44, 45, 48).

The first qualitative study addressing perspectives on preventive treatments for RA found that most participants would accept a prophylactic treatment if their risk of developing RA was 30% or greater (42). However, the participants in that study were FDRs enrolled in a prospective observational cohort and their views may not be representative of other at-risk groups. Other studies of FDRs and RA patients (45–47) suggested that lifestyle interventions would be preferred over pharmaceutical therapies, highlighting concerns about medication side effects and beliefs that drug treatment is appropriate only after symptoms have developed. Such beliefs were echoed by Mosor et al. (2020) who reported that seropositive individuals without symptoms were less inclined

TABLE 1 | Summary of published qualitative studies exploring perceptions/preferences of RA prediction/prevention.

Participants	Study Objectives	Methods	Key findings	Authors
20 FDRs* taking part in an observational cohort study (Switzerland)	Explore perceptions of preventive treatments and participation in interventional trials to prevent RA	Interviews	Preventive treatments with low risk of serious adverse effects were acceptable when risk of RA was above 30%.	Novotny et al. (42)
4 CSA** patients taking part in an observational cohort study (Netherlands)	Explore perceptions of CSA and prognostic information about RA	Focus Groups	Negative views about numerical risk estimates	Newsum et al. (43)
32 FDRs recruited *via* RA patients (UK, Germany, Austria)	Explore perceptions of RA risk and predictive testing	Interviews	Unmet information needs and concerns about uncertainty/anxiety	Stack et al. (44)
22 RA patients (UK)	Explore perceptions of predictive testing, preventive intervention and communicating with relatives about RA risk	Interviews	Positive views associated with misperceptions about risk information. Selective family communication about risk	Falahee et al. (45)
32 FDRs recruited *via* RA patients in secondary care clinics (UK, Germany, Austria)	Explore perceptions of preventive interventions for RA	Interviews	Lifestyle interventions preferred. Drugs appropriate after symptom onset. Concerns about drug side effects.	Simons et al. (46)
25 participants (13 patients, 5 FDRs and 7 rheumatologists	Define attributes of treatments to prevent RA to be assessed in a quantitative study	Focus groups	Role of healthcare professional recommendation in treatment decisions highlighted	Munro et al. (47)
34 seropositive individuals (24 CSA patients and 10 asymptomatic individuals attending extended health examination) (Austria, Germany, UK)	Explore perspectives and information needs around predictive test results and preventive treatment	Interviews	Symptomatic individuals more likely to accept preventive intervention and experience anxiety	Mosor et al. (48)
18 seropositive CSA patients invited to take part in interventional trial to prevent RA development (Netherlands)	Identify barriers and facilitators to participation in trial to prevent RA development	Focus groups	Identified information needs of trial participants highlighting potential for benefit and addressing concerns about burden of trial participation	Van Boheemen et al. (34)
21 members of the public (UK)	Perceptions of predictive testing for RA, breast cancer and early onset Alzheimer's disease	Focus groups	Concerns around predictive testing less pronounced for RA. RA perceived to be less serious than other diseases.	Singhal et al. (49)

*FDR, First-degree relative; **CSA, Clinically suspect arthralgia.

to consider preventive treatments than those who were experiencing arthralgia (48). The focus group study by Munro et al. (2020) involving participants who were either RA patients, FDRs or rheumatologists also found that the precision of disease risk estimates and endorsement by a trusted healthcare professional would be important considerations when deciding whether to accept a preventive treatment for RA (47). No other qualitative studies published to date have addressed the perspectives of healthcare professionals.

Many of the themes described above were also found in interviews with autoantibody positive individuals with CSA who had been invited to take part in a trial of a treatment to reduce their risk of developing RA (34). Whilst potential for personal and societal benefit, along with detailed information and support from the individual's physician, facilitated trial participation, barriers included beliefs about personal risk status and the need for treatment, and concerns about treatment-related harms and the perceived burden of trial participation.

QUANTITATIVE INVESTIGATIONS

Table 2 summarizes published quantitative investigations. A survey study found that over 50% of FDRs were definitely interested in taking a predictive test to quantify their risk of developing RA (52). Predictors of levels of interest included attitudes about risk knowledge, information-seeking preferences and beliefs that predictive testing could cause psychological harm. No other quantitative studies have addressed preferences for predictive testing for RA.

Van Boheemen et al. (2020) surveyed willingness to use 100% effective preventive medications amongst seropositive arthralgia patients and rheumatologists (54). At 30% baseline risk of developing RA, 53% of patients and 74% of rheumatologists would be willing to use a preventive therapy with no side effects. At 70% baseline risk, this increased to 69% for patients and 92% for rheumatologists. A drug with minor side effects was acceptable to 26% of patients and 31% of rheumatologists when the baseline risk of RA was 30%; and to 40% of patients and 76% of rheumatologists when risk of RA was 70%. Patients' willingness to make preventive lifestyle changes was high, though this was not often the focus of rheumatologists' consultations (54).

Stated choice methods, where participants choose between hypothetical treatment options described by treatment attributes (e.g., risks, benefits, method of administration, etc.) with pre-specified levels that are varied systematically, provide quantitative information about the relative importance of treatment attributes, benefit/risk tradeoffs, preference heterogeneity, and predicted uptake. Such information can inform selection of outcomes and endpoints in clinical trials and also support stakeholder (e.g. regulator, HTA) decision-making (38, 39). Whilst stated preferences for RA treatments have been widely assessed (56) there are limited examples for RA prevention (57).

A best-worst scaling study of 32 FDRs enrolled in a prospective cohort in Switzerland reported that treatment

TABLE 2 | Summary of published quantitative studies assessing perceptions/preferences of RA prediction/prevention.

Participants	Study Objectives	Methods	Key findings	Authors
32 FDRs* taking part in an observational cohort study (Switzerland)	Assess impact of treatment efficacy, mode of administration, severe side effects and mild side effects on likelihood of acceptance of preventive treatment for RA	Stated choice survey (best-worst scaling)[1]	Hypothetical RA risk status affected likelihood that treatment chosen. Treatment effectiveness and severe side effects significantly affected choices, mild side effects and mode of administration did not.	Finckh et al, (51)
288 self-reported FDRs recruited *via* Amazon's Mechanical Turk platform	Assess relative importance of, and trade-offs between, preventive treatment effectiveness, side effects, mode of administration, certainty in evidence for effectiveness, and healthcare professional endorsement	Stated choice survey (discrete choice experiment)[2]	Method of administration, effectiveness, healthcare professional endorsement and serious side effects were most influential determinants of choices. Predicted uptake of biological therapies was low.	Harrison et al, (52)
108 participants (78 RA patients, 30 of their FDRs and 39 rheumatologists) (Canada)	Assess relative importance of, and trade-offs between, preventive treatment effectiveness, side effects, mode of administration, certainty in evidence for effectiveness, and rheumatologist/patient endorsement	Stated choice survey (discrete choice experiment)[2]	Rheumatologist/patient endorsement most important attribute. Non-biologic therapies preferred. Preferences of patients and FDRs differed from those of rheumatologists	Harrison et al, (53)
187 participants (100 seropositive CSA** patients, 38 FDRs of patients with axial spondylitis, 49 rheumatologists) (Netherlands)	Assess willingness to accept 100% effective preventive treatments with no/minor side effects at 30%/70% disease risk	Survey	Lifestyle interventions were acceptable to participants, but rarely discussed by rheumatologists. Acceptability of drug treatment was higher amongst rheumatologists than at risk individuals. Treatment acceptability increased with hypothetical risk of RA	Van Boheemen et al, (54)
396 FDRs of RA patients (UK)	Assess interest in taking a predictive test for RA, and predictors of interest.	Survey	FDRs interest in predictive testing was high. Predictors of interest included information-seeking preferences and beliefs that predictive testing would increase empowerment or cause anxiety	Wells et al, (55)

*FDR, First-degree relative; **CSA, Clinically suspect arthralgia.
[1]Stated choice study design informed by Novotny et al. (2013) qualitative study (42).
[2]Stated choice study design informed by Munro et al. (2018) qualitative study (47).

effectiveness to reduce risk of RA and the likelihood of serious adverse effects were significant determinants of the likelihood that participants would choose a preventive treatment (51). Mild adverse events and the method of drug administration did not influence participants' decisions. Preventive therapies were chosen 7%, 30% and 38% of the time when participants assumed a baseline risk status of 1%, 20%, and 40%, respectively (51).

A larger sample of self-reported FDRs took part in a Canadian discrete choice experiment (DCE) (52). Participants were asked to assume a 60% risk of developing RA. Method of administration, treatment effectiveness, healthcare professional preference and risk of serious side effects were the treatment attributes that most influenced participants' choices. Latent class analysis identified three sub-groups of participants whose preferences were driven not only by treatment effectiveness, but also by safety aspects, healthcare professional endorsement and treatment convenience, respectively. Predicted uptake was high for non-biologic drugs such as hydroxychloroquine (84%), but low for atorvastatin and biologics (52).

Nonbiologic drugs were also preferred in a similar survey in Canada of a sample including RA patients, FDRs and rheumatologists (53). 38% of patients/FDRs preferred no preventive treatment, compared with 12% of rheumatologists. The most important drivers of participants' choice were shared decision-making (whether the treatment option was supported by the rheumatologist/patient), risks of serious side effects, and treatment effectiveness (53).

Finally, the protocol of a stated choice survey employing both a DCE and a probabilistic threshold technique to assess preferences for preventive treatments for RA has been published (58). That study recruits large samples of the general population *via* survey panels in the UK, Germany and Romania, and also recruits FDRs of confirmed RA patients. Initial findings from the DCE of the general population indicated that treatment effectiveness was the most important determinant of choice across countries, and the sample in Romania was more sensitive to treatment risks (59). Predicted uptake of profiles resembling RA prevention candidate therapies varied across countries, with a profile chosen to estimate abatacept being most likely treatment to be chosen in all three (59).

DISCUSSION

The studies described in this narrative review highlight significant progress in our understanding of preferences for risk assessment and preventive interventions for RA (60). There are now a number of qualitative explorations across a range of stakeholder groups indicating perceived potential for benefit that is sometimes outweighed by concerns around the probability of RA development, treatment harms, uncertainty about effectiveness, and perceptions that preventive intervention with pharmaceutical products are not warranted for RA. The latter finding may reflect commonly held public misperceptions that RA is not a serious condition, and/or that it is a natural part of human ageing (61–63). Taken together these studies highlight an urgent need to provide at risk groups with accurate information about RA, RA risk and the risks and benefits associated with potential preventive strategies to support shared decision-making in the context of trial participation and effective clinical translation. Little is known about the perspectives of healthcare professionals in this context. As the implementation of preventive strategies for RA would require considerable reconfiguration of healthcare services, further studies are needed.

Whilst several studies have described a preference for lifestyle interventions over pharmaceutical therapies, and personalized risk education has been shown to increase risk-reducing health behaviours amongst FDRs of RA patients (64), interventional trials of potential preventive lifestyle interventions for RA (such as smoking cessation, periodontal treatment, weight loss and dietary change) are currently lacking.

There are fewer examples of quantitative studies. Choice-based methods have been applied to samples of FDRs and the public and provide initial evidence that preventive treatments for RA are acceptable to those assuming a hypothetical high-risk status. However, no quantitative studies have used stated choice methods to directly elicit the preferences of very high-risk populations (e.g., seropositive individuals with CSA) for either predictive tools or preventive treatments. Further research in this area is therefore needed to enable quantification of the relative importance of outcomes/intervention attributes, benefit/risk tradeoffs and predicted uptake of treatment profiles for this group. Such information would support patient-focused development of preventive therapies and enhance the likelihood of clinical impact. Importantly, no stated choice studies have quantified the degree of benefit required from preventive lifestyle interventions for RA in exchange for sustained behavioral change. This, is an important area for future research given that several studies have indicated that lifestyle interventions are preferred for prevention of RA. No studies to date have assessed preferences for combined lifestyle and pharmacological intervention.

All preference studies undertaken to date have focused on a single aspect of treatment effectiveness: reduction of the risk of RA development. None have investigated preferences for outcomes such as delay of the onset of RA, or reduction of subsequent RA severity. For symptomatic at-risk groups, important additional benefits may include reduction of symptoms such as arthralgia and fatigue. Further research is therefore needed to quantify the relative importance of these outcomes in high-risk populations. All existing studies were undertaken in Europe or North America. Further investigation is needed to assess preferences in different countries with different types of healthcare provision and also in low and middle income countries. Existing choice-based studies have not yet identified participant characteristics (e.g., gender; health literacy; and numeracy) associated with preference heterogeneity (52), though this is currently under investigation (58).

Comparisons across quantitative studies are limited by methodological heterogeneity. For example, where a treatment attribute describing healthcare professional endorsement or

certainty of risk estimates is included in the experimental design it is likely to be an important determinant of participants choices (52, 53). Such considerations can be held constant in the treatment scenario to allow assessment of the relative importance of additional treatment characteristics.

The emergence of evidence-based recommendations to guide the use of preference studies for decision-making in the medical product lifecycle, such as those produced by the PREFER consortium (35), provides a framework for future studies in this area. PREFER has also contributed to an agenda for further refinement of stated preference study methodology. For example, the application of measures of psychological constructs to explain preference heterogeneity (65, 66), and the development of scenario-based interactive educational tools to deliver background information and training to preference study participants to support informed choices (67). These methodological considerations are particularly relevant in the context of RA prevention, where decision making by those at risk of developing RA about accepting treatment is likely to be highly preference sensitive, and influenced by underlying beliefs about RA, personal risk status and treatment risks and benefits. Therefore, the development of innovative educational tools to obtain informed preferences within preference elicitation studies of preventive interventions for RA could also be usefully applied to support shared decision-making in clinical settings.

Preventive strategies for other chronic conditions are routinely integrated into clinical practice, and many asymptomatic individuals accept preventive pharmaceutical treatments (e.g., statins and antihypertensive medications are widely prescribed to reduce risk of cardiovascular disease). A similar approach to RA could dramatically improve clinical outcomes with considerable cost savings. The development of treatments to achieve this that are acceptable to those at risk would represent an important paradigm shift. Such an achievement is more likely to be realized if it is informed by an understanding of stakeholder perspectives and underpinned by evidence that aligns with the treatment preferences of at-risk populations.

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Both authors contributed to the conception, writing and finalization of this article. MF wrote an initial draft. All authors contributed to the article and approved the submitted version.

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Precursors to Systemic Sclerosis and Systemic Lupus Erythematosus: From Undifferentiated Connective Tissue Disease to the Development of Identifiable Connective Tissue Diseases

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The pathogenesis of connective tissue diseases (CTDs), such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), is characterized by derangements of the innate and adaptive immune system, and inflammatory pathways leading to autoimmunity, chronic cytokine production, and chronic inflammation. The diagnosis of these diseases is based on meeting established criteria with symptoms, signs and autoantibodies. However, there are pre-clinical states where criteria are not fulfilled but biochemical and autoimmune derangements are present. Understanding the underlying processes responsible for disease pathogenesis in pre-clinical states, which place patients at increased risk for the development of established connective tissue diseases, represents an opportunity for early identification and potentially enables timely treatment with the goal of limiting disease progression and improved prognosis. This scoping review describes the role of the innate and adaptive immune responses in the pre-clinical states of undifferentiated CTD at risk for SSc and prescleroderma, the evolution of antibodies from nonspecific to specific antinuclear antibodies prior to SLE development, and the signaling pathways and inflammatory markers of fibroblast, endothelial, and T cell activation underlying immune dysregulation in these pre-clinical states.

Keywords: systemic sclerosis, scleroderma, prescleroderma, pathogenesis, innate immunity, adaptive immunity, systemic lupus erythematosus, autoimmunity

INTRODUCTION

Systemic sclerosis (SSc) is a rare multisystem autoimmune connective tissue disease (CTD) characterized by fibrosis of the skin and internal organs, vasculopathy, and autoimmunity with distinct antibodies. SSc is classified using the American College of Rheumatology/European League of Rheumatism (ACR/EULAR) 2013 criteria (1). However, there are pre-morbid clinical states, including Undifferentiated Connective Tissue Disease at risk for Systemic Sclerosis (UCTD-risk-SSc) and prescleroderma, where autoimmunity and dysregulation of inflammatory pathways occur without the presence of clinical symptoms (2). UCTD-risk-SSc, also known as very early/early SSc, is a label given to patients who do not meet the ACR/EULAR 2013 criteria, but who present with Raynaud's Phenomenon (RP) and either typical SSc capillaroscopic findings (megacapillaries or avascular areas) or serum marker antibodies (anti-centromere, anti-topoisomerase I, anti-RNA polymerase III, anti-Th/To, and anti-Pm-Scl) (3, 4). UCTD-risk-SSc patients have a 35-79% risk of developing definite SSc over time (5–7). Prescleroderma is diagnosed in patients with RP who present with serum marker autoantibodies (anti-centromere or anti-topoisomerase I) and immunofluorescence derived antinuclear antibodies (ANA) at titre >1:320 or serum antibodies and avascular capillaroscopic changes or ANA positivity at 1:320 and avascular areas (7). Moreover, patients with prescleroderma have an even higher risk of developing established SSc than UCTD-risk-SSc (7). Making a diagnosis and intervening early may change the trajectory of disease in these patients.

Another CTD with pre-clinical stages progressing to identifiable disease is systemic lupus erythematous (SLE). SLE which is characterized by features such as arthritis, rash, photosensitivity, serositis, cytopenias, mucositis, glomerulonephritis, fevers and fatigue, may onset insidiously and can be difficult to differentiate from other autoimmune diseases initially (8, 9). Commonly ANA will pre-date SLE diagnosis by years during undifferentiated pre-clinical stages termed "incomplete SLE" or "possible SLE" when ACR criteria for SLE are not met (10, 11). Approximately 55% of patients with incomplete SLE (iSLE) develop SLE (12). Furthermore, as disease progression occurs, more specific antibodies for SLE are produced such as anti-double stranded DNA and anti-Smith antibodies (10, 13).

Ultimately, the changes observed in these pre-clinical stages with varying likelihood of progression to full-blown disease are insidious and driven by derangements in inflammatory signalling and autoimmunity. The purpose of our scoping review was to elucidate the role of the innate and adaptive immune systems and dysregulated signaling pathways in pre-clinical states, and their contribution to the establishment of full-blown disease.

SEARCH STRATEGY

Our search strategy was developed with an experienced information specialist (Supplementary Material). We searched the databases EMBASE and MEDLINE with restrictions for the English language and included peer-reviewed manuscripts as well as conference abstracts. We sought to include studies which provided information regarding the role of adaptive and innate immune systems and the dysregulation of pathways which contributed to the development of classifiable SSc or SLE. Therefore, we included studies which explicitly studied individuals termed as UCTD-risk-SSc, Very early/early SSc, prescleroderma, pre-SLE, incomplete SLE, or lupus-like. Studies were excluded if they provided information regarding inflammatory pathways where patients with established disease were investigated. The search and inclusion of studies was performed by one reviewer (LMC) with review of included studies performed by both authors (LMC & JEP). Our search yielded 2313 manuscripts after duplicates were removed on August 10, 2021 and pertinent manuscripts have been included (**Figure 1**).

SYSTEMIC SCLEROSIS

Dysregulated Signalling Pathways and Autoimmunity

Progressive inflammation, vasculopathy and fibrosis orchestrated by aberrant cytokine production is a hallmark of SSc. Chemokines involved in extracellular matrix deposition, erroneous activation of fibroblasts, and anomalous immune system activation, including CCL2, MIP-1α/CCL3, CCL4, CCL7/MCP-3, and CXCL8, have been observed to be significantly upregulated in the serum of established SSc patients when compared to healthy controls (14–16). However, the presence of these chemokines is more nuanced in pre-clinical disease. Vettori et. al., compared the serum of UCTD-risk-SSc patients to fibromyalgia and/or osteoarthritis controls without RP, and definite SSc patients for soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), CCL2, CXCL8, IL-13, IL-33, and transforming growth factor-β (TGF-β) (17). A significant increase was observed in sICAM-1, CCL2, CXCL8, and IL-13 along a disease spectrum gradient from UCTD-risk-SSc to limited cutaneous SSc (lcSSc) to diffuse cutaneous SSc (dcSSc). sICAM-1 is involved in the transmigration of leukocytes from vessels to endothelium and promotes inflammation through T cell activation and cytokine production (18, 19). CXCL8 and CCL2 are pro-fibrotic alter angiogenesis, and affect the migration of monocytes, T cells, and neutrophils (20–22). IL-13 contributes to fibrogenesis through fibroblast activation and TGF-β stimulation (23). Consequently, chemokines increase as disease severity worsens highlighting the progressive derangement of vasculature and autoimmune changes in SSc. Interestingly, higher IL-33 levels were found in UCTD-risk-SSc patients compared to controls and established SSc. IL-33 induces IL-4, IL-5, and IL-13 production leading to arterial vessel media hypertrophy and eosinophilic and mononuclear cell infiltration (24). Therefore, IL-33 functions as a very early mediator in the progression to established SSc, is involved in the fibrotic stage of

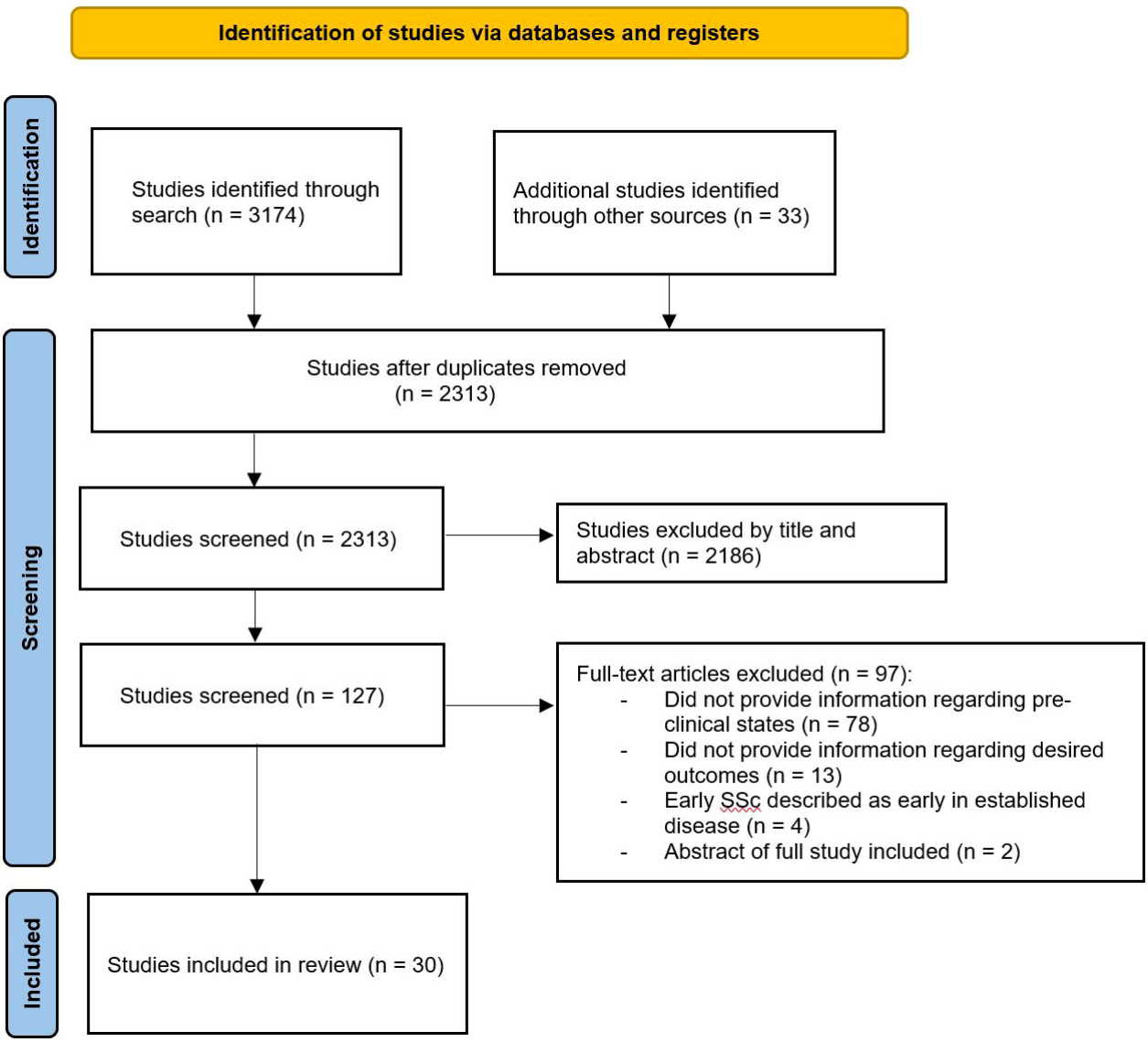

FIGURE 1 | Flow diagram of scoping review selection process.

SSc through IL-13 stimulation; and serves as a predictive marker to elucidate which patients will develop established disease (25).

Other cytokines are abnormal in UCTD-risk-SSc including soluble IL-2 receptor alpha (sIL-2Rα), aminoterminal propeptide of type III collagen (PIIINP), and CXCL4 (7, 26, 27). sIL-2Rα functions as a marker of T-cell activation, whereas PIIINP functions as a marker of collagen formation and fibroblast activation (28, 29). CXCL4 functions as a potent anti-angiogenic chemokine and serves to inhibit endothelial cell proliferation and migration (30). Additionally, CXCL4 has pro-fibrotic capabilities through inhibiting interferon-gamma (IFN-γ) expression and stimulating IL-13 and IL-4 production (31). CXCL4 levels, measured from serum, were higher in UCTD-risk-SSc than controls and were associated with anti-Scl 70 antibodies and sICAM-1 (27, 32). Furthermore, CXCL4 levels, drawn from non-platelet poor plasma, were reported to correlate with extent of skin fibrosis and were predictive of pulmonary arterial hypertension and lung and skin fibrosis progression in SSc (33).

Type I IFN represents another significant contributor to the pathogenesis of SSc through the upregulation of genes involved in the activation of the innate and adaptive immune systems. The increased expression of these type I IFN regulated genes, termed the type I IFN signature, has been previously observed in SLE and other autoimmune diseases (34, 35). Brkic et al., investigated the whole-blood samples of healthy controls without RP, patients with primary RP, UCTD-risk-SSc, and definite SSc patients to determine the expression of 11 type I IFN inducible genes (36).

Authors report increased type I IFN related gene expression in UCTD-risk-SSc patients compared to healthy controls, but not in primary RP compared to controls. This finding eludes to the early contribution of the type I IFN pathway in the pathogenesis of SSc. Furthermore, the presence of polymorphisms of IFN regulated genes have been found to confer increased risk of SSc (37).

Vasculopathy and Fibrogenesis

Cossu et al. investigated angiogenetic and endothelial dysfunction markers involved in vasculopathy (38). Authors sampled the serum of healthy controls without RP, UCTD-risk-SSc, lcSSc, and dcSSc patients for angiopoietin-2 (ang-2), CXCL16, e-selectin, sICAM-1, CXCL8, sVCAM-1, and VEGF. There was a significant trend along a disease spectrum from controls to UCTD-risk-SSc to lcSSc and to dcSSc for ang-2, CXCL16, e-selectin, and sICAM-1. Authors also observed a significant difference in ang-2 between controls and UCTD-risk-SSc. Ang-2's functioning is contextual as it facilitates angiogenesis if VEGF is present, but causes blood vessel regression if pro-angiogenic stimuli are absent (35). Clinically, ang-2 correlates with the extent of skin involvement in SSc as measured by the modified Rodnan skin score (mRSS), disease activity, and C-reactive protein (39). Tabata et. al., found that IGF-1, VEGF, and RANTES levels are significantly higher in mild established SSc compared to pre-clinical SSc (40).

Fibrogenic inflammatory pathways resulting from chronic inflammation and orchestrated through fibroblast dysfunction lead to excessive accumulation of extracellular matrix components, including hyaluronic acid, fibronectin, and proteoglycans, in SSc (41). Sera of healthy controls without RP, UCTD-risk-SSc, and non-fibrotic SSc patients were analyzed whereby elevated markers (CXCL10/IP-10, CXCL11/I-TAC, tumor necrosis factor receptor type II (TNFRII), and chitinase 3-like protein 1) were higher in UCTD-risk-SSc patients compared to controls (42). CXCL10 and CXCL11 are angiostatic and migration chemokines which drive smooth muscle cell proliferation, and recruit T cells, monocytes, and natural killer cells (43–45). Importantly, CXCL10 and CXCL11 levels are associated with UCTD-risk-SSc patients most at risk for developing established SSc (25, 46). Furthermore, CXCL10 and CXCL11 are observed to be correlated with type I IFN signature and decrease with type I IFN receptor blockade with anifrolumab (47).TNFRII has a role in the proliferation and activation of regulatory T cells (48). Additionally, TNFRII co-stimulated lymphocytes secrete pro-fibrotic cytokines in patients with SSc (49). Chitinase 3-like protein 1 has been implicated in regulating and stimulating angiogenesis and fibrogenesis through activation of Syndencan-1 and focal adhesion kinase (50). Furthermore, in SSc patients, chitinase 3-like protein 1 has been correlated with articular involvement and T cell activation (51). These findings highlight the interplay between the adaptive and innate immune systems alongside fibrogenesis.

Alterations of natural killer (CD 56+) and natural killer T cells (CD56+ CD3+) in early SSc compared to controls, primary RP, and established SSc were found and thought to be related to differential Toll-like receptor (TLR) 1/2 stimulation (52). Early SSc demonstrated an intermediate activation pattern regarding CD56+ secretion of IL-6, TNF-a, and MIP-1α/CCL3 compared to controls with significant differences of IL-6 secretion. An increasing trend in CD56+ activation for TNF-α and CCL3 occurred between early SSc and controls. This pattern of elevated IL-6, TNF-α, and CCL3 alludes to the role of underlying innate immune mechanisms in prescleroderma or early SSc; which, may eventually lead to established SSc. The development of SSc is shown over time (**Figure 2**).

SYSTEMIC ERYTHEMATOSUS LUPUS

Autoimmunity and Dysregulated Pathways

Antibodies predate the diagnosis of SLE by multiple years in a characteristic pattern evolving from non-specific ANA to more specific SLE antibodies prior to diagnosis. In a large serology study, a cohort of 130 military personnel who ultimately developed SLE were followed from first detection of ANA to diagnosis of SLE a median of 9.2 years later (11). Furthermore, anti-Ro, anti-La, anti-phospholipid, anti-double stranded DNA, anti-Smith, and anti-nuclear ribonucleoprotein (anti-RNP) antibodies were reported to have a time of first detection to diagnosis of 9.4 years, 8.1 years, 7.6 years, 9.3 years, 8.1 years, and 7.2 years, respectively. This observed pattern, corroborated by further studies, reflects progressive antibody evolution towards more specific SLE antibodies over time in patients ultimately diagnosed with SLE as ANA, anti-double stranded DNA, and anti-Smith antibodies have 86%, 94.7%, and 99% specificity, respectively (53–56). The presence and development of SLE specific antibodies can also serve as predictive makers of developing established disease. Munroe et. al., investigated unaffected blood relatives of SLE patients to identify risk factors of disease establishment (57). Relatives who developed SLE had elevated ANA and anti-Ro titers, and were likely to be anti-dsDNA and anti-RNP positive at baseline and follow up compared to those who did not transition. Anti-cardiolipin antibody positive patients also had more risk of developing SLE (58–60).

Cytokine changes in pre-clinical SLE have been studied (61). Interferon-α, IL-4, IL-9, IL-10, CXCL10 and monocyte chemotactic protein-1 (MCP-1/CCL2) were studied in sera of 35 patients prior to established SLE. CXCL10 was significantly higher in pre-clinical sera compared to controls and was correlated with interferon-α. One of the drivers of innate and adaptive immune dysregulation occurs through an up-regulation of interferon regulated genes, which is also known as the IFN signature of SLE (62). IFN-α, a type I IFN, stimulation leads to increased dendritic cell maturation, increased Th1 cell development and response, and enhanced NK, B, and T cell proliferation and survival (62). IFN-α correlates positively with IgG, and negatively with IgM autoantibodies (63). CXCL10 and IFN-α concentrations are higher in pre-clinical patients who are positive for any antibody compared to antibody negative patients.

Type II IFN (IFN-γ) is additionally implicated in SLE development (64). IFN-γ leads to production of IFN-α and the B-lymphocyte stimulator (BLyS) (65, 66). BLyS, otherwise

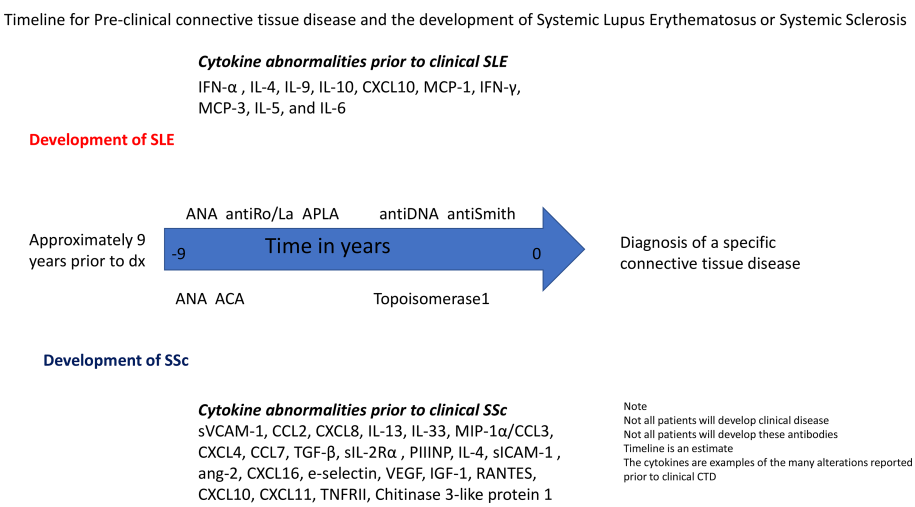

FIGURE 2 | Timeline for Pre-clinical connective tissue disease and the development of Systemic Lupus Erythematosus or Systemic Sclerosis. Legend: SLE, Systemic lupus erythematosus; SSc, Systemic sclerosis; ANA, antinuclear antibody; APLA, antiphospholipid antibodies; ACA, anticentromere antibodies.

known as B-cell activating factor of the TNF family (BAFF), is produced by innate immune cells and serves as a mediator of B cell proliferation and survival (67–69). BLyS induces a Th1 cellular response which coordinates both innate and cytotoxic immunity (65). Munroe et al., studied the timing and role of type I and II IFN, IFN-associated mediators, and antibody formation in pre-clinical patients who would later develop established SLE (70). Elevated IFN-γ, CXCL10, and MCP-3 levels occurred prior to IFN-α activity and antibodies. IFN-γ and MCP-3 are abnormal more than 4 years prior to the development of SLE. Therefore, though type I IFN is observed to be elevated in association with antibody positivity prior to SLE diagnosis, type II IFN and IFN-associated mediators seem to represent the pathogenetic intermediaries altering innate and adaptive immune system derangements through elevation of IFN-α and autoantibody formation. These findings agree with a finding that IFN-γ, IL-5, and IL-6 were elevated at least 3.5 years prior to classification (71). Importantly, these observed temporal differences may be secondary to the measurement techniques used in these studies and further investigations with direct measurements tools, such as single-molecule arrays, may further elucidate the temporal relationship between type I and II IFN. **Figure 2** shows a timeline for the development of SLE and SSc.

DISCUSSION

Understanding the immunological and inflammatory perturbations involved in the development of CTDs such as SSc and SLE provides clinicians with an opportunity to recognize pre-clinical patients that may benefit from close monitoring, investigations, and potentially early intervention to limit disease progression. Pre-clinical disease states, such as UCTD-risk-SSc, prescleroderma, and incomplete SLE, present with underlying aberrations, often years before clinical disease is present, of the innate and adaptive immune systems, and inflammatory pathways which drive pathogenesis and increase risk of developing established disease.

The pathogenic mechanisms present in UCTD-risk-SSc and prescleroderma include immune signal dysregulations, erroneous immune system recruitment, aberrant angiogenesis leading to vasculopathy, and inappropriate fibroblast activation leading to tissue fibrosis. Multiple cytokines are observed to increase along a disease spectrum from UCTD-risk-SSc to classified SSc and include sICAM-1, CCL2, CXCL8, ang-2, CXCL16, e-selectin, and IL-13 (**Table 1**). The mechanism of action of these cytokines includes transmigration of lymphocytes endothelium, innate immune cell activation and signal propagation, and extracellular matrix deposition. Furthermore, there are disease markers which are observed to be predictive of SSc and include sIL-2Rα, PIIINP, CXCL4, CXCL10, and CXCL11 (**Table 2**). Patients with SSc who have the limited cutaneous SSc subset frequently develop RP and anti-centromere antibody 8 years before other manifestations of SSc often followed by dilated nailfold capillaries, then puffy fingers or

TABLE 1 | Elevated chemokines observed in UCTD-risk-SSC orchestrating SSc pathogenesis.

Cytkine	Function
sICAM-1	Transmigration of leukocytes, T cells activation
CCL2	Chemotaxis of monocytes, T cells, neutrophils
CXCL8	Angiogenesis induction, immune cell proliferation
IL-13	Fibroblast activation, TGF-β secretion stimulation
Ang-2	Angiogenesis induction, monocyte activation
TNFRII	Regulatory T cell proliferation, profibrotic cytokine secretion
CHI3L1/YKL-40	Angiogenesis and fibrogenesis regulation

TABLE 2 | Taxa Cytokines observed to be predictors of SSc development.

Cytkine	Function
sIL-2Rα	Marker of T cell activation and proliferation
PIIINP	Marker of collagen formation and fibroblast activation
CXCL4	IL-13 and IL-4 stimulation
CXCL10	Smooth muscle cell proliferation, immune cell chemotaxis
CXCL11	T cell, monocyte, natural killer recruitment

sclerodactyly and other features of SSc (2–4). The presence or absence of these features is significant in risk stratification where patients with RP but without antibodies or nailfold capillary changes are at 1.8% risk of definite SSc compared to 79.5% in those with RP and positive antibodies and nailfold capillary changes (5). At this point in time, other than treating RP to try to prevent ischemic changes, there is no specific treatment to change the natural history of future development of SSc. Also, 1/3 may develop SSc over the next 5 years (so 2/3 won't) and this can lead to over-diagnosis, and patient anxiety. Interventions such as smoking cessation and reducing RP attacks and encouraging a healthy lifestyle including a diet high in omega3 fatty acids may be appropriate but this is speculation. Patients with diffuse cutaneous SSc do not develop RP until close to their diagnosis (often 1 to 2 years before or at the time of other signs and symptoms of SSc), so finding prescleroderma clinical features in the majority of these patients has not been possible.

Likewise, SLE development is rooted in aberrations of the innate and adaptive immune systems. Pre-clinical SLE is characterized by an evolving IFN signature and progressive SLE-specific antibody formation prior to disease classification. IFN-γ and IFN associated mediators can predate diagnoses by 3.5 years, and are present prior to and alongside antibody positivity. Throughout pre-clinical SLE, antibody formation occurs in a pattern that evolves from non-specific ANA to more specific SLE antibodies. Namely, ANA and anti-Ro formation can predate diagnosis by 9 years or more but are considered less specific. Whereas, the more specific anti-Smith and anti-dsDNA develop closer to disease onset. The development of SLE specific antibodies can function as predictive markers of transformation to clinical SLE.

Clinically, it is difficult to ascertain what to do with the findings. Other than close monitoring of patients at risk, it is not feasible to check cytokine panels (with high variability) and redoing antibodies is likely not cost effective. However, the changes in immune regulation that predate clinical CTD help in the understanding of pathogenesis and may in future provide targeted treatment for patients with a high probability of converting to chronic debilitating disease. It has been suggested that treating patients at risk for SLE with hydroxychloroquine may change the disease trajectory but large controlled studies are needed to determine if there is benefit in this approach (72); one such multicenter, randomized, placebo-controlled, double-blind clinical trial is currently underway (NCT0303118). Interestingly, there are already drug targets in clinically active SLE targeting signalling that has been shown to be abnormal prior to disease onset such as BlyS (belimumab) and type I interferon with anifrolumab. Intervening prior to clinical disease would not be appropriate with the knowledge we have but in future, personalized medicine may help to give a more robust prediction of who will develop chronic autoimmune CTD.

CONCLUSION

Ultimately, the coordinated dysregulation of the innate and adaptive immune systems, and inflammatory signalling pathways leads to the pathogenesis of connective tissue disease. Our improved understanding of these underlying aberrations in pre-clinical stages of disease will serve to better identify patients at increased risk.

AUTHOR CONTRIBUTIONS

LM and JP were involved in study design, review of literature, and manuscript writing. All authors reviewed the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.869172/full#supplementary-material

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REVIEW
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A Roadmap for Investigating Preclinical Autoimmunity Using Patient-Oriented and Epidemiologic Study Designs: Example of Rheumatoid Arthritis

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Background & Aims: Rheumatoid arthritis (RA) is a prototypic autoimmune disease causing inflammatory polyarthritis that affects nearly 1% of the population. RA can lead to joint destruction and disability along with increased morbidity and mortality. Similar to other autoimmune diseases, RA has distinct preclinical phases corresponding to genetic risk, lifestyle risk factors, autoantibody development, and non-specific symptoms prior to clinical diagnosis. This narrative review will detail observational studies for RA risk and clinical trials for RA prevention as a roadmap to investigating preclinical autoimmunity that could be applied to other diseases.

Methods: In this narrative review, we summarized previous and ongoing research studies investigating RA risk and prevention, categorizing them related to their design and preclinical phases.

Results: We detailed the following types of studies investigating RA risk and prevention: retrospective population-based and administrative datasets; prospective studies (case-control and cohort; some enrolling based on genetics, first-degree relative status, elevated biomarkers, or early symptoms/arthritis); and randomized clinical trials. These correspond to all preclinical RA phases (genetic, lifestyle, autoimmunity, early signs/symptoms). Previous and ongoing randomized controlled trials have enrolled individuals at very elevated risk for RA based on biomarkers, symptoms, imaging abnormalities, or early signs/symptoms.

Conclusion: We detailed the rich variety of study designs that is necessary to investigate distinct preclinical phases of an autoimmune disease such as RA. However, further progress is needed to fully elucidate the pathogenesis of RA that may ultimately lead to prevention or delay of disease onset.

Keywords: rheumathoid arthritis, epidemiology, autoimmunity, biomarkers, preclinical, prevention

INTRODUCTION

Rheumatoid arthritis (RA) is a prototypic autoimmune disease characterized by inflammatory polyarthritis, affecting nearly 1% of the population (1). RA is characterized by painful, swollen joints that can severely impair physical function and quality of life and associated with increased mortality (2). About 70% of patients with RA are women, and peak incidence is between ages 50 and 60 years (1). RA is a clinical diagnosis, but about two-thirds of patients have elevated anti-citrullinated protein antibodies (ACPA) or rheumatoid factor (RF) (1).

Numerous genetic, lifestyle, and serologic risk factors have been identified that predict the future development of RA. Many patients develop non-specific symptoms prior to the clinical diagnosis. Some patients may present with undifferentiated inflammatory arthritis that may not meet research criteria for RA. Thus, distinct preclinical phases have been proposed leading up to clinical RA diagnosis (3). These correspond to genetic, lifestyle, autoimmunity, and early signs/symptoms (**Figure 1**). Some of these phases may be amenable to behavioral (4) or pharmacologic interventions to delay or even prevent the onset of RA.

In this narrative review, we detail previous and ongoing research studies that have elucidated the preclinical phases of RA. Since other immune-mediated inflammatory diseases may have similar preclinical phases, the experience may serve as a roadmap to epidemiologic and investigations that lead to intervention studies for prevention of autoimmune diseases.

GENETIC STUDIES

The interaction of genetic and environmental risk factors underlies the model for pathogenesis of many autoimmune diseases, including RA. In this paradigm, individuals genetically predisposed to an autoimmune disease are exposed to environmental risk factors throughout the life course, which may eventually manifest as clinical disease. Since many autoimmune diseases are more likely to occur within the same family, this suggests both shared genetic and environmental components for autoimmune disease susceptibility. Twin studies including those for RA (5), have shown that most autoimmune diseases have moderate to strong hereditability (6).

RA, like most other autoimmune diseases, is a complex, polygenic diseases, meaning many genetic loci are linked, each of which usually has only a modest association with a specific condition. Unlike monogenic diseases, the genetic components of complex diseases are not usually deterministic. Rather, complex chronic diseases such as autoimmune diseases alter the probability of disease development only slightly. For example, the strongest genetic risk factor for RA is the "shared epitope" at *HLA-DRB1* and is linked to a three-fold increased RA risk compared to not having any shared epitope allele (7). The shared epitope was initially linked to RA in the 1970s using the major histocompatibility complex as a set of candidate genes (8). More recently, specific amino acid haplotypes have been implicated as strongly affecting RA risk at peptide-binding grooves of the HLA-DRβ1 protein (9), offering biologic explanation to the genetic association studies. However, the shared epitope is relatively common even in the general population, so the absolute risk of RA is relatively low even among individuals who do have this genetic factor.

While the shared epitope remains the strongest risk factor, the era of genome-wide association studies (GWAS) has identified additional single nucleotide polymorphisms related to RA. Over 100 independent genetic loci are currently associated with risk of RA, although the risk of any one of these single nucleotide polymorphisms is modest compared to the shared epitope (10). Since common genetic factors typically have modest effect size, very large sample sizes are typically needed to identify these

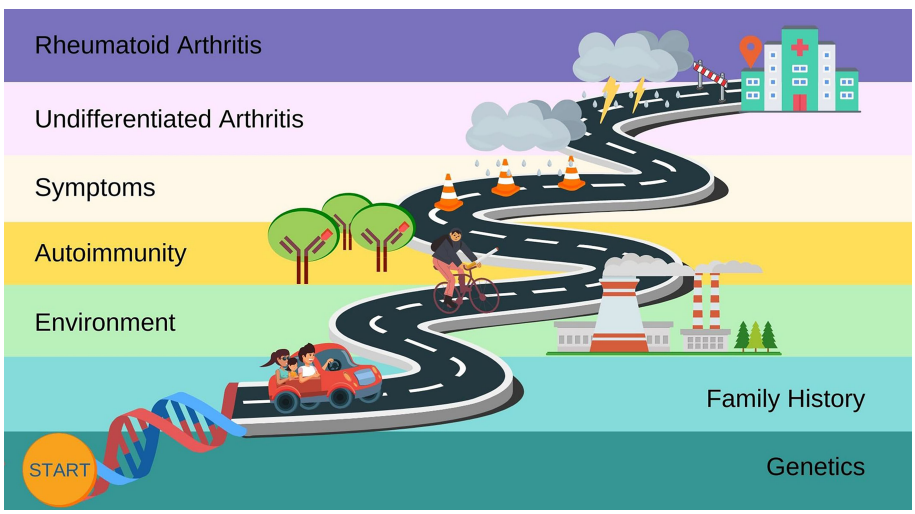

FIGURE 1 | Schematic illustrating the roadmap to the preclinical phases of rheumatoid arthritis.

signals (11). Thus, international efforts are needed to collect the necessary sample size, which can be logistically difficult. Since germline genetics should not change appreciably, patients with prevalent RA and population-based controls can be used to investigate risk factors. Thus, patients can be enrolled after diagnosis to investigate this time-invariant set of genetic factors. For many of the other study designs to be detailed later, either patient recall or enrollment prior to RA diagnosis is required to investigate preclinical phases of RA. Another practical advantage of genetic studies is that they are relatively unconfounded from many factors since they were in place since conception. Thus, future events such as cigarette smoking should not affect the genetic risk of RA. However, differences in ancestry can confound genetic studies as population stratification. Early studies only investigated a single ancestry, typically European (12). Modern studies have now moved to trans-ethnic GWAS both to increase inclusion across marginalized groups and to identify potentially novel genetic factors (13). RA is a clinically heterogeneous disease which may make it difficult to identify genetic signals. Thus, some GWAS focused on seropositive RA as a more homogeneous phenotype (14). Other genetic studies have investigated seronegative RA (15), using the genetics to eliminate signals from masquerading disease such as spondyloarthritis (known to be strongly related to HLA-B27).

The latest trans-ethnic GWAS included over 275,000 participants across five ancestral populations to identify an additional 34 novel variants associated with RA (currently in preprint form) (13). Even larger future studies may identify even more common variants. Future genetic studies are needed to integrate rare variants (through whole exome or whole genome sequencing) with GWAS data. In addition, epigenetic studies may link either inherited or acquired environmental triggers with RA risk by gene regulation changes (16). Somatic mutations have not yet been linked with RA risk, but Clonal Hematopoiesis of Indeterminate Potential (CHIP) has been associated with other chronic diseases (17), while VEXAS syndrome was recently defined as a clinical entity based on a specific somatic mutation (18).

Both twin studies and GWAS have potential limitations. Twin studies primarily are limited in their lack of generalizability and inability disentangle the effects of shared environment and the gene-environment interactions. Both twin studies and GWAS can have selection bias, specifically recruitment or volunteer bias of individuals who are willing to donate biospecimens. This can lead to disproportionate sample populations, particularly greater proportions with European ancestry that could affect generalizability across different ancestries and lead to inequities in discovery of genetic architecture in marginalized populations. Focusing on specific populations with high rates of RA may identify novel genetic factors since RA prevalence varies by geography (19). For example, North American indigenous groups have a high rate of RA (20), but sample size large enough for GWAS has not yet been performed. GWAS in particular require very large international sample sizes are needed to detect effects of genetic factors. This can pose logistical limitations across centers.We now detail specific research programs that have elucidated preclinical RA phases (**Table 1**).

RETROSPECTIVE COHORT STUDY: ROCHESTER EPIDEMIOLOGY PROJECT

The Rochester Epidemiology Project (REP) is a medical record-linking system for residents of Olmsted County, MN, USA to perform population-based studies (21). A unique resource for chronic disease epidemiology, the REP's enrollment includes approximately 95% of Olmsted County's residents who have allowed their medical record to be used for research (21). As a result, the REP has accumulated approximately 700,000 participants since its inception in 1966 (22). REP's linked medical records from both inpatient and outpatient providers include a standardized index for diagnoses codes and surgical interventions (21, 23). These data enable accurate assessments of disease incidence, risk, causes and outcomes at the population level, using REP's databases (21, 23).

Retrospective cohorts to identify trends of RA incidence are readily available using REP as RA cases and controls can be sampled from the same population (24). Cases are identified using the 1987 ACR criteria for RA by medical record review. An increase in RA prevalence – from 0.62% in 1995 to 0.72% in 2005 – and incidence in women was reported between 1995 and 2007 (24). A population-based incidence cohort of 466 patients that fulfilled 1987 ACR criteria for RA between 1995 and 2007 was compared with another 2005 cohort of patients with prevalent RA. The cause for this increase is unknown, but potentially could be due to environmental factors (24). Furthermore, retrospective cohorts for serological status, preclinical risk factors and social determinants can be assembled and compared to determine incidence and risk (25). A 2005-2014 cohort showed RF-negative RA incidence significantly increased and RF-positive RA decreased compared to previous decades in Olmsted County. These cohorts were age and sex-adjusted to the white population in the US, and prevalence rates were estimated (25). Since REP relies on clinical data, patients diagnosed with RA prior to the early 2000s only had RF available since ACPA was not available prior to then. For RA patients diagnosed later, both RF and ACPA are available (25).

With REP, entire non-RA patient groups in Rochester, Minnesota and Olmstead County can be followed to determine preclinical risk for RA. For instance, asthmatics and patients with proinflammatory conditions were found to not have statistically increased risk for RA; however, asthmatics showed increased risk for diabetes mellitus and coronary heart disease (26). Moreover, environmental and demographic factors like socioeconomic status (SES) have also been analyzed using REP (27). Residents of lower SES in Olmsted County were found to have increased risk of RA than their higher SES counterparts, comparing a population-based cohort of cases with RA to their controls without RA from 1988 to 2007 (27). Thus, REP serves as a unique resource and exemplar for retrospectively assessing preclinical autoimmunity.

TABLE 1 | Selected observational studies investigating rheumatoid arthritis risk.

Study name	Region, country Year initiated	Cohort description	Preclinical RA phase(s) studied	RA phenotyping	Data elements
Rochester Epidemiology Project (REP)	Olmsted County, MN, USA 1966	All residents of Olmsted County	Overall incidence	Medical record review meeting 1987 ACR or 2010 ACR/EULAR criteria	Medical records
Nurses' Health Study (NHS) and Nurses' Health II (NHSII)	USA 1976 (NHS) 1989 (NHSII)	Female working nurses at baseline	Genetics, lifestyle, biomarkers	Incident RA after baseline; Self-report and confirmed to meet either 1987 ACR or 2010 ACR/EULAR criteria on medical record review	Repeated biennial surveys, banked blood and cheek cells prior to/after RA onset
Etude Epidémiologique auprès des femmes de la Mutuelle Générale de l'Education (E3N)/European Prospective Investigation into Cancer and Nutrition (EPIC)	France	Females aged 40-65 at study initiation in 1990	Lifestyle	Incident RA: self-reported on surveys, and validated by medication reimbursement As, physicians, autoantibody positivity, or ACR criteria	Surveys, banked blood/saliva prior to RA
Taiwan's National Health Insurance Research Database (NHIRD)	Taiwan	Residents in Taiwan enrolled in the National Health Insurance Program	Overall incidence	Medical record review and diagnosis by two rheumatologists	Administrative claims and geocoded data
Studies of the Etiology of RA (SERA)	USA 1996	Individuals without RA at who have risk factors for RA: (1) Elevated ACPA or RF; or (2) first-degree relative or presence of shared epitope	All	RA-related autoantibodies, RA features on joint examination, 1987 and 2010 ACR/EULAR criteria or diagnosed by a board-certified rheumatologist	Surveys, physical exam, blood, sputum, saliva; substudies with chest imaging and spirometry
Evaluation of a SCREENing strategy for Rheumatoid Arthritis (SCREEN-RA)	Switzerland 2009	First degree relatives and high risk individuals	All	Incident RA after baseline	Surveys, blood, stool sample, dental/plaque samples
Indigenous North American Family Studies	Manitoba, Canada Alaska, USA 2005	Relatives of Indigenous North Americans with RA	All	Inflammatory arthritis assessed by a study rheumatologist	Joint examinations, symptom report questionnaire, and antibody testing
Mexican family Studies	Guadalajara, Mexico 2007	First- and second-degree relatives who do not have RA	Genetics, biomarkers	Inflammatory arthritis assessed by a study rheumatologist	Joint examinations, symptom report questionnaire, bloods
Colombia FDR Cohort	Colombia	FDRs of individuals with RA, healthy controls, individuals diagnosed with early RA	All	2010 ACR/EULAR criteria or DMARD use	Surveys, periodontal exams, questionnaires, blood sample, inflammatory marker
Early arthritis clinics	Leiden, Netherlands Leeds, UK Birmingham, UK	Individuals presenting with arthralgia or undifferentiated inflammatory arthritis, not meeting 2010 ACR/EULAR criteria for RA	All	2010 ACR/EULAR criteria; DMARD use; inflammatory arthritis assessed by a study rheumatologist	Magnetic resonance imaging, ultrasound, synovial fluid/tissue, blood, surveys, other imaging

ACPA, anti-citrullinated protein antibodies; ACR, American College of Rheumatology; EULAR, European Alliance of Associations for Rheumatology; FDR, first-degree relative; RA, rheumatoid arthritis.

RETROSPECTIVE COHORTS: TAIWAN NATIONAL DATABASES

Taiwan's National Health Insurance Research Database (NHIRD) is one of the largest administrative health care databases in the world, enabling high quality population-based research to be conducted on a nationwide scale. With 99.99% of Taiwan's population enrolled under the National Health Insurance (NHI) Program, the NHIRD stores Taiwan's insurance claims data and specifically for research purposes (28, 29). All data, since 2000, from both outpatient and inpatient facilities are included in the database and since 2016, research-approved datasets are released as either sampling datasets, disease-specific databases, and full population datasets (28, 29). The NHIRD has thus helped produce numerous retrospective epidemiological studies identifying environmental RA risk factors, as well as various patient populations at risk for RA.

RA cases can be identified in the NHIRD *via* the Registry of Catastrophic Illness Patient Database (28, 30). Taiwan is unique in that its NHI Program classifies RA as a statutory major disease (28, 30). RA diagnoses are validated by at least two rheumatologists after review of clinical data and individuals who fulfill diagnostic criteria get issued a catastrophic illness certificate that exempts them from healthcare insurance copay (28, 30). Cases for RA can additionally be verified using ICD codes or other clinical data like medications. Thus, cases of RA are generally accurate and can be accessed with ease.

Numerous patient populations have been assessed for RA risk using the NHIRD. For instance, retrospective cohort studies suggest that patients with sleep disorders, endometriosis, Mycoplasma pneumonia, hepatitis C virus infection, multiple sclerosis, and periodontitis exposure have an increased risk of RA (31–36). This is a strength of the NHIRD; these patient populations are also well defined and have strong follow up within the database. Certain treatments have been found to be associated with a decrease in RA risk, such as thiazolidinedione use among patients with type 2 diabetes mellitus, and interferon-based therapy for patients with hepatitis C virus using the NHIRD (35, 37). Additionally, analyses of NHIRD demographic, and environmental risk factors have also been assessed such as the use of insurable monthly income as a measure for socioeconomic status, as well as other national databases like the Taiwan Air Quality-Monitoring Database to assess the effect of air pollution on RA risk (38–40). Taiwan's NHIRD is, therefore, an immense asset to identifying determinants of RA and risk.

Retrospective cohort studies are limited by missing data and, as a result, the inability to fully adjust for potential confounders or investigate factors not routinely measured. Data used for retrospective studies are often collected without specific research questions in mind, for instance, clinical data from electronic health records. Some administrative data may be inaccurate or be used to rule out diseases. Therefore, careful attention is needed to ensure validity of factors being studied. Other missing data, such as lifestyle factors, may include confounders for the RA risk factors being studied. Additionally, patient-reported data is prone to recall bias. This can lead to under- or over-reporting of RA and other variables.

PROSPECTIVE CASE-CONTROL STUDY: EPIDEMIOLOGICAL INVESTIGATION OF RHEUMATOID ARTHRITIS (EIRA)

EIRA is a Swedish population-based prospective case-control study initiated in 1996 that enrolls newly diagnosed RA patients and matching each to general population controls based on sex, age, and location (41). The study population was also restricted to middle and southern parts of Sweden, allowing investigators to study geographic variables (41). Participants with RA were identified by collective recruitment efforts from rheumatology departments within hospitals, as well as some private rheumatology clinics, totaling 21 separate recruitment teams (41). Thus, a practical advantage of EIRA is that RA patients can be enrolled just after diagnosis, when they are already interacting with the medical system. However, some of the survey data may be prone to recall bias and biomarkers may have emerged after clinical diagnosis.

Participants in EIRA respond to standardized questionnaires about lifestyle factors and environmental exposures (41). Some of the variables of interest include physical activity, smoking habits, family, and occupation. Participation from both cases and controls was successful, 95% and 80% response rate to the questionaries, respectively (42). Nearly all participating cases provide a blood sample as well for genetic and biomarker studies. In cases, RA was classified according to the 1987 American College of Rheumatology (ACR) criteria and confirmed by a rheumatologist (41). Most rheumatologists in Sweden are recruiting centers for EIRA. Data are also linked to a national RA register to identify additional cases that were not identified through routine clinical care (43).

EIRA was instrumental identify a gene-smoking interaction for seropositive RA risk, one of the seminal epidemiologic findings in RA (44). EIRA phenotypes RA cases based on serostatus and genotyped all cases and controls for the shared epitope (44). Padyukov et al. reported a strong interaction between smoking and the shared epitope, which helped build the foundation for the mucosal paradigm for seropositive RA pathogenesis (44).

EIRA investigators have analyzed many other factors obtained from surveys for RA risk. For example, oral contraceptive (OC) use was associated with RA risk among women (42). Ever and past users of OC had a decreased risk of ACPA-positive RA when compared to never users (42). Another EIRA study found that silica exposure was associated with increased RA risk (45). Occupations often associated with silica exposure include rock drilling and stone crushing (45). Another EIRA study showed that vaccinations received within 5 years of index year were not associated with RA risk (46),.

EIRA is particularly valuable because the study population has detailed geographic data. This minimizes variability in environmental surroundings, as factors such as pollution or physical working environments can be easily compared (47). Hart et al. found no increase in risk of RA based on particulate matter pollution in Stockholm, Sweden (47). They did, however, derive an increase in RA from nitrogen dioxide produced by local traffic and sulfur dioxide from heating sources, specifically in ACPA-negative RA (47).

A disadvantage of case-control studies is the reliance on recall to determine past events preceding the outcome. Since RA cases are aware they have RA, this may influence how they remember behaviors. Circulating biomarkers may also be influenced by treatment factors after RA diagnosis, so there are logistical challenges in enrolling newly diagnosed RA patients into a research study prior to the use of any medications. Since genetics are generally time-fixed, incorporating genetic factors in studies is not dependent on the timing of RA onset to enrollment. It can also be logistically challenging to prospectively match each RA case to healthy controls in a real-time manner, particularly with many matching factors. A solution may be to over-recruit controls and then match later, but that comes with resource costs. Identifying suitable healthy controls can be challenging, either from healthy volunteer effect or from recruiting patients with other health conditions that may impact causal inference.

PROSPECTIVE COHORT AND NESTED CASE-CONTROL STUDIES: NURSES' HEALTH STUDIES

The Nurses' Health Study (NHS) and the NHSII are large prospective cohort studies that have been integral resources

used to identify and confirm lifestyle, genetic, and serologic risk factors for RA. The NHS follows women who were between the ages of 30-55 and were working as registered nurses in the United States when enrolled in 1976 (n=121,700) (48). The NHSII is a similarly designed large nationwide cohort of working US nurses that were between the ages 25-42 when enrolled in 1989 (n=116,429). All women receive biennial surveys gathering data on lifestyle, diseases, medications, family history, and other data. Repeated measures of food frequency questionnaires have been obtained in both cohorts. The NHS and NHSII are characterized by very high follow-up rates (>90%) (48). Plasma and cheek swabs have been utilized for RA investigations (49). These detailed data with repeated measures allow investigators to integrate lifestyle, family history, genetics, and biomarkers with RA investigations. Another strength of this cohort is that the participants are medically sophisticated because of their occupation as nurses, leading to more accurate reporting and high retention rates. The biennial surveys are modified and expanded in content at each cycle to gather data on other factors such as sleep patterns and physical activity (48). While most of the data are collected using surveys, teams of investigators also carefully phenotype other chronic disease outcomes by obtaining medical records to confirm disease onset (2). The large sample size and lengthy follow-up also allow for investigations of incident diseases, even for relatively uncommon diseases such as RA and systemic lupus erythematosus.

Investigators in the NHS and NHSII identify incident cases of RA and other systemic rheumatic diseases using a 2-stage procedure. First, all participants that self-report a new diagnosis of RA are mailed the Connective Tissue Disease Screening Questionnaire (CSQ), previously validated to have high sensitivity for many types of systemic rheumatic diseases (50). For those who screen positive on the CSQ, medical records dated near the time of diagnosis are obtained. Two study rheumatologists independently collect components of the 1987 ACR and 2010 ACR/EULAR criteria to confirm all incident RA cases (2). Thus, all RA cases have high validity. In addition, reviewers collect dates of symptom onset and clinical diagnosis as well as clinical results on rheumatoid factor and anti-cyclic citrullinated peptide (51).

The NHS and NHSII investigate several preclinical RA phases using a variety of study designs. For exposure data that were prospectively collected from the surveys, investigators perform prospective cohort analyses. An advantage of this dataset is that data were collected prior to RA onset, reducing the potential for recall bias. For example, one of the earliest NHS papers linked breastfeeding with reduced RA risk and irregular menstrual cycles with increased risk of RA (52). Another paper confirmed that cigarette smoking was associated with risk of seropositive RA using data from the NHS (53). More recent papers have been able to analyze the NHSII once enough incident RA cases had accrued during follow-up. For example, long-term healthier diet was associated with reduced RA risk in data analyzing women who had answered repeated food frequency questionnaires in the NHS and NHSII (54). A recent updated analysis on smoking and seropositive RA risk identified sustained smoking cessation as a behavior that may reduce RA risk (55). Some analyses in the NHS and NHSII incorporate a latency period (or "lag") between when exposures are measured and when RA risk is being assessed to limit the potential for reverse causation. For example, changes in physical activity and low mood may immediately precede the formal diagnosis of RA. In studies on physical activity and depression as risk factors for RA, investigators in the NHS included a lag of at least 4 and up to 8 years to exclude the time period immediately before RA diagnosis when these changes may have been due to early, undiagnosed RA (56, 57). Recent papers have employed the causal inference methods to adjust for potential confounding and mediating relationships between variables in the preclinical RA phases. A study investigating passive smoking and RA risk used the life course epidemiology approach to study *in utero*, childhood, and adult passive smoking while adjusting for the confounding and mediating effect of personal smoking using marginal structural models (58). Beyond lifestyle factors, investigators have used the NHS and NHSII to investigate a variety of other potential RA risk factors that include diseases such as asthma and chronic obstructive pulmonary disease, family history, medication use such as proton pump inhibitors, and geocoded variables such as ambient air pollution (47, 59–61).

Studies in the NHS and NHSII investigate biomarkers for RA using genetics and banked blood in nested case-control studies. For genetic studies, both incident and prevalent RA are included since germline genetic factors do not change over time. Controls are also readily available from the same population. The NHS have contributed data to several large genome-wide association studies (GWAS) (11, 62). Investigators also constructed genetic risk scores (GRS) weighted by the effect size estimate of GWAS. Rather than analyzing many genetic factors, each with small effect sizes, the RA GRS is able to incorporate the genetic data into a single variable (63).These scores have been periodically updated to include newer variants (64, 65). Finally, an RA GRS incorporated the amino acid haplotype model of the HLA-DRB1 shared epitope to examine gene-smoking interactions, confirming that smoking interacts with specific amino acid haplotypes in the peptide-binding groove (66). Therefore, the NHS has been an important study to identify gene-environment interactions.

The NHS and NHSII have also been crucial in biomarker studies for RA risk. These nested case-control studies use blood banked prior to the onset of RA to identify circulating biomarkers. For example, investigators found that ACPA appeared in blood up to 10 years prior to RA onset (51). Follow-up studies showed that women with asthma were more likely to have elevated ACPA in pre-RA suggesting that pulmonary mucosal inflammation may influence RA-related autoantibody production prior to RA onset (67, 68). Other biomarkers examined in the NHS and NHSII have included inflammatory markers, Epstein-Barr virus antibodies, carotenoids, vitamin D, leukocyte telomere length, metabolomic profiles, and adipokines (49, 69–75).

Finally, some studies have incorporated many risk factors to build prediction models for RA. An initial prediction model that incorporated RA GRS, lifestyle factors, and gene-environment interactions had an area under the receiver operating characteristic curve (AUROC) of up to 0.738 for seropositive RA (64). A follow-up paper that incorporated an updated RA GRS had an AUROC of 0.82 for seropositive RA among those with positive family history (65). A more recent paper used machine learning methods to select covariates that included metabolomic factors associated with future RA risk (76). Thus, the Nurses' Health Studies have been a rich resource to investigate RA risk across the spectrum of preclinical phases.

PROSPECTIVE COHORT STUDY: EPIC-E3N

The Etude Epidémiologique auprès des femmes de la Mutuelle Générale de l'Education (E3N) is a prospective cohort study based on nearly 100,000 French women (77). The study was initiated in 1990 and the participants were aged 40-65 years old at study start (77). E3N collects information on lifestyle habits and reproductive factors, as well as general health status approximately every 2-3 years by collecting questionnaires (77). E3N is a study nested in the more broad European Prospective Investigation into Cancer and Nutrition (EPIC) which is comprised of a larger, and broader European cohort, recruiting participants from 10 European nations (78). EPIC was introduced as a means of investigating the most pressing and prevalent health issues facing women in the 1990s (78). These included cancer and severe chronic conditions. E3N emerged as a sub-study investigating lifestyle habits, behaviors, and trends in women's health and how they relate to disease outcome and wellbeing (78). Like other large prospective cohorts, E3N collects periodic surveys from participants, and blood and saliva samples from subjects as well (78). This allows for the reinforcement of findings with both qualitative reports and genetic and biological findings. The investigators have also been able to link samples and questionnaires to health data, specifically drug reimbursement files from the insurance group which covered all of the study's participants (78).

E3N has also been used to investigate incident RA (78). Women self-report new diagnoses of RA, but this was only accurate for 42% of cases (79). The validity of RA cases from this cohort increased to between 75.6 to 90.1%, depending on whether an inflammatory rheumatic disease questionnaire or medication reimbursement match was made, in addition to the self-report (79).

The E3N study group has also allowed investigators to examine additional habits and conditions that may increase or decrease RA risk using prospectively collected data that is less prone to recall bias than retrospective studies. Nguyen et al. found that ever smokers who adhered to the Mediterranean diet had lower RA risk (80). The E3N cohort has also helped expand on established environmental risk factors such as smoking (78, 81). For example, passive exposure to smoke in childhood was associated RA risk in non-smokers or ever smokers (81, 82). The investigation observed RA onset earlier in those exposed to passive smoking, compared to those without this same exposure (82).

Prospective cohort studies have some possible limitations. First, survey data from participants may be subject to recall bias or inaccuracy. However, in many of these studies, data were collected prior to clinical onset of RA, limiting potential for recall bias. Another possible limitation relates to stringency of case identification methods and loss to follow-up. For example, relying solely on self-report may lead to over-diagnosis. Conversely, requiring a high threshold of criteria to identify true cases may eliminate ambiguous cases and may be prohibitive to pursue from a cost and effort perspective. Cohorts with high rates of loss to follow-up may not identify cases due to loss of contact. Since RA is a relatively rare outcome, large prospective cohorts are needed to investigate this. Most of the prospective cohort studies were originally constructed to investigate other factors (e.g., female reproductive factors), so may not be the ideal study population for RA and may not have collected all data elements relevant for RA. It is also crucial to acknowledge that that causation between an exposure and RA as an outcome cannot be established with a prospective cohort study due to the observational nature of the study design.

PROSPECTIVE CASE-CONTROL AND BIOMARKER STUDIES AMONG FIRST-DEGREE RELATIVES: STUDIES OF THE ETIOLOGY OF RA (SERA)

First-degree relatives have been fruitful to investigate since they are interested in RA prevention due to awareness and also are at increased risk due to genetics and environmental factors. Established in 2002 in the United States, the Studies of the Etiology of RA (SERA) project enrolls and follows at-risk individuals for RA onset (83). SERA aims to identify the lifestyle, demographic, environmental, biomarker, and genetic factors of preclinical RA (83). Participants do not have RA and are recruited based on their genetic and serological risk (83). Participants in SERA are either (1) first-degree relatives (FDR) of RA probands (2), have the shared epitope, or (3) have elevated RA-related autoantibodies such as ACPA or RF (83). Healthy controls are also recruited and are confirmed to not have RA or RA-related autoantibodies (83). Some of these participants are found through health fair screening that offers ACPA testing to the general population. Within SERA, a prospective cohort of FDRs has been assembled to study preclinical RA as FDRs have uniquely relevant genetic and environmental risk factors for RA. This cohort's utility lies both in increasing the yield of identifying individuals with preclinical RA and in potentially identifying additional biomarkers (83). Questionnaires, medical history, interview data, joint count examination by a study physician or trained nurse, and blood and urine are collected during research visits for all FDRs (83). Sputum and saliva have also been collected for some later participants, allowing RA-related

autoantibodies to be evaluated in the lungs and contributing to the mucosal paradigm of RA (84–86). For seropositive FDRs, follow up visits occur annually, whereas for seronegative FDRs, they are seen every other year (83). Some SERA substudies obtain other measures such as chest imaging and spirometry (87). FDRs are also instructed to notify the investigators if they develop any signs or symptoms of RA diagnosis.

SERA recruits FDRs *via* their RA probands who must meet ≥4 ACR classification criteria upon medical record review or have a diagnosis of RA from a board-certified rheumatologist (83). FDRs and other at-risk subjects are confirmed to not meet the 1987 ACR or 2010 ACR/EULAR criteria for RA at the time of recruitment (83). SERA often utilizes RA-related autoantibody positivity as a surrogate outcome for RA development (83). Physical examination may reveal features of RA such as joint tenderness and/or swelling in prototypic joints involved in RA (22, 88). Additionally, genetic testing for the shared epitope in FDRs are also performed (83). Incident inflammatory arthritis after baseline has also been examined, and a subset of these participants have classifiable RA (89).

Studies from SERA have produced seminal environmental and genetic risk findings in preclinical RA. Elevation of RA-related autoantibodies at baseline were strongly associated with future development of inflammatory arthritis in a prospective cohort study (89). Erythrocyte membrane-bound omega-3 fatty acid levels as a marker of dietary intake were found to be inversely associated with RF-positivity in SE positive subjects in a nested case-control study (90). Survey data also showed that SE positive subjects who took omega-3 supplements at baseline were found to have lower RF-positivity prevalence in a cross-sectional study (90, 91). For instance, higher odds for inflammatory joint signs, either prevalent at baseline or incident during follow-up, was found in smokers compared to non-smokers (92, 93). Additionally, the effects of air pollution, stress obesity and oral contraceptive use in RA development have also been investigated using the SERA dataset in a variety of study designs (22, 92, 94).

Biomarkers of preclinical RA have been identified as well in SERA studies, such as increased lipid mediators which are associated with risk of developing inflammatory arthritis (95). In addition, autoantibody positivity has been associated with other markers in the blood such as elevated cytokines/chemokines in FDRs, illuminating overall circulating inflammation in at-risk populations (96). A seminal study that incorporated chest imaging and spirometry was one of the first studies to show high proportion of autoantibody-positive participants without RA had airway abnormalities, one of the first to suggest that RA-related autoantibodies may originate in pulmonary mucosa and helped to form the foundation of the "mucosal paradigm" of RA pathogenesis. SERA's sputum collection has further expanded identifying RA risk factors to the lungs (22, 97). Namely, sputum autoantibodies are present in the absence of seropositivity, elucidating the importance of the lungs in the development in RA and garnering future investigation (84, 85).

PROSPECTIVE COHORT STUDY AMONG FIRST-DEGREE RELATIVES: SCREEN-RA

This Swiss study also enrolls first-degree relatives and high-risk individuals for RA risk (98). This population was featured as these individuals are considered more likely to develop RA due to likely predisposition to genetic factors associated with RA risk (98). The cohort, termed SCREEN-RA or Evaluation of a SCREENing strategy for RA, began in 2009 and followed initially "healthy, asymptomatic individuals" predisposed to developing RA due to familial history (98). At baseline, all individuals were undiagnosed with RA, but were at various stages of presentation with some attesting to arthralgias, while others had high autoantibodies without symptoms, and some who only identified as FDRs without additional risk indicators or suggestion of early disease onset (98). With the founding of the study, the team hoped to strategically build a tool, combining various preclinical RA features, that could forecast a likely RA diagnosis within 3-5 years of baseline (98).

SCREEN-RA recruitment involved 10 centers across Switzerland (98). In addition to first degree relatives, the study team included people with other, previously diagnosed autoimmune diseases, since certain RA biomarkers are also notable in other autoimmune diseases. Because the investigators were interested in broadly addressing preclinical RA phases, multiple investigational elements were collected at study start. To address environmental habits and factors, genetics, and autoimmunity, questionnaires, DNA and RNA, and serum samples were collected, respectively (98). In a subpopulation of more "high risk" FDRs, presenting with 2 copies of the notorious shared epitope, elevated autoimmunity markers at baseline, or undifferentiated arthritis, additional stool samples were collected, and oral exams were performed to assess dental microbiota (98). After each FDR or high-risk individual was enrolled, follow-up questionnaires, built in tandem with SERA questionnaires to increase reproducibility of results, were mailed annually to monitor incident case development and track environmental and lifestyle conditions (98). "High risk" participants are seen clinically each year and provide a blood sample during follow up as well (98).

Data from the SCREEN-RA cohort has produced notable findings that have linked novel factors to specific RA phenotype, as well as increased likelihood of symptom onset. Of note, Wells et al. found that the microbial presence of Prevotella copri in the gut microbiotica was found more often in stool samples from those with high RA genetic risk (99). Similarly, Alpizar-Rodriguez et al. found that Prevotella was more often found in stool samples of RA-FDRs with RA symptoms or autoantibodies compared to asymptomatic subjects (100). This may suggest that changes in the composition of the gut microbiota preceding RA onset may be causal to disease development (99). Additionally, high risk subjects at study start were subject to periodontal exams. Blinded examiners searched for evidence of periodontitis, or shrinking of gums and loosening of teeth (101). Presence of this dental disease was associated with seropositivity of ACPA in RA cases, while high risk individuals

without periodontal disease were more likely to be seronegative for ACPA in this nest-control sub study of SCREEN-RA (101). Highly expanded T-cell clones (HEC) were also increased in concentration as RA diagnosis approached (102). T-cells communicate with and activate B-cells at the mucosal level, so this increase of HEC supports the model that a local immune reaction could spur RA onset (98, 102).

PROSPECTIVE COHORT STUDY AMONG FIRST-DEGREE RELATIVES: INDIGENOUS NORTH AMERICAN STUDIES

Researchers at the University of Manitoba have assembled a cohort of Indigenous North Americans (INA) with RA and their relatives since 2005 (103). This prospective cohort was recruited from Cree and Ojibwe populations at urban and rural medical centers in Manitoba and Saskatchewan, Canada. The relative risk of RA is estimated to be 2-3 times higher in these INA populations of Central Canada than other populations (104). The study population being enriched for RA risk factors such as genetics, smoking, and socioeconomic factors, the investigators were able to focus on a population well at risk for developing RA. Probands had a diagnosis of RA according to the ACR 1987 criteria and both probands and relatives were over the age of 18 and self-identified as Indigenous North Americans (103). A cohort of controls without RA and with no first-degree relatives with RA was recruited from the same population (103).

The recruitment of probands; their family members, who were primarily first-degree relatives (75.5%); and unrelated, unaffected members of the same relatively homogenous population allowed the investigators to examine the potential genetic causes of RA, including the shared epitope (20). The shared epitope is more common among INAs, which may in part explain a higher prevalence of ACPA-positive RA. Moreover, familial clustering of RA is frequent in these populations and the age of RA onset is younger (105), suggesting a genetic predisposition to RA development, which may also be influenced by similar sociodemographics and environmental exposures.

Samples from this cohort of INAs were used to examine ACPA isotypes (IgA, IgG1, IgG2, IgG3, IgG4, and IgM) in RA patients and their unaffected family members. Among RA patients, 91.4% had ACPA antibodies, as did 19.0% of their healthy relatives and 8.8% of healthy INA controls, much higher than non-INA populations. The IgM isotype was more common in RA patients than in their family members, indicating a more current immune response in those with clinical disease (20). Fine specificity assays performed on serum obtained at baseline for IgG ACPA-positive members of this cohort revealed that about half of RA patients had anti-Sa or anti-citrullinated fibrinogen antibodies, while the IgG ACPAs of healthy relatives did not react against either antigen (20). Thus, serologic studies from this cohort have provided valuable insight into the environmental exposures contributing to RA onset. Longitudinal serology studies in this cohort have also been investigated. Participants who were positive for either ACPA or RF at baseline were followed annually, while those who tested negative for both were followed every three years (106). The stability of autoantibody titers was assessed over time, and further fine specificity were performed 10 years later (106). Among those that progressed to clinical RA, ACPA levels increased in quantity over time and became increasingly reactive. Recently, a proteomic signature implicating specific immune pathways was able to accurately differentiate progressors to RA from individuals at-risk due to family history or elevated ACPA but did not progress to RA using longitudinal measures of prospectively collected data (107).

Physical and joint exams from this cohort provide valuable insights into RA disease and symptom onset in those genetically and immunologically at risk for RA. A cross-sectional study within this cohort included a musculoskeletal symptom questionnaire, as well as collection of demographic and cultural data (108). White controls were recruited from the same geographic area for this substudy for further comparison. Study rheumatologists or trained study nurses evaluated subjects for swollen and tender joints. FDRs showed more RA symptoms in the hand joints than did INA controls, who in turn showed more hand symptoms than White controls. RA symptoms in other joints were increased in FDRs, but not in INA controls compared to White controls (108). A longitudinal study within this cohort assessed ACPA or RF-positive FDRs at yearly intervals and ACPA and RF-negative participants every 3 years, assessing for swollen joints at each visit (106). The clinical follow-up of these patients allowed the investigators to probe the development of RA symptoms in a population with an increased likelihood of developing RA.

OTHER PROSPECTIVE COHORT STUDIES AMONG FIRST-DEGREE RELATIVES

Investigators at the Unidad de Investigacion en Enfermedades Cronico-Degenerativa in Guadalajara, Mexico, conducted a large prospective cohort study to investigate the risk and mechanisms of developing RA in close relatives of RA patients (109). RA patients and their first- and second-degree blood relatives were invited to join the longitudinal cohort to evaluate the risk of these relatives of developing RA. Probands were recruited from rheumatology clinics at three centers, and two study physicians confirmed the RA diagnosis by ACR 1987 criteria. Relatives were healthy individuals older than 15 years without RA or any rheumatic or chronic disease, which was confirmed by joint exam. Relatives received follow-up calls every four months for five years. Participants whose responses on the Community Oriented Program for Control of Rheumatic Disease (COPCORD) indicated possible inflammatory arthritis, or those who requested in-person exams, were evaluated by study rheumatologists (109). Evaluations included joint exams, laboratory measures, and radiographic imaging. These were repeated by the same rheumatologist two weeks later if the first joint exam found no evidence of inflammatory arthritis, allowing for greater detection of early disease. Subjects who moved to

other cities continued participation and were examined by local rheumatologists if needed. The investigators succeeded in following 90% of study participants to study completion. They found that baseline elevated ACPA was strongly associated with future RA development (109).

The same group has used samples from RA patients and their relatives in several cross-sectional studies to conduct genetic and biomarker analyses. In one study, investigators compared samples from established RA patients, early RA patients, their ACPA+ and ACPA- relatives, and healthy controls to evaluate differences in expression of genes in the type I interferon signature (110). Recruiting at-risk family members with and without ACPAs, while evaluating early and established RA separately, allowed the researchers to demonstrate differences in gene expression across a spectrum of RA risk. Using the same approach, the group was able to demonstrate differences in TLR7 and TLR9 across these levels of risk and progression (111). Another study investigated transcriptomics in early RA patients and their ACPA+ and ACPA- relatives, identifying candidate biomarkers for RA progression in this genetically at-risk population (112). A fourth study used levels of TNF and IL-6 as measures of subclinical inflammation in asymptomatic FDRs of RA patients to investigate the role of the bone biomarkers Dkk1 and sclerostin in joint damage prior to onset of clinical RA (113). Using samples from RA patients and their genetically similar, at-risk relatives allowed investigators to explore the biological mechanisms of RA onset.

A study in Colombia follows first degree relatives (FDR) of individuals with RA, matching study subjects 2:1 to healthy controls from the general population (114). The controls and FDRs were matched by gender and age (114). Subjects in this cross-sectional study were 18 years or older (114). This is a critical study population because the link between genetics, and RA development have been heavily considered due to the increased conversion to RA diagnosis among FDRs (115). Previous studies have estimated the increase in risk of developing RA to be approximately 4 times higher in FDRs of people diagnosed with RA than in individuals that are not FDRs (116). FDRs were defined according to 2012 EULAR recommendations (117). People with early RA (eRA), diagnosed within the last 2 years and fulfilling 2010 EULAR criteria, were also studied in this cohort. These eRA subjects were additionally taking conventional synthetic drugs.

Investigators utilized this cohort to examine adipokine association and periodontal disease in individuals diagnosed with early RA and their FDRs (118). The authors found that high leptin, presence of Porphyromonas gingivalis, a pathogen with an enzyme that is able cause citrullination in the periodontium (118). The pathogen, itself, is not a marker of periodontitis, however the presence of "antibodies against P. gingivalis before the onset of RA symptoms are associated with ACPAs and RA disease activity markers" (118). Swollen joints were also suggested as potentially relevant identifiers associated with RA development in FDRs (118). Another study using this same subject population included 124 FDRs (117). This investigation examined anti-post-translationally modified protein antibodies (AMPA), which are staples of RA (117). The AMPA examined by the group was the anti-carbamylated protein antibodies (anti-CarP) (117). The Colombia-based study found thar anti-CarP antibodies are more often observed in FDRs than healthy controls (117). It is important, however, the note that other studies did not find that this AMPA's presence added additional risk for developing RA (115).

Family-based studies are limited by the ability to recruit a large enough sample to enable investigations. However, the advantage is that family members are familiar with RA so may be interested in prevention efforts. It is also possible that they could have large attrition rates after enrollment since most remain healthy. Thus, longitudinal studies can be challenging, particularly since the incidence rate of RA is low even among family history. Many studies use surrogate markers of RA such as autoantibody measurements or RA traits such as tender or swollen joints that are on the causal pathway toward RA. As in other studies, they may be prone to recall bias. However, this may be less of a threat than case-control studies since included participants do not have RA at time of enrollment.

BIOBANKS, SECONDARY ANALYSES OF LARGE TRIALS, AND OTHER STUDIES

Some large biobanks have been particularly to perform research of circulating markers predicting future RA. One of the earliest studies in Sweden found that elevated RF and ACPA preceded clinical RA onset by years and were strongly associated with RA onset and interact with genetic factors including the shared epitope (119, 120). The Department of Defense biorepository has also identified the temporal expansion of inflammatory biomarkers and autoantibodies prior to clinical RA onset (121–124). The Dutch Lifelines study was used to investigate RA-related autoantibodies in individuals without RA (125). The Guangzhou Biobank Cohort used survey data to identify reproductive factors associated with RA (126). The UK Biobank has been used to perform Mendelian randomization studies to identify lifestyle behaviors with RA risk using genetic markers as instrumental variables (127–129). MyEIRA is a Malaysian prospective population-based case-control study enrolling incident RA patients, similarly designed as the Swedish EIRA study (130). The Swedish Mammography cohort and the Malmö Preventive Medicine Program have been used to investigate RA risk using survey and spirometric data (131, 132). The Iowa Women's Health Study is another large prospective cohort study that used survey data to investigate RA risk (133, 134). Nested case-control study within European Prospective Investigation into Cancer and Nutrition (EPIC) have also examined biomarkers and RA risk (135). The Health Improvement Network is a large population-based study in the United Kingdom that has also been used to investigate RA risk (136). The Norfolk Arthritis registry has produced some of the most important case-control studies to identify RA risk factors (137, 138). Pharmacy claims data have also been used for pharmacoepidemiologic studies of RA risk (139, 140). Several large placebo-controlled randomized trials, including the Women's Health Study (investigating vitamin E and aspirin)

(141, 142), Women's Health Initiative (investigating postmenopausal hormones) (143), and VITAL trial (investigating vitamin D and omega-3 fatty acids) (144) have investigated RA risk as a secondary outcome, the latter suggesting that vitamin D may have potential protection of incident RA and other autoimmune diseases. Finally, the Mayo Clinic and Mass General Brigham Biobanks have been harnessed to analyze electronic health record (145) and survey data collected prior to RA onset and will use banked blood for future studies (75, 146–149).

PROSPECTIVE COHORT STUDIES AMONG THOSE WITH SYMPTOMS OR UNDIFFERENTIATED ARTHRITIS: EARLY ARTHRITIS CLINICS

Early Arthritis Clinics are central in their investigational utility due to the cohorts' high conversion rates to RA diagnosis and because of the unique data collected. European Early Arthritis Clinics have been established in Leeds and Birmingham in the United Kingdom and Leiden in the Netherlands, respectively, enroll patients with early arthralgias and undifferentiated arthritis with high potential to evolve into RA (150). Initially, beyond the immense potential for research into the early disease progression, EACs were established to treat patients in the period prior to irreversible, destructive damage to the joints that is often associated with established RA (150). Another particularly outstanding component of these clinics is their short referral to assessment timeline, which aims to be converted within 2 weeks (150). Patients at EACs are referred by their general practitioners to the clinics in a streamlined manner (150). "Ideal" referrals would display inflammatory arthritis features but not yet meet clinical criteria for RA (150). Referring providers may be asked to submit details including familial history, NSAID response, and joints effected to correctly funnel patients and preserve effective and efficient treatment once admitted to the EAC (150). EACs may employ physicians, trainees, occupational therapists, nurses, and other healthcare providers to contribute more holistically to caring for, educating, and diagnosing the patient (150). EAC inclusion criteria differs among sites but is predominantly symptom driven. The Leiden clinic integrates patients with less than 2 years of symptoms and with evident arthritis upon physical exam (151). The Leeds clinic narrowed their criteria to limit enrollment to patients with symptom duration under 1 year.

EACs collected patient data on turnover from pre-RA cohort induction to RA development within 1 year. Leiden and Leeds reported rates of 31% and 15%, respectively, which demonstrates that patients and providers accurately identified early RA symptoms (151). EACs consent patients at induction into the clinics and collect quantitative and qualitative measures periodically. These procedures and study measures include reproducible methods such as DAS, HAQ, and RAQoL (150). Subjects also report on symptoms, demographics, and medical history (150). Blood samples are collected to measure inflammatory markers and genetics, while imaging, including ultrasound (US) and magnetic resonance imaging (MRI) tools are used to demonstrate evidence of erosion and bony changes (150). Innovatively, samples of synovial fluid from swollen joints have also been collected. Many of these data points, including imaging and synovial fluid are unique to these EAC cohorts and can thus contribute to novel methods of predicting and potentially influencing preclinical RA prevention measures.

Previously completed studies suggesting a correlation between early RA and Vitamin D deficiency were reexamined using data from the Birmingham Early Arthritis Clinic Cohort (BEACON) (152). Using samples from 790 patients enrolled in the cohort, the authors, including Karim Raza and Andrew Filer, the primary investigators of the BEACON cohort, found no clear relationships between early RA and 25OHD, or low serum 25-ydroxyvitamin D) (152). By using synovial fluid, Raza and his team recognized that the make-up of joint fluid in early RA patients was distinct from that of other inflammatory diseases (153). This RA joint fluid profile, including CXCL4 and CXCL7, appeared approximately 3 months into symptom onset, but was not present in established RA fluid profiles (153).

The Leiden Early Arthritis Clinic performed 589 hand and foot MRIs in their study cohort between August 2010 and October 2014 (154). These included patients with undifferentiated arthritis (UA), established RA, and yet others have other forms of arthritis (154). This group's MRIs were compared to a group of 193 symptom-free volunteers who established the "norm" for the MRIs (154). Within subgroups of UA, MRIs were most predictive of progression to RA in those with oligoarthritic disease (effecting 2-4 joints) compared to monoarthritis (1 joint) and polyarthritis (effecting 5 or more joints) (154). Another conclusion was that if inflammation was not detected on the MRI, then progression to RA was highly unlikely (154).

Early arthritis clinic studies are limited by the infrastructure needed to efficiently identify patients early in their disease course and enroll into research studies. Early arthritis clinics are uncommon in North America likely due to relative fragmented care here compared to those in Europe where patients with early arthritis are funneled to the same academic center. Success of early arthritis clinic often depends on providers other than rheumatologists to identify patients quickly and appropriately refer to rheumatology. Early arthritis may present ambiguously so there is potential for over-diagnosis if all patients with hand or foot arthralgias are referred. Thus, close communication and education between rheumatology and other providers is needed. Providers need to feel invested in the research topic to develop this expertise. Point of care ultrasound in primary care may be helpful to identify the patients most at risk of progressing to RA. Finally, the timeline of when a patient with very early arthritis becomes RA can be difficult to discern, and research definitions have evolved. Thus, some patients deemed as "at risk of RA" may actually have RA at baseline. Careful attention to the current research guidelines and accurate data collection is essential to classify patients correctly.

CLINICAL TRIALS

Clinical trials crucially serve to assess lifestyle changes and identify preventative medications in populations at-risk for RA (**Table 2**). For preclinical RA, clinical trials have been conducted using health education tools, glucocorticoids, disease-modifying antirheumatic drugs (DMARDs), and atorvastatin (3). Pharmaceutical randomized controlled trials for RA prevention generally recruit at-risk individuals based on autoantibody positivity and arthralgias/early inflammatory arthritis in the joints. Clinical trials can collect surveys, biospecimens, physical exam and joint count data, disease activity assessments, and imaging results, which inform RA diagnoses made using ACR/EULAR criteria. However, trials that utilized the 1987 ACR/EULAR criteria may have enrolled participants already with RA according to the 2010 criteria, affecting previously reported results (155). Nonetheless, clinical trials contribute immensely to our understanding of RA pathogenesis and inform clinical treatments and practices. Here, we provide an overview of different clinical for RA prevention. We first discuss a behavioral intervention among FDRs. We then discuss completed trials in the order they were completed. We then detail some ongoing trials that do not yet have results.

The Personalized Risk Estimator for RA (PRE-RA) Family study was a prospective, randomized controlled trial that assessed willingness to change behaviors after an RA risk education intervention. RA FDRs were randomized to one of three education arms where the PRE-RA arm and the PRE-RA Plus arm received personalized RA risk educations *via* a web-based tool or a one-on-one session with a health educator, respectively (156). The Comparison arm received a standard RA education. Participants' RA risk was calculated and assessed based on participants' demographic, genetic, and biomarker data, as well as their RA-related behaviors (smoking, obesity, dental health, and diet and supplement intake) (156). Participants' willingness to change RA related behaviors was evaluated over 1 year (156). Willingness to change was most apparent among the PRE-RA arm which utilized the web-based education tool, and for both the PRE-RA and PRE-RA plus arms, concern for developing RA significantly decreased compared to that of the Comparison group (157, 158). Thus, the PRA-RA trial found that personalized RA-risk education increases willingness to modify RA-related behaviors, ultimately RA risk, as well as provides reassurance for individuals at-risk for RA (157, 158). The PRE-RA Family Study serves as a proof-of-concept that an educational intervention may modify RA risk-related behaviors that could lead to lower RA risk.

Several multi-center, randomized, double-blind placebo-controlled trials have been conducted to evaluate the efficacy and appropriateness of glucocorticoids for preventing RA. These trials include the Stop Arthritis Very Early (SAVE) trial for methylprednisolone, the Steroids In Very Early Arthritis (STIVEA) trial for methylprednisolone acetate, and the Dexamethasone in seropositive arthralgias trial (159, 160). SAVE was a multi-national trial that recruited individuals with inflammatory arthritis of at least one joint for <16 weeks duration and were randomized to receive a single injection of methylprednisolone or placebo, intramuscularly (160). Data elements collected include 66/68 joint counts, visual analogue scales (VAS) of patient-reported joint pain and global disease activity, and biospecimens. No significant difference in remission between the groups was found (160). STIVEA was a British trial that examined the effects of intramuscular (IM) injections of glucocorticoids in participants with early inflammatory polyarthritis (IP) (159). In contrast to SAVE, participants must have had IP of 4-10 weeks with tenderness and soft tissue swelling in two or more joints (159). Additionally, at least one of the joints must have been the wrist, metacarpophalangeal or proximal interphalangeal joint (159). STIVEA participants were randomized to receive three weekly injections of either methylprednisolone acetate or placebo (159). Moreover, STIVEA's primary outcome, the need to start DMARDs within the 6 months following the first injection, was met (159). The placebo group was more likely to need DMARDS at 6 months than the glucocorticoid group (159). The authors thus conclude that STIVEA's intervention (a 3-week course of IM methylprednisolone acetate) prevents approximately one in 10 patients from progressing into RA within the following 12 months (159). However, differences in disease activity measures, joint damage and clinical diagnoses for RA did not differ between groups (159). These secondary findings in line with those of SAVE. Bos et al. conducted another trial on glucocorticoid efficacy in early RA (161). This Dutch trial randomized participants to receive either IM injections of dexamethasone or placebo (161). The primary outcome of this trial was a 50% decrease in autoantibody levels or eventual normalization at 6 months in ACPA-negative and/or IgM-RF-positive participants with arthralgias (161). A significant decrease in antibody levels was observed among the dexamethasone group; however, no participants became seronegative (161). Additionally, a greater percentage in the dexamethasone group actually progressed to developing IA than the placebo group, and 3 subjects in each arm progressed according to the 1987 ACR/EULAR criteria (161).

Methotrexate has been investigated in several preventative RA clinical trials (155, 162–164). The Probable Rheumatoid Arthritis: Methotrexate versus Placebo Treatment (PROMPT) trial in the Netherlands followed participants with undifferentiated IA, randomized into either a methotrexate arm or placebo arm (162). The primary outcome, RA diagnosis meeting 1987 ACR criteria, did not differ between arms (162). This could have been affected by participants already having RA using 2010 ACR/EULAR criteria. Despite this, an exploratory subgroup of ACPA positive participants benefited from methotrexate more than those receiving placebo (155). Thus, PROMPT's results suggest that methotrexate may be a strong treatment option for individuals with early RA who are ACPA positive (155, 162). Methotrexate was also used in the multi-national trial, the Definitive Intervention in New Onset Rheumatoid Arthritis (DINORA) study (164). DINORA's key

TABLE 2 | Selected clinical trials investigating rheumatoid arthritis prevention.

Study name	Region, country/ Year initiated	Main eligibility criteria	Intervention arm	Control arm	Primary outcome	Notes
Stop Arthritis Very Early (SAVE)	Europe, Mexico, Japan, Austria	Individuals with IA of <16 weeks duration	Methylprednisolone 120 mg IM x1	Placebo	Drug-free clinical remission at both weeks 12 and 52	No difference in primary outcome
Steroids in Very Early Arthritis (STIVEA)	UK 2002	Individuals with IP of 4-10 weeks duration, ACR1958 criteria for probable RA	Methylprednisolone 80mg IM every week x3	Placebo	DMARD initiation by 6 months	Statistically lower DMARD initiation in methylprednisolone group
Dexamethasone in Seropositive Arthralgias	Netherlands 2004	Individuals with ACPA- and/or RF-positivity with arthralgia and presence of shared epitope	Dexamethasone 100 mg IM at baseline and 6 weeks	Placebo	50% reduced antibody or normalization at 6 months	No difference in primary outcome; dexamethasone group had decreased antibody levels
Probable Rheumatoid Arthritis: Methotrexate versus Placebo Treatment (PROMPT)	Netherlands 2001	Symptoms of arthritis < 2 years duration, undifferentiated arthritis diagnosed using ACR 1958 criteria for probable RA	Methotrexate titrated to maximum of 30 mg PO weekly	Placebo	RA by 1987 ACR criteria	No difference in primary outcome; subgroup of ACPA+ with reduced RA risk
Treat Early Arthralgia to Reverse or Limit the Exacerbation of RA (TREAT EARLIER)	Netherlands 2014	Clinically suspect arthralgia with onset <1 year, subclinical inflammation of hand or foot joints at 1.5 T MRI	Methylprednisolone 120 mg IM then methotrexate titrated to maximum of 25 mg weekly	Placebo	RA by 2010 ACR/EULAR criteria	Ongoing
Definitive Intervention in New Onset Rheumatoid Arthritis (DINORA)	Austria 2007	Symptom duration of 2- 12 weeks, synovial swelling present in 2+ joints (at least joint must have been a metacarpophalangeal, proximal interphalangeal, or metatarsophalangeal joint)	Infliximab + methotrexate combination Methotrexate monotherapy	Placebo	Clinical remission after 1 year	Higher proportion in intervention groups than placebo group
Abatacept study to Determine the effectiveness in preventing the development of rheumatoid arthritis in patients with Undifferentiated inflammatory arthritis and to evaluate Safety and Tolerability (ADJUST)	North America, Europe, South America 2004	ACPA-positive patients with UA (not fulfilling the ACR criteria for RA) and synovitis of two or more joints	Abatacept	Placebo	RA by 1987 ACR criteria	Primary outcome not met; suggestion of delay in progression to RA in abatacept group
Abatacept Reversing Subclinical Inflammation by MRI in ACPA-positive Arthralgia (ARIAA)	Germany, Czech Republic, Spain 2014	ACPA positive, MRI signs of inflammation	Abatacept	Placebo	Improvement in at least one of the MRI inflammation parameters	Preliminary results favor abatacept group (peer review publication pending)
Arthritis Prevention in the Preclinical Phase of RA with Abatacept (APIPPRA)	United Kingdom 2018	Individuals with arthralgias, RF and ACPA positivity, or arthralgias with ACPA positive >3x ULN	Abatacept	Placebo	RA by 2010 ACR/EULAR criteria	Ongoing
Prevention of Clinically Manifest Rheumatoid Arthritis by B cell Directed therapy in the earliest phase of the disease (PRAIRI)	Netherlands 2010	Individuals with ACPA and RF positivity with arthralgias, never used DMARDs, no IA	Rituximab + Solumedrol	Placebo + Solumedrol	Inflammatory arthritis	No difference in primary outcome; secondary analysis suggested delay in inflammatory arthritis for rituximab group
Statins to Prevent Rheumatoid Arthritis (STAPRA)	Netherlands 2015	Individuals with arthralgia, ACPA positivity >3x ULN or ACPA and RF, without arthritis	Atorvastatin	Placebo	Clinical arthritis	No difference in primary outcome
Strategy to Prevent the Onset of Clinically-Apparent Rheumatoid Arthritis (StopRA)	USA 2016	ACPA >2x ULN, no IA, never used DMARDs	Hydroxychloroquine	Placebo	RA by 2010 ACR/EULAR criteria	Ongoing

ACPA, anti-citrullinated protein antibodies; ACR, American College of Rheumatology; DMARD, disease-modifying antirheumatic drug; EULAR, European Alliance of Associations for Rheumatology; FDR, first-degree relative; IA, inflammatory arthritis; IM, intramuscular; MRI, magnetic resonance imaging; RA, rheumatoid arthritis; RF, rheumatoid factor; UA, undifferentiated arthritis; ULN, upper limit of normal.

finding was that treating early RA with infliximab in addition to methotrexate can lead to sustained remission when compared to a placebo group (164). Moreover, the ongoing Treat Early Arthralgia to Reverse or Limit the Exacerbation of RA (TREAT EARLIER) trial based in the Netherlands continues to evaluate methotrexate's potential as a preventative pharmaceutical (163).

Biologic DMARDs, such as abatacept and rituximab, have been used in several preventative clinical trials. The UK trial, Abatacept Study to Determine the Effectiveness in Preventing the Development of Rheumatoid Arthritis in Patients with Undifferentiated inflammatory Arthritis (ADJUST) study enrolled ACPA positive, individuals with UA to receive 8 intravenous (IV) injections of abatacept or placebo for 6 months with two years of follow up (165). Using the 1987 ACR criteria, the abatacept group progressed to RA insignificantly less than the placebo group; however, the authors found a decrease in ACPA positivity and inhibition of erosive development (165). Similarly, the ongoing Arthritis Prevention in the Preclinical Phase of RA with Abatacept (APIPPRA) trial is another UK study which enrolled ACPA-positive individuals with arthralgias and is evaluating the effectiveness of subcutaneous abatacept in RA prevention (166). Abatacept was found to significantly improve subclinical arthritis in high RA-risk individuals in the Abatacept Reversing Subclinical Inflammation as Measured by MRI in ACPA-positive arthralgia (ARIAA) trial based in Europe. The primary endpoint was met with participants in the abatacept group improving in MRI parameters compared to the placebo group. The Prevention of Clinically Manifest Rheumatoid Arthritis by B cell Directed Therapy (PRAIRI) study in the Netherlands evaluated the efficacy of rituximab in ACPA-positive participants with arthralgias (167). Participants were randomized into a single infusion of rituximab and methylprednisolone arm or a placebo and methylprednisolone arm (167). There was no significant difference between arms in time to developing IA, the primary outcome. The authors argue; however, that rituximab delayed arthritis development as the timepoints for when 25% of all participants developed arthritis was 12 months for the placebo group, and 24 months for the rituximab group (167).

Other pharmacologic randomized controlled trials have used atorvastatin and Hydroxychloroquine. Atorvastatin was used in the Statins to Prevent Rheumatoid Arthritis (STAPRA) trial in the Netherlands which ended prematurely due to low recruitment. The primary endpoint was clinical arthritis, and no significant findings were made. In the United States, the multi-site Strategy to Prevent the Onset of Clinically-apparent Rheumatoid Arthritis (StopRA) trial is ongoing. ACPA-positive participants, without IA, who have never used DMARDs, are randomized to receive either HCQ or placebo for 1 year and are monitored for 2 years for follow up. HCQ was previously found to reduce risk in individuals with palindromic rheumatism in a retrospective cohort study (168).

The main disadvantage of clinical trials is cost and time. Due to the large financial and time commitment, care is needed at all stages to ensure that the trial will reach a definitive conclusion to the research question. Strict eligibility criteria may make it difficult to meet recruitment goals. Conversely, loose eligibility criteria may dilute the ability to find a true effect and lower the outcome rate that could also be a threat to validity. Study design considerations such as choice, dose, and duration of study drug and the appropriate control group are essential. There is also a balance between the depth of data collected and the time commitment for the participant. Protocols with lengthy study visits and frequent follow-up may be prone to missing data and loss to follow-up. This also could impose selection bias if only enthusiastic and health literate individuals agree to participate. Efforts should be made to include marginalized populations into research studies.

CONCLUSIONS

We detailed the rich variety of study designs that is necessary to investigate distinct preclinical phases of an autoimmune disease such as RA. These studies have formed a complementary approach using epidemiologic and patient-oriented study designs. This has led to several intervention studies, some of which have been successful at delaying the onset of RA. However, further progress is needed to fully elucidate the pathogenesis of RA that may ultimately lead to prevention or delay. Many of the phases have indistinct transition points that may not apply to all individuals. This may also be related to underlying heterogeneity of phenotypes within a disease. The European Alliance of Associations for Rheumatology recently published their points to consider related to conducting clinical trials and observational studies in individuals at risk of RA to establish best practices and standardize nomenclature (169). This and other similar initiatives may lead to more consistent recruitment and data collection methods that may allow for more collaborative and definitive studies with larger sample size. Also, the global interest in RA prevention may lead to larger, international trials to allow for sufficient sample size to identify and implement behavioral and pharmacologic interventions for RA prevention. Overall, epidemiologic and biomarker approaches should be integrated with genetic risk factors to understand etiologies of complex autoimmune diseases such as RA. These lessons can be applied to other immune-mediated inflammatory diseases that arise from a similar paradigm.

AUTHOR CONTRIBUTIONS

EK and GQ share first authorship and contributed equally to this work. Conceptualization: All authors. Writing — original draft preparation: All authors. Writing — review and editing: JS. Supervision: JS. All authors contributed to the article and approved the submitted version.

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Relationship Between a Vitamin D Genetic Risk Score and Autoantibodies Among First-Degree Relatives of Probands With Rheumatoid Arthritis and Systemic Lupus Erythematosus

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Objective: Higher 25-hydroxyvitamin D (25(OH)D) levels have been associated with reduced risk for autoimmune diseases and are influenced by vitamin D metabolism genes. We estimated genetically-determined vitamin D levels by calculating a genetic risk score (GRS) and investigated whether the vitamin D GRS was associated with the presence of autoantibodies related to rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in those at increased risk for developing RA and SLE, respectively.

Methods: In this cross-sectional study, we selected autoantibody positive (aAb+) and autoantibody negative (aAb-) individuals from the Studies of the Etiologies of Rheumatoid Arthritis (SERA), a cohort study of first-degree relatives (FDRs) of individuals with RA (189 RA aAb+, 181 RA aAb-), and the Lupus Family Registry and Repository (LFRR), a cohort study of FDRs of individuals with SLE (157 SLE aAb+, 185 SLE aAb-). Five SNPs known to be associated with serum 25(OH)D levels were analyzed individually as well as in a GRS: rs4588 (*GC*), rs12785878 (*NADSYN1*), rs10741657 (*CYP2R1*), rs6538691 (*AMDHD1*), and rs8018720 (*SEC23A*).

Results: Both cohorts had similar demographic characteristics, with significantly older and a higher proportion of males in the aAb+ FDRs. The vitamin D GRS was inversely

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associated with RA aAb+ (OR = 0.85, 95% CI = 0.74-0.99), suggesting a possible protective factor for RA aAb positivity in FDRs of RA probands. The vitamin D GRS was not associated with SLE aAb+ in the LFRR (OR = 1.09, 95% CI = 0.94-1.27). The *SEC23A* SNP was associated with RA aAb+ in SERA (OR = 0.65, 95% CI = 0.43-0.99); this SNP was not associated with SLE aAb+ in LFRR (OR = 1.41, 95% CI = 0.90 – 2.19).

Conclusion: Genes associated with vitamin D levels may play a protective role in the development of RA aAbs in FDRs of RA probands, perhaps through affecting lifelong vitamin D status. The GRS and the *SEC23A* SNP may be of interest for future investigation in pre-clinical RA. In contrast, these results do not support a similar association in SLE FDRs, suggesting other mechanisms involved in the relationship between vitamin D and SLE aAbs not assessed in this study.

Keywords: vitamin D, autoantibody positive (aAb+), autoantibody negative (aAb-), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), genetic risk score (GRS)

INTRODUCTION

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are chronic inflammatory autoimmune diseases (ADs) thought to develop *via* a complex interplay between inherent genetic risk and environmental exposures that ultimately trigger autoimmunity (1). While there is a subset of RA/SLE patients that are seronegative, the majority of patients exhibit disease specific autoantibodies (aAb) that can be elevated years prior to the clinical diagnosis of disease during a period that can be termed 'preclinical autoimmunity' (2, 3). However, the complete etiology of both RA and SLE remains unknown; in particular, it is not known what factors may drive the development of aAbs during the preclinical period.

Genetic factors are thought to account for 40-50% of RA (4, 5) and 55-77% of SLE (6, 7) risk, leaving approximately half of the risk for disease development unexplained. Epidemiologic studies have identified many environmental factors associated with the risk and severity of disease for both RA and SLE (8, 9). Vitamin D (25-hydoxyvitamin D; 25(OH)D) is one environmental factor that has been studied, since vitamin D deficiency is a common finding in patients who have a clinical diagnosis of an AD including RA and SLE (10). The major role of vitamin D is maintaining normal blood levels of calcium and phosphorus. In addition, 25(OH)D has been shown to have immune-modulatory properties, such as preventing antigen expression, regulating T cell activity and inhibiting cytokine abundance (11–14).

Exposure to natural light is the most common source of vitamin D levels (14–16); and dietary intake of fortified foods or fatty fish is another way in which people gain vitamin D levels (17). However, sunlight exposure and diet can fluctuate throughout an individual's lifetime, such that a single 25(OH)D measure may not adequately reflect long-term vitamin D status. There is a known genetic influence on 25(OH)D serum levels (18–20). Twin studies have estimated the heritability of vitamin D serum levels to be between 50% and 80% (21, 22). Jiang et al. (19) recently conducted a genome-wide association study (GWAS) on 79,366 individuals of European ancestry and found a select number of single nucleotide polymorphisms (SNPs) that explained 38% of the variance in serum 25(OH)D concentrations. A genetic risk score that predicts vitamin D concentrations (i.e., genetically determined 25(OH)D) may provide a more stable estimate of lifetime vitamin D levels or status.

In this paper, we focus on investigating if genetic determinants of vitamin D levels are inversely associated with autoantibody positivity prior to clinical symptoms in two at-risk populations: first-degree relatives (FDRs) of RA and SLE probands. FDRs of people with an AD are at an increased risk for that AD compared to the general public (23–25). We generated a genetic risk score (GRS) for serum 25(OH)D levels to evaluate the relationship between vitamin D and autoantibody positivity status in at-risk individuals.

MATERIALS AND METHODS

Study Population

We utilized two at-risk cohorts in which we identified RA and SLE probands and their respective unaffected FDRs. Both cohorts have been approved by their institutional review boards (University of Colorado and Oklahoma Medical Research Foundation) and had written informed consent prior to any procedures.

RA FDRs were selected from the Studies of the Etiologies of Rheumatoid Arthritis (SERA), a prospective cohort study that enrolled FDRs of probands with RA (26). RA probands met ≥4 1987 American College of Rheumatology (ACR) RA classification criteria (27). FDRs were tested for rheumatoid factor (RF) isotypes (IgA, IgG, IgM), RF by nephelometry, anti-cyclic citrullinated peptide 2 (CCP2), and/or anti-CCP3.1, as described in James et al. (28). An FDR testing positive for any one of these autoantibodies (aAb) was selected as an aAb+ RA FDR (n = 189). An FDR testing negative for these autoantibodies was selected as an aAb- RA FDR (n = 181). To be consistent with Jiang (19) and reduce confounding due to ethnic and racial difference, all RA FDRs selected for genotyping were non-Hispanic white, and one FDR was randomly chosen from each family so that no FDRs were related to other FDRs (28).

The SLE FDRs were selected from the Lupus Family Registry and Repository (LFRR), a prospective study of FDRs of probands with SLE (29). SLE probands met ≥4 ACR SLE classification criteria (30). FDRs were tested for autoantibodies to Sm, Sm/RNP, RNP, dsDNA, chromatin, ribosomal P, Ro/SSA, La/SSB and/or anti-cardiolipin autoantibodies: IgA, IgG, and IgM, as described in James et al. (28). An FDR testing positive for any one of these aAb was selected as an aAb+ SLE FDR (n=157). An FDR testing negative for these autoantibodies was selected as an aAb- SLE FDR) (n=185). For similar reasons as mentioned above, all SLE FDRs were non-Hispanic white, and one FDR was randomly chosen from each family so that no FDRs were related to other FDRs (28).

Genotyping & Genetic Risk Score Calculation

RA and SLE FDR DNA samples were genotyped using the Illumina MEGA^{EX} BeadChip and the ImmunoChip v1.0., respectively, per Illumina protocols starting with 250 ng of genomic DNA and read on an Illumina iSCAN. Genome Studio (Illumina) was used for quality control (QC) which included removing SNPs and samples with missing call rates >10%, minor allele frequency < 0·00001, and Hardy Weinberg Equilibrium < 0·001. SNPs that indicated known QC errors (e.g., poor clustering) were also removed.

Jiang et al. (19) identified six SNPs associated with circulating 25(OH)D concentrations in a European ancestry genome-wide association study. Of these SNPs, five SNPs (or their proxies) had been genotyped in the RA FDRs using the MEGA^{EX} BeadChip: rs3755967 (in *GC*, chr4: 71743681), rs12785878 (in *NADSYN1*, chr11:71456403), rs10741657 (in *CYP2R1*, chr 11:14893332), rs10745742 (in *AMDHD1*, chr12:95964751), and rs8018720 (in *SEC23A*, chr14:39086981). For markers rs3755967 and rs10745742, we used proxy SNPs with 100% linkage disequilibrium (LD) according to the 1000 Genomes Project CEU population, whom are Utah residents of Northern and Western European ancestry, and (rs4588 located on chr4:71752606 and rs6538691 located on chr12:95959729, respectively). These five SNPs had not been genotyped with the ImmunoChip, so in order to measure these in the SLE FDR population, we directly genotyped them using the rhAMP^{TM} SNP Genotyping assay (Integrated DNA Technologies) per manufacturers protocols using the forward and reverse primers shown in **Supplemental Table S1**. **Supplemental Table S2** shows details on the markers (and proxy markers) used in the analysis.

To calculate the vitamin D GRS, we summed the number of effect alleles for each of the five markers. For each SNP, an individual would have the potential to have either 0, 1 or 2 effect alleles, leaving the potential GRS of any individual to be an integer between 0 and 10. The effect allele is the allele which was associated with a higher circulating 25(OH)D concentration as reported in Jiang et al. (19). We also dichotomized the vitamin D GRS into high (≥ 5 effect alleles) and low (< 5 effect alleles).

Statistical Analyses

All analyses were performed within cohort (RA FDRs or SLE FDRs). For genetically determined vitamin D, we tested vitamin D associated SNPs individually under an additive genetic model and the vitamin D GRS as both a continuous and a categorical (high/low) variable. Covariates for further statistical analyses were selected if they were significantly associated (p-value < 0.05) with aAb+ status. A logistic regression was used to identify the genetically determined vitamin D association with autoantibody positivity status while adjusting for sex and age. To address population stratification, we examined ancestry principal components (PCs) that were available for all RA FDRs (using the MEGA^{EX} BeadChip) and for a subset of 304 SLE FDRs (using the ImmunoChip). Because we were concerned about needing to eliminate 59 SLE FDRs from the analyses if we adjusted for the PCs, we performed sensitivity analyses to show that there was no significant change in effect size estimates in both cohorts when the first three ancestry PCs were included in the models (**Supplemental Table S3**). To optimize sample size in the SLE FDRs and keep methods comparable across cohorts, we did not adjust for ancestry PCs in the final statistical models.

RESULTS

Demographics of the Study Populations

Table 1 depicts the demographics of aAb+ and aAb- FDRs in each cohort. In both cohorts, aAb+ FDRs are significantly older than aAb- FDRs; and aAb- FDRs are more likely to be female than aAB+ FDRs.

Vitamin D GRS Allele Distribution Across Cohorts

The frequencies of the effect alleles of the vitamin D SNPs and the distributions of the vitamin D GRS were similar across the

TABLE 1 | Demographic characteristics for the RA FDR and SLE FDR cohorts.

Characteristic	RA aAb+ FDR	RA aAb- FDR	p-value	SLE aAb+ FDR	SLE aAb- FDR	p-value
N	189	181		157	185	
Sex: % female	75.7	86.2	0.01	73.9	83.8	0.03
Age: mean ± SD	51.7 ± 16.2	47.4 ± 15.5	0.01	59.2 ± 15.3	55.7 ± 14.6	0.03
BMI: mean ± SD	26.8 ± 5.5	26.9 ± 6.1	0.88	28.1 ± 5.8	27.3 ± 5.8	0.09
*Ever Smoker: % yes	40.2	41.7	0.78	48.5	48.1	0.91
25(OH)D3 GRS: mean ± SD	4.6 ± 1.4	4.9 ± 1.4	0.03	4.86 ± 1.33	4.67 ± 1.51	0.22
25(OH)D3 GRS High: % yes	49.2	60.2	0.03	42.0	44.3	0.67

**1 SERA FDR missing smoking data.*

All patients selected for both cohorts were non-Hispanic white.

two cohorts (**Figure 1**). The effect allele frequencies ranged from 0.156 and 0.161 for the *SEC23A* SNP to 0.719 and 0.727 for the *NADSYN1* SNP in the RA and SLE FDR cohorts respectively. Both cohorts had a median vitamin D GRS of 5, and a mean (SD) of 4.76 (1.43) and 4.74 (1.45) for RA and SLE FDRs respectively.

Vitamin D GRS Association With Autoantibody Positivity (aAb+)

In the SERA RA FDR cohort, the vitamin D GRS (as a continuous variable and as a high/low category) was significantly associated with RA aAb+ status, adjusting for age and sex (**Figure 2**). The presence of a higher number of effect alleles (potentially reflecting a higher lifetime levels of vitamin D) was associated with a lower odds of being aAb+ in the RA FDRs. In addition to the vitamin D GRS, the *SEC23A* was significantly associated with RA aAb+ status in RA FDRs, adjusting for age and sex (OR 0.65; 95% 0.43 to 0.99; p = 0.046). Neither the vitamin D GRS nor any of the vitamin D SNPs were associated with SLE aAb+ status in the SLE FDRs.

Genetic Risk Score and 25(OH)D Levels: A Sub-Analysis

To investigate whether the vitamin D GRS was associated with 25 (OH)D levels, we identified a subset of FDRs in the RA and SLE populations that had had plasma 25(OH)D concentrations measured previously. Twenty-eight of the RA FDRs in the

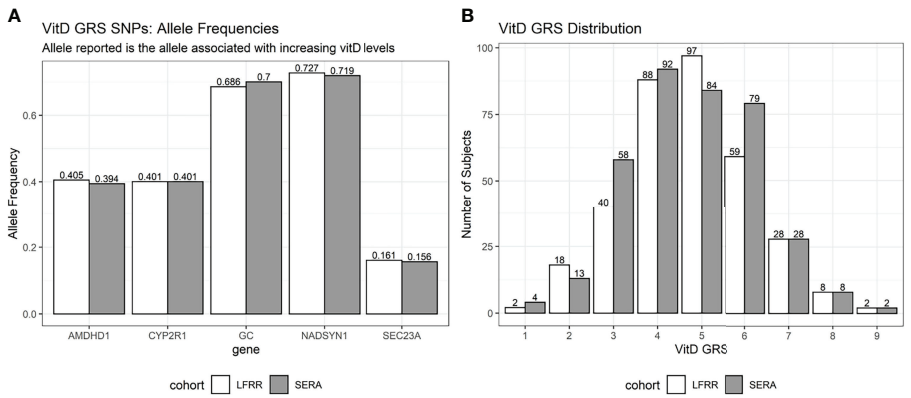

FIGURE 1 | Effect allele frequencies and vitamin D GRS distribution. Effect alleles are those that were associated with an increased 25(OH)D. **(A)** The allele frequency for each of the 5 SNPs used in the vitamin D GRS calculation are shown. The SLE FDRs are shown in white bars (LFRR cohort) and RA FDRs are in gray bars (SERA cohort). **(B)** The distribution of the vitamin D GRS is shown in for each cohort.

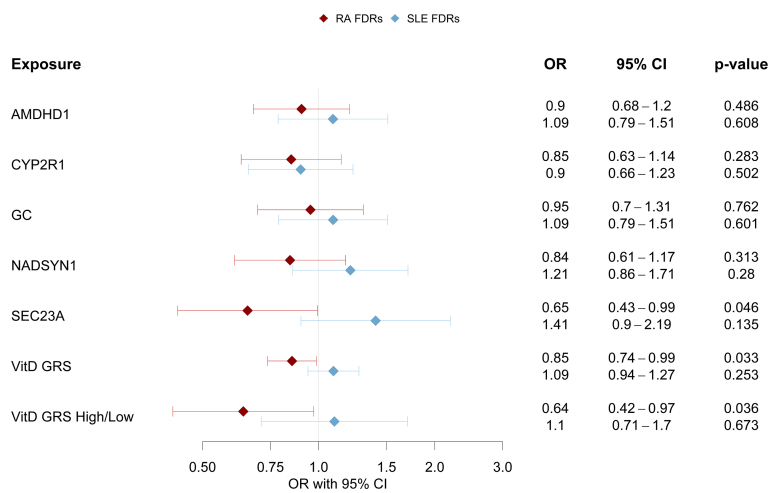

FIGURE 2 | Vitamin D GRS Association with Cohort Specific aAb+. For different vitamin D level measures (either individual SNPs, vitamin D GRS, or dichotomized vitamin D GRS), the odds ratio (OR) is shown as the dot and the corresponding 95% confidence interval is the line. The RA FDR cohort is in red and SLE FDR cohort in blue.

current analysis had plasma 25(OH)D concentration measured previously by radioimmunoassay (DiaSorin, Inc) (31). Sixty-four of the SLE FDRs had plasma 25(OH)D concentrations measured previously using a commercial enzyme immunoassay (Immunodiagnostic Systems, Inc., Scottsdale, AZ) according to manufacturer instructions. To compare 25(OH)D concentrations in high and low vitamin D GRS groups, we performed a Welch's t-test, which accounts for different variances within the groups. In the RA FDRs, those with a high vitamin D GRS had significantly higher 25(OH)D concentration at a single point in time than those with a low vitamin D GRS {25(OH)D mean [standard deviation (SD)]: 29.3 (2.97) and 24.2 (9.01) ng/mL for high and low GRS, respectively; p-value = 0.04} (**Supplemental Figure S1A**). In SLE FDRs, there was no association between the vitamin D GRS and 25(OH)D concentration [25(OH)D mean (SD): 26.1 (10.2) and 24.3 (8.38) ng/mL for high and low GRS, respectively; p-value = 0.47] (**Supplemental Figure S1B**).

DISCUSSION

Association of Vitamin D GRS and RA aAb+ Status

We observed in RA FDRs that a higher vitamin D GRS was associated with lower risk of RA aAb positivity. If indeed the GRS is indicative of longer-term adequate vitamin D levels, this may suggest that long-term adequate vitamin D levels area a possible protective factor for RA aAb+ among individuals at-risk for developing RA. Our results are consistent with the hypothesis that increased 25(OH)D levels may protect again RA through the suppression of cytokines and inflammation (reviewed in (32)). Moreover, supplementation of vitamin D and omega-3 fatty acids was associated with a decreased risk of rheumatoid arthritis in the recently reported VITAL randomized controlled trial (33). Our finding, along with others, suggest that long term vitamin D supplementation may be needed in individuals at-risk for RA, particularly those lacking the effect alleles of SNPs that lead to a higher genetically determined vitamin D level.

Lack of Association of Vitamin D GRS and SLE aAb+ Status

In contrast, all associations between the vitamin D GRS and the individual SNPs with aAb+ in the SLE FDRs were non-significant. This does not necessarily mean vitamin D levels are not associated with SLE aAb+ but potentially the genetically-regulated component is not associated, or perhaps more complex mechanisms are involved in disease etiology. Young et al. (34) has shown that the relationship between circulating 25(OH)D levels and SLE was modified by a CYP24A1 polymorphism, with each minor allele copy presenting a stronger inverse relationship between 25(OH)D and SLE. Bae and Lee (35) performed a mendelian randomization on vitamin D levels and found no causal association between vitamin D and risk for either RA or SLE. However, this study only assessed SNPs in *SSTR4*, *NADSYN1* and *GC*, and did not examine *SEC23A*, which contained our strongest effect allele.

Additionally, the SLE aAb+ FDRs could possibly be a more heterogenous population than the RA aAb+ FDRs. More autoantibodies were considered for one to be defined as a SLE aAb+ (8 autoantibodies) compared to RA aAb+ (6 autoantibodies). Not only are there various types of autoantibodies for SLE, but it is well noted that patients with SLE have a variety of symptoms occurring in different combinations (31) leading to within-disease heterogeneity (36). This greater heterogeneity may suggest that the vitamin D GRS should be investigated within sub-types of SLE autoimmunity, which requires a larger sample size than that available to the current study.

Interestingly, our vitamin D GRS was not robustly associated with circulating 25(OH)D levels in the SLE FDRs, which may also be an explanation as to why we did not see an association with SLE aAb+ status. And finally, there may be disease-specific effects of vitamin D in AD development. For example, it is possible that the vitamin D GRS is associated with production of RA-related autoantibodies in the preclinical period of RA development as we have observed herein; this may be in contrast to SLE where vitamin D may play a role in the transition from autoantibody positivity to clinical disease onset. Future studies should follow Ab+ individuals for progression to clinical disease to examine this hypothesis.

SEC23A Role in Immune Response

The *SEC23A* SNP was the only SNP in the vitamin D GRS with a significant protective association with RA aAb+ on its own. This is of interest as the allele represents a missense variant that alters the protein's amino acid sequence from a leucine to valine and could result in a functional change in the protein. *SEC23A* is a component of the coat protein complex II which is required for the translocation of insulin-induced glucose transporter SLC2A4/GLUT4 to the cell membrane (32). *SEC23A* also has a role in immune function as it is part of the GO Biological Process GO:0002474: antigen processing and presentation of peptide antigen *via* MHC class I. Antigen presentation is a major process in activating both B and T cells, a necessary component for the inflammation process in general (37). In addition, this process has been shown to be important in the pathogenesis of RA (38) and could function differently based on one's genetic background. Therefore, it is possible that the effect of *SEC23A* on immune function may or may not work through vitamin D levels and requires further exploration.

Strengths and Limitations

A strength of our study is that we included a large number of at-risk individuals for both RA and SLE; and that these individuals did not have classified disease, which allowed a unique opportunity to examine whether vitamin D SNPs are relevant in the preclinical phase of disease. A limitation of our study is its focus on non-Hispanic whites exclusively, which limits its generalizability. In addition, we only assessed five of the six vitamin D SNPs reported from Jiang (19). Additional genetic markers may be needed to adequately assess the complex relationship of vitamin D and SLE aAb+, as reported by Young et al. (34). Additional limitations include that only a small subset of samples had circulating 25(OH)D levels measured, and that two different 25(OH)D assays were utilized in the two cohorts.

Conclusion and Future Directions

These findings suggest that a high vitamin D GRS may have a protective role in the development RA-specific autoantibodies in individuals at-risk for RA. We speculate that this may be due to higher lifetime levels of vitamin D or other immune effects of this GRS. Future studies need to expand on the complex role of vitamin D in the preclinical phase of ADs, including assessment of additional vitamin D associated SNPs, longitudinal assessment of 25(OH)D levels, and the study of larger more diverse study populations. An important next step would be to replicate our findings in a more generalizable population. Examining potential modifiable factors for the effect of vitamin D levels (e.g., gene-environment interactions), could lead to new understanding of vitamin D in AD etiology. Finally, as there are an increasing number of prevention studies in pre-clinical RA populations, a therapeutic trial of vitamin D supplementation in this population may be warranted. In addition, we do not have consistently collected vitamin D supplement use across our two populations. And since the point of our GRS analysis was to investigate an estimate of long-term vitamin D levels rather than levels based on current sun exposure (i.e., season), we did not include season of blood draw in our models of RA or SLE aAb outcomes. We note that season of blood draw was not associated with the GRS, so it would not be considered a confounder in the analysis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by (SERA cohort) University of Colorado; (LFRR cohort) Oklahoma Medical Research Foundation. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LV completed data analysis across both studies and wrote manuscript. EB carried out experiments in SERA, completed data analysis in SERA and contributed to writing manuscript. JS provided patient data and samples in SERA and carried out experiments in SERA. JG, WD, and SM carried out experiments in LFRR. MF carried out experiments in SERA. JK provided patient data and samples in LFRR. KY, MD, TM, JO, MW, JB, RK, PG, CL, KD, JJ, and VH provided experimental, analytical and editorial guidance. JN designed experiments and wrote manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.881332/full#supplementary-material

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REVIEW
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Understanding the Concept of Pre-Clinical Autoimmunity: Prediction and Prevention of Systemic Lupus Erythematosus: Identifying Risk Factors and Developing Strategies Against Disease Development

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There is growing evidence that preceding the diagnosis or classification of systemic lupus erythematosus (SLE), patients undergo a preclinical phase of disease where markers of inflammation and autoimmunity are already present. Not surprisingly then, even though SLE management has improved over the years, many patients will already have irreversible disease-related organ damage by time they have been diagnosed with SLE. By gaining a greater understanding of the pathogenesis of preclinical SLE, we can potentially identify patients earlier in the disease course who are at-risk of transitioning to full-blown SLE and implement preventative strategies. In this review, we discuss the current state of knowledge of SLE preclinical pathogenesis and propose a screening and preventative strategy that involves the use of promising biomarkers of early disease, modification of lifestyle and environmental risk factors, and initiation of preventative therapies, as examined in other autoimmune diseases such as rheumatoid arthritis and type 1 diabetes.

Keywords: systemic lupus erythematosus, prevention, biomarkers, risk factors, pathogenesis

1 INTRODUCTION: PREDICTION AND POSSIBLY PREVENTION OF SLE IN THE NEAR FUTURE

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by immune dysregulation and systemic inflammation, leading to progressive and irreversible multi-organ damage. Although SLE is relatively uncommon [SLE affects ~25 to 50 per 100,000 persons in the United States (1, 2)], it disproportionately affects young women during their prime reproductive years, particularly those of non-White ancestry (3, 4). SLE remains among the leading causes of mortality in young females, underscoring its impact as an important public health issue (5, 6).

With the discovery of more risk factors for SLE including genetics and environmental/lifestyle risk factors our ability to estimate SLE risk is improving, and thus so is the identification of patients who are at high versus low risk of this complex autoimmune disease.

A better understanding of SLE pathogenesis may enable earlier and more accurate identification of at-risk patients, as well as the discovery of therapeutic targets, and the design of prevention trials. However, since the breakthrough and serendipitous discovery of the Lupus Erythematous (LE) cell and its role in SLE pathogenesis in 1948 (7), are we any closer to achieving this goal? The LE cell provided evidence that autoantibodies are a key player in SLE pathogenesis, which are generated by a dysregulated immune system leading to immune complex formation and deposition, and subsequent inflammation and organ damage. In the 75 years that followed the LE cell identification, there was an explosion of serologic tests and technologies developed to detect autoantibodies, most centrally the antinuclear antibody (ANA) test, to aid in the diagnosis or classification of SLE [reviewed in (8)].

SLE is notoriously difficult to diagnose and classify because of the heterogeneity and non-specificity of clinical signs and symptoms in early disease. The diagnosis of SLE is thus frequently delayed such that by the time a formal diagnosis is confirmed, irreversible organ damage has already occurred. There are reports that the diagnosis of SLE is delayed by a median of 47 months, with patients submitting to an average of 10 consultations and evaluation by three different physicians before a diagnosis is finally made (9). A delay in SLE diagnosis has been associated with worse outcomes including higher disease activity, organ damage, lower quality of life, and remarkably increased healthcare costs (9). Organ damage occurring early in the disease course also has a negative impact on SLE patients, as it is associated with further damage, development of comorbidities and early mortality (10, 11). The classification criteria for SLE have been through several iterations to improve sensitivity and specificity, with the most recent criteria being the American College of Rheumatology (ACR)/EULAR (European League Against Rheumatism) 2019 criteria (12, 13). Unlike the others, one of the major differences with the new criteria is that it uses the "ANA at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test at least once" as an entry criterion.

Despite advances in therapy, such as the recent approval of several new drugs (anifrolumab, voclosporin, and a new indication for belimumab) (14–16), without timely and accurate diagnosis to allow the initiation of evidence-based therapy, patients with SLE will continue to be at increased risk for morbidity, disability, and premature death secondary to cardiovascular events (e.g., strokes and myocardial infarction), malignancy, and infection, driven by uncontrolled inflammation (6, 17). Furthermore, antimalarials continue to be the mainstay therapy in SLE. Hydroxychloroquine (HCQ) has been shown to reduce SLE flares (lupus nephritis in particular), organ damage, pregnancy complications, cardiovascular events and survival (18–23). There is also evidence to suggest it can delay the onset of SLE, prompting a clinical trial that is currently underway to answer whether it can be used as a preventative therapy (18).

Emerging research suggests that our increasing knowledge about risk factors and biomarkers for SLE could lead to the identification of those at highest risk, and potentially then to early interventions *prior to the onset of symptoms*, to intercept and prevent this often-devastating disease. We review how current understanding of the development of SLE is contributing to progress in the identification of those who are developing disease, and how genetic and population risk factor studies are leading to the potential for disease prevention through early identification, environmental or lifestyle changes, and therapeutic interventions.

2 THE PATHOGENESIS OF PRE-CLINICAL SLE AND IMPORTANT BIOMARKERS AND RISK FACTORS

Understanding of the etiopathogenesis of SLE is evolving [reviewed in (24)]. The currently accepted model for multiple complex autoimmune diseases is that development takes place over time prior to diagnosis and in several stages (**Figure 1**). This next section will review the three phases that precede the diagnosis of SLE: 1) genetic risk, 2) asymptomatic autoimmunity and inflammation, and 3) early symptoms of lupus. As we discuss each phase, we will describe potential avenues of disease prevention including biomarkers for early disease detection and modifiable risk factors.

2.1 Genetic Risk

SLE likely begins and is accelerated by a complex interplay between genetic risk, lifestyle and environmental risk factors and immune dysregulation. When individuals who possess SLE genetic risk alleles are exposed to environmental risk factors throughout their lives, synergistic interactions may take place, accelerating the onset of autoimmunity and inflammation. About 5-12% of subjects with a first-degree relative with SLE will develop the disease in their lifetime, whereas in persons with a congenital deficiency of the complement component C4, this risk can increase to 90% (25). Children who develop SLE appear to have a larger contribution of known SLE genetic risk, in particular non-HLA genes, than do adults with SLE, and thus the contribution of environmental exposures to SLE susceptibility may be increasingly important with advancing age (26, 27).

A series of landmark genome-wide association studies (GWAS) over the past decade in SLE have greatly expanded our understanding of the genetic basis of SLE [reviewed in (28, 29)]. To date, over 100 SLE susceptibility loci have been identified, predominantly in European and Asian populations, explaining up to 30% of SLE heritability (30–44). These include alleles in the Major Histocompatibility Complex (MHC) region (multiple genes), some of the Fcγ receptors, *ATG5, BLK, BANK1, IRF5 (interferon regulatory factor 5), ITGAM, PDCD1, PTPN22, PXK, SPP1, STAT4, TNFSF4, TNFAIP3*, *XKR6*, and deficiencies

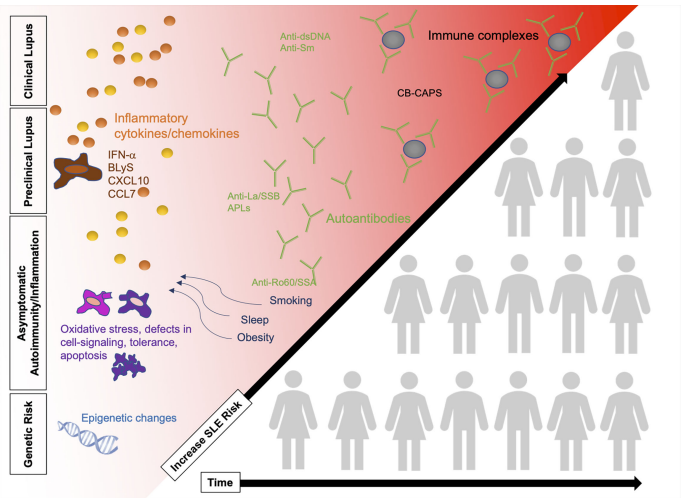

FIGURE 1 | SLE pathogenesis in four phases, increasing in SLE risk over time as patients accumulate risk factors. Changes in the immune system are detected prior to the diagnosis of clinical SLE including presence of autoantibodies, cytokines, and immune complex deposition. Some patients (illustration not representative of actual pre-clinical/clinical SLE population) will progress over time to clinical SLE while others remain in the earlier stages of preclinical SLE. Refer to **Figure 2** for potential points for early risk assessment and intervention opportunities. BLyS, B-cell lymphocyte stimulator; IFN, interferon; SLE, systemic lupus erythematosus.

in complement components (29). Many of these genes belong to important pathways involved in immune complex clearance, host immune signal transduction, and pathways involving interferon (IFN), a key driving cytokine in many cases of SLE.

SLE-associated genes involved in the innate immune system have been gaining interest because of the "IFN signature". Patients with SLE and high levels of IFN-α tend to have more severe disease manifestations (45). Normally, type I IFNs are produced during early response to viral infections and promote dendritic maturation and proinflammatory cytokines. This has several important effects on the immune system including the stimulation of the Th1 pathways, promotion of B-cell activation for autoantibody production, and regulation of apoptosis. One of these genes is *IRF5*, which regulates type I IFN-responsive genes. Outside of the MHC, it is one of the most strongly and consistently SLE-associated with a modest contribution to SLE risk (odds ratio 1.5) (46). The rs7574865 SNP risk variant of *STAT4* has also shown to confer increased sensitivity to IFN-α signaling in peripheral blood mononuclear cells of SLE patients, and is associated with more severe disease, early disease onset and production of antibodies to double-stranded DNA (dsDNA) (47). Together, *IRF5* and *STAT4* have an additive effect for increased risk of SLE development (48). Additional genes that influence the IFN pathway and innate immune signaling include *IRAK1*, which is found on the X chromosome and therefore is thought to contribute increased SLE risk among females (49), and osteopontin, which is also associated with early disease onset (50), as well as *IRF7, IFIH1*, and *TYK2*.

Other risk factors for SLE development are genes linked to the MHC, primarily *HLA-DRB1* in the MHC class II region (51). HLA molecules play a key role in autoantibody production as demonstrated by one Japanese study that identified both SLE risk signature and autoantibodies to ribonucleoprotein (RNP), SSA/Ro60, SSB/La, cardiolipin were localized to the peptide binding groove of *HLA-DRB1* and anti-Sm to *HLA-DPB1* (52). Multiple genes involved in the adaptive immune response and autoantibody production have also been linked to SLE risk such as *PTPN22* (53) and *BANK1* with three functional variants that lead to an altered B cell activation threshold to increase SLE risk (41).

Given that SLE is multifactorial and multigenic, an individual's risk for SLE development cannot be well estimated using only known genetic risk factors. Several similar weighted genetic risk scores (GRS) have been developed to try to estimate an individual's cumulative genetic susceptibility to SLE risk (54). A high GRS has been associated with earlier onset SLE and more severe disease phenotypes (55). Overall, men with SLE also appear to have slightly higher GRS than do women with SLE, suggesting that there is a stronger genetic component of disease among families with male SLE patients and perhaps that environmental or hormonal factors contribute to lowering the threshold for the development of SLE more among females than males (or, conversely, environmental, or hormonal factors may raise this threshold in males) (56). Other studies have also demonstrated greater SLE risk when genetic and environmental interactions are combined such as vitamin D status in those with *CYP24A1* alleles (57), current/recent smoking and GRS (54).

Future genetic studies will likely reveal increased numbers of genetic biomarkers, further refining our understanding of SLE risk and pathogenesis. Large genetic studies in more diverse racial and ethnic groups are still necessary, as most SLE GWAS to date have studied subjects of European or Asian ancestry. Research and development of models that incorporate

environmental risk factors will hopefully hone our ability to identify those who are at high risk of developing SLE, and lead to new therapeutic targets.

2.2 Asymptomatic Autoimmunity and Inflammation

Some individuals genetically susceptible to SLE will transition into a period of asymptomatic autoimmunity and inflammation prior to the development of overt clinical manifestations. Which individuals will progress and why? These are key questions that we are still trying to answer. Thus far, studies have pointed to environmental risk factors, some known and others yet to be discovered, as potential triggers for this transition. These events likely act by both separate and overlapping biological pathways, including but not limited to increasing oxidative stress, loss of immune tolerance, autoantibody formation, complement activation and immune complex deposition, epigenetic modifications, and upregulation in cytokine expression (58). In this pre-symptomatic phase where there is already evidence of early immune changes, can we use this our advantage to identify these at-risk patients earlier? And if we could identify the earliest changes of SLE, could we "turn it off" or move a person "backwards" on their trajectory towards SLE? In this next section, we will highlight important biomarkers and potential interventions as we review the different pathways of autoimmunity and inflammation in SLE pathogenesis.

2.2.1 Increased Oxidative Stress

Oxidative stress, which is defined by an imbalance between the production and neutralization of reactive oxygen intermediates (ROI), is normally utilized by phagocytic cells to eliminate pathogenic organisms. However, in SLE, this is increased leading to abnormal activation and processing of cell-death signals and autoantibody production [reviewed in (59)]. Endogenous sources of oxidative stress include increased ROI production in mitochondria, NADPH oxidase enzymes in phagocytes, endothelial cells, T cells, and B cells (60, 61). Ultra-violet (UV) radiation, viral and bacterial infections, and chemical exposure have been implicated to be environmental sources of oxidative stress. Oxidative stress not only induces T-cell dysfunction and propagation of oxidative modification of self-antigens leading to systemic inflammation, but it also damages various organ systems resulting in renal, cardiovascular, and cutaneous disease/comorbidities in SLE (62–64).

Currently, there are no biomarkers of oxidative stress in routine clinical use. Potential biomarkers that have been correlated with disease activity in established SLE patients include increased modification of serum albumin (65), urinary levels of F2 isoprostane (66), and serum nitric oxide levels (67). Future studies are still needed to determine if these biomarkers and others can help diagnose pre-symptomatic disease. Potential antioxidant therapies for SLE include N-acetylcysteine and rapamycin, but their role in preclinical disease is unclear (68, 69). On the other hand, dietary intake of antioxidant vitamins (vitamins A, C, and E and α-carotene, β-carotene, cryptoxanthin, lycopene, lutein, or zeaxanthin) has not been found to decrease SLE risk in epidemiologic studies (70, 71).

2.2.2 Break in Immunological Tolerance

Loss of self-tolerance occurs in SLE when autoantibodies target nuclear self-antigens that are released into the extracellular space and exposed to the immune system [reviewed in (72)]. Abnormalities in apoptosis, NETosis, and histone modifications are thought to be involved in this process. Apoptosis is an important source of autoantigens in SLE and it has been shown that many of the nuclear autoantigens (e.g., DNA, Ro, La, and small nuclear RNP) that are targeted in SLE are clustered in blebs at the surface of apoptotic cells where oxidative modification can occur (63, 73). NETosis is a specialized form of neutrophil cell death that has also been implicated as another potential source of autoantigens (74). During NETosis, structures termed neutrophil extracellular traps (NETs) are extruded by neutrophils to entrap and dismantle bacteria, viruses, fungi, and parasites. These NETs include fibrillary networks of DNA, citrullinated histones, and granule peptides such as cathepsin G, neutrophil elastase, and myeloperoxidase. In SLE, apoptosis and NETosis are increased, resulting in an excess load of nuclear autoantibodies (72, 74).

However, these on their own are unlikely to break immunological tolerance as several studies were not able to induce immune activation by immunizing mice with apoptotic cells/blebs or NETs (75, 76). A deficiency in clearance of apoptotic cells and/or NETs due to intrinsic phagocyte defects and absent/deficient serum factors are thought to lead to an enduring exposure of modified proteins such as histones in the immune system (77). These modified proteins are regarded as neoantigens that are no longer perceived as endogenous and subsequently elicit an autoimmune response. It can also stimulate an inflammatory response through the activation of nucleic acid recognition receptors (e.g., members of the Toll-like receptor (TLR) family), which are important in viral and bacterial defense and associated with type I IFN production (discussed in *2.2.4 Cytokines/Chemokines*). Improving the clearance of apoptotic cells and/or NETs may therefore be potential therapeutic targets for SLE or SLE prevention.

2.2.3 Autoantibodies

In addition to apoptotic cells and NETs, other important sources of autoantigens include neoantigens generated from necrotic cells under the influence of processes like oxidation and cleavage and infectious agents (e.g., single-stranded RNA, double-stranded RNA, and DNA). Autoantibodies and cytokines are produced by B lymphocytes that process and present these antigens. Autoantibodies can form immune complexes with their antigen, which can lead to organ damage through immune complex deposition and local and systemic inflammation. In a positive feedback loop, autoantibodies can then induce NETosis, and immune complexes can stimulate plasmacytoid dendritic cells to produce pro-inflammatory cytokines including IFN-α which can incite further NETosis. In SLE, intrinsic abnormalities of B-cell and T-cell interaction

also contributes to the production of autoantibodies [reviewed in (78)]. In SLE, these cells are hyperresponsive to stimuli resulting in the production of higher quantities of autoantibodies and cytokines. Furthermore, defects in immune tolerance permit the survival of dangerous autoreactive B cells that lead to further production and diversification of harmful autoantibodies in a process called epitope spreading (79, 80). Early in the disease course, an antibody response might begin with a particular epitope, and this is then later followed by a spread of the response to other epitopes in the same polypeptide (intramolecular) and in other distinct but structural similar molecules (intermolecular) (81). In **Table 1**, we summarize common SLE autoantibodies, their clinical associations, and onset prior to the diagnosis of SLE (82–85).

SLE is thus a paradigmatic autoimmune disease, with formation and detection of a wide range of autoantibodies, some of which are more SLE-specific and more pathologic than others. Autoantibody detection has long been a valuable and effective approach to the diagnosis, classification and prognostication with a wide range of established systemic autoimmune rheumatic diseases (SARD), including SLE (87). However, the exact contribution of autoantibody testing to the identification of subclinical and very early SLE is still to be determined. In a seminal study by Arbuckle et al. (83), a serum biobank and database established by the American military was queried and SLE-related autoantibodies were found in stored blood up to 9.4 years (mean 3.3 years) before the onset of SLE symptoms and eventual diagnosis. Other studies have confirmed similar findings (84, 88–92). Anti-SSA/Ro60 antibodies typically appeared first (83, 91, 92). Anti-SSB/La and anti-phospholipid antibodies have been reported to appear next (83). IgG and/or IgM anti-cardiolipin antibodies were detected in 18.5% of patients with mean onset of 3.0 years prior to the diagnosis of SLE and up to 7.6 years before SLE diagnosis (93). Anti-dsDNA anti-Sm, and anti-RNP antibodies (mean 3.4 vs. 1.2 years; p=0.005) appear later (83, 91, 92). Other studies have also demonstrated that anti-dsDNA and anti-Sm antibodies in non-SLE or early undifferentiated connective tissue disease patients are predictive of SLE evolution (88, 94, 95). A positive ANA test, a test used to screen for the presence of autoantibodies, has been reported to appear up to 9.2 years (mean 2.25 years) prior to SLE diagnosis or classification. As SLE progressed before and after diagnosis or classification, new autoantibodies steadily accumulated, consistent with other literature supporting increased epitope spread over time (85, 92, 96, 97).

The *absence* of specific autoantibodies in SLE or the presence of others may also help to identify those who are at *lower risk* of progression to SLE. ANAs are non-specific and found in up to 20% of healthy subjects, and are more common in females, with increasing age, and in the setting of infection, lung, and autoimmune thyroid disease (98–100). Anti-dense fine speckled 70 (DFS70) antibodies may be a useful biomarker to *rule out* the diagnosis of SLE as they are rarely found in SLE patients. In an international study of 1137 patients with SLE followed from inception in the Systemic Lupus International Collaborating Clinics (SLICC) cohort, only 1.1% had monospecific (no other detectable autoantibodies) anti-DFS70 antibodies (101). Thus, the presence of anti-DFS70 antibodies may help to discriminate between those who are ANA-positive healthy subjects versus those with SLE. Anti-C1q autoantibodies, which are associated with lupus nephritis (102), were infrequently found in patients with incomplete SLE in a small cross-sectional study of 70 patients (86). The authors suggest that although it remains undetermined whether this autoantibody could be a predictor of SLE risk, the presence of an elevated anti-C1q antibody in a patient with incomplete SLE might raise concerns for SLE or more specifically, lupus nephritis (86).

One of the challenges of identifying novel predictive autoantibodies for SLE development is that although over 200 different autoantibodies have been described in SLE, only 10% have been made widely available as diagnostic assays approved by regulatory authorities; most are still for research purposes only (10). Furthermore, most studies of these novel autoantibodies in SLE have been small and cross-sectional in design, without consideration of hallmarks of early disease or variable longitudinal disease course and outcomes, even though autoantibody test results may vary over time. The parameters associated with this longitudinal variation, such as the impact of medical therapies on antibody responses, also have not been well studied.

There has been a call for future exploration of novel autoantibody biomarkers given the non-specificity of ANA for SLE (11, 12). Investigators at the University of Toronto examined approximately 200 ANA-positive patients without established SARD, using a custom antigen microarray of 144 established and novel autoantibodies (85). They found that the majority of patients who tested negative for most current commercially available autoantibodies were positive for autoantibodies on their custom microarray. Anti-Ro52/Tripartite motif containing-21 (TRIM21) autoantibodies were predictive of SARD progression over the next two years (defined by the 1997 ACR criteria for SLE (103), 2013 ACR-EULAR criteria for systemic sclerosis (104) or 2016 ACR–EULAR criteria for Sjögren's syndrome (105)), with positive predictive value of 46% and negative predictive value of 89%. To close the 'seronegative gap', more studies of novel disease-specific autoantibody biomarkers are needed and will help to identify valid predictors of disease evolution, potentially enabling identification and treatment of patients with SLE in these early stages (10).

2.2.4 Cytokines/Chemokines

Increased IFN-α activity is an important contributor to SLE pathogenesis because of its involvement in the induction of B-lymphocyte stimulator (BLyS) and DNA- and RNA- protein binding autoantibody specificities. BLyS plays a key role in regulating B cell survival and differentiation, which is central to autoantibody production and class switching. Drugs blocking BLyS activity (belimumab), and more recently, the type I IFN receptor subunit 1 (anifrolumab), have reduced disease activity in patients with SLE in large clinical trials and are now approved therapies for SLE treatment (14, 16).

TABLE 1 | SLE autoantibodies, clinical significance, and time to SLE onset.

Antibody Target	SLE Clinical Significance	Time to SLE Onset[1]
SSA/Ro60	• Subacute cutaneous SLE • Lymphopenia • Neonatal lupus • In pediatric SLE, milder disease (cutaneous, musculoskeletal) • Protective with SSB/La (less renal and neurologic disease)	Up to 8.1-9.4 years (mean 2.3-2.97 years)
SSB/La	• Subacute cutaneous SLE • Neonatal lupus • Leukopenia • Serositis • Protective with SSA/Ro60 (less renal and neurologic disease)	Up to 7.0-8.1 years (mean 0.6-2.83 years)
Cardiolipin	• Part of classification criteria • Antiphospholipid syndrome • Pulmonary hypertension • Decreased survival	Up to 7.6 years (mean 2.29 years)
dsDNA	• Part of classification criteria • Lupus nephritis • Disease activity • Pathogenic	Up to 6.6-9.3 years (mean 1.24-2.0 years)
U1-RNP	• Leukopenia • Neuropsychiatric SLE • Raynaud's • Musculoskeletal involvement • Lung involvement	Up to 7.2-7.5 years (mean 0.20-1.2 years)
Histone	• Drug-induced SLE • Neuropsychiatric SLE • Pathogenic	Up to 6.5 years (mean 1.9 years)
Sm (U2-U6 RNP)	• Part of classification criteria • Serositis • Lupus nephritis • Neuropsychiatric SLE	Up to 1.1-8.1 years (mean 0.47 years)
Ro52/TRIM21	• Hematologic involvement with SSA/Ro60 • Neonatal lupus • More severe disease (renal)	Predictive of progression to SLE in patients followed over two years
C1q	• Lupus nephritis • Hypocomplementemic urticarial vasculitis with or without SLE	Detected in incomplete SLE patients but infrequently, timing unknown
β2GP1	• Part of classification criteria • Antiphospholipid syndrome • Pathogenic	Unknown
β2GP1 domain 1	• Antiphospholipid syndrome	Unknown
High Mobility Group Proteins	• Disease activity	Unknown
Ku	• Raynaud's • Myositis • Arthritis	Unknown
Nucleosomes and Chromatin	• Lupus nephritis with more severe renal failure • Disease activity • Pathogenic	Unknown
PCNA	• Lupus nephritis • Neuropsychiatric SLE • Thrombocytopenia	Unknown
PS/PT	• Antiphospholipid syndrome	Unknown
Ribosomal P	• Lupus nephritis • Neuropsychiatric SLE • Lupus hepatitis • Disease activity	Unknown

1. Based on Arbuckle et al. (83), Eriksson et al. (84), Munoz-Grajales et al. (85), and Olsen et al. (86).

2. β2GP1, beta 2 glycoprotein 1; dsDNA, double-stranded DNA; PCNA, proliferating cell nuclear antigen; PS/PT, Phosphatidylserine/Prothrombin; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; TRIM21, Tripartite motif containing-21.

In a case-control study by Munroe et al. of SLE patients and matched healthy controls, serum collected prior to and at/after SLE classification were analyzed (92). Prior to SLE classification (average timespan of 4.3 years), upregulation of IFN-associated mediators, as observed with autoantibodies, accumulated over a period of years, and then plateaued close to the time of disease classification ($p<0.001$). The most important predictor of increased IFN-α activity was the number of positive autoantibodies ($p<0.001$). Increased circulating IFN-α activity and BLyS levels were also detected shortly before subjects met SLE classification criteria ($p\leq0.005$), suggesting that this may be a turning point in SLE pathogenesis where immune dysregulation is amplified by positive feed-forward mechanisms. Other studies have also showed that early SLE patients have exacerbated type I IFN signatures, their autoantibodies specificities have already class-switched to IgG isotypes (106), and autoantibody containing immune complexes drive type I IFN activation (107–110).

Although IFN-α activity may be an important contributor to SLE progression, not all SLE patients (only ~25%) have increased IFN-α activity preceding SLE diagnosis or classification (92). Hence, other forms of immune dysregulation likely accompany IFN-α activity, such as type II IFN (IFN-γ). IFN-γ is important in mediating the crosstalk between innate cells and lymphocytes, breaking self-tolerance and enabling the activation and persistence of autoreactive B cells (111). It modulates TLR regulation to facilitate autoantibody production, antigen presentation, and recruitment of lymphocytes to germinal centers (111). It can also drive the production of IFN-α and BLyS levels, leading to inflammation, B cell activation and autoantibody production. Munroe et al. further found increased levels of circulating IFN-γ in pre-clinical SLE patients prior to detectable upregulation of IFN-α and autoantibody positivity, as well as dysregulation of the chemokines IP-10 (CXCL10) and MCP-3 (CCL7) (92). Other mediators that have been implicated in SLE pathogenesis and are elevated years before SLE classification include IL (interleukin)-12p70, MIG, IL-4, IL-5, and IL-6 (91). These chemokines, which aid in the recruitment of cells to sites of inflammation, may also be important biomarkers in early pathogenesis of SLE.

2.2.5 Complement Activation

Complement activation is responsible for much of the systemic inflammation and tissue damage in SLE [reviewed (112)]. All three pathways of complement activation are involved in SLE, with the classical pathway, activated by antigen-antibody complexes, being the most important in SLE pathogenesis. Low complement C3, C4 and CH50, levels are diagnostic and disease activity biomarkers in SLE (113). However, they are not always reliable as they are influenced by the acute phase response, individual differences in complement gene copy number and expression, and variability in protein catabolism and synthesis (114).

To overcome the limitations of measuring C3 and C4, assays to measure cell-bound activation (split) products (CB-CAPS), such as erythrocyte-bound C4d (EC4d) and B lymphocyte-bound C4d (BC4d), have recently been developed. These are formed upon activation of the complement cascade and reflect complement activation rather than the levels of the individual protein. These are measured using EDTA anti-coagulated blood by flow cytometry which can be labor intensive, but on the other hand, sample processing is usually minimal, no centrifugation is needed, and it does not require low temperature for storage and transportation.

CB-CAPS are promising SLE biomarkers, shown to be more sensitive than C3, C4, and anti-dsDNA for the SLE diagnosis (115, 116), and more prevalent in patients with probable SLE. When used in combination with a proprietary panel of other autoantibodies, one study reported these biomarkers were able to identify patients with a greater than three-fold increased risk of developing SLE and were slightly better than complements or anti-dsDNA alone at predicting transition to SLE among patients with undifferentiated connective tissue disease [reviewed in (117, 118)]. These results suggest that complement activation may also occur early in the evolution of SLE and be an important feature in patients with suspected SLE.

2.2.6 Lifestyle and Environmental Risk Factors Related to SLE Risk (With a Focus on Those That Are Potentially Modifiable)

The number of factors beyond age, race, sex, family history, and genetics that are strongly associated with risk of developing SLE has been growing in recent years. Multiple large cohort studies have contributed to our understanding of how lifestyle, behavioral, psychosocial, and environmental risk factors may converge and synergize with underlying genetic risk. This likely leads to an acceleration of underlying and brewing autoimmunity, allowing it to manifest in SLE. These factors include current cigarette smoking, obesity (in particular, at younger ages), childhood and adult trauma, stress, post-traumatic stress disorder, low or no alcohol intake, environmental air pollution, environmental silica, and hormonal exposures and reproductive factors among women [reviewed in (58, 119)]. While is it not known whether these environmental risk factors work *via* similar or disparate biologic pathways, nor whether they are perhaps also inextricably linked to other societal risk factors that are more difficult to measure, the picture of how and the extent to which they contribute to SLE susceptibility is coming into focus. Gene-environment interactions likely contribute to SLE risk, and only a handful of these specific interactions have been discovered to date (54, 57).

In a recent, large, prospective evaluation of healthy lifestyle behaviors and SLE risk using the Nurses' Health Study (NHS) and NHSII, adherence to multiple healthy behaviors (healthy diet (highest 40th percentile of the Alternative Healthy Eating Index), regular exercise (performing at least 19 metabolic equivalent hours of exercise per week), never smoker or past smoker, moderate alcohol consumption [drinking ≥5 gm/day alcohol), and maintaining a healthy body weight (body mass index <25 kg/m^2)] was associated with a lower risk of SLE development overall (120). There was a 19% reduction for each additional healthy behavior and an even greater reduction (22%) was observed for the risk of dsDNA positive SLE. Strikingly, the risk of SLE was *half as high* among those with the best adherence

to healthy lifestyle behaviors compared to among those with the poorest adherence. Overall, the population attributable risk, or the proportion of the risk in this population that could be attributed to these five modifiable lifestyle risk factors was 47.7% [95% confidence interval (CI) 23.1-66.6%]. These results suggest that lifestyle behaviors likely work synergistically to influence the risk of SLE and potentially produce stronger effects together than individually *via* common biological pathways including production of autoantibodies and dysregulation of pro-inflammatory cytokines. Moreover, although much work remains to be done in disentangling the specific pathways by which these environmental risk factors may be related to SLE pathogenesis, this also suggest that much of SLE may be preventable with lifestyle change, a somewhat revolutionary concept.

Many potential biologic mechanisms and synergies are possible. For example, exposure to obesity and toxic components of cigarette smoke both cause oxidative stress (121). This, in turn, increases intracellular levels of reactive oxygen species to damage DNA forming immunogenic DNA adducts, thereby promoting dsDNA antibody production (**section 2.2.3**) (122–124). In the NHS and NHSII cohorts, cigarette smoking was associated with a higher risk of anti-dsDNA positive SLE than never smokers [hazard ratio 1.86 (95% CI 1.14-13.04)] (125), a finding confirmed in other studies (126, 127). In addition to causing oxidative stress (*section 2.2.1*), the by-products of smoking could also augment autoreactive B cells in the native repertoire (126) and induce pulmonary ANA in the lungs of exposed mice (128). Alcohol consumption, on the other hand, contains several compounds such as ethanol and antioxidants, that can potentially counteract the changes induced by smoking and obesity including inhibiting key enzymes in DNA synthesis (129, 130). Moderate alcohol intake (≥5 gm/day or >0.5 drinks/day) was associated with a decreased risk of incident SLE in NHS and other studies [hazard ratio 0.61 (95%CI 0.41-0.89)] (131).

Although the association between SLE risk and various diets is less clear in humans (132–134), murine models have demonstrated that low dietary fiber intake and Western-type diet (i.e., high in sugar, fat, refined grains, and red meat) were associated with increased autoantibody production in SLE-prone mice (135, 136). A murine study also demonstrated that in mice genetically susceptible to SLE, sleep deprivation was associated with an earlier onset of disease and accelerated production of autoantibodies (137). Among women followed in the Black Women's Health study, a diet high in carbohydrates was associated with increased risk of developing SLE (132). The association between lack of sleep (less than the recommended 7 hours a night) and SLE risk in humans has been reported in several studies (138, 139). In a prospective study of 436 non-SLE relatives of SLE patients, relatives were more likely to transition to SLE if they reported sleeping less than seven hours a day [odds ratio 2.8 (95%CI 1.6-5.1)] (138).

Many lifestyle factors associated with SLE development increase levels of pro-inflammatory cytokines (*section 2.2.4*). Smoking increases BLyS expression (128), Tumor necrosis factor alpha (TNF-α), and IL-6 (140, 141). Among positive ANA women, elevated BLyS and lower IL-10 (an anti-inflammatory cytokine) levels could be found among current smokers (142). Both TNF-α and IL-6 also play important roles in the modulation of insulin resistance (121). Adipose tissue, in particular visceral fat, secretes pro-inflammatory adipocyte-derived cytokines and exhibit higher levels of C-reactive protein (CRP), TNF-α receptor 2, and IL-6 than non-obese individuals (143). Alcohol, on the other hand, suppresses TNF-α, IL-6, IL-8, and IFN-γ to counteract systemic inflammation (129, 130). In sleep-deprived individuals, increased levels of IL-6, TNF-α have been observed in addition to its role in impairing the function of T cells and CD4 regulatory T cells, which are important in self-tolerance (**section 2.2.2**) (144–148). Sleep disturbances in individuals who have had childhood or adult trauma, post-traumatic stress disorder or occupational stress from working nightshifts or rotating shifts, may also explain why these factors have also been linked to SLE onset (149–155). Systemic inflammation with elevated TNF, IL-6 and CRP levels is also found in these conditions (150, 156–164).

Other environmental and occupational related risk factors, including chemical and physical exposures, have also been linked to SLE onset and mechanisms involving stimulation of cellular necrosis and relate to intracellular antigens with resulting inflammation and IFN upregulation. These exposures include crystalline silica dust (165–168), air pollution and other respiratory particulates (169, 170), heavy metals such as mercury (149), and agricultural pesticides (149, 171, 172). UV radiation is also thought to trigger SLE onset, and it has been shown in SLE patients and lupus-prone mice, that there is a rise in type I IFN signaling and expansion and prolonged activation of T cells following UVB exposure (173–175). The association of UV radiation and SLE risk however is likely complicated by its role in vitamin D3 synthesis in the skin, which has been hypothesized to *reduce* SLE risk (176). A more detailed discussion about vitamin D and its role in preventing SLE is found in **section 3.**

Use of exogenous hormones, oral contraceptive pills, and hormone replacement therapy have been associated with risk of SLE (177–179). Among recent oral contraceptive pill users, a dose response between oral contraceptive pill dose of ethinyl estradiol and SLE risk has been demonstrated (178). Estrogen is thought to induce autoreactivity by upregulating several genes involved in B cell activation and survival (*cd22*, *shp-1*, *bcl-2*, and *vcam-1*) and preventing B cell receptor-mediated apoptosis (180).

The association between infection and SLE is the Epstein-Barr virus (EBV) has been of interest for many years. The data on whether prior EBV infection is a risk factor for SLE development are still unclear [reviewed in (181)]. The release of EBV-encoded small RNA from infected cells is thought to induce type 1 interferon and proinflammatory cytokines *via* activating TLR-3 signaling (182). Another potential mechanism is through molecular mimicry between EBV and SLE antigens and epitope spreading. In a systematic review and meta-analysis of 25 case-control studies, a higher seroprevalence of anti-viral

capsid antigen IgG [odds ratio 2.08 (95%CI 1.15-3.76)] and anti-early antigen antibody, a marker of viral replication, was observed in patients with *existing* SLE compared to health or nonhealthy controls [odds ratio 4.5 (95%CI 3.00-11.06)] (183). However, the results should be interpreted with caution given there was publication bias regarding recruitment, matching and reporting of blinded laboratory analysis and these studies do not address whether EBV is causally related to SLE. On the other hand, in a Danish population-based study, it was the EBV-serologic *negative* individuals that had an increased risk for SLE, particularly one to four years after serologic testing [standardized incidence rate 6.6 (95%CI 3.3–13.2)] (184). This may reflect surveillance bias as those patients who go on to develop SLE may have had EBV testing as part of their workup for early SLE symptoms. More recently, there are data to suggest that EBV reactivation is associated with SLE disease onset. In a prospective study of unaffected relatives of SLE patients (n=436), SLE relatives who transitioned to classifiable SLE had increased levels of EBV IgG antibodies prior to SLE transition compared to relatives who did not transition (185). Furthermore, increasing levels of EBV antibodies were associated with SLE disease transitioning, particularly among those with variants in genes that are associated with SLE and implicated in EBV infection.

The association between vaccinations and SLE risk remains to be elucidated, but thus far, epidemiological studies in SLE suggest that there is no association (186). It is thought that vaccines could potentially trigger autoimmunity through molecular mimicry, autoantibodies, and response to adjuvants in the vaccine. There have been emerging reports of new-onset autoimmune diseases including rheumatoid arthritis (187), immune thrombotic thrombocytopenia (188), autoimmune liver disease (189), IgA nephropathy (190), and Guillain-Barré Syndrome [reviewed in (191)] after vaccination. However, the evidence is from mainly case reports or cross-sectional studies demonstrating a temporal association. There have also been a few case reports of SLE and lupus nephritis 1-2 weeks following COVID-19 vaccination (192–194). Without more substantive evidence, however, individuals should be encouraged to get vaccinated as it remains one of the most effective interventions to prevent COVID-19 infection and related morbidity and mortality.

2.3 Early or Preclinical SLE

During the next phase of SLE pathogenesis, still pre-diagnosis, individuals may start to develop early non-specific symptoms of SLE, but not yet enough to be diagnosed or classified with the disease (12, 103). These patients are sometimes referred to as incomplete lupus or undifferentiated connective tissue disease (195). Eventually, some people with early and non-specific breakdown of immune tolerance and signs and symptoms of systemic inflammation and autoimmunity will develop more disease features and organ damage and diagnosed or classified as SLE. The duration of this early phase is highly variable from individual to individual. Some may have smoldering disease onset over years, while others experience a rapidly explosive onset of SLE with multiple simultaneous and severe clinical manifestations and autoantibodies. The rapidity of SLE onset likely relates to the specific combination of genetic and environmental SLE risk factors and their interactions, and has been shown to vary by racial ancestry (196). Depending on the cohort and setting, it has been reported that up to half of undifferentiated SARD patients with very early connective tissue disease evolve to fulfill diagnostic and classification criteria of a SARD, including SLE (197). Identifying those at high risk of developing SLE, or in early phases of its development, would enable a "window of opportunity" whereby interventions could be targeted at intercepting disease and halting or slowing the progression to SLE (87).

3 DISCUSSION: PROPOSAL OF A CLINICAL CARE PATHWAY TO SCREEN AND PREVENT SLE

Even before patients are diagnosed with SLE, some may suffer irreversible organ damage, including pulmonary arterial hypertension, cardiovascular disease, renal, and neurological damage (198). Studies have also demonstrated that prior to being diagnosed by an astute clinician or meeting formal classification criteria for SLE, patients are already at higher risk of hospitalizations and lupus-related complications (199, 200). If these patients who are developing SLE could be identified at an early stage, decision-making regarding preventative strategies and therapeutic interventions could be improved.

An appropriate screening and prevention program for SLE has great potential to improve public health outcomes. When organized effectively, it would be targeted to identifying those at risk for SLE to prevent disease development, reduce disability, and cut mortality through early detection and treatment. This will be challenging however, given that SLE is a rare disease in the general population. Here we proposed a clinical care pathway for the screening and prevention of SLE (**Figure 2**) involving four different levels that start with targeting patients who are at genetic risk, the asymptomatic autoimmunity stage, pre-clinical, and finally clinical disease states as discussed in the section above.

3.1 Risk Assessment and Early Detection

Currently, there is no consensus concerning how to identify individuals at high risk for SLE or at what preclinical phase of disease should a patient be referred to see a rheumatologist. Given that SLE is a relatively rare disease with an incidence of about 1/2000 in the general population, most hypothetical screening programs would have to rely on inexpensive, readily available, and accurate tests (201). Population studies have used a 30-item questionnaire that can be completed within 30 minutes called the Connective Tissue Disease Screening Questionnaire (CSQ) to screen populations for SLE and other connective tissue diseases (202). It has high sensitivity for SLE (96%, 95%CI 90-99%) but moderate specificity (86%, 95%CI 81-91%) and has been validated among African American women (203). It is best employed in a two-stage screening method followed by medical record review or in-person assessment and should not be used as

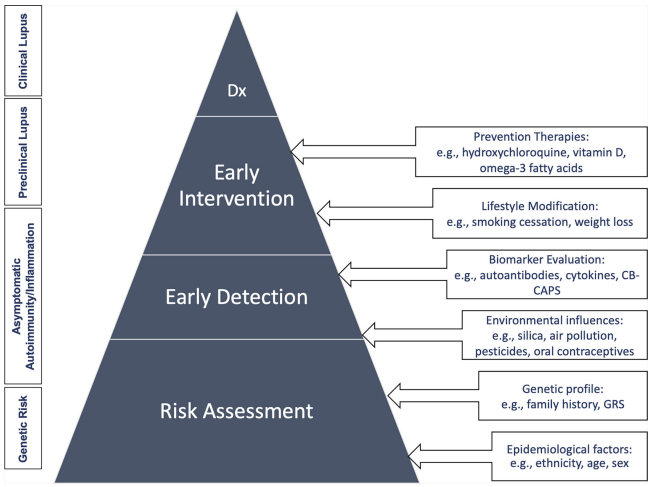

FIGURE 2 | Clinical care pathway for the screening and prevention of SLE. Dx, diagnosis; GRS, genetic risk score.

a test on its own due to the high false-positive rate. The ANA is the biomarker that we utilize today to "screen" for autoimmune connective tissue diseases, including SLE (204–206). However, as the ANA test is most usually performed when patients already have symptoms, it is not really a population-based screening test. As some patients may already have organ damage, ideally, those patients should be caught earlier in the asymptomatic autoimmunity and early preclinical phases, *prior* to clinical signs and symptoms. In this review, we highlighted numerous biomarkers that have shown promise in the identification of at-risk patients that could be detected in these earlier phases (**section 2**). These included autoantibodies such as ANA and anti-SSA/Ro60, genetic susceptibility loci, and upregulated cytokines/chemokines that coincide with timing of the initial appearance of autoantibodies, as well as markers of complement activation.

While some of these tests are readily available and accessible, there are several questions related to their use for screening purposes that need to be clarified. To better understand what makes a screening program appropriate, there are ten principles laid out by the 1968 World Health Organization that prompt important discussion about the benefits, harm, costs and ethics of a screening and prevention programs (207). If a program for SLE were implemented today, it would likely satisfy many of the criteria such as 1) "the condition should be an important health problem"; 2) "there should be an accepted treatment for patients with recognized disease"; 3) "facilities for diagnosis and treatment should be available". However, there is still uncertainty surrounding some of the other criteria. Specifically related to testing, for instance, it is unclear if a biomarker test or panel were administered to screen for SLE in the general population that, "the cost of case-finding (including a diagnosis and treatment of patients diagnosed) [would] be economically balanced in relation to possible expenditure on medical care as a whole." We have yet to determine the population that should be targeted for screening. However, it may be reasonable to narrow the screening eligibility criteria, based on the evidence from epidemiological studies to individuals from high-risk populations.

Preliminary data using the NHS and NHSII cohorts demonstrate that a weighted GRS in combination with lifestyle and environmental risk factors predicted future SLE risk with a good area under the curve of 0.77 (208). Therefore, using a GRS in combination with other risk factors assessment may be a valuable tool that may feasibly be employed in at-risk populations for predicting disease (**Table 2**). Once these patients have been identified, they could then be referred and potentially enrolled in prevention trials (discussed in *3.2.2 Preventative Therapies*). Other prevention efforts targeting individuals at high genetic risk for lifestyle modification type of prevention trials could also be envisioned.

3.2 Early Intervention

3.2.1 Lifestyle Modification

We discussed several modifiable risk factors that health care providers should encourage their patients who may be at risk for SLE to address, including smoking cessation, moderate alcohol consumption, regular exercise, avoidance of certain occupational and environmental exposures, medications, and maintaining a healthy weight and good sleep hygiene. The cost-effectiveness of adopting a healthy lifestyle is clear in that it is not only the risk of SLE that would be reduced, but that of many other chronic and complex diseases. To test the effectiveness of lifestyle interventions in actually reducing SLE risk, a primary prevention clinical trial would be necessary, but would be very challenging.

It is important to recognize that while the evidence suggests providers should encourage patients to adhere to as many healthy behaviors as possible for the greatest reduction in SLE and other chronic disease risk, there are many structural and

TABLE 2 | SLE risk stratification chart.

Types of Risk Factors: Epidemiological, immune biomarkers, lifestyle and environmental	Genetic Risk				
	Low Risk -No high-risk alleles -Low GRS -No family history		↔	High Risk -Multiple high-risk alleles -High GRS -Positive family history	
No risk factors	Low Risk	Low Risk	Moderate Risk	High Risk	Very High Risk
1-2 types of risk factors	Low Risk	Low Risk	Moderate Risk	High Risk	Very High Risk
All 3 types of types of risk factors present	Moderate Risk	Moderate Risk	Moderate Risk	High Risk	Very High Risk
All 3 types of types of risk factors present with 1-2 SLE features	High Risk	High Risk	High Risk	High Risk	Very High Risk
3 or more types of risk factors with multiple SLE features but not enough to meet classifiable disease	Very High Risk	Very High Risk	Very High Risk	Very High Risk	Very High Risk

GRS, genetic risk score; SLE, systemic lupus erythematosus.

institutional factors that affect an individual's ability to adhere or achieve a healthy lifestyle. These include poverty, pollution, toxins, stress, and institutional and structural racism, among others, which have disproportionately affected non-White groups in the United States, who are also the same groups with the highest incidence and severity of SLE. Future studies should examine how to improve adherence to lifestyle interventions and address barriers that prevent or limit ability to meet healthy goals, especially among sociodemographic groups that are medically vulnerable.

3.2.2 Preventative Therapies

The first prevention trial in SLE is the Study of Anti-Malarials in Incomplete Lupus Erythematosus (SMILE), a multi-center, randomized, double-blind, placebo-controlled trial of HCQ compared to placebo, a 24-month clinical study (209). The purpose of this trial is to evaluate the efficacy and safety of HCQ intervention to prevent future onset of clinically apparent SLE. The inclusion criteria are patients 15-49 years of age with a positive ANA and at least one (but not three or more) additional clinical or laboratory criterion from the 2012 SLICC classification criteria (210). This study is expected to be completed in 2023. This study was initiated after James et al. demonstrated in a retrospective study on 130 United States military personnel that individuals who were treated with HCQ prior to SLE diagnosis had delayed the onset of complete SLE compared to untreated patients (median: 1.08 years versus 0.29 years) (18). Furthermore, individuals who had received HCQ in that study had slower accumulation of new autoantibodies. Other small studies showed that patients with incomplete SLE or new-onset, mild SLE treated with HCQ had lower levels of IFN-inducible genes, serum BLyS levels (also known as B cell–activating factor or BAFF), anti-C1q antibodies, IL-9, and better self-reported health status scores (86, 211). These results support the hypothesis that HCQ could influence SLE disease progression. Therefore, the SMILE trial will not only inform clinicians as to whether HCQ can be used to prevent SLE, but it will be the first step towards testing feasibility of disease prevention studies in SLE.

Recently, the results of a large (25,871 participants) randomized, double-blind, placebo-controlled, two-by-two factorial design trial examined the impact of vitamin D (cholecalciferol; 2000 IU/day) and marine omega 3 fatty acids (1 g/day as a fish oil capsule containing 460 mg of eicosapentaenoic acid and 380 mg of docosahexaenoic acid) on the incidence of various autoimmune diseases (212). The investigators found a reduction in autoimmune disease by 22% with vitamin D supplementation for five years, with or without omega 3 fatty acids, reduction by 15% with omega-3 fatty acid supplementation with or without vitamin D (not statistically significant). While there were too few new cases of SLE to be examined in this older population (men age 50 and older and women age 55 and older), vitamin D deficiency is common in SLE (213) and is important for regulating numerous genes involved in inflammation and the immune system through IL-2 inhibition, antibody production, and proliferation of lymphocytes (214, 215). Additionally, prior small cohort studies in SLE on specialized pro-resolving mediators (SPMs), a family of omega-3 fatty acid-derived lipid mediators, suggest that specific SPMs, such as the resolvins and lipoxins, may counter-regulate the production of inflammatory mediators and promote resolution of inflammation (216, 217). Further studies to examine whether omega-3 fatty acid supplementation can affect SPM levels and thereby forestall the development of SLE in at-risk populations will be needed.

Another potential therapy to decrease SLE risk that has been proposed is melatonin. Disrupted melatonin production in nightshift workers has been proposed as an important mechanism of increasing risk for autoimmune diseases including SLE [reviewed in (218)]. In lupus-prone mice, abnormal circadian rhythm of melatonin levels in response to light/dark cycle has been observed (219). When melatonin was administered to lupus-prone mice, there was decreased levels of autoantibodies, inflammatory cytokines, reduce renal injury, and increased levels of anti-inflammatory cytokine IL-10 (220, 221), particularly for females. Further studies in humans are called for to investigate the mechanism by which melatonin may be related to SLE risk and whether it could be a potential therapeutic strategy.

It is important to recognize that there are significant barriers to conducting prevention trials in SLE. A major challenge faced by past SLE prevention trials is low patient recruitment and retention. A lack of enthusiasm among clinicians and patients due to risk aversiveness and misunderstanding or misinterpretation of the purpose of prevention trials have resulted in underenrollment and selective enrollment, poor adherence, and attrition in some studies (222–224). Whereas good health status, encouragement from one's physicians, desire to learn and contribute to research are positive factors for participation in SLE prevention trials (225). Therefore, future prevention trials in SLE should employ strategies such as health education about the clinical problem and importance of the trial, and involving the patients personal physicians to improve recruitment of SLE patients into prevention trials (225).

4 CONCLUSION

Developing a deeper understanding of SLE pathogenesis, its preclinical stages, and risk factors, will ultimately enable effective screening and potentially prevention. This may appear to be a daunting task; however, tremendous progress has been made over the last few decades with greater insights into the etiopathogenesis of SLE, identification of novel biomarkers for early SLE detection, epidemiologic and genetic studies that have revealed important risk factors, and the first prevention trial in SLE is already underway. Well-designed prospective clinical studies to further elucidate the mechanisms of disease development and more clinical prevention trials are needed.

AUTHOR CONTRIBUTIONS

All authors have participated drafting the work or revising it critically for important intellectual content, final approval of the version published, agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved,

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Pre-Clinical Autoimmunity in Lupus Relatives: Self-Reported Questionnaires and Immune Dysregulation Distinguish Relatives Who Develop Incomplete or Classified Lupus From Clinically Unaffected Relatives and Unaffected, Unrelated Individuals

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Systemic lupus erythematosus (SLE) is propelled by pathogenic autoantibody (AutoAb) and immune pathway dysregulation. Identifying populations at risk of reaching classified SLE is essential to curtail inflammatory damage. Lupus blood relatives (Rel) have an increased risk of developing SLE. We tested factors to identify Rel at risk of developing incomplete lupus (ILE) or classified SLE vs. clinically unaffected Rel and healthy controls (HC), drawing from two unique, well characterized lupus cohorts, the lupus autoimmunity in relatives (LAUREL) follow-up cohort, consisting of Rel meeting <4 ACR criteria at baseline, and the Lupus Family Registry and Repository (LFRR), made up of SLE patients, lupus Rel, and HC. Medical record review determined ACR SLE classification criteria; study participants completed the SLE portion of the connective tissue disease questionnaire (SLE-CSQ), type 2 symptom questions, and provided samples for assessment of serum SLE-associated AutoAb specificities and 52 plasma immune mediators. Elevated SLE-CSQ scores were associated with type 2 symptoms, ACR scores, and serology in both cohorts. Fatigue at BL was associated with transition to classified SLE in the LAUREL cohort ($p \leq 0.01$). Increased levels of BLyS and decreased levels of IL-10 were associated with type 2 symptoms ($p < 0.05$). SLE-CSQ scores, ACR scores, and accumulated AutoAb specificities correlated with levels of multiple

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inflammatory immune mediators ($p<0.05$), including BLyS, IL-2Rα, stem cell factor (SCF), soluble TNF receptors, and Th-1 type mediators and chemokines. Transition to SLE was associated with increased levels of SCF ($p<0.05$). ILE Rel also had increased levels of TNF-α and IFN-γ, offset by increased levels of regulatory IL-10 and TGF-β ($p<0.05$). Clinically unaffected Rel (vs. HC) had higher SLE-CSQ scores ($p<0.001$), increased serology ($p<0.05$), and increased inflammatory mediator levels, offset by increased IL-10 and TGF-β ($p<0.01$). These findings suggest that Rel at highest risk of transitioning to classified SLE have increased inflammation coupled with decreased regulatory mediators. In contrast, clinically unaffected Rel and Rel with ILE demonstrate increased inflammation offset with increased immune regulation, intimating a window of opportunity for early intervention and enrollment in prevention trials.

Keywords: autoimmunity, systemic lupus erythematosus, autoantibodies, cytokines, pre-clinical disease, family studies, follow-up studies, risk assessment

1 INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifaceted autoimmune disease associated with chronic, underlying immune dysregulation. Altered immune pathways and the development of SLE-associated autoantibodies have been noted prior to the development of clinical disease, with continued expansion and accumulation as patients move toward disease classification (1, 2). Observed benefits of early intervention for patients at high risk of other autoimmune diseases such as type 1 diabetes mellitus (3) 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 (5–8). Fundamental to successful early intervention is the identification of preclinical factors that signal and differentiate disease transition from states of latent autoimmunity that may never progress. This may be particularly true for relatives of SLE patients, who have an increased risk of developing SLE compared to the general population (9, 10).

Autoantibody specificities alone are insufficient to identify relatives at highest risk of developing lupus (11), as other forms of immune dysregulation both preface and coincide with autoantibody production to give rise to clinical sequelae and SLE transition (1, 2). Type I IFN (IFN-α) genetic polymorphisms and activity are associated with SLE pathogenesis (12) in lupus relatives (13), with enhanced IFN activity particularly associated with DNA- and RNA-protein binding autoantibody specificities (14, 15). In addition to type I IFN, multiple genes that contribute to activation of type II IFN (IFN-γ) pathways are associated with SLE (16, 17), with IFN-γ being among the earliest dysregulated mediators noted in pre-clinical SLE (1, 2), promoting a chronic pro-inflammatory cascade contributing to SLE disease pathogenesis (18, 19). Furthermore, IFN-γ can drive both type I IFN (20) and B-lymphocyte stimulator (BLyS) production (21–27). Bridging innate and adaptive immunity, IFN-γ perpetuates Th1-type adaptive cellular responses, recruiting cells to sites of inflammation by stimulating the secretion of such chemokines as MCP-1 (CCL2), MCP-3 (CCL7), MIG (CXCL9), and IP-10 (CXCL10) (20, 28–30). Another consistently detected pro-inflammatory mediator detected as patients transition to SLE (1, 11) and a marker of impending lupus disease flare (18, 19) is stem cell factor (SCF), associated with hematopoiesis, T-cell differentiation, and chemokine release (31, 32). Other immunoregulatory mechanisms, including levels of circulating IL-10 and TGF-β, also appear to be altered in SLE disease pathogenesis (1, 11, 18, 19).

Although immune dysregulation is a key precipitating factor to clinical disease development, affected individuals may or may not be aware of the ongoing immunological imbalance. Despite their sometimes difficult discernment, patient-reported symptoms are being increasingly recognized as a valuable focus to bridge the patient-provider disconnect noted in SLE (33, 34). A number of "type 2" manifestations noted in SLE that are unclear in origin and have an uncertain connection to underlying inflammation (33, 35), particularly fatigue, but also anxiety, depression, cognitive dysfunction/headaches, and sleep disturbances, are reported by patients early in disease development (36, 37). In addition, the connective tissue disease screening questionnaire (CSQ) was developed as a patient-reported screening tool for various connective tissue diseases (CTD), including SLE (38). Although validated in the general population (39, 40), the SLE portion of the questionnaire (SLE-CSQ) is based on ACR classification criteria for SLE and has the potential for identification of lupus relatives who may remain clinically unaffected vs. being at increased risk of developing ILE or transitioning to classified SLE (11, 41).

A number of SLE inception cohorts have noted the presence of organ damage by the time patients reach disease classification (42–45), and such early damage is predictive of early mortality (42, 44). Identifying early SLE signs and symptoms coupled with markers of altered immunity may be beneficial to developing a screening strategy to identify lupus relatives who would most benefit from early intervention trials compared to those who may

remain in a state of latent autoimmunity without developing clinical disease. To this end, we assessed clinical, serologic, and immunological factors prior to and after SLE disease transition in two unique cohorts of lupus relatives: the lupus autoimmunity in relatives (LAUREL) follow-up cohort allowed for assessment before and after disease transition, and the lupus family registry and repository (LFRR) cohort, a confirmatory cohort assessed after the LAUREL cohort, consisting of patients with classified SLE and their blood relatives.

2 MATERIALS AND METHODS

2.1 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 (46–48). One subset of study participants were selected from the Lupus Autoimmunity in Relatives (LAUREL) follow-up cohort (11), with inclusion criteria consisting of lupus patient relatives meeting < 4 ACR SLE classification criteria (47, 48) at baseline (SLE relatives meeting ≥4 ACR criteria after medical record/serological assessment were excluded from the study) (46, 49). LAUREL cohort participants were recruited at their baseline time point from 1992-2011 and at their respective follow-up time point from 2009-2012 (**Figure S1**), an average of 6.4 years, to identify lupus relatives who transitioned to classified SLE (11). Select individuals in the LAUREL cohort were matched by sex, race, and age (± 5 years) to unaffected HC.

A confirmatory subset of study participants was selected from the Lupus Family Registry and Repository (LFRR) cohort (46), recruited from 1992-2008 (**Figure S1**), with inclusion criteria consisting of patients meeting American College of Rheumatology (ACR) classification for SLE (meeting ≥4 cumulative ACR criteria) (47, 48), relatives of SLE patients not reaching disease classification (meeting <4 ACR criteria), and unaffected healthy controls (HC). All study participants provided written informed consent along with demographic and clinical information, as well as serum and plasma samples at the time of enrollment in the LAUREL and LFRR cohorts; LAUREL cohort participants also provided serum and plasma samples at follow-up (11). Samples were stored at -20°C and assays performed on freshly thawed samples.

As outlined in the flow chart in **Figure S1**, for each nested cohort, information regarding cumulative clinical and laboratory features for each case was obtained by appropriately consented medical record review by a rheumatology-trained physician or nurse. Clinical manifestations evaluated in this protocol were determined according to criteria set by the ACR (47, 48). Stringent documentation requirements were used for review of the medical record. Each ACR criterion was recorded as being either present or absent. The date of occurrence and the presence or absence of each ACR criterion was recorded for each patient. In addition to ACR criteria, lupus relatives were assessed and scored with a modified version of the recently published SLE Risk Probability Index (mSLERPI) (50), including the following ACR criteria: malar rash, discoid rash, oral ulcers, arthritis, serositis, leukopenia, thrombocytopenia or hemolytic anemia, neurological disorder, proteinuria, ANA, and immunological disorder; alopecia, low C3 and C4, and interstitial lung disease were excluded due to insufficient data.

In addition to questionnaires to obtain demographic, education, socioeconomic, family pedigree, medical history, and medication data, participants completed the SLE-specific portion of the Connective Tissue Disease Screening Questionnaire (CSQ) (38, 40). The SLE portion of the CSQ (SLE-CSQ) was scored using an algorithm based on ACR classification criteria (38). The SLE-CSQ refers to nine criteria from the 1982 revised ACR criteria for SLE: malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, proteinuria, hematologic disorder (anemia, leukopenia, low platelet count), and positive antinuclear antibody (ANA) titer. In addition, the SLE-CSQ refers to two criteria from the 1971 American Rheumatism Association criteria for SLE (alopecia and Raynaud's phenomenon). The CSQ instrument has been validated in community-based cohorts across multiple ethnicities (38–40).

2.2 Detection of SLE-Associated Autoantibody Specificities

Serum samples were screened for SLE-associated autoantibodies for the purposes of determining immunologic and ANA SLE classification criteria (47, 48) in OMRF's College of American Pathologists certified Clinical Immunology Laboratory, as previously described (51). ANAs (HEp-2 cells) and anti-double-stranded DNA (anti-dsDNA by *Crithidia luciliae*) were measured using indirect immunofluorescence (Inova Diagnostics); a positive result was defined as detection of ANAs at a titer of ≥1:120 and anti-dsDNA antibodies at a titer of ≥1:30. Precipitin levels of autoantibodies directed against Ro/SSA, La/SSB, Sm, nRNP, and ribosomal P were detected by immunodiffusion. Anticardiolipin (aCL) antibodies were measured by enzyme linked immunosorbent assay, with a titer of >10 IgG or >10 IgM units considered positive.

In addition, serum samples were screened for autoantibody specificities using the BioPlex 2200 multiplex system (Bio-Rad Technologies, Hercules, CA). The BioPlex 2200 ANA kit uses fluorescently dyed magnetic beads for simultaneous detection of 11 autoantibody specificity levels, including reactivity to dsDNA, chromatin, ribosomal P, Ro/SSA, La/SSB, Sm, the Sm/RNP complex, RNP, Scl-70, centromere B, and Jo-1, with anti-Factor XIII level serving as a control for sample integrity (51). Autoantibodies to dsDNA, chromatin, Ro/SSA, La/SSB, Sm, Sm/RNP complex, and RNP were used for analysis in the current study. Anti-dsDNA (IU/mL) has a previously determined positive cutoff of 10 IU/mL; an Antibody Index (AI) value (range 0-8) is reported by the manufacturer to reflect the fluorescence intensity of each of the other autoantibody specificities with a positive cutoff as AI=1.0. The AI scale is standardized relative to calibrators and control samples provided by the manufacturer.

2.3 Detection of Soluble Plasma Mediators

After verification of SLE classification criteria and status, study participants in the LAUREL cohort at follow-up and in the confirmatory LFRR nested cohort with classified SLE (≥4 cumulative ACR criteria; n=56 at follow-up in LAUREL; n=100 from LFRR), as well as lupus relatives meeting 3 ACR classification criteria (incomplete lupus, ILE; n=34 at follow-up in LAUREL; n=72 from LFRR; also verified as ILE by SLICC criteria (52)) were matched by sex and race to clinically unaffected lupus relatives (n=154 from LAUREL; n=159 from the LFRR), as well as to unaffected HC with no family history of SLE (n=77 matched to LAUREL participants; n=127 matched to LFRR participants).

Plasma levels of BLyS (R&D Systems, Minneapolis, MN) and APRIL (eBioscience/Invitrogen/ThermoFisher Scientific, Waltham, MA) were determined by enzyme-linked immunosorbent assay (ELISA), per the manufacturer protocol. An additional fifty analytes, including innate and adaptive cytokines, chemokines, and soluble TNFR superfamily members (**Table S1**), were assessed by xMAP multiplex assays (Affymetrix/eBioscience/ThermoFisher, Waltham, MA) (1, 2, 11, 18, 19).

Data were analyzed on the Bio-Rad BioPlex 200® array system (Bio-Rad Technologies, Hercules, CA), with a lower boundary of 100 beads per analyte per sample. Median fluorescence intensity for each analyte was interpolated from 5-parameter logistic nonlinear regression standard curves. Analytes below the detection limit were assigned a value of 0.001 pg/mL. A known control serum was included on each plate (Cellgro human AB serum, Cat#2931949, L/N#M1016) to control for batch-effects. Well-specific validity was assessed by AssayCheX™ QC microspheres (Radix BioSolutions, Georgetown, TX, USA) to evaluate non-specific binding. Mean inter-assay coefficient of variance (CV) of multiplexed bead-based assays for cytokine detection has previously been shown to be 10-14% (53, 54) and a similar average CV (11%) was obtained across the analytes in this assay was obtained using healthy control serum. Intra-assay precision of duplicate wells averaged <10% CV in each 25-plex assay.

2.4 Statistical Analyses

Chi-square or Fisher's exact test were used, as appropriate, to determine categorical differences in sex, race, and familial relationship, as well as the presence of ACR criteria, medication usage, SLE-CSQ questionnaire components, lupus-associated autoantibody specificities, and Youden index (55) determined soluble mediator positivity based on Rel vs. SLE, with Bonferroni adjusted p-values. Categorical variables significant after Bonferroni correction for multiple comparison were assessed for size effect differences, comparing odds ratios with Haldane-Anscombe correction (56). 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 Kruskal-Wallis test with Dunn's multiple comparison correction. Correlations between plasma soluble mediator levels and SLE-CSQ or number of autoantibody specificities were determined by Spearman rank correlation. All statistical analyses were performed using GraphPad Prism version 9.3.1.

3 RESULTS

3.1 Demographic and Pedigree Characteristics in Clinically Unaffected Lupus Relatives vs. Relatives With ILE or SLE

We utilized two unique and well characterized cohorts of lupus relatives to determine differences in self-reported, clinical, and serologic/immunologic features that distinguish those relatives who developed incomplete (ILE) or classified SLE vs. demographically matched, clinically unaffected lupus relatives (Rel) and unaffected healthy controls (HC). Of the 436 lupus relatives meeting <4 ACR classification criteria enrolled in the lupus autoimmunity in relatives (LAUREL) follow-up cohort at baseline, 56 (12.8%) transitioned to classified SLE and 34 (7.8%) developed ILE, meeting 3 ACR criteria at their follow-up visit, an average of 6.4 years later. These individuals were demographically matched by sex, race, and age (± 5 years) to 154 clinically unaffected Rel and 77 unaffected HC, with no demographic difference between the groups (**Table 1**) (11, 46, 49).

As a confirmatory cohort to the follow-up visit in the LAUREL cohort, a subset of 100 SLE patients and 72 with ILE in the LFRR were demographically matched by sex and race to 159 clinically unaffected lupus relatives and 127 unaffected HC. SLE patients in the LFRR were significantly younger (37.8 ± 11.3 years) than those in the LAUREL cohort (53.5 ± 12.0 years, $p<0.0001$). This was also true for clinically unaffected relatives (56.4 ± 14.8 years in LFRR vs. 52.5 ± 13.6 years in LAUREL, $p<0.0001$, **Table 1**).

Of interest, although the frequency of multiplex families (>1 SLE patient/family) in the LAUREL cohort was similar across ILE (26%), SLE (27%), and Rel (31%) groups (**Table 1**, $p\geq0.8148$), SLE patients in the LFRR (20%) were less likely to come from multiplex families than those with ILE (42%) or clinically unaffected relatives (30%) (**Table 1**, $p\leq0.0036$).

3.2 Lupus Type 2 Symptoms Associated With SLE-CSQ Scores and Altered BLyS and IL-10 Levels in Lupus Relatives

Recently categorized Type 2 SLE symptoms, including chronic fatigue, anxiety, depression, chronic headaches, and associated sleep disturbances are present within the context of both active and inactive SLE in patients with classified disease (33, 35). Many of these same symptoms, particularly fatigue (36, 37), often occur in the initial presentation of patients who transition to classified disease (36, 37, 57).

We evaluated baseline (prior to SLE transition) questionnaire (46) responses of self-reported chronic fatigue, anxiety, depression, chronic headaches, and hours of sleep/night (46)

TABLE 1 | Demographic Characteristics of Nested Lupus Relatives Study.

LAUREL[a] Follow-up Nested Cohort	–>ILE	–>SLE	Lupus Relatives (Rel)	Unaffected HC	ILE/SLE	ILE/SLE/Rel	ILE/SLE/Rel/HC	Rel/HC
Demographics (n, %)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Gender					*0.4741*	*0.4654*	*0.6566*	*1.0000*
Female	32 (94%)	49 (88%)	142 (92%)	71 (92%)				
Race					*0.1645*	*0.5302*	*0.7374*	*0.7073*
European American	25 (74%)	43 (77%)	125 (81%)	60 (78%)				
African American	4 (12%)	9 (16%)	18 (12%)	9 (12%)				
Native American	4 (12%)	4 (7%)	8 (5%)	7 (9%)				
Asian	1 (2%)	0	3 (2%)	1 (1%)				
Age (SD)	48.9 (13.2)	47.7 (12.0)	49.3 (14.9)	52.5 (13.6)	*0.6382*	*0.7548*	*0.2161*	*0.1172*
Multiplex Pedigree (n, %)	9 (26%)	15 (27%)	47 (31%)	–	*1.0000*	*0.8148*	–	–
Relationship Status (n, %)					*0.5242*	***0.0002***	–	–
Parent of SLE patient	6 (18%)	10 (18%)	62 (40%)	–	*1.0000*	***0.0014***	–	–
Child of SLE patient	2 (6%)	10 (18%)	13 (8%)	–	*0.1239*	*0.0918*	–	–
Sibling of SLE patient	13 (38%)	21 (38%)	89 (58%)	–	*1.0000*	***0.0105***	–	–
Non-FDR of SLE Patient	9 (26%)	22 (39%)	23 (15%)	–	*0.2573*	***0.0007***	–	–
LFRR[a] Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE**	**ILE/SLE/Rel**	**ILE/SLE/Rel/HC**	**Rel/HC**
Demographics (n, %)	**n=72**	**n=100**	**n=159**	**n=127**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Gender					***0.0292***	*0.0642*	*0.1532*	*1.0000*
Female	68 (94%)	100 (100%)	155 (97%)	123 (97%)				
Race					***0.0421***	*0.0686*	*0.1374*	*0.4704*
European American	48 (67%)	50 (50%)	97 (61%)	72 (57%)				
African American	24 (33%)	50 (50%)	62 (39%)	55 (43%)				
Age (SD)	49.1 (13.9)	37.8 (11.3)	56.4 (14.8)	42.0 (14.7)	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***
Multiplex Pedigree (n, %)	30 (42%)	20 (20%)	48 (30%)	–	***0.0036***	***0.0087***	–	–
Relationship Status (n, %)					*0.5279*	***<0.0001***	–	–
Parent of SLE patient	11 (15%)	4 (4%)	120 (75%)	–	***0.0130***	***<0.0001***	–	–
Child of SLE patient	3 (4%)	1 (1%)	6 (4%)	–	*0.3100*	*0.3635*	–	–
Sibling of SLE patient	18 (25%)	8 (8%)	42 (26%)	–	***0.0043***	***0.0010***	–	–
Non-FDR of SLE Patient	17 (24%)	14 (14%)	17 (11%)	–	*0.1129*	***0.0351***	–	–

[a]*LAUREL, Lupus Autoimmunity in Relatives; LFRR, Lupus Family Registry and Repository cohort.*

Categorical significance determined by [b]*Chi-square test or* [c]*Fisher's Exact test.*

p-values in bold are significant at ***p≤0.05****.*

Rel, lupus relatives; HC, healthy controls; ILE, incomplete lupus erythematosus; LAUREL, Lupus Autoimmunity in Relatives; LFRR, Lupus Family Registry and Repository; SLE, systemic lupus erythematosus.

from lupus relatives in the nested LAUREL cohort vs. matched HC (n=77, **Table 1**). Lupus relatives were divided into those meeting no ACR criteria (No; n=61), only serologic (immunologic and ANA) ACR criteria (Ser, n=116), or clinical ACR criteria (Clin, n=67) (**Table 2**, top panel). The most consistent and significant differences were among those who reported having chronic fatigue, most frequent among lupus relatives meeting clinical ACR criteria (78%), similar among lupus relatives meeting no ACR criteria or only serologic ACR criteria (28% and 31%, respectively), yet all more frequent than matched HC (8%, p≤0.0024). Lupus relatives meeting clinical ACR criteria at baseline were also more likely to report anxiety (49%), depression (66%), chronic headaches (66%), and <7 hours of sleep/night (55%), *p≤0.0323*. Lupus relatives meeting no ACR criteria or only serologic criteria were similar to HC with respect to reporting anxiety, yet reported more chronic headaches (**Table 2**, top panel).

In addition, lupus relatives at baseline who transitioned to SLE at follow-up had the highest reported rate of fatigue (82%) compared to those who developed ILE (56%) or remained clinically unaffected (Rel, 26%) (**Table 2**, 2nd panel, *p≤0.0141*). Yet those who transitioned to SLE at follow-up had similar frequency of reported anxiety, depression, chronic headaches, and <7 hours of sleep/night (45-64%) as those who developed ILE (47-65%), with increased frequency compared to lupus relatives who remained clinically unaffected (23-44%, **Table 2**, 3rd panel, *p≤0.0124*). With the exception of anxiety and depression, where Rel had similar reported frequency as HC, lupus relatives had higher frequencies of type 2 symptoms at baseline than matched HC. This trend continued *after* transition to SLE in both the LAUREL (at follow-up) and LFRR cohorts (**Table 2**, 3rd and 4th panels, respectively), where SLE patients and lupus relatives with ILE had similar reported frequencies of type 2 symptoms, which were greater than clinically unaffected relatives and HC.

Given that lupus relatives meeting clinical ACR criteria were more likely to report type 2 symptoms, particularly fatigue, we asked if there were differences in either the SLE portion of the self-reported connective tissue disease questionnaire [SLE-CSQ; (38, 39)] or in SLE-associated immune mediators (1, 2, 11) in lupus relatives who reported fatigue at baseline in the LAUREL cohort, prior to disease transition (**Figures 1**, **2**). We observed greater SLE-CSQ scores in lupus relatives meeting no ACR criteria (No), only serologic criteria (Ser), or clinical criteria

TABLE 2 | Type 2 Symptoms in Lupus Relatives Who Transition to ILE or SLE.

LAUREL Nested Cohort	No ACR Criteria	Serologic ACR Criteria Only	Meets Clinical ACR Criteria	Unaffected HC	No/Ser/Clin[d]	No/Ser	No/Clin	Ser/Clin	No/HC	Ser/HC[d]
Baseline (Prior to SLE Transition)	**n=61**	**n=116**	**n=67**	**n=77**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**
Chronic Fatigue	17 (28%)	36 (31%)	52 (78%)	6 (8%)	***<0.0001***	*0.7314*	***<0.0001***	***<0.0001***	***0.0024***	***<0.0001***
Anxiety	14 (23%)	29 (25%)	33 (49%)	11 (14%)	***<0.0001***	*0.1119*	***<0.0001***	***0.0011***	*1.0000*	*0.1019*
Depression	21 (34%)	48 (41%)	44 (66%)	18 (23%)	***0.0006***	*0.4191*	***0.0007***	***0.0021***	*0.1840*	***0.0129***
Chronic Headaches	28 (46%)	52 (45%)	44 (66%)	12 (16%)	***0.0168***	*1.0000*	***0.0323***	***0.0008***	***0.0001***	***<0.0001***
Sleep <7 hours/night[a]	21 (37%)	29 (26%)	35 (55%)	–	***0.0001***	***0.0019***	*0.7176*	***0.0002***	–	–
LAUREL Nested Cohort	**–>ILE**	**–>SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE/Rel/HC**	**ILE/SLE/Rel**	**ILE/SLE**	**ILE/Rel**	**SLE/Rel**	**Rel/HC**
Baseline (Prior to SLE Transition)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**
Chronic Fatigue	19 (56%)	46 (82%)	40 (26%)	6 (8%)	***<0.0001***	***<0.0001***	***0.0141***	***0.0018***	***<0.0001***	***0.0008***
Anxiety	16 (47%)	25 (45%)	35 (23%)	11 (14%)	***<0.0001***	***0.0010***	*0.8311*	***0.0057***	***0.0031***	*0.1624*
Depression	22 (65%)	36 (64%)	55 (36%)	18 (23%)	***<0.0001***	***<0.0001***	*1.0000*	***0.0034***	***0.0003***	*0.0715*
Chronic Headaches	20 (59%)	36 (64%)	68 (44%)	12 (16%)	***<0.0001***	***0.0216***	*0.6574*	*0.1326*	***0.0124***	***<0.0001***
Sleep <7 hours/night[a]	14 (47%)	31 (57%)	40 (27%)	–	–	***0.0002***	*0.1848*	*0.0954*	***<0.0001***	–
LAUREL Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE/Rel/HC**	**ILE/SLE/Rel**	**ILE/SLE**	**ILE/Rel**	**SLE/Rel**	**Rel/HC**
Follow-up (After SLE Transition)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**
Chronic Fatigue	21 (62%)	43 (77%)	43 (28%)	6 (8%)	***<0.0001***	***<0.0001***	*0.1536*	***0.0003***	***<0.0001***	***0.0003***
Anxiety	16 (47%)	24 (43%)	35 (23%)	11 (14%)	***<0.0001***	***0.0017***	*0.8272*	***0.0057***	***0.0055***	*0.1624*
Depression	18 (53%)	35 (63%)	64 (42%)	18 (23%)	***<0.0001***	***0.0223***	*0.3868*	*0.2546*	***0.0081***	***0.0084***
Chronic Headaches	17 (50%)	36 (64%)	47 (31%)	12 (16%)	***<0.0001***	***<0.0001***	*0.1940*	***0.0443***	***<0.0001***	***0.0162***
Sleep <7 hours/night[a]	20 (67%)	26 (48%)	57 (40%)	–	–	***0.0223***	*0.1155*	***0.0084***	*0.3323*	–
LFRR Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE/Rel/HC**	**ILE/SLE/Rel**	**ILE/SLE**	**ILE/Rel**	**SLE/Rel**	**Rel/HC**
LFRR (After SLE Transition)	**n=72**	**n=100**	**n=159**	**n=127**	***p-value*[b]**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**	***p-value*[c]**
Chronic Fatigue	55 (76%)	73 (73%)	37 (23%)	19 (14%)	***<0.0001***	***<0.0001***	*0.7237*	***<0.0001***	***<0.0001***	*0.0524*
Anxiety	32 (44%)	34 (34%)	33 (21%)	31 (24%)	***0.0010***	***0.0007***	*0.1646*	***0.0002***	***0.0178***	*0.4783*
Depression	43 (60%)	65 (65%)	50 (31%)	43 (34%)	***<0.0001***	***<0.0001***	*0.4799*	***<0.0001***	***<0.0001***	*0.7040*
Chronic Headaches	41 (57%)	60 (60%)	51 (32%)	39 (31%)	***<0.0001***	***<0.0001***	*0.6880*	***0.0003***	***<0.0001***	*0.8981*
Sleep <7 hours/night[a]	32 (52%)	52 (52%)	60 (39%)	59 (46%)	*0.1353*	*0.0626*	*1.0000*	*0.0923*	*0.0522*	*0.2760*

[a]out of 33 (ILE), 53 (SLE), and 147 (Rel) reported at BL; out of 30 (ILE), 54 (SLE), and 144 (Rel) reported at FU; out of 61 (ILE), 100 (SLE), 154 (Rel), and 126 (Healthy Controls [HC]) reported in LFRR.

Categorical significance determined by [b]Chi-square test or [c]Fisher's Exact test.

*[d]**p<0.0001** No/Ser/Clin/HC all group comparisons; **p<0.0001** Clin/HC all group comparisons.*

*p-values in bold are significant at **p<0.05**.*

Clin, relatives meeting clinical criteria; Rel, lupus relatives ;HC, healthy controls; LAUREL, Lupus Autoimmunity in Relatives; LFRR, Lupus Family Registry and Repository; No, relatives meeting no ACR criteria; Ser, relatives meeting only serologic criteria.

(Clin) who reported chronic fatigue ($p<0.05$, **Figure 1A**), with the highest SLE-CSQ scores, irrespective of chronic fatigue, in lupus relatives meeting clinical ACR criteria ($p<0.01$, **Figure 1A**). Of note, among the multiple serum SLE-associated autoantibody specificities and plasma immune mediators assessed, BLyS levels were *increased* in lupus relatives and HC who reported chronic fatigue, while IL-10 levels were *decreased*, irrespective of ACR criteria status ($p<0.05$, **Figures 1B, C**).

We noted similar patterns of elevated SLE-CSQ scores in lupus relatives assessed by classification status who reported fatigue (**Figure 2**). Of note, BLyS levels were increased in lupus relatives who developed ILE or remained clinically unaffected and HC who reported chronic fatigue in both cohorts. However, this increase was not present in relatives who reported chronic fatigue and transitioned to SLE, either prior to disease transition (**Figure 2A**) or after reaching disease classification (**Figures 2B, C**). Once again, IL-10 levels were largely decreased in lupus relatives who reported chronic fatigue in both cohorts (**Figure 2**). With respect to other type 2 symptoms, SLE-CSQ scores are likely to be increased in lupus relatives and HC who reported anxiety (**Figure S2**), depression (**Figure S3**), or chronic headaches (**Figure S4**). SLE-CSQ scores were highest in those with clinical ACR criteria prior to SLE transition (panel A), as well as those lupus relatives who transitioned to SLE, either before (panel B), or after (panels C-D) reaching SLE classification, $p<0.05$. BlyS levels were likely to be elevated in lupus relatives reporting these type 2

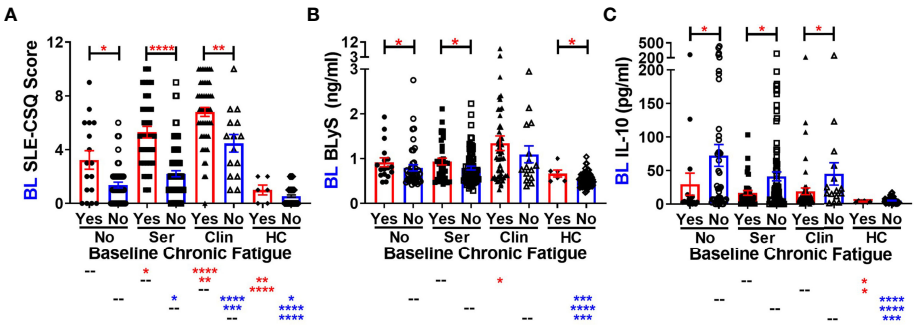

FIGURE 1 | Altered SLE-CSQ scores and BLyS and IL-10 levels associated with reported chronic fatigue in lupus relatives prior to disease transition in the LAUREL cohort. Lupus relatives in the LAUREL cohort at baseline meeting No ACR criteria (No), only serologic ACR criteria (Ser), or clinical ACR criteria (Clin) vs. matched, unaffected healthy controls (HC) who did (Yes) or did not (No) report chronic fatigue on the LFRR questionnaire were evaluated for **(A)** SLE-CSQ scores, **(B)** plasma BLyS levels, and **(C)** plasma IL-10 levels. Mean ± SEM. *****p<0.0001*; ****p<0.001*; ***p<0.01*; **p<0.05* by Kruskal-Wallis with Dunn's multiple comparison.

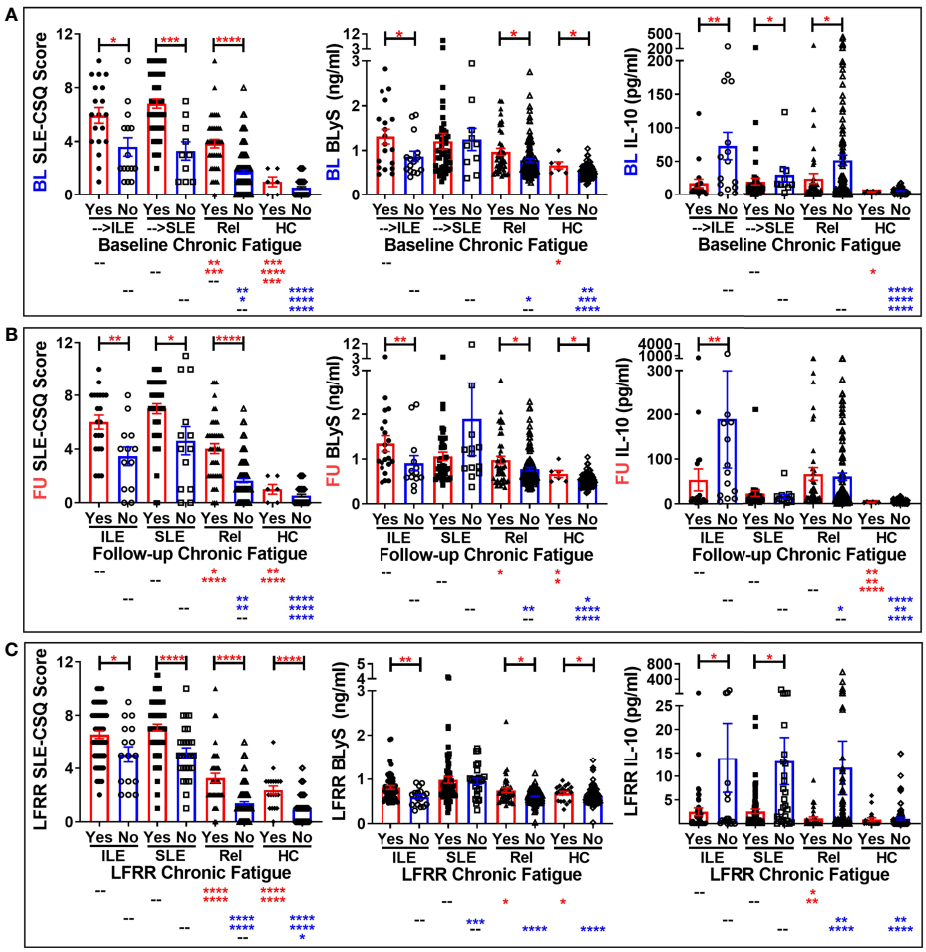

FIGURE 2 | Altered SLE-CSQ scores and BLyS and IL-10 levels associated with reported chronic fatigue in lupus relatives prior to and after disease transition in the LAUREL and LFRR confirmatory cohorts. Lupus relatives who developed ILE (ILE), transitioned to SLE (SLE), or remained clinically unaffected (Rel) vs. matched, unaffected healthy controls (HC) who did (Yes) or did not (No) report chronic fatigue on the LFRR questionnaire were evaluated for SLE-CSQ scores (*1st column*), plasma BLyS levels (*2nd column*), and plasma IL-10 levels (*3rd column*) in **(A)** LAUREL cohort at baseline (pre-transition), **(B)** LAUREL cohort at follow-up (post-transition), and **(C)** LFRR confirmatory cohort (post-transition). Mean ± SEM. *****p<0.0001*; ****p<0.001*; ***p<0.01*; **p<0.05* by Kruskal-Wallis with Dunn's multiple comparison.

symptoms except those who transitioned to classified SLE, where BLyS levels were not associated with type 2 symptoms (**Figures S2–S4**). Although not necessarily significant, IL-10 levels trended higher in lupus relatives who did not report type 2 symptoms (**Figures S2–S4**). With respect to sleep (**Figure S5**), there was no consistent pattern of altered SLE-CSQ scores nor BLyS and IL-10 levels noted in either lupus relatives or HC.

3.3 Increased Clinical and Serologic Features Pre-Classification in Lupus Relatives Who Develop ILE or Transition to Classified SLE

In addition to Type 2 symptoms, individuals who develop ILE or transition to SLE are likely to report and/or present with serologic and/or clinical ACR criteria for SLE *prior* to disease transition (1, 2, 11, 58, 59). This may be particularly true for lupus relatives, who are at increased risk for developing SLE (9, 10, 60). At the baseline visit in the LAUREL cohort (prior to disease transition), expectedly, lupus relatives meeting clinical ACR criteria had higher ACR scores (number of ACR criteria) and modified SLE Risk Probability Index (mSLERPI) (50) scores than those meeting only serologic criteria ($p<0.0001$, **Figure 3A**, 1st and 2nd columns, respectively). Of interest, those relatives who were destined to develop ILE or transition to SLE at follow-up met a similar number of ACR and mSLERPI criteria at baseline (**Figure 3B**, 1st and 2nd columns, respectively). This is reflective of the lack of significant difference in the clinical and serologic (immunologic and ANA) ACR criteria met at baseline, as well as frequency of immune modulating treatments, in the LAUREL cohort for those relatives who developed ILE or transitioned to SLE at follow-up (**Table 3**). However, despite the lack of significance ($p\geq0.2390$), it was noted that only those relatives who transitioned to SLE at follow-up presented with serositis (n=4, 7%) or neurologic (n=1, 2%) criteria at baseline. Also of note, relatives who remained clinically unaffected, or met only serologic criteria at baseline, had higher baseline ACR scores than matched HC, likely due to the higher rate of ANA positivity (IIF titer ≥1:120) in clinically unaffected relatives (51%) vs. HC (18%), both of which were significantly lower than those who developed ILE (88%) or transitioned to SLE (91%) ($p<0.0001$, **Table 3**).

At the follow-up time point (post-SLE transition) in the LAUREL cohort, those relatives who had ILE had similar frequency of accumulated hematologic and serologic (immunologic/ANA) criteria as those who transitioned to SLE,

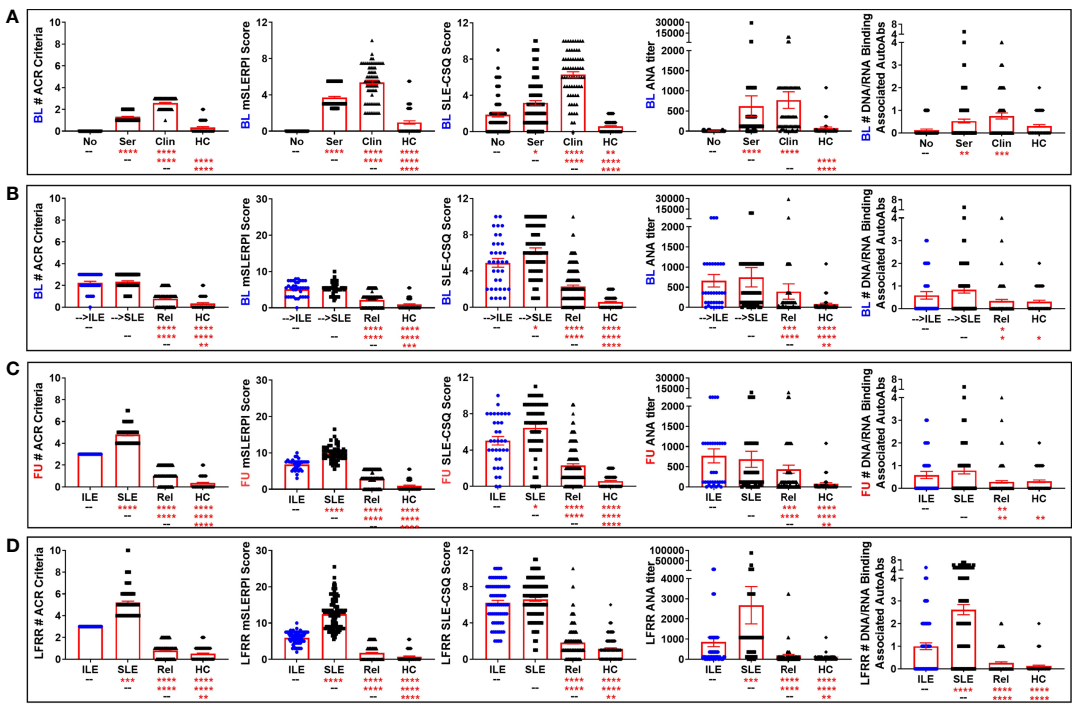

FIGURE 3 | Altered ACR and SLE-CSQ scores as well as ANA titers and autoantibody accumulation in lupus relatives who develop ILE or transition to SLE. Lupus relatives and matched healthy controls (HC) were evaluated for # of ACR criteria for SLE (*1st column*), modified SLE Risk Probability Index (mSLERPI) scores (*2nd column*), SLE-CSQ scores (*3rd column*), ANA titer (*4th column*), and # of SLE-associated autoantibody specificities (*5th column)* in **(A)** LAUREL cohort at baseline meeting No ACR criteria (No), only serologic ACR criteria (Ser), or clinical ACR criteria (Clin) vs. matched, unaffected HC and **(B–D)** lupus relatives who developed ILE (ILE), transitioned to SLE (SLE), or remained clinically unaffected (Rel) vs. matched healthy controls (HC) in **(B)** LAUREL cohort at baseline (pre-transition), **(C)** LAUREL cohort at follow-up (post-transition), and **(D)** LFRR confirmatory cohort (post-transition). Mean ± SEM. ****$p<0.0001$; ***$p<0.001$; **$p<0.01$; *$p<0.05$ by Kruskal-Wallis with Dunn's multiple comparison.

TABLE 3 | ACR Criteria and Medication in LAUREL Nested Cohort at Baseline (Prior to SLE Transition).

	–>ILE	–>SLE	Lupus Relatives (Rel)	Unaffected HC	ILE/SLE	ILE/SLE/Rel	ILE/SLE/Rel/HC	Rel/HC
ACR Classification Criteria (n,%)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Malar Rash	3 (8%)	7 (13%)	–	–	*0.7368*	–	–	–
Discoid Rash	1 (3%)	1 (2%)	–	–	*1.0000*	–	–	–
Photosensitivity	7 (21%)	14 (25%)	–	–	*0.7981*	–	–	–
Oral Ulcers	1 (3%)	2 (4%)	–	–	*1.0000*	–	–	–
Arthritis	10 (29%)	20 (36%)	–	–	*0.6465*	–	–	–
Serositis	0	4 (7%)	–	–	*0.2930*	–	–	–
Pericarditis	0	1 (2%)	–	–	*1.0000*	–	–	–
Pleuritis	0	3 (5%)	–	–	*0.2689*	–	–	–
Renal	1 (3%)	1 (2%)	–	–	*1.0000*	–	–	–
Proteinuria	1 (3%)	1 (2%)	–	–	*1.0000*	–	–	–
Cellular Casts	0	0	–	–	*1.0000*	–	–	–
Neurologic	0	1 (2%)	–	–	*1.0000*	–	–	–
Seizure	0	1 (2%)	–	–	*1.0000*	–	–	–
Psychosis	0	0	–	–	*1.0000*	–	–	–
Hematologic	6 (18%)	5 (9%)	–	–	*0.3200*	–	–	–
Hemolytic Anemia	0	0	–	–	*1.0000*	–	–	–
Thrombocytopenia	0	0	–	–	*1.0000*	–	–	–
Leukopenia	4 (12%)	3 (5%)	–	–	*0.4260*	–	–	–
Lymphopenia	4 (12%)	4 (7%)	–	–	*0.7070*	–	–	–
Immunologic[a]	17 (50%)	25 (45%)	41 (27%)	14 (18%)	*0.8241*	***0.0004***	***<0.0001***	*0.1904*
anti-dsDNA	5 (15%)	6 (11%)	1 (1%)	0	*0.7415*	***0.0002***	***<0.0001***	*1.0000*
anti-Sm	0	1 (2%)	0	0	*1.0000*	–	–	–
anti-cardiolipin (aCL)	14 (41%)	18 (32%)	40 (26%)	14 (18%)	*0.4962*	*0.1885*	*0.0611*	*0.2481*
ANA	30 (88%)	51 (91%)	78 (51%)	14 (18%)	*0.7249*	***<0.0001***	***<0.0001***	***<0.0001***
Medications (n, %)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Steroid	15 (44%)	33 (59%)	6 (4%)	1 (1%)	*0.1963*	***<0.0001***	***<0.0001***	*0.4292*
Hydroxychloroquine	16 (47%)	34 (61%)	5 (3%)	0	*0.2744*	***<0.0001***	***<0.0001***	*0.1723*
Immunosuppressant[d]	6 (18%)	14 (25%)	1 (1%)	0	*0.4485*	***<0.0001***	***<0.0001***	*1.0000*
Major Immunosuppressant[d]	1 (3%)	4 (7%)	0	0	*0.6462*	***0.0326***	***0.0072***	*1.0000*
Biologic	0	0	0	0	–	–	–	–

[a]*Seropositivity determined by Crithidia luciliae assay (anti-dsDNA; titer≥1:30), gel precipitation assay (anti-Sm), or ELISA (aCL; >10 IgG or IgM units).*

Categorical significance determined by [b]*Chi-square test or* [c]*Fisher's Exact test.*

[d]*Immunosuppressant = methotrexate, azathioprine; Major Immunosuppressant = mycophenolate mofetil, cyclophosphamide.*

p-values in bold are significant at ***p≤0.05****.*

ANA, antinuclear antibodies; Rel, lupus relatives; HC, healthy controls; ILE, incomplete lupus erythematosus; SLE, systemic lupus erythemtosus.

while those with classified SLE had accumulated a higher frequency of mucocutaneous (malar rash, discoid rash, photosensitivity, and oral ulcers), arthritis, serositis, and neurologic criteria (*p≤0.0273*, **Table 4**). This was reflective of both the expected increase in number of ACR and mSLERPI criteria (*p<0.0001*, **Figure 3C**, 1st and 2nd columns, respectively) and increase in hydroxychloroquine use (*p=0.0051*, **Table 4**), but not other immune modulating treatments, in those lupus relatives who transitioned to classified SLE compared to those relatives with ILE at follow-up. While relatives who remained clinically unaffected also had lower rates of meeting immunologic criteria (36%) or being ANA positive (64%) compared to relatives who developed ILE (62% and 97%, respectively) or transitioned to SLE (55% and 96%, respectively) at follow-up in the LAUREL cohort (*p≤0.0451*, **Table 4**), they were also significantly higher than matched, unaffected HC, with 18% frequency in meeting immunologic criteria and ANA positivity (*p≤0.0061*, **Table 4**).

We wanted to know if lupus relatives with classified SLE or ILE, as well as clinically unaffected relatives and matched HC in the confirmatory LFRR nested cohort had a similar profile of ACR criteria as those at follow-up in the LAUREL cohort. The number of ACR and mSLERPI criteria met in the lupus relative groups and HC were similar between the LFRR (**Figure 3D**, 1st and 2nd columns, respectively) and follow-up, post-SLE transition visit in the LAUREL cohort (**Figure 3C**, 1st and 2nd columns), including increased ACR and mSLERPI scores in clinically unaffected relatives vs. HC (*p<0.01*). However, relatives with classified SLE in the confirmatory LFRR nested cohort had a greater frequency of renal (59% vs. 9% in LAUREL, *p<0.0001*), hematologic (54% vs. 14%, *p<0.0001*), and immunologic (94% vs. 55%, *p<0.0001*) ACR criteria (**Tables 5**, **6**). In contrast, relatives who transitioned to SLE in LAUREL at follow-up were more likely to meet mucocutaneous ACR criteria, including malar rash (59% vs. 35% in LFRR, *p=0.0044*), photosensitivity (52% vs. 35%, p=0.0440), oral ulcers (45% vs. 25%, *p=0.0195*).

Arthritis, serositis, and neurologic clinical criteria, as well as rate of ANA positivity, were similar between relatives with classified SLE in the LFRR (13-68%) vs. LAUREL (13-75%) follow-up cohorts (**Tables 4**, **5**). Similar to the LAUREL cohort, SLE patients (12-86%) in the LFRR cohort were more likely than relatives with ILE (8-61%) to meet mucocutaneous,

TABLE 4 | ACR Criteria and Medication in LAUREL Nested Cohort at Follow-up (After SLE Transition).

	ILE	SLE	Lupus Relatives (Rel)	Unaffected HC	ILE/SLE	ILE/SLE/Rel	ILE/SLE/Rel/HC	Rel/HC
ACR Classification Criteria (n,%)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Malar Rash	5 (15%)	33 (59%)	–	–	***<0.0001***	–	–	–
Discoid Rash	1 (3%)	10 (18%)	–	–	***0.0469***	–	–	–
Photosensitivity	9 (26%)	29 (52%)	–	–	***0.0273***	–	–	–
Oral Ulcers	5 (15%)	25 (45%)	–	–	***0.0052***	–	–	–
Arthritis	19 (56%)	42 (75%)	–	–	*0.0677*	–	–	–
Serositis	0	25 (45%)	–	–	***<0.0001***	–	–	–
Pericarditis	0	7 (13%)	–	–	***0.0418***	–	–	–
Pleuritis	0	23 (41%)	–	–	***<0.0001***	–	–	–
Renal	1 (3%)	5 (9%)	–	–	*0.4026*	–	–	–
Proteinuria	1 (3%)	5 (9%)	–	–	*0.4026*	–	–	–
Cellular Casts	0	0	–	–	–	–	–	–
Neurologic	0	7 (13%)	–	–	***0.0418***	–	–	–
Seizure	0	5 (9%)	–	–	*0.1523*	–	–	–
Psychosis	0	2 (4%)	–	–	*0.5246*	–	–	–
Hematologic	8 (24%)	8 (14%)	–	–	*0.2734*	–	–	–
Hemolytic Anemia	1 (3%)	1 (2%)	–	–	*1.0000*	–	–	–
Thrombocytopenia	1 (3%)	0	–	–	*0.3778*	–	–	–
Leukopenia	5 (15%)	5 (9%)	–	–	*0.4942*	–	–	–
Lymphopenia	5 (15%)	4 (7%)	–	–	*0.2899*	–	–	–
Immunologic[a]	21 (62%)	31 (55%)	55 (36%)	14 (18%)	*0.6810*	***0.0031***	***<0.0001***	***0.0061***
anti-dsDNA	6 (18%)	9 (16%)	1 (1%)	0	*1.0000*	***<0.0001***	***<0.0001***	*1.0000*
anti-Sm	0	2 (4%)	0	0	*0.5246*	–	–	–
anti-cardiolipin (aCL)	13 (38%)	14 (25%)	29 (19%)	14 (18%)	*0.2437*	***0.0451***	*0.0636*	*1.0000*
ANA	33 (97%)	54 (96%)	98 (64%)	14 (18%)	*1.0000*	***<0.0001***	***<0.0001***	***<0.0001***
Medications (n, %)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Steroid	17 (50%)	21 (38%)	7 (5%)	1 (1%)	*0.0861*	***<0.0001***	***<0.0001***	*0.2745*
Hydroxychloroquine	11 (32%)	35 (64%)	8 (5%)	0	***0.0051***	***<0.0001***	***<0.0001***	*0.0546*
Immunosuppressant[d]	13 (38%)	17 (30%)	5 (3%)	0	*0.4935*	***<0.0001***	***<0.0001***	*0.1723*
Major Immunosuppressant[d]	1 (3%)	4 (7%)	1 (1%)	0	*0.6462*	***0.0265***	***0.0097***	*1.0000*
Biologic	2 (6%)	0	0	0	*0.1401*	***0.0020***	***0.0007***	*1.0000*

[a]*Seropositivity determined by Crithidia luciliae assay (anti-dsDNA; titer≥1:30), gel precipitation assay (anti-Sm), or ELISA (aCL; >10 IgG or IgM units).*

Categorical significance determined by [b]*Chi-square test or* [c]*Fisher's Exact test.*

[d]*Immunosuppressant = methotrexate, azathioprine; Major Immunosuppressant = mycophenolate mofetil, cyclophosphamide.*

p-values in bold are significant at ***p≤0.05****.*

ANA, antinuclear antibodies; Rel, lupus relatives; HC, healthy controls; ILE, incomplete lupus erythematosus; SLE, systemic lupus erythemtosus.

serositis, and neurologic ACR criteria, as well as be prescribed hydroxychloroquine. However, SLE patients (49-94%) in the LFRR cohort were also more likely than their counterparts with ILE (3-47%) to meet arthritis, renal, and immunologic criteria (*p<0.0001*, **Table 5**), reflected with increased rates of immune modulating treatments, including steroids (94% SLE vs. 77% ILE, *p=0.0033*, **Table 5**). Clinically unaffected relatives (1-37%) in the LFRR had similar rates of immunologic criteria and immune modulating treatments as matched HC (1-30%), but were once again more likely than HC to be ANA positive (43% Rel vs. 21% HC, *p<0.0001*, **Table 5**), reinforcing an important difference between lupus relatives who remain clinically unaffected and demographically matched healthy individuals in the general population.

3.4 Participant-Reported SLE-CSQ Increased in Lupus Relatives and Reflects Future SLE Classification Status

ACR scores for SLE classification reflect a cumulative combination of currently observed and previously documented clinical and serologic criteria (47). The SLE portion of the CSQ is based on the ACR classification criteria for SLE and may serve as a useful screening tool for identifying individuals at risk of developing SLE (11, 34, 38–41). Although validated only in the general population (38, 40), we sought to determine if the SLE-CSQ scores and reported symptoms were reflective of medical record confirmed SLE classification status in lupus relatives. At the baseline visit in the LAUREL cohort, we noted that lupus relatives had significantly higher SLE-CSQ scores than matched HC (**Figure 3A**, 3rd column), with the highest scores in relatives meeting clinical ACR criteria (*p<0.0001*), followed by serologic criteria only (*p<0.0001*) and no classification criteria (*p=0.0021*). Relatives who would transition to SLE at follow-up had higher SLE-CSQ scores than those who will develop ILE (*p=0.0354*, **Figure 3B**, 3rd column). Post-transition, relatives with classified SLE continued to have higher SLE-CSQ scores than those with ILE (*p=0.0142*, **Figure 3C**, 3rd column) in the LAUREL cohort, while relatives with classified SLE in the LFRR cohort had similar SLE-CSQ scores in the LFRR cohort (**Figure 3D**, 3rd column).

Of note, clinically unaffected relatives in both the LAUREL (baseline and follow-up) and LFRR confirmatory cohorts had lower SLE-CSQ scores than those who developed ILE or

TABLE 5 | ACR Criteria and Medication in LFRR Confirmatory Nested Cohort (After SLE Transition).

	ILE	SLE	Lupus Relatives (Rel)	Unaffected HC	ILE/SLE	ILE/SLE/Rel	ILE/SLE/Rel/HC	Rel/HC
ACR Classification Criteria (n,%)	**n=72**	**n=100**	**n=159**	**n=127**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Malar Rash	13 (18%)	35 (35%)	–	–	***0.0162***	–	–	–
Discoid Rash	6 (8%)	12 (12%)	–	–	*0.6146*	–	–	–
Photosensitivity	26 (36%)	35 (35%)	–	–	*1.0000*	–	–	–
Oral Ulcers	5 (7%)	25 (25%)	–	–	***0.0020***	–	–	–
Arthritis	27 (38%)	68 (68%)	–	–	***<0.0001***	–	–	–
Serositis	7 (10%)	37 (37%)	–	–	***<0.0001***	–	–	–
Pericarditis	4 (6%)	17 (17%)	–	–	***0.0322***	–	–	–
Pleuritis	4 (6%)	28 (28%)	–	–	***0.0001***	–	–	–
Renal	2 (3%)	49 (49%)	–	–	***<0.0001***	–	–	–
Proteinuria	2 (3%)	48 (48%)	–	–	***<0.0001***	–	–	–
Cellular Casts	0	20 (20%)	–	–	***<0.0001***	–	–	–
Neurologic	1 (1%)	13 (13%)	–	–	***0.0085***	–	–	–
Seizure	0	8 (8%)	–	–	***0.0214***	–	–	–
Psychosis	1 (1%)	5 (5%)	–	–	*0.4027*	–	–	–
Hematologic	25 (35%)	54 (54%)	–	–	*0.5829*	–	–	–
Hemolytic Anemia	0	7 (7%)	–	–	***0.0424***	–	–	–
Thrombocytopenia	2 (3%)	20 (20%)	–	–	***0.0008***	–	–	–
Leukopenia	16 (22%)	30 (30%)	–	–	*0.2969*	–	–	–
Lymphopenia	11 (15%)	31 (31%)	–	–	***0.0198***	–	–	–
Immunologic[a]	34 (47%)	94 (94%)	59 (37%)	38 (30%)	***<0.0001***	***<0.0001***	***<0.0001***	*0.2114*
anti-dsDNA	6 (8%)	75 (75%)	1 (1%)	0	***<0.0001***	***<0.0001***	***<0.0001***	*1.0000*
anti-Sm	1 (1%)	33 (33%)	0	0	***<0.0001***	–	–	–
anti-cardiolipin (aCL)	31 (43%)	63 (63%)	59 (37%)	38 (30%)	***0.0129***	***0.0002***	***<0.0001***	*0.2114*
ANA	67 (93%)	91 (91%)	69 (43%)	27 (21%)	*0.7799*	***<0.0001***	***<0.0001***	***<0.0001***
Medications (n, %)	**n=66**	**n=100**	**n=135**	**n=100**	***p-value*[b]**	***p-value*[c]**	***p-value*[c]**	***p-value*[b]**
Steroid	51 (77%)	94 (94%)	5 (4%)	7 (7%)	***0.0033***	***<0.0001***	***<0.0001***	*0.3695*
Hydroxychloroquine	40 (61%)	86 (86%)	1 (1%)	0	***0.0003***	***<0.0001***	***<0.0001***	*1.0000*
Immunosuppressant[d]	14 (21%)	55 (55%)	1 (1%)	1 (1%)	***<0.0001***	***<0.0001***	***<0.0001***	*1.0000*
Major Immunosuppressant[d]	5 (8%)	51 (51%)	0	0	***<0.0001***	***<0.0001***	***<0.0001***	–
Biologic	0	4 (4%)	0	0	*0.1522*	***0.0142***	***0.0030***	–

[a]*Seropositivity determined by Crithidia luciliae assay (anti-dsDNA; titer≥1:30), gel precipitation assay (anti-Sm), or ELISA (aCL; >10 IgG or IgM units).*

Categorical significance determined by [b]*Chi-square test or* [c]*Fisher's Exact test.*

[d]*Immunosuppressant = methotrexate, azathioprine; Major Immunosuppressant = mycophenolate mofetil, cyclophosphamide.*

p-values in bold are significant at ***p≤0.05****.*

ANA, antinuclear antibodies; Rel, lupus relatives; HC, healthy controls; ILE, incomplete lupus erythematosus; SLE, systemic lupus erythemtosus.

transitioned to SLE ($p<0.0001$), yet significantly higher than unaffected HC ($p<0.0001$, **Figures 3B–D**, 3rd column). This was also true across the individual component responses, where clinically unaffected relatives were less likely to note individual symptoms than their SLE and ILE counterparts ($p<0.05$) in both LAUREL (baseline and follow-up) and LFRR cohorts (**Table 6**), yet more likely than matched, unaffected HC to report symptoms, particularly sun sensitivity ($p\leq0.0098$), pleurisy ($p\leq0.0001$), and positive ANA ($p\leq0.0431$). Lupus relatives who transitioned to SLE were more likely than those who developed ILE to report cheek rash ($p=0.0134$), mouth sores ($p=0.0011$), and pleurisy ($p=0.0496$) at baseline (LAUREL), mouth sores and protein in the urine at follow-up (LAUREL), and protein in the urine, seizure, and low blood counts (LFRR). In contrast, relatives with ILE in the LFRR cohort were more likely to report cold sensitivity ($p=0.0422$, **Table 6**)

Overall, SLE-CSQ scores closely correlated with the number of ACR criteria documented in the medical record across the LAUREL (baseline and follow-up) and LFRR cohorts (Spearman r≥0.526 [0.426-0.614 95% CI], $p<0.0001$, **Table 7**), as well as ANA titer (Spearman r≥0.238 [0.113-0.367], $p=0.0002$, **Table 7**) and number of autoantibody specificities (Spearman r≥0.140 [0.011-0.265], $p=0.0286$, **Table 7**). The number of autoantibody specificities detected in both the LAUREL (baseline and follow-up) and LFRR cohorts also correlated with number of ACR criteria documented in the medical record (Spearman r≥0.238 [0.113-0.357], $p\leq0.0002$, **Table 7**) and ANA titers (Spearman r≥0.313 [0.191-0.425], $p<0.0001$, **Table 7**). Lupus relatives meeting clinical criteria at baseline in the LAUREL cohort had similar ANA titers and number of SLE-associated autoantibody specificities as those meeting only serologic criteria, yet higher ($p<0.0001$) than matched relatives with no ACR criteria and unrelated HC, which had similar profiles (**Figure 3A**, 4th-5th columns). This was also true when comparing relatives who developed ILE or transitioned to SLE, with similar ANA titers and number of SLE-associated autoantibody specificities at baseline and follow-up in the LAUREL cohort that were higher ($p<0.001$) than matched, clinically unaffected relatives and unaffected HC (**Figures 3B, C**, 4th-5th columns).

However, relatives with classified SLE in the confirmatory LFRR cohort had the highest ANA titers and number of SLE-associated autoantibody specificities, followed by relatives who

TABLE 6 | SLE-CSQ Components in Lupus Relatives Who Transition to ILE or SLE.

LAUREL Nested Cohort	–>ILE	–>SLE	Lupus Relatives (Rel)	Unaffected HC	ILE/SLE	ILE/SLE/Rel	ILE/SLE/Rel/HC	Rel/HC
Baseline (Prior to SLE Transition)	**n=34**	**n=56**	**n=154**	**n=77**	***p*-value[a]**	***p*-value[b]**	***p*-value[b]**	***p*-value[a]**
Cheek rash	10 (29%)	29 (52%)	13 (8%)	1 (1%)	***0.0134***	***<0.0001***	***<0.0001***	***0.0387***
Discoid lupus	2 (6%)	0	0	0	*0.1401*	***0.0020***	***0.0007***	*1.0000*
Sun sensitivity	15 (44%)	35 (63%)	42 (27%)	1 (1%)	*0.1255*	***<0.0001***	***<0.0001***	***<0.0001***
Mouth sores	9 (26%)	35 (63%)	32 (21%)	3 (4%)	***0.0011***	***<0.0001***	***<0.0001***	***0.0004***
Arthritis	23 (68%)	43 (77%)	62 (40%)	13 (17%)	*0.4613*	***<0.0001***	***<0.0001***	***<0.0001***
Pleurisy	12 (35%)	33 (59%)	35 (23%)	2 (3%)	***0.0496***	***<0.0001***	***<0.0001***	***<0.0001***
Protein in urine	16 (47%)	26 (46%)	21 (14%)	2 (3%)	*1.0000*	***<0.0001***	***<0.0001***	***0.0089***
Seizure	6 (18%)	7 (13%)	5 (3%)	0	*0.5457*	***0.0036***	***0.0001***	*0.1723*
Low blood counts	26 (76%)	39 (70%)	62 (40%)	17 (22%)	*0.6284*	***<0.0001***	***<0.0001***	***0.0078***
Positive ANA	19 (56%)	38 (68%)	20 (13%)	0	*0.4895*	***<0.0001***	***<0.0001***	***0.0003***
Cold sensitivity	14 (41%)	32 (57%)	37 (24%)	6 (8%)	*0.1923*	***<0.0001***	***<0.0001***	***0.0023***
Rapid hair loss	13 (38%)	30 (54%)	24 (16%)	1 (1%)	*0.1941*	***<0.0001***	***<0.0001***	***0.0005***
LAUREL Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE**	**ILE/SLE/Rel**	**ILE/SLE/Rel/HC**	**Rel/HC**
Follow-up (After SLE Transition)	**n=34**	**n=56**	**n=154**	**n=77**	***p*-value[a]**	***p*-value[b]**	***p*-value[b]**	***p*-value[a]**
Cheek rash	10 (29%)	28 (50%)	14 (9%)	1 (1%)	*0.0781*	***<0.0001***	***<0.0001***	***0.0233***
Discoid lupus	5 (15%)	4 (7%)	3 (2%)	0	*0.2899*	***0.0053***	***0.0005***	*0.5526*
Sun sensitivity	18 (53%)	41 (73%)	35 (23%)	1 (1%)	*0.0675*	***<0.0001***	***<0.0001***	***<0.0001***
Mouth sores	11 (32%)	37 (66%)	33 (21%)	3 (4%)	***0.0024***	***<0.0001***	***<0.0001***	***0.0004***
Arthritis	26 (76%)	44 (79%)	66 (43%)	13 (17%)	*0.8006*	***<0.0001***	***<0.0001***	***<0.0001***
Pleurisy	12 (35%)	31 (55%)	27 (18%)	2 (3%)	*0.0829*	***<0.0001***	***<0.0001***	***0.0006***
Protein in urine	10 (29%)	30 (54%)	18 (12%)	2 (3%)	***0.0302***	***<0.0001***	***<0.0001***	***0.0238***
Seizure	4 (12%)	8 (14%)	6 (4%)	0	*1.0000*	***0.0224***	***0.0012***	*0.1822*
Low blood counts	22 (65%)	38 (68%)	55 (36%)	17 (22%)	*0.8195*	***<0.0001***	***<0.0001***	***0.0362***
Positive ANA	25 (74%)	48 (86%)	32 (21%)	0	*0.1734*	***<0.0001***	***<0.0001***	***<0.0001***
Cold sensitivity	16 (47%)	32 (57%)	43 (28%)	6 (8%)	*0.3894*	***0.0002***	***<0.0001***	***0.0003***
Rapid hair loss	11 (32%)	29 (52%)	25 (16%)	1 (1%)	*0.0838*	***<0.0001***	***<0.0001***	***0.0003***
LFRR Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE**	**ILE/SLE/Rel**	**ILE/SLE/Rel/HC**	**Rel/HC**
Follow-up (After SLE Transition)	**n=72**	**n=100**	**n=159**	**n=127**	***p*-value[a]**	***p*-value[b]**	***p*-value[b]**	***p*-value[a]**
Cheek rash	39 (54%)	57 (57%)	10 (7%)	3 (2%)	*0.7568*	***<0.0001***	***<0.0001***	*0.1520*
Discoid lupus	0	0	0	0	–	–	–	–
Sun sensitivity	47 (65%)	59 (59%)	26 (16%)	8 (6%)	*0.4305*	***<0.0001***	***<0.0001***	***0.0098***
Mouth sores	38 (53%)	50 (50%)	13 (8%)	10 (8%)	*0.7586*	***<0.0001***	***<0.0001***	*1.0000*
Arthritis	58 (81%)	73 (73%)	72 (45%)	29 (23%)	*0.3724*	***<0.0001***	***<0.0001***	***0.0001***
Pleurisy	44 (61%)	57 (57%)	25 (9%)	15 (12%)	*0.6393*	***<0.0001***	***<0.0001***	*0.5631*
Protein in urine	31 (43%)	76 (76%)	21 (13%)	8 (6%)	***<0.0001***	***<0.0001***	***<0.0001***	*0.0748*
Seizure	8 (11%)	25 (25%)	7 (4%)	4 (3%)	***0.0301***	***<0.0001***	***<0.0001***	*0.7598*
Low blood counts	51 (71%)	89 (89%)	59 (37%)	42 (33%)	***0.0081***	***<0.0001***	***<0.0001***	*0.5341*
Positive ANA	46 (64%)	68 (68%)	11 (7%)	2 (2%)	*0.6252*	***<0.0001***	***<0.0001***	***0.0431***
Cold sensitivity	47 (65%)	60 (60%)	22 (14%)	13 (10%)	***0.0422***	***<0.0001***	***<0.0001***	*0.3416*
Rapid hair loss	33 (46%)	48 (48%)	17 (11%)	8 (6%)	*0.8771*	***<0.0001***	***<0.0001***	*0.2126*

Categorical significance determined by [a]Chi-square test or [b]Fisher's Exact test.

p-values in bold are significant at ***p≤0.05****.*

Rel, lupus relatives; HC, healthy controls; ILE, incomplete lupus erythematosus; SLE, systemic lupus erythemtosus.

developed ILE, clinically unaffected relatives, and matched HC, with significant differentiation between the groups ($p<0.01$, **Figure 3D**, 4^{th}-5^{th} columns). This was associated with an increased likelihood of LFRR SLE patients to be positive for autoantibody specificities to dsDNA (44%, $p<0.0001$), chromatin (49%, $p\leq0.0002$), and nucleosome antigens, including Sm (35%, $p<0.0001$), SmRNP (43%, $p\leq0.0001$), and RNP (41%, $p\leq0.0003$) compared to relatives with ILE (1-21%), clinically unaffected relatives (1-9%), and unaffected HC (0-3%, **Table 8**). In contrast, relatives who transitioned to SLE had similar rates of autoantibody positivity to Ro/SSA (25-38%) and La/SSB (11-12%) compared to those with ILE (24-26%, Ro/SSA; 15%, La/SSB) in both LAUREL (baseline and follow-up) and LFRR cohorts (**Table 8**), while being increased compared to matched, clinically unaffected relatives (9-11% Ro/SSA, 1-4% La/SSB) and unaffected HC (2-3% Ro/SSA, 2-3% La/SSB, $p\leq0.0117$, **Table 8**). Although clinically unaffected relatives had similar ANA titers and number of SLE-associated autoantibody specificities detected (**Figure 3**), they were more likely than unaffected HC to be positive for autoantibody specificities toward chromatin (10% Rel vs. 0 HC, $p=0.0017$) at baseline (LAUREL), Ro/SSA (11% Rel vs. 3% HC, $p=0.0393$) at follow-up (LAUREL), and Ro/SSA (9% Rel vs. 2% HC, $p=0.0319$) in the LFRR cohort (**Table 8**).

TABLE 7 | Correlation Between SLE-CSQ Score, ACR Score, and SLE-Associated Autoantibody Specificities in Lupus Relatives.

SLE-SCQ Score vs.	LAUREL (BL) Nested Cohort			LAUREL (FU) Nested Cohort			LFRR Nested Cohort		
	Spearman r	95% CI	*p-value*[a]	Spearman r	95% CI	*p-value*[a]	Spearman r	95% CI	*p-value*[a]
ACR Score	0.526	0.426 to 0.614	***<u>≤0.0001</u>***	0.562	0.467 to 0.645	***<u>≤0.0001</u>***	0.710	0.650 to 0.761	***<u>≤0.0001</u>***
ANA titer	0.328	0.208 to 0.439	***<u>≤0.0001</u>***	0.238	0.113 to 0.357	***<u>0.0002</u>***	0.428	0.332 to 0.514	***<u>≤0.0001</u>***
# of SLE-associated AutoAbs	0.190	0.062 to 0.311	***0.0029***	0.140	0.011 to 0.265	***0.0286***	0.340	0.237 to 0.434	***≤0.0001***
# SLE-associated AutoAbs vs.	**Spearman r**	**95% CI**	***p-value***[a]	**Spearman r**	**95% CI**	***p-value***[a]	**Spearman r**	**95% CI**	***p-value***[a]
# ACR Criteria	0.238	0.113 to 0.357	***<u>0.0002</u>***	0.296	0.173 to 0.409	***<u>≤0.0001</u>***	0.525	0.440 to 0.601	***<u>≤0.0001</u>***
ANA titer	0.313	0.191 to 0.425	***<u>≤0.0001</u>***	0.376	0.259 to 0.482	***<u>≤0.0001</u>***	0.561	0.480 to 0.633	***<u>≤0.0001</u>***

[a]*Spearman correlation Bonferroni corrected* ***p≤0.0017****.*

All p-values ***≤0.05*** *in* ***bold****. All p-values* ***<u>≤0.0017</u>*** ***<u>bold and underlined</u>*** *to denote continued significance with Bonferonni correction.*

ANA, antinuclear antibodies; BL, baseline; FU, follow-up; ACR, American College of Rheumatology; AutoAbs, autoantibodies.

TABLE 8 | SLE-Associated Autoantibody Specificities in Lupus Relatives Who Transition to ILE or SLE[a].

LAUREL Nested Cohort	–>ILE	–>SLE	Lupus Relatives (Rel)	Unaffected HC	ILE/SLE	ILE/SLE/Rel	ILE/SLE/Rel/HC	Rel/HC
Baseline (Prior to SLE Transition)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value***[b]	***p-value***[c]	***p-value***[c]	***p-value***[b]
dsDNA	0	6 (11%)	5 (3%)	6 (8%)	*0.0793*	***0.0275***	*0.0595*	*0.1868*
Chromatin	4 (12%)	7 (13%)	16 (10%)	0	*1.0000*	*0.9024*	***0.0226***	***0.0017***
Ro/SSA	9 (26%)	14 (25%)	15 (10%)	2 (3%)	*1.0000*	***0.0044***	***<0.0001***	*0.0613*
La/SSB	5 (15%)	6 (11%)	6 (4%)	2 (3%)	*0.7415*	***0.0370***	***0.0215***	*0.7220*
Sm	0	2 (4%)	0	1 (1%)	*0.5246*	***0.0339***	*0.1073*	*0.3333*
SmRNP	1 (3%)	4 (7%)	4 (3%)	2 (3%)	*0.6462*	*0.2937*	*0.4168*	*1.0000*
RNP	1 (3%)	8 (14%)	7 (5%)	11 (14%)	*0.1451*	***0.0273***	***0.0164***	***0.0166***
LAUREL Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE**	**ILE/SLE/Rel**	**ILE/SLE/Rel/HC**	**Rel/HC**
Follow-up (After SLE Transition)	**n=34**	**n=56**	**n=154**	**n=77**	***p-value***[b]	***p-value***[c]	***p-value***[c]	***p-value***[b]
dsDNA	1 (3%)	4 (7%)	7 (5%)	6 (8%)	*0.6462*	*0.6305*	*0.6306*	*0.3671*
Chromatin	3 (9%)	5 (9%)	2 (1%)	0	*1.0000*	***0.0156***	***0.0028***	*0.5536*
Ro/SSA	8 (24%)	15 (27%)	17 (11%)	2 (3%)	*0.8068*	***0.0117***	***0.0001***	***0.0393***
La/SSB	5 (15%)	6 (11%)	6 (4%)	2 (3%)	*0.7415*	***0.0370***	***0.0215***	*0.7220*
Sm	0	2 (4%)	1 (1%)	1 (1%)	*0.5246*	*0.1846*	*0.3425*	*1.0000*
SmRNP	1 (3%)	6 (11%)	4 (3%)	2 (3%)	*0.2469*	***0.0386***	*0.0512*	*1.0000*
RNP	2 (6%)	6 (11%)	7 (5%)	11 (14%)	*0.7051*	*0.2575*	*0.0626*	***0.0166***
LFRR Nested Cohort	**ILE**	**SLE**	**Lupus Relatives (Rel)**	**Unaffected HC**	**ILE/SLE**	**ILE/SLE/Rel**	**ILE/SLE/Rel/HC**	**Rel/HC**
Follow-up (After SLE Transition)	**n=72**	**n=100**	**n=159**	**n=127**	***p-value***[b]	***p-value***[c]	***p-value***[c]	***p-value***[b]
dsDNA	1 (1%)	43 (44%)	5 (3%)	3 (3%)	***<0.0001***	***<0.0001***	***<0.0001***	*1.0000*
Chromatin	15 (21%)	48 (49%)	10 (6%)	3 (3%)	***0.0002***	***<0.0001***	***<0.0001***	*0.2560*
Ro/SSA	19 (26%)	37 (38%)	14 (9%)	2 (2%)	*0.1387*	***<0.0001***	***<0.0001***	***0.0319***
La/SSB	11 (15%)	12 (12%)	2 (1%)	2 (2%)	*0.6518*	***0.0001***	***<0.0001***	*0.6504*
Sm	4 (6%)	34 (35%)	2 (1%)	0	***<0.0001***	***<0.0001***	***<0.0001***	*0.5194*
SmRNP	11 (15%)	42 (43%)	4 (3%)	2 (2%)	***0.0001***	***<0.0001***	***<0.0001***	*1.0000*
RNP	11 (15%)	40 (41%)	5 (3%)	1 (1%)	***0.0003***	***<0.0001***	***<0.0001***	*0.4074*

[a]*Seropositivity determined by Bioplex 2200 ANA xMAP assay.*

Categorical significance determined by [b]*Chi-square test or* [c]*Fisher's Exact test.*

p-values in bold are significant at ***p≤0.05****.*

Rel, lupus relatives; HC, healthy controls; ILE, incomplete lupus erythematosus; SLE, systemic lupus erythemtosus.

3.5 Alteration of Select Immune Mediators Associated With SLE-CSQ, Serology, and Classification Status in Lupus Relatives

We have previously demonstrated that circulating immune mediator levels are altered prior to the appearance of autoantibody specificities (1, 2) and clinical disease (1, 2, 11) in the development of SLE, and the number and heterogeneous nature of altered immune pathways increases as patients transition to classified SLE (1, 2).

Given the differences in clinical and serologic profiles, as well as participant-reported SLE-CSQ scores in clinically unaffected lupus relatives vs. those who develop ILE or transition to SLE, we assessed which immune mediators were altered relative to these parameters (**Table 9** [lupus relatives only] and **Table S2** [lupus relatives + HC]). We observed most consistent correlation with plasma levels of the pro-inflammatory mediator SCF, soluble TNF superfamily members, particularly the B-lymphocyte activator BLyS, IFN-associated chemokines, and select adaptive mediators,

TABLE 9 | Correlation Between SLE-CSQ Score, ACR Score, or SLE-Associated Autoantibody Specificities and Immune Parameters in Lupus Relatives.

SLE-SCQ Score vs.	LAUREL (BL) Nested Cohort			LAUREL (FU) Nested Cohort			LFRR Nested Cohort		
	Spearman r	95% CI	*p-value*[a]	Spearman r	95% CI	*p-value*[a]	Spearman r	95% CI	*p-value*[a]
SCF	0.246	0.121 to 0.364	***0.0001***	0.252	0.127 to 0.369	***<0.0001***	0.160	0.050 to 0.266	***0.0036***
BLyS	0.237	0.111 to 0.355	***0.0002***	0.275	0.151 to 0.390	***<0.0001***	0.318	0.214 to 0.414	***<0.0001***
TNF-α	-0.051	-0.179 to 0.079	*0.4320*	-0.159	-0.283 to -0.031	***0.0127***	0.121	0.010 to 0.229	***0.0281***
TNFRI	0.083	-0.047 to 0.210	*0.1955*	0.154	0.025 to 0.278	***0.0161***	0.162	0.051 to 0.268	***0.0033***
TNFRII	0.142	0.012 to 0.266	***0.0271***	0.182	0.054 to 0.304	***0.0045***	0.161	0.051 tp 0.268	***0.0033***
MCP-1/CCL2	0.134	0.047 to 0.259	***0.0367***	0.085	-0.045 to 0.212	*0.1856*	0.180	0.070 to 0.285	***0.0010***
MCP-3/CCL7	0.182	0.054 to 0.304	***0.0043***	0.043	-0.087 to 0.171	*0.5034*	0.088	-0.023 to 0.197	*0.1108*
MIG/CXCL9	0.165	0.037 to 0.289	***0.0096***	0.008	-0.121 to 0.138	*0.5034*	0.048	-0.063 to 0.159	*0.3830*
IP-10/CXCL10	0.049	-0.081 to 0.177	*0.4452*	-0.071	-0.198 to 0.059	*0.2724*	0.158	0.048 to 0.265	***0.0039***
IL-2Rα	0.148	0.019 to 0.272	***0.0210***	0.189	0.061 to 0.310	***0.0031***	0.225	0.117 to 0.328	***<0.0001***
IL-12p70	-0.021	-0.150 to 0.108	*0.7416*	-0.119	-0.244 to 0.011	*0.0641*	0.186	0.077 to 0.291	***0.0007***
IFN-γ	-0.035	-0.163 to 0.095	*0.5902*	-0.087	-0.214 to 0.043	*0.1767*	0.164	0.054 to 0.270	***0.0028***
IL-10	-0.078	-0.205 to 0.052	*0.2265*	-0.148	-0.272 to -0.019	***0.0206***	0.201	0.092 to 0.305	***0.0002***
Active TGF-β	-0.138	-0.262 to -0.009	***0.0314***	-0.127	-0.252 to 0.002	***0.0474***	0.062	-0.050 to 0.172	*0.2648*
ACR Score vs.	**Spearman r**	**95% CI**	***p-value***[a]	**Spearman r**	**95% CI**	***p-value***[a]	**Spearman r**	**95% CI**	***p-value***[a]
SCF	0.298	0.176 to 0.412	***<0.0001***	0.271	0.147 to 0.387	***<0.0001***	0.081	-0.030 to 0.190	*0.1411*
BLyS	0.214	0.087 to 0.334	***0.0008***	0.264	0.139 to 0.380	***<0.0001***	0.398	0.300 to 0.487	***<0.0001***
TNF-α	0.013	-0.117 to 0.142	*0.8426*	-0.177	-0.299 to -0.049	***0.0057***	-0.008	-0.119 to 0.103	*0.8809*
TNFRI	0.017	-0.113 to 0.146	*0.7911*	0.093	-0.037 to 0.219	*0.1497*	0.227	0.117 to 0.329	***<0.0001***
TNFRII	0.062	-0.068 to 0.189	*0.3385*	0.103	-0.026 to 0.230	*0.1071*	0.205	0.097 to 0.309	***0.0002***
MCP-1/CCL2	0.129	0.000 to 0.254	***0.0439***	0.183	0.055 to 0.305	***0.0041***	0.059	-0.052 to 0.169	*0.2806*
MCP-3/CCL7	0.187	0.059 to 0.309	***0.0034***	0.101	-0.029 to 0.227	*0.1157*	-0.085	-0.194 to 0.027	*0.1243*
MIG/CXCL9	0.063	-0.067 to 0.191	*0.3258*	0.032	-0.097 to 0.161	*0.6155*	0.078	-0.034 to 0.187	*0.1584*
IP-10/CXCL10	-0.045	-0.173 to 0.085	*0.4838*	-0.061	-0.189 to 0.069	*0.3412*	0.216	0.107 to 0.319	***<0.0001***
IL-2Rα	0.119	-0.011 to 0.244	*0.0642*	0.212	0.085 to 0.332	***0.0009***	0.288	0.183 to 0.386	***<0.0001***
IL-12p70	0.020	-0.109 to 0.149	*0.7526*	-0.180	-0.303 to -0.052	***0.0047***	0.198	0.089 to 0.302	***0.0003***
IFN-γ	0.001	-0.129 to 0.130	*0.9928*	-0.175	-0.298 to -0.047	***0.0061***	0.048	-0.063 to 0.158	*0.3833*
IL-10	-0.064	-0.192 to 0.065	*0.3163*	-0.219	-0.339 to -0.093	***0.0006***	0.252	0.145 to 0.353	***<0.0001***
Active TGF-β	-0.113	-0.239 to 0.017	*0.0788*	-0.192	-0.314 to -0.065	***0.0026***	0.021	-0.090 to 0.132	*0.7017*
# SLE-associated AutoAbs vs.	**Spearman r**	**95% CI**	***p-value***[a]	**Spearman r**	**95% CI**	***p-value***[a]	**Spearman r**	**95% CI**	***p-value***[a]
SCF	0.136	0.007 to 0.261	***0.0339***	0.789	-0.051 to 0.206	*0.2194*	0.068	-0.043 to 0.178	*0.2182*
BLyS	0.326	0.205 to 0.437	***<0.0001***	0.199	0.071 to 0.320	***0.0018***	0.328	0.225 to 0.424	***<0.0001***
TNF-α	0.036	-0.094 to 0.164	*0.5799*	0.010	-0.119 to 0.139	*0.8749*	0.124	0.013 to 0.231	***0.0246***
TNFRI	0.063	-0.067 to 0.190	*0.3296*	0.570	-0.073 to 0.185	*0.3750*	0.182	0.072 tp 0.287	***0.0009***
TNFRII	0.155	0.026 to 0.279	***0.0153***	0.083	-0.047 to 0.210	*0.1961*	0.230	0.122 to 0.333	***<0.0001***
MCP-1/CCL2	0.194	0.066 to 0.315	***0.0024***	0.899	-0.040 to 0.217	*0.1617*	0.086	-0.026 to 0.195	*0.1198*
MCP-3/CCL7	0.260	0.136 to 0.377	***<0.0001***	0.104	-0.028 to 0.288	*0.1141*	-0.024	-0.133 tp 0.089	*0.6842*
MIG/CXCL9	0.207	0.079 to 0.327	***0.0012***	0.200	0.073 to 0.321	***0.0017***	0.255	0.148 to 0.356	***<0.0001***
IP-10/CXCL10	0.222	0.095 to 0.341	***0.0005***	0.138	0.008 to 0.262	***0.0318***	0.366	0.265 to 0.458	***<0.0001***
IL-2Rα	0.238	0.112 to 0.356	***0.0002***	0.244	0.119 to 0.362	***0.0001***	0.192	0.083 to 0.297	***0.0004***
IL-12p70	-0.005	-0.134 to 0.125	*0.9405*	0.090	-0.216 to 0.040	*0.1636*	0.252	0.145 to 0.353	***<0.0001***
IFN-γ	0.032	-0.098 to 0.160	*0.6230*	-0.059	-0.187 to 0.071	*0.3586*	0.132	0.021 to 0.239	***0.0166***
IL-10	0.017	-0.112 to 0.146	*0.7864*	-0.078	-0.205 to 0.052	*0.2269*	0.285	0.180 to 0.384	***<0.0001***
Active TGF-β	0.053	-0.077 to 0.181	*0.4086*	-0.091	-0.218 to 0.038	*0.1551*	0.164	0.053 to 0.270	***0.0029***

[a]*Spearman correlation Bonferroni corrected* ***p≤0.0036.***

p-values in bold are significant at ***p≤0.05****.*

BL, baseline; BLyS, B lymphocyte stimulator; FU, follow-up; HC, healthy controls; MCP-1, monocyte chemoattractant protein -1; MIG, monokine induced by gamma interferon; IP-10, interferon-γ-inducible protein-10; SCF, stem cell factor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TGF-β, transforming growth factor-β ; LAUREL, Lupus Autoimmunity in Relatives; LFRR, Lupus Family Registry and Repository.

including Th1-type mediators that help drive the production of such chemokines and regulatory mediators IL-10 and active TGF-β. SCF was more likely to be associated with the presence of ACR classification criteria, both prior to (LAUREL baseline) and after SLE classification (LAUREL follow-up and LFRR) whether self-reported (SLE-CSQ score) or medical record confirmed (ACR score), while BLyS was consistently associated with both the presence of ACR classification criteria and the accumulation of autoantibody specificities, both before and after disease classification was reached (**Tables 9** and **S2**). This was also true of IFN-associated chemokines, particularly if healthy individuals were included in the correlation analysis (**Table S2**). The most consistently correlated Th1-type mediator associated with both ACR classification criteria and autoantibody accumulation before and after disease transition was soluble IL-2Rα, while IL-12p70 and IFN-γ had increased correlation with clinical disease after

disease transition, particularly in the LFRR cohort (**Tables 9** and **S2**). Curiously, the regulatory mediators IL-10 and active TGF-β presented with a mix of negative correlations to clinical criteria in the LAUREL cohort and positive correlations with both clinical and serologic features in the LFRR cohort (**Tables 9** and **S2**).

We compared levels of these apparently altered immune mediators prior to (LAUREL baseline) and after disease transition (LAUREL FU and LFRR) in lupus relatives who remained clinically unaffected, developed clinical symptoms that either resulted in ILE or SLE classification, as well as matched healthy individuals (**Figures 4**, **5** and **S5**). Prior to disease transition, levels of pro-inflammatory mediators SCF, BLyS, MCP-3, and IL-2Rα (**Figure 4A**), as well as MCP-1 and MIG (**Figure S5A**) were highest in those lupus relatives in the LAUREL cohort who met clinical ACR criteria at baseline ($p<0.05$). With the exception of MCP-1, these mediators remained elevated pre- and post-transition in lupus relatives who developed ILE or SLE in both the LAUREL (**Figures 4B, C**) and LFRR (**Figure 4D**) cohorts. Of note, IFN-associated chemokines MCP-1 and IP-10, as well as Th1-type mediator IL-12p70, were increased in lupus relatives irrespective of disease transition status, while MIG was more likely to be increased in lupus relatives who developed ILE. TNFRII was increased in all lupus relatives, while TNFRI was equally increased in relatives developing ILE or SLE in the LAUREL cohort, with both further differentiating relatives who entered the LFRR with classified SLE (**Figure S5**).

Conversely, the regulatory mediators IL-10 and active TGF-β, as well as IFN-γ, were lowest in HC and lupus relatives in the LAUREL cohort who met clinical ACR criteria at baseline (**Figure 5A**). These mediators, as well as TNF-α, were highest in the LAUREL cohort at baseline and follow-up in those lupus relatives who remained clinically unaffected or only developed ILE and did not transition to classified SLE (**Figures 5B, C**). In the LFRR cohort, IL-10 was highest in lupus relatives who were clinically unaffected, while active TGF-β, as well as IFN-γ and TNF-α, were elevated in lupus relatives with ILE (**Figure 5D**). These data suggest that some pro-inflammatory mediators are able to possibly overwhelm immune regulation to drive the development and pathogenesis of SLE, while others may be offset by regulatory mediators to either prevent clinical disease or stall it from transitioning to classified SLE.

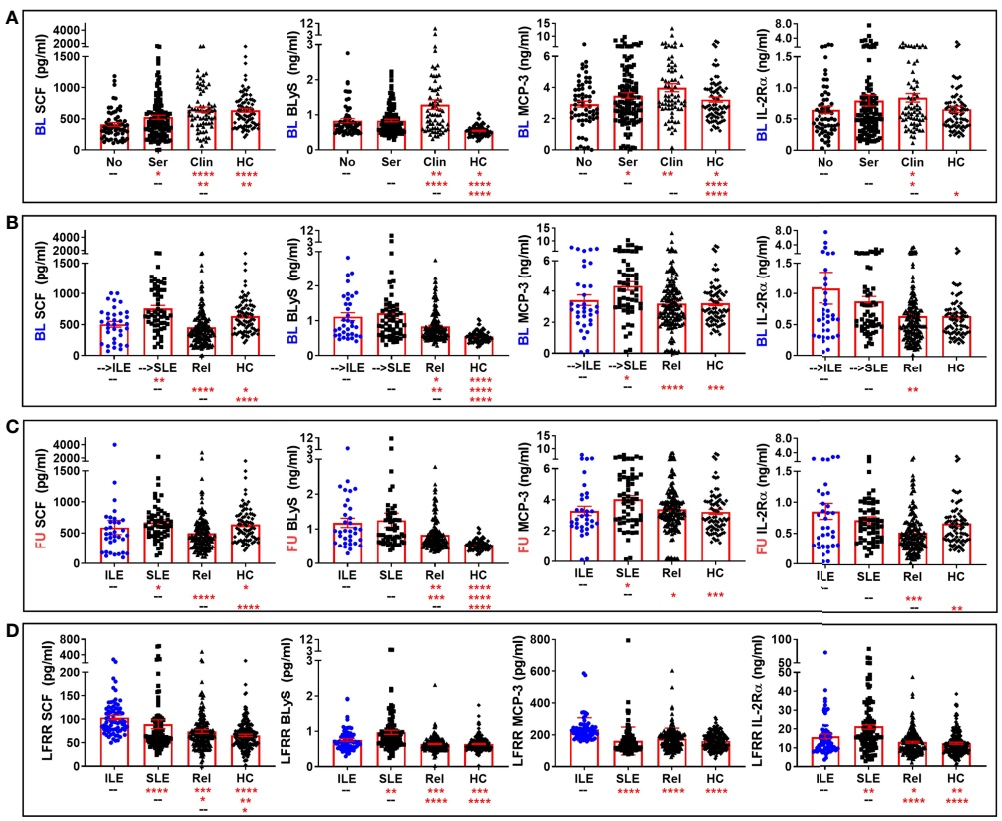

FIGURE 4 | Altered pro-inflammatory mediators in lupus relatives who develop ILE or transition to SLE. Lupus relatives and matched healthy controls (HC) were evaluated for plasma levels of stem cell factor (SCF; *1st column*), BLyS (*2nd column*), MCP-3 (*3rd column*), and soluble IL-2Rα (*4th column*) in **(A)** LAUREL cohort at baseline meeting No ACR criteria (No), only serologic ACR criteria (Ser), or clinical ACR criteria (Clin) vs. matched, unaffected HC and **(B-D)** lupus relatives who developed ILE (ILE), transitioned to SLE (SLE), or remained clinically unaffected (Rel) vs. matched, unaffected healthy controls (HC) in **(B)** LAUREL cohort at baseline (pre-transition), **(C)** LAUREL cohort at follow-up (post-transition), and **(D)** LFRR confirmatory cohort (post-transition). Mean ± SEM. *****p<0.0001*; ****p<0.001*; ***p<0.01*; **p<0.05* by Kruskal-Wallis with Dunn's multiple comparison.

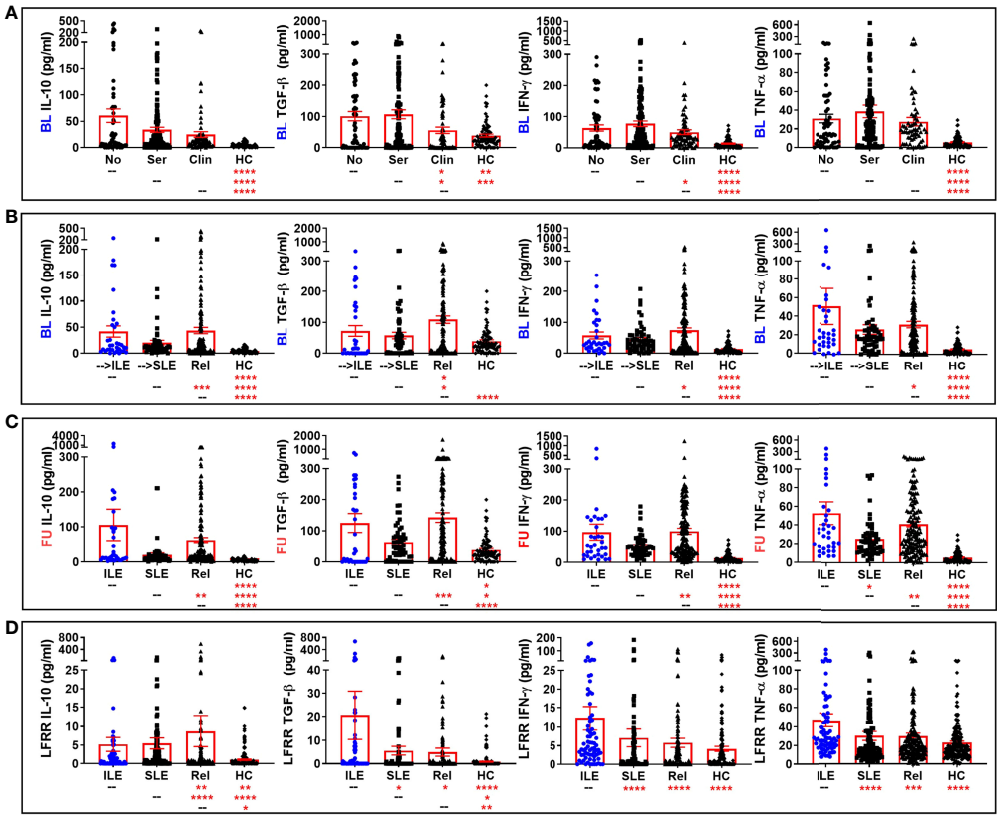

FIGURE 5 | Altered regulatory and select Th1-type mediators in lupus relatives who develop ILE or transition to SLE. Lupus relatives and matched, unaffected healthy controls (HC) were evaluated for plasma levels of evaluated for plasma levels of IL-10 (*1st column*), active TGF-β (*2nd column*), IFN-γ (*3rd column*), and soluble TNF-α (*4th column*) in **(A)** LAUREL cohort at baseline meeting No ACR criteria (No), only serologic ACR criteria (Ser), or clinical ACR criteria (Clin) vs. matched, unaffected HC and **(B–D)** lupus relatives who developed ILE (ILE), transitioned to SLE (SLE), or remained clinically unaffected (Rel) vs. matched healthy controls (HC) in **(B)** LAUREL cohort at baseline (pre-transition), **(C)** LAUREL cohort at follow-up (post-transition), and **(D)** LFRR confirmatory cohort (post-transition). Mean ± SEM. *****p<0.0001*; ****p<0.001*; ***p<0.01*; **p<0.05* by Kruskal-Wallis with Dunn's multiple comparison.

To determine how well soluble mediators differentiated unaffected relatives vs. those who developed ILE or transitioned to SLE, we determined positive/negative cut-off values between Rel and SLE in each cohort based on the Youden Index that maximizes sensitivity and specificity (55). We then compared size effects (odds ratios) across 14 parameters across type 2 symptoms, ACR criteria, SLE-CSQ scores, and soluble mediators that remained significant after Bonferroni correction ($p \leq 0.0036$) when comparing unaffected relatives vs. relatives in the LAUREL cohort at baseline who would transition to SLE (**Figure 6A**, left panel). SCF, IFN-γ, IL-10, and BLyS, alongside reported type 2 symptoms chronic fatigue, depression, and sleep disturbances, probable SLE (SLE-CSQ score ≥4) based on the SLE-CSQ questionnaire, as well as ACR criteria arthritis, photosensitivity, immunologic criteria, and ANA positivity differentiated unaffected Rel vs. relatives who would transition to SLE prior to disease classification. Eleven out of 14 parameters remained significant post-SLE classification in both the LAUREL cohort at follow-up (**Figure 6B**, left panel) and the confirmatory LFRR cohort (**Figure 6C**, left panel). Clinical ACR criteria, positive ANA, and a probable SLE-CSQ score, alongside SCF and BLyS, consistently differentiated unaffected relatives vs. those who developed ILE (**Figures 6A–C**, middle panel), while IL-10, SCF, and ACR criteria best differentiated ILE vs. SLE across the cohorts (**Figures 6A–C**, right panel).

4 DISCUSSION

Reliably identifying those at highest risk of developing lupus clinical features and/or transitioning to classified SLE for early intervention vs. those who do not advance beyond latent autoimmunity remains challenging. Despite the presence of familial genetics (61) and more than two-fold increased frequency of antinuclear antibody (ANA) positivity (51) compared to the general population (62), a considerable majority of lupus relatives will never transition to classified SLE (63, 64). Many will remain clinically unaffected in a state of persistent latent autoimmunity that does not progress beyond serologic features (65, 66). Others may also develop clinical features of SLE with heightened risk of permanent organ damage (67), yet never reach disease classification (41). In both

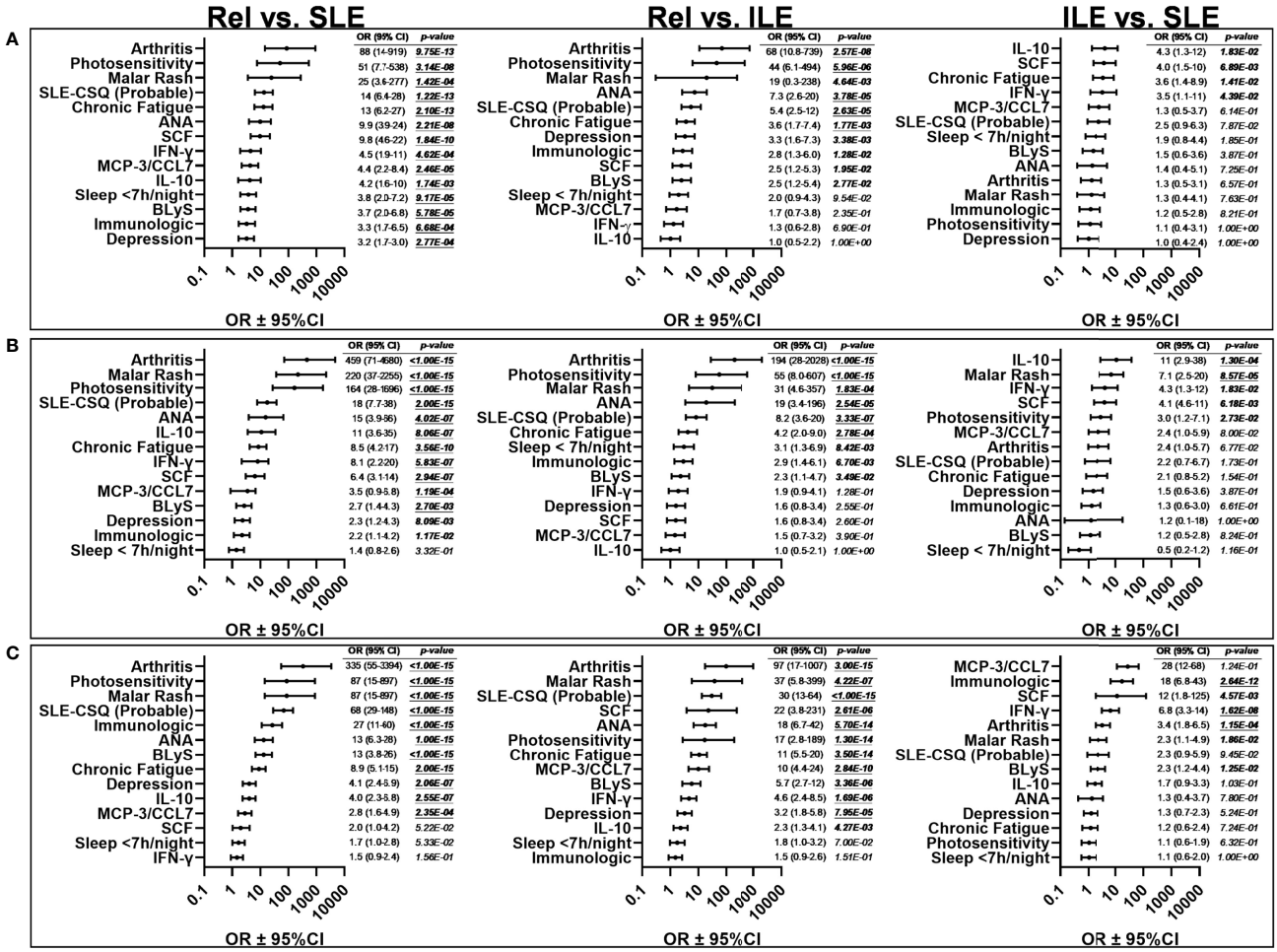

FIGURE 6 | Effect size of informative variables that distinguish lupus relatives prior to and after disease transition in the LAUREL and LFRR cohorts. Odds ratios (± 95% CI) were determined by Fisher exact test for lupus relatives (Rel) vs. relatives who transitioned to SLE, prior to SLE classification in the LAUREL baseline cohort **(A**, Rel vs. SLE**)**, for clinical, serologic, and immunologic differentiating variables as outlined in **Table S3**. Bonferroni's correction for multiple comparison was applied to all significant variables ($p<0.05$); the 14 variables with $p \leq 0.0036$ were considered significant for differentiating Rel vs. SLE **(A)** prior to disease transition. These same variables were assessed for effect size and significance comparing Rel vs ILE and ILE vs SLE prior to disease transition in the LAUREL cohort at baseline **(A)**, as well as Rel vs SLE, Rel vs ILE, and ILE vs SLE after disease transition in the LAUREL cohort at follow-up **(B)**, as well as the LFRR confirmatory cohort **(C)**.

unique cohorts evaluated in the current study (11, 46, 68), lupus relatives without classified disease were more likely to be parents, children, or siblings of SLE patients, while those who had transitioned to classified SLE were noted to be more distant relatives. Although somewhat surprising, other studies have noted similar findings, with adult-onset SLE among families increased among non-first degree relatives (9, 10, 60).

Also of note was that lupus relatives who transitioned to SLE in the LAUREL cohort were older than those with classified disease in the confirmatory LFRR nested cohort, possibly because they were recruited into the LAUREL cohort prior to disease transition at baseline and were more likely to be of European American descent (11, 68). Similar to other studies, we noted in the current study that the potentially later-onset SLE in the LAUREL cohort included more males (69) and more European Americans (70), with a somewhat milder presentation vs. SLE patients evaluated from the LFRR confirmatory cohort, including less renal, hematologic, and immunologic criteria and more mucocutaneous criteria post-transition in the LAUREL cohort (70–73). Yet, those with classified SLE in the LAUREL and LFRR cohorts met roughly the same number of ACR criteria, and others have shown that damage accrual is similar between early- and late-onset SLE (69, 72), with the potential for more co-morbidities in late-onset SLE (70). These findings reinforce the need for astute long-term follow-up of lupus relatives at highest risk of disease transition.

For many, transition to classified SLE has an insidious clinical onset that can be difficult to pinpoint (70), especially since some of the first patient-reported symptoms may include non-specific "type 2" (33, 35) SLE-associated symptoms such as fatigue,

anxiety, depression, chronic headaches, and sleep disturbances (36, 37, 74, 75). Although these were more likely to be present in pre- and post-classification lupus relatives who also met clinical ACR criteria in the LAUREL and LFRR cohorts, with fatigue more prevalent in pre-SLE relatives at baseline, they were also more frequent at baseline and follow-up in clinically unaffected relatives compared to HC in the LAUREL cohort. These findings reinforce the notion of intertwining of type 2 and type 1 (inflammatory/clinical) features in SLE (33, 35), and justify the need for more SLE-specific symptom screening in lupus relatives. Of note, SLE-CSQ scores were consistently increased in lupus relatives and HC in both the LAUREL and LFRR cohorts who reported type 2 symptoms, with the highest scores in relatives who also presented with clinical ACR criteria at baseline and developed ILE or transitioned to SLE at follow-up in LAUREL and the LFRR. Yet, SLE-CSQ scores were also increased in clinically unaffected relatives compared to matched HC.

SLE-CSQ scores were highly correlative with number of medical record confirmed ACR criteria met in both cohorts, even before SLE transition, supporting the utility of SLE-CSQ as a clinical screening tool (11, 41). The increase in SLE-CSQ scores associated with type 2 symptoms suggests that there may also be additional underlying alternate or concurrent non-SLE processes. One candidate that may be present in both clinically unaffected relatives and those who develop ILE or SLE is fibromyalgia, which has been previously noted in SLE patients with either active or inactive disease who experience type 2 symptoms (33). Many fibromyalgia patients are also ANA positive, yet previous studies suggest that ANA positivity is not necessarily predictive of SLE or other autoimmune disease development (76, 77), similar to what we have observed in lupus relatives [(11) and current study]. Another candidate, with or without fibromyalgia, is undifferentiated connective tissue disease (UCTD) (78), particularly in unaffected lupus relatives. Unlike their ILE counterparts, who met both serologic and clinical classification criteria for SLE, and a number of whom were being treated with immunosuppressive medication, clinically unaffected lupus relatives exhibited only ANA positivity and immunologic/serologic manifestations, usually anti-cardiolipin autoantibody positivity. That both ILE and clinically unaffected lupus relatives exhibited increased levels of regulatory immune mediators suggests that the presence of clinical classification criteria may differentiate ILE from UCTD (37, 78) and is supported by the presence of arthritis or photosensitivity being among the greatest differentiators of lupus relatives who remained clinically unaffected or developed ILE, whether in the LAUREL cohort at baseline or follow-up or in the confirmatory LFRR cohort.

Although differences in ANA titer or autoantibody specificity accumulation were not noted with the presence of type 2 symptoms (data not shown), except for sleep disturbances, where no patterns of immune mediator changes were found, there was a consistent increase in plasma BLyS levels, particularly among lupus relatives reporting type 2 symptoms who remained clinically unaffected or only developed ILE. Conversely, increased plasma levels of IL-10 were found in lupus relatives who did *not* report type 2 symptoms, particularly for fatigue. These findings suggest a unique opportunity for intervention in lupus relatives reporting type 2 symptoms with elevated BLyS and/or decreased IL-10 levels, as belimumab has been shown to improve fatigue and quality of life measures in SLE patients (79, 80), while non-pharmacologic modalities such as physical (81, 82) and mindfulness (83) exercises have been shown to increase anti-inflammatory IL-10 levels and decrease fatigue and other type 2 symptoms. Although no immune mediators were found to be associated with sleep disturbances, we observed in the current study that sleep disturbances were more prevalent in lupus relatives meeting clinical ACR criteria at baseline (pre-transition) and that those averaging less than seven hours of sleep/night were more likely to transition to SLE [(57, 84) and current study].

Given that lupus relatives who remain clinically unaffected with respect to SLE classification may have other underlying symptoms that would benefit from clinical assessment and intervention, and that individuals with ILE, even if they never reach SLE classification, are at risk for accumulating organ damage (69, 72), screening approaches to identify lupus relatives for early intervention trials and longitudinal assessment studies would be beneficial to both more closely dissect and address immune dysregulation prior to disease classification (85) and potentially reduce the socioeconomic burden of SLE (86). ANA positivity alone, whether in familial (9–11, 66) or non-familial (1, 87) cohorts, is not predictive of who will develop ILE or transition to SLE. Additionally utilizing the SLE-CSQ, that was found to be strongly associated with medical record confirmed cumulative ACR scores, would add specificity for SLE and negative predictive value without substantial increase in administrative burden, particularly if screening for lupus relatives with SLE-CSQ scores of 3 (possible lupus) or more (probable lupus) (68).

In addition, screening for immune pathway dysregulation in conjunction with ANA positivity may improve our ability to identify individuals at high risk for developing clinical disease (1, 11, 41). In a more limited subset of lupus relatives in the LAUREL cohort, we have previously shown that the pro-inflammatory mediator SCF was an independent predictor of transition to classified SLE (41), with confirmation of enhanced SCF levels in relatives who developed ILE or transitioned to SLE in the expanded group of relatives in the LAUREL and LFRR cohort in the current study. SCF interacts with the receptor, c-kit, to enhance pro-inflammatory adaptive immunity (32, 88) that drives downstream effector mediators that include MCP chemokines, MCP-1 and MCP-3 (31), that were increased in lupus relatives, including those with clinical disease. In addition to being associated with reported type 2 symptoms, plasma levels of BLyS were also observed to be elevated in lupus relatives, particularly those meeting clinical disease criteria who developed ILE or transitioned to SLE. BLyS is produced in response to both type I IFN (IFN-α) (89), a heritable risk factor in SLE (13), and type II IFN (IFN-γ) (21), a Th1-type cytokine affected by signaling through IL-2Rα (90, 91), the soluble form of which was similarly increased in the current study. In addition to its

association with SLE pathogenesis (22) and disease activity and flare (92, 93), BLyS has been shown in previous studies to be elevated as patients transition from autoantibody positivity to clinical disease and transition to classified SLE (1, 2), with blockade of BLyS (23, 24), as well as type I IFN receptors (25, 26) and IFN-γ (27) that drive BLyS, having the potential to improve disease outcomes in subsets of SLE patients.

In contrast, the regulatory mediator IL-10, observed to be decreased in lupus relatives with type 2 symptoms, along with active TGF-β, previously shown to be a negative predictor of SLE transition in a more limited subset of lupus relatives in the LAUREL cohort (41), were both increased in lupus relatives *without* clinical ACR criteria at baseline (LAUREL), as well as clinically unaffected relatives and relatives who only developed ILE, but did not have classified SLE at follow-up (LAUREL and LFRR). That lupus relatives who only developed ILE also had elevated levels of regulatory mediators may explain the mix of negative and positive correlations to SLE-CSQ scores, ACR scores, and autoantibody specificity accumulation in the LAUREL and LFRR cohorts in the current study. Curiously, we observed similar increased levels of TNF-α and IFN-γ in clinically unaffected relatives and relatives with ILE, but not classified SLE, in the current study. One possible explanation is that relatives with classified SLE were more likely to be on immune modifying treatments that may decrease these mediators, particularly if these patients were well managed. We have previously shown that both TNF-α and IFN-γ are maintained at lower levels in the periphery during periods of non-flare, with rising levels precipitating imminent clinical disease flare (18, 19). For clinically unaffected relatives and those who developed ILE, the Th1-type adaptive mediator IFN-γ is among the earliest dysregulated mediators detected in pre-clinical SLE (1, 2), with TNF-α belonging to the same Th1-type cytokine group. The concurrent upregulation of regulatory mediators in these same lupus relatives has the potential to offset underlying basal inflammation in these individuals, while a likely feed-forward effect of accumulating altered inflammatory pathways takes place in those who transition to classified SLE (1, 2).

There are a number of limitations in the current study. Due to the vast majority of lupus relatives entering both the LAUREL and confirmatory LFRR cohorts years before either the SLICC (52) or EULAR/ACR (94) SLE classification criteria were published, it was necessary to utilize the 1997 ACR classification criteria (47, 48) in the current study. Yet, there were similarities in both ACR scores and the recently published SLERPI (50) scores across both LAUREL and the confirmatory LFRR cohorts. The use of unique cohorts necessitated utilization of the nested LFRR cohort as a confirmatory cohort for the follow-up findings in LAUREL. The difference in timing of biological assessments between the cohorts, particularly soluble immune mediators requiring research-use-only multiplex immunoassay platforms that are highly sensitive and specific while sample sparing, but known for inter-user and inter-lot variability (95), precluded the combining of datasets for analysis. Despite this caveat, immune dysregulation noted in LAUREL was largely recapitulated in the confirmatory LFRR cohort. Despite being able to tease out type 2 symptoms in both cohorts, other self-reported data, such as smoking (96) and alcohol consumption (97), were not widely available for analysis in the current study. That being said, a previous study assessing a subset of SLE patients, lupus relatives, and healthy controls with available self-reported smoking data in the LFRR found no association with increased autoantibody production (98). Finally, the LAUREL cohort only provided a single follow-up time point, and unlike the Department of Defense SLE cohort (1, 2), was not able to provide serially collected longitudinal samples for assessment as lupus relatives transition to classified SLE.

Identifying lupus relatives at risk of transitioning to SLE vs. those who may remain in a state of latent autoimmunity is necessary to decrease the rate of early organ damage for those who transition (5) while reducing the necessity for multiple and/or immunosuppressant treatments that perpetuate morbidity and increased healthcare costs (86). In addition to self-reported symptoms as well as serologic and clinical classification criteria, we found in the current study that immune mediator alterations also differentiate lupus relatives who develop ILE or SLE compared to clinically unaffected relatives and HC. Early intervention in SLE may be most effective before the immune system enters a feed-forward, self-sustaining cycle of broken tolerance that results in clinical disease and transition to classified SLE (99). In addition to its potential for treating lupus relatives with type 2 symptoms, discussed above, increased levels of BLyS associated with classification status and the success of belimumab in subsets of SLE patients with classified disease (23) makes this drug a potential steroid-sparing candidate for early intervention in lupus relatives at increased risk of developing clinical disease, particularly those without pre-existing organ damage (100). For those lupus relatives with ILE who meet some clinical ACR criteria, but have not reached SLE classification, hydroxychloroquine may be a viable early intervention candidate (101), with evidence of delayed transition to classified SLE (7) and clinical improvement in patients with ILE (8). Adequate screening using a combination of self-reported assessments and serological immune components, coupled with longitudinal monitoring and early intervention strategies may be the key to maintain clinically unaffected lupus relatives and delaying or preventing disease transition in relatives who already meet clinical classification criteria.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, OMRF and MUSC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MEM designed and carried out experiments, completed data analysis, and principally wrote manuscript. KAY, JMG, and JMN provided experimental and editorial guidance. DLK, GSG, MHW, MLI, DJW, DRK, JBH, and JAJ provided patient data and samples for the LAUREL and LFRR cohorts, as well as editorial guidance. JAJ provided additional support in addition to experimental and editorial guidance. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.866181/full#supplementary-material

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Antibodies to Citrullinated Protein Antigens, Rheumatoid Factor Isotypes and the Shared Epitope and the Near-Term Development of Clinically-Apparent Rheumatoid Arthritis

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Background/Purpose In rheumatoid arthritis (RA) autoantibodies including antibodies to citrullinated protein antigens (ACPA) and rheumatoid factor (RF) can be predictive of incident clinical RA. However, there is limited understanding of how antibody changes over time impact prediction of the likelihood and timing of future clinical RA.

Materials and Methods: We evaluated relationships between ACPA, the shared epitope (SE), RF isotypes and incident RA in a prospective cohort of 90 ACPA(+) individuals without baseline arthritis identified through health-fair testing (i.e. Healthfair). We also evaluated ACPA and RF isotypes and time-to-diagnosis of RA in a retrospective cohort of 215 individuals with RA from the Department of Defense Serum Repository (DoDSR).

Results: Twenty-six of 90 (29%) of ACPA(+) Healthfair participants developed incident RA. Baseline or incident dual RF-IgA and RF-IgM positivity was associated with increased risk for incident RA (HR 3.09; 95% CI 1.15 to 8.29) although RFs were negative in ~50% of individuals with incident RA. SE was associated with increased risk of RA (HR 2.87, 95% CI 1.22-6.76). In the DoDSR cohort, triple positivity for ACPA, RF-IgA and RF-IgM was present a median of 1-2 years prior to RA diagnosis, with some sex-specific differences.

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Conclusion: These findings can be used to counsel individuals at-risk for future RA and to design clinical trials for RA prevention. The findings also suggest that RF could be a surrogate outcome as a success of an immunologic intervention in RA prevention. Additional studies are needed to understand the biologic of different patterns of autoantibody elevations in RA evolution.

Keywords: rheumatoid arthritis (RA), pre-rheumatoid arthritis (pre-RA), antibodies to citrullinated protein antigens (ACPA), rheumatoid factor (RF), prediction of future rheumatoid arthritis, shared epitope (SE)

INTRODUCTION

A number of studies demonstrate that there is a period of seropositive rheumatoid arthritis (RA) development that can be termed 'Pre-RA' during which there are elevations of circulating autoantibodies including antibodies to citrullinated protein antigens (ACPA) and rheumatoid factor (RF) in absence of and prior to the appearance of clinically-apparent inflammatory arthritis (IA) as well as a clinical diagnosis of RA (clinical RA) that may further classifiable by established criteria (1–3). Importantly, these autoantibodies may play a pathogenic role in the development of RA (4, 5); furthermore, the diagnostic accuracy of these autoantibodies for the future onset of clinical IA/RA has underpinned the development of several clinical prevention trials (1, 6–10).

A key aspect of these trials is to use as a component of the inclusion criteria a biomarker profile that is highly predictive for future RA onset (i.e. likelihood of RA) as well as incident RA within a defined time interval to optimize clinical trial design and duration by having highly accurate estimates of expected incidence rates.

Notably, some published data suggest that combinations of ACPA and RF are highly predictive of future RA within a relatively short time period (11–15). In addition, several studies have reported that the presence of the shared epitope (SE) in the setting of ACPA positivity is associated with higher risk of progression to future IA/RA (16, 17). However, many prospective studies evaluating the prediction of future RA have only utilized autoantibody positivity at a single time point or not found conclusive improvements in prediction based on changing autoantibody levels over time (14, 18–20). As such, there is a limited understanding of how longitudinal changes of autoantibody positivity for ACPA and RF may further inform the likelihood and timing of incident clinical IA/RA, as well as potentially provide insights into how various 'endotypes' of RA may develop (e.g. ACPA and RF positive RA, versus ACPA positive alone). To address this gap, herein we have utilized two separate cohorts to evaluate the role of autoantibody positivity over time, as well as the presence of the SE, to define the likelihood and timing of incident clinical IA/RA.

MATERIALS AND METHODS

Study Populations

Two separate cohorts were used in these analyses. The first cohort was created in Colorado from individuals identified with ACPA positivity through health-fair based testing and is termed the 'Healthfair' cohort. As described previously, at a series of Colorado-based health-fairs, individuals who did not have a prior diagnosis of RA were offered the opportunity for blood testing for ACPA (17, 21). Individuals who were positive for the ACPA test anti-cyclic citrullinated peptide (anti-CCP3, Inova Diagnostics Inc., San Diego, CA) were invited to an additional follow-up research visit. If at that visit they were confirmed to be ACPA(+) on repeat testing and did not have prior or current clinically-apparent IA/RA, they were enrolled into a longitudinal follow-up study where questionnaires were administered, serial joint examinations performed (66/68 count by a rheumatologist or trained personnel) and serial autoantibody biomarker testing was performed. Incident clinical IA/RA was identified at scheduled research visits or at *ad hoc* visits if there were changing symptoms, and individuals with IA were classified as having RA by the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria (2). Notably, none of the Healthfair cohort was treated with disease modifying anti-rheumatic therapy prior to the onset of incident RA.

The second cohort is a retrospective case-control cohort created from the Department of Defense Serum Repository (DoDSR) and is termed the 'DoDSR cohort'. The DoDSR is part of a program to monitor the health of US military personnel (22–24) and the creation of the cohort of RA cases and controls that is used herein has been previously described (25–27). In brief, 215 individuals who had a diagnosis of clinical RA were identified based on documentation in the medical record and at least one rheumatologist encounter, and confirmation of diagnosis by medical chart review by a rheumatologist or trained rheumatology nurse from Walter Reed National Military Medical Center (WRNMMC), with 212 (~99%) of cases meeting 1987 RA classification criteria. Material for genetic studies was not available from the DoDSR. Notably, we have previously used this DoDSR cohort to evaluate the relationship between various biomarkers including ACPA. A single isotype of RF (IgM) and calprotectin and the timing of a future diagnosis of RA (27). However, we are including this cohort in these new analyses to validate the findings in the Healthfair cohort, and furthermore we will present new analytic approaches and biomarker findings (e.g. combinations of RF-IgA and RF-IgM isotypes) not previously reported in this cohort.

Autoantibody Testing

Serum samples from the Healthfair and DoDSR cohorts were tested using enzyme linked immunoabsorbent assays (ELISA) for

anti-cyclic citrullinated peptide-3 (anti-CCP3 IgG, Inova Diagnostics Inc., San Diego, CA), and RF-IgA and RF-IgM isotypes (QUANTA Lite platform, Inova Diagnostics Inc., San Diego, CA). Notably, we did not evaluate RF-IgG given it is not widely available for routine clinical testing. All autoantibody testing was performed at the University of Colorado in the Exsera Biolabs, with the technician blinded to the case-control status of samples. Anti-CCP3 positivity was evaluated based on the manufacturer established cut-off of ≥20 units. Following a guideline from the 1987 classification criteria for RA (3), RF-IgA and RF-IgM positivity was determined based on levels present in <5% of two control groups. Specifically, for the Healthfair cohort, we determined the RF cut-offs in a group of 491 randomly selected blood donors from Colorado. For the DoDSR cohort, we used a group of 156 controls selected from the DoDSR who did not have a diagnosis of RA based on chart review; furthermore, these controls were matched to the RA cases on age, sex, race and region of enlistment in the military (26).

Shared Epitope Testing

Genetic material was only available from the Healthfair cohort and it was typed for the presence of HLA alleles containing the shared epitope (SE) using methods previously described (28). Participants were considered SE positive (dichotomous variable yes/no) if one or more allele included the following subtypes: DRB1*0401, *0404, *0405, *0408, *0409, *0410, *0413; *0101, *0102 and *1001.

Statistical Analyses

Healthfair Cohort

We evaluated baseline characteristics between participants who did or did not develop incident IA/RA using Fishers exact test or two sample t-tests as appropriate, and computed descriptive transition rates between different RF positivity statuses for all samples. In addition, we created graphical representations of progression to RA based on baseline factors (e.g. autoantibodies) using Kaplan-Meier curves. For our main analysis, we present time-to-RA from study entry as an outcome in a series of Cox regression models with a time-varying covariate denoting baseline or incident positivity for autoantibodies, with adjustment for SE status and anti-CCP3 levels <=60/>60 units. Differences in IA-free probabilities are tested *via* log-rank tests with type I error rate of 0.05. Finally, we plotted predicted survival curves under several realistic hypothetical trajectories from baseline to repeat testing at 1 year and accounting for changes in various anti-CCP3 and RF isotype states (and stratified by the presence/absence of the SE) using the technique of Smith and colleagues (29).

DoDSR Cohort

Given this cohort was retrospectively created and all cases developed RA we did not utilize it to replicate exactly the analyses in the prospective Healthfair cohort; instead, we focused on analyses that evaluated the relationship between combinations of autoantibodies and the timing of a future diagnosis of RA. We produced summary statistics for variables of interest, and sex-based differences at each sample collection time were conducted using Fisher's Exact tests. For each sample, the time-to-RA was calculated and is presented stratified by positivity status in boxplots. For inference between these strata, time-to-RA was treated as a time-to-event variable and modeled *via* a Cox regression with positivity status as a time-varying covariate (a Markov renewal model), thus the hazard of developing RA after each measurement is assumed to be independent of previous encounters. Additionally, these models are stratified by (e.g. a different baseline hazard estimated for) the number of pre-RA diagnosis samples each person had in the data set to account for the fact that certain patients did not have all measurements. The output of this method is hazard ratios; the factor increase in the hazard of developing RA for each 1-unit increase (or positivity) in each covariate, holding other covariates constant. Finally, to assess pairwise group differences in the time-to-RA among those who had: 1) no positivity, 2) anti-CCP3 positivity, 3) any RF positivity, or 4) anti-CCP3 and dual RF-IgA and RF-IgM positivity, we used a series of pairwise Wald tests. These tests are adjusted for differences in age and gender, and the p-values are adjusted for multiple comparisons using the false discovery rate method of Benjamini-Hochberg (30). Aside from these latter pairwise comparisons, nominal (unadjusted) p-values are presented in the results.

Ethical Considerations

Study activities using the DoDSR data and samples were approved by institutional review boards at the University of Colorado and WRNMMC, and study activities using the Healthfair data and samples were approved by institutional review board at the University of Colorado.

RESULTS

Healthfair Cohort

Descriptive Characteristics

The descriptive characteristics of the Healthfair cohort are reported in **Table 1**. Of the 90 subjects, 26 (29%) developed incident IA/RA after a mean of 731 days (~2 years) and over a mean of 1111 days (~3 years) of follow-up of the entire cohort. All 26 (100%) of those with incident IA met 2010 ACR/EULAR classification criteria for RA at the time of initial identification of their IA.

Baseline Factors and Incident IA/RA

In univariate analyses, compared to individuals who did not develop incident IA/RA, at their baseline visit the individuals who developed incident IA/RA had a higher prevalence of positivity for at least one allele containing the shared epitope, a higher prevalence of an anti-CCP3 level >2 and >3 times the upper limit of normal as well as a higher prevalence of positivity for both RF-IgA and RF-IgM (**Table 1**). There were no significant associations at the baseline visits between incident RA and the presence/absence of joint pain or smoking status (**Table 1**). In addition, at baseline the prevalence of RF-IgM positivity was significantly higher in current and ever smokers, although the prevalence of RF-IgA positivity was not (**Supplemental Table 1**).

TABLE 1 | Characteristics of the Healthfair cohort.

	No incident IA/RA (n=64)	Incident IA/RA (n=26)	P-value
Days to incident IA/RA or last follow-up visit, mean (SD)	1265 (887)	731 (836)	-
Age at baseline visit, mean (SD)	58 (12)	55 (12)	0.263
Age at diagnosis of IA/RA, mean (SD)	-	57 (11)	-
Number of total visits or number of visits prior to incident IA/RA, mean (SD)	**5 (3)**	**3 (2)**	**<0.001**
Female, n (%)	39 (61%)	20 (77%)	0.221
Non-Hispanic white, n (%)	54 (84%)	20 (77%)	0.600
At least 1 allele containing the shared epitope, n (%)	**24 (38%)**	**18 (69%)**	**0.005**
Ever smoker (Baseline visit), n (%)	24 (38%)	11 (42%)	0.812
Current smoker (Baseline visit), n (%)	3 (5%)	1 (4%)	0.114
Self-reported number of painful joints (Baseline visit), median (range)	0 (0-18)	1 (0-24)	0.142
Self-reported presence of >=1 painful joint (Baseline visit), n (%)	30 (47%)	18 (69%)	0.065
Anti-CCP3 positive at standard cut-off level (>=20 units) at baseline visit, n (%)	64 (100%	26 (100%)	1.000
Anti-CCP3 >2 times the upper limit of normal (>40 units) at baseline visit, n (%)	**39 (60%)**	**22 (85%)**	**0.045**
Anti-CCP3 >3 times the upper limit of normal (>60 units) at baseline visit, n (%)	**24 (38%)**	**17 (65%)**	**0.020**
Anti-CCP positive at last visit, or visit prior to incident IA/RA, n (%)	55 (86%)	26 (100%)	0.055
Anti-CCP3 >2 times the upper limit of normal at last visit or visit prior to incident IA/RA, n (%)	40 (63%)	20 (77%)	0.224
Anti-CCP3 >3 times the upper limit of normal at last visit or visit prior to incident IA/RA, n (%)	26 (41%)	16 (62%)	0.102
RF patterns at baseline visit, n (%)			
RF-IgA(-) RF-IgM(-)	49 (77%)	17 (65%)	0.301
RF-IgA(-) RF-IgM(+)	11 (17%)	3 (12%)	0.749
RF-IgA(+) RF-IgM(-)	2 (3%)	0 (0%)	1.000
RF-IgA(+) RF-IgM(+)	**2 (3%)**	**6 (23%)**	**0.007**
RF patterns at last visit, or visit prior to incident IA/RA, n (%)			
RF-IgA(-) RF-IgM(-)	44 (69%)	13 (50%)	0.226
RF-IgA(-) RF-IgM(+)	10 (16%)	6 (23%)	0.543
RF-IgA(+) RF-IgM(-)	5 (8%)	1 (4%)	0.668
RF-IgA(+) RF-IgM(+)	5 (8%)	6 (23%)	0.145
Autoantibody patterns at or after developing incident IA/RA, n (%)			
Anti-CCP3 positive standard cut-off (>=20 units)	n/a	26/26 (100%)	n/a
Anti-CCP3 >2 x upper limit of normal (>40 units)		23/26 (89%)	
Anti-CCP3 >3x upper limit of normal (>60 units)		18/26 (69%)	
RF-IgA(-) RF-IgM(-)		14/26 (54%)	
RF-IgA(-) RF-IgM(+)		5/26 (19%)	
RF-IgA(+) RF-IgM(-)		1/26 (4%)	
RF-IgA(+) RF-IgM(+)		6/26 (23%)	

IA, inflammatory arthritis; RA, rheumatoid arthritis; SD, standard deviation; anti-CCP, anti-cyclic citrullinated peptide; RF, rheumatoid factor; Ig, immunoglobulin; n/a, not applicable. Bold means statistically significant results (i.e. p < 0.05).

In survival models and Kaplan-Meier curves there was a significantly higher incidence of IA/RA in individuals who at baseline were dual positive for RF-IgA and RF-IgM when compared to those who were positive for only one RF isotype, or no RF isotypes (**Figure 1A**). In addition, because the presence of an anti-CCP3 level of >60 units was associated with increased risk for RA in univariate analysis, and that high level is also given additional points towards RA classification in the 2010 ACR/EULAR criteria, and the presence of the SE was also associated with increased risk for incident IA/RA (**Table 1**), we further evaluated the relationship between RF positivity and incident IA stratified by baseline anti-CCP3 levels (<=60 or >60), and the presence/absence of the SE (**Figures 1B–E**). In these analyses, in both SE positive and negative individuals the incidence of IA was significantly higher in individuals who were dual RF-IgA and RF-IgM positive (**Figures 1B, C**), although the lowest incidence of IA/RA was in individuals who were SE negative and did not have at baseline dual positivity for RF-IgA and RF-IgM (**Figure 1C**). In addition, in participants with baseline anti-CCP3 levels >60, the incidence of IA/RA was significantly greater in those with dual positivity for RF-IgA and RF-IgM (**Figure 1D**). However, in participants with baseline anti-CCP3 levels of <=60, while the survival curves visually differed, there were no significant differences in IA/RA incidence between those who developed dual positivity for RF-IgA and RF-IgM (**Figure 1E**).

Longitudinal Biomarker Changes and Incident IA/RA

Descriptions of autoantibody positivity at the last follow-up visit or visit immediately prior to incident IA/RA are presented in **Table 1**, and in more detail in **Supplemental Table 2** and **Supplemental Figure 1**. Overall, most (>50%) of individuals and samples maintained their original pattern of autoantibody positivity over time. However, there were non-significant trends for the individuals who did not develop IA/RA to have lower prevalence of autoantibody positivity than those who developed incident IA/RA. In particular, 9/64 (14%) individuals who did not develop IA/RA lost positivity for anti-CCP3 compared to 0/26 (0%) in those who developed incident IA/RA (p>0.05).

To address the effect of changing autoantibody positivity over time on incident IA/RA, we used a Cox regression model and a time-varying covariate to evaluate the role of baseline and incident RF positivity and risk for incident IA/RA, and

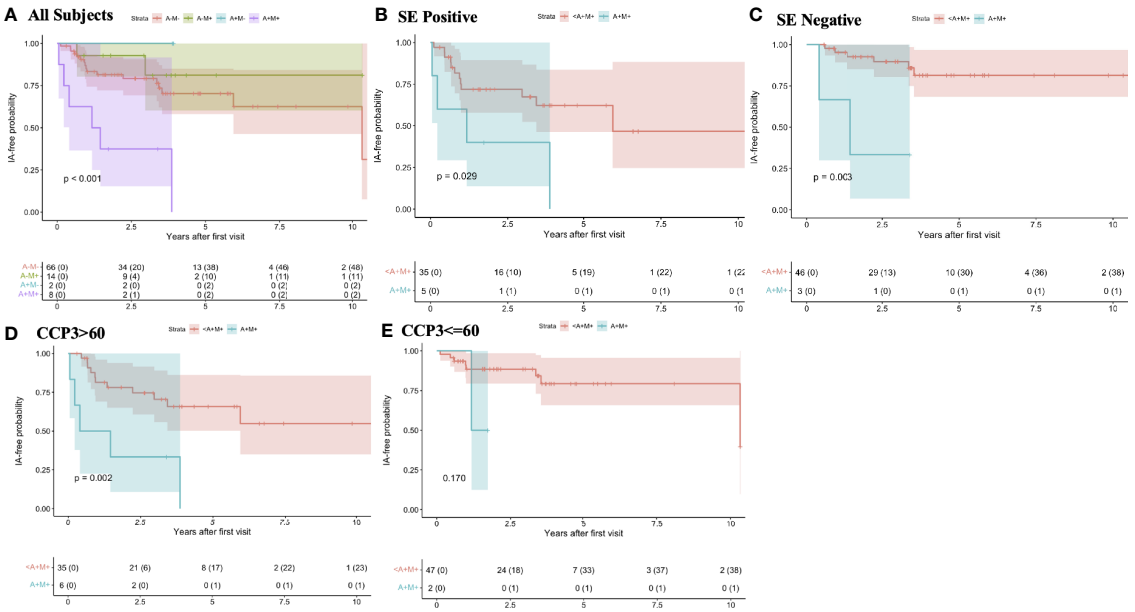

FIGURE 1 | Rates of progression to inflammatory arthritis/rheumatoid arthritis by baseline rheumatoid factor isotype positivity and stratified by shared epitope positivity and baseline anti-CCP3 levels In this cohort, at baseline, all individuals are anti-CCP3 positive at the standard cut-off (>=20 units). In all subjects **(A)** the individuals who were additionally dual positive at baseline for RF-IgA and RF-IgM (purple line) had a significantly greater rate of progression to IA/RA than individuals who were positive for only one RF isotype (blue and green lines), or who were negative for both (red line). In individuals stratified by the presence **(B)** and absence **(C)** of at least one allele containing the shared epitope, baseline dual positivity for RF-IgA and RF-IgM was associated with increased rate of progression to IA/RA (**B**, green lines) compared to individuals who were positive for only one RF isotype or who were negative for both isotypes (**B**, red lines). The lowest incidence rate of IA/RA was in participants who were SE negative and who did not have dual positivity for RFIgA and RF-IgM (**C**, red line). In individuals who had a baseline anti-CCP3 level of >60 units (3 times the upper limit of normal), baseline dual positivity for RF-IgA and RF-IgM was associated with increased rate of progression to IA/RA **(D)**, green line. There was a similar trend in those with anti-CCP3 levels <=60, although this was not statistically significant **(E)**. The colored bands around each line represent 95% confidence intervals. A, rheumatoid factor IgA; M, rheumatoid factor IgM; <A+M+, positive for RFIgA or RFIgM, or neither but not both; SE, shared epitope; IA, inflammatory arthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, anti-cyclic citrullinated peptide antibody; Ig, immunoglobulin.

adjusting for the presence of the shared epitope and anti-CCP3 level positive at >60. In these analyses (the results of which are presented in detail in **Supplemental Table 3**) baseline or incident dual RF-IgA and RF-IgM positivity was associated with a significantly higher risk for incident IA/RA (Hazard Ratio 3.09, 95% Confidence Interval 1.15 to 8.29, p=0.025). The presence of the SE was also significantly associated with increased risk for RA (HR 2.87, 95% CI 1.22 to 6.76, p=0.016); however, positivity for only one RF isotype (RF-IgA or RF-IgM) not associated with a significantly increased risk for incident IA/RA (RF-IgA positive only: HR 1.20, 95% CI 0.16 to 9.32; RF-IgM positive only: HR 1.33, 95% CI 0.47 to 3.78, p=0.5990). In contrast to the univariate analyses, in these multivariate analyses, positivity for anti-CCP3 >60 was not significantly associated with incident RA (HR 1.45, 95% CI 0.62 to 3.39, p=0.390).

We also created hypothetical models to visualize the relationships between various 'states' of autoantibody positivity at baseline as well as at a repeat visit at 1 year, as this could approximate a clinical situation. In these analyses, individuals who were positive for the SE and persistently positive at baseline and 1 year for anti-CCP3 >60 units, and dual RF-IgA and RF-IgM had the highest rate of incident clinical IA/RA (**Figure 2A**). Individuals that transitioned at 1 year from antibody negative to positive (either double RF-IgA and RF-IgM, CCP high, or both), had higher rates of incident clinical IA/RA than the negative at baseline group, while also having lower incidence than hypothetical individuals that were antibody positive from baseline (**Figures 2A**, **B**). In contrast, individuals who had the lower incidence of RA were negative for the SE, persistently had an anti-CCP3 level of <=60 and were persistently negative for RF-IgA and RF-IgM (**Figure 2B**).

DoDSR Cohort

We also evaluated the relationship between anti-CCP3, RF-IgA and RF-IgM positivity and the timing of incident IA in the DoDSR cohort that is described in **Supplemental Table 4**. Notably, this cohort differed from the Healthfair in that pre-RA samples were selected retrospectively from individuals with a known 'future' diagnosis of RA and therefore we could not evaluated likelihood of future RA; furthermore, in the DoDSR cohort the earliest or 'baseline' visit, an individual did not have to be positive for anti-CCP3. In addition, compared to the Healthfair cohort, the participants in the DoDSR cohort had a higher percentage of males, the age of diagnosis of RA is younger, and there was less clinical data available including smoking status, and no genetic tests were available. Moreover, we identified in the DoDSR cohrt that women had a higher prevalence than men of RF-IgA and RF-IgM

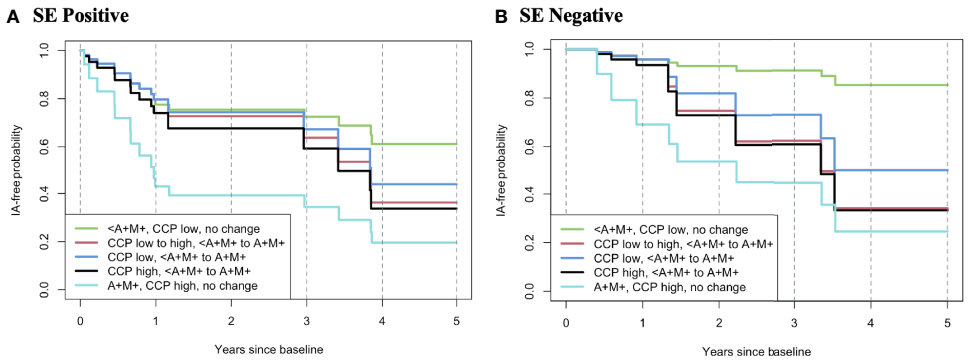

FIGURE 2 | Hypothetical model of rates of progression to inflammatory arthritis/rheumatoid arthritis based on change of autoantibody profile from baseline to 365 days. In this model, all individuals are anti-CCP3 positive at baseline. The rates of progression to IA/RA are modelled using data from the Healthfair cohort and based on a change from a baseline state of autoantibody positivity to a state at 365 day s as this can approximate a clinical care pathway where an individual who has autoantibody positivity without IA/RA is re-evaluated for changes in autoantibody positivity at 1 year. The figures also present models stratified by positivity/negative for the shared epitope. Overall, the highest rate of progression to IA/RA was in individuals who were SE positive and had high anti-CCP3 (>60 units) and dual positivity for RF-IgA and RF-IgM at baseline that persisted at 365 days (**A**, light blue line), with the lowest rate of incident IA/RA in SE(-) individuals with baseline and follow-up low anti-CCP3 (<=60 units) and who were positive for one or less RF isotype (**B**, green line). A, rheumatoid factor IgA; M, rheumatoid factor IgM; <A+M+, positive for RFIgA or RFIgM, or neither but not both; SE, shared epitope; IA, inflammatory arthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, anti-cyclic citrullinated peptide antibody; Ig, immunoglobulin.

positivity at the earliest available time point pre-RA diagnosis as well as a higher prevalence of RF-IgA and RF-IgM positivity post-RA diagnosis (**Supplemental Table 4**), although there were no sex-specific differences in autoantibody positivity in the Healthfair cohort (**Supplemental Table 5**).

In these analyses (**Figure 3**), in women, samples that were negative for anti-CCP3 and both RF isotypes were a median of 5.90 years from a diagnosis of RA compared to samples that were 'triple' positive for anti-CCP3, RF-IgA and RF-IgM that were a median of 1.08 years prior to a diagnosis of RA. In men, samples that were negative for anti-CCP3 and RF were a median of 5.41 years from a diagnosis of RA compared to samples that were triple positive for anti-CCP3, RF-IgA and RF-IgM that were a median of 1.12 years prior to a diagnosis of RA.

DISCUSSION

In the prospectively evaluated Healthfair cohort of anti-CCP3 positive subjects without IA at baseline, we have identified that

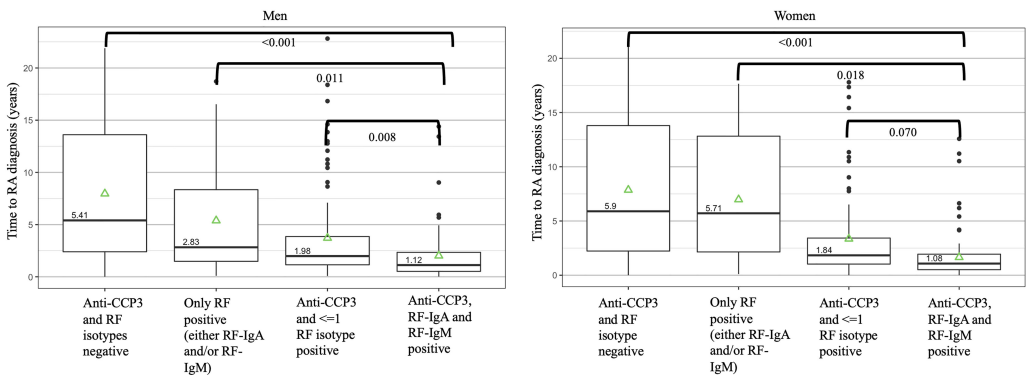

FIGURE 3 | Autoantibody positive states and median time to a future diagnosis of rheumatoid arthritis in the Department of Defense Serum Repository cohort. The times to diagnosis are stratified by men (n=113) and women (N=103) as women had a higher overall prevalence of rheumatoid factor (RF) positivity than men. Overall, positivity for anti-CCP3, RF-IgA and RF-IgM in a sample was seen closest to diagnosis. Of note, while not in the figure, in men, anti-CCP3 positivity at >60 units (with or without positivity for ≤1 RF isotype) was present a median of 1.93 years prior to diagnosis; in women, anti-CCP3 positivity at >60 units (with or without positivity for ≤1 RF isotype) was present a median of 1.64 years prior to diagnosis. P-values represent comparisons between autoantibody positive states using pairwise contrasts and age-adjusted Cox regression model as well as adjusting using the false-discovery method of Benjamini-Hochberg. The green triangles represent the mean time of autoantibody positivity prior to RA diagnosis. DoDSR, Department of Defense Serum Repository; RA, rheumatoid arthritis; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; Ig, immunoglobulin.

baseline or incident dual positivity for RF-IgA and RF-IgM is indicative of a subset of individuals who have a greater likelihood of developing near-term incident IA/RA. Importantly, this was true for 'all comers' who were anti-CCP3 positive at baseline at standard cut-off levels, as well as in individuals stratified by at baseline by the presence of either high-positive anti-CCP3 levels or the SE, although the loss of significance of an associations of high positive anti-CCP3 levels in multivariate analyses suggest that the dual positivity for RFs and SE are stronger predictors of incident IA/RA. Furthermore, in the DoDSR cohort 'triple' positivity of anti-CCP3, RF-IgA and RF-IgM was present closer to diagnosis. In aggregate, these findings support that a combination of positivity of anti-CCP3 and these two RF isotypes, including persistent 'dual' positivity for these RFs over time, is strongly associated with the future onset of clinical IA/RA, as well as imminent RA, with additional influence from the SE.

If an ACPA positive individual is identified who has these factors (e.g. dual RF isotype positivity, SE positivity, potentially high-positive ACPA), it may aid in counseling them as to their overall risk and potential timing of development of future IA/RA as well as referral to clinical rheumatologic care (15). In particular, the hypothetical model presented in **Figure 2** suggests that repeat evaluation for evolving autoantibody positivity at 1 year can be informative, and this may be a 'real life' clinical scenario and follow-up period. Furthermore, these findings may be applied going forward in clinical trial development for RA prevention to identify individuals who are at particularly high-risk for imminent onset of clinical IA/RA – and indeed several existing clinical prevention trials have as inclusion criteria either high-positive ACPA levels, or positivity for ACPA plus combinations of RF isotypes (7–9). Importantly, many prospective studies of pre-RA have utilized individuals who have initially presented to health-care with arthralgia and were subsequently found to have autoantibody positivity (14, 16); while the Healthfair cohort studied herein still had a substantial portion of individuals with some joint symptoms at baseline and therefore may be somewhat comparable to individuals identified through clinics, ~30% of ACPA(+) individuals who later developed RA did not report joint pain at baseline. As such, these findings suggest that approaches such as health-fair ACPA testing can identify individuals at higher risk for development of future RA, and these approaches may be incorporated into future clinical studies.

In addition, most of the current prevention trials in RA are using as primary endpoints clinical IA and classifiable RA. Those are reasonable outcomes given the appearance of clinical IA is currently a key clinical decision point in RA diagnosis and management. However, it may be that incident RF positivity could also be an important surrogate endpoint in preventive interventions in individuals who are ACPA positive. Specifically, while we do not yet know the complete pathophysiologic processes that may drive RF generation in pre-RA, ACPA and dual RF-IgA and RF-IgM positivity is likely indicative of an expansion of autoimmune processes towards a state where initiation of synovitis may be more likely and more imminent (4, 31). As such, an intervention that decreases prevalent or incident dual RF positivity in an ACPA positive individual may potentially decrease an overall risk for future RA. Supporting this notion, in the prospective Healthfair cohort the findings herein suggest that maintenance of RF negativity or the loss of RF positivity is associated with a 'state' that is at lower risk for progression to IA/RA – at least within the duration of the study. Moreover, these findings are similar to what has been described in a longitudinal study of a cohort of indigenous North American People where loss of ACPA and/or RF positivity occurred in individuals who did not develop incident IA/RA (18). Therefore, the 'disappearance' of RA-related autoantibody positivity may be truly associated with decreased risk for progression to clinical RA for some individuals.

A caveat, however, is that while autoantibodies are informative in identifying risk for future RA, autoantibody testing alone provides a limited understanding of the underlying pathophysiologic processes in RA development. In particular, ~77% of those who developed RA within the Healthfair cohort did not have dual RF-IgA and RF-IgM positivity, and an additional subset with incident RA were negative for both RF's and/or had anti-CCP3 levels <=60. Furthermore, while SE was associated with incident RA, ACPA, RFs and incident RA still developed in SE negative individuals in the Healthfair cohort, and ~8% of those who did not develop incident RA were ACPA and dual RF-IgA and RF-IgM positive. Moreover, we have previously published that in the DoDSR cohort described herein a percentage (~20%) of individuals who developed clinical RA were positive for ACPAs and/or RF's at some point in pre-RA yet lost positivity for at least one of those autoantibodies post-RA diagnosis (26). In aggregate, these points support that there are various 'endotypes' of RA risk and development that may be defined by autoantibodies and certain genetic factors (e.g. SE); however, these features are not comprehensive, and furthermore the loss of detectable autoantibodies may not be indicative of a reduced risk for future RA in all individuals. More broadly, these points highlight that additional studies are needed in order to understand the drivers of pathogenic autoimmune processes, autoantibody-related and otherwise (e.g. T cell autoreactivity), that are related to various aspects of RA development including early symptoms and transitions to clinical RA (4, 5, 32–34). These other factors may include environmental factors, mucosal and/or microbial influences (e.g. viral or bacterial) that importantly may also be targets for preventive interventions (33, 35, 36). Notably, in the Healthfair subjects smoking was associated with RF-IgM positivity but not RF-IgA, although smoking was not associated with incident RA; given prior studies associating smoking with RA-related autoantibodies as well as potentially incident RA (37), this will need further exploration.

Notably, the ACPA assay utilized herein was the anti-CCP3 assay and therefore it is not clear that findings herein are applicable to all ACPA assays which may have differing predictive values for future IA/RA (38, 39) In addition, there are multiple other factors including other autoantibody systems [e.g. antibodies to carbamylated antigens and/or other modified proteins (40)], inflammatory markers [e.g. C-reactive protein, serum calprotectin (27)], cytokines, chemokines and cellular

assays (13, 34, 41) as well as clinical features such as joint symptoms (42) that may be incorporated into the prediction of the likelihood and timing of future IA/RA, and these will need further investigation.

A final item of interest was within the DoDSR cohort, women had a higher rate of positivity for RFs than men, although this was not the case in the Healthfair cohort. The reasons for this are not clear, and published studies of rates of RF positivity in patients with clinical RA are conflicting and often not reported in a sex-stratified manner (43). However, a consideration is that the mean age of diagnosis of RA in the DoDSR cohort was younger than most published cohorts, and indeed was ~20 years younger than the mean age at incident RA in the Healthfair cohort. With that, it may be that there is an age-related sex effect on RF development; this needs further exploration to understand the biology of RF development as well as potentially to develop more age and sex-specific prediction models for future RA.

In conclusion, in ACPA(+) individuals dual RF-IgA and RF-IgM positivity as well as the presence of the SE and can be an indicators of a higher likelihood and more imminent onset of clinical seropositive RA. Further studies are needed into the 'endotypes' of RA as well as the biologic relationships between ACPA, RFs, SE in the natural history of RA development.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not publicly available due to institutional review board requirements. Specific requests for data can be requested from corresponding author Kevin D. Deane. Requests to access the datasets should be directed to Kevin.deane@cuanschutz.edu.

ETHICS STATEMENT

Study activities using the DoDSR data and samples were approved by institutional review boards at the University of Colorado and WRNMMC, and study activities using the Healthfair data and samples were approved by institutional review board at the University of Colorado. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB, RP, WT, and KD performed analyses and wrote the paper. MF, LM, and EB performed data and sample management. MP, MF, LM, and EB performed sample testing and results management. DB, MF, CS, MD, LM, EB, JN, VH, and KD recruited and evaluated subjects for the Healthfair cohort. MF, LM, EB, VH, JE, GT, TM, and KD constructed the DoDSR cohort and data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Construction and Application of Polygenic Risk Scores in Autoimmune Diseases

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Genome-wide association studies (GWAS) have identified hundreds of genetic variants associated with autoimmune diseases and provided unique mechanistic insights and informed novel treatments. These individual genetic variants on their own typically confer a small effect of disease risk with limited predictive power; however, when aggregated (e.g., *via* polygenic risk score method), they could provide meaningful risk predictions for a myriad of diseases. In this review, we describe the recent advances in GWAS for autoimmune diseases and the practical application of this knowledge to predict an individual's susceptibility/severity for autoimmune diseases such as systemic lupus erythematosus (SLE) *via* the polygenic risk score method. We provide an overview of methods for deriving different polygenic risk scores and discuss the strategies to integrate additional information from correlated traits and diverse ancestries. We further advocate for the need to integrate clinical features (e.g., anti-nuclear antibody status) with genetic profiling to better identify patients at high risk of disease susceptibility/severity even before clinical signs or symptoms develop. We conclude by discussing future challenges and opportunities of applying polygenic risk score methods in clinical care.

Keywords: autoimmune diseases, genome wide association studies (GWAS), multi-ancestry genetic study, polygenic risk score (PRS), electronic health record (EHR)

INTRODUCTION

There are nearly 100 autoimmune diseases, many of which are rare with prevalence of less than 5 per 100,000 individuals (1, 2). Yet, the prevalence of autoimmune diseases is increasing in recent years. The National Institutes of Health estimates that 14.7-23.5 million people (around 4-7% of the population) are affected in the United States overall (3).

Autoimmune diseases arise from a combination of genetic predispositions and environmental factors that result in the loss of self-tolerance and may cause the immune system to mount a response against the body's own healthy cells and tissues (4). Genetic effects can alter both the innate and adaptive immune systems (5). Likewise, altered immune responses can be triggered by

environmental factors like microbial antigens or environmental toxins, although triggers in many of these disorders, remain unclear. This often leads to the production of autoantibodies and activation of cell-mediated autoimmunity. Some autoimmune diseases target specific cell types (e.g., pancreatic ß-cells in type-1 diabetes or thyroid-stimulating hormone (TSH) receptor in Hashimoto thyroiditis), while others can target a common antigen present in a wide range of cell types (e.g., nuclear antigens in systemic lupus erythematous or systemic sclerosis) (6).

The clinical presentation and severity of most autoimmune diseases are heterogenous due to their complex etiology (7). Moreover, symptoms of different disorders can overlap. As a result, autoimmune disease diagnosis remains challenging. Misdiagnoses of autoimmune diseases are common (8–10) and a correct diagnosis can take several years and multiple physician visits (e.g., rheumatology, endocrinology, hematology, etc.). Delayed diagnoses and treatment can allow disease to progress to advanced stages, affecting multiple organ systems, and even leading to fatality. As a result, early diagnosis and proper treatment management of autoimmune diseases is a clinical necessity.

In this review, we discuss the current states of genome wide association studies for a number of autoimmune diseases and how we can leverage those results to develop polygenic risk scores (PRS) for disease risk prediction based on one's genetic information. We discuss various methods and strategies used to derive PRS models. Finally, in the era of precision using electronic health records, we discuss the clinical utility of combining conventional lab tests with genetic data to improve risk prediction.

GWAS OF AUTOIMMUNE DISEASES REVEALS GENETIC ARCHITECTURE

Genome wide association studies (GWAS) have significantly changed our understanding of the genetic landscape underpinning autoimmune diseases. In this review, we look into 16 autoimmune diseases or traits: ankylosing spondylitis (AS), celiac disease (CEL), Crohn's disease (CD), Grave's disease (GD), Hashimoto thyroiditis (HT), multiple sclerosis (MS), primary biliary cirrhosis (PBC), psoriasis vulgaris (PSO), psoriatic arthritis (PSOAR), rheumatoid arthritis (RA), Sjögren's syndrome (SS), systemic lupus erythematous (SLE), systemic sclerosis (SSC), type 1 diabetes (T1D), ulcerative colitis (UC), and vitiligo (VIT). At the time of this review, there are 179 published GWAS studies that have identified over 350 loci across these 17 autoimmune traits (11).

Due to linkage disequilibrium, significantly associated variants may be correlated and dependent. To properly count GWAS discoveries, we define loci iteratively using the following algorithm. For a given trait, we first rank variants with p-values < $5 \times 10_{-8}$ from the GWAS catalog based on their p-values, from small to large. We define the first locus as a 1 million basepair window surrounding the most significant variant. We then remove all variants in the locus from the list of significant variants and repeat the above procedure to define the next locus until we exhaust all significant variants for the trait. SLE and MS have the most loci identified (159 and 155 loci respectively), while PSOAR and SS have the least (9 and 10 loci respectively) (**Figure 1A**). This disparity could be due to the number of reported studies, sample sizes of each study, heritability of the disorder. It also depends on the effect sizes of causative genetic variants. Some variants involved in certain disorders may have large effect sizes. Individuals carrying the variants will almost surely develop disease. Most other variants have moderate effect sizes, and only slightly increase the disease risk.

GWAS have found pervasively shared genetic basis among autoimmune traits (12, 13). This finding has led to great interest in jointly analyzing GWAS results from different autoimmune traits. For example, Acosta-Herrera et al. conducted the first cross-disease meta-analysis of seropositive rheumatic diseases (SSC, SLE, RA, and idiopathic inflammatory myopathies) (14). This joint analysis enabled identification of five shared immune-related loci that had not been previously associated with these individual diseases. As another example, Márquez et al. performed meta-analysis on data from CEL, RA, SSC, and T1D. This not only allowed them to identify novel genome-wide associations, but also to propose new candidate treatments through drug repositioning analysis (15).

GWAS has also helped reveal the genetic etiology of disease subtypes, which is important given the extensive clinical heterogeneity. For example, Chung et al. performed a GWAS to identify risk loci associated with anti-dsDNA autoantibody production in SLE patients (16). They observed that previously identified SLE susceptibility loci are associated with higher autoantibody production in anti-dsDNA positive SLE patients compared to anti-dsDNA negative SLE patients. This study also importantly underscores the need to identify genetic loci and non-genetic factors in autoantibody-negative SLE patients.

Despite the success of GWAS in characterizing autoimmune diseases, there are areas for further improvement. For example, it is important to identify sex-specific variants, particularly as many autoimmune diseases have a sex bias that are not fully explained by hormonal differences between males and females. For example, the incidence of SS, SLE, HT, GD, scleroderma, myasthenia gravis, PBC, and RA are female biased (17), while T1D and AS are male biased (18). There are also disorders that are not sex biased, such as UC and CD (19). Currently, most studies still pool both sexes together, with little effort to identify whether there is heterogeneity in disease susceptibility variants between female and male (20). Very few studies include chromosome X in their analysis, which is an important omission that needs to be further studied (**Figure 1B**). Inclusion and in-depth analysis of chromosome X and its relation to autoimmune diseases are especially important for sex-biased diseases, e.g., most of SLE and SS cases are females.

In addition, current GWAS studies primarily focused on samples of European ancestry, and thus lack ancestral diversity (**Figure 1C**). This is a rather unfortunate omission, as many autoimmune diseases are more prevalent in non-European populations (21). The lack of diversity hinders our understanding

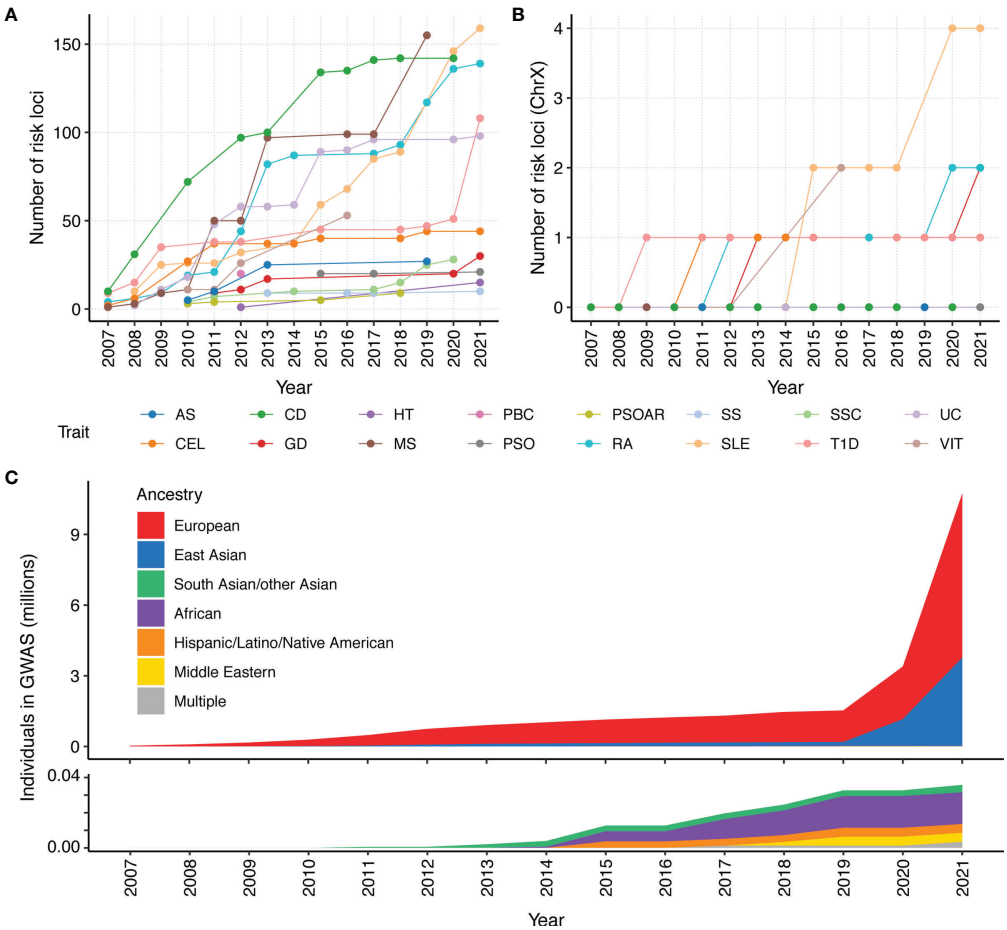

FIGURE 1 | Number of risk loci identified by GWAS for 16 autoimmune traits and ancestry composition per year since 2007. We count the cumulative number of reported loci in GWAS catalog. Each locus is defined as a 1 million basepair window surrounding a genome-wide association signal ($p < 5\times10^{-8}$). All significant variants within a 1 million basepair window are attributed to a single locus. The cumulative number of unique loci that were identified in a year were calculated for the **(A)** whole genome and **(B)** chromosome X. Given that the X chromosome represents approximately 5% of the genome, the paucity of X GWAS loci for most autoimmune disorders makes it clear that the X chromosome is understudied. **(C)** Cumulative assessment of GWAS participants by ancestry over time, according to GWAS catalog. A majority of current GWAS studies are from European ancestry. As people of European ancestry only account for 16% of the population, the non-European population remain under-represented.

of the etiology of autoimmune diseases. Multi-ancestry genetic studies are in great need for further discovery and refinement of disease-associated loci (22). There have been limited multi-ancestry meta-analysis efforts for SLE, RA, CEL, SSC, and T1D. These studies have helped identify novel risk loci (15, 23–32) and improve our understanding of these autoimmune diseases (23, 26, 30, 33).

STATISTICAL METHODS FOR GENETIC RISK PREDICTION

Advances in GWAS of autoimmune diseases have helped reveal biological mechanisms underlying autoimmunity. Another application for GWAS results is to predict whether an individual is at a risk of developing a disease using his/her genotype. A polygenic risk score (PRS) aggregates many risk variants identified from GWAS to formulate a score that predicts an individual's risk for a certain disease. If the score is high in comparison to the population of healthy individuals, the patient has a high probability of developing the disease. Identifying individuals at risk can influence clinical decisions, including frequent monitoring, early detection and/or early intervention before the disease fully develops.

Several methods and strategies existed for creating PRS models (**Figure 2** and **Table 1**). In general, a base GWAS summary statistic and ancestry-matched linkage disequilibrium (LD) reference panel are necessary to develop the ancestry-specific PRS model. When LD information is not available for the individuals analyzed in the GWAS, a LD reference panel from major public genomic resources [e.g. 1000 Genomes Project (61), Haplotype Reference Consortium (62)] can be

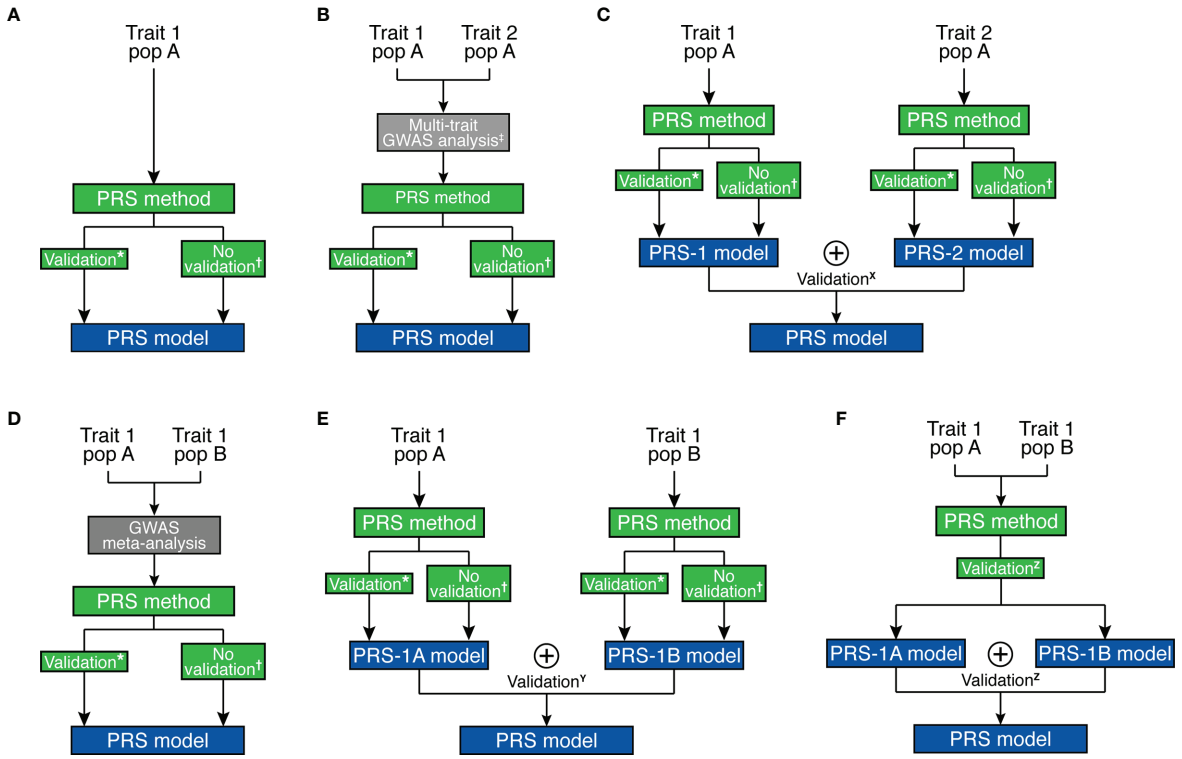

FIGURE 2 | Overview of strategies for polygenic risk score model development. **(A)** Single-trait and single-ancestry framework. **(B)** Multi-trait (at GWAS level) and single-ancestry framework. **(C)** Multi-trait (at PRS model level) and single-ancestry framework. **(D)** Single-trait and multi-ancestry (at GWAS level) framework. **(E)** Single-trait and multi-ancestry (at PRS model level) framework. **(F)** Single-trait and multi-ancestry (at both levels) framework. *Pruning and Thresholding, PRSice, Pruning and Thresholding with functionally-informed LASSO shrinkage, AnnoPred, BayesR, GBLUP, JAMPRED, LDpred/LDpred2, LDpred-funct, PRS-CS, LASSOSUM. †PUMAS, GCTA/SBLUP, GCTB/SBayesR, LDpred-inf, LDpred-funct-inf, PRS-CS-auto, LASSOSUM-pseudovalidation. ‡MTAG, wMT-GWAS, Genomic SEM. X MPS, wMT-SBLUP. Y MultiPRS, PolyPred+. Z PRS-CSx. ⊕ represents the "stacking" method to combine different risk scores.

used as a proxy. Some PRS methods require estimating tuning parameters, thus need an additional validation dataset (**Table 1**).

For the remaining of the section, we will review some methodological advances and challenges of the calculation of PRS for interested readers. Readers who are more interested in applications can safely ignore them and advance to the next section.

The most basic PRS method is pruning and thresholding, also known as clumping and thresholding, which involves two filtering steps. Specifically, the algorithm iteratively: 1) removes variants that are correlated with the top variant within the locus [pruning (37)] and 2) removes variants with a P-value larger than a certain threshold [thresholding (38)]. More sophisticated methods, such as LDpred (46), LDpred2 (47), BayesR (43), and PRS-CS (49) also perform shrinkage estimation by fitting the model using Bayesian methods and using a prior to model the effect size distribution of SNPs in the genome, which allows borrowing strength across different variants. More recently, AnnoPred (42) and LDpred-funct (48) methods further allow incorporation of functional priors to prioritize SNPs located within functionally-annotated regions. Another important class of methods uses penalized regression to build prediction models [e.g. LASSO regression in LASSOSUM (50)], which can be computationally more efficient than Bayesian methods.

Due to the pervasive genetic sharing between different autoimmune diseases, incorporating GWAS datasets from genetically correlated traits may improve the accuracy of genetic effect estimates, which will in turn improve the prediction accuracy of the PRS model. This is particularly appealing for autoimmune diseases with low prevalence. As it is often difficult to collect enough number of cases for less prevalent disorders, borrowing strength from other genetically-correlated autoimmune diseases is beneficial. For example, SLE is a rare autoimmune disease that is clinically and genetically known to overlap with RA and SSC (63, 64). Multi-trait PRS analysis can be performed at two different stages. First, multi-trait association methods [e.g., MTAG (34), wMT-GWAS (35), Genomic SEM (36)] can be used to improve marginal effect estimates, which we can use with other prediction methods to improve prediction accuracy (**Figure 2B**). Alternatively, "stacking" based methods create a weighted combination of PRS for different traits to enhance prediction accuracy, e.g., MPS (57), wMT-SBLUP (35). Stacking-based methods require

TABLE 1 | A list of polygenic risk score and other relevant methods.

Multi-trait GWAS methods	
- MTAG (34)	
- wMT-GWAS (35)	
- Genomic SEM (36)	
Single-ancestry PRS methods	
PRS methods requiring validation dataset	*PRS methods not requiring validation dataset*
Pruning and Thresholding	***Pruning and Thresholding***
- Pruning (37) + Thresholding (38)	- PUMAS (51)
- PRSice (39, 40)	
- Pruning + Thresholding with functionally-informed LASSO shrinkage (41)	
Bayesian Framework	***Bayesian Framework***
- AnnoPred (42)	- GCTA (52)/SBLUP (53)
- BayesR (43)	- GCTB (54)/SBayesR (55)
- GBLUP (44)	- LDpred-inf (46)
- JAMPred (45)	- LDpred-funct-inf (48)
- LDpred (46)/LDpred2 (47)	- PRS-CS-auto (49)
- LDpred-funct (48)	- SDPR (56)
- PRS-CS (49)	
Others	***Others***
- LASSOSUM (50)	- LASSOSUM-pseudovalidation (50)
Multi-trait PRS methods	
- MPS (57)	
- wMT-SBLUP (35)	
Multi-ancestry PRS methods	
Linear combination	
- MultiPRS (58)	
- PolyPred+ (59)	
Bayesian Framework	
- PRS-CSx (60)	

a validation dataset to estimate weights to combine different PRS (**Figure 2C**).

Another important aspect of the PRS model is the transferability of the model across all populations. Currently, ~79% of all GWAS participants are of European descent (**Figure 1C**), which only make up for 16% of the global population. The PRS models developed for individuals of European ancestry often have reduced accuracy for prediction in non-European ancestries (65). Poor PRS transferability may be due to linkage disequilibrium differences, allele frequency differences, causal effect-size differences, and heritability differences between ancestries (59). There is great interest to develop transferable PRS integrating multi-ancestry genetic studies. There are several approaches to integrate multi-ancestry datasets for PRS prediction.

First, multi-ancestry meta-analysis of GWAS can improve marginal genetic effect estimates, which is used for a prediction model to improve prediction accuracy (**Figure 2D**). A second possible approach also uses "stacking" methods to combine PRS models [e.g., MultiPRS (58), PolyPred+ (59)] similar to multi-phenotype analysis (**Figure 2E**). Finally, multi-ancestry meta-analysis and stacking methods can both be applied [e.g., PRS-CSx (60)] (**Figure 2F**). The transferability of PRS depends on the target population and can be improved by prioritizing functional variants (66). For example, Ishigaki et al. demonstrated that the PRS performance for rheumatoid arthritis is comparable between European and East Asian populations when incorporating functional information to prioritize causal variants (67). Importantly, it still remains an open question how to best combine multi-ancestry genetic data to create a better and more transferable PRS model. Despite the advances brought by these methodologies, it is essential to enlarge non-European GWAS sample sizes. For further discussion on development, evaluation, and application of PRS, readers may refer to more thorough reviews on this topic, e.g., Chatterjee et al. (68) and Choi et al. (69).

AVAILABILITY, ACCURACY AND UTILITY OF POLYGENIC RISK SCORE MODELS

At the time of this review, 48 PRS models have been deposited in Polygenic Score (PGS) Catalog for risk prediction for 16 autoimmune traits (**Figure 3**) (70). CEL, T1D, and SLE have the most PRS models, while to date ATD has no PRS models yet (**Figure 3A**). The most commonly used method for building the PRS model across these studies is penalized regression (50, 71–73), followed by weighted sum of the variants from established genes (e.g., from variants that reach genome-wide significance, candidate genes, etc., in contrast to scores constructed based on all variants from GWAS) (**Figure 3B**). The least used methods were pruning and thresholding (37, 38) (**Figure 3B**). Lastly, depending on the method, the number of SNPs used in the PRS model varied. LDpred2, a method assuming polygenicity, retained the most SNPs, ranging from 22,026 to as many as 566,637, while other variable selection methods used less than 2,000 SNPs in the PRS. The number of retained SNPs also critically depends on the genetic architecture of the disease. PRS of highly polygenic traits tend to contain many SNPs, while the traits that are more similar to a monogenic disorder use fewer SNPs in the PRS (**Figure 4**). Using GWAS data from UK biobank (74) along with LASSOSUM method (50), we demonstrated that the Spearman's correlations between number of loci and number of genetic variants in polygenic risk score models are significantly and positively correlated for both quantitative/ordinal traits (**Figure 4A**; Spearman's correlation = 0.74, $p<2.2\times10^{-16}$) and binary/categorical traits (**Figure 4B**; Spearman's correlation = 0.29, $p=4.8\times10^{-10}$). Interestingly, a few outlier traits have many SNPs in the PRS model but relatively few GWAS loci. They are often the ones that were not extensively studied, and the sample sizes are relatively smaller. Thus, the number of known loci were relatively modest.

The most common PRS model performance metric reported is classification accuracy, as measured by the area under the curve of receiver-operating characteristic curve (ROC-AUC). Other studies report risk prediction performance as odds ratio or fold change of the proportion of cases to control in the top

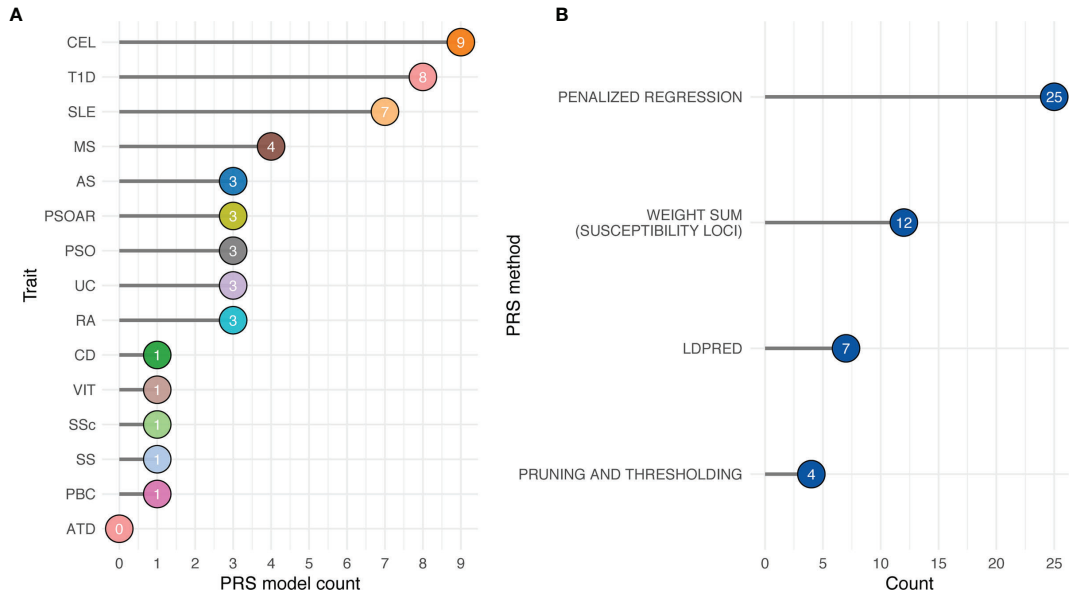

FIGURE 3 | Availability of autoimmune PRS models from Polygenic Score Catalog. **(A)** Number of available PRS models by trait. **(B)** Number of available PRS models by PRS method. *Penalized regression*: LASSOSUM, snpnet, L1-penalized support vector machine. *Weighted sum (susceptibility loci)*: GWAS significant variants, HLA-specific significant variants, GWAS fine-mapped variants, and SNPs curated from literatures. *LDpred*: LDpred and LDpred2.

X^{th} percentile (e.g., top 20^{th} percentile) of the PRS distribution and compare it with the middle or bottom X^{th} percentile of the PRS distribution. Odds ratio or fold change are hard to compare between studies, as different studies use different percentile thresholds. We will only discuss PRS model performance for the studies that reported ROC-AUC.

The PRS models for T1D and CEL showed the best performance when compared to other diseases, which can be attributable to their relatively simple genetic architectures. Every PRS model of T1D had a ROC-AUC greater than 0.75, and some models had a ROC-AUC value greater than 0.9. PRS models for other autoimmune traits had moderate performance, with ROC-

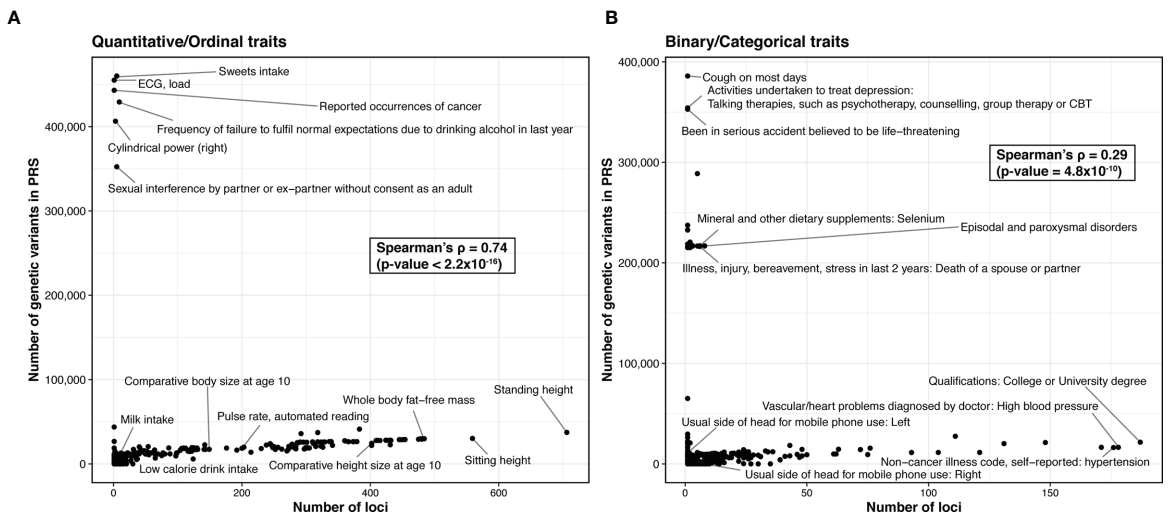

FIGURE 4 | Comparison of the trait polygenicity and the PRS model size. **(A)** Quantitative/ordinal traits. **(B)** Binary/categorical traits. We apply LASSOSUM across GWAS analysis of the UK biobank data (round 2) from http://www.nealelab.is/uk-biobank/. We exclude traits that have no significant variant ($p < 5\times10^{-8}$). For binary/categorical traits, we further excluded traits with number of cases ≤5000. In total, we created polygenic risk score models for 338 quantitative/ordinal traits and 454 binary/categorical traits. We used number of loci identified in UK Biobank as a proxy for the degree of trait polygenicity.

AUC that were greater than 0.6 but usually below 0.75. Almost all PRS models included age, sex, array type (when available), and genetic principal components as covariates in their models.

In addition to utilizing PRS for predicting disease incidence, there is also great interest in investigating the association between a high PRS and disease severity. Reid et al. observed that a high PRS for SLE was associated with earlier disease onset, increased risk of organ damage, renal dysfunction, and all-cause mortality (75). Chen et al. also observed that a high PRS for SLE correlates with poorer prognostic factors like earlier age-of-onset and lupus nephritis (76). Oram et al. observed the PRS for T1D predicted progression to insulin deficiency in diabetic young adults (77). These studies validate the clinical utility of PRS to identify individuals with high risk and susceptible to poor outcomes.

The performance of the PRS models should be interpreted with caution. Most of the PRS models were developed and evaluated using data from European ancestry populations. Due to this bias, several studies have reported decreased predictive performance when applying PRS models from European ancestry to other ancestries. Wang et al. conducted a GWAS for SLE using the Chinese population with a sample size that matches the levels of European studies (78). They developed Chinese and European specific PRS models, and these ancestry-matched models significantly outperformed ancestry-mismatched models by an average ROC-AUC of 0.14. Similarly, a PRS for T1D developed using a European ancestry population performed comparably in non-Hispanic European and Hispanic ancestries (ROC-AUC 0.86 and 0.90 respectively), but it did not perform as well in African Americans (ROC-AUC 0.75) (79). Following this observation, Onengut-Gumuscu et al. conducted a GWAS for T1D on African-ancestry participants and an African-specific PRS model improved prediction (ROC-AUC 0.87) compared to a European-based PRS model (80). Privé et al. investigated the portability of PRS models for 245 traits developed using individuals from Northwestern European ancestry in 9 different ancestry groups (72). Their analysis included several autoimmune traits: hypothyroidism, T1D, MS, UC, CD, SLE, and PSO. They observed an overall significant reduction in the accuracy of PRS models when applied to individuals from other ancestries and the performance systematically decreased as the ancestries became genetically distant from the training data used to train PRS models. Furthermore, some studies had a small number of cases in the external validation dataset (less than 100 samples). Performance metrics like ROC-AUC could be unreliable when there is a substantial imbalance between cases and controls.

FUTURE DIRECTIONS

GWAS to date have identified numerous loci associated with different autoimmune diseases, most of which have small effect sizes. PRS enabled by large GWAS have provided an essential tool for early diagnosis and risk prediction. However, PRS only accounts for a portion of the genetic contribution, and does not fully capture other demographic, lifestyle, environmental, and clinical risk factors that may influence disease risk over time.

Besides PRS, it is also important to incorporate other clinical and demographic variables in the prediction models. For example, many autoimmune diseases have different prevalence between sexes, age group, and ancestries (81): CD and UC affect men and women equally, while SS, SLE, GD, HT, RA, and MS have a greater incidence in female (17). CD and UC have a high incidence in Caucasians and Hispanics (82), while GD is more frequent in the Asian population and less in Sub-Saharan Africans (83). Lifestyle and environmental features also modulate autoimmune disease risk. For instance, cigarette smoking is associated with increased risk of developing GD (84), SLE (85), RA (86), CD (87), and AS (88), but has shown to be associated with reduced risk of SS (89), UC (90), and CEL (91). Other factors like alcohol consumption and exercise habits also play an important role in the risk of developing autoimmune disorders (92). Some of these data are included in electronic health records (EHRs) that are now being adopted worldwide. EHRs are also a valuable source of patient history and clinical data, especially measurements for biological features that are associated with over disease onset. Physical measurements like blood pressure or body mass index, or serological measurements of antibodies or protein biomarkers provide a set of complementary information that we can use to predict the risk of disease development in addition to genetics. We believe integration of these factors with PRS could provide further improvement in estimation of disease risk.

Although limited, efforts are already underway to integrate clinical risk factors with PRS. Knevel et al. developed genetic probability tool (G-PROB) to calculate the genetic-probability (G-probabilities) of multiple related inflammatory arthritis-causing conditions (rheumatoid arthritis, systemic lupus erythematosus, spondyloarthropathy, psoriatic arthritis, and gout) in patients with unexplained joint swelling, as these patients are often misdiagnosed (10). By jointly analyzing probabilities from all diseases, their method was able to attain a reasonable diagnostic accuracy with ROC-AUC of 0.84. They further observed 35% of the patients were misclassified at the initial visit. In comparison, in 53% of patients, the disease with the highest G-probability corresponded to the final diagnosis. In 77% of patients, the final diagnosis was within the top two diseases with highest G-probabilities. This demonstrated that integration of their method with clinical information could significantly improve differential diagnosis.

Similarly, by combining a PRS of SSC with demographic and immunological parameters, Castillo et al. increased model performance by achieving ROC-AUC = 0.787 compared to ROC-AUC = 0.673 with PRS alone (93). Abraham et al. developed a PRS for CEL specific to high-risk individuals with HLA-DQ2.5 risk haplotypes, a marker that is sensitive but not specific (94). The targeted PRS model (ROC-AUC = 0.718) outperformed a PRS model that had been constructed to distinguish all CEL patients (ROC-AUC = 0.679). These studies demonstrate the utility of integrating additional risk factors with PRS, as it allows stratification of the population into different risk categories that will allow better and personalized clinical decision making.

Finally, we have provided a list of routine clinical biomarkers that are typically screened to help autoimmune

TABLE 2 | A list of clinical biomarkers for each autoimmune disease.

Autoimmune disease	Clinical biomarkers
Ankylosing spondylitis	HLA-B27
Celiac disease	Anti-gliadin antibody, anti-endomysial antibody, anti-tissue transglutaminase, deamidated gliadin peptide, HLA-DQ2, HLA-DQ8
Crohn's disease	Anti-Saccharomyces cerevisiae antibody, perinuclear antineutrophil cytoplasmic
Grave's disease	Anti-thyroid-stimulating hormone receptor antibody, thyroid-stimulating hormone, free thyroxine, triiodothyronine, HLA-B8, HLA-DR3
Hashimoto thyroiditis	Anti-thyroglobulin, anti-thyroid peroxidase, anti-thyroid-stimulating hormone receptor antibody, anti-nuclear antibody, HLA-DR3, HLA-DR5
Multiple sclerosis	Oligoclonal IgG bands, HLA-DR2
Primary biliary cirrhosis	Anti-mitochondrial antibody, anti-nuclear antibody, alkaline phosphatase
Psoriasis vulgaris	Rheumatoid factor, anti-nuclear antibody, HLA-B17, HLA-C06
Psoriatic arthritis	HLA-B27
Rheumatoid arthritis	Rheumatoid factor, anti-cyclic citrullinated peptide antibody, HLA-DR4
Sjögren's syndrome	Anti-Ro/SSA antibody, anti-La/SSB antibody, rheumatoid factor, anti-nuclear antibody
Systemic lupus erythematosus	Anti-nuclear antibody, anti-dsDNA antibody, anti-Smith antibody, anti-phospholipid antibodies, C3, C4, HLA-DR2, HLA-DR3
Systemic sclerosis	Anti-nuclear antibody, anti-centromere antibody, anti-topoisomerase I antibody
Type 1 diabetes	Islet autoantibodies, anti-glutamic acid decarboxylase, HLA-DR3, HLA-DR4
Ulcerative colitis	Anti-Saccharomyces cerevisiae antibody, perinuclear antineutrophil cytoplasmic
Vitiligo	Anti-thyroperoxidase antibody, anti-thyroglobulin antibody

disease diagnosis (**Table 2**). Systematic integration of PRS with routine clinical biomarkers is an important next step for PRS to become a useful clinical screening tool.

AUTHOR CONTRIBUTIONS

CK, HM, and DL wrote the first draft of the manuscript. LC, NO, and BJ wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Preclinical Autoimmune Disease: a Comparison of Rheumatoid Arthritis, Systemic Lupus Erythematosus, Multiple Sclerosis and Type 1 Diabetes

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The preclinical phase of autoimmune disorders is characterized by an initial asymptomatic phase of varying length followed by nonspecific signs and symptoms. A variety of autoimmune and inflammatory manifestations can be present and tend to increase in the last months to years before a clinical diagnosis can be made. The phenotype of an autoimmune disease depends on the involved organs, the underlying genetic susceptibility and pathophysiological processes. There are different as well as shared genetic or environmental risk factors and pathophysiological mechanisms between separate diseases. To shed more light on this, in this narrative review we compare the preclinical disease course of four important autoimmune diseases with distinct phenotypes: rheumatoid arthritis (RA), Systemic Lupus Erythematosus (SLE), multiple sclerosis (MS) and type 1 diabetes (T1D). In general, we observed some notable similarities such as a North-South gradient of decreasing prevalence, a female preponderance (except for T1D), major genetic risk factors at the HLA level, partly overlapping cytokine profiles and lifestyle risk factors such as obesity, smoking and stress. The latter risk factors are known to produce a state of chronic systemic low grade inflammation. A central characteristic of all four diseases is an on average lengthy prodromal phase with no or minor symptoms which can last many years, suggesting a gradually evolving interaction between the genetic profile and the environment. Part of the abnormalities may be present in unaffected family members, and autoimmune diseases can also cluster in families. In conclusion, a promising strategy for prevention of autoimmune diseases might be to address adverse life style factors by public health measures at the population level.

Keywords: rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), type 1 diabetes (T1D), prodromal phase, genetic risk factors, environmental risk factors, pathophysiological process

INTRODUCTION

Autoimmune disorders are diseases in which the immune system recognizes and reacts against self-antigens. Clinical onset is often preceded by low grade inflammation (1), disease-specific autoimmune features and nonspecific signs and symptoms. Little is known about similarities and differences between these diseases concerning the time course, nature and extent of inflammatory or autoimmune events. In the last 20 years, the number of individuals affected by autoimmune diseases has increased, especially in the more economically developed countries (2–4). Various mechanisms have been proposed to explain the increased incidence and prevalence, some of which might be shared between different autoimmune diseases.

Individuals in the pre-clinical phase have an initial asymptomatic phase of varying length in which the immune system is activated and the autoimmune process is started. Oftentimes, this phase is followed by nonspecific signs and symptoms and it might take years for the disease to manifest itself. To shed more light on this matter, we here compare the preclinical disease course of a selection of four important autoimmune diseases: rheumatoid arthritis (RA), Systemic Lupus Erythematosus (SLE), multiple sclerosis (MS) and type 1 diabetes (T1D). Although these are clinically distinct diseases, involving different autoimmune reactions and target organs, in some cases they appear to share certain genetic and environmental risk factors as well as pathophysiological mechanisms (5, 6). These insights may help to design strategies to prevent the development or progression of autoimmune diseases in general.

This review describes the evolution of disease manifestations from the pre-clinical phase up to clinical disease when the diagnosis can be made. We thereby focus on similarities and differences between the selected diseases rather than provide an in-depth review per disease. The review is not intended to give an overview of intervention studies in the at-risk phase, since these are discussed in another article of the present issue. The data were collected from literature *via* PubMed and Medline (**Box 1**).

OVERVIEW OF AUTOIMMUNE DISEASES

RA, SLE, MS and T1D are autoimmune diseases which affect specific organs (**Figure 1**), with a later shift towards systemic compromise due to complications and comorbidity. They may also be associated to varying degrees with systemic inflammation. In the pre-clinical stage of autoimmune diseases, individuals have risk factors, both genetic and environmental, which predispose them to the disease. In the next paragraphs, we'll present an overview of those risk factors, and how they might be similar or differ between diseases.

Rheumatoid Arthritis

RA is an organ-specific autoimmune disease mainly characterized by a symmetrical peripheral polyarthritis, in which systemic inflammation and other manifestations may also be associated. RA affects 0,5-1% of the population worldwide, with a higher prevalence in regions at greater distance from the equator (7, 8). RA is seen as an autoimmune disease due to the presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) in the majority of cases, which is then associated with a more severe disease course (9, 10).

In RA there is a familial clustering of disease, and a family history of RA increases the risk of disease by three to ten times (11, 12). This indicates an important role of genetic factors in disease risk. Indeed, more than 100 loci have been found associated to RA (13). The most relevant alleles are the 'shared epitope' (SE) at the *HLA-DRB1* locus and Protein tyrosine phosphatase (*PTPN22*) (9, 14, 15). *HLA-DRB1* codes for a cell surface molecule with a peptide-binding groove that has high affinity towards citrullinated proteins (16, 17). Other important genetic factors in RA are *CLTA4* and *PADI4*, involved in the immune system regulation and post-translational conversion of arginine to citrulline residues, respectively (9, 18). RA has also higher incidence in women, with a female-to-male ration of 2-3:1 (19).

Several environmental factors contribute to the risk of RA. The most prominent one is smoking (20–22), which as a risk factor interacts with SE (17, 23, 24). The increased risk of RA associated with smoking requires long term exposure to manifest, but moderate cigarette consumption is enough to affect disease risk and individuals will have a high RA risk even years after smoking cessation (22, 25). Similarly, other airway irritants such as silica and textile dust exposure are associated with increased risk of RA (21, 26, 27). Additional lifestyle behavior, such as lack of exercise, stress, and an unhealthy diet, all contribute to increasing the risk of developing RA (28–33). Studies that have shown an association between high birth weight and RA suggest that even environmental exposures *in utero* may contribute to the risk for RA (34, 35).

The average age of onset of clinically manifest RA is around 50 years old. The onset is preceded in many cases by a preclinical phase characterized by activation of the immune system and production of autoantibodies. Circulating autoantibodies together with low level inflammation as measured by high sensitive CRP are found on average 5 years before the onset of symptoms (36, 37). In one prediction model (38) using demographic, clinical and serological characteristics, individuals in the highest risk category had an 80% probability of developing RA within 5 years. Immune cell recruitment is usually followed by non-specific musculoskeletal symptoms and fatigue (39). Moreover, pain and transient swelling of the joints are common symptoms in at risk individuals (30-60% of seropositive individuals) (40).

In the years preceding symptoms, the autoantibody response broadens to include more and more ACPA specificities (37, 41–43), anti-acetylated peptide antibodies (AAPA), anti-carbamylated (anti-CarP), and RF, which is referred to as epitope spreading

BOX 1 | Search strategy and selection – We searched MEDLINE for publications in English using the terms "rheumatoid arthritis", "systemic lupus erythematosus", "multiple sclerosis", and "type 1 diabetes", "risk factors", "preclinical", "prodromal", "asymptomatic", and MEDLINE subheadings. We selected articles based on our opinion of their scientific importance. We focused on original research articles, and selected reviews from highly authorative journals. We provide an overview of four autoimmune diseases, comparing their similarities and differences in their preclinical stage.

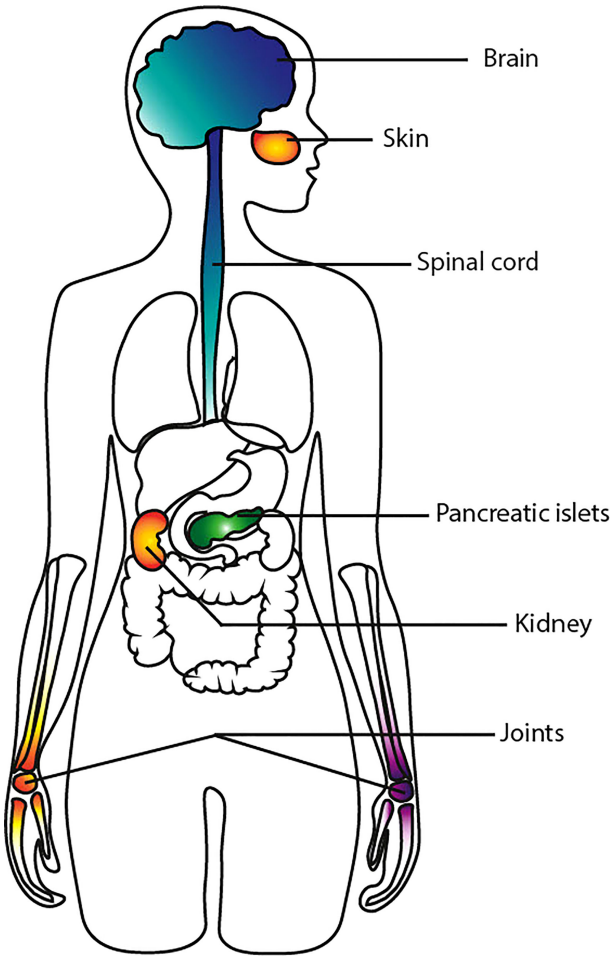

FIGURE 1 | Primary site of onset. This illustration shows the primary site of initiation of the autoimmune process in RA, SLE, MS, and T1D. RA is represented in purple, SLE in orange, MS in blue and T1D in green.

(44–48). In the months before clinical onset, ACPA additionally undergo glycosylation changes both of the Fc part and the Fab part of the ACPA-IgG molecule, leading to a more pro-inflammatory phenotype (49–51). It has been hypothesized that environmental exposure related to the respiratory tract (smoking, dust, respiratory infections) might be involved in antibody production and disease pathogenesis (21, 26, 27). Those environmental triggers could cause low-level inflammation of the lung mucosae, leading to protein citrullination (23, 52).

In patients with early RA, studies of low level inflammation at mucosal sites such as the gums and the lungs have revealed that these inflammatory lesions can be involved in local ACPA production. Transfer of ACPA to the joints may then be one mechanism that incites inflammation at the joint level. Soluble factors also regulate the immune response, and both pro-inflammatory and anti-inflammatory cytokine levels are altered in the preclinical phase (53). Markers of inflammation, such as C-Reactive Protein (CRP), are also increased up to 5 years before RA onset and positively correlate with antibody levels. Individuals with both elevated CRP and autoantibodies are more likely to develop RA (36, 37, 54). Increased plasma levels of polyunsaturated fatty acid-derived lipid mediators such as 5-Hydroxyeicosatetraenoic acid (5-HETE) are seen in ACPA positive individuals who later develop inflammatory arthritis (IA), further increasing the risk and pointing to a low omega3 fatty acid status. Cytokines associated with 5-HETE, such as IL-1β, IL-6, IL-8, and TNF, are also altered in preclinical RA individuals (55, 56).

In the phase with vague symptoms such as stiffness or arthralgia, inflammation of the joints can sometimes already be detected by imaging modalities as ultrasound, MRI and PET scan. In particular, subclinical inflammation of the joints detected by MRI predicted RA onset by a few months (57–60). At the time RA is diagnosed, patients usually have symmetrical polyarthritis in the hands and/or feet, which when left untreated can progress to joint destruction (61, 62). In established RA there is an overlap with other diseases, or comorbidity, including cardiovascular disease, chronic lung disease and periodontitis (63, 64).

Systemic Lupus Erythematosus

SLE is characterized by a great variety of clinical manifestations, including inflammatory skin lesions, arthritis, pleurisy and pericarditis, inflammation in the internal organs, involvement of the central or peripheral nervous system, hematological manifestations, and others. The disease course is highly variable, some patients experiencing long periods of remission (the absence of disease manifestations), but many more experiencing frequent flares of disease activity and/or chronic symptoms. For many patients, the general feeling of illness, accompanied by fatigue, lassitude, and minor cognitive difficulties is the most burdensome feature of the disease.

SLE is an uncommon disease with wide geographic variation in distribution, with high frequency in North America; SLE also has higher frequency in the Afro-American population compared to Caucasians, which may be due to both genetic and environmental differences (65). There is a genetic component of disease, with concordance in monozygotic twins of 24-35% as compared to 2-5% in dizygotic twins (66). In the Caucasian population, *HLA-DRB1*1501* and *HLA-DRB1*0301* are associated with a 2-to-3 fold increase risk of SLE (67, 68). Other genes strongly associated with SLE are those coding for the complement system and the Fc-γ receptor (FcγR), all of which have a role in immune regulation (69–71). Genes that are involved in the IFN pathway, such as Interferon Regulatory Factor 8 (*IRF8*), *IFIH1*, Toll-like receptor 7 (*TLR7*), and Tyrosine Kinase 2 (*TYK2*) are also risk loci for the disease (72, 73). SLE also affects women much more frequently than men (female-to-male ratio 9:1) (65, 74, 75).

Smoking is a risk factor for SLE and is associated with higher anti-double strand (anti-dsDNA) antibody production (76). In the Nurses' Health Study, nurses that smoked had a 67% increased risk of developing SLE compared to non-smokers, although the intensity of smoking did not influence disease risk. This association is time-sensitive, and the increased SLE risk persists for up to five years after quitting (77). It has been suggested that vitamin D may have a role in SLE pathogenesis and progression, and vitamin D supplementation might ameliorate inflammatory and hemostatic markers, however, this is controversial (78, 79). Lack of sleep is also associated with the transition to SLE in one study (80).

SLE occurs at all ages but the peak incidence is in the 3rd and 4th decades of life, and men have a later peak incidence compared to women (81, 82). Studies of the evolution of SLE from a healthy state through a preclinical phase to full-blown disease are complicated by the fact that the diagnosis of SLE cannot be made until sufficient clinical manifestations have occurred, to give the clinician the confidence that the diagnosis is correct. Thus, it is quite common for individuals to experience some joint pains and skin lesions for several years without a diagnosis. But then, an episode of pleurisy and the discovery of antinuclear (ANA) and anti-dsDNA antibodies leads to the diagnosis of SLE. No serious observer can doubt that the earlier joint and skin symptoms were manifestations of the same disease *process*, yet it would not have been correct to make the diagnosis of SLE at that time. In some cases, intermediate disease categories are used, such as "incomplete lupus" or "undifferentiated connective tissue disease", but lack of uniform definitions and the variety of clinical and laboratory manifestations that are seen have hampered further progress.

Thus, it has been challenging to investigate the pre-clinical phase of SLE. A landmark study by Arbuckle et al. found that the emergence of autoantibodies preceded the clinical disease by many years, and there seemed to be a strict order by which they manifest: the first antibodies to appear are ANA, antiphospholipid, anti-Ro (SS-A) and anti-La antibodies (SS-B), which manifest at the same time. Anti-Ro antibodies are detectable in the serum approximately four years before SLE clinical manifestations. Subsequently, anti-dsDNA antibodies become manifest months before clinical diagnosis, followed by anti-Sm and anti-nuclear ribonucleoprotein (anti-RNP) antibodies, whose levels start increasing exponentially up to a year before diagnosis and are highest just before the disease is diagnosed (83, 84).

SLE also has alterations in levels of pro-inflammatory cytokines long before the onset of clinical signs and symptoms, with increased type I and II interferon (IFN-I and IFN-II), IL-5, IL-6, IL-17, and TNF (84, 85).

Multiple Sclerosis

MS is an inflammatory demyelinating disorder of the central nervous system (CNS) with a presumed autoimmune pathogenesis. Several genetic, environmental and lifestyle risk factors are reported. A latitudinal gradient has been found, i.e. the farther away from the equator the frequency of MS increases. This latitudinal risk factor may reflect differences in UV radiation, sun exposure, vitamin D levels and epigenetic interactions. Migration from a higher to a lower latitude after puberty has an impact on disease risk: migrants retain its original risk (6, 86, 87). Genetic predisposition has a role in disease susceptibility, with 5% disease concordance in dizygotic twins that increases to 25% in monozygotic twins (87). HLA alleles exert the most common genetic risk factors, in particular the *HLA-DRB1*1501* haplotype has been demonstrated to be the most significant genetic risk factor to develop MS (odds ratio approximately 3) (5, 88). More than 500 small nucleotide polymorphisms (SNPs) are associated with MS risk, involving mostly immune associated genes, such as IL-2 receptor subunit alpha *(IL2RA)*, *IL7R*, *CLEC16A* and *CD226* (5). MS is more frequent in women, with a female-to-male ratio of 2-3 (89).

Cigarette smoking contributes to the risk of MS, with a 50% higher risk in ever smokers compared to never smokers (86, 87). Two environmental factors that influence MS risk are vitamin D levels and Epstein-Barr virus (EBV) infection (6, 86, 87, 90, 91). Vitamin D deficiency in earliest stages of life is associated with increased risk of MS, while high sun exposure during childhood correlates with lower risk of disease (5, 6). In addition, the Nurses' Health Study showed a 40% decrease risk of MS in women that had at least 400 international unit (IU) of vitamin D intake per day. Childhood obesity is associated with a higher risk to develop MS (86, 87, 92). Although the mechanism of action has not been fully elucidated yet, EBV infection seems to be a causative and necessary but not sufficient agent to develop MS

(5, 86, 87, 90, 91). Recently, Lanz et al. demonstrated a high-affinity molecular mimicry between the glial cell adhesion molecule (GlialCAM) in the CNS and EBV nuclear antigen 1 (EBNA1). Considering that nearly 100% of MS patients has detectable anti-EBNA1 antibodies before clinical symptoms, it suggests that molecular mimicry may play a role in the pathophysiological mechanism to induce MS (93). Age of infection also influences disease risk, with 2-to-3 fold higher risk in individuals with EBV infection at later age (87).

In general patients are identified when they first manifest signs and symptoms characteristic for this disease (94). Most patients are diagnosed between age 20 and 40 year, however children and people of older age may also be diagnosed with MS (89). The clinical phase of MS is preceded by a latent period, in which a prodromal phase of MS can be identified (95). The prodromal phase can manifest 10-15 years before symptom onset, even up to 20 years in primary progressive MS (PP-MS). In this phase, an early set of sign and symptoms that predates classical MS symptoms start to manifest (96). A subclinical inflammation (SCIN) phase seems to be the first step of disease pathogenesis (94). While no formal biomarkers of the prodromal stage are available, the radiologically isolated syndrome (RIS) might be considered a neuroimaging biomarker (96, 97). In RIS, the CNS shows lesions similar to those identified in MS patients without clinical symptoms suggestive of MS, with areas of the brain and the spinal cord that show signs of damage and scarring (97).

Serum neurofilament light chain (sNfl) is indicative of ongoing neuraxonal degeneration, and can be used as a biomarker for neuronal injury. MS patients usually have high levels of sNfl that decrease after treatment with disease modifying therapies, and MS risk positively correlates with higher sNfl levels in a time-dependent manner, starting several years before MS (median of 6 years) (96, 98). In the earliest stages of disease, the adaptive immune system is mostly involved in pathogenesis, in particular with autoreactive T cells, B cells, and autoantibody production against myelin proteins (99, 100). T and B cell in spinal fluid are altered in prodromal MS, and present a pro-inflammatory cluster, with high percentage of expanded CD8+ T cells within the neuronal lesion (94).

MS can either manifest as episodes of inflammation with neurological symptoms followed by partial or total remission (relapsing remitting MS, RRMS, 85% of patients), or as a gradually progressive disease (PPMS). In time, RRMS may evolve into a progressive phase of the disease called secondary progressive MS (SPMS). Depending on the site of the lesion, patients may have different clinical pictures. Common presenting symptoms in RRMS are optic neuritis and ascending sensory symptoms, whereas PPMS in general presents with progressive motor impairment (88).

Type 1 Diabetes

In T1D autoimmunity targets the beta-cells of the pancreas eventually resulting in absolute insulin deficiency. Similarly to the diseases mentioned above, T1D incidence is also affected by the latitudinal gradient and migration, with increased disease risk when populations move from low-incidence to high-incidence countries (6, 101). However, genes have a relevant role in disease risk, and relatives of T1D patients have a 15-20 times higher risk of developing T1D, rising from about 0,4% in the general population to 25-50% in monozygotic twins (102). Familial risk is mostly linked to HLA genes, and decreases to 1% in non-HLA genes (5). HLA are the most common alleles involved in T1D, but other relevant genetic risk factors include genes involved in the insulin and metabolism, as well as regulators of the immune response (5, 103).

In contrast with the other diseases discussed here, there is no demonstrated association between smoking and T1D (6), which might be explained more by the young age of patients at disease onset than by a true lack of a role of smoking in disease pathogenesis. Low physical activity, psychological stress and psychological trauma are associated with T1D risk (101). Vitamin D supplementation leads to lower autoantibody levels which may be beneficial in the early stages of disease (5, 15).

Diet may also influence T1D risk, as there is an increased risk in overweight children (101). Cow's milk consumption is associated with islet autoimmunity (IA) and pancreatic beta cell destruction (15, 101). Other possible risk factors for T1D are viral infections, such as enterovirus, Coxsackie B viruses (CBVs), and respiratory viruses. Viral infections seem to correlate with incidence of islet autoimmunity (5, 101).

T1D present two peaks of incidence at 4-7 years old and – more commonly – 10-14 years of age (104, 105). T1D pathogenesis is characterized by three stages, two of which compose the preclinical phase. The first, asymptomatic stage involves immune recognition and activation with autoantibody production, initial beta cell destruction, but absence of dysglycaemia. In the second stage, progressive islet destruction and loss of beta cell mass leads to impaired insulin production and eventually dysglycaemia. Individuals in this stage are still asymptomatic (15), however, this stage evolves gradually. When approximately 80% of beta cell mass is destructed, glucose will rise and patients will become symptomatic. The percentage beta cell loss needed before symptoms arise decreases with age (106). The insulitis, persistent inflammation of pancreatic cells, is associated with functional impairment in the latest stages of preclinical disease (107). However, functional biochemical testing might already show impaired glucose tolerance.

Biomarkers of the T1D preclinical phase, and its progression towards clinical manifestation, are also the proinsulin to c-peptide (PI:C) ratio and reduced pancreatic volume. The first is indicative of beta cell stress, while the latter seems to correlate with reduced pancreatic islets and loss of exocrine volume. At-risk individuals, especially children younger than 10 years old, that progress to T1D have higher serum PI:C ratio than those who never progress to T1D. Moreover, FDR of T1D patients have reduced pancreatic volume compared to seronegative individuals, although it is still higher than patients with recent onset T1D (108, 109).

High levels of CD4+ and CD8+ T cells with specificity for beta cell autoantigens are now found in the islets of asymptomatic individuals. This antigen recognition might be mediated by B cell antigen

presentation to T cells (107, 110). As said, autoantibodies are the first markers of disease. There are five main autoantibodies directed against insulin and islet cells. They precede clinical manifestations of T1D and are markers of beta-cell autoimmunity: autoantibodies against insulin (IAA), autoantibodies against insulinoma-associated antigen-2 (IA-2), autoantibodies against glutamic acid decarboxylase (GAD or GADA), autoantibodies against zinc-transporter 8 (ZnT8), and islet cell antibodies (ICA). The distribution of the different antibodies is age-related as IAA is the main antibody found in children, while GADA is most commonly found in young adults (111). Post-translation modification of insulin causes the formation of new epitopes that are recognized by autoantibodies involved in T1D pathogenesis (15, 103, 112, 113). The probability of diabetes development is dependent on the number of islet antibodies found in one person (114).

The symptomatic stage of T1D manifests as polyuria, polydipsia due to hyperglycemia, and eventually ketoacidosis caused by excessive lipolysis due to insulin deficiency and can only be treated with insulin replacement therapy (106).

COMPARISON BETWEEN DISEASES

The four diseases included in this review can affect a wide range of organs and tissues, that may be the initial site of an attack by the immune system. In line, the resulting pathology is diverse and one could easily conclude that the diseases have little in common. However, when one looks beyond the clinical manifestations to the genetic, environmental and behavioral determinants, it appears that apart from the differences there are also some notable similarities (**Table 1**). These include (in the majority of diseases) aspects such as a

TABLE 1 | Overview of selected major characteristics, risk factors, immunological and clinical features of four autoimmune diseases.

Variable		Disease			
		RA	***SLE***	***MS***	***T1D***
Characteristics		North-South declining gradient Familial clustering Female-to-male ratio 2-3:1 Average onset age 55 yrs	High frequency in North America Familial clustering Female-to-male ratio 9:1 Average onset age 35 yrs	North-South gradient Familial clustering Female-to-male ratio 2-3:1 Average onset age 30 yrs	North-South gradient Familial clustering Female-to-male ratio 1:1.8 Average onset age 5 yrs (peak 1) or 12 yrs (peak 2)
Genetic risk factors	*HLA*	HLA-DRB1 (SE)	HLA-DRB1 HLA-DQ HLA-DR	HLA-DRB1 HLA-DR3 HLA-DR4 HLA-DR6	HLA-DRB1 HLA-DR4
	Non-HLA	PTPN22 CLTA4 PADI4	Complement system (C1q, C2, and C4) FcγR (FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16)) MAVS IFN pathway (IFIH1, IRF5, TLR7, TYK2)	IL2RA IL7R CLEC16A CD226	INS PTPN22 CTLA4 SH2B3 BACH2 IL2RA IL7R CLEC16A CD226
Environmental risk factors		Smoking Dust Lack of exercise Obesity Stress	Smoking Vitamin D – controversial [Obesity] [Lack of sleep] EBV infection	Smoking Vitamin D/Lack of UV radiation Obesity EBV infection	Vitamin D Obesity Infections Psychological stress/trauma Diet – [cow's milk]
Preclinical immune system		Autoantibodies (ACPA, RA, anti-CarP, AAPA) Cytokines (↑IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ) T cells (Th2, low Treg cells)	Autoantibodies (ANA, antiphospholipid, anti-Ro, anti-La, anti-dsDNA, anti-Sm, anti-RNP) Cytokines (↑IFN-γ, IL-5, IL-6, IL-17, TNF)	Antibodies against myelin protein T cells (expanded CD8+ T cells, altered Treg cell function) B cells and plasma cells in CNS lesion	Autoantibodies (IAA, IA-2, GADA, anti-ZnT8, ICA)Complement (C4d increased in pancreas of seropositive individuals) T cells (CD4+ and CD8+ T cells)
Early clinical manifestations		Symmetrical polyarthritis	Skin lesions Arthritis CNS and peripheral nervous system inflammation Internal organ inflammation Hematological manifestations	Neuronal inflammation Monocular visual loss Sensory and motor limb symptoms	Polyuria Polydipsia Hyperglycemia

This table summarizes selected major genetic and environmental risk factors, the involvement of the immune system in the preclinical stage, and early disease manifestations. In brackets [] are risk factors which association has been found weak, either for lack of evidence or for weakness of the association itself.

North-South gradient of decreasing prevalence (6, 86, 87, 101), a female preponderance (19, 89, 115), major genetic risk factors at the HLA level, partly overlapping cytokine profiles and lifestyle risk factors such as obesity, smoking and stress. Of note, T1D has predominance in males (116).

The North-South gradient may point to genetic differences, but can also be partly due to different climatic influences or dietary habit differences between more Northern and more Southern regions. Likewise, the observed female preponderance may be related to reproductive hormonal factors or alternatively to X-linked genetic factors. For both explanations, the available data do not fully explain the predominance of females (117). The importance of the environment is illustrated by the effect of migration, as an example children that move from Nordic countries to southern countries in younger years have the same prevalence of MS and T1D as is present in the new country (6, 86, 87). Moreover, an increased prevalence of RA was observed after migration from rural to urban areas in South Africa (118). A central characteristic remains the lengthy period of asymptomatic to undifferentiated disease which can cover many years, suggesting a gradually evolving interaction between the genetic profile and the environment. Differently from RA, SLE, and MS, T1D symptomatology is dependent on the amount of beta cell destruction, with residual hormonal function preceding symptoms (106).

When we thus suppose there may be a partly shared pathophysiology between the four diseases, one might expect this to become apparent in a clustering of diseases in the same individual. RA and SLE can indeed occur together, a situation called "rhupus", however, this is quite uncommon (119). MS and T1D also tend to have a lower overlap than expected by their prevalences, partially due to an opposite role of HLA haplotypes (5, 6). T1D on the other hand seems to predispose affected persons to develop RA, possibly due to shared genetic risk factors (120).

On the level of antibodies, we see that RA patients might express ANA antibodies, while SLE and T1D patients can also express RF and/or ACPA (121). Relatives of patients also have risk of developing autoimmunity, not necessarily the same as their affected relative. This might be due to both shared genetic and environmental risk factors, and a more pro-inflammatory state of the immune system. Indeed, the presence of autoantibodies and their related autoimmune disease predispose patients to manifest non-disease specific antibodies, in a process called poly-autoimmunity. Hence, the mechanisms involved and the timeline of autoantibody production are still not clear, but both genetic and environmental factors might be involved (121). Taken together, the four diseases show a modest overlap in occurrence but more overlap in autoimmune phenomena.

In this sections below, we look further into these overlapping aspects.

Genetic Risk Factors

RA, SLE, MS, and T1D all have a genetic component, with familial clustering and higher risk of disease in first degree relatives of patients (FDR) (12, 66, 87, 102, 122). Several of these genetic risk factors are shared between the diseases, with similarities being most apparent between RA and SLE on one hand, and between MS and T1D on the other hand.

The most prominent genetic risk factors are alleles within the HLA class, in particular *HLA-DRB1*. HLA contributes to nearly 33% of RA risk (123).The HLA-associated risk in RA with an odds ratio of around 6 is almost entirely due to a small peptide sequence present in a number of *HLA-DRB1* haplotypes, the 'shared epitope' (124, 125). In the Caucasian population, *HLA-DRB1* alleles are associated with a 2-to-3 fold increase risk of SLE, however, this association has not been seen consistently in the Afro-American population (67, 68, 126). On the other hand, specific HLA haplotypes might have a protective role in MS and T1D, such as *HLA-DRB1*01*, *HLA-DRB1*10*, *HLA-DRB1*11* and *HLA-DRB1*14* (5). *HLA-DRB1*04* is a risk factor in both RA and T1D (5, 24), while *HLA-DRB1*1501/DQB1*0602* have an opposite effect in MS and T1D, with an increased risk for MS but a protective role in T1D (5, 87). However, several other SNPs of the HLA gene associated with T1D also seem to be associated with MS (5, 6). Both MS and T1D have an epistasis effect, with haplotype-specific interactions between alleles of different parental origins (5).

RA, SLE, and T1D also share non-HLA risk factors with other autoimmune diseases, such as celiac disease, psoriasis, and autoimmune thyroid disease (5, 127, 128). Moreover, loci on the chromosome 3 have a 16% relative contribution to the risk of RA (123). A large number of non-HLA genes involved in autoimmune diseases are interlinked in a network that regulates interferon signaling and dendritic cell (DC) and T cell function. The tyrosine kinase cell-surface receptor *FLT3*, also known as CD135, is expressed on DC, and lymphoid and myeloid progenitors, and is involved in the regulation of monocyte and DC maturation. A specific intron variation in *FLT3* causes the production of a truncated protein, with decreased levels of FLT3 receptor and increased circulating FLT3 ligand, which could lead to autoimmunity. *FLT3* is associated with increased risk of RA, SLE, and T1D, and high levels of FLT3 ligand are found in both serum and synovial fluid of inflamed joints of RA patients (127).

Both RA and SLE show an association between disease risk and genes that are involved in type I interferon production, signaling, and response, such as *IRF5*, Interleukin 1 receptor associated kinase 1 (*IRAK1*), and Signal transducer and activator of promoter 4 (*STAT4*) (9, 14). In SLE, in presence of anti-RNA binding proteins (RBP) and anti-dsDNA antibodies, *IRF5* is associated with higher levels of circulating type I interferon activity. Additionally, *IRF5* variants are associated with higher antibody production predisposition in healthy individuals, which could form immune complexes that activate innate immune cells through over activation of the toll-like receptor (14).

A SNP haplotype of the *STAT4* gene in the third intron is associated with both RA and SLE, with higher risk when this SNP is present in both alleles. *STAT4* is involved in the signaling of cytokines, such as IFN-I, IL-12, and IL-23, which promote differentiation of effector T cells towards a Th17 phenotype. However, *STAT4* has different roles in RA and SLE at least

according to animal models: while in RA STAT4 deficiency in mice is protective, with inability of those mice to develop RA, in SLE STAT4-deficient mice have accelerated nephritis and higher mortality (129).

PTPN22, which codes for a protein involved in both T and B cell signaling, is also an important risk factor for RA, SLE, and T1D (9, 14, 15). In RA, *PTPN22* has a stronger association risk in male compared to female seropositive individuals, and gene carriers have an earlier onset of disease (9). *PTPN22* is one of the common non-HLA genes associated with T1D, together with *IL2RA*, which in turn is also associated with SLE and MS (15, 128). *IL2RA* is involved in lymphocyte activity regulation and confers a 28% and 33% increased risk of developing MS and T1D, respectively (6, 130).

Other non-HLA genetic factors involved in autoimmunity are small nucleotide polymorphisms (SNP) in immune associated genes, such as *IL7R*, *SH2B3*, *CTLA4*, *BACH2*, *CLEC16A* and *CD226*, and the latter are involved in both MS and T1D risk (5, 6, 9, 131–134). These SNP can either give a predisposition to both diseases, or be mutually exclusive, and some of the shared genetic risk factors between MS and T1D are directly associated with disease development (5, 6). Both in MS and T1D, the weight of the genetic predisposition in disease development depends on the family member affected by the disease, with a parent-of-origin effect (5, 135). In T1D, there is an higher risk associated with paternal heredity, while in MS the increased risk is associated with maternal heredity (5).

Lifestyle and Environmental Factors

Although genetic factors play an important role in risk of autoimmunity, genetic predisposition is able to explain only up to 50% of the risk of developing RA and T1D, leaving half of the patients without any known genetic marker (122, 135). Numerous studies have investigated the role of environmental factors in disease development including lifestyle factors, comorbidities, external agent exposure and bacterial and viral infections (77, 136, 137).

Smoking is one of the most prominent environmental risk factors, and has a role in RA, SLE, and MS (20, 77, 87). Although no association has been described between smoking and T1D, this is more likely due to the young age of T1D onset. Smoking causes citrulline autoimmunity in the lung in genetically susceptible individuals (24, 52, 138) and also triggers the production of RF (20, 22, 23), explaining an association between smoking and seropositive RA. In SLE, smoking is a risk factor for anti-dsDNA production (77), while in MS smoking induces an increased axonal demyelination and disruption of the blood-brain barrier, in parallel with an immunomodulatory effect mediated by increasing both nitric oxide levels and its metabolites (87). Both in RA and MS, but not in SLE, smoking has a dose-response relation with disease risk (22, 25, 86). After smoking cessation, the increased risk for RA and SLE remains present for several years (22, 25).

Occupational exposure seems also to be a risk factor for autoimmune diseases; silica and other inorganic dust exposure have been reported to increase the risk of RA and SLE (27, 139, 140). However, these associations are not as strong as for smoking.

Additional lifestyle factors are exercise, alcohol consumption, diet and body mass index (BMI). Exercise and moderate alcohol consumption have been associated with decreased risk of RA and SLE (33, 141), while obesity is associated with higher risk of RA, SLE, MS and T1D (76, 141–145). In persons at risk for RA, the combination of obesity and smoking seems to synergistically increase the risk of RA (146). In the Nurses' Health Study, overweight and obese women had higher risk of developing RA, MS, and T1D (86, 147, 148). Similarly, being overweight is associated with higher risk of T1D (101). Consequently, dietary factors may be expected to play a role in disease risk. The overall dietary quality influences the risk for RA, amounting to a 40% decrease in risk for seropositive RA in women in the highest versus the lowest quartile of dietary quality (32). As for MS, a highly enriched fish diet seems to be protective; populations in Northern countries with a diet high in fish and fish oils show a similar MS incidence to those in lower-latitude countries (86, 87). In the case of T1D, cow's milk has been suggested to trigger an autoimmune response in genetically at-risk individuals that leads to the destruction of pancreatic beta cells (15, 101). This correlation has been also found in the Diabetes Autoimmunity Study in the Young (DAISY), in which children with low and moderate genetic risk that had higher cow's milk intake also had higher risk of islet autoimmunity (IA) (149).

Either chronic stress or the presence or post-traumatic stress disorder (PTSD) have both been related to the subsequent occurrence of autoimmune diseases (28). In a study covering the whole population of Sweden, a diagnosis of a stress-related disorder increased the risk of any autoimmune disease by 50% in the whole period of 35 years thereafter, including the diseases discussed here. Furthermore, a large study on US veterans of the Iraq war showed a doubled risk of RA, SLE and MS in individuals affected by PTSD (150). An increased risk for RA was also found by the Nurses' Health Study in nurses that had PTSD symptoms (151), and chronic stress and psychological trauma had also been suggested to be associated with T1D risk. At least for the effect of stress, this might be due to higher levels of cortisol, inducing insulin resistance while also modulating the immune response (101).

It is important to consider that many of the associations mentioned above have a tendency to cluster within the population. Unhealthy diet, lack of physical activity, obesity, chronic stress, as well as environmental exposure, low socio-economic status and low income, all co-segregate, making it hard to identify if the causal association found by observational studies is caused by one specific factor or a combination of them.

Vitamin D levels have been suggested to influence disease severity in both MS and T1D in a seasonal way, with higher relapses in MS and diagnostic rate in T1D linked to vitamin D status (5). The mechanisms behind this association are not clear. However, 25-hydroxy vitamin D (25(OH)D) levels, which reflect vitamin D absorption by UV light exposure, inversely correlate with MS risk in white individuals (86, 87). 25(OH)D levels also inversely correlate with BMI, especially above 30, which might

suggest an indirect mechanism of BMI as a risk factor (86). The onset of the first demyelinating event in at-risk-of-MS individuals correlates with both sun exposure and vitamin D levels. Sun exposure is measured by the degree of actin damage, which was lower at the time of onset of disease (87). While there is no correlation between T1D and 25(OH)D levels at birth, a birth-cohort study in Finland showed that 1 year of supplementation of dietary vitamin D, at a dose of 2000 IU daily was associated with a reduced risk of developing T1D in children. This might indicate a role of vitamin D in the pathogenesis of T1D between birth and early childhood (152, 153).

Another factor that may play a role in disease risk are viral infections. In RA there is no consistent evidence of infections involved in the pathogenesis. Epstein-Barr (EBV) infection has been suggested to increase the risk of SLE (154, 155) and is a major environmental risk factor for MS development (91, 96). While individuals with elevated immunoglobulin levels against EBV have a 2-fold increased risk of developing MS, EBV seronegative individuals have a disease risk near zero. Moreover, this mechanism seems to be specific to EBV, since cytomegalovirus infection does not influence MS risk, suggesting that EBV infection may be partially necessary for MS onset (5, 86, 96, 156). It has been postulated that EBV infection either increases activation and expansion of T and B cells, or is responsible for B cell immortalization, in particular of B cells that produce antibodies against EBV, leading to antigen presentation to pathogenic T cells (87).

While the association between MS and EBV infection is strong, the role of infections inT1D pathogenesis is not yet well defined. The Diabetes Prediction and Prevention (DIPP) study demonstrated a correlation between first autoantibody appearance and enterovirus infection, and serological studies suggest a link between Coxsackie B virus, in particular CBV4 serotype, and T1D. Moreover, the Teddy study described a possible correlation between respiratory infections, with a common peak between 6 and 9 months of age, and increased risk of islet autoimmunity, which follow a similar trend (15, 157). In summary, there is evidence for a role of viral infections in the pathogenesis mainly of MS and T1D, with a very specific role of EBV in MS.

Activation of the Immune System

RA, SLE, MS, and T1D all have a latent phase that precedes formal clinical diagnosis (**Figure 2**). The length of this phase can vary between diseases and within individuals at risk for the same disease, but a common feature is the activation of the immune system, which is visible to a varying degree in the different diseases and precedes the onset of symptoms.

Humoral Immunity

As described above, the majority of RA patients is seropositive, and these antibodies develop over many years before the clinical disease, with increasing concentrations as well as specificities

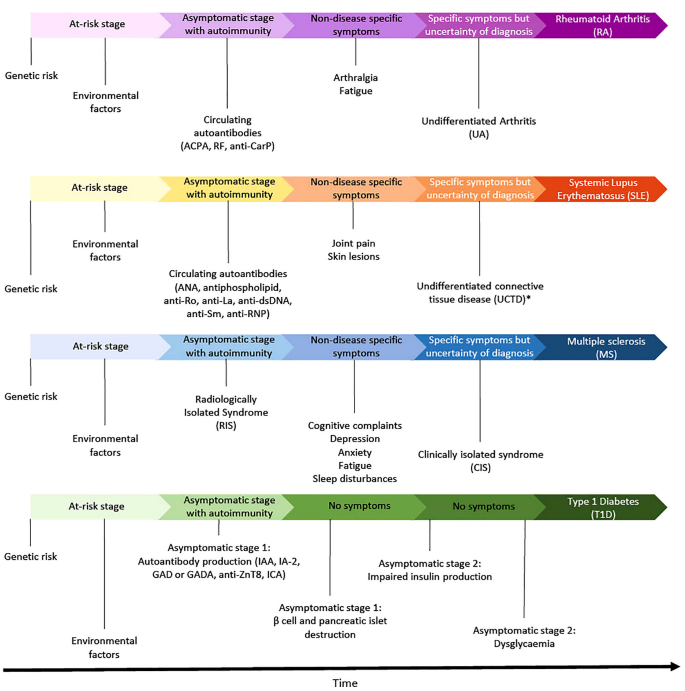

FIGURE 2 | This illustration shows an overview of the transition from at-risk to disease diagnosis. In purple is represented RA, in orange SLE, in blue MS, and in green T1D. *Also known as "incomplete Lupus". ACPA, Anti-citrullinated protein antibody; RF, Rheumatoid factor; anti-CarP, anti-carbamylated; ANA, antinuclear antibody; anti-dsDNA, anti-double strand DNA; anti-RNP, anti-nuclear ribonucleoprotein; IAA, autoantibodies against insulin; IA-2, autoantibodies against insulinoma-associated antigen-2; GAD or GADA, autoantibodies against glutamic acid decarboxylase; anti-ZnT8, autoantibodies against zinc-transporter 8; ICA, islet cell antibodies.

(59, 158). In particular ACPA are thought to be involved in the development of synovitis and bony erosions.

In contrast, in SLE autoantibodies are uniformly found in all patients. This is in part due to the conceptions and definitions used for making the diagnosis of SLE in clinical practice, codified by the recent EULAR/ACR classification criteria for SLE where the presence of ANA is required (159).

Some autoantibodies in SLE play an important role in the pathogenesis; this is most convincing for anti-DNA antibodies. Furthermore, there is a strong association between the combination of multiple antibodies, such as anti-dsDNA and anti-C1q, decreased complement levels, and lupus nephritis (LN). The most reproducible autoantibodies for diagnostic purposes are those reflecting renal involvement (160, 161).

So far, in MS no specific autoantibody has been found, however, autoantibodies against several CNS cells have been reported in this disease (162, 163).

As noted above, also persons at risk for T1D can develop several types of autoantibodies. There is a combination effect of multiple antibodies, with 70% risk of disease in children with multiple (three or four) circulating antibodies. Young children preferably develop IAA, while GAD autoantibodies are most commonly found in teenagers. However, the conversion from single to multiple antibodies can be slow (111, 114, 164–166).

Nearly 60% of children with single autoantibodies will lose antibody production over time and convert to seronegative (15). This mechanism is unique to T1D and differs from RA and SLE.

Of note, FDR of RA, SLE, and T1D patients may have autoantibodies detectable in their serum in absence of any signs or symptoms of disease (83, 121).

Cellular Immunity

The cellular component of the immune system also has an active role in disease pathogenesis. In preclinical RA, ACPA+ individuals have decreased T regulatory (Treg) cell levels and a shift of CD4+ T cells towards pro-inflammatory subsets, in particular T helper (Th) 2 cells (53, 167). In SLE, numerous abnormalities of cellular immunity have been described (168) but it has been difficult to determine whether these are necessary elements of the pathophysiology of the disease itself or the consequences of long-standing inflammation or of the treatments used to control it. In established MS altered activity and levels of Treg cells and predominance of CD8+ T cells are found within the neuronal lesion (94, 169, 170). In the prodromal phase of MS, the frequency of expanded CD8+ T cells within the CNS increases, and cells show alterations of their markers towards a more pro-inflammatory phenotype (94). Autoreactive T cells target the myelin in MS and the pancreatic islets autoantigens in T1D patients and T1D relatives, respectively (171). In the asymptomatic phase of T1D, the high levels of CD4+ and CD8+ T cells that are specific for beta cell autoantigens cause persistent inflammation of the pancreatic islet, called insulitis. This antigen recognition might be mediated by B cell antigen presentation to T cells (107, 110).

The B cell component is also altered in individuals at risk for RA, who have higher levels of IgA plasmablasts than the general population (172). The importance of the B cell component in the evolution from at-risk individuals to RA has also been demonstrated in the PRAIRI study, a clinical trial in ACPA-positive at-risk individuals, in which B cell depletion through a single dose of rituximab significantly delayed disease onset compared to placebo (173). In SLE, patients have decreased levels of CD27-IgD-IgM B cells, which represent an activated and auto-reactive state (174). Expanded B cells are also found in the neuronal lesions of prodromal MS individuals, where they correlate with oligoclonal immunoglobulin bands (94). Moreover, B cells also have a role in the pathogenesis of T1D, as demonstrated by B cell depletion after 1 year treatment with rituximab. Patients that received the treatment had reduced impairment of beta cell function compared to placebo, and required less insulin for disease management (175).

Soluble Factors

Soluble factors have a role in disease pathogenesis, inducing immune activation, recruitment, and regulation of the immune response. They are also responsible for direct pathogenic manifestations and can be used in some cases as biomarkers of disease progression. Soluble factors involve a variety of molecules, such as cytokines, complement and markers of inflammation.

Preclinical RA individuals on average have increased levels of both pro-inflammatory and anti-inflammatory cytokines, such as IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ. Cytokine levels change over time, IL-4 and IL-14 levels being higher at the earliest stages of disease, and IL-17 levels increasing before disease onset and decreasing after RA becomes established (53, 176). Type I interferon (IFN-I) is detectable in the blood of both at-risk and established RA individuals, and has also a role in SLE initiation of SLE. While in RA there is higher production of IFNβ, in SLE there is abundance of circulating IFNα. Treatment of viral hepatitis with INF-α has been associated with *de novo* onset of SLE, the symptoms of which would improve after the treatment is stopped. Serum levels of IFN-I increase drastically one year before SLE onset, and circulating IFN-I is considered an hereditary risk factor for SLE (14). IFN-γ is also increased in SLE individuals more than 3.5 years before diagnosis and is associated with increased anti-RNA antibody production, inflammation, and transition from undifferentiated disease to connective tissue disease (84, 177). First-degree relatives of patients with MS have on average a more pro-inflammatory cytokine profile (higher TNFα, lower IL-10), this suggests that differences in cytokine profile may contribute to the pathogenesis of MS (178)

The complement system is involved in both SLE and T1D. The presence of C1q deficiency in at-risk SLE individuals, together with increased IgG : IgM anti-dsDNA ratio, may be indicative of disease development (179). C4d has been found to be increased in the pancreas of 25% of T1D patients, while in non-diabetic individuals this percentage decreases to 7% of T1D associated autoantibody positive and 2% of autoantibody negative individuals (180).

Preclinical Signs and Symptoms

In RA, autoantibody production precedes the first disease manifestations by years (158). In time, the low lover

inflammation and ACPA and/or RF titers increase, followed by non-specific musculoskeletal symptoms (39). Other common symptoms in pre-RA are arthralgia, fatigue, reduced mental health due to limited functionality and work absence, and non-articular manifestations, such as cardiovascular diseases (181, 182). More than 60% of seropositive individuals tend to have pain, stiffness and swelling of the joint, and nearly 30% had joint tenderness, even before RA onset (40). Another study showed an increased frequency of primary care visits for musculoskeletal symptoms, infections and comorbidities in the years prior to the diagnosis of IA (183).

As explained above, the identification of a preclinical stage of SLE and the diagnosis itself is complicated by the need of sufficient clinical manifestation and the time elapse that this entails. Individuals in this phase may experience joint or skin symptoms for several years, associated with ANA and anti-DNA antibody production.

During the prodromal phase of MS decreased cognitive performance, fatigue, pain, depression, anxiety, bowel, and bladder disorders are more often reported in the 5 years before the diagnosis of MS. Individuals in the prodromal phase are also more likely to seek healthcare and present health deterioration 5-10 years before the first clinical event (96, 98, 184). Nearly one third of RIS individuals develop MS-related neurological symptoms within 5 years. Age younger than 35 years old, male gender, thoracic or cervical spinal cord lesion, and the presence of oligoclonal bands in the cerebrospinal fluid are major predictors of RIS conversion to MS (98, 185).

The preclinical phase of T1D can be divided into two stages, with an initial stage of immune recognition and antibody production, beta cell and pancreatic destruction, followed by an exacerbation of islet destruction that leads to insulin production impairment and dysglycaemia (15). Functional tests are able to detect an impairment of insulin production and dysregulation of glucose metabolism in the preclinical phase, however specific signs or symptoms are only shown with manifest hyperglycemia, the clinical stage (15, 186, 187).

DISCUSSION

The necessarily incomplete overview of the preclinical phase of four distinct autoimmune diseases presented in this narrative review naturally highlights several differences in pathophysiology and clinical manifestations, but also shows that many of their etiologic and pathophysiological features actually overlap. The picture that emerges of these autoimmune diseases is that of a genetically determined increased sensitivity to breach immune tolerance to certain body parts, that is triggered under the influence of often multiple environmental factors during many years. Highly prevalent environmental factors such as smoking, obesity and stress are related to all four of these diseases and are known in general to produce a state of chronic systemic low grade inflammation (1). Thus, although the genetic basis and clinical features of the diseases are quite specific, the trigger for their manifestation in many cases is quite general.

The preclinical or prodromal phase of these diseases is characterized by nonspecific symptoms and in some cases more specific signs of autoimmunity at laboratory testing, which increase towards the onset of clinically manifest disease and subsequent diagnosis. Thus a high risk of future clinical disease can mostly be measured accurately only shortly, typically in the last year or so, before onset of clinical disease. Such a high risk of imminent disease then provides the setting in which preventive interventions with drug therapy could be tested, a situation resembling very early treatment of the same disease.

Attempts at prevention at an earlier stage would then involve interventions directed at life style factors. However, since it is difficult to identify individuals with an only slightly increased risk for autoimmune diseases, preventive efforts for autoimmune diseases would then become part of the public health domain. Indeed, increased public health or legislation actions to reduce smoking and obesity, as well as other unhealthy behaviors, while being completely nonspecific, could have a huge impact on the incidence and burden of not only the autoimmune diseases discussed here, but chronic non-communicable diseases in general. Meanwhile, physicians treating persons with increased risk of these diseases will have to await further advances in the prediction of clinical disease and in the (cost-)effectiveness of preventive therapy in high risk individuals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Altered Balance of Pro-Inflammatory Immune Cells to T Regulatory Cells Differentiates Symptomatic From Asymptomatic Individuals With Anti-Nuclear Antibodies

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Systemic Autoimmune Rheumatic Diseases (SARDs) are characterized by the production of anti-nuclear antibodies (ANAs). ANAs are also seen in healthy individuals and can be detected years before disease onset in SARD. Both the immunological changes that promote development of clinical symptoms in SARD and those that prevent autoimmunity in asymptomatic ANA$^+$ individuals (ANA$^+$ NS) remain largely unexplored. To address this question, we used flow cytometry to examine peripheral blood immune populations in ANA$^+$ individuals, with and without SARD, including 20 individuals who subsequently demonstrated symptom progression. Several immune populations were expanded in ANA$^+$ individuals with and without SARD, as compared with ANA$^-$ healthy controls, particularly follicular and peripheral T helper, and antibody-producing B cell subsets. In ANA$^+$ NS individuals, there were significant increases in T regulatory subsets and TGF-ß1 that normalized in SARD patients, whereas in SARD patients there were increases in Th2 and Th17 helper cell levels as compared with ANA$^+$ NS individuals, resulting in a shift in the balance between inflammatory and regulatory T cell subsets. Patients with SARD also had increases in the proportion of pro-inflammatory innate immune cell populations, such as CD14$^+$ myeloid dendritic cells, and intermediate and non-classical monocytes, as compared to ANA$^+$ NS individuals. When comparing ANA$^+$ individuals without SARD who progressed clinically over the subsequent 2 years with those who did not,

Abbreviations: ANA, Anti-nuclear antibody; Ab, Antibody; ELISA, Enzyme-linked immunosorbent assay; HC, Healthy control; IFN, Interferon; IFN-α, Interferon-alpha; IL, Interleukin; mDC, myeloid dendritic cell; NS; asymptomatic; pDC, plasmacytoid dendritic cell; PBMC, Peripheral blood mononuclear cell; SARD, Systemic autoimmune rheumatic disease; SjD, Sjogren's Disease; SLE, Systemic lupus erythematosus; SSc, Systemic sclerosis; Tfh, T follicular helper;TGF-ß1, Transforming growth factor beta-1; Tph; T peripheral helper; Treg, T regulatory; UCTD, Undifferentiated connective tissue disease.

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we found that progressors had significantly increased T and B cell activation, as well as increased levels of LAG3$^+$ T regulatory cells and TGF-ß1. Collectively, our findings suggest that active immunoregulation prevents clinical autoimmunity in ANA$^+$ NS and that this becomes impaired in patients who progress to SARD, resulting in an imbalance favoring inflammation.

Keywords: b cells, monocytes, t cells, dendritic cells, anti-nuclear antibodies, systemic autoimmune rheumatic diseases, interferon-alpha, t regulatory cells

INTRODUCTION

The anti-nuclear antibody (ANA)-associated Systemic Autoimmune Rheumatic Diseases (SARD), which include Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SS), and Systemic Sclerosis (SSc), are chronic multi-system autoimmune diseases with a significant morbidity and mortality. Although each of these conditions has some distinctive autoantibodies (autoAbs) and clinical features, there is considerable overlap in the types of autoAbs produced and clinical symptoms, suggesting a shared etiology. This is supported by studies showing numerous shared genetic risk factors (1–5) and a high prevalence of elevated levels of interferon (IFN)-induced gene expression (6–12).

Since SARD can often present with life-threatening inflammation and/or irreversible damage, there is tremendous interest in defining at-risk individuals and initiating therapy early to prevent these poor outcomes. To achieve this, it is necessary to have a highly accurate biomarker for impending disease and knowledge of the key immune events to target. A characteristic feature of SARD is a prolonged preclinical phase in which ANAs can be seen in the absence of clinical symptoms (13–16). While this observation suggests that ANAs could be used to identify at-risk individuals, ANAs, as detected by immunofluorescence using HEp-2 as a substrate, are seen in ~20% of healthy women (12), only a small subset of whom (estimated at 5-8%) will transition to SARD. Thus, additional biomarkers are required to identify ANA positive (ANA$^+$) individuals at high risk of impending progression. In addition, little is known about the immunologic features that differentiate asymptomatic ANA$^+$ individuals from those with SARD, and progressors from non-progressors.

To address these knowledge gaps, our laboratory has been recruiting and longitudinally following a unique cohort of ANA$^+$ individuals lacking a SARD diagnosis. In a previous study, we characterized several B and T cell phenotypes in the peripheral blood of these subjects, contrasting them with those seen in ANA$^-$ healthy controls and early SARD patients (17). This led to the surprising observation that ANA$^+$ individuals lacking a SARD diagnosis had increased proportions of activated B and T cells, similar to that observed in early SARD. Indeed, in that original study, except for a trend to increased activation in ANA$^+$ individuals with SARD as compared to those without, no distinctive immunologic differences were seen between these two groups. In this study, we examined a broader array of immune populations in an effort to define the key immunologic differences that discriminate between ANA$^+$ individuals with and without a SARD diagnosis, and to characterize the immunologic changes that distinguish ANA$^+$ individuals who demonstrate subsequent clinical progression from those who do not.

MATERIALS AND METHODS

Subjects and Data Collection

ANA$^+$ individuals (≥1:160 or 1:80 with a specific autoAb) were recruited from the Toronto Western and Mount Sinai Hospital Rheumatology Clinics, where they had been referred for evaluation because of a positive ANA test. Following assessment by one of the participating rheumatologists, patients were stratified into three groups based upon the presence of SARD clinical diagnostic criteria [1997 American College of Rheumatology (ACR) criteria for SLE (18), 2013 ACR/European League Against Rheumatism (EULAR) criteria for SSc (19), or the revised 2016 ACR/EULAR criteria for SS (20)], as follows: (1) asymptomatic ANA$^+$ (ANA$^+$ NS), with no clinical SARD criteria; (2) undifferentiated connective tissue disease (UCTD), with at least one clinical symptom of SARD but who did not meet criteria for SARD diagnosis; or (3) early SARD. All SARD patients included within the study met disease classification criteria, were within the first 2 years of diagnosis, and were not taking corticosteroids or disease-modifying anti-rheumatic drugs, with the exception of hydroxychloroquine. For patients seen after 2015, yearly follow-up was offered to monitor any potential disease progression, and all patients with at least 2 years of follow-up care were included in the study, contrasting progressors and non-progressors. Clinical progressors were defined based upon development of new clinical SARD criteria or new organ involvement characteristic for SARD, within 2 years of initial assessment. Sex-matched ANA$^-$ healthy controls (ANA$^-$ HC) were recruited from hospital and laboratory personnel. Patients provided information on a family history of rheumatic disease using a validated questionnaire (21). The study was approved by the Research Ethics Boards of the two hospitals and all participants signed informed consent.

Cellular Characterization

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood collected in sodium-heparin tubes over a Ficoll/Hypaque (GE Healthcare) gradient, treated to remove residual red blood cells, and immediately stained, or archived in Liquid N_2 (in CryoStor®) and subsequently stained immediately following thawing. Prior to staining with various combinations of directly-conjugated monoclonal Abs, the cells (5 x 10^5/stain) were incubated with viability dye (Fixable Far-Green Dead Cell Stain, Invitrogen) for 30 minutes on ice. The Abs used for staining were as follows: mouse anti-human, TBET-PE (4B10), FOXP3-PE (206D), CD56-

PE (5.1H11), CD4-PerCP (SK3), IgD-PerCP (IA6-2), CD123-PerCPCy5.5 (6H6), CD11c-PeCy7 (3.9), CD38-PeCy7 (HB-7), CD21-APC (Bu32), CXCR3-APC (G025H7), HELIOS-APC (22F6), CD16-APC (B73.1), CD27-APC/Fire750 (M-T271), CD3-APC/Fire750 (SK7), CD19-BV421 (H1B19), PD1-BV421 (EH12.2H7), CD138-BV605 (MI15), CD20-BV605 (2H7), CXCR5-BV605 (J252D4), CD25-BV605 (2A3), and CD86-BV605 (BU63) from Biolegend; and mouse anti-human CCR6-PE (11A9), CD3-PeCy7 (SK7), CD19-APC-H7 (SJ25C1), CD45RA-APC/Fire750 (HI100), CD20-APC-H7 (2H7), LAG3-BV421 (T47-530), CD14-BV421 (MøP9), and HLADR-BV605 (646-6) from BD Biosciences. Staining for intracellular FOXP3 and HELIOS was performed using the Human FOXP3 Buffer Set (BD Biosciences) for fixation and permeabilization, according to the manufacturer's protocol. Events were acquired using a three-laser LSRII or FACSCanto (BD Biosciences) flow cytometer, with fluorescence-minus-one (FMO) controls being used as negative staining controls. The data was analyzed using FlowJo software (BD Biosciences).

Cytokine Measurements

For measurement of transforming growth factor beta-1 (TGF-β1), freshly thawed heparinized plasma (stored at -80°C and not previously thawed) was activated by adding 5 µL of 1.0 M HCl to 10 µL of plasma, and incubated for 10 minutes at room temperature. The reaction was then neutralized by addition of 5 µL of 1.2 M NaCl/0.5M HEPES and the resultant mixture was diluted to a final volume of 400 µL with diluent reagent. The concentration of TGF-ß1 in the diluted activated plasma (100 µL per well, in duplicate) was measured using a human TGF- ß1 DuoSet ELISA Kit and Ancillary Reagent Kit 1 (R&D Systems), with the optical density being read at 450 nm using a FLUOStar® Omega microplate reader (BMG Labtech). IFN5 scores were determined by measuring the expression levels of five IFN-induced genes (*EPSTI1, IFI44L, LY6E, OAS3*, and *RSAD2*) in whole peripheral blood archived in Tempus tubes (Applied Biosystems), using a custom NanoString (NanoString Technologies) (12, 17). Log_2 normalized expression levels of the 5 genes were summed to generate a composite IFN5 score. Serum IFN-α was measured using patient serum collected and archived at −80°C at the time of recruitment, as previously described (12).

Measurement of autoAbs

ANAs were quantified by indirect immunofluorescence using the Kallestad® HEp-2 kit (BioRad), through the University Health Network laboratory. The serum levels of 11 specific autoAbs (anti-dsDNA, -chromatin, -Ro, -La, -Sm, -SmRNP, -RNP, -Jo-1, -Scl-70, -centromere, and -ribosomal P), were quantified using the Bioplex® 2200 ANA Screening System (BioRad), with the company's suggested cut-offs being used to define a positive test. AutoAb testing was performed on all HCs, and those meeting the entrance criteria were re-classified into the asymptomatic ANA^+ group. HCs with a positive ANA <1:160 or found to have any specific autoAb in the absence of a positive ANA were excluded from the study. Ro60 and Ro52 Abs were measured using an autoantigen microarray, as previously reported (22).

Data Analysis

The Kruskal-Wallis test was used for statistical comparisons of differences between three or more groups, followed by Dunn's post-test for multiple comparisons. Comparisons between two groups were performed using the Mann-Whitney test. The strength of correlation between two variables was assessed using Spearman's correlation coefficient, with the lines that visually display these associations being computed by linear regression analysis. All statistical analyses were performed using GraphPad Prism Software, Version 8 (San Diego, CA, USA), except for the correlation matrices, which were produced in R using the corrplot (v0.84) package. For statistical tests, asterisks indicate a p value of <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

RESULTS

The T Helper Cell Phenotype Differs Between ANA^+ Individuals With and Without a SARD Diagnosis

We have previously shown that ANA^+ NS and UCTD patients share a number of B cell activation phenotypes and increases in the proportion of T follicular helper cells with early SARD patients (17). However, the functional characteristics of the expanded Tfh population and many innate immune populations were not examined. Therefore, to further explore the immunologic differences between symptomatic and asymptomatic ANA^+ individuals, the current study was performed. **Supplementary Table 1** outlines the demographic characteristics of the subjects, the majority of whom did not overlap with the previously published study.

Although our ANA^+ NS subjects lacked clinical SARD criteria, they could have other clinical symptoms not attributable to SARD. The ANA testing for these individuals was performed for the following reasons: non-inflammatory arthritis/arthralgias (40%, mostly osteoarthritis and fibromyalgia), sicca symptoms in the absence of objective signs of dryness (15%), healthy mother with a child with congenital heart block or neonatal lupus (14%), urticaria/non-specific rash (11%), family history of autoimmunity (7%), recruitment to the study as a healthy control (6%), and other (7%). All UCTD patients had a least one clinical symptom of SARD, but lacked sufficient disease classification criteria for a diagnosis of SARD. These symptoms included: Raynaud's phenomenon (38%), inflammatory arthritis (19%), abnormal nailfold capillaries (17%), objective ocular signs (12%), photosensitivity (10%), objective oral signs (8%), puffy fingers (6%), pericarditis (4%), interstitial lung disease (4%), malar rash (4%), ITP/TTP (4%), alopecia (4%), oral ulcers (2%), chilblains (2%), calcinosis (2%), esophageal dysmotility (2%), calcinosis (2%), and oral ulcers (2%). SARD patients had to meet objective disease classification criteria for diagnosis (see *Materials and Methods*).

The subjects were predominantly female with similar proportions in all groups. However, ANA^- HCs were significantly younger than ANA^+ NS and UCTD patients.

There were no significant differences between groups in the ethnicity of the subjects, with the majority of subjects in each group being Caucasian. In all of the ANA^+ groups, the majority of subjects had an ANA titer of 1:640 or greater, but SARD patients had a larger number of nuclear antigen autoantibody specificities (as determined by the Bioplex©) when compared to the other ANA^+ groups.

Although most studies have shown an increase in Tfh cells in SARD, there has been inconsistency between studies as to which sub-populations of cytokine-producing cells are increased (23–29). To determine whether the cytokine profile of Tfh cells in ANA^+ NS and UCTD patients is similar to that seen in early SARD, PBMCs were stained to identify Tfh ($CD3^+CD4^+CD45RA^-PD1^{hi}CXCR5^+$) cells. The proportion of cells with a Th1, Th2 or Th17 phenotype was then determined by staining with anti-CXCR3 and CCR6 monoclonal Abs, with the $CXCR3^+CCR6^-$, $CXCR3^-CCR6^-$, and $CXCR3^-CCR6^+$ populations being enriched for Th1, Th2, and Th17 cells (representative gating shown in **Figures 1A, B**), as previously reported (30).

Compatible with previous reports of increased Tfh cells in SLE, SS, and SSc, there was a significant expansion of Tfh cells in early SARD patients as compared to ANA^- HC, and as observed in our previous study, this was also seen to a lesser extent in ANA^+ NS or UCTD patients (**Figure 1C**). The increases in Tfh

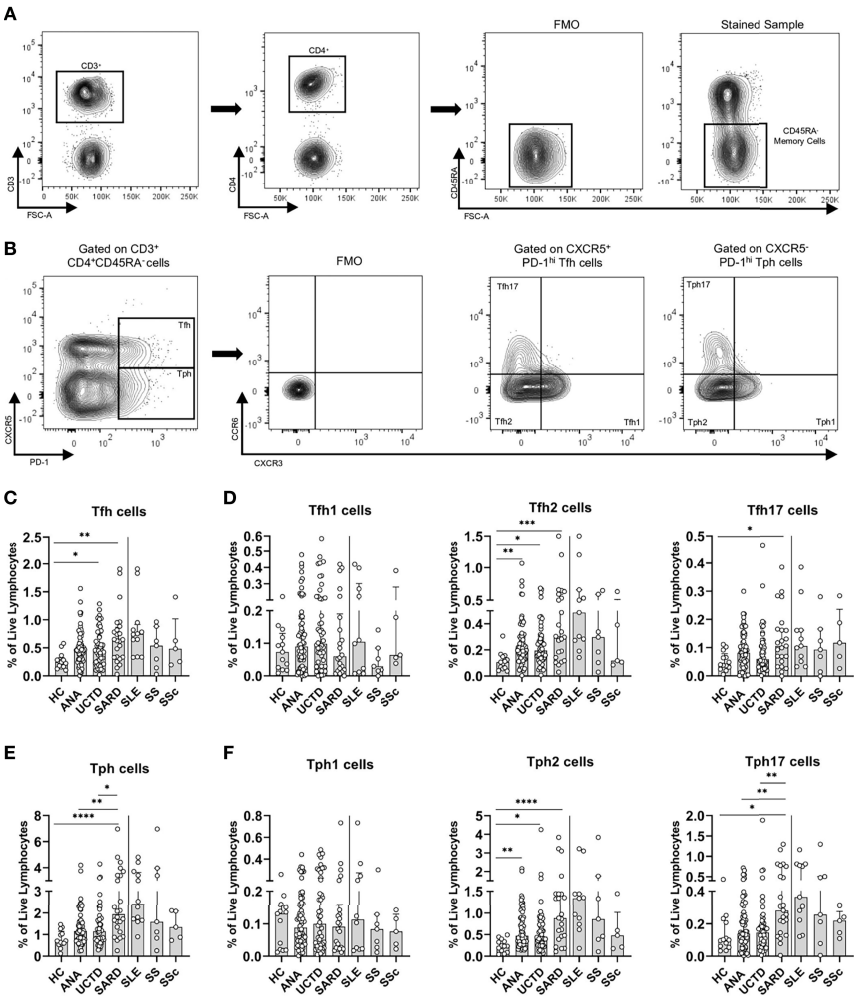

FIGURE 1 | Asymptomatic anti-nuclear antibody positive (ANA^+) individuals lacking a diagnosis of systemic autoimmune rheumatic diseases (SARD) have abnormalities in T helper subsets that are amplified in symptomatic patients with early SARD. **(A)** Gating strategy for identification of ($CD3^+CD4^+CD45RA^-$) memory T cells from the peripheral blood mononuclear cells of a representative ANA^+ patient. **(B)** Gating strategy for identification of T follicular helper (Tfh, $PD\text{-}1^{hi}CXCR5^+$) and T peripheral helper (Tph, $PD\text{-}1^{hi}$, $CXCR5^-$) cells and the Th1 ($CXCR3^+$, $CCR6^-$), Th2 ($CXCR3^-$, $CCR6^-$), and Th17 $CXCR3^-$, $CCR6^+$) subsets within these populations. **(C, D)** The proportions of Tfh cells and the individual Tfh subsets within the memory T compartment stratified by subject group. **(E, F)** The proportions of Tph cells and the individual Tph subsets within the memory T compartment stratified by subject group. The solid vertical line in each plot separates the groups that were statistically compared to one another from the individual SARD on the right, which were not statistically compared to any group. Bars represent the median with interquartile range. Each data point represents an individual subject. Statistical significance was determined using the Kruskal-Wallis test with Dunn's *post-hoc* test for multiple comparisons; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. HC, ANA^- healthy control; ANA, asymptomatic ANA^+; UCTD, undifferentiated connective tissue disease; SARD, systemic autoimmune rheumatic disease; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic sclerosis.

cells in early SARD occurred in the Th2 and Th17 subsets, with no difference in the proportion of Th1 cells, as compared to ANA^- HC. ANA^+ NS and UCTD patients also showed a trend to increased proportions of Tfh cells, which was smaller than that seen in SARD, and which appeared to result from small increases in the Th1 and Th17 subsets, together with a significant increase in the Th2 cell subset (**Figure 1D**).

Recently, a novel extra-follicular T helper subset termed T peripheral helper (Tph) cells that shares many properties with Tfh cells but lacks expression of CXCR5 (representative Tph gating shown in **Figure 1B**) was found to be increased in SLE and SS (31–33). This cell subset was increased in early SARD, at significantly higher levels than those seen in ANA^+ NS and UCTD patients (**Figure 1E**). As was observed for Tfh in early SARD, the increase in Tph cells was attributable to increases in the proportion of the Th2 and Th17 subsets within this population (**Figure 1F**). The proportion of Tph2 cells was also significantly increased in ANA^+ NS and UCTD patients, but the magnitude of this increase was less than that seen in SARD (**Figure 1F**). In contrast, there was only a slight trend to increased Tph17 cells in these non-SARD groups, which was significantly less than that seen in early SARD (**Figure 1F**).

Both Tfh and Tph cells are reported to provide support for differentiation of B cells to Ab-producing plasma cells and/or plasmablasts (17, 32, 33). We previously showed that there is a trend to increased proportions of plasma cells and plasmablasts in ANA^+ individuals lacking a SARD diagnosis (17), and similar findings were seen in this study (**Supplementary Figure 1**). When all subjects were included, there was a weak correlation between the proportion of Tfh and Tph cells and the proportion of plasma cells and/or plasmablasts. As might be expected based on the literature, the correlation with plasma cells was slightly stronger for Tfh than Tph (Tfh ρ=0.221, p=0.011; Tph ρ=0.210, p=0.016), whereas the opposite was seen for plasmablasts (Tfh ρ=0.164, p=0.059; Tph ρ=0.222, p=0.010).

Age-associated B cells (ABCs) are increased in SLE (34, 35) and have features suggesting that they are precursors of plasmablasts (34, 36). Consistent with previous studies, the levels of these cells were increased in early SLE, and in SARD overall. However, no substantive increases were seen in ANA^+ individuals lacking a SARD diagnosis. As previously reported, blood ABC levels were significantly correlated with the proportion of plasmablasts, and to a lesser extent, plasma cells (plasmablasts ρ=0.265, p=0.008; plasma cells ρ=0.255, p=0.011) (32). However, in contrast to previous reports, ABC levels correlated with Tfh (ρ=0.270, p=0.007) and not Tph levels.

Taken together, the data indicates that Tfh and Tph cell activation differs between ANA^+ individuals with and without SARD, with increases in both the Th2 and Th17 subsets of these populations in early SARD patients relative to those lacking a SARD diagnosis.

T Regulatory Cell Subsets Are Increased in ANA^+ NS and UCTD, Relative to Early SARD

Although there is some inconsistency regarding the proportion and function of T regulatory (Treg) cell populations in SARD, possibly due to heterogeneity in defining these populations and the markers used for their identification, available evidence suggests that Treg cells are reduced and/or functionally impaired in SARD patients (37–45). It has also been proposed that Tregs act to prevent symptoms in ANA^+ individuals lacking a SARD diagnosis (46). To explore whether there are differences in the proportions of various Treg populations between symptomatic and asymptomatic ANA^+ individuals, we examined extra-follicular, follicular, and $LAG3^+$ Treg populations, gated as shown in **Figures 2A–C**. For all three populations, there was a consistent trend to increase in asymptomatic ANA^+ NS and UCTD patients as compared to ANA^- HC and early SARD patients (**Figures 2D–F**), which variably achieved statistical significance. In contrast, these populations were either similar or somewhat reduced in SARD patients as compared to ANA^- HC. As a result, there was a significant increase in the ratio of Tph2 and Tph17 cells to extra-follicular Tregs in SARD patients when compared with ANA^+ individuals lacking a SARD diagnosis (**Figure 2G**).

One of the mechanisms by which Tregs, particularly $LAG3^+$ cells, exert their function is through secretion of TGF-β1 (47). Consistent with enhanced immunoregulation in ANA^+ NS, there were significantly elevated plasma levels of this cytokine relative to ANA^- HC (**Figure 2H**), with a progressive trend to normalization in UCTD and SARD patients. As expected, there was a moderate positive correlation between the proportion of $LAG3^+$ Tregs, but not extra-follicular or follicular Tregs, and TGF-β1 (**Figure 2I**).

Collectively, these findings suggest that there is a shift from predominant T cell regulation to predominant pro-inflammatory T cell activation that discriminates asymptomatic ANA^+ NS individuals from early SARD.

Accumulation of Innate Immune Populations Favoring Production of Pro-Inflammatory Factors Differentiates Early SARD From Asymptomatic ANA^+ Individuals

Dendritic cells (DC) play an important role in supporting immune activation in SARD, both through the production of type I IFN by plasmacytoid DCs (pDCs) and activation of T cell subsets by myeloid DCs (mDCs). Studies have shown that in SARD patients with active ongoing inflammation, there is a trend to reduced levels of these cells in the peripheral blood, which is associated with their increased localization to the tissues (48–50). To assess how these populations differ between symptomatic and asymptomatic ANA^+ individuals, pDCs and mDCs were examined (gating shown in **Figures 3A, B**). mDCs were further divided into $CD14^+$ and $CD14^-$ subsets, as previous studies have shown that $CD14^+$ mDCs are expanded in SARD, express a variety of pro-inflammatory cytokines, and are very effective inducers of Th2 and Th17 differentiation (51). As shown in **Figure 3C**, no differences were seen in the proportion of pDCs between any of the ANA^+ subject sub-groups and ANA^- HC. However, there was a significant increase in the proportion of $CD14^-$ mDCs in ANA^+ individuals lacking a SARD diagnosis as

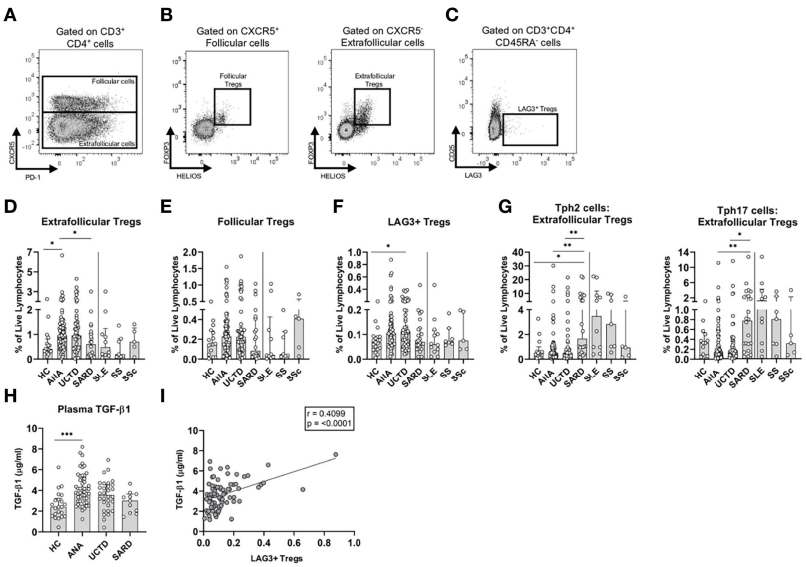

FIGURE 2 | T regulatory (Treg) subsets and transforming growth factor beta-1 (TGF-β1) are increased in anti-nuclear antibody positive (ANA^+) individuals lacking a systemic autoimmune rheumatic diseases (SARD) diagnosis. **(A)** Gating strategy for identification of ($CD3^+CD4^+$) follicular ($CXCR5^+$) and extra-follicular ($CXCR5^-$) T cells for a representative ANA^+ patient. Gating strategy for identification of **(B)** ($HELIOS^+FOXP3^+$) follicular and extra-follicular Tregs and **(C)** memory ($CD45RA^-$) $LAG3^+$ T regulatory cells ($LAG3^+$ Tregs, $LAG3^+CD25^-$). **(D–F)** The proportions of Treg subsets stratified by subject group. **(G)** The ratio of memory T peripheral helper 2 cells to extra-follicular Tregs; and the ratio of memory T peripheral helper 17 cells to extra-follicular Tregs stratified by subject group on a log10 scale. **(H)** Plasma TGF-β1 levels stratified by subject group. **(I)** The correlation between the proportion of memory $LAG3^+$ Tregs and TGF-β1 levels. The solid vertical line in each plot separates the groups that were statistically compared to one another from the individual SARD on the right, which were not statistically compared to any group. Bars represent the median with interquartile range. Each data point represents an individual subject. Statistical significance was determined using the Kruskal-Wallis test with Dunn's *post-hoc* test for multiple comparisons; $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. The strength of association was determined using a non-parametric Spearman correlation analysis. The solid line of best fit was computed from linear regression. HC, ANA^- healthy control; ANA, asymptomatic ANA^+; UCTD, undifferentiated connective tissue disease; SARD, systemic autoimmune rheumatic disease; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic sclerosis.

compared to ANA^- HC, with a trend to decrease in SARD patients as compared to the other ANA^+ groups (**Figure 3D**). Conversely, the proportion of $CD14^+$ mDCs was significantly increased in SARD as compared to both ANA^- HC and ANA^+ NS (**Figure 3E**). These findings suggest that there is a relative depletion of $CD14^-$ mDCs and accumulation of the more pro-inflammatory $CD14^+$ mDCs in the circulation of patients with early SARD, as compared to ANA^+ individuals lacking symptoms.

Previous studies indicate that SARD patients have increased proportions of monocytes in their peripheral blood, particularly those of the intermediate and non-classical type (52–55). Non-classical monocytes have been shown to have an increased capacity to secrete pro-inflammatory molecules and present antigens to T cells, as compared to classical monocytes (56, 57). To determine whether similar changes were observed in ANA^+ individuals lacking a SARD diagnosis, classical ($CD14^{hi}CD16^-$), non-classical ($CD14^{lo}CD16^+$) and intermediate monocytes ($CD14^{hi}CD16^+$), were gated as shown in **Figure 3F**. All three subsets were significantly expanded in early SARD when compared to ANA^- HC (**Figure 3G**). Although there was a slight trend to an increase in these populations in ANA^+ NS and UCTD patients compared to ANA^- HC, the proportion of these cells was significantly lower in ANA^+ NS individuals than in SARD patients (**Figure 3G**). Thus, individuals with SARD show significant expansion of both pro-inflammatory DC and pro-inflammatory monocyte populations that support T cell activation as compared to asymptomatic ANA^+ individuals.

Cellular Phenotypes Seen in ANA^+ Individuals Lacking a SARD Diagnosis Correlate With autoAb and IFN Levels

As shown in **Supplementary Table 1**, the group of ANA^+ individuals lacking a SARD diagnosis had significant variation in the type and number of autoAbs seen, as well as the ANA titer. We have previously shown that a subset of these individuals have elevated levels of IFN-induced gene expression in their peripheral blood, as measured by a composite score derived from the levels of 5 IFN-induced genes, termed the IFN5 score (12). We further demonstrated that the levels of this score correlate with the levels of IFN-α, as measured by high sensitivity ELISA (12), as well as anti-Ro60 and -Ro52 antibodies, and that ANA^+ individuals lacking a SARD diagnosis with high levels of anti-Ro52 antibodies or IFN-α are at an increased risk of clinical progression over the subsequent 2 years (22, 58). To investigate the association between these serologic changes and the peripheral blood cellular profile in these individuals, a Spearman correlation

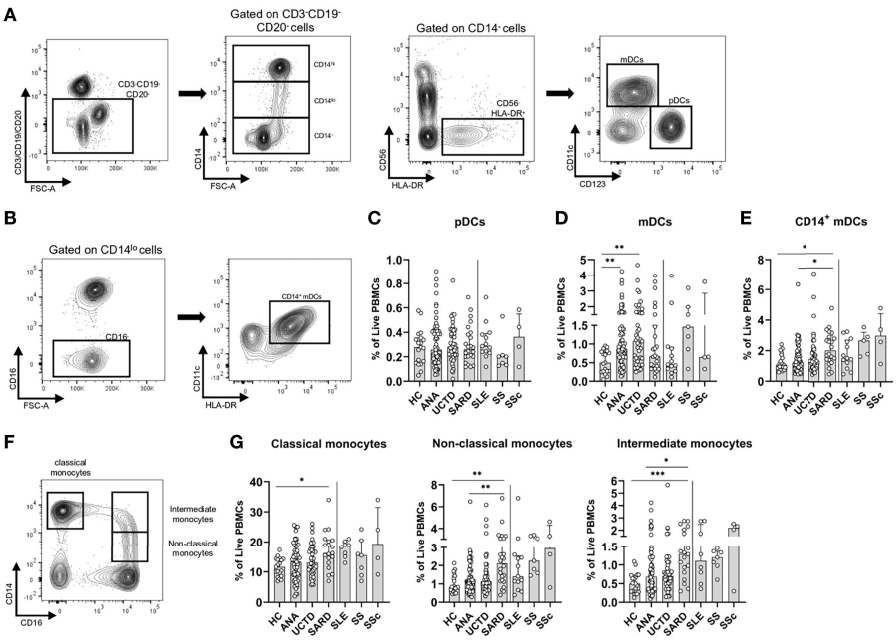

FIGURE 3 | Differences in the frequencies of innate immune populations distinguish anti-nuclear antibody positive (ANA^+) individuals lacking a systemic autoimmune rheumatic diseases (SARD) diagnosis from early SARD patients. **(A)** Gating strategy for identification of $CD14^-HLA\text{-}DR^+CD56^-$ plasmacytoid dendritic cells (pDCs, $CD123^+CD11c^-$) and myeloid dendritic cells (mDCs, $CD123^-CD11c^+$) from the lineage negative compartment ($CD3^-CD19^-CD20^-$) in a representative ANA^+ patient. **(B)** Gating strategy for identification of $CD14^+$ mDCs ($CD14^{lo}CD123^-CD11c^+$). **(C–E)** The proportion of pDCs, mDCs, and $CD14^+$ mDCs stratified by subject group. **(F)** Gating strategy for identification of classical monocytes ($CD16^-CD14^{hi}$); non-classical monocytes ($CD16^+CD14^{lo}$); and intermediate monocytes ($CD16^+CD14^-$). **(G)** The proportion of the monocyte subsets stratified by subject group. The solid vertical line in each plot separates the groups that were statistically compared to one another from the individual SARD on the right, which were not statistically compared to any group. Bars represent the median with interquartile range. Each data point represents an individual subject. Statistical significance was determined using the Kruskal-Wallis test with Dunn's *post-hoc* test for multiple comparisons. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. HC, ANA^- healthy control; ANA, asymptomatic ANA^+; UCTD, undifferentiated connective tissue disease; SARD, systemic autoimmune rheumatic disease; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic sclerosis.

matrix was produced (**Figure 4**). Although **Figure 4** shows the data for the pooled analysis of all ANA^+ individuals lacking a SARD diagnosis, very similar results were observed when ANA^+ NS and UCTD patients were examined independently (**Supplemental Material**; **Figure 2**).

As noted in our previous study, there was a moderate positive correlation between two markers of IFN levels, the IFN5 score and/or serum levels of IFN-α, and all of the serologic markers of autoAb production (17). IFN levels also correlated, moderately to strongly, with multiple markers of B cell activation, including activated memory B cell subsets and plasmablasts/plasma cells. This finding is compatible with previous work indicating that IFN acts to enhance B cell activation and differentiation to Ab-producing cells (59–62), and suggests that it may play an important role in driving autoAb production in ANA^+ individuals lacking a SARD diagnosis. The observation that the levels of plasmablasts/plasma cells correlate with serologic markers of autoAb production supports this concept. AutoAb production also demonstrated a weak correlation with Tfh and Tph cells, together with several of the subsets within these populations, consistent with the role of these cells in supporting Ab production. In general, the proportions of these T cells and their subpopulations did not correlate with IFN levels.

Unlike the pro-inflammatory T cell subsets, the proportion of $LAG3^+$ Tregs positively correlated with both autoAb and IFN levels, suggesting that the same immune processes that lead to activation of other immune populations may act to expand $LAG3^+$ Tregs, which may act in turn to suppress development of symptomatic autoimmunity. In contrast, the proportions of extra-follicular and follicular Tregs did not correlate with autoAb production, and in the case of extra-follicular Tregs demonstrated negative correlations with some of the activated immune populations.

Although the majority of innate immune subsets did not correlate with autoAb production, a number of populations correlated with IFN levels. Notably, the proportion of pDCs correlated inversely with markers of elevated IFN levels, suggesting that, similar to what is observed in SARD (48–50), pDCs are depleted from the circulation when high levels of IFN-α are produced, possibly as a result of recruitment to the tissues. In contrast, the levels of $CD14^+$ mDCs, intermediate monocytes, and non-classical monocytes all showed a moderate positive correlation with IFN levels. These findings suggest that one of the mechanisms by which high levels of IFN may promote progression is through facilitating development of these pro-inflammatory innate immune populations.

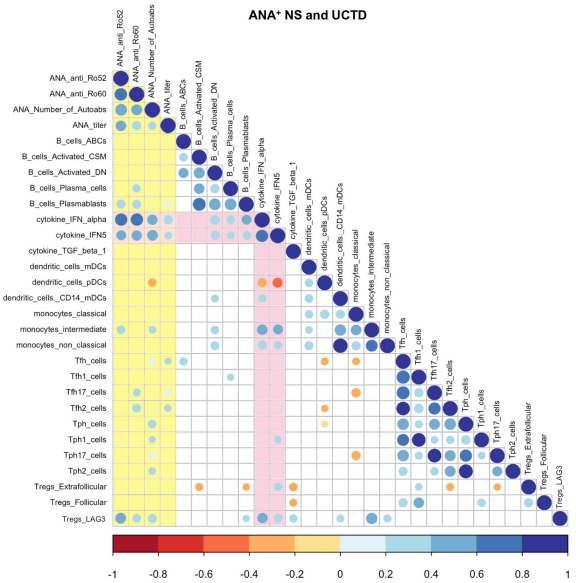

FIGURE 4 | Spearman correlation matrix between cellular and selected serologic/cytokine phenotypes in anti-nuclear antibody positive (ANA$^+$) individuals lacking a systemic autoimmune rheumatic diseases (SARD) diagnosis. The color and size of the dots represents the ρ value, with the scales shown at the bottom of each matrix. Non-significant ($p \geq 0.05$) correlations are not displayed. Associations with autoAb levels are highlighted in yellow and those with IFN levels are highlighted in pink. ANA, anti-nuclear autoantibody; CSM, class-switched memory; DN, double-negative; IFN, interferon; TGF, transforming growth factor; mDCs, myeloid dendritic cells; pDCs, plasmacytoid dendritic cells; Tfh, T follicular helper; Tph, T peripheral helper; Tregs, T regulatory cells.

Progressors Have More B and T Cell Activation Than Non-Progressors

As some of the immune cell populations correlated with elevated autoAb/IFN levels, which had been reported to be associated with an increased risk of clinical progression (22, 58, 63), it was of interest to us to determine the cellular immunologic features that distinguish ANA$^+$ individuals without SARD who will progress clinically from those who will not. To address this question, yearly longitudinal follow-up was offered to all of these individuals, with the option of attending clinic earlier if new symptoms developed. At present, there are 20 ANA$^+$ individuals who demonstrated symptomatic progression within 2 years of recruitment, defined as the development of new SARD diagnostic criteria or new organ involvement characteristic for SARD. Non-progressors were defined as participants who were followed for at least two years and remained stable without development of new symptoms during that period. The clinical characteristics of the progressors and non-progressors are outlined in **Supplementary Table 2** and an outline of disease progression in patients who progressed is given in **Supplementary Table 3**.

As shown in **Figure 5A**, within the B cell lineage, progressors had a significant increase in the proportion of plasmablasts as compared to non-progressors. Trends to increased proportions of activated class-switched memory and CD27$^-$IgD$^-$ double negative memory B cells, as well as ABCs and plasma cells, were also seen in progressors. These findings suggest that higher levels of B cell activation may be associated with an increased likelihood of progression.

Similar findings were observed for T cells, with higher percentages of Tfh and Tph cells in progressors as compared to non-progressors (**Figures 5B, C**). This increase was not associated with an expansion of any particular cytokine-producing subset. Although there were trends to an increase in the Tfh2, Tfh17, Tph1 and Tph2 subsets in progressors as compared to non-progressors, none of these achieved statistical significance. Thus, despite evidence for higher levels of Th2- and Th17-type cells in early SARD, increased levels of these populations do not appear to occur prior to or predict symptomatic progression.

Although the levels of the various Treg subsets were generally reduced in SARD as compared to ANA$^+$ individuals lacking a SARD diagnosis, no differences were seen in the proportions of extra-follicular or follicular Tregs between progressors and non-progressors (**Figure 5D**). However, there were significantly higher levels of LAG3$^+$ Tregs and TGF-ß1 in progressors when compared with non-progressors (**Figures 5D, E**). These findings suggest that the induced T regulatory pathway appears to be activated and expanded in progressors, but ultimately fails to prevent development of symptomatic autoimmunity.

In contrast to the findings observed for adaptive immune populations, the majority of innate immune populations showed no differences between progressors and non-progressors. A significant difference was only observed for the CD14$^-$ mDC population, which was reduced in progressors relative to non-progressors, mirroring the difference observed between SARD and ANA$^+$ individuals lacking a SARD diagnosis (**Figure 5F**). Very minor trends to decreased pDCs and to increased CD14$^+$ mDCs and intermediate monocytes were also seen in progressors (**Figures 5F, G**). Thus, significant accumulation of pro-inflammatory monocytes/DC populations does not appear to precede clinical progression.

DISCUSSION

While a considerable number of studies have examined the cellular immunologic changes in patients with well-established SARD, often on treatment, studies examining these immunologic changes in ANA$^+$ individuals lacking a SARD diagnosis are scarce. In a previous study examining predominantly T and B cell subsets, we found that many of the changes ascribed to SARD are also seen in asymptomatic ANA$^+$ individuals (ie. lacking SARD symptoms), suggesting that they are associated with the development of benign autoimmunity rather than the transition to symptomatic disease (17). These findings were validated in the current study, in a largely independent cohort, indicating the robustness of this phenotype. However, it remained to be determined what the key differences were between symptomatic and asymptomatic ANA$^+$ individuals. Here we show, by performing a more in-depth analysis of T

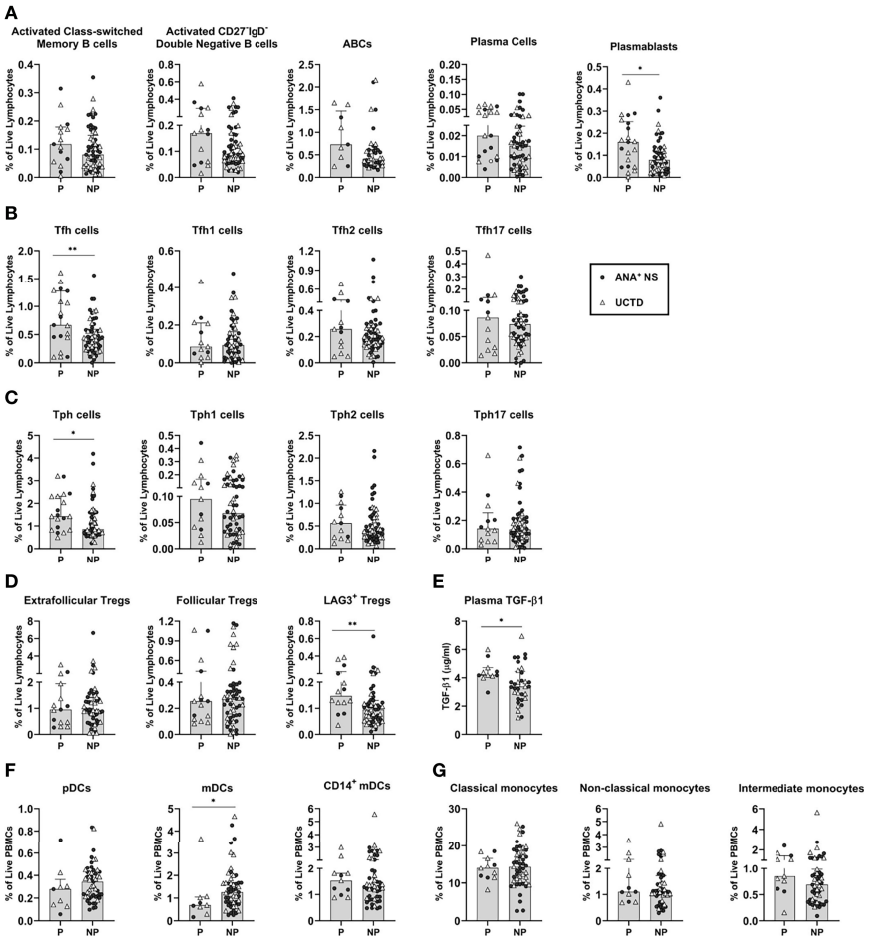

FIGURE 5 | Antinuclear antibody positive (ANA$^+$) individuals lacking a systemic autoimmune rheumatic diseases (SARD) diagnosis who demonstrated symptomatic progression demonstrate differences in adaptive and innate immune populations, relative to non-progressors. All graphs compare progressors and non-progressors at baseline (initial assessment). Patients diagnosed as ANA$^+$ NS or UCTD at initial assessment are represented by the closed circles and the open triangles, respectively. **(A)** B cell subsets. **(B, C)** T helper cell subsets. **(D)** T regulatory cell subsets. **(E)** Plasma transforming growth factor beta-1 (TGF-β1) levels. **(F)** Dendritic cell subsets. **(G)** Monocyte subsets. Bars represent the median with interquartile range. Each data point represents an individual subject. For each set of comparisons, statistical significance was determined using the Mann-Whitney test. *p ≤ 0.05, **p ≤ 0.01. P, Progressors; NP, Non-Progressors.

helper and regulatory cells together with innate immune populations, that these key differences lie in the balance between pro-inflammatory and regulatory immune cell subsets.

We have previously shown that Tfh cells are increased in ANA$^+$ NS individuals (17). We report here that this increase is predominantly due to an increase in Th2 cells and that there is a similar increase in Tph2 cells. These findings indicate that both germinal center and extra-follicular T cell responses are enhanced in ANA$^+$ NS, and given their correlation with autoAb levels, support autoAb production. Currently, the tissues where the extra-follicular T cell response arise are unknown. The observation that Th2 cells are increased in asymptomatic ANA$^+$ individuals, most of whom will never develop SARD, is consistent with previous work showing small but significantly elevated levels of Th1- and Th2-associated cytokines in these individuals (46) and studies showing that these cytokines can be seen years in advance of the transition to disease in SLE patients (64–66). However, in contrast to these serum cytokine studies, increases in circulating Th1 cells were not seen in the current study, nor in our previous study where we examined IFN-γ-producing cells in the CD4$^+$ T cell compartment (17). The reason for this disparity is unclear; however, it is possible that cytokine-producing Th1 cells are activated in ANA$^+$ NS individuals but remain localized within the tissues, and thus may only be detectable in the circulation through their cytokine secretion.

SARD patients had increased levels of Tph cells and a trend to increased Tfh cells, with increases in both the Th2- and Th17-subsets of these populations, relative to ANA$^+$ NS and UCTD patients. These findings suggest that the transition to SARD is associated with increases in the T cell populations that support B cell differentiation to Ab-producing cells. This observation is compatible with previous studies by ourselves and others showing that the number of anti-nuclear autoAbs and/or titers

of autoAbs are higher in early SARD than in ANA^+ individuals lacking a SARD diagnosis (22, 67, 68). In SLE, it has previously been shown that the transition to disease is associated with progressive increases in T cell-derived cytokines, with IL-17 in particular increasing concurrent with disease onset (64). Our findings provide additional support for the concept that significant increases in the Th17-type cells occur concomitantly with early disease, and indicates that this feature extends to the other SARD conditions.

T regulatory cell populations were highest in ANA^+ NS and appeared to drop to more normal levels in SARD, suggesting that these cells may be actively regulating inflammation to prevent symptomatic disease in ANA^+ NS. Previous studies examining the cytokine profile of asymptomatic ANA^+ individuals or SLE patients prior to their transition to symptomatic disease reached a similar conclusion (46, 64). As was seen in those studies, we found that the levels of TGF-ß1 were increased in ANA^+ NS patients as compared to ANA^- healthy controls, and normalized in SARD patients. However, the Treg populations that accompanied these increases were not examined in the earlier studies. Here, we show that ANA^+ NS and UCTD patients have increases in multiple Treg populations, but only the $LAG3^+$ population correlates with TGF-ß1. This observation is compatible with the function of $LAG3^+$ Tregs, which have been shown to regulate autoimmunity through secretion of IL-10 and TGF-ß1, as well as through direct cellular contact (47). Notably, $LAG3^+$ Tregs are induced in response to multiple environmental stimuli at barrier sites such as the gut, respiratory tract and skin, and have been shown to migrate to remote sites of autoimmune inflammation (69). Whether the expansion of this population indicates a role for environmental triggers in the development of autoimmunity in ANA^+ NS is currently unknown.

The shift in the balance of Treg to Tfh/Tph cells in early SARD, as compared to ANA^+ individuals lacking a SARD diagnosis, indicates that the onset of symptomatic autoimmunity is accompanied by a shift from predominant immunoregulation to a more pro-inflammatory pattern. A similar type of shift has been reported for UCTD patients as they transition to SARD, with an increase in the ratio of Th17 to Treg cells (70). The immune mechanisms leading to this shift remain to be definitively determined; however, one possibility is that the expansion of $CD14^+$ mDCs seen in SARD facilitates this shift. In SLE, this population has been shown to have an enhanced ability to support Th17 differentiation and, through OX40L expression, to augment Tfh cell differentiation and impair Treg function (51, 71). The non-classical and intermediate monocytes that are expanded in SARD have also been reported to support T cell activation/differentiation (56, 57). Alternatively, the balance of Treg to Tfh/Tph cells could be affected by changes in immune function at barrier sites, such as the gastrointestinal tract. Previous studies have shown that there are alterations in the gut microbiome in SARD that can be associated with enhanced gut permeability (72), which have been shown to facilitate a shift in the Treg to Th17 balance (73, 74).

In ANA^+ individuals lacking a SARD diagnosis, there was an inverse correlation between the levels of pDCs and serum levels of IFN-α and IFN-induced gene expression. These findings contrast with the results of a previous study of ANA^+ 'at-risk' individuals where decreased levels of pDCs were seen when compared with healthy controls (75). In that study, there was no correlation between the levels of pDCs and peripheral blood IFN-induced gene expression. Based upon this lack of correlation, together with RNAseq and functional data suggesting that the pDCs are functionally impaired in 'at risk' individuals, it was argued that pDCs are not a source of the IFN that induces the altered gene expression in the peripheral blood. Our findings argue for an alternate explanation for this lack of responsiveness, specifically that it reflects prior activation of this population. Along these lines, we and others have previously shown that pDCs transiently produce IFN-α and then become refractory to further activation with Toll-like receptor (TLR) stimulation (76, 77), a phenomenon termed TLR tolerance. TLR signaling in pDCs also induces their migration to the tissues, which may account for their depletion from the blood.

Comparison of progressors and non-progressors prior to progression indicated that progressors had elevated levels of B and T cell activation, with changes reflecting increased follicular and extra-follicular (tissue) responses, as compared to non-progressors. Progressors also had increases in the proportion of $LAG3^+$ Treg cells and TGF-ß1, suggesting that these cells are expanded during the immune response that leads to progression, but fail to prevent development of symptoms. Whether this failure results from impaired function of this or other Treg populations, as has been reported for SARD (40, 43–45, 71, 78), remains to be determined. Surprisingly, progressors had reduced levels of mDCs as compared to non-progressors. mDCs shuttle from the blood stream through the tissues and are retained in the tissue and/or draining lymph nodes when there is localized inflammation. Thus, the depletion of these cells may indicate the presence of sub-clinical inflammation prior to the onset of overt clinical symptoms in progressors.

In summary, we have identified a number of immunologic features that discriminate asymptomatic ANA^+ individuals from early SARD patients, and ANA^+ symptom progressors from non-progressors. Our findings provide insight into the immune mechanisms that lead to clinical symptoms in SARD, and raise the possibility of targeting these mechanisms to block development of SARD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Boards of the University Health

Network and Mount Sinai Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. JW and RG had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: RG, EV, KM, and JW. Acquisition of data: RG, EV, KM, DB, MK, CM-G, CN, SJ, LH, ZA, ZT, DB, AB, and JW. Analysis and interpretation of data: RG, EV, CM-G, CN, and JW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.886442/full#supplementary-material

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