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Risk and protective factors in the natural history of autoimmunity

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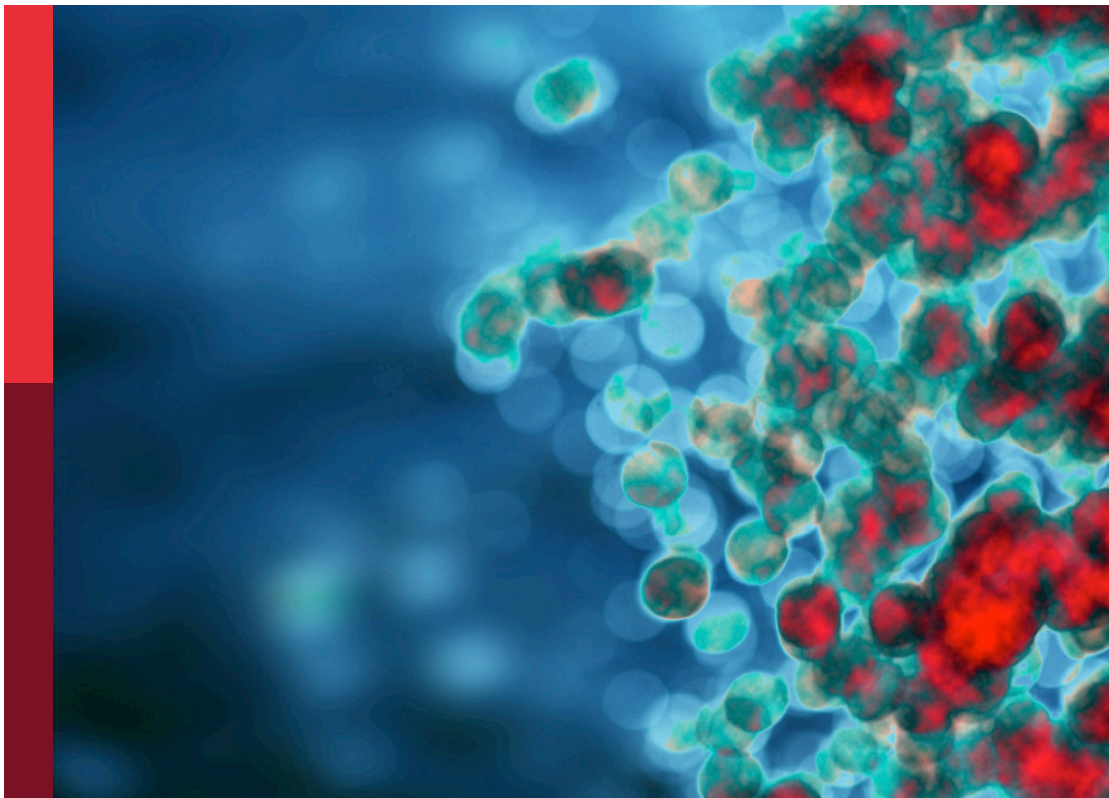

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Risk and protective factors in the natural history of autoimmunity

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Editorial: Risk and protective factors in the natural history of autoimmunity

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Editorial on the Research Topic
Risk and protective factors in the natural history of autoimmunity

Autoimmune diseases, the third-most common category after cancer and heart disease, affect at least 5% of the U.S. population (1) and are severe, chronic, and costly to individuals and society. Preclinical or asymptomatic autoimmunity may arise years before diagnosis, occurs in the general population, and appears to be increasing; an example is the rising prevalence of antinuclear antibodies in the U.S. in recent decades (2). However, only some individuals will develop symptoms and pathologies. The articles in this Research Topic focus on risk and protective factors for asymptomatic or preclinical autoimmunity and disease. The relationship between autoimmunity and other diseases, especially cancer and infections, also has important clinical implications. These questions take on greater urgency, given the apparent rise in rates and costs of many autoimmune diseases (3).

Autoimmunity

Clinical suspicion may lead to autoantibody testing; however, a low predictive probability can result in repeated, costly, and unnecessary testing. Barnado et al. addressed this problem using electronic health records of antinuclear antibody (ANA)-positive individuals, finding a greater likelihood of developing autoimmune diseases among those who were younger, female, with higher-titer ANAs, higher platelet counts, disease-specific autoantibodies, and more billing codes for relevant symptoms. In sum, this model is a useful clinical tool for identifying high-risk ANA-positive patients who should undergo further evaluation, while reassuring lower-risk individuals and reducing unnecessary referrals.

While autoantibodies are known to precede numerous autoimmune diseases, the majority of studies lack longitudinal sampling, and the factors that determine progression or regression are poorly understood. In children at risk of developing type 1

diabetes with disease-specific autoantibodies, Carry et al. found differences in DNA methylation, comparing those who progressed to disease, those who maintained autoantibodies, and those who sero-reverted. The candidate genes were related to diet, glucose levels, and immune and pancreatic beta cells. This suggests that environmental factors may contribute to disease risk. Further studies are needed that include exposure data and biomarkers in the progression of preclinical autoimmunity.

In a cross-sectional analysis of cotinine (a marker of cigarette smoke exposure) and ANA prevalence among a representative population sample of the U.S. population, Dinse et al. observed that, over the study periods (1988-1991, 1999-2004, and 2011-2012), the percentage of individuals with ANA was highest (13.3-19.2%) among nonsmokers but non-trending, lower (11.1-15.5%) for "passive" smokers but steadily increasing, and lowest for active smokers, increasing from 7.4% in 1999–2004 to 13.3% in 2011-2012. These findings imply the presence of unmeasured environmental influences on ANA prevalence.

Autoimmunity and cancer

In their review of the cancer risk associated with connective tissue disease, Tonutti et al. explored the multiple, complex interrelationships between these entities. The long-recognized increased cancer risk in many rheumatic conditions may develop for various reasons, including loss of immune tolerance due to oncogenesis, proinflammatory immune activation/autoimmunity that may promote oncogenesis, or immunosuppressive therapies that may decrease cancer surveillance. Conversely, autoimmunity may contribute to the removal of constantly generated neoplasms. Incomplete data support all these theories, and further research is needed. In response, Chen highlighted the need for multidisciplinary collaborations that synthesize different diseases and harmonize methods for detecting autoantibodies.

Sex differences in autoimmunity

Female sex is associated with ANA prevalence and an increased risk of many autoimmune diseases. Investigating a polygenetic risk score for juvenile idiopathic arthritis (JIA), Haftorn et al. examined scores in a population-based study of 238 JIA cases vs. over 73,000 controls. Their investigations into how to best model genetic susceptibilities revealed strong sex differences, suggesting that generalized additive models (GAM) should employ sex stratification, although general linear models can also be applied successfully.

Scofield et al. examined the mechanisms underlying sex differences in immune cells' Toll-Like Receptor (TLR7) signaling using published studies among subjects with SLE (along with other autoimmune diseases). The authors found that the sex bias among patients was explained by specific gene expressions, while inactivations of the X chromosome were also observed. Examined environmental factors included EBV infections and hormonal, mainly estrogen, effects on B cells, suggesting potential molecular pathways.

Environmental and genetic risk factors for autoimmune diseases

In their overview, Choi et al. highlighted diverse non-genetic risk and protective factors for systemic autoimmune rheumatic disorders and the complex interactions that may occur prior to disease development. These risk factors include airborne, waterborne, workplace/occupational, social, and behavioral factors, many of which have changed dramatically in recent decades, which may help explain the increase in autoimmunity and disease. Machine learning methods and multiomics have paved the way for a better understanding of these risk factors, and expansions of these and other new technologies could allow for better preventive approaches in the future.

In a study of JIA, Dåstøl et al. explored the role of seafood and dietary contaminants in the context of a polygenic risk score. While they did not find evidence of associations between estimated intakes of environmental contaminants and risk of JIA based on quantiles of fish intake or proxies for potential heavy metal exposure, patients with low genetic predisposition had stronger, significant associations with environmental toxicants, suggestive of environmentally induced JIA.

Some environmental factors may be considered triggers. Concerns have been raised that autoimmunity may develop following vaccine-specific immune activation and inflammatory responses. In their study of myositis patients, Alhassan et al., in the pre-COVID era, found genetic risk and protective factors for developing myositis within 6 months of vaccination. These factors included human leukocyte antigen (HLA) alleles and immunoglobulin (Ig) allotypes. Large-scale studies with greater genotyping and phenotyping are needed to personalize risk assessment and enhance vaccine safety.

Infections are also possible triggers. In a global network of 74 healthcare organizations and nearly 4 million patients, Hileman et al. investigated the incidence of autoimmune diseases up to 1 year after a diagnosed infection. They found an elevated risk of eight autoimmune diseases in patients diagnosed with COVID-19, especially cutaneous vasculitis, polyarteritis nodosa, and hypersensitivity angiitis. A positive ANA was also more likely and predictive of risk following infection. The authors concluded that SARS-CoV-2 may be a potential trigger for some autoimmune diseases, but the risk may diminish over time, as seen in this study following infection with Omicron variants.

Summary

Taken together, these studies highlight the importance of considering environmental factors and genetic susceptibility in the context of autoimmunity and disease. These contributions suggest the need for well-designed, multidisciplinary studies of

asymptomatic autoimmunity, exposome-genome interactions, and relationships with cancer and infections. The external exposome includes a broader range of features than represented here, including heavy metals, other xenobiotics, along with the psychosocial environment and natural disasters (4, 5), all of which warrant focused future research.

Author contributions

CP: Writing – original draft, Writing – review & editing, Conceptualization. EE: Conceptualization, Writing – review & editing, Writing – original draft. FM: Writing – review & editing, Conceptualization, Writing – original draft.

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New-onset autoimmune disease after COVID-19

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Introduction: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) may trigger autoimmune disease (AD) through initial innate immune activation with subsequent aberrations in adaptive immune cells leading to AD. While there are multiple reports of incident AD diagnosed after COVID-19, the risk in the context of key circulating strains is unknown.

Methods: TriNetX, a global, federated, health research network providing access to electronic medical records across 74 healthcare organizations, was utilized to define an adult cohort between January 1, 2020, and March 3, 2023. Exposure was defined as COVID-19 diagnosis (ICD-10 code or positive laboratory test). Age- and sex-propensity score-matched controls never had COVID-19 diagnosed. Outcomes were assessed 1 month to 1 year after the index date. Patients with AD prior to or within 1 month after the index date were excluded from the primary analysis. Incidence and risk ratios of each AD were assessed.

Results: A total of 3,908,592 patients were included. Of 24 AD patients assessed, adjusted risk ratios for eight AD patients who had COVID-19 were higher compared to those who had no COVID-19. Cutaneous vasculitis (adjusted hazard ratio (aHR): 1.82; 95% CI 1.55–2.13), polyarteritis nodosa (aHR: 1.76; 95% CI 1.15–2.70), and hypersensitivity angiitis (aHR: 1.64; 95% CI 1.12–2.38) had the highest risk ratios. Overall, psoriasis (0.15%), rheumatoid arthritis (0.14%), and type 1 diabetes (0.13%) had the highest incidence during the study period, and of these, psoriasis and diabetes were more likely after COVID-19. The risk of any AD was lower if COVID-19 was diagnosed when Omicron variants were the predominant circulating strains. A positive antinuclear antibody was more likely and predictive of AD after COVID-19.

Discussion: SARS-CoV-2 may be a potential trigger for some AD, but the risk for AD may decrease with time given the apparent lower risk after infection with Omicron variants.

KEYWORDS

autoimmune diseases, COVID-19, autoantibodies, risk factors, antinuclear antibodies

Introduction

Viral infections are often cited as important environmental triggers for autoimmune disease. In the setting of the global COVID-19 pandemic, this is highly relevant, as millions of individuals have been infected with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Indeed, there have been multiple reports of newly diagnosed autoimmune diseases after COVID-19 (1, 2). With the breadth of autoimmune disease manifestations, the rarity of many autoimmune diseases, and the lack of accumulated data in the context of COVID-19 variants up to this point, the overall risk of autoimmune disease after COVID-19 including recent key COVID-19 variants is not yet known.

The pathophysiology of autoimmune disease is complex, and the interplay of multiple factors, including genetic and environmental, likely contribute. Simplistically, the host immune response to viral infection has been postulated as a trigger for autoimmunity and includes the production of both interferons (especially alpha interferon), presentation of nuclear contents by "netting" neutrophils, and subsequent maturation of plasmacytoid dendritic cells that act as potent antigen-presenting cells. These virus-induced T cell-mediated autoimmune responses in the right host may lead to autoimmune disease *via* activation of the adaptive immune system resulting in B- and T-cell activations as evidenced first by autoantibodies and later by dysregulated T cells that contribute to overall loss of tolerance to self-antigen.

Interestingly, autoantibodies have been detected in patients with COVID-19 (3, 4). Further, some human proteins have homologous regions with SARS-CoV-2 peptides that could function as autoantigens (5). Additionally, it is clear that in some people with COVID-19, SARS-CoV-2 infection mediates a hyperinflammatory state. Dysregulated inflammasome activation has been implicated in autoimmune disease pathogenesis, and SARS-CoV-2 can activate the inflammasome (nod-like family, pyrin domain-containing 3, or NLRP3), which regulates the secretion of proinflammatory cytokines interleukin 1 beta (IL-1β) and IL-18 (6). More research is needed in this area; however, there is biological plausibility linking SARS-CoV-2 with autoimmunity.

The purpose of this study was to assess the risk of new-onset autoimmune disease within the first year after COVID-19 diagnosis in the context of the predominate circulating variants at the time of infection. We hypothesized that autoimmune disease diagnoses would be higher after COVID-19 infection than in age- and sex-matched controls and that risk would be attenuated when COVID-19 diagnosis occurred when the predominate circulating strains were the Omicron variants. While positive antinuclear antibodies (ANAs) are associated with a variety of autoimmune diseases, a positive ANA test alone is neither sufficient for rheumatologic diagnosis nor predictive of disease development. Therefore, our secondary aim was to evaluate the risk of ANA positivity after COVID-19 and how well ANA positivity predicted the development of new autoimmune diseases within the first year after COVID-19 diagnosis.

Materials and methods

This was a retrospective and population-based cohort study utilizing TriNetX. TriNetX is a global, federated, health research network providing access to electronic medical records including diagnoses, procedures, medications, laboratory values, and genomic information across large healthcare organizations. TriNetX provides de-identified data, transformed into a proprietary data schema, including an extensive data quality and accuracy assessment. This analysis was performed on data drawn from 74 healthcare organizations and completed on March 3, 2023. The study population was defined as adults 18 years of age or older, seen on or after January 1, 2020, with at least one follow-up visit after the index date. Patients with any of the autoimmune diseases evaluated as outcomes in this study diagnosed prior to the index date or within 1 month after the index date were excluded from the primary analysis. The exposure of interest was COVID-19 diagnosis defined by ICD-10 code or positive laboratory test (see Supplementary Table 1 for ICD-10 codes and laboratory tests included). Controls did not have COVID-19 diagnosis (defined by the same criteria) and were propensity score-matched to patients with COVID-19 by age and sex. The index date was defined as the date of COVID-19 diagnosis for the exposed group or first provider visit for any reason during the study period for controls. ANA positivity was defined as nuclear antibody presence in serum by immunofluorescence. This study was approved by the Institution Board Review Committee at Case Western Reserve University/University Hospitals Cleveland Medical Center (STUDY20231104). Written informed consent was waived, as the TriNetX system safeguards patients' privacy in reporting de-identified data.

Outcomes, i.e., incident autoimmune diseases, selected for inclusion were those previously reported in case reports and case series as well as additional autoimmune diseases to attempt to develop as complete a list as possible. Outcomes were defined by ICD-10 codes (see Supplementary Table 1 for ICD-10 codes utilized for each autoimmune disease included). Outcomes were assessed starting 1 month after the index date until 1 year after.

Statistical analysis

The two groups, the exposed or COVID-19 group and the controls or no COVID-19 group, were propensity score-matched by age and sex. Demographics were described by frequency and percent for categorical variables and by mean ± standard deviation for continuous variables for each group. Incidence of each autoimmune disease and risk ratios were assessed for each outcome, i.e., patients with outcome/total patients per group with 95% confidence intervals. Incidence and risk ratios were adjusted for age and sex through propensity score matching as described above. In the primary analyses, patients with any of the autoimmune diseases evaluated as outcomes in this study diagnosed prior to the index date or within 1 month after the index date were excluded. As part of the secondary analyses, patients with the specific outcome being analyzed were excluded

from the analysis for that outcome only. For the secondary analyses, the cohort was propensity score-matched by age and sex prior to excluding the patients with known disease.

Results

Data were available from 1,954,296 adults from January 1, 2020, to March 3, 2023, who lacked prior autoimmune disease and who were diagnosed with COVID-19. Adults without prior autoimmune disease and a diagnosis of COVID-19 during the same time period were propensity score-matched by age and sex at birth to these adults to generate a cohort of 3,908,592 people. Overall, the mean age ± standard deviation (SD) was 48.7 ± 17.9, and 57.7% were women. There were more people from racial and ethnic minorities among those who had COVID-19; however, there were also more people with unknown race and/or ethnicity among those who did not have COVID-19 (see Table 1).

Risk of incident autoimmune disease after COVID-19

The risk of being diagnosed with any autoimmune disease was higher within 1 year following COVID-19 compared to a similar time period in age- and sex-matched controls who did not have COVID-19 diagnosis (adjusted risk ratio (aRR) for any autoimmune disease 1.09 (95% confidence interval or CI 1.07–1.12)). In evaluating each type of autoimmune disease individually, one-third (8 out of 24) of the autoimmune diseases assessed were more likely to be diagnosed after COVID-19. Figure 1 shows adjusted risk ratios for each autoimmune disease assessed. Cutaneous vasculitis (aRR 1.82 (95% CI 1.55–2.13)), polyarteritis nodosa (aRR 1.76 (1.15–2.70)), and hypersensitivity angiitis (aRR 1.64 (1.12–2.38)) were associated with the highest risk. The three autoimmune diseases with the highest incidence during the study period were psoriasis (diagnosed in 5,690 or 0.15%), rheumatoid arthritis (5,618 or 0.14%), and type 1 diabetes mellitus (5,015 or 0.13%). Of these, both psoriasis (aRR 1.23 (95% CI 1.17–1.30)) and type 1 diabetes mellitus (aRR 1.38 (1.31–1.46)) were more common after COVID-19. Graves' disease (0.88 (0.80–0.97)), systemic lupus erythematosus (0.88 (0.80–0.97)), and Crohn's disease (0.84 (0.76–0.92)) were the only diseases less likely to be diagnosed after COVID-19. See Table 2 for the incidence of each autoimmune disease assessed overall as well as by group and adjusted risk ratios.

Of those with COVID-19, the risk of having been hospitalized within 10 days of COVID-19 diagnosis was higher for people who developed autoimmune disease after COVID-19 than people who did not (aRR for hospitalization 1.54 (95% CI 1.44–1.63)) (see Supplementary Figure 1).

In the secondary analysis, people with a specific autoimmune disease prior to or within 1 month after the index date were excluded from the analysis for that outcome. Overall, 4,407,892 individuals were included in this cohort. Supplementary Table 2 shows demographics overall and by COVID-19 exposure group, which were similar to the primary analysis. In this analysis, the risk of being diagnosed with 18 out of the 24 autoimmune diseases

TABLE 1 Demographics overall and by COVID-19 exposure group.

	Overall N = 3,908,592	COVID-19 n = 1,954,296	No COVID-19 n = 1,954,296	p-Value
Age (years) at index	48.7 ± 17.9	48.7 ± 17.9	48.7 ± 17.9	>0.99
Sex, n (%)				
Female	2,253,498 (57.7%)	1,126,749 (57.7%)	1,126,749 (57.7%)	>0.99
Male	1,654,160 (42.3%)	827,080 (42.3%)	827,080 (42.3%)	>0.99
Unknown	934 (<1%)	467 (<1%)	467 (<1%)	>0.99
Race, n (%)				
White	2,171,935 (55.6%)	1,139,355 (58.3%)	1,032,581 (52.8%)	<0.001
Black/African American	516,815 (13.2%)	280,842 (14.4%)	235,973 (12.1%)	<0.001
Asian	94,298 (2.4%)	42,225 (2.2%)	52,073 (2.7%)	<0.001
American Indian, Alaskan Native	13,447 (0.3%)	7,038 (0.4%)	6,409 (0.3%)	<0.001
Pacific Islander	4,947 (0.1%)	2,654 (0.1%)	2,293 (0.1%)	<0.001
Unknown	1,107,149 (28.3%)	482,182 (24.7%)	624,967 (32%)	<0.001
Ethnicity, n (%)				
Not Hispanic/Latino	2,133,897 (54.6%)	1,143,516 (58.5%)	990,381 (50.7%)	<0.001
Hispanic/Latino	296,358 (7.6%)	171,282 (8.8%)	125,076 (6.4%)	<0.001
Unknown	1,478,337 (37.8%)	639,498 (32.7%)	838,839 (42.9%)	<0.001

Groups are matched by propensity score. Propensity scoring included age, male sex, and female sex. People with any prevalent autoimmune diseases prior to or within 1 month after the index date were excluded prior to propensity score matching. Values shown are mean ± standard deviation for continuous variables and frequency (column percent) for categorical variables.

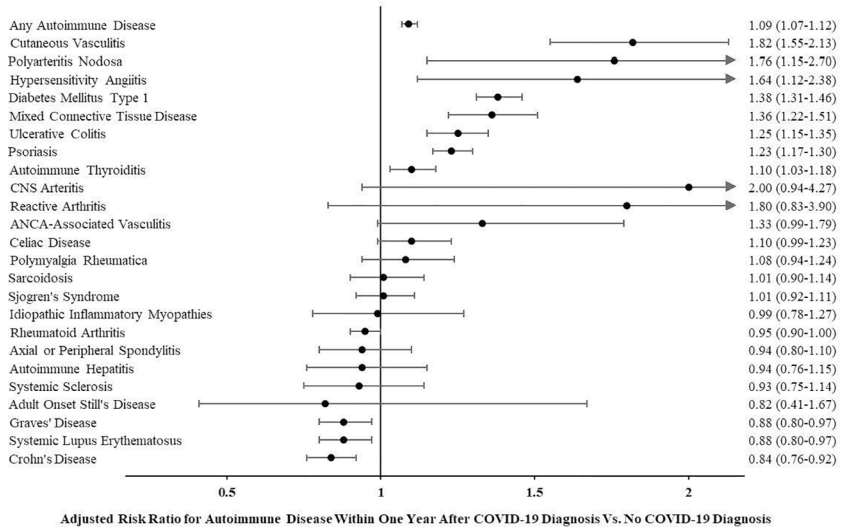

FIGURE 1
Adjusted risk ratio for autoimmune disease within 1 year after COVID-19 diagnosis *vs.* no COVID-19 diagnosis.

TABLE 2 Incident autoimmune diseases overall and by COVID-19 exposure group.

	Overall N = 3,908,592	COVID-19 n = 1,954,296	No COVID-19 n = 1,954,296	Adjusted risk ratio (95% CI)
Any autoimmune disease	31,052 (0.794%)	16,199 (0.829%)	14,853 (0.760%)	1.09 (1.07–1.12)
Autoimmune diseases more likely after COVID-19				
Cutaneous vasculitis	674 (0.017%)	435 (0.022%)	239 (0.012%)	1.82 (1.55–2.13)
Polyarteritis nodosa	91 (0.002%)	58 (0.003%)	33 (0.002%)	1.76 (1.15–2.70)
Hypersensitivity angiitis	116 (0.003%)	72 (0.004%)	44 (0.002%)	1.64 (1.12–2.38)
Type 1 diabetes mellitus	5,014 (0.128%)	2,908 (0.149%)	2,106 (0.108%)	1.38 (1.31–1.46)
Mixed connective tissue disease	1,407 (0.036%)	811 (0.041%)	596 (0.030%)	1.36 (1.22–1.51)
Ulcerative colitis	2,447 (0.063%)	1,359 (0.070%)	1,088 (0.056%)	1.25 (1.15–1.35)
Psoriasis	5,690 (0.146%)	3,137 (0.161%)	2,553 (0.131%)	1.23 (1.17–1.30)
Autoimmune thyroiditis	3,625 (0.093%)	1,902 (0.097%)	1,723 (0.088%)	1.10 (1.03–1.18)
Autoimmune diseases less likely after COVID-19				
Graves' disease	1,524 (0.039%)	713 (0.036%)	811 (0.041%)	0.88 (0.80–0.97)
Systemic lupus erythematosus	1,596 (0.041%)	746 (0.038%)	850 (0.043%)	0.88 (0.80–0.97)
Crohn's disease	1,737 (0.044%)	792 (0.041%)	945 (0.048%)	0.84 (0.76–0.92)
Autoimmune diseases with no associated increased or decreased risk after COVID-19				
CNS arteritis	30 (0.001%)	20 (0.001%)	≤10 (0.001%)	2.00 (0.94–4.27)
Reactive arthritis	28 (0.001%)	18 (0.001%)	≤10 (0.001%)	1.80 (0.83–3.90)
ANCA associated vasculitis	177 (0.005%)	101 (0.005%)	76 (0.004%)	1.33 (0.99–1.79)
Celiac disease	1,313 (0.034%)	689 (0.035%)	624 (0.032%)	1.10 (0.99–1.23)
Polymyalgia rheumatica	834 (0.021%)	433 (0.022%)	401 (0.021%)	1.08 (0.94–1.24)
Sarcoidosis	1,129 (0.029%)	568 (0.029%)	561 (0.029%)	1.01 (0.90–1.14)

(Continued)

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TABLE 2 Continued

	Overall N = 3,908,592	COVID-19 n = 1,954,296	No COVID-19 n = 1,954,296	Adjusted risk ratio (95% CI)
Sjögren's syndrome	1,811 (0.046%)	910 (0.047%)	901 (0.046%)	1.01 (0.92–1.11)
Idiopathic inflammatory myopathies	261 (0.007%)	130 (0.007%)	131 (0.007%)	0.99 (0.78–1.27)
Rheumatoid arthritis	5,618 (0.144%)	2,740 (0.140%)	2,878 (0.147%)	0.95 (0.90–1.00)
Axial or peripheral spondylitis	616 (0.016%)	298 (0.015%)	318 (0.016%)	0.94 (0.80–1.10)
Autoimmune hepatitis	370 (0.009%)	179 (0.009%)	191 (0.010%)	0.94 (0.76–1.15)
Systemic sclerosis	358 (0.009%)	172 (0.009%)	186 (0.010%)	0.93 (0.75–1.14)
Adult-onset Still's disease	31 (0.001%)	14 (0.001%)	17 (0.001%)	0.82 (0.41–1.67)

Groups are matched by propensity score. Propensity scoring included age, male sex, and female sex. People with any prevalent autoimmune diseases prior to or within 1 month after the index date were excluded from this analysis prior to propensity score matching.
CNS, central nervous system; ANCA, anti-neutrophil cytoplasmic antibodies.

evaluated was higher during the 1 year after COVID-19 diagnosis than during a similar time period in controls. The other autoimmune diseases had similar incidences over 1 year in both groups. See Supplementary Table 3 for the incidence of each autoimmune disease assessed overall and by group with adjusted risk ratios.

Effect of different timeframes on incident autoimmune disease risk after COVID-19

People diagnosed with COVID-19 from July 1, 2021, to November 30, 2021 (during which time the predominant circulating strain of SARS-CoV-2 was the Delta variant), as well as people diagnosed from January 1, 2020, to June 30, 2021 (pre-Delta variant timeframe), had a higher risk of any autoimmune disease when compared to people diagnosed with COVID-19 on or after December 1, 2021. Following December 1, 2021, Omicron SARS-CoV-2 variants were the predominant circulating strains in the USA. The adjusted risk ratio was 0.62 (95% CI 0.59–0.66) for incident autoimmune disease during Omicron *vs.* Delta variant timeframes and 0.66 (95% CI 0.64–0.69) during Omicron *vs.* pre-Delta variant timeframes. See Figure 2 for autoimmune diseases more commonly diagnosed during Delta and pre-Delta than Omicron variant timeframes. None of the autoimmune diseases were more likely to be diagnosed in the first year following COVID-19 infection when the predominant circulating strains were the Omicron variants.

The association of positive ANA test and incident autoimmune disease after COVID-19

In those without a history of autoimmune disease or a positive ANA test, the risk of having a positive ANA test was higher after COVID-19 (980 out of 1,949,921) than for those who did not have COVID-19 (578 out of 1,949,921), adjusting for age and sex (adjusted risk ratio 1.70 (95% CI 1.53–1.88)). Among those with COVID-19, the risk of developing an autoimmune disease was higher for those with a positive ANA test after COVID-19 diagnosis than those without a positive ANA test after adjusting for age and sex (adjusted risk ratio 11.90 (95% CI 6.28–22.55)) (see Supplementary Figure 2 for flowchart with absolute numbers). Specifically, a positive ANA test after COVID-19 was predictive of a new diagnosis for each of the following autoimmune diseases: systemic lupus erythematosus, rheumatoid arthritis, mixed connective tissue disease, Sjögren's syndrome, cutaneous vasculitis, hypersensitivity angiitis, autoimmune thyroiditis, Graves' disease, Crohn's disease, celiac disease, polymyalgia rheumatica, idiopathic inflammatory myopathies, autoimmune hepatitis, and systemic sclerosis (see Table 3).

Effect of any COVID-19 vaccination on incident autoimmune disease risk after COVID-19

Of 1,953,971 patients with COVID-19 and without a history of autoimmune disease, 159,306 (8.2%) had documentation of any COVID-19 vaccination in the TriNetX database. The adjusted risk ratio of any new autoimmune disease diagnosis within 1 year of the index date was 1.18 (95% CI 1.10–1.27) for those who received vaccination *vs.* those with no documentation of vaccination (see Supplementary Figure 3 for flowchart with absolute numbers). In assessing this for each separate autoimmune disease, increased risk post-COVID-19 in those vaccinated compared to those with no documentation of vaccination was only apparent for celiac disease (adjusted risk ratio 1.80 (95% CI 1.22–2.65)), autoimmune thyroiditis (1.70 (1.37–2.11)), Sjögren's syndrome (1.54 (1.16–2.04)), psoriasis (1.42 (1.21–1.66)), and ulcerative colitis (1.40 (1.09–1.80)). The risk of polymyalgia rheumatica was similar regardless of vaccination status. No autoimmune disease was less common post-COVID-19 in those who received vaccination when compared with those with no documentation of vaccination. See Supplementary Table 4 for the incidence of each autoimmune disease assessed overall and by group and adjusted risk ratios.

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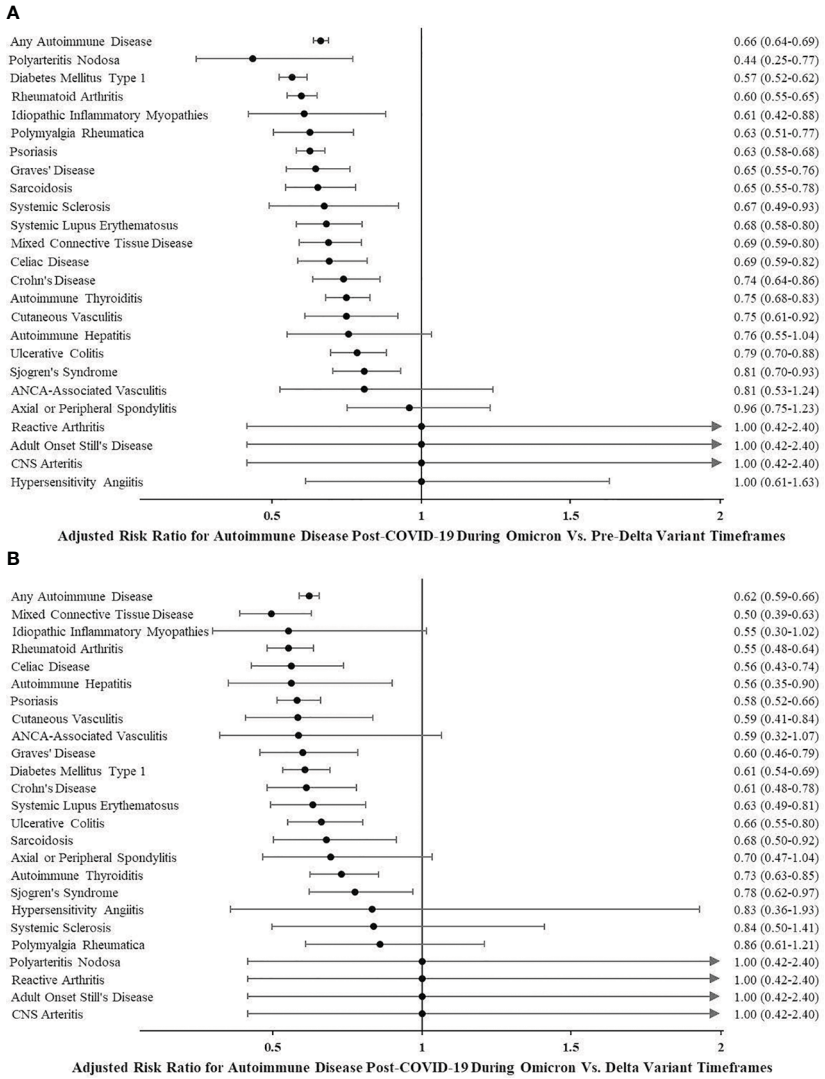

FIGURE 2
(A) Adjusted risk ratio for autoimmune disease post-COVID-19 during Omicron *vs.* pre-Delta variant timeframes. **(B)** Adjusted risk ratio for autoimmune disease post-COVID-19 during Omicron *vs.* Delta variant timeframes.

TABLE 3 Risk of autoimmune disease by ANA status.

	Positive ANA n = 991	Negative or no ANA n = 991	Adjusted risk ratio (95% CI)
Any autoimmune disease	119 (12.000%)	≤10 (1.009%)	11.90 (6.28–22.55)
Systemic lupus erythematosus	28 (2.825%)	0 (0%)	–
Rheumatoid arthritis	32 (3.229%)	≤10 (1.009%)	3.20 (1.58–6.47)
Mixed connective tissue disease	19 (1.917%)	0 (0%)	–
Sjögren's syndrome	17 (1.715%)	0 (0%)	–
Cutaneous vasculitis	≤10 (1.009%)	0 (0%)	–
Hypersensitivity angiitis	≤10 (1.009%)	0 (0%)	–
Autoimmune thyroiditis	≤10 (1.009%)	0 (0%)	–
Graves' disease	≤10 (1.009%)	0 (0%)	–
Crohn's disease	≤10 (1.009%)	0 (0%)	–

(Continued)

TABLE 3 Continued

(Continued)

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TABLE 3 Continued

	Positive ANA n = 991	Negative or no ANA n = 991	Adjusted risk ratio (95% CI)
Celiac disease	≤10 (1.009%)	0 (0%)	–
Polymyalgia rheumatica	≤10 (1.009%)	0 (0%)	–
Idiopathic inflammatory myopathies	≤10 (1.009%)	0 (0%)	–
Autoimmune hepatitis	≤10 (1.009%)	0 (0%)	–
Systemic sclerosis	≤10 (1.009%)	0 (0%)	–
Type 1 diabetes mellitus	≤10 (1.009%)	≤10 (1.009%)	1 (0.42–2.39)
Ulcerative colitis	≤10 (1.009%)	≤10 (1.009%)	1 (0.42–2.39)
Psoriasis	≤10 (1.009%)	≤10 (1.009%)	1 (0.42–2.39)
Sarcoidosis	≤10 (1.009%)	≤10 (1.009%)	1 (0.42–2.39)
Axial or peripheral spondylitis	≤10 (1.009%)	≤10 (1.009%)	1 (0.42–2.39)
Polyarteritis nodosa	0 (0%)	0 (0%)	–
CNS arteritis	0 (0%)	0 (0%)	–
Reactive arthritis	0 (0%)	0 (0%)	–
ANCA associated vasculitis	0 (0%)	0 (0%)	–
Adult-onset Still's disease	0 (0%)	0 (0%)	–

Groups are matched by propensity score. Propensity scoring included age, male sex, and female sex. People with any prevalent autoimmune diseases or positive ANA test prior to or within 1 month after the index date were excluded from this analysis prior to propensity score matching.
ANA, antinuclear antibody; CNS, central nervous system; ANCA, anti-neutrophil cytoplasmic antibodies.

Discussion

This is the first study of this magnitude of incident autoimmune disease including timeframes where circulating SARS-CoV-2 strains including more recent Omicron variants predominated. We demonstrate that COVID-19 diagnosis was associated with an increased risk of autoimmune disease in the year after infection, and notably, a positive ANA test was more likely after COVID-19 and predicted risk of new-onset autoimmune diseases.

Our finding of an increase in cutaneous vasculitis and polyarteritis after COVID-19 infection is not unexpected given that cutaneous small-vessel vasculitis or capillaritis such as leukocytoclastic vasculitis is frequently associated with perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) and antibodies against myeloperoxidase (anti-MPO) and is seen after a variety of infections (streptococcal and hepatitis infection in Henoch–Schonlein purpura) and environmental insults (levamisole in therapeutic and illicit drugs) (7, 8). These diseases also may be accompanied by autoantibodies to cytoplasmic ANCA (c-ANCA) as well as anti-phospholipid antibodies (a major cause of clots following COVID-19 infection). Cutaneous and systemic polyarteritis have both been reported in association with genetic deficiency of adenosine deaminase-2 (DADA2). Adenosine deaminase-2 (ADA2) function(s) are not entirely known, but the protein does appear to contribute to vascular integrity. High levels of ADA2 have been reported in association with infectious and inflammatory illnesses (9) including macrophage activation syndrome in systemic-onset juvenile idiopathic arthritis (10). DADA2 also has been associated with the activation of alpha interferon-associated genes, but any interrelationship between these two states has not been described yet in COVID-19.

Of those with COVID-19, the risk of having been hospitalized within 10 days of COVID-19 diagnosis was higher for people who developed autoimmune disease after COVID-19 than people who did not develop autoimmune disease. This suggests that those who developed autoimmune disease may have had more severe manifestations of COVID-19 than people who did not develop autoimmune disease. Further, prior to vaccination and treatment availability, individuals with genetic risk factors for systemic lupus erythematosus (similar to those with pre-formed anti-cytokine antibodies) may have been at increased risk of life-threatening COVID-19 infection and mortality, potentially resulting in the underrepresentation of systemic lupus erythematosus in COVID-19 survivors when analyzing later timeframes in context of predominant SARS-CoV-2 circulating strains.

The effect of differing circulating strains on the advent of post-acute sequelae of SARS-CoV-2 (PASC) has been previously investigated. Whether PASC is defined by the persistence of symptoms months after a COVID-19 infection or by new-onset health conditions linked to COVID-19, such as new-onset diabetes and cardiovascular disease, Omicron variants appear to be associated with lesser risk than earlier strains (11–15). Our study extends these observations of the potentially less pathogenic nature of Omicron variants to new-onset autoimmune diseases following COVID-19.

Another interesting observation in our study is the apparent increased risk of certain autoimmune diseases after vaccination. In contrast to our observation of autoimmune disease, studies have shown that vaccination is protective against PASC symptoms and incident diabetes after COVID-19 infection (11, 16, 17). New-onset autoimmune phenomena have been described post-COVID-19 vaccination (including immune-mediated hepatitis after COVID-19 vaccination), not all of which have a clear causal relationship established (18–20). Using real-world electronic health record data is more prone to underreporting of vaccination status, which may explain the low vaccination numbers in our study. That said, more studies are needed to better define the risk of autoimmune disease after vaccination.

The finding that ANA positivity is more common after COVID-19 infection and is predictive of new-onset autoimmune disease is

noteworthy. In contrast to the often transient positivity of antiphospholipid antibodies, p-ANCA, anti-MPO, and autoantibodies to rheumatoid arthritis and systemic lupus erythematosus may be present for 8 years or more prior to the onset of incident autoimmune disease (21, 22). This implies that if autoantibodies are present at increased frequency, the incidence of autoantibody disease may rise over longer periods of time, and our estimates of the frequency of autoimmunity may vastly underestimate the effect of COVID-19 on incident autoimmunity in long-term studies. Further, if Omicron variants overly induce lower levels of innate immune activation and subsequently less stimulation of B and T cells, it may take longer to induce similar levels of autoantibodies and T-cell derangements. It is therefore impossible to exclude the possibility that there will be a longer lag in the onset of new autoimmune disease following infection with the Omicron variants compared to Alpha/Delta SARS-CoV-2 and that ultimately, the rates of autoimmune disease may be similar to those seen with all the SARS-CoV-2 variants. Our report undoubtedly includes some patients in the control group who were asymptomatic for COVID-19 and who were neither tested for COVID-19 nor recognized as having COVID-19. This could lead to type II error, as some patients who developed COVID-19-related autoimmune disease may have been misclassified as having been COVID-19 uninfected, leading to smaller effect sizes regarding the risk of autoimmune disease after COVID-19. Importantly, our study also differs from prior reports from TriNetX that required either a positive or negative polymerase chain reaction test to be available for the analyses and focused only on the pre-Omicron era of COVID-19 (January 2020–December 2021) (23). In that way, our results are more generalizable, as they reflect the aggregation of the effects of pre-Delta, Delta, and Omicron variants of COVID-19 with comparisons for incident autoimmune disease and can be re-run at intervals for many years to come.

In addition, ANAs have been classified historically using indirect immunofluorescence assays (IFAs) mostly on the human epidermoid carcinoma (Hep2) cell line to detect nuclear localization, and ANA by IFA was used to define ANA positivity in our study. However, many laboratories have switched to a multiplex assay to measure autoantibodies directly by the target antigen. Clinicians may conclude that an ANA is positive when autoantibodies measure an antigen in the cocktail and may or may not obtain concomitant or subsequent ANA by IFA on the Hep2 cell line to detect nuclear autoantibodies. This is important, as multiplex assays may result in overdiagnosis of autoimmune disease based on a single autoantibody specificity, as positive autoantibody status is sometimes equated to a clinical diagnosis of autoimmunity by non-rheumatologists. This is particularly relevant to anti-U1-ribonucleoprotein (anti-RNP), which accompanies a high-titer ANA by IFA for classification as mixed connective tissue disease but is seen frequently at low levels in the current multiplex technology used across multiple centers. The specificity of low-titer reactivity by multiplex as predictive of future autoimmune disease has never been established, but such low-titer antibodies are observed frequently after COVID-19. Whether any of the anti-SARS-CoV-2 protein antibodies cross-react with antigens in the multiplex assays and therefore wane over time also is a topic ripe for exploration.

A strength of this analysis included the use of TriNetX to analyze data from a large population encompassing 74 healthcare organizations throughout the globe. However, we should recognize that we were unable to adjust for all potential confounders. Limitations of our study are similar to other large studies using electronic health record-derived data and include reliance on ICD-10 diagnoses for disease classification, which may have led to some misclassification. Similarly, we relied on electronic health record data for diagnosis of COVID-19 infection, which may have misclassified some asymptomatic COVID-19 infections as uninfected controls. However, if the latter is true, the effect of COVID-19 infection on incident autoimmune disease may have been underestimated. Further, as with all studies assessing new health conditions after COVID-19, we cannot rule out the possibility that some apparent incident autoimmune diseases were actually flares of previously undiagnosed disease, nor can we rule out potential relation with disproportionate stress. Finally, while Omicron may relate to reduced pathogenesis regardless of vaccination and prior infection status when compared to Delta (23), the role of innate and adaptive immunity in new-onset autoimmune disease after COVID-19 in the context of key variants is yet to be determined. Despite these limitations, however, the major strengths of our study lie in the fact that we have carefully captured the emergence of new-onset autoimmune disease following COVID-19 in a large-scale study. Importantly, our study differs from a prior report from TriNetX that required either a positive or negative polymerase chain reaction test to be included in the analyses and focused only on the pre-Omicron era of COVID-19 (January 2020–December 2021) (24), whereas our report reflects comparison and aggregation of the effects of pre-Delta, Delta, and Omicron variants of COVID-19 on incident autoimmune disease.

In summary, several autoimmune diseases were more likely to be diagnosed within the first year after COVID-19 than in age- and sex-matched controls. The risk of new-onset autoimmune diseases after COVID-19 appears to be attenuated with the more recent Omicron strains. Positive ANA test is more common after COVID-19 and is predictive of incident autoimmune diseases. This suggests that SARS-CoV-2 may be a trigger for certain autoimmune diseases. Future work must focus on longer-term observational cohorts and should assess the persistence and predictive value of different measured autoantibodies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Institution Board Review committee at Case Western Reserve University/ University Hospitals Cleveland Medical Center (STUDY20231104). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because data from the TriNetX system safeguards patient's privacy in reporting deidentified data.

Author contributions

CH: Writing – original draft, Writing – review & editing. SM: Writing – original draft, Writing – review & editing. NP: Data curation, Formal analysis, Writing – review & editing. NS: Supervision, Writing – review & editing. GM: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

CH has served as consultant for Theratechnologies and Gilead and has received research grant support from Gilead. GM has served as consultant for Gilead, Merck, Theratechnologies, Janssen, GSK/ViiV, and has received research grants from Gilead, Merck, Janssen and Theratechnologies.

The remaining 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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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1337406/full#supplementary-material

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Identifying antinuclear antibody positive individuals at risk for developing systemic autoimmune disease: development and validation of a real-time risk model

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Objective: Positive antinuclear antibodies (ANAs) cause diagnostic dilemmas for clinicians. Currently, no tools exist to help clinicians interpret the significance of a positive ANA in individuals without diagnosed autoimmune diseases. We developed and validated a risk model to predict risk of developing autoimmune disease in positive ANA individuals.

Methods: Using a de-identified electronic health record (EHR), we randomly chart reviewed 2,000 positive ANA individuals to determine if a systemic autoimmune disease was diagnosed by a rheumatologist. *A priori*, we considered demographics, billing codes for autoimmune disease-related symptoms, and laboratory values as variables for the risk model. We performed logistic regression and machine learning models using training and validation samples.

Results: We assembled training (n = 1030) and validation (n = 449) sets. Positive ANA individuals who were younger, female, had a higher titer ANA, higher platelet count, disease-specific autoantibodies, and more billing codes related to symptoms of autoimmune diseases were all more likely to develop autoimmune diseases. The most important variables included having a disease-specific autoantibody, number of billing codes for autoimmune disease-related symptoms, and platelet count. In the logistic regression model, AUC was 0.83 (95% CI 0.79-0.86) in the training set and 0.75 (95% CI 0.68-0.81) in the validation set.

Conclusion: We developed and validated a risk model that predicts risk for developing systemic autoimmune diseases and can be deployed easily within the EHR. The model can risk stratify positive ANA individuals to ensure high-risk individuals receive urgent rheumatology referrals while reassuring low-risk individuals and reducing unnecessary referrals.

KEYWORDS

antinuclear antibodies, electronic health record, risk model, autoimmune disease, rheumatology

1 Introduction

Positive antinuclear antibodies (ANAs) cause diagnostic dilemmas for clinicians across multiple specialties (1–3). Currently, no clinically available or validated tools exist to help clinicians determine the significance of a positive ANA. While a positive ANA serves as a diagnostic criterion for multiple autoimmune diseases, the test alone only has a 11% positive predictive value for systemic autoimmune disease (4). In US studies, rates of positive ANAs in the general population without autoimmune disease range from 14% to 27% (5, 6).

Frequent, inappropriate ordering of ANA testing has been recognized as a clinical problem by the American Board of Internal Medicine and the American College of Rheumatology in their "Choosing Wisely" campaign. Specifically, it is recommended to not order an ANA test unless specific symptoms for an autoimmune disease are present (7, 8). Up to 22% of all rheumatology referrals are for a positive ANA (1, 9). Only 11-20% of individuals with a positive ANA have an autoimmune disease diagnosed at referral (4, 10–13). Frequent ANA referrals in the setting of an international shortage of pediatric and adult rheumatologists (14–16) contribute to inefficient use of limited resources and lengthen wait times for rheumatology consultation (1, 9, 12).

Triage systems and electronic consultations have attempted to tackle the problem of frequent ANA referrals with limited success (12, 17–20). Risk models have been developed for systemic lupus erythematosus (SLE) (21, 22) but not for multiple systemic autoimmune diseases associated with a positive ANA. We aimed to develop and validate a robust risk model for use in the rheumatology clinic that uses readily available data in the electronic health record (EHR) to identify which individuals with a positive ANA are at high and low risk for developing systemic autoimmune disease.

2 Methods

2.1 Data source and patient selection

After receiving approval from the Vanderbilt University Medical Center (VUMC) IRB (#210189), we used the Synthetic Derivative, a de-identified version of the EHR that contains billing code and clinical data on over 3.6 million individuals spanning across three decades (23). Records from outside VUMC are not available.

We assembled all individuals within the Synthetic Derivative who had a positive ANA, defined as a titer ≥ 1:80 (Supplementary Figure 1). For ANA testing, the Hep-2 immunofluorescence assay was used for the entire study period (Appendix). We selected a random sample of 2,000 individuals with a positive ANA to perform chart review to assess for the model outcome and collect covariates. Model outcome was defined as developing a systemic autoimmune disease diagnosed by a rheumatologist, as EHR notes often lack systematic documentation of disease criteria (24). We performed chart review for development of systemic autoimmune disease from time of first positive ANA up to ten years later or individual's last EHR interaction. We allowed up to ten years, as individuals with autoimmune diseases can face significant diagnostic delays (25). Systemic autoimmune diseases are listed in Supplementary Table 1. In addition to diseases classically associated with a positive ANA (i.e., SLE, Sjogren's, systemic sclerosis, mixed connective tissue disease, and idiopathic inflammatory myopathies), we included other systemic autoimmune diseases such as rheumatoid arthritis (RA) and seronegative conditions (i.e., psoriatic arthritis, ankylosing spondylitis). Since the risk model will be used for triage to the rheumatology clinic, we aimed to include individuals with systemic autoimmune diseases who would be followed in that setting. While the ANA is not part of clinical criteria for these conditions, the ANA test is still frequently ordered in the evaluation of symptoms for these conditions (26). We excluded individuals with organ-specific autoimmune diseases such as autoimmune thyroiditis and autoimmune hepatitis, who would not be primarily managed by a rheumatologist. Individuals diagnosed outside of VUMC were included only if notes documented the individual was seen by an outside rheumatologist. For our primary analysis, we only analyzed individuals who were incident cases, defined as newly diagnosed with systemic autoimmune diseases at VUMC.

2.2 Model development

Based on clinical relevance and published SLE risk models (21, 22), prespecified predictors included demographics, laboratory values, and billing codes up to the time of first positive ANA

(Supplementary Table 2). Specifically, billing codes captured signs and symptoms for autoimmune diseases. A collection timeline for model covariates and outcome is detailed in Figure 1. Model outcome was developing a systemic autoimmune disease diagnosed by a rheumatologist within 10 years of first positive ANA (25).

Age was defined as age at first positive ANA documented at VUMC. The Synthetic Derivative defines race and ethnicity using a mixture of self-report and administrative entry with a fixed set of categories in accordance with NIH terminology. Studies have validated that these race and ethnicity assignments reflect self-report and genetic ancestry (27). For our primary analysis, race was initially excluded from the model as it was not significant in univariate analyses. Studies have shown that risk models that include race could potentially disadvantage high-risk groups from receiving appropriate care (28, 29). We performed a sensitivity analysis where race was included in the model, as studies demonstrate an increased risk of developing autoimmune disease in racial and ethnic underserved populations (1, 5).

We examined laboratory values one year prior to the date of the first positive ANA to allow for adequate data capture for individuals in the EHR and up to one month after to ensure capture of send-out studies such as the myositis antibody panel. We included autoantibodies associated with multiple autoimmune diseases (Supplementary Table 3). Autoantibodies were measured via enzyme-linked immunosorbent assays with manufacturer values to determine positivity (Appendix). We selected white blood cell count, platelet count, and serum creatinine as leukopenia, thrombocytopenia, and elevated serum creatinine have all been associated with autoimmune diseases (22, 30, 31). In SLE risk models (21, 22) and studies assessing presence of autoimmune diseases in positive ANA individuals (30, 31), leukopenia and thrombocytopenia were important predictors. Therefore, when examining multiple laboratory values for an individual, we selected the lowest white blood cell and platelet counts within the study period. For serum creatinine, we used the highest value within the study period to simulate how a rheumatologist might review lab trends. These values were treated as continuous variables. For missing laboratory values, we used median value imputation, as this method has been shown to be comparable to multiple imputation and is more feasible in real-time predictive models (32). We included ANA titer, as higher ANA titers are associated with risk of developing autoimmune disease (9, 30). Reporting of ANA titers are detailed in the Appendix. Briefly, ANA titer was dichotomized to 1:80 and ≥ 1:160 categories due to limited reporting of titers in some of the historical data. While different ANA patterns may have associations with different systemic autoimmune diseases (33), we did not include ANA pattern. ANA patterns are not reported in a standardized fashion at our institution according to the International Consensus on ANA patterns (33). Multiple or inconsistent patterns are often reported, particularly in the setting of changing technology over the study period. Further, as pattern is reported as a text variable, extraction from the EHR in real-time to input into the risk model would be challenging.

We used both ICD-9 and ICD-10-CM billing codes to capture signs and symptoms for systemic autoimmune diseases (Supplementary Table 4). These codes were significant in a UK SLE risk model (21) and were expanded upon to ensure capture of signs and symptoms for multiple autoimmune diseases in addition to SLE. Similar to the UK model, we searched for billing codes up to five years prior to the date of first positive ANA (21). In model development, we had an insufficient sample size to fit a model with a unique predictor for each billing code, so we created a single aggregated variable (Supplementary Table 5).

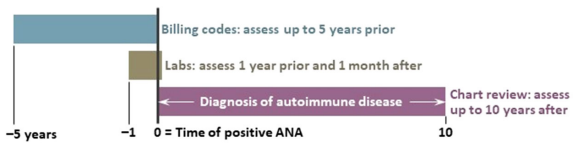

FIGURE 1
Timeline of model covariates. We assessed billing codes up to 5 years prior to the first positive antinuclear antibody (ANA) test. Laboratory values were assessed up to 1 year and 1 month after the ANA test. We conducted chart review for the model outcome of developing a systemic autoimmune disease diagnosed by a rheumatologist up to 10 years after the first positive ANA test.

2.3 Statistical analysis

We derived separate training and validation sets using 2,000 positive ANA individuals. We estimated that 10-15% of our 2,000 positive ANA individuals would have an incident autoimmune disease (4, 10–13), leading to 200-300 cases for the training and validation sets combined. To prevent overfitting and applying the rule of 10-15 outcomes per one degree of freedom (34), we fit a logistic regression model with 13 degrees of freedom. Prespecified variables are shown in Supplementary Table 2. Total number of visits, white blood cell count, and serum creatinine were collinear with included model variables and were removed from the final model. We performed logistic regression using the following predictors: age at time of first positive ANA, sex, ANA titer, platelet count, and billing codes. Final model formula is in Supplementary Figure 2. We also performed machine learning methods including extreme gradient boosting (XGB) (35–37) and neural networks. Hyperparameters are in the Appendix. We assessed model performance in the training and validation sets using c-statistic, Brier score, and calibration curves.

2.4 Model validation

We conducted an internal validation of the logistic regression model using a bootstrap with 200 replications (38, 39). The bootstrap validation can test the stability of a model across different samples. In addition, a random selection of individuals, separate from the training set, was set aside as a “hold-out” for model validation (Supplementary Figure 1). Specifically, we estimated needing 100-200 incident autoimmune disease cases to avoid overfitting our model. To achieve this sample, we used 1384

individuals of which 1030 incident individuals were used for analysis, resulting in 152 incident cases. We then used the remainder of the original 2,000 set for a validation set with 616 individuals, of which 449 incident individuals were used for analysis, resulting in 74 incident cases.

2.5 Sensitivity analyses and deployment feasibility assessment

For our primary analysis, we excluded subjects with "unclear" autoimmune diagnoses. In a sensitivity analysis, we treated "unclear" subjects as not cases. We also included a sensitivity analysis where race was included with categories of White, Black, and Other. To account for longitudinal and censored data, we conducted a Cox proportional-hazard model using the same variables as the logistic regression model. Outcome was time from first positive ANA to either autoimmune disease diagnosis or last EHR follow-up (Appendix). We initially dichotomized ANA titer to 1:80 and ≥ 1:160 categories due to historical reporting in some of our data (Appendix). We then conducted a sensitivity analysis using more recent data (2017-2021) that incorporated multiple categories for the ANA titer (1:80, 1:160, 1:320, 1:640, 1:1280, and ≥ 1:2560). We also conducted sensitivity analyses where seronegative conditions were not counted as a case (Appendix).

We applied our logistic regression model to data extracted from our EHR-provided data warehouse (Epic Clarity) to assess feasibility of deploying the model in real-time. We calculated risk probabilities for systemic autoimmune disease for individuals with a positive ANA from 2017-2021. This time period captured the updated ANA titer reporting to the most current data available at time of analysis.

3 Results

3.1 Individual characteristics

Training (n = 1030) and validation (n = 449) sets are compared in Table 1 with individuals having similar characteristics. In the training set, 15% (n = 152) of individuals with a positive ANA developed a systemic autoimmune disease. Individuals with systemic autoimmune diseases were younger (41.8 ± 21.5 vs. 47.9 ± 19.3 years, p = 0.003), more likely to be female (84% vs. 70%, p <

TABLE 1 Characteristics of incident positive ANA individuals in training and validation sets.

Characteristics	Training set n = 1030	Validation set n = 449	*p* value*
Autoimmune disease % (n)	15% (152)	16% (74)	0.40
Age at positive ANA, years mean ± SD	47.0 ± 19.8	48.0 ± 20.3	0.44
Race % (n)† White	85% (807)	85% (355)	0.88
African American	12% (113)	12% (50)	
Asian	2% (16)	1% (5)	
Other	1% (11)	1% (5)	
Ethnicity† Hispanic	3% (32)	3% (11)	0.46
Not Hispanic or Latino/a	97% (889)	97% (397)	
Sex			
Female	72% (739)	74% (333)	0.34
ANA titer‡			
1:80	20% (202)	19% (87)	0.92
≥ 1:160	80% (828)	81% (362)	
White blood cell count† K/uL, Mean ± SD	6.9 ± 3.4	6.9 ± 2.9	0.88
Platelet count† K/uL, Mean ± SD	235 ± 100	233 ± 92	0.58
Serum creatinine† mg/dL, Mean ± SD	1.1 ± 0.9	1.2 ± 1.4	0.25
Ever present autoantibody§ **% (n)**	15% (155)	15% (68)	0.96

(Continued)

TABLE 1 Continued

Characteristics	Training set n = 1030	Validation set n = 449	*p* value*
Total any billing codes mean ± SD	30 ± 60	37 ± 71	0.27
Count of specific billing codes‖ mean ± SD	0.7 ± 0.8	0.8 ± 0.9	0.01
Alopecia % (n)	2% (21)	1% (6)	0.35
Arthritis	26% (264)	31% (140)	0.03
Fatigue	20% (207)	23% (104)	0.18
Interstitial Lung Disease	1% (14)	2% (11)	0.14
Pulmonary Hypertension	1% (11)	1% (6)	0.66
Rash	9% (97)	9% (42)	0.97
Raynaud's	2% (19)	3% (12)	0.31
Serositis	4% (40)	5% (23)	0.28
Sicca	0.3% (3)	1% (5)	0.05

*Mann-Whitney U test for continuous variables and chi-square test for categorical variables. P values calculated with excluding missing observations.
†Race, ethnicity, and lab values have missing data with 81 (8%) for race, 109 (11%) for ethnicity, 201 (20%) for white blood cell count, 211 (20%) for platelet count, and 210 (20%) for serum creatine in the training set. In the validation set, 32 (7%) for race, 41 (9%) for ethnicity, 91 (20%) for white blood cell count, 95 (21%) for platelet count, and 100 (22%) for serum creatine.
‡For ANA titer, up until July 1, 2016, titers were reported as 1:40 (negative), 1:80, and ≥ 1:160. After this date, titers were then reported as 1:40 (negative), 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560.
§Presence of other autoantibodies included rheumatoid factor, cyclic citrullinated peptide, SSA (Ro), SSB (La), scl-70, centromere, RNP, Smith, dsDNA, ANCA, Jo-1, or any antibody from the myositis antibody panel.
‖See Supplementary Table 4 for full list of ICD-9 and ICD-10-CM billing codes and Supplementary Table 5 for details on scoring. For each individual, we counted if any billing code was ever present (1 for present, 0 for absent) for each of the nine categories (i.e., arthritis, fatigue) and then summed this up across the nine prespecified billing code categories for a maximum score of nine.

0.001), have a higher ANA titer (≥1:160 vs. 1:80) (90% vs. 79%, p = 0.002), lower serum creatinine (0.9 ± 0.6 vs. 1.2 ± 1.0 mg/dL, p < 0.001), higher platelet count (274 ± 113 vs. 229 ± 96 K/uL, p < 0.001), and a disease-specific autoantibody (51% vs. 9%, p < 0.001) (Table 2). No significant differences were found in race, ethnicity, or white blood cell count in individuals with vs. without systemic autoimmune diseases. Individuals with systemic autoimmune disease had a higher count of the nine billing code categories (scale 0 to 9) compared to individuals without disease (0.9 ± 0.9 vs. 0.6 ± 0.8, p < 0.001). Individuals with systemic autoimmune

TABLE 2 Characteristics of positive ANA individuals with vs. without systemic autoimmune disease in the training set.

Characteristics	No systemic autoimmune disease n = 878	Systemic autoimmune disease n = 152	Proportion with systemic autoimmune disease*	*p* value†
Age at positive ANA, years, mean ± SD	47.9 ± 19.3	41.8 ± 21.5	..	0.003
Race % (n)‡ White	85% (680)	85% (127)	16%	0.26
African American	12% (94)	13% (19)	17%	
Asian	2% (16)	0% (0)	0%	
Native American	0.1% (1)	1% (1)	50%	
Other	1% (10)	1% (1)	9%	
Ethnicity‡ Hispanic	4% (30)	1% (2)	6%	0.13
Not Hispanic or Latino/a	96% (744)	99% (145)	16%	
Sex Female	70% (612)	84% (127)	17%	< 0.001
Male	30% (266)	16% (25)	9%	

(Continued)

TABLE 2 Continued

Characteristics	No systemic autoimmune disease n = 878	Systemic autoimmune disease n = 152	Proportion with systemic autoimmune disease*	*p* value†
ANA titer§ 1:80	21% (186)	11% (16)	8%	0.002
≥ 1:160	79% (692)	90% (136)	16%	
White blood cell count‡				
K/uL, mean ± SD	6.9 ± 3.4	7.1 ± 3.2	..	0.49
Platelet count‡ K/uL, mean ± SD	229 ± 96	274 ± 113	..	<0.001
Serum creatinine‡ mg/dL, mean ± SD	1.2 ± 1.0	0.9 ± 0.6	..	<0.001
Ever present autoantibody‖				
No	91% (800)	49% (75)	9%	<0.001
Yes	9% (78)	51% (77)	50%	
Total any billing codes, mean ± SD	32 ± 62	23 ± 43	..	0.02
Count of specific billing codes,¶ mean ± SD	0.6 ± 0.8	0.9 ± 0.9	..	< 0.001
Alopecia	2% (16)	3% (5)	24%	0.24
Arthritis	23% (203)	40% (61)	23%	< 0.001
Fatigue	19% (169)	25% (38)	18%	0.10
Interstitial Lung Disease	2% (13)	1% (1)	7%	0.42
Pulmonary Hypertension	1% (9)	1% (2)	18%	0.26
Rash	9% (81)	11% (16)	17%	0.61
Raynaud's	1% (12)	5% (7)	37%	0.006
Serositis	4% (34)	4% (6)	15%	0.97
Sicca	0.3% (3)	0% (0)	0%	0.47

*Overall percentage of individuals with systemic autoimmune disease is 14.8%. P values calculated with excluding missing observations.
†Mann-Whitney U test for continuous variables and chi-square test for categorical variables.
‡Race, ethnicity, and lab values have missing data with 81 (8%) for race, 109 (11%) for ethnicity, 201 (20%) for white blood cell count, 211 (20%) for platelet count, and 210 (20%) for serum creatine.
§For ANA titer, up until July 1, 2016, titers were reported as 1:40 (negative), 1:80, and ≥ 1:160. After this date, titers were then reported as 1:40 (negative), 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560.
‖Presence of other autoantibodies included rheumatoid factor, cyclic citrullinated peptide, SSA (Ro), SSB (La), scl-70, centromere, RNP, Smith, dsDNA, ANCA, Jo-1, or any antibody from the myositis antibody panel.
¶See Supplementary Table 4 for full list of ICD-9 and ICD-10-CM billing codes and Supplementary Table 5 for details on scoring. For each individual, we counted if any billing code was ever present (1 for present, 0 for absent) for each of the nine categories (i.e., arthritis, fatigue) and then summed this up across the nine prespecified billing code categories for a maximum score of nine.

disease were more likely to have billing codes for arthritis (40% vs. 23%, p < 0.001) and Raynaud's phenomenon (5% vs. 1%, p = 0.006) but not the other seven code categories.

Of the 152 individuals with systemic autoimmune diseases, the most frequent diagnoses were SLE at 18% (n = 28) followed by other at 16% (n = 24), undifferentiated connective tissue disease at 16% (n = 24), and RA at 15% (n = 22) (Supplementary Table 6). Other consisted of psoriatic arthritis, unspecified inflammatory arthritis, and inflammatory bowel disease (Supplementary Table 6). Individuals with unclear diagnoses of systemic autoimmune disease (n = 66) were excluded from the primary analysis but are described in Supplementary Table 7. For individuals without systemic autoimmune diseases, when available alternative diagnoses were documented by rheumatologists, the most frequent diagnoses were fibromyalgia (n = 18), osteoarthritis (n = 11), and gout (n = 6) (Supplementary Table 8).

3.2 Model description and validation

The final model included age at first positive ANA, sex, ANA titer, presence of another autoantibody, platelet count, and billing code category count. Age was fit with a three-knot restricted cubic spline and interacted with sex and was prespecified based on prior

literature (21). Our data demonstrated a higher probability of systemic autoimmune disease in female vs. male individuals at younger ages but a similar probability at older ages (Supplementary Figure 3). The most important variables in the model were presence of another autoantibody (i.e., dsDNA), billing code category count, and platelet count (Figure 2). Model AUC was 0.83 (95% CI 0.79-0.86) (Figure 3A) with a Brier score of 0.10 and calibration shown in Figure 3B. XGBoost resulted in an AUC of 0.94 (95% CI 0.91-0.95) and neural networks with an AUC of 0.83 (95% CI 0.79-0.87).

Based on the internal bootstrap validation, the logistic regression model was stable and robust (Appendix). For the validation set (n = 449), 16% of individuals had systemic autoimmune disease (Supplementary Table 9). For the logistic regression model, AUC was 0.75 (95% CI 0.68-0.81) (Figure 3C) with a Brier score of 0.12 with calibration shown in Figure 3D. XGBoost resulted in an AUC of 0.72 (95% CI 0.65-0.78) and neural networks with an AUC of 0.74 (95% CI 0.68-0.81).

3.3 Sensitivity analyses

Race was included in the model with categories of White, Black, and Other resulting in an AUC of 0.83 (95% CI 0.79-0.87). When individuals of unclear case status for systemic autoimmune disease were counted as non-cases, model AUC was 0.80 (95% CI 0.76-0.83). When these unclear individuals were counted as cases, model AUC was 0.74 (95% CI 0.71-0.77). The distribution of model risk scores for these unclear individuals most closely matched individuals who were not cases (Supplementary Figure 4). For the Cox model with the outcome time to autoimmune diagnosis, model predictors behaved similarly to the logistic regression model (Supplementary Figure 5).

To reflect more updated ANA titer reporting, we used a cohort of individuals with a positive ANA from 2017 to 2021 (n = 584) (Appendix) to perform additional sensitivity analyses. For the 2017-2021 cohort, there was a significant difference in the distribution of ANA titers between cases and non-cases ($p < 0.001$). Of the cases, 40% had an ANA titer greater than 1:640, while 18% of non-cases had a titer greater than 1:640 (Supplementary Table 10). In this cohort, using a dichotomized ANA titer (1:80 vs. ≥1:160), model AUC was 0.85 (95% CI 0.81 – 0.90). For the model with full ANA titer reporting (i.e., 1:80, 1:160, 1:320, 1:640, 1:1280, ≥ 1:2560), model AUC was 0.89 (95% CI 0.84 – 0.92). Lastly, we assessed if a higher ANA titer cutoff would impact model performance using the above 2017-2021 cohort. We fit a model using an ANA cutoff at 1:160, which had an AUC of 0.83 (95% CI 0.78-0.87), identical to the performance of the model using the original ANA cutoff at 1:80 (AUC of 0.83 (95% CI 0.78-0.87)).

For using an alternative case definition for systemic autoimmune disease that did not count seronegative conditions (i.e., psoriatic arthritis, ankylosing spondylitis) as cases, model AUC was 0.86 (95% CI 0.83-0.89).

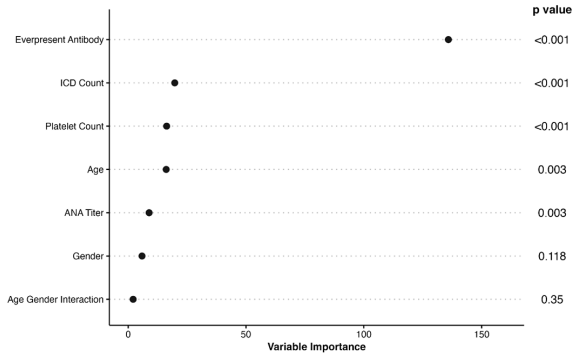

FIGURE 2
Importance of Variables in ANA Risk Model. The list of variables in the final ANA risk model are shown to the left with p values to the right. The x axis shows variable importance using a Wald statistic. Ever-present antibody refers to having a disease-specific autoantibody such as a rheumatoid factor or dsDNA. ICD count refers to billing code category count that ranges from 0 to 9.

3.4 Distribution of risk scores by type of autoimmune disease

We examined the distribution of model risk scores by type of autoimmune disease (Supplementary Figure 6). Individuals with SLE had the highest risk scores with a median of 0.481 and IQR of 0.312-0.685 followed by RA with 0.423 (0.144-0.582). Individuals labeled as other, with predominantly seronegative conditions, had the lowest median risk score of 0.107 (0.061-0.269). Seronegative conditions included psoriatic arthritis, and inflammatory bowel disease. Individuals with seropositive diseases had a higher median risk score compared to individuals with seronegative diseases (0.385 vs. 0.107, difference in medians = 0.278, 95% CI 0.195 – 0.332, $p < 0.001$).

3.5 Deployment feasibility

We assessed the feasibility of implementing the logistic regression risk model in our Epic EHR using data for all individuals with a positive ANA from 2017-2021 (n = 22,234). We observed a similar distribution of risk scores in Epic compared to our training set that used a de-identified EHR database (Synthetic Derivative) (Supplementary Figure 7). A demonstration of how the risk model works can be accessed at https://cqs.app.vumc.org/shiny/AutoimmuneDiseasePrediction/ (Figure 4). A disclaimer is included that the application is not intended for clinical practice.

4 Discussion

We developed and validated a risk model that predicts risk for developing systemic autoimmune disease in individuals with a positive ANA. The model is important because it utilizes readily available clinical data in the EHR, can be deployed easily within clinical practice, and helps risk stratify individuals with a positive ANA, a source of frequent rheumatology referrals. Our risk model

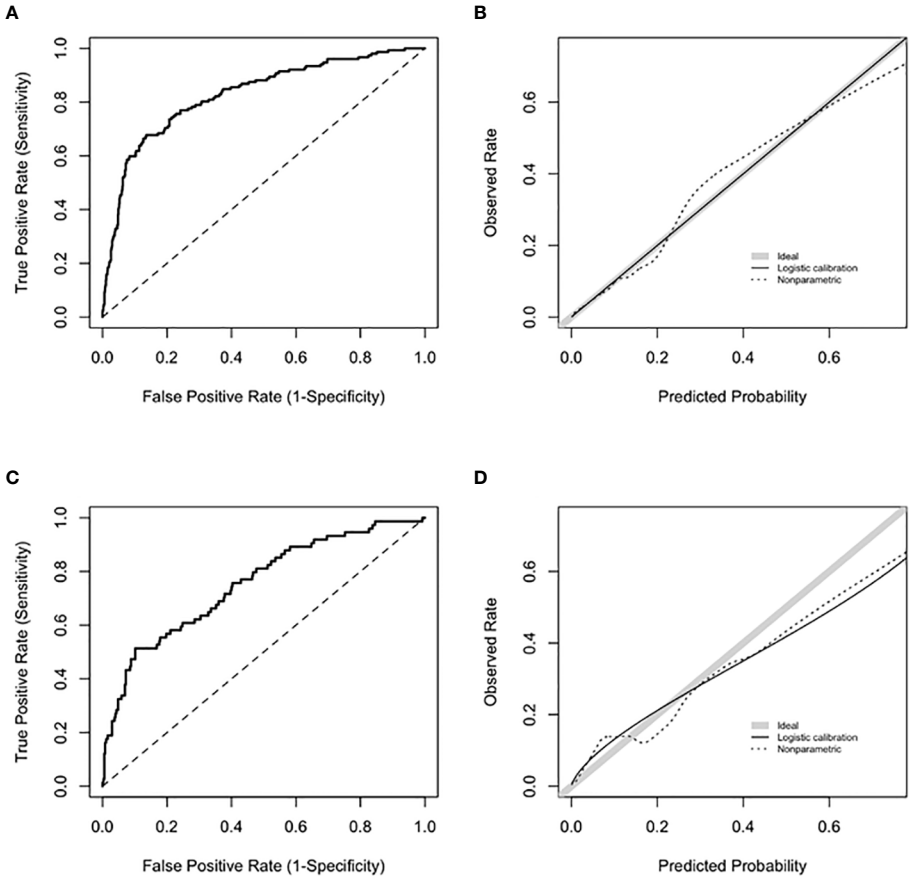

FIGURE 3
Model performance for training and validation sets. **(A)** shows ROC for the training set with an AUC 0.83 (95% CI 0.79-0.86). **(B)** shows calibration curve with a slope of 1 and intercept of 0 for the training set. Slopes that approach 1, as shown by the shaded grey line, demonstrate ideal calibration, agreement between predicted risk for systemic autoimmune disease and observed rate. **(C)** shows ROC for the validation set with an AUC 0.75 (95% CI 0.68-0.81). **(D)** shows calibration curve for the validation set. Calibration slope was equal to 0.71 and intercept was equal to 0.08.

identifies high-risk individuals, who are most likely to develop a systemic autoimmune disease, to ensure they are seen urgently for prompt diagnosis and treatment. Our risk model also identifies low-risk individuals who could be reassured, reducing unnecessary rheumatology referrals.

To our best knowledge, a risk model that focuses on individuals with a positive ANA and predicts risk for multiple systemic autoimmune diseases does not currently exist. One SLE risk model used UK EHR data (21) but did not focus on positive ANA individuals or examine risk for other autoimmune diseases.

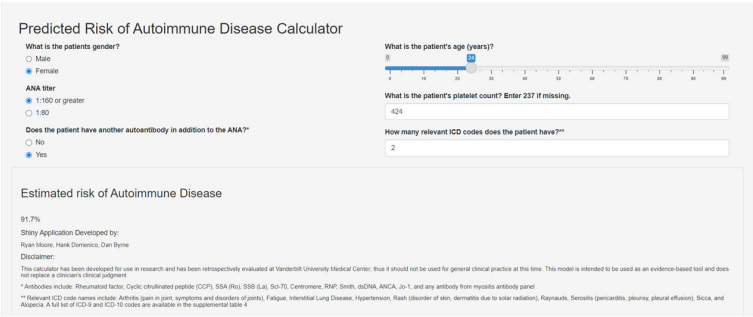

FIGURE 4
Screenshot of Shiny app for risk model for systemic autoimmune disease. The screenshot shows the risk model covariates used to estimate risk for systemic autoimmune disease. This app demonstrates how the risk score is calculated and is not intended for clinical practice. The Shiny app can be accessed at the following link: https://cqs.app.vumc.org/shiny/AutoimmuneDiseasePrediction/.

In this model, billing codes such as arthritis, rash, sicca, and fatigue were most significantly associated with risk of developing SLE along with female sex, younger age, and a higher number of clinic visits. We found similar results in our model and used similar billing codes but expanded our codes to identify not just SLE but also other systemic autoimmune diseases. Similar to the UK SLE model, we used a non-linear age and an age-sex interaction term. Despite its strengths, the UK SLE model had limited performance with a positive predictive value of 7-9%, a sensitivity of 24-34%, and an AUC of 0.75. Further, this model was not deployed in the EHR. Our model attained a higher AUC of 0.83 and can be easily deployed in real-time in the EHR.

Another SLE risk model from a Greek center (22) used random forests and Lasso-LR models. Not surprisingly, clinical items from the ACR SLE classification criteria accurately identified SLE cases with a high model AUC. While this study had a relatively large sample and a validation set, the model was developed using rheumatology clinic individuals and not in a general practice setting where there is often diagnostic dilemma. This model would be challenging to deploy in the EHR as it relies on SLE diagnostic criteria that may not be documented systematically, even in rheumatology notes (24).

The most important variable in our model was having another autoantibody in addition to the positive ANA, which is more specific for autoimmune diseases (1–3). Individuals with disease-specific autoantibodies may have a higher pretest probability for autoimmune disease by simply having these tests ordered. We tried to mitigate this bias by only including incident positive ANA individuals without established diagnoses of systemic autoimmune disease. Further, our institution conducts reflex testing where disease-specific autoantibodies are sent if an ANA is positive. Disease-specific autoantibodies may not be available fully in real-time at centers that do not perform reflex testing with a positive ANA, which may impact the performance of the model. The next most important variable was count of the nine prespecified billing code categories. *A priori*, we selected billing codes that captured signs and symptoms for autoimmune diseases and were significant in the UK SLE risk model (21). As expected, a higher count of these billing codes was predictive for systemic autoimmune disease. While billing codes may not always adequately capture an individual's symptoms, ICD billing codes allow for automation of the risk model in real-time and allow for portability of the model to other EHRs and databases that use common data models. Platelet count was also an important variable in our model. We originally hypothesized that a lower platelet count would be associated with systemic autoimmune disease. Prior SLE risk models identified thrombocytopenia as an important model predictor (21, 22), and other studies demonstrated an association of thrombocytopenia with autoimmune disease in positive ANA individuals (30, 31). Instead, we found a higher value of an individual's lowest platelet count was associated with systemic autoimmune disease. Higher platelet counts have been observed in individuals with RA and correlate with increased disease activity (40) and may also signal inflammation (41). *A priori*, we elected to not include inflammatory markers such as sedimentation rate (ESR) and C-reactive protein (CRP), as we had significant missingness of these values in the EHR. Further, these markers are nonspecific and can fluctuate widely in an individual (42–44). Elevations in these markers can be unrelated to an underlying systemic autoimmune disease, for example, in the setting of infection and malignancy (42–45).

A priori, we included race and ethnicity in our risk model. African American and Hispanic individuals have higher frequencies of positive ANAs compared to White individuals and are at higher risk of developing autoimmune disease, particularly SLE (1, 5). In univariate analysis, neither race nor ethnicity were significantly associated with systemic autoimmune disease, so race and ethnicity were not initially included. Studies have shown that risk models that include race could potentially disadvantage high-risk groups from receiving appropriate care (28, 29). For our model, this could include Black individuals. In a sensitivity analysis, we included race and found a similar model AUC of 0.83.

Our logistic regression model demonstrated robustness in both an internal bootstrap validation and a separate validation set. A successful bootstrap validation demonstrates the model can hold up when it encounters different samples. With predicting a clinically complex outcome where no current tools or risk models exist, our model validation demonstrated an improvement over usual care. To assess alternative approaches, we developed models using XGBoost and neural networks. XGBoost had a higher apparent AUC compared to the training set logistic regression model, likely due to overfitting, but did not hold up in validation. Neural networks performed similarly to the logistic regression model but with added complexity that would limit interpretability and deployment in the EHR.

While we developed, validated, and deployed a robust risk model to predict risk of systemic autoimmune disease in positive ANA individuals, our study has limitations. Our model was developed at a single academic medical center with more complex patients being evaluated, so may not generalize to other practice settings. Further, our study population was predominantly White, so it may not generalize to individuals with different race and ethnicity backgrounds and in other geographic areas. Our data encompasses an almost 30-year study period that included changes in ANA titer reporting. As a result, our primary analysis for the risk model included dichotomized reporting of the ANA titer to capture historical data. Sensitivity analyses using a more recent cohort of positive ANA individuals using both the dichotomized and full reporting of the ANA titer had similar model AUCs with overlapping confidence intervals. For future versions of the risk model, full reporting of the ANA titer can be used. We purposely defined systemic autoimmune disease based on a rheumatologist's diagnosis instead of classification criteria, as classification criteria are not systematically documented in clinical notes (24). Case definition by a rheumatologist could contribute to heterogeneity of cases (i.e. calling an individual with mild SLE and SLE nephritis both SLE).

Interestingly, our model did not perform as well in individuals with seronegative conditions not typified by autoantibodies, as

presence of these autoantibodies was the strongest predictor in our model. This limitation should be considered when interpreting risk scores. Seronegative conditions encompass overlapping diseases including plaque psoriasis, psoriatic arthritis, and inflammatory bowel diseases. These conditions have different HLA-based risk alleles, disease mechanisms, and disease presentations compared to seropositive conditions (46). While these seronegative conditions are not classically associated with a positive ANA, individuals with these conditions can have higher rates of ANA positivity compared to the general population (47–49) and often have an ANA test ordered as part of their clinical evaluation (26). In a sensitivity analysis, not counting the individuals with seronegative conditions as cases did not greatly impact the performance of the model.

Our model achieved a robust AUC of 0.83, but it does not discriminate perfectly between individuals with and without systemic autoimmune diseases. We found this AUC to be an improvement over usual care, where no current risk models exist to help risk stratify positive ANA individuals. The risk model was not designed to diagnose systemic autoimmune disease but to serve as a tool to identify positive ANA individuals who are at risk of developing systemic autoimmune disease within the next 10 years. The risk model can complement the clinician's judgment as well as the patient history and physical exam. The risk model could also assist the ordering physician in identifying individuals at lower risk that may not need rheumatology referral. This reassurance may reduce unnecessary referrals and expenses to the healthcare system. We purposefully created a continuous risk score, which is more rigorous than commonly used dichotomous or "cut-off" scores. Without a "cut-off score," we cannot currently estimate a positive predictive value. We are currently conducting a prospective validation of the risk model in real-time in the EHR to inform which individuals are low vs. high risk. While we created an application to demonstrate how the model incorporates variables and calculates a risk score, this application is not intended to be used in clinical practice yet or identify individuals as low vs. high risk.

In summary, we developed, validated, and deployed a risk model to identify which positive ANA individuals will develop systemic autoimmune disease. This risk model can be automated and deployed in real-time with no input needed from a clinician. In the setting of an international shortage of rheumatologists (14–16), a risk-stratifying tool for positive ANA individuals is critical. For future directions, we are assessing our risk model in real-time in the EHR prospectively and its impact on time to diagnosis and treatment for autoimmune diseases. Pending prospective validation, we envision our risk model would predict risk of autoimmune diseases within 10 years of a positive ANA similar to the FRAX that predicts 10-year fracture risk (50) or the ASCVD risk algorithm that predicts 10-year cardiovascular event risk (51). Risk scores from our model could then directly inform management of individuals with positive ANAs. High-risk individuals could be seen urgently by rheumatologists to ensure prompt diagnosis and treatment, and low-risk individuals could be reassured, reducing unnecessary rheumatology referrals.

Data availability statement

Raw data and R code used in analyses will be available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Vanderbilt University Medical Center. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

AB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RM: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. HD: Conceptualization, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SG: Data curation, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. AC: Data curation, Investigation, Writing – original draft, Writing – review & editing. AS: Data curation, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. BH: Data curation, Investigation, Writing – original draft, Writing – review & editing. KW: Data curation, Investigation, Writing – original draft, Writing – review & editing. AA: Data curation, Investigation, Writing – original draft, Writing – review & editing. LC: Data curation, Investigation, Writing – original draft, Writing – review & editing. AK: Data curation, Investigation, Writing – original draft, Writing – review & editing. AM: Investigation, Methodology, Project administration, Resources, Software, Writing – original draft, Writing – review & editing. DB: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1384229/full#supplementary-material

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Longitudinal changes in DNA methylation during the onset of islet autoimmunity differentiate between reversion versus progression of islet autoimmunity

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Background: Type 1 diabetes (T1D) is preceded by a heterogenous pre-clinical phase, islet autoimmunity (IA). We aimed to identify pre vs. post-IA seroconversion (SV) changes in DNAm that differed across three IA progression phenotypes, those who lose autoantibodies (reverters), progress to clinical T1D (progressors), or maintain autoantibody levels (maintainers).

Methods: This epigenome-wide association study (EWAS) included longitudinal DNAm measurements in blood (Illumina 450K and EPIC) from participants in Diabetes Autoimmunity Study in the Young (DAISY) who developed IA, one or more islet autoantibodies on at least two consecutive visits. We compared *reverters* - individuals who sero-reverted, negative for all autoantibodies on at least two consecutive visits and did not develop T1D (n=41); *maintainers* - continued to test positive for autoantibodies but did not develop T1D (n=60); *progressors* - developed clinical T1D (n=42). DNAm data were measured before (pre-SV visit) and after IA (post-SV visit). Linear mixed models were used to test for differences in pre- vs post-SV changes in DNAm across the three groups. Linear mixed models were also used to test for group differences in average DNAm. Cell proportions, age, and sex were adjusted for in all models. Median follow-up across all participants was 15.5 yrs. (interquartile range (IQR): 10.8-18.7).

Results: The median age at the pre-SV visit was 2.2 yrs. (IQR: 0.8-5.3) in progressors, compared to 6.0 yrs. (IQR: 1.3-8.4) in reverters, and 5.7 yrs. (IQR: 1.4-9.7) in maintainers. Median time between the visits was similar in reverters 1.4

yrs. (IQR: 1-1.9), maintainers 1.3 yrs. (IQR: 1.0-2.0), and progressors 1.8 yrs. (IQR: 1.0-2.0). Changes in DNAm, pre- vs post-SV, differed across the groups at one site (cg16066195) and 11 regions. Average DNAm (mean of pre- and post-SV) differed across 22 regions.

Conclusion: Differentially changing DNAm regions were located in genomic areas related to beta cell function, immune cell differentiation, and immune cell function.

KEYWORDS

DNA methylation, type 1 diabetes (T1D), DAISY, islet autoimmunity, reversion

1 Introduction

T1D is an autoimmune disorder with significant long-term morbidity. The pre-clinical phase is defined by the appearance of autoantibodies against pancreas cell antigens, termed islet autoimmunity (IA). There is strong evidence to support autoantibodies as a biomarker of T1D risk (1). However, IA is dynamic. While progression to T1D or multiple autoantibodies has been well characterized, a subset of individuals lose autoantibody positivity (2) and revert back to an autoantibody negative state. Autoantibody reversion was first described by Spencer et al (3) in a cohort of 685 individuals with a first degree relative affected by T1D. After 5 years, 7/20 developed T1D, 1 remained AB positive and 12/20 reverted. Transient autoantibody positivity has been described in several additional studies (4–6). However, these historical studies describing the transient nature of autoantibodies are difficult to interpret due to the development of more accurate autoantibody tests as well as differences in the definition of reversion. Vehik et al (2) conducted the most comprehensive and rigorous study of reversion in current literature. Among 596 individuals enrolled in The Environmental Determinants of Diabetes in the Young (TEDDY) study who developed one or more persistent autoantibodies, 21% reverted to an antibody negative state. Seroreversion was associated with significantly decreased risk of T1D (hazard ratio: 0.14, 95% CI: 0.04-0.59). Understanding the unique protective mechanisms occurring prior to or following IA that lead to IA reversion may have important implications for development of interventions that delay or prevent progression to T1D.

Genetic variation is a well-established risk factor for T1D (7). However, heterogeneity in disease concordance among monozygotic twins (8) as well as temporal changes in both T1D incidence (9) and age at T1D onset (10) in population studies have created a strong interest in the role of the environment in the etiology of T1D. Epigenetic modifications such as DNA methylation (DNAm) may represent a mechanistic pathway between genetic susceptibility, environmental exposures, and progression or reversion of IA. Epigenetics broadly describes a class of modifiable mechanisms that can regulate gene expression and are sensitive to external stimuli (11). DNAm is a frequently studied epigenetic biomarker that is postulated to play a role in autoimmune diseases as epigenetic mechanisms are important regulators of immune cell differentiation, plasticity and function (12, 13). DNAm changes prior to and during the IA phase may provide key information about underlying epigenetic profiles that explain progression or reversion from IA.

Previous epigenome wide studies have identified significant associations between DNAm and T1D (14–17). However, associations have been inconsistent and many of the studies have focused on static and/or post-T1D differences in DNAm between cases and controls (14–16). Although important in understanding the etiology of T1D, DNAm differences obtained from a single time point are difficult to interpret as it is not possible to determine when the changes occurred and moreover, whether they are the cause or consequence of the disease process. Understanding the timing of the changes is key to identifying external factors that cause these changes and therefore, may be amenable to preventative interventions. The purpose of this study was to test DNAm obtained before and after IA seroconversion (SV) in the Diabetes Autoimmunity Study in the Young (DAISY). We aimed to identify pre vs. post-SV changes in DNAm that differed across three distinct IA progression phenotypes, those who lose autoantibodies (reverters), progress to clinical T1D (progressors), or maintain autoantibody levels (maintainers).

2 Materials and methods

2.1 Study population

We reviewed individuals from the Diabetes Autoimmunity Study in the Young (DAISY) who developed islet autoimmunity (IA) between February 1994 and February 2019. DAISY is a longitudinal birth cohort study that includes n=2544 children at high risk for T1D. Subjects are recruited from two high risk populations, those with a first degree relative (FDR) with T1D or

those with a high-risk genotype, [defined as DRB1*04, DQB1*0302/DRB1*0301, DQB1*0201 (DR3/4 DQ8)]. Subjects complete study visits at 9, 15, and 24 months. Following the 24-month visit, study visits occur annually. As described previously (18), radioimmunoassays were used to test serum samples for autoantibodies to insulin (IAA), GAD65 (GAA), and IA-2 (IA-2A). Prior to 2010, GADA and IA-2A were tested using a combined radioassay (19). The National Institute of Diabetes and Digestive and Kidney Diseases harmonized assay was used to test for GADA and IA-2A after 2010 (20). Serum samples from individuals positive for GAD65, IAA, or IA-2 were tested for ZnT8A following development and implementation of the ZnT8 assay (21). If autoantibodies are detected, participants return for study visits every 3-6 months.

Islet autoimmunity (IA) was defined as the presence of one or more autoantibodies (see above) on at least two consecutive visits 3-6 months apart. The first visit among these consecutive autoantibody positive visits designated the start of IA, referred to as seroconversion (SV) throughout the remainder of the manuscript. We defined the three autoimmune progression phenotypes based on the autoantibody testing. The *reverter group* was defined as individuals who reverted for all autoantibodies during two or more consecutive visits, did not develop T1D, and were autoantibody negative for all autoantibodies at their last DAISY visit. The *maintainer group* was defined as individuals who continued to test positive for islet autoantibodies and did not develop T1D at the time of their last visit. The *progressor group* was defined as individuals who developed clinical T1D.

Among individuals who developed IA during DAISY and underwent autoantibody testing for a minimum of two or more study visits (n=213), we excluded individuals for the following: missing a pre- or post-SV blood sample (n=54), onset of IA unclear due to gaps (>365 days) in study visits (n=2), missing study visit prior to initial pre-SV positive visit (n=14). The Colorado Multiple Institutional Review Board approved all DAISY protocols (COMIRB 92-080). Informed consent and assent, if appropriate, was obtained from the parents/legal guardians of all children prior to participation in any research related activities.

2.2 Methylation measurements

Methylation measurements were obtained from peripheral whole blood samples collected at multiple time-points in individuals from DAISY. The Infinium HumanMethylation 450K Beadchip platform (Illumina, San Diego, CA, USA) was used to obtain methylation measurements on a subset of samples. The 850K Infininium MethylationEPIC BeadChip (Illumina, San Diego, CA, USA) was used to obtain measurements on the remaining samples. Two platforms were used due to changes in technology during the course of the study. Samples were randomly assigned to the two platforms making sure all timepoints from the same individual were included on the same platform.

DNA was bisulfite converted using the Zymo EZ DNA Methylation kit (Zymo Research, CA, USA). The bisulfite-converted DNA was labeled with fluorescent dyes and hybridized to 450K and 850K DNAm arrays. Samples were arranged on the plates in a specific sequence to minimize within and between batch effects (plate effects are represented by first 11 digits of the array variable on GEO). The minfi (v1.12.0) package (22) in R (v3.5.2) was used to perform quality control (QC) checks at the sample level. The processing pipeline is described in greater detail in Vanderlinden et al (23).

The DNAm probes were annotated to the genome based on the hg19 genome build using the Illumina annotation manifest files. Non-autosomal CpGs or CpGs located within or near (<2 base pairs) known single nucleotide polymorphisms (SNPs) were excluded. CpG sites with a beta range <3% on both platforms were removed from analysis. A total of n=198,008 overlapping DNAm probes met our filtering criteria and were used in subsequent analyses. Normalized M-values (SeSAMe (v1.0.0) pipeline with Noob normalization) were used in all statistical analyses. We use the term DNAm probe and the probe identifier when referring to the data in the Methods and Results. However, each probe is designed to measure DNAm at a single CpG site which is used as a more general term in the Discussion. See Figure 1 for an overview of the study methods.

2.3 Overlapping gene expression measurements

Gene expression data were available in a subset of individuals (n=36) at the post-SV visit. RNA processing and quantification is described in greater detail in Carry et al (24). In brief, paired end sequencing was performed using the Illumina NovaSEQ 6000™ system and samples were quantified against the Ensembl reference transcriptome (hg19, version 87) using the RSEM algorithm (25). Data were quantile normalized using DESeq2 (26), re-normalized using RUV (27), and then transformed using the regularized log function (26). The transformed data were used in all subsequent statistical analyses.

2.4 Overlapping metabolomics measurements

Untargeted metabolomics data were available in a subset (n=110) of individuals at both the pre-SV and post-SV visits. Metabolomics processing and quantification is described in greater detail in Carry et al (28). In brief, non-fasting plasma samples were used to quantify metabolite levels using three untargeted panels, HILIC panel: HILIC-QTOF MS/MS (29), GCTOF panel: GC-TOF-MS (30), and Lipid panel: CSH-QTOF MS/MS (31). BinBase (32) was used to process and annotate the GC-TOF-MS data. MS-Dial (33) was used to process and annotate the liquid chromatography (LC), CSH-QTOF-MS and HILIC-QTOF-MS, data. LipidBlast (34) and Massbank of North America were also used to annotate the complex lipids (http://mona.fiehnlab.ucdavis.edu/). Metabolomic data were normalized using the systematic error removal using random forest (SERRF) algorithm (35). All metabolites were Box-Cox transformed prior to statistical analysis.

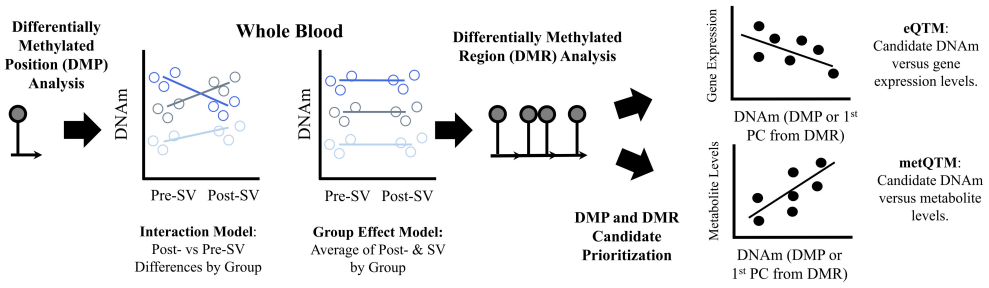

FIGURE 1
Summary of methods used to identify and prioritize DNAm candidates. Description: We used an epigenome wide association study design to identify differentially methylated positions (DMP) associated with the three islet autoimmunity progression phenotypes, reverters, maintainer, or progressors. We used two DMP models (1) an interaction model that tested whether changes in DNA methylation (DNAm) levels at single CpGs pre-IA versus post-IA differed across groups and (2) a group effect model that tested whether average methylation levels (pre- and post-IA) differed across groups. We also performed regional analyses (differentially methylated regions or DMRs) based on single CpG sites from the two models to identify regions with consistent methylation effects. We identified regions where average regional methylation levels differed between groups (μDMRs) as well as regions where changes in regional methylation levels pre- vs post-IA differed across groups (ΔDMRs). In order to prioritize regions, we tested whether the DNAm candidates identified in our analysis were associated with gene expression levels post-SV, an expression quantitative trait methylation analysis (eQTM). To account for the multiple CpGs within each DMR, we used a principal component analysis to capture common patterns across all CpGs included in the DMR. We identified cis-eQTMs (midpoint of region +/- 500 KB of the TSS of the gene) by testing the correlation between gene expression and the 1st principal component. We also tested the correlation between DNAm candidates and metabolite levels obtained from overlapping samples, a metabolite quantitative trait methylation analysis (metQTM). We used a principal component analysis to capture common patterns across all CpGs included in the candidate DMRs. We tested the correlation between metabolite levels and the 1st principal component. CpGs are represented by lollipop plots in the figure.

2.5 Genetic ancestry

Ancestry principal components (PC) were estimated for all study participants from genetic data collected in DAISY. Sample processing and genotyping were performed at the University of Virginia School of Medicine Center for Public Health Genomics based on exome genotyping (Illumina HumanCoreExome-24 BeadChip, N=283) or whole genome sequencing (N=162) from the larger DAISY population, see Buckner et al (36) for a more complete description of the genetic processing and calculation of the genetic ancestry PCs.

2.6 Statistical analyses

The overall methods workflow is summarized in Figure 1. Linear mixed models were used to test for differences in DNAm between the pre- and post-SV visit across reverters, maintainers, and progressors (autoimmune phenotype*visit interaction). Separate linear mixed models were also used to test for differences in average DNAm (mean of the DNAm levels at the pre- and post- SV visits) between the autoimmune phenotypes (group effect). Platform (EPIC vs 450K), age, sex, and cell proportions (estimated using the minfi (v1.12.0) package (22) implementation of the Houseman method) were adjusted for in all models. The group effect models were also adjusted for population ancestry (see Supplementary Material for complete description of ancestry data). Ancestry data (1st 2 PCs) were unavailable for 2 individuals in the group effect model and thus, these individuals were not included in this analysis. See Appendix 1 (Data Sheet 1) for the linear mixed model code. We did not adjust for ancestry in the interaction (autoimmune phenotype*visit) models because the interaction models test for within individual differences, and thus are less likely to be impacted by time invariant confounders such as population ancestry. The Benjamini Hochberg false discovery rate (FDR), was used to correct for multiple comparisons (37). Significance was assessed based on the FDR adjusted p-value <0.10. Model diagnostics are described in the Supplementary Files (Data Sheet 2), see Appendix 2, Figures A–C and Table A.

The comb-p python software package (38) was used to identify differentially methylated regions (DMRs). Within the comb-p pipeline, we used a seed p-value of 0.1 and then searched for adjacent probes within a window of 500 bases, using a step size of 50 bases. Comb-p combines probes within this window and then calculates an overall, spatially corrected p value for the entire region based on the Stouffer-Liptak method. The Sidak method is used to adjust the overall regional p values for multiple testing. Regional analyses were performed based on the individual DNAm probes from the interaction (post- vs pre-SV changes by autoimmune phenotype), referred to as differentially changing DMRs (ΔDMR) throughout the remainder of the manuscript. Regional analyses were also performed based on DNAm probes from the main effect model (differences in average of pre- and post-SV DNAm between groups), referred to as average DMRs (μDMR) throughout the remainder of the manuscript. For both regional analyses, we reviewed all regions with ≥4 DNAm probes that were significant at the combined Sidak adjusted region p value of 0.10. Because the interaction and group effect p values are based on a two degree of freedom test (numerator degrees of freedom for the overall F-test), it is possible for the DMR to capture a set of DNAm probes with similar p values but substantial heterogeneity in the directions of effect within the three groups. Therefore, for the ΔDMRs, we retained regions with a consistent direction of effect, defined as a region where the direction of change in DNAm between the two visits (hyper methylation or hypo methylation) was consistent across 100% of the DNAm probes within the region in one or more of the study groups. For the μDMRs, we retained regions where the direction of effect (hypo or

hypermethylation) for one or more of the pairwise group comparisons was consistent across 100% of the DNAm probes included in the region.

2.7 Expression quantitative trait methylation analysis: correlation between gene expression and DNAm candidates

In order to better understand our primary DNAm results, we tested the correlation between gene expression levels and our DNAm candidates, one DMP, 11 ΔDMRs, and 22 μDMRs in a subset of individuals (n=36, see Appendix 3, Table B) with methylation data pre- and post-SV as well as gene expression data post-SV. First, linear mixed models were used to regress out age, sex, platform, and cell proportions from the DNAm values at each of the candidate CpG sites. Ancestry PC1 and ancestry PC2 were also regressed out from all CpG sites included in the μDMRs candidate regions. Next, using the residuals from the linear mixed models, the within individual differences in DNAm (post-SV minus pre-SV) were used to represent changes in DNAm between the study visits for each of the CpG sites included in the ΔDMRs. The average residual values from the post-SV and pre-SV study visits were used to represent average methylation for each of the CpG sites within the μDMRs. Next, we performed a principal component analysis of DNAm levels across the region-specific CpG sets. For each DMR, the first PC was extracted for subsequent testing, allowing us to consider all CpG sites together rather than testing many individual sites separately. Linear regression models were then used to regress out the effects of age and sex from the gene expression levels. Finally, Spearman correlation coefficients were used to test the correlation between DNAm and gene expression residuals. We looked for cis-eQTMs, defined as genes significant at the FDR adjusted p value of 0.10 where transcription start site was +/- 500 KB of the midpoint of the DMR. FDR adjustment was based on the total number of DNAm cis-gene pairs (256 transcript DNAm pairs for the ΔDMR candidates and 544 transcript DNAm pairs for the μDMR candidates).

2.8 Metabolite quantitative trait methylation analysis: correlation between metabolite levels and DNAm candidates

We tested the correlation between DNAm and untargeted metabolite levels in a subset of our study population (n=110, see Appendix 3, Table B) with DNAm and metabolomics data available both pre- and post-SV. Only data from overlapping samples was included in this supplementary analysis. Linear models were used to regress age and sex from the Box-Cox transformed metabolite levels at each visit. Consistent with the DNAm methods, using the residuals from the linear mixed models, the difference between metabolite residuals at each visit (post-SV minus pre-SV residuals) was used to represent change in metabolites and the average residual values (average of post-SV and pre-SV residuals) were used to represent average metabolite values. For the ΔDMR candidates and the single DMP candidate, linear regression models were then used to test the correlation between the change in metabolites versus the ΔDMR PCs (described above) as well as the single DMP candidate. For the μDMR candidates, linear regression models were then used to test the correlation between average metabolite levels versus the μDMR PCs (described above). False discovery (FDR) rate adjusted p values were calculated for all individual metabolite DNAm candidate pairs according to methods described by Benjamini and Hochberg (37). FDR adjusted p values were calculated separately for each platform. Only annotated metabolites from the HILIC (81 metabolites), Lipid (373 metabolites), and GC-TOF (98 metabolites) panels were evaluated in subsequent analyses. Metabolites were evaluated at an FDR adjusted p value of 0.10.

3 Results

3.1 Study population

The final study population included 60 individuals in the maintainer group, 42 individuals in the progressor group, and 41 individuals in the reverter group. At both the pre-SV and post-SV visits, age differed by group, and the estimated cell proportions differed by group at the post-SV visit (Table 1). At the time of data analysis, duration of follow-up, defined as median time from the initial visit to the development of T1D or last study visit, was 9.3 years (IQR: 6.1 to 12.3 years) for the progressors, 16.5 years for the maintainers (IQR: 14.3 to 20.9 years) and 16.6 years for the reverters (IQR: 15.2 to 20.2 years).

The specific autoantibody subgroups present at the onset of seroconversion in the three groups are described in greater detail in Appendix 4 (Data Sheet 4), Table C. As expected, the prevalence of multiple autoantibodies at serconversion was higher in progressors (31%) relative to maintainers (18%) and reverters (0%). Across the entire islet autoimmunity follow-up period, the occurrence of multiple autoantibodies at one or more study visit(s) following IA seroconversion was also higher in progressors (86%) compared to maintainers (58%). Among reverters, 10% developed multiple autoantibodies at one of more study visit(s) during the time period between seroconversion (IA onset) and seroreversion.

3.2 Differentially methylated position analysis

Change in methylation at the DNAm site cg16066195 on chr 7 was significantly (FDR adjusted p value=0.0174) different across groups. The reverter group was characterized by an increase in DNAm between pre- and post-SV visits (ie, a positive slope) whereas the progressor and maintainer groups were characterized by no change or a decrease in DNAm (Figure 2). This site is an island CpG site (CpG island *chr7:73703458-73704127)* that maps to an area near the *CLIP2* gene.

We also tested whether average DNAm (mean of DNAm levels pre- and post-SV) differed across groups. No DNAm probe was significant at the FDR adjusted alpha level of 0.10.

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TABLE 1 Demographics and clinical characteristics.

	Maintainer n=60		Progressor n=42		Reverter n=41		P Value
	Median | Freq	IQR | %	Median | Freq	IQR | %	Median | Freq	IQR | %	
Pre-Islet Autoimmunity Visit							
Age at Visit, median (IQR)	5.7	1.4-9.7	2.2	0.8-5.3	6.0	1.3-8.4	0.0079
CD8T, median (IQR)	13.3%	9.4-16.6%	14.6%	11.8-15.9%	12.2%	9.7-16.1%	0.3864
CD4T, median (IQR)	22.0%	15.6-26.1%	23.4%	17.3-31.8%	19.3%	16.1-25.5%	0.1959
NK, median (IQR)	1.4%	0.0-4.7%	0.0%	0.0-1.5%	1.3%	0.0-3.1%	0.0653
Bcell, median (IQR)	15.3%	10.6-18.5%	17.9%	13.4-22.6%	14.9%	10.2-19.7%	0.1599
Mono, median (IQR)	8.3%	6.9-10.3%	7.5%	5.2-9.4%	7.6%	6.2-9.5%	0.3390
Gran, median (IQR)	38.5%	30.6-50.9%	35.5%	24.7-44.6%	42.8%	32.0-52.0%	0.2205
Post-Islet Autoimmunity Visit							
Age at Visit, median (IQR)	8.0	5.2-11.3	4.9	2.4-9.4	7.1	3.1-10.0	0.0087
CD8T, median (IQR)	11.8%	9.5-15.6%	14.6%	11.3-16.7%	12.3%	8.9-16.4%	0.1183
CD4T, median (IQR)	17.6%	13.1-22.1%	21.7%	17.3-26.9%	17.6%	13.0-21.7%	0.0061
NK, median (IQR)	2.7%	0.0-6.0%	0.0%	0.0-3.5%	1.3%	0.0-4.1%	0.0018
Bcell, median (IQR)	11.2%	8.6-15.0%	16.5%	12.7-19.7%	13.1%	8.3-16.7%	0.0011
Mono, median (IQR)	9.1%	7.8-10.8%	7.8%	4.8-9.3%	8.5%	7.0-10.1%	0.0293
Gran, median (IQR)	46.0%	39.6-52.7%	37.9%	28.6-44.4%	47.8%	38.4-53.6%	0.0025
Non-Hispanic White Ethnicity, freq (%)	43	71.7%	38	90.5%	29	70.7%	0.0458
Female Sex, freq (%)	34	56.7%	19	45.2%	21	51.2%	0.5224
HLDR3/4 High Risk Genotype, freq (%)	16	26.7%	19	45.2%	10	24.4%	0.0711
First Degree Relative with T1D, freq (%)	38	63.3%	25	59.5%	19	46.3%	0.2242

IQR, interquartile range; CD8T, cytotoxic T cells; CD4T, T helper cells; NK, natural killer T cells; Mono, monocytes; Gran, granulocytes.

3.3 Differentially methylated region analysis

We also tested for genomic regions (Figure 1). In contrast to the single CpG site (DMP) analysis, the regional analysis allowed us to identify multiple CpG sites that demonstrated similar DNAm changes between the pre- and post-SV visits across the three study groups (ΔDMRs). We focused on FDR significant regions of ≥4 DNAm probes where the direction of the change in DNAm (between the pre-SV and post-SV visits) was consistent (100% of probes changed in a similar direction) within one or more of the groups. We identified 11 candidate DMRs (Table 2; Figure 3).

We also tested for regions where the average DNAm levels at the pre- and post-SV visits differed across the groups (μDMRs). We identified 22 FDR significant μDMRs of ≥4 DNAm probes where the direction of the pairwise group differences in DNAm was consistent across all CpG sites included in the region (Table 3; Figure 4).

3.4 eQTM candidate prioritization

We tested the correlation between DNAm and cis- gene expression levels in a subset of overlapping samples. The availability of individual level DNAm data allowed us to look at the entire DMR together. Based on the ΔDMR candidates, we identified two FDR significant cis eQTMs representing one DMR and two gene transcripts, GNAS and ATP5E (ΔDMR1, region on chromosome 20, see Table 4). Within this region, increased DNAm post- vs pre-SV was positively associated with expression of GNAS and ATP5E (see Table 4).

3.5 Metabolite quantitative trait methylation analysis candidate prioritization in overlapping samples

We tested whether the single DMP candidate, cg16066195, as well as the candidate DNAm regions identified in our primary

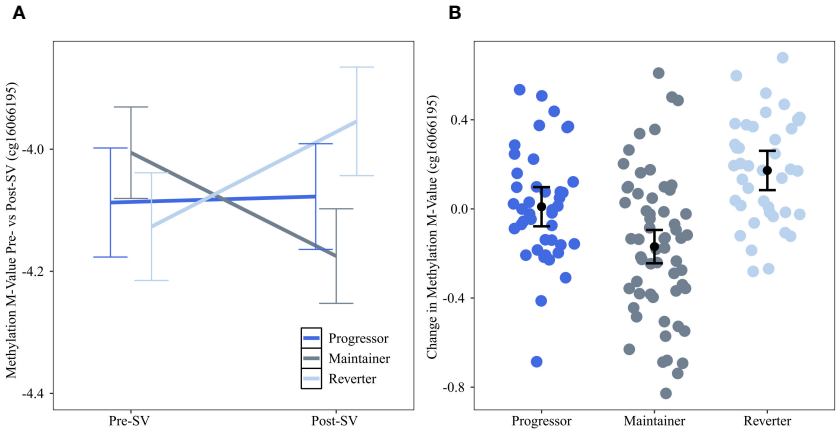

FIGURE 2
Changes in DNAm between the pre- and post-SV visits at cg16066195 across the three IA progression phenotypes. Description: **(A)** provides the average methylation M-values and corresponding 95% confidence intervals within the three IA progression phenotypes pre- and post-SV. **(B)** describes the individual level changes in methylation m-values (y-axis) between the post-SV visit relative to the pre-SV visit in the three IA progression phenotypes (x-axis). Positive values represent increasing DNAm whereas negative values represent decreasing methylation between visits. All DNAm values in **(A, B)** have been adjusted for age, sex, and cell proportions.

analysis were associated with metabolite levels. Consistent with the eQTM analysis, we regressed out age and sex from annotated metabolites and then tested the correlation between annotated metabolites versus DNAm regional PCs. Based on the ΔDMR candidates, we identified 26 annotated metabolites from the Lipid panel that were correlated with 4 DMRs (see Table 5; Figure 5). ΔDMR 8 was correlated with multiple lipids, primarily PCs, ΔDMR 5 was also correlated with multiple lipids, primarily correlated with TGs (fats). ΔDMR 9 and ΔDMR 2 were correlated with a single lipid, an ether lipid, and a TG, respectively. Metabolite candidates primarily consisted of odd-chain fatty acid containing lipid species (OCFA). Furthermore, the majority of the metabolites (29/30) were positively correlated with increasing DNAm levels. The μDMR candidate regions as well as the single DMP candidate were not significantly associated with metabolite levels at our FDR adjusted cutoff of 0.10.

4 Discussion

Epigenetic biomarkers are appealing in the study of complex diseases such as T1D based on their heritability, role in gene expression, and responsiveness to external stimuli. Epigenetic effects in observational studies are challenging to interpret because it is often not possible to determine whether DNA methylation (DNAm) is causative or secondary to the disease process. A strength of our study is the longitudinal analysis of DNAm levels both before and after the onset of IA. We identified a single CpG site as well as genomic regions where changes in DNAm between the post-SV and pre-SV visits were significantly different across the IA progression phenotypes. We also identified regions where average DNAm levels pre- and post-SV differed across the progression phenotypes. Together, the DNAm regions have potential biological relevance to T1D etiology based on their potential role in immune and beta cell function.

We identified a DNAm site, cg16066195, on chromosome 7 where DNAm levels increased between the pre- and post-SV visits among individuals who reverted to an IA negative state (reverters) compared to progressors (who showed no change in DNAm) and maintainers (who showed decreasing DNAm, Figure 2). This island CpG is located near the transcription start site for the protein coding gene *CLIP2*. In a mouse model of diet induced changes in beta cell expression, *CLIP2* gene expression was significantly downregulated among mice fed a carbohydrate containing diabetogenic high-fat diet relative to mice fed a diabetes-protective carbohydrate free high-fat diet (39). Furthermore, SNPs within *CLIP2* (rs2528994 and rs512023) have demonstrated modest associations with T2D in both the Diabetes Genetics Initiative (40) and the Wellcome Trust Case Control Consortium (41).

Our methylation analysis also identified numerous regions where average methylation post- and pre-SV differed across the autoimmune phenotypes in areas of the genome potentially relevant to T1D etiology. We identified a DMR on chromosome 12, μDMR4, characterized by hypermethylation in the reverter group relative to the progressor and maintainer groups (Figure 4). This includes 4 probes that, based on the ENCODE Project Consortium (42), are located in a known enhancer region. Three of the four probes within this region are located within the transcription start site for *NRIP2*, predicted to act upstream or within the notch signaling pathway (43). This pathway is relevant to T1D (44) based on its role in immune cell differentiation and function (45) as well as pancreas development (46), islet cell function (47), and islet cell survival (48). All four probes within μDMR4 are also located within the 5'UTR region for *ITFG2*, a gene expressed in numerous tissues including immune cells. Mouse and *in vitro* models have demonstrated that *ITFG2* deficiency alters B cell maturation and migration (49). In a lupus mouse model, MRL/lpr, autoimmunity development occurred earlier and was more severe in *ITFG2* deficient mice (49). Together, these findings suggest a potential role for *ITFG2*

TABLE 2 Regions where DNAm changes between the post- and pre-SV visits were consistently different across groups (group*visit interaction).

DMR ID	Chr.	Start	Stop	Gene	N CpG Sites	Sidak Adj. Region P	Leading CpG Site	Slope % R*	Slope % P*	Slope % M*	Median Slope R†	Median Slope P†	Median Slope M†
ΔDMR 1	chr20	57426538	57427974	GNAS; GNASAS; GNAS-AS1	29	8.33E-05	cg26496204	69%	100%	100%	0.01	-0.03	-0.05
ΔDMR 2	chr20	36148604	36149751	BLCAP; NNAT	30	1.37E-04	cg24675557	100%	80%	80%	0.05	-0.02	-0.02
ΔDMR 3	chr1	75198582	75199118	TYW3; CRYZ; RP11-17E13.3	8	3.40E-03	cg00121533	100%	88%	100%	0.06	0.04	-0.08
ΔDMR 4	chr14	101291068	101293727	MEG3	25	6.74E-03	cg14034270	96%	85%	100%	0.02	-0.02	-0.02
ΔDMR 5	chr11	1296469	1297386	TOLLIP	7	1.81E-02	cg11095027	86%	57%	100%	0.03	0.03	-0.07
ΔDMR 6	chr15	91473059	91473570	UNC45A	8	2.00E-02	cg03291024	75%	100%	100%	0.01	0.09	-0.09
ΔDMR 7	chr5	1245669	1246292	SLC6A18	4	3.38E-02	cg09075844	100%	100%	100%	-0.03	0.03	-0.06
ΔDMR 8	chr6	170597377	170597899	DLL1	4	3.66E-02	cg05228964	50%	100%	100%	<0.01	0.10	-0.04
ΔDMR 9	chr6	28945322	28945493	RN7SL471P‡	4	6.09E-02	cg10919664	100%	100%	100%	0.07	0.06	-0.16
ΔDMR 10	chr6	27647713	27648355	RP1-15D7.1‡	4	7.14E-02	cg25106913	75%	75%	100%	<0.01	0.06	-0.05
ΔDMR 11	chr5	1867978	1868694	IRX4‡	6	8.71E-02	cg14773178	83%	100%	100%	0.04	0.08	-0.08

DMRs limited to regions with a minimum of 4 probes and 100% of within group slopes in the same direction for one or more groups.
Chr., chromosome.
Start/Stop, DMR start and stop position.
Gene, Gene annotation from the Illumina manifest file, based on UCSC reference genes mapped to CpG sites within DMR and/or genes mapped to CpG sites within known regulatory regions, if gene was not annotated within the Illumina manifest file, noted with ‡, gene name based on closest transcription start site.
Leading CpG site, most significant DMP within the region.
Sidak Adj. Region P, regional p value corrected for multiple testing based on number similarly sized regions possible based on genomic coverage in the DMR analysis.
*R, reverters; P, progressors; M, maintainers, Percent of within group slopes (Pre-SV vs Post-SV) in the same direction (hypo (–) or hyper (+) methylation) across all the probes included in the DMR.
†Median slope (Pre-SV vs Post-SV) across all probes included in the DMR for each group, (+) values indicate increasing DNAm (–), indicate decreasing DNAm.

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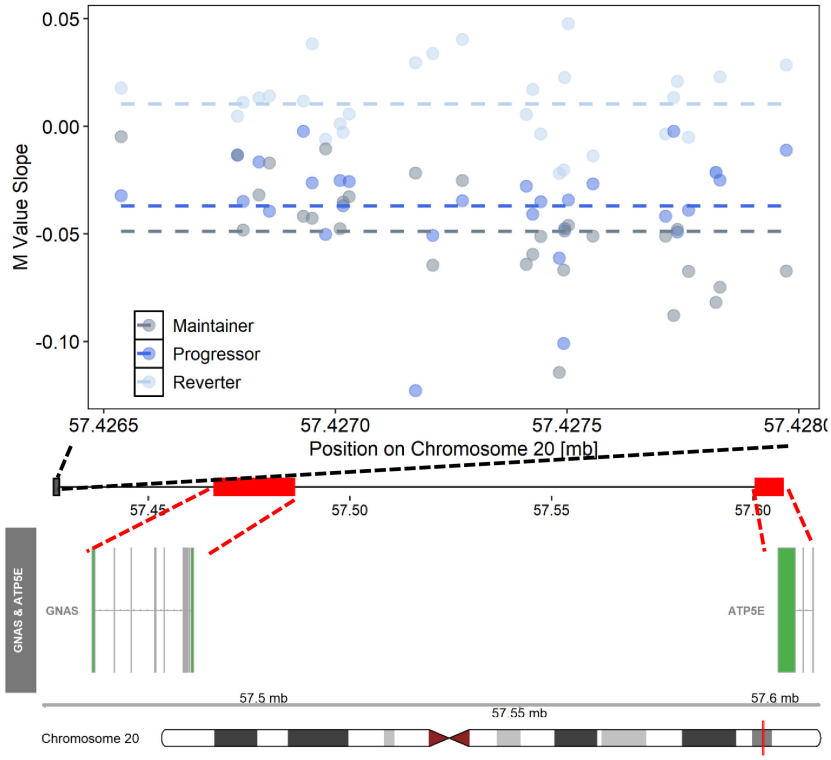

FIGURE 3
Differentially changing methylation region on chromosome 20 where changes in DNAm (pre- vs post-SV) differed across the three IA progression phenotypes. Description. Region on chromosome 20 loc 57426538 to 57427974 (ΔDMR1) where the change in DNA methylation (DNAm) post- vs pre-SV differed across groups. In the top panel, each dot represents the within group slopes (y-axis) or changes in DNAm m-values between the post-SV and pre-SV visit at each of the CpG sites included ΔDMR 1. The x-axis represents the position (mb) of the CpGs within the region. All slope values were adjusted for age, sex, and cell proportions. Positive values indicate methylation values increased following IA seroconversion whereas negative values indicate methylation decreased following IA seroconversion. The dashed lines represent the average slope value within each group across the entire region. The middle panel represents the location of the region (black solid square) relative to the closest genes, *GNAS* and *ATP5E* (red solid boxes). There are multiple known isoforms for *GNAS* and *ATP5E*, the bottom panel displays the most biologically relevant or consensus transcript based on the Ensembl database. The red line on the ideogram, bottom of the figure, represents the location of *GNAS* and *ATP5E* on chromosome 20.

TABLE 3 Regions where average of post- and pre-SV DNAm levels were consistently different across groups (group main effect).

DMR ID	Chr.	Start	Stop	Gene	N Probes	Sidak Adj. Region P	Leading CpG Site	Median PvR‡	Median RvM‡	Median PvM‡
µDMR 1	chr1	180922636	180923341	RP11-46A10.4; RP11-46A10.5	4	1.38E-05	cg00579423	0.09	0.37	0.46
µDMR 2	chr10	99338056	99338241	ANKRD2	4	1.75E-04	cg27469738	-0.11	0.26	0.17
µDMR 3	chr10	52008360	52008906	ASAH2	4	6.45E-03	cg24123634	-0.07	-0.02	-0.11
µDMR 4	chr12	2943902	2944481	NRIP2; ITFG2	4	7.06E-03	cg02852959	-0.15	0.19	0.04
µDMR 5	chr12	75784855	75785098	GLIPR1L2; CAPS2	6	7.59E-03	cg12351126	0.10	0.24	0.34
µDMR 6	chr12	51566379	51567113	TFCP2	7	1.24E-02	cg19016289	0.05	0.15	0.2
µDMR 7	chr1	1289835	1290713	MXRA8	6	1.61E-02	cg07284273	-0.16	0.33	0.15
µDMR 8	chr15	72766637	72767333	ARIH1; RP11-1007O24.3	4	1.93E-02	cg26880891	0.09	0.02	0.14
µDMR 9	chr19	45206843	45207560	CEACAM16	4	2.78E-02	cg24091949	-0.09	-0.04	-0.13
µDMR 10	chr19	2250901	2251068	AMH	4	2.83E-02	cg23218559	-0.18	0.38	0.21

(Continued)

TABLE 3 Continued

DMR ID	Chr.	Start	Stop	Gene	N Probes	Sidak Adj. Region P	Leading CpG Site	Median PvR‡	Median RvM‡	Median PvM‡
µDMR 11	chr18	7567426	7568266	PTPRM	5	3.44E-02	cg05870479	0.09	0.04	0.11
µDMR 12	chr15	85524778	85525674	PDE8A	4	4.02E-02	cg02839273	0.05	0.05	0.13
µDMR 13	chr2	85765644	85766105	MAT2A	4	4.39E-02	cg06978067	0.08	0.05	0.13
µDMR 14	chr19	48048129	48049234	ZNF541	4	4.90E-02	cg22341310	-0.12	0.17	0.06
µDMR 15	chr4	4861683	4862241	MSX1	4	5.94E-02	cg11930592	0.12	-0.04	0.08
µDMR 16	chr11	598325	599091	PHRF1	5	7.14E-02	cg12921473	-0.06	-0.05	-0.10
µDMR 17	chr5	101119084	101119767	OR7H2P*	4	7.67E-02	cg12197752	0.09	0.18	0.29
µDMR 18	chr13	42031761	42032737	C13orf15; RGCC	4	8.16E-02	cg18495682	0.06	0.02	0.09
µDMR 19	chr3	38206610	38207525	OXSR1	4	8.20E-02	cg19728055	0.07	0.05	0.11
µDMR 20	chr10	14372431	14372914	FRMD4A	5	8.45E-02	cg05755354	-0.16	-0.02	-0.18
µDMR 21	chr8	145550361	145551157	DGAT1	5	8.72E-02	cg11127482	0.06	0.04	0.11
µDMR 22	chr11	128693473	128694916	FLI1*; KCNJ1*	9	9.44E-02	cg15509024	-0.12	-0.09	-0.18

DMRs limited to regions with a minimum of 4 probes and direction of pairwise comparison was consistent across all probes in the region.
Chr., chromosome.
Start/Stop, DMR start and stop position.
Sidak Adj. Region P, regional p value corrected for multiple testing based on number similarly sized regions possible based on genomic coverage in the DMR analysis
Leading CpG site = most significant DMP within the region
Gene, Gene annotation from the Illumina manifest file, based on UCSC reference genes mapped to CpG sites within DMR and/or genes mapped to CpG sites within known regulatory regions, if gene was not annotated within the Illumina manifest file, noted with *gene name based on closest transcription start site.
‡R, reverters; P, progressors; M, maintainers, Median effect size across the region representing difference in methylation M values between groups.

in B cell differentiation and as a potential regulator of autoimmunity. Although, average methylation within DMR4 was not correlated with expression of ITFG2 or NRIP2 in our secondary eQTM analysis, three probes within µDMR4 (cg05194726; cg06997549; cg02852959) were correlated with expression of both ITFG2 and NRIP2 in whole blood based on the BIOS QTL browser (50), an online resource that provides a searchable database of FDR significant associations between DNAm and gene expression (eQTM). Additional work is needed to understand the connections between methylation within this region on chr 12, ITFG2 expression, NRIP2 expression, and T1D etiology.

We also identified several regions of differentially changing DNAm that are potentially relevant to T1D etiology based on known associations between DNAm in these regions and relevant environmental risk factors. We identified a region on chr 20 near the *GNAS/GNASAS* loci, ΔDMR 1, that was characterized by decreasing DNAm pre- vs post-SV in maintainers and progressors relative to reverters (Table 2; Figure 3). Based on the ENCODE Project Consortium (42), 25 of the 29 probes in ΔDMR 1 are located within a DNAase hypersensitivity region and 4 probes are known to interact with transcription factor binding. DNAm in this region is responsive to environmental stressors. Umbilical cord blood DNAm near *GNAS* was altered among infants born to a mother affected by gestational diabetes (GDM), a disorder characterized by glucose intolerance during pregnancy (51). Based on the Dutch Hunger Winter Families Study (52), siblings exposed to the war-time Dutch Hunger Winter famine were associated with persistent changes in DNAm in a region near the *GNASAS* locus relative to their unexposed siblings (53). The direction and magnitude of effect depended on timing of exposure and sex of the exposed individual (53). DNAm among exposed siblings was also altered near another gene implicated in metabolic disease *MEG3* (53), a gene that mapped to ΔDMR4 which was also characterized by decreasing methylation among progressors and maintainers relative to reverters (Table 2). Interestingly, both the GNAS (54) and MEG3 (55) genes are maternally imprinted. Loss of maternal imprinting should be investigated as a potential mechanism in the etiology of T1D using whole-genome bisulfite sequencing in order to provide a higher density representation of DNAm changes within imprinted areas of the genome.

The secondary eQTM analysis in a subset of overlapping samples confirmed that changes in methylation within ΔDMR1 were associated with expression of GNAS. Increased methylation post- versus pre-SV was associated with higher levels of GNAS expression at the post-SV visit in a subset of overlapping samples. GNAS is an important regulator of insulin secretion in beta cells (56). GNAS silencing results in decreased insulin secretion and insulin content (56). GNAS encodes the G protein subunit alpha which also plays a role in the interaction between antigen presenting cells and T helper cell differentiation (57). Mice with dendritic cells deficient for GNAS result in a phenotype characterized by preferential Th2 differentiation, Th2 type inflammation, and subsequent development of allergic asthma (57). Overlap between autoimmunity and atopic conditions have long been hypothesized based on disruptions in similar immune pathways (58). Positive associations between childhood asthma and subsequent T1D development have been observed in several countries (59–61). Overall, our results suggest that maintenance of DNAm levels

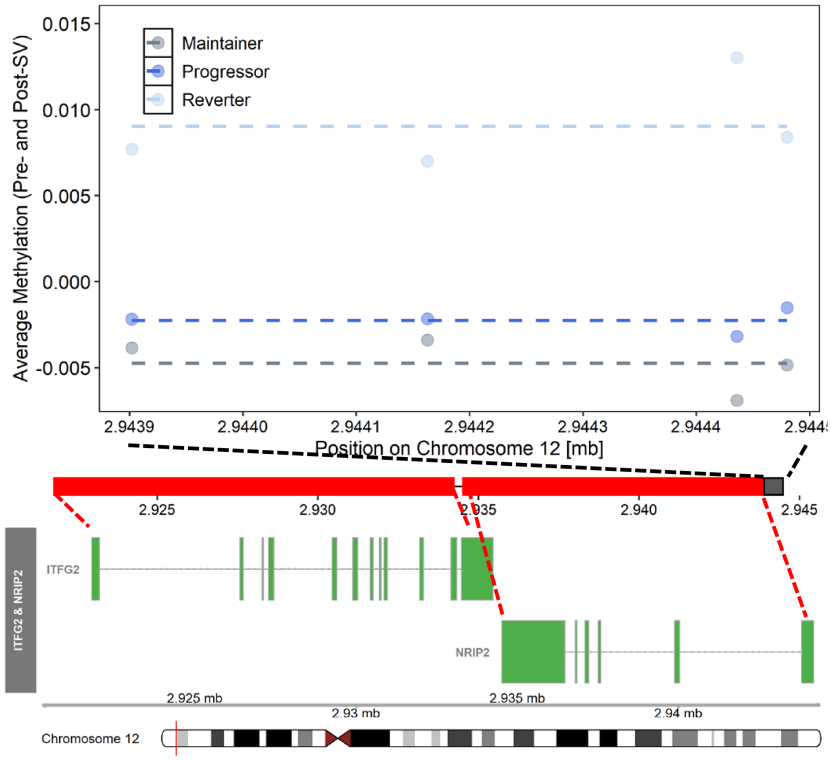

FIGURE 4
Differentially methylated region on chromosome 12 where average (pre- and post-SV) methylation levels differed across the three IA progression phenotypes. Description. Region on chromosome 12 loc 2943902 to 2944481 (µDMR4) where average DNA methylation (DNAm) levels, post- and pre-SV, differed across groups. In the top panel, each dot represents the average DNAm value (y-axis) at each of the CpG sites included µDMR4. The x-axis represents the position (mb) of the CpGs within the region. All DNAm values were adjusted for age, sex, cell proportions, and genetic ancestry. The dashed lines represent the average methylation value within each group across the entire region. The middle panel represents the location of the region (black solid square) relative to the closest genes, *ITFG2* and *NRIP2* (red solid squares). There are multiple known isoforms for *ITFG2* and *NRIP2*, the figure displays the most biologically relevant or consensus transcript based on the Ensembl database. The red line on the ideogram, bottom of the figure, represents the location of *ITFG2* and *NRIP2* on chromosome 12.

near GNAS during IA may represent a unique protective mechanism in reverters.

In order to further characterize the DNAm regions identified in the primary analysis, we tested the correlation between changes in DNAm and changes in annotated metabolites (metQTM). Four differentially changing DMRs were correlated with changes in 26 unique lipid metabolites (Table 5). ΔDMR 8, characterized by increasing methylation in progressors (Figure 5), was correlated with 18 of the 26 lipid metabolites. This region of differentially changing methylation is notable based on its location in an open chromatin region within the body of the *DLL1* gene on chr. 6. As a notch signaling ligand, DLL1 controls the differentiation of pancreatic progenitor cells into exocrine versus endocrine cells (46). The loss of DLL1 results in early progenitor cell differentiation and an

TABLE 4 Summary of FDR significant cis-eQTMs representing correlation between differentially changing methylation regions and gene expression post- SV.

Methylation DMR Information					Cis-Gene Expression Information						
DMR ID	Chr.	DMR Start	DMR Stop	N Probes	Gene Symbol	Ensembl ID	Strand	Gene Start	Gene End	Corr*	FDR
ΔDMR 1	20	57426538	57427974	29	GNAS	ENSG00000087460	1	57414773	57486247	0.559	0.0667
ΔDMR 1	20	57426538	57427974	29	ATP5E	ENSG00000124172	-1	57600522	57607437	0.557	0.0667

DNAm levels for all probes identified in the DMR analysis (Tables 1, 2) were included in a PCA. We then tested the association between the 1st PC and RNA seq data from overlapping visit at the post-SV visit. Only significant cis (TSS +/- 500KB of midpoint of DMR) expression quantitative trait methylation (cis-eQTM) associations are presented.
*Spearman correlation coefficient.
Chr., chromosome.
DMR Start/End, DMR start and end position.
Gene Start/End, Gene start and end positions (based on annotation file for GEO, GSE50244).
Beta, beta coefficient from linear regression model (adjusted for age and sex) representing association between 1st PC from DNAm probes in each DMR and islet cell pancreas expression.
FDR, Benjamini-Hochberg FDR adjusted p value.

TABLE 5 Secondary metQTM analysis of the association between pre- versus post-SV change in methylation across the DMRs and pre- versus post-SV change in metabolite levels.

DMR ID	Chr.	DMR Start	DMR Stop	Metabolite Name†	Standardized Beta	FDR Adj. P Value
ΔDMR 2	chr20	36148604	36149751	TG (49:2)	0.320	0.0992
ΔDMR 5	chr11	1296469	1297386	TG (53:2)	0.411	0.0121
				Phosphatidylcholine (33:1)	0.361	0.0469
				TG (53:3)	0.353	0.0627
				PE (38:4)	0.339	0.0826
				TG (49:2)	0.330	0.0948
				TG (47:0)	0.329	0.0952
				TG (51:3)	0.327	0.0954
				PC (33:1)	0.327	0.0954
				Phosphatidylcholines (35:1)	0.325	0.0954
				TG (53:1)	0.320	0.0992
ΔDMR 8	chr6	170597377	170597899	Phosphatidylcholine (35:4)	0.438	0.0078
				Phosphatidylcholines (33:1)	0.404	0.0121
				Phosphatidylcholines (33:0)	0.403	0.0121
				Phosphatidylcholines (33:1)	0.402	0.0121
				Phosphatidylcholines (35:3)	0.396	0.0138
				LPC (15:0)	0.393	0.0139
				Phosphatidylcholines (38:5)	0.375	0.0527
				Phosphatidylcholines (33:2)	0.366	0.0445
				Phosphatidylcholines (35:4)	0.365	0.0445
				Phosphatidylcholines (31:0)	0.350	0.0647
				Phosphatidylcholines (35:1)	0.347	0.0647
				Phosphatidylcholines (36:3)	0.347	0.0647
				Phosphatidylcholines (p-34:0) or Phosphatidylcholines (o-34:1)	-0.334	0.0940
				TG (49:3)	0.332	0.0940
				Phosphatidylcholines (33:2)	0.332	0.0940
				Phosphatidylcholines (36:3) B	0.325	0.0954
				Phosphatidylcholines (37:6)	0.324	0.0954
				Phosphatidylcholines (35:1)	0.323	0.0975
ΔDMR 9	chr6	28945322	28945493	*Phosphatidylcholine* (p-38:2) or *Phosphatidylcholine*(o-38:3)	0.345	0.0662

DNAm levels for all probes identified in the DMR analysis (Tables 1, 2) were included in a PCA. We then tested the association between the 1st PC changes in metabolites between the pre- and post-SV visits.
Chr., chromosome.
DMR Start/End, DMR start and end position.
Gene Start/End, Gene start and end positions (based on annotation file for GEO, GSE50244).
Standardized Beta, beta coefficient from linear regression model testing the association between change in DNAm and change in metabolites pre-SV vs post-SV. The slopes have been standardized to represent a 1 stdev change in metabolite per 1 standard deviation change in DNAm regional PC levels.
FDR Adj. P value, Benjamini-Hochberg FDR adjusted p value.
†See Appendix 5 (Data Sheet 5) (Tables D, E) for complete annotation for all metabolites included in Table 5.

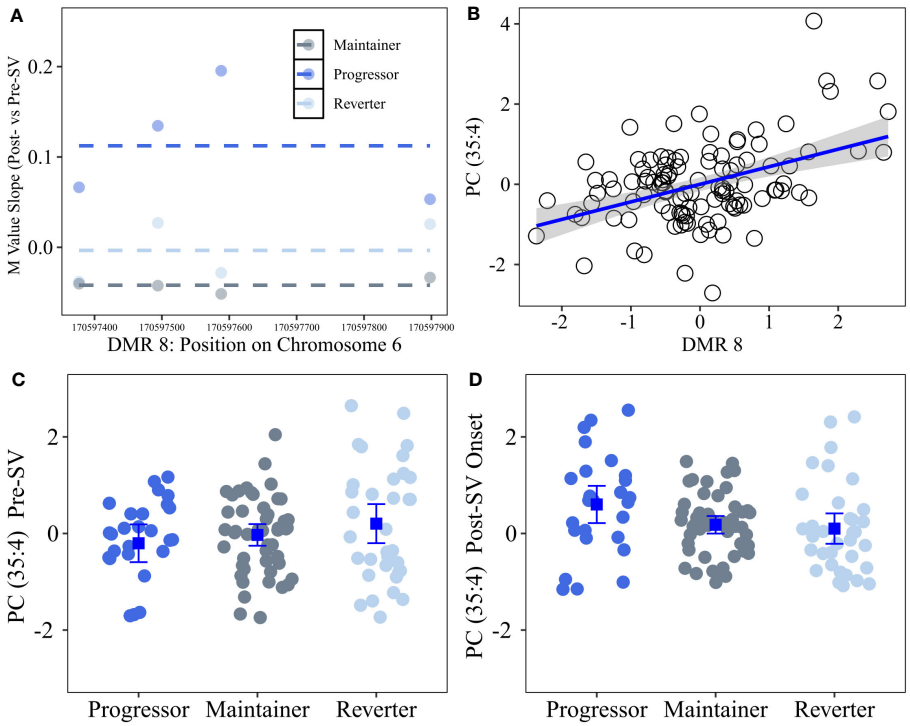

FIGURE 5
Differentially changing region on chromosome 6 (post- vs pre-SV) that was positively correlated with changes in lipid metabolites (post- vs pre-SV). Description: Region on chromosome 6 loc 170597377 to 170597899 (ΔDMR8) where the change in DNA methylation (DNAm) post- vs pre-SV differed across groups. In the top left **(A)**, each dot represents the within group slopes (y-axis) or changes in methylation m-values between the post-SV and pre-SV visit at each of the CpG sites included ΔDMR 8. The x-axis represents the position (mb) of the CpGs within the region. Positive values indicate DNAm values increased following IA seroconversion whereas negative values indicate DNAm decreased following IA seroconversion. The dashed lines represent the average slope value within each group across the entire region. The top right **(B)** represents the association between DMR wide DNAm captured by the 1st PC (x-axis) and changes in metabolite values (y-axis) between the post- and pre-SV visits. DNAm and metabolite expression values have been standardized to facilitate the interpretation of the slope as a 1 standard deviation increase in the change in metabolite levels between the post- and pre-SV visits per 1 standard deviation increase in the change in methylation between post- and pre-SV visits. The bottom panels **(C, D)** represent the average metabolite levels and corresponding 95% confidence intervals within the three groups pre- and post-SV. All DNAm and metabolite values were adjusted for age, sex, and cell proportions.

overabundance of endocrine cells (46). A recent mouse model confirmed DLL1 is also relevant to islet cell function in the mature pancreas based on its high level of expression in beta cells and corresponding role in insulin secretion (47). Furthermore, DLL1 plays an important role in differentiation of B cells and the development of antigen secreting cells; the presence of DLL1 influences AB titer levels and isotype switching (45). Additional work is needed to understand the connection between a concordant increase in lipid levels and DNAm within the *DLL1* gene following seroconversion.

Our secondary metQTM was unique in that DNAm and metabolite levels were available pre- and post-SV in a subset of overlapping samples. This analysis revealed a consistent positive association between increasing lipid metabolite levels, post- vs pre-SV, and increasing DNAm levels across several regions (25 of the 26 unique lipid metabolites were positively correlated with DNAm changes, see Table 5). Numerous studies (62–68) have reported associations between dysregulation in lipid levels and T1D. Although lipid levels have been shown to be influenced by age at sample collection/timing of sample collection relative to onset of IA and type of first appearing autoantibody, prior research suggests lower lipid levels, including sphingomyelins and phosphatidylcholines, are generally associated with increased risk of T1D and/or IA (62–68). In our study, increasing lipid levels, in particular phosphocholines, following the onset of IA were strongly correlated with increasing methylation within ΔDMR8. This region was characterized by increasing methylation within the progressor group. However, as demonstrated in Figure 5, the lipid metabolite most strongly correlated with DNAm changes in this region, *Phosphatidylcholine* (35:4), was lower in the progressor group prior to SV and then subsequently increased following the onset of IA, suggesting higher levels of lipids within the progressor group may be unique to changes that occur following seroconversion.

There was a high prevalence of odd-chain fatty acid (OCFA) containing lipid species among the metabolites correlated with DNAm changes. Recently, there has been increased recognition of OCFA in plasma and their potential biological relevance (69). OCFA levels have been associated with glucose homeostasis, insulin resistance, T2D, and BMI (69, 70). Pfleuger et al (71) observed higher levels of odd-chain triglycerides among autoantibody positive versus negative children in BABYDIAB. This parallels the concordant post-seroconversion increase in OCFA levels and DNAm near the DLL1 gene (ΔDMR 8)

among progressors (Figure 5) in the current study. OCFA have been proposed a marker of dairy intake which has been positively correlated with progression to T1D in prior work in DAISY (72). However, dairy intake contributes modestly to OCFA levels. These lipids primarily originate endogenously from adipocytes as well as from dietary intake of numerous foods including dairy, poultry, and fiber (70, 73, 74). Additional work is needed to understand connections between increasing methylation and increasing OCFA as well as the source of these lipid species.

A major strength of our study was the inclusion of DNAm measurements prior to T1D as well as the multi-omics work used to identify correlations between DNAm and gene expression as well metabolite levels. We measured DNAm before and after SV (ie, the appearance of IA) which builds on prior studies that have included DNAm measures after T1D and/or after IA onset only (14–16). A novel feature of our longitudinal methodology was our group*visit interaction modelling strategy that allowed us to identify changes in DNAm before and after the onset of IA, a critical window in T1D pathogenesis. These within individual effects are essential to understanding the etiology of T1D as they are robust to individual level confounders such as sex, genetic predisposition, and/or family history. Johnson et al (17) also used a longitudinal case-control analysis of T1D cases vs. unaffected controls in DAISY. In contrast, the current study design focused on individuals who developed IA and furthermore, tested for differences in DNAm post- vs pre-SV (group*visit interaction) rather than testing for differences in methylation by age (group*age interaction). Comparing the DMRs identified by this study versus Johnson et al (17), only two regions were located within 1 MB of each other–one on chr 6 ΔDMR 9 (28945322–28945493) in the current study vs chr 6 28973328-28973521 in Johnson et al (17), and one on chr 20 ΔDMR 2 (36148604–36149751) in the current study vs chr 20 36148954-36149232 in Johnson et al (17). Consistent with prior work, ΔDMR 9 and ΔDMR 2 were both associated with differential changes in DNAm in progressors relative to maintainers and/or reverters.

4.1 Limitations

We obtained DNAm from whole blood, which means we were unable to identify cell subtype specific effects. Similarly, our study focused on blood tissue only. DNAm changes within the blood may not reflect DNAm changes within other tissues that contribute to T1D, such as the pancreas. Due to advancements in technology during the study, DNAm was measured on two platforms. Individuals were randomly assigned to the platforms to minimize bias. We looked for cis-eQTMs. Given that it is possible that regions act over larger areas of the genome, we may have missed larger effects that occurred outside of our 500 KB window. Due to the small sample size, the eQTM was underpowered to identify FDR significant DMR vs gene transcript pairs. This limitation may explain lack of concordance between eQTM results and BIOS QTL results (μDMR4). Furthermore, among the two gene transcripts that were correlated with changes in methylation within ΔDMR1, gene expression data were only available at the post-SV visit. Therefore, it was not possible to determine whether gene expression also changed pre- versus post-SV. Finally, metabolite levels are influenced by age and dietary patterns. Although we adjusted for age, the large differences in age between the progressor group and the reverter and maintainer groups creates challenges in interpreting the metabolite vs methylation correlations. Additional work is need to replicate the metabolite vs DNAm regional effects.

5 Conclusion

T1D is an autoimmune disease characterized by immune mediated destruction of beta cells. Beta cell stress has been proposed as a mechanism connecting environmental perturbations such as infection, inflammation, diet, and increased insulin secretion to disease progression (75). Our EWAS identified DNAm candidates known to be modified by diabetes relevant environmental factors including diet and glucose levels (*CLIP2*, *GNAS/GNAS-AS*, *MEG3*). Our results also implicated genes (*DLL1* and *GNAS*) with functional roles in both beta and immune cells. Our results build upon prior work by identifying specific areas of the genome where DNAm changes pre- and post-SV visits differentiated between reversion versus progression of IA. The correlation between changes in DNAm and changes in lipid levels reveal common connections between DMRs in different areas of the genome that may be related to disruptions in lipid metabolic pathways. Additional work is needed to replicate these findings, test for cell-specific changes in DNAm pre- vs post-seroconversion, and to identify modifiable factors that lead to these DNAm changes; ideally, the first step in the development of preventative strategies that delay or prevent progression of IA.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA597238.

Ethics statement

The studies involving humans were approved by Colorado Multi-institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

PC: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Conceptualization. LV: Writing – review & editing, Software, Methodology, Data curation. RJ: Writing – review & editing, Methodology, Data curation. TB: Writing – review & editing. AS: Writing – review & editing, Supervision. KK: Writing – review & editing, Supervision, Methodology. IY: Writing –

review & editing, Supervision. TF: Writing – review & editing, Supervision. OF: Writing – review & editing, Resources, Data curation. MR: Writing – review & editing, Supervision, Resources, Funding acquisition. JN: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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.2024.1345494/full#supplementary-material

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Environment and systemic autoimmune rheumatic diseases: an overview and future directions

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Introduction: Despite progress in our understanding of disease pathogenesis for systemic autoimmune rheumatic diseases (SARD), these diseases are still associated with high morbidity, disability, and mortality. Much of the strongest evidence to date implicating environmental factors in the development of autoimmunity has been based on well-established, large, longitudinal prospective cohort studies.

Methods: Herein, we review the current state of knowledge on known environmental factors associated with the development of SARD and potential areas for future research.

Results: The risk attributable to any particular environmental factor ranges from 10-200%, but exposures are likely synergistic in altering the immune system in a complex interplay of epigenetics, hormonal factors, and the microbiome leading to systemic inflammation and eventual organ damage. To reduce or forestall the progression of autoimmunity, a better understanding of disease pathogenesis is still needed.

Abbreviations: aHR, adjusted hazards ratio; AI, artificial intelligence; ANA, antinuclear antibody; BWHS, Black Women's Health Study; CI, confidence interval; COVID-19; coronavirus disease 2019; BlyS, B-cell lymphocyte stimulator; CCP, cyclic citrullinated peptide; CRP, C-reactive protein; dsDNA, anti-double-stranded DNA; DNAm, DNA methylation; EBV, Epstein-Barr virus; GRS, genetic risk score; HCQ, hydroxychloroquine; HLA, human lymphocyte antigen; HR, hazard ratio; ML, machine learning; IFN, interferon; IL, interleukin; IIM, idiopathic inflammatory myopathies; IU, international units; NHS, Nurses' Health Study; OR, odds ratio; NHSII, PTSD, post-traumatic stress disorder; RA, rheumatoid arthritis; rRNA, ribosomal RNA; SARD, systemic autoimmune rheumatic diseases; SARS-CoV2, severe acute respiratory syndrome coronavirus 2; SjD, Sjögren disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; TNF, tumor necrosis factor; UV, ultraviolet; VH3 BCR, VH3 B Cell Repertoire.

Conclusion: Owing to the complexity and multifactorial nature of autoimmune disease, machine learning, a type of artificial intelligence, is increasingly utilized as an approach to analyzing large datasets. Future studies that identify patients who are at high risk of developing autoimmune diseases for prevention trials are needed.

KEYWORDS

autoimmunity, autoimmune diseases, environment, autoantibodies, epigenetics, microbiome, machine learning, artificial intelligence

Introduction

Environmental factors operating on the background of hormonal factors and genetic vulnerability may be accelerating factors included in a long-held paradigm that helps explain the etiology of systemic autoimmune rheumatic disease (SARD), including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), Sjögren's disease (SjD), idiopathic inflammatory myopathies (IIM) and others (1). On the backdrop of an increasing prevalence of SARD and other autoimmune diseases (2–6), potential accelerating factors include several environmental and socioeconomic factors that include alterations of foods, increasing exposure to xenobiotics due to water and air pollution, heat and other extreme weather events (i.e., climate change), biodiversity loss, ultraviolet (UV) light exposure, pandemics and infections, and socioeconomic factors such as changes in personal lifestyles and psychological stress.

Extensive research over the past three to four decades has elucidated the environmental factors associated with SLE (7) and other SARD. In general, the environmental factors can be classified as airborne, waterborne, workplace/occupational, social, and behavioral (8). While it has not been possible to identify a universal environmental "pathogen" for all SARD, there is compelling evidence that some environmental exposures clearly serve as risk factors for disease onset. The central importance of identifying these factors is that many of these factors are actionable and modifiable through intervention and remediation. Expanding the use of machine learning (ML), a form of artificial intelligence (AI), to analyze large datasets including environmental exposures may lead to the identification of other modifiable environmental risk factors, and allow the development of new disease-specific remediation programs (2).

Environmental factors and autoimmunity

The development of SARD has been associated with several lifestyle behaviors. For instance, cigarette smoke (9–11), obesity (12), alcohol use (moderate consumption being protective) (10, 13–15), poor nutrition and intake of ultra-processed foods (16), psychosocial factors (e.g., major depression (17), sleep deprivation (18), child abuse, personal trauma, post-traumatic stress disorder [PTSD]) (19, 20), and reproductive factors (21–23) have been associated with SLE development. Environmental exposures such as air pollution (24), occupational hazards (25), residential proximity to hazardous waste sites or pesticide exposure (26, 27), UV light (28–33), vitamin D deficiency (34), and exposure to viruses (35, 36) have also been linked to increased SLE risk. Similar lifestyle factors have been reported for increased risk of developing RA (moderate alcohol consumption decreases RA risk), SSc, IIM, other SARD, and autoinflammatory conditions (Tables 1, 2).

Precisely how and the extent to which these lifestyle factors contribute to individual risk of autoimmune disease likely varies (57, 58). This has been particularly well-studied using large cohort studies including cohorts enrolled in the Nurses' Health Study (NHS) and Black Women's Health Study (BWHS). In SLE, each factor independently increases the risk of disease development by 10-200%, but they likely interact with each other and with genetic risk, potentially synergistically, to accelerate brewing autoimmunity in SLE [reviewed in (57–60)]. Using SLE as an example below, we discuss several potential biologic pathways involving epigenomics, the microbiome, and immune dysregulation that lead to inflammation and organ damage, mechanisms that may also apply to the development of other SARD (Figure 1).

Common pathways of pathogenesis: immune dysregulation, epigenomics, the microbiome

Immune dysregulation

Inflammation is an adaptive response to stressors that involves multiple physiological processes that include the innate and adaptive immune systems. In turn, inflammation regulates – and is regulated by – several highly interconnected systems including the epigenome and microbiome (64). Unhealthy lifestyle behaviors (i.e., smoking, sedentary lifestyle, and consumption of ultra-

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TABLE 1 Environmental factors that increase risk for systemic autoimmune rheumatic diseases.

Lifestyle Exposure	Disease Association	Reported Risk from Select Key References (Citation)
Air Pollution	RA	• HR 1.31 (95%CI: 0.98–1.74) living near traffic pollution (road) vs. not (24)
	SLE	• Increases in air pollutants nitrogen dioxide (NO_2), carbon monoxide (CO), and fine particles ($PM_{2.5}$) (HR 1.21 [95% CI: 1.08–1.36], HR 1.44 [95% CI: 1.31–1.59], and HR 1.12 [95% CI: 1.02–1.23], respectively) (37)
	SARD[1]	• OR 1.13 (95%CI: 1.02-1.25) for lowest vs. highest satellite fine particulate air pollution level (38)
Cigarette Smoke	RA	• RR 3.8 (95%CI: 2.0-6.9) in current smokers vs. never smokers (39) • OR 1.65 (95%CI: 1.03–2.64) for >20 versus 0 pack-years) for anti-CCP-positive RA (40)
	SLE	• OR 1.50 (95%CI: 1.09–2.08) for current smokers compared with non-smokers (11) • HR 1.86 (95%CI: 1.14–3.04) for current vs. never smokers for dsDNA+ SLE risk (9)
Diet	SLE	• Women in the highest tertile of cumulatively updated dietary ultra-processed food (UPF) intake/day were at almost 50% greater risk of developing SLE vs. women in the lowest tertile of UPF daily intake (16)
Hazardous Waste Sites	SLE	• Exposure to volatile organic compounds ($P < 0.05$) (26)
Obesity	RA	• History of obesity (OR 1.24 [95%CI: 1.01–1.53]) (41)
	SLE	• An 85% (HR 1.85 [95%CI: 1.17-2.91]) significantly increased risk of SLE among obese compared to normal BMI women in the more recent NHSII cohort (12), but not NHS
Organic Solvents, Pesticides and Heavy Metal	RA	• Application of chemical fertilizers (adjusted OR 1.7 [95%CI: 1.1-2.7]) and cleaning with solvents (OR 1.6 [95%CI: 1.1-2.4]) (42)
	SLE	• Pesticide exposure (adjusted OR 2.24 [95%CI: 1.28–3.93]) (27) • Association with SLE risk seen with mercury (OR 3.6 [95%CI: 1.3-10.0]) and mixing pesticides for agricultural work (OR 7.4 [95%CI: 1.4-40.0]) (43)
	SSc	• OR 2.9 (95%CI: 1.1-7.6) for solvent organic solvent exposure (male SSc vs controls) (44)
Periodontitis	RA	• OR 1.16 (95%CI: 1.13-1.21) history of periodontitis (45)
Psychosocial	SLE	• Probable PTSD (HR 2.94 [95%CI: 1.19–7.26]) and trauma exposure (HR 2.83 [95%CI: 1.29–6.21]) (19) • Women with a history of depression vs. no depression (HR 2.67 [95%CI: 1.91-3.75]) (17) • Adverse childhood experiences (abuse, neglect, and household challenges) associated with increased risk of SLE. Exposure to the highest vs. lowest physical and emotional abuse was associated with 2.57 times greater risk of SLE (95%CI: 1.30–5.12) (46). HR for ≥2 episodes of severe sexual abuse compared to no abuse was 2.51 (95%CI: 1.29–4.85) and ≥5 episodes of severe physical abuse was 2.37 (95%CI: 1.13–4.99) among Black women) (20).
Reproductive/ Hormonal Factors	SLE	• Pooled RR 1.5 (95%CI: 1.1-2.1) oral contraceptive use and use of postmenopausal hormones RR 1.9 (95%CI: 1.2-3.1) (21)
Silica	RA	• Silica exposed men OR 2.2 (95%CI: 1.2-3.9) among men aged 18 to 70 years and 2.7 (95%CI: 1.2-5.8) among those aged 50 to 70 years (47)
	SLE	• Medium silica exposure was OR 2.1 (95%CI: 1.1–4.0), high exposure OR 4.6 (95%CI: 1.4–15.4) (25)
	Vasculitis	• Overall significant summary effect estimate of silica "ever exposure" with development of AAV (OR 2.56 (95%CI: 1.51–4.36) (48)
	SSc	• The combined estimator of relative risk for studies in females was 1.03 (95%CI: 0.74–1.44) and was 3.02 (95%CI: 1.24–7.35) for males (49).
Sleep Deprivation	SLE	• HR 2.47 (95%CI: 1.29-4.75) for chronic low sleep duration (≤5 hours/night versus >7–8 hours) (18)
UV Radiation	SLE	• History of more than one serious sunburn before the age of 20 years (OR 2.2, 95%CI: 1.2–4.1) and sunburn-susceptible skin type (OR 2.9, 95%CI: 1.6–5.1) (32)
Viruses	SLE	• Epstein-Barr virus serologic reactivation among unaffected SLE relatives (viral capsid antigen IgG OR 1.28 [95%CI: 1.07-1.53], p=0.007 and early antigen IgG OR 1.43 [95%CI: 1.06-1.93], p=0.02) (36)
	SARD	• Higher risk of RA (adjusted HR (aHR) 2.98 [95%CI: 2.78–3.20]), SLE (aHR 2.99 [95%CI: 2.68–3.34]), dermatopolymyositis (aHR 1.96 [95%CI: 1.47–2.61]), SSc (aHR 2.58 [95%CI: 2.02–3.28]), SjD (aHR 2.62 [95%CI: 2.29–3.00]), mixed connective tissue disease (aHR 3.14 [95%CI: 2.26–4.36]), Behçet's disease (aHR 2.32 [95%CI: 1.38–3.89]), polymyalgia rheumatica (aHR 2.90 [95%CI: 2.36–3.57]), and vasculitis (aHR 1.96 [95%CI: 1.74–2.20]) among COVID-19 vs. non-COVID-19 exposed unvaccinated individuals (50).

AAV, anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis; CI, confidence interval; CCP, cyclic citrullinated peptide; HR, hazard ratio; NHSII, Nurses' Health Study Cohort 2; OR, odds ratio; RA, rheumatoid arthritis; RR, relative risk; SARD, systemic autoimmune rheumatic diseases; SjD, Sjögren disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; UV, ultraviolet.
1. SARD included systemic lupus erythematosus, Sjögren's disease, scleroderma, polymyositis, dermatomyositis, or undifferentiated connective tissue disease.

TABLE 2 Environmental factors that decrease risk for systemic autoimmune rheumatic diseases.

Lifestyle Exposure	Disease Association	Reported Risk from Select Key References (Citation)
Alcohol	RA	• HR 0.78 (95%CI: 0.61–1.00) for alcohol use of 5.0–9.9 gm/day (51)
	SLE	• HR 0.65 [95%CI: 0.45–0.96] among women who drank 2 or more servings of wine had significantly decreased SLE risk compared to women who did not drink wine (13)
Diet	RA	• HR 0.67 (95%CI: 0.51-0.88) among women aged ≤55 years, better quality diet was associated with lower RA risk, particularly seropositive RA (52)
Exercise	SLE	• Regular exercise (performing at least 19 metabolic equivalent hours of exercise per week) assessed with other healthy behaviors (never or past smoker, healthy diet, moderate alcohol consumption, healthy body weight) was associated with a 19% reduction in SLE risk per additional healthy behavior, such that women with four or more healthy lifestyle factors had the lowest risk (HR 0.42 [95%CI: 0.25-0.70]) (53).
	RA	• Similar to the SLE study above, a lower risk of RA was also observed with a healthier lifestyle including regular exercise, i.e., women with five healthy lifestyle factors had the lowest risk (HR 0.42 [95% CI: 0.22-0.80]) (54).
Reproductive/ Hormonal Factors	RA	• RR 0.8 (95%CI: 0.6–1.0) for breastfeeding for 2–23 total months (55)
Vitamin D	SARD[1]	• Vitamin D 2000IU daily supplementation was associated with a 22% reduction in the development of autoimmune disease (HR 0.78 [95% CI: 0.61, 0.99], P=0.05) (56).

CI, confidence interval; HR, hazard ratio; RA, rheumatoid arthritis; RR, relative risk; SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; 1. This included RA, polymyalgia rheumatic, autoimmune thyroid disease, psoriasis, inflammatory bowel disease, and many others (e.g., SLE, systemic sclerosis).

processed foods) promote systemic inflammation leading to chronic inflammatory diseases, including SARD. Before developing overt clinical manifestations, individuals developing SARD have a period of asymptomatic autoimmunity and inflammation of variable intensity and duration, characterized by increasing oxidative stress, loss of immune tolerance, autoantibody formation, immune complex deposition and complement activation, epigenetic modifications, and upregulation and/or downregulation of cytokine expression [reviewed in (65)].

In SLE, both obesity and exposure to the toxic components of cigarette smoke induce oxidative stress (66). This, in turn, raises intracellular levels of reactive oxygen species that damage DNA producing immunogenic DNA adducts that can lead to the production of 'pathogenic' anti-double-stranded DNA antibodies (dsDNA) (67–69). In the NHS and NHSII cohorts, smokers were at higher risk of developing anti-dsDNA positive SLE compared to never-smokers (hazard ratio [HR] 1.86 [95% confidence interval (CI): 1.14-13.04]), while there were no significant associations between smoking status or pack-years and overall SLE or anti-dsDNA negative SLE (9). In addition to elevated oxidative stress, the byproducts of smoking could also augment native autoreactive B cells (11) and induce pulmonary antinuclear antibody (ANA) as demonstrated in the lungs of exposed mice (70). Smoking may also influence specific genes in the pathogenesis of SLE (57). An individual with a high SLE genetic risk score or GRS (score based on 86 single-nucleotide polymorphisms and 10 classic HLA alleles previously associated with SLE) and a status of current/recent smoking was strongly associated with SLE risk (odds ratio [OR] 1.5, p=0.0003 versus more distant past/never smoking) and even stronger in the presence of anti-dsDNA antibodies. Not surprisingly, smoking also affects circulating cytokines. Elevated SARD-related cytokines including the B-cell lymphocyte stimulator (BlyS) (70), tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-6 (71, 72), but lower IL-10 (an anti-inflammatory cytokine) have been detected in smokers (73). These cytokines affect the function of T cells and $CD4^+$ regulatory T cells, which are important in maintaining self-tolerance. Similarly, adipose tissue, in particular visceral fat, secretes pro-inflammatory adipocyte-derived cytokines and exhibits higher levels of C-reactive protein (CRP), TNF-α receptor 2, and IL-6 than non-obese individuals (74).

The association between SLE risk and diet is less clear in humans (75–77) compared to other autoimmune diseases such as RA [reviewed in (78)]. There is evidence from SLE-prone mice models that low dietary fiber intake and a Western-type diet (i.e., high in sugar, fat, refined grains, and red meat) are associated with increased autoantibody production (79, 80). In the BWHS, a diet high in carbohydrates and low in fats was associated with an increased risk of developing SLE in African American women (HR 1.88 [95%CI: 1.06-3.35]) (75). Consumption of ultra-processed foods, in particular sugar and artificially sweetened beverages, has been associated with an increased risk of developing SLE among women (16). Low to moderate alcohol consumption (approximately 1/2 drink a day), on the other hand, has been shown to reduce the risk of SLE development among women (10, 13–15). Alcohol (e.g., ethanol) and antioxidants may counteract the changes induced by smoking and obesity, i.e., inhibiting key enzymes in DNA synthesis and suppressing TNF-α, IL-6, IL-8, and interferon (IFN)-γ that lower systemic inflammation (81, 82).

Several studies have reported an association between lack of sleep and SLE risk in humans (18, 83, 84). In the NHS and NHSII cohorts, chronic low sleep duration (</=5 hours/night versus the recommended >7-8 hours) was associated with increased SLE risk (adjusted HR 2.47 [95% CI: 1.29, 4.75]), with stronger effects among those with body pain and depression. In sleep-deprived individuals, increased levels of IL-6 and TNF-α have been reported (85–89).

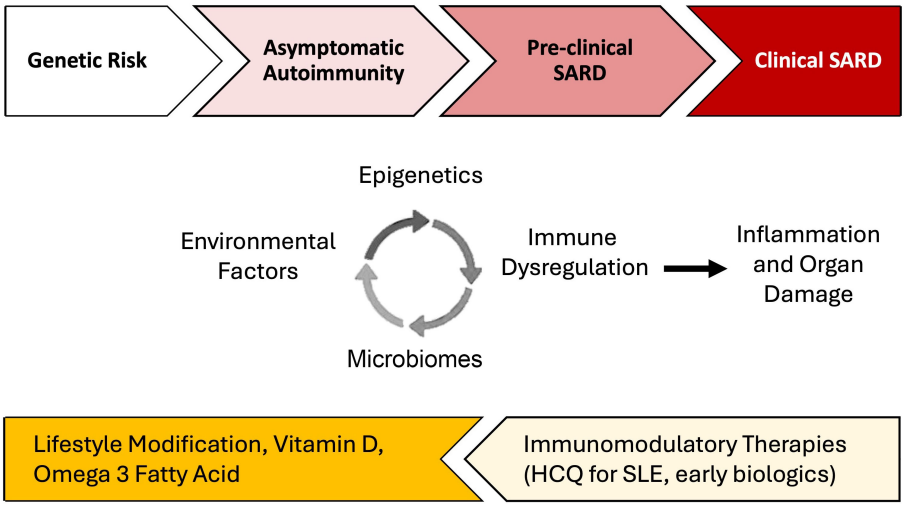

FIGURE 1
Environmental factor-associated pathogenesis and personalized preventative vs. treatment interventions for systemic autoimmune rheumatic diseases (SARD). Among individuals genetically predisposed to SARD development, unhealthy lifestyle behaviors and other environmental factors can trigger dysregulation in the microbiome, epigenetic changes, and immune dysregulation which, together, drive inflammation. In turn, inflammation can drive further derangements in the microbiome, cause distinct epigenetic changes, and lead to additional immune dysregulation. During the periods of asymptomatic autoimmunity and pre-clinical SARD, this positive feedback leads to a process wherein inflammation becomes chronic and self-sustaining, ultimately driving autoimmunity and eventually leading to organ damage and clinical disease. Effective lifestyle interventions, supplementation, and early introduction of immunomodulatory therapies may help prevent disease progression. There may be a potential role for treatments such as hydroxychloroquine for pre-SLE [SMILE trial underway (61)] and Abatacept, a T-cell co-stimulation inhibitor, for pre-RA (62, 63).

In SLE-prone mice, sleep deprivation was associated with accelerated production of autoantibodies and earlier disease onset (90). Sleep disturbances arising in individuals who have had childhood or adult trauma, PTSD, or occupational stress from working night or rotating shifts, may also explain why these factors have also been linked to SLE onset (17, 19, 20, 43, 91, 92). In the NHSII, PTSD, a condition arising after exposure to trauma and marked by severe psychological stress, was associated with increased SLE risk (HR 2.94 [95% CI: 1.19-7.26], p<0.05) compared to women with no trauma, even after adjusting for other SLE risk factors smoking, body mass index (BMI), and oral contraceptive use (19). In the NHS and NHSII, women with a history of depression had a higher risk of SLE (HR 2.67 [95:CI: 1.91-3.75] p<0.001) compared to women with no depression (17). Systemic inflammation, denoted by elevated TNF, IL-6, and CRP levels, has been repeatedly reported in individuals with emotional stress and distress (91, 93–102).

There is also evidence that sex hormones are important in SLE development (21, 22), a disease, like some other SARD, that predominantly affects females. In SLE, a population-based nested case-control study using the UK's General Practice Research Database demonstrated that there was a dose-response in oral contraceptive pill (ethinyl estradiol) and SLE risk (adjusted rate ratio [aRR] 1.42, 1.63, and 2.92 for < or =30 microgram, 31-49 microgram, and 50 microgram, respectively) (22). They also reported that the rate was particularly increased among females who recently started taking oral contraceptive pills (aRR 2.52 [95% CI: 1.14-5.57]) compared with longer-term current users. Estrogen prevents B cell receptor-mediated apoptosis and upregulates several genes that contribute to B cell activation and survival (cd22, shp-1, bcl-2, and vcam-1) (103).

Chemical and physical exposures have also been historically linked to SLE onset, including crystalline silica dust (25, 33, 104, 105), heavy metals such as mercury (43), air pollution and other respiratory particulates (38, 106), residential proximity to hazardous waste sites (26), agricultural pesticides (27, 43, 107), and organic solvents (42, 44). Proposed mechanisms of pathogenesis include stimulation of cellular necrosis and release of intracellular antigens resulting in systemic inflammation and IFN upregulation. These environmental exposures have also been described as important risk factors in the development of RA (42), SSc (44), and vasculitis (48). A comprehensive review of the literature (~1980-2010) on environmental factors and SARD development concluded that among these chemical factors, crystalline silica exposure, solvent exposure, and smoking had the strongest level of evidence (108). Since then, however, multiple studies have been published. The evidence for metal exposure and SARD development including mercury at that time was felt to be insufficient, although there is renewed interest in mercury-induced autoimmunity in more recent studies (109, 110). Mercury exposure has been associated with autoimmune features that are more consistent with pre- or sub-clinical autoimmunity in humans, and in animal studies, acts independently of type I IFN to induce milder disease (111).

UVB radiation can exacerbate pre-existing SLE, however, whether it contributes to SLE disease onset or pathogenesis is less

clear. While UVB radiation can up-regulate Th2 cells and down-regulate Th1 cells, induce IL-10 production, increase type I IFN expression, and prolong T cell activation to increase SLE risk (29–31), another subset of UV radiation, UVA, is used as a phototherapy modality to treat cutaneous forms of lupus (112). UVB also has an important role in vitamin D3 synthesis in the skin, which has been hypothesized to *lower* SLE risk (28, 113). Vitamin D deficiency is reportedly common among SLE patients (34) and is important in the regulatory pathways of numerous genes involved in inflammation and immunity including IL-2 inhibition, antibody production, and lymphocyte proliferation (114, 115). We will later discuss a large, randomized, double-blind, placebo-controlled clinical trial called the vitamin D and omega 3 trial (VITAL) trial, where vitamin D 2000 IU daily supplementation was associated with a 22% reduction in the development of autoimmune disease (HR 0.78 [95% CI: 0.61, 0.99], p=0.05) (56).

Viral triggers, particularly Epstein-Barr Virus (EBV), have also been associated with SLE development (35). In a recent study of 436 unaffected SLE patient relatives who were followed for 6.3 ± 3.9 years and evaluated for interim transitioning to SLE, increased serological reactivation of EBV was associated with higher risk of transitioning to SLE (viral capsid antigen IgG OR 1.28 [95%CI: 1.07-1.53], p=0.007 and expression of EBV early antigen IgG (OR 1.43 [95%CI: 1.06-1.93], p=0.02) (36). Proposed mechanisms include molecular mimicry and the release of EBV-encoded small RNAs from infected cells resulting in the induction of type-1 IFN and proinflammatory cytokines via activating toll-like receptor (TLR)-3 signaling (116). The interest in triggering of autoimmune conditions by viral infections was renewed during the coronavirus disease 2019 (COVID-19) pandemic when there were outbreaks of pediatric inflammatory multisystemic syndrome [PIMS also referred to as multisystem inflammatory syndrome in children (MIS-C)] that reportedly followed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in children. These reports included cases of Kawasaki-like disease, Kawasaki disease shock syndrome, toxic shock syndrome, myocarditis and macrophage activation syndrome (117–119). In adults, SARS-CoV-2 infection has also been linked to a higher risk of developing a diverse spectrum of new-onset autoimmune diseases as highlighted by two large retrospective studies (50, 120). Chang et al. used data from the TriNetX network and propensity score matching (two cohorts [COVID-19 and non-COVID-19] of 887,455 SARS-CoV-2 unvaccinated individuals) to identify the incidence of autoimmune conditions during the study period (1 January 2020 to 31 December 2021) (50). Unlike EBV, there was a wider spectrum of SARD seen including higher risk of RA (adjusted hazard ratio (aHR) 2.98 [95%CI: 2.78–3.20]), SLE (aHR 2.99 [95%CI: 2.68–3.34]), dermato/polymyositis (aHR 1.96 [95%CI: 1.47–2.61]), SSc (aHR 2.58 [95%CI: 2.02–3.28]), SjD (aHR 2.62 [95% CI: 2.29–3.00]), and other autoimmune diseases. Future studies that elucidate how viruses, such as SARS-CoV-2, increase the risk of SARD development may help implement preventive measures and early treatment in individuals who have had these infections to prevent morbidity and mortality.

A key pathway involved in both anti-viral response and the pathogenesis of SLE and other SARD including IIM and SSc is the type I IFN pathway (121). Approximately 50-70% of adult and pediatric SLE patients have an upregulated IFN signature, a cluster of IFN-stimulated genes, that correlates with disease activity and severity (122). A recent study demonstrated that type-1 IFN inhibits the aryl hydrocarbon receptor (AHR) pathway. Suppressed AHR signaling promotes T cell production of CXC ligand 13 (CXCL13), a chemokine that regulates B cell recruitment and lymphoid aggregation in inflamed tissues (123). AHR is important for sensing changes in the cellular milieu provided by the environment, diet, commensal flora, and host metabolism (124). In response to these environmental ligands, AHR has a protective role against inflammation by downregulating pro-inflammatory pathways (124). In the gut, AHR is expressed in epithelial cells and immune cells in the lamina propria to also stabilize the gut epithelial barrier (124). In the central nervous system, AHR is upregulated in astrocytes and microglia in response to ligands that cross the blood-brain barrier (124). Lower AHR expression has been described as a potential mechanism of pathogenesis for several autoimmune conditions including inflammatory bowel disease (125), multiple sclerosis (126), and psoriasis (127). In SLE, deficits in the AHR-driven immunoregulation exacerbated by the type-1 IFN may explain how alterations in the environment lead to the development of autoimmunity and uncontrolled inflammation. Moreover, polycyclic aromatic hydrocarbons, smoking, air pollution, and other environmental exposures cause DNA methylation changes in the AHR repressor genes, potentially linking these exposures to the development of autoimmunity (128–130). Future studies are warranted to elucidate the pathways by which regulation of the AHR pathway is related to lymphocyte activation status in the pathogenesis of autoimmunity.

Epigenetic changes

The currently accepted etiologic model for SARD implicates an interaction of inherited genetic factors and environmental exposures over time. DNA methylation (DNAm), an epigenetic change controlling gene expression, is influenced by both genetics and environmental exposures and therefore, may provide a critical link between them [reviewed in (131–133)]. For instance, UV light exposure, infections, silica, heavy metals and pesticide exposures, cigarette smoking, and air pollution are all thought to inhibit DNAm by oxidative stress, which could promote SARD onset specifically or non-specifically (134). In addition to DNAm, cigarette smoking is linked to the activation of enzymes that regulate other types of epigenetic modifications (i.e., post-translational modifications of histones via methylation, acetylation, phosphorylation, ubiquitination, and regulation of non-coding RNA sequences) to mediate the expression of multiple inflammatory genes, thereby participating in the onset development of autoinflammatory diseases (135).

DNAm occurs when a methyl group is added to a cytosine base in a cytosine-phosphate-guanine dinucleotide (CpG) which, in general, silences nearby gene expression. By comparison, demethylation activates gene expression. These changes, mainly

demethylation and in particular IFN gene hypomethylation, have been observed in various cell subsets, including CD4 T cells in patients affected by SLE (136–145). Upregulation of type I IFN in SLE is thought to induce an "IFN epigenomic signature", activating latent enhancers and "bookmarking" chromatin, reprogramming genes to be hyper-responsive, amplifying the inflammatory cascade (146–148). Emerging data reveal that some of these epigenetic changes are correlated with SLE disease manifestations (malar and discoid rash, dsDNA autoantibodies, lupus nephritis) and disease severity (137, 139, 144, 149), and are highly specific to SLE such that they distinguish individuals with existing SLE from controls and other SARD (141, 150). Well-designed epidemiologic studies are still needed to determine whether other epigenetic changes precede the development of SARD and whether such changes could be modified to abrogate disease.

Microbiome influences

There is mounting evidence that imbalances in the microbiota contribute to metabolic and immune regulatory dysfunction, which may contribute to the pathogenesis of chronic inflammatory diseases such as SARD [reviewed in (151)]. Several independent reported studies of 16S rRNA libraries have identified characteristic patterns of gut dysbiosis in SLE, in which there is an inverse relationship between disease activity and overall biodiversity of the intestinal microbiota (152–154). In studies of 61 female SLE patients, there was an eight-fold increase in *Ruminococcus gnavus* abundance compared to the healthy subjects, and most patients with high R. *gnavus* abundance had active nephritis (152). Increases in *R. gnavus* abundance have also been observed in other diseases including allergies and spondyloarthropathies with inflammatory bowel disease (155–157). Importantly, many strains of *R. gnavus* express a VH3 B cell repertoire (BCR) targeted B cell superantigen, particularly relevant to SLE given the importance of B cell activation in disease pathogenesis (158).

Evidence suggests that SLE patients may suffer chronic microbial translocation through impaired gut barrier integrity contributing to immunologic dysregulation (159). Oral microbiome studies confirm that SLE patients have a distinct microbiome signature compared to healthy controls, with evidence of translocation of bacteria, e.g., *Veillonella* species, from the oral cavity to the intestine (160, 161).

In healthy adults, the microbiome, even at the level of strains, is relatively stable over many years (162). However, the microbiome can be altered by diet, sleep, exercise, stress, medications (antibiotics and non-antibiotics), and the environment (163). Perturbations in the gut microbiome composition have been suggested to trigger SLE onset or disease flares and *vice versa* (164). In-depth studies examining the impact of lifestyle and environmental factors on changes to the microbiome and subsequent risk of autoimmune diseases are needed.

Other host barriers should also be considered as potential targets for prevention including the oral cavity and lung mucosa as these have been identified as sites of pathogenic autoreactive immune responses that contribute to autoimmune disease. The initiation of RA by inflammation characterized by an aberrant Th-17-dominated immune response, neutrophil activation, antigen citrullination, and anti-cyclic citrullinated peptide (CCP) production is exacerbated by microbial dysbiosis, the presence of oral pathobionts (e.g., *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*), and periodontitis has been described (45, 165–167).

The lung mucosa is another site of protein citrullination leading to RA development, promoted by microbial infection or dysbiosis and the inhalation of pollutants such as tobacco smoke or other pollutants (168, 169). This anti-CCP production and translocation into the systemic circulation has been proposed to accelerate the development of RA with interstitial lung disease for individuals who are genetically predisposed (e.g., gain-of-function MUC5B promoter variant reducing mucociliary function in small airways responsible for clearing inhaled particles in the lungs (170)). It is difficult to be certain that microbiome alterations observed in recent studies of SARD patients are not due to established and treated disease. Additional studies of the microbiome before disease onset are warranted.

Mitigation of environmental factors

Traditional cohort studies

Our current understanding of lifestyle factors and autoimmune diseases has largely depended on large observational epidemiological studies (53, 54, 171). Many of these studies used self-reported data including the use of validated and standardized questionnaires. These studies also relied on the retention of subjects in the long term to enable repeated measurement of lifestyle behaviors. Nevertheless, these studies have filled important knowledge gaps in our understanding of the link between environmental exposures and autoimmunity.

In the NHS and NHSII cohorts, our group demonstrated that 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 or past smoker, moderate alcohol consumption (drinking ≥5 gm/day alcohol), and maintaining a healthy body weight (body mass index <25 kg/m2) was associated with a 19% reduction in SLE risk per additional healthy behavior, such that women with four or more healthy lifestyle factors had the lowest risk (HR 0.42 [95%CI: 0.25-0.70]) (53). An even greater reduction per healthy behavior (22%) was observed for the risk of anti-dsDNA-positive SLE. 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%CI: 23.1-66.6%]. Using the same cohorts and similar modeling, a lower risk of RA was also observed with a healthier lifestyle, i.e., women with five healthy lifestyle factors had the lowest risk (HR 0.42 [95%

CI: 0.22-0.80]) (54). Therefore, a significant proportion of the risks of both SLE and RA may be preventable by adhering to healthy lifestyles.

Intervention and prevention trials

There is a scarcity of clinical trials examining lifestyle and environmental interventions and prevention strategies to reduce the risk of autoimmune disease development. One of the challenges in designing a strong and well-powered prevention study is identifying which at-risk individuals to study. Our group has previously developed SLE risk prediction models having 76% accuracy by combining family history, genetic factors, and lifestyle, medical and behavioral exposures that classify a woman's risk of SLE in the next two years (172). There is also a rapidly growing panel of potential biomarkers of SLE risk or early disease including anti-dense fine speckled 70 (DFS70) as a rule-out SARD test (173), anti-C1q antibodies as a rule-in test (174), cytokines and chemokines (175, 176), IFN signature (177), as well as markers of complement activation (178). Therefore, identifying individuals for screening, risk-stratifying, assessing biomarkers, and testing intervention and prevention strategies before clinical disease onset has recently become possible (65, 179).

In a pivotal randomized, double-blind, placebo-controlled vitamin D and omega 3 trial (VITAL) trial with a two-by-two factorial design (n=25 871 participants followed for a median of 5.3 years), vitamin D (2000IU/day) supplementation for five years [with or without omega 3 fatty acid (1000 mg/day)] had a significant reduction in the risk of confirmed autoimmune disease of 22% (HR 0.78 [95% CI: 0.61, 0.99], p=0.05) (56). This included RA, polymyalgia rheumatica, autoimmune thyroid disease, psoriasis, inflammatory bowel disease, and others (e.g., SLE, SSc). Individuals who received an omega-3 fatty acid supplementation (with or without vitamin D supplementation) had a reduced rate of incident autoimmune disease by 15% but this was not statistically significant. However, the two-year post-intervention observation study where participants were no longer provided with any supplements but were invited to continue being observed while off assigned supplements, demonstrated that the protective effects of the 5.3 years of randomized exposure to 2000 IU/day of vitamin D dissipated, but the randomized supplementation with 1,000 mg/day of omega-3 fatty acids for the 5.3 years was seen to have a sustained effect in reducing autoimmune disease incidence (180). The results suggest that vitamin D supplementation of 2000 IU/day should be given continuously for long-term prevention of autoimmune disease, while the beneficial effects of omega-3 fatty acids may be more sustained.

The only SLE-specific prevention trial to date is the "Study of Anti-Malarials in Incomplete Lupus Erythematosus (SMILE)" (61), which was set to determine whether SLE progression can be abrogated by using hydroxychloroquine (HCQ) among patients with a positive ANA test and at least one (but not three or more) additional clinical or laboratory criterion from the 2012 Systemic Lupus Inception Collaborating Clinics (SLICC) classification criteria (181). This highly anticipated, multicenter, randomized, double-blind, placebo-controlled, 24-month trial is expected to be completed soon.

A similar HCQ prevention trial in RA ("Strategy to Prevent the Onset of Clinically-Apparent Rheumatoid Arthritis" or STOP-RA) was halted early due to the futility of the treatment (182). In the interim analysis it was observed that in individuals who were anti-CCP positive but without inflammatory arthritis at baseline, one year of HCQ was not superior to placebo in preventing or delaying the development of inflammatory arthritis, and the classification of individuals as having RA at 3 years (probabilities of RA development were 34% in the HCQ arm and 36% in the placebo; p=0.844). Therefore, in RA, HCQ did not help prevent or delay the onset of clinical disease compared to placebo. The study did suggest however that anti-CCP at levels of ≥40 units will be an important enrolment criterion in future RA prevention studies. Therefore, as we strive towards a future of prevention over cure in any SARD, a better and more standardized approach to identifying the timing of intervention and which patients are at the highest risk is urgently needed to ensure the success of prevention trials.

Other RA prevention trials such as the "TREAT Early Arthralgia to Reverse or Limit Impending Exacerbation to Rheumatoid arthritis" (TREAT EARLIER) trial examining one year of methotrexate also did not meet its endpoint of development of clinical arthritis among individuals with arthralgia clinically suspected of progressing to RA and magnetic resonance imaging (MRI)-detected subclinical joint inflammation (183). The T-cell co-stimulation inhibitor abatacept has shown greater promise in delaying RA development in two different at-risk populations. In the "Abatacept inhibits inflammation and onset of rheumatoid arthritis in individuals at high risk" or ARIAA trial, abatacept treatment for six months among RA-at-risk individuals (anti-CCP positive and showing MRI signs of inflammation) reduced subclinical joint inflammation and delays the development of RA (62). In the "Arthritis Prevention In the Pre-clinical Phase of RA with Abatacept" (APIPPRA) trial, at-risk individuals were defined as individuals with arthralgia, anti-CCP plus rheumatoid factor (RF) positive or high anti-CCP titers ≥3 x upper limit of normal plus RF negative, without synovitis at baseline (63). In this randomized, double-blind, multicenter, parallel, placebo-controlled, phase 2b clinical trial, 52 weeks of abatacept treatment reduced RA development over two years compared to placebo. However, by 24 months, the effect of abatacept treatment on symptom burden and subclinical inflammation as determined by ultrasound was not sustained. Therefore, longer treatment with abatacept beyond 12 months might be required. These studies again highlight the need for criteria that identify at-risk individuals from patients with early RA and the most appropriate time to target preventative interventions (184).

Future technologies for research on environmental exposures and SARD

In the last decade, there has been an exponential uptake of AI technologies to study diseases including SARD [reviewed in (185–

187)]. Much of this is due to greater access to a variety of data sources, e.g., images, efficient data collection tools, and supercomputer and analytic methods to rapidly compute. ML is a type of AI that refers to utilizing computers to perform specific tasks by learning from the data rather than being explicitly programmed with instructions such as traditional statistical tests. Within ML, different algorithms are generally categorized into supervised, unsupervised, reinforcement, and deep learning.

In the study of SARD, ML has proven useful in developing prediction models for diagnosis and disease outcomes and in elucidating pathogenesis [reviewed in (185)]. As SARD are highly complex, multifactorial, and heterogeneous diseases, ML is an ideal approach because it can reveal patterns and interactions between variables in large and complex datasets more accurately and efficiently than traditional statistical methods. As we enter an era of 'multi-omics', information on our patients is becoming increasingly 'layered' and challenging to interpret and ML holds promise for new insights and interpretations.

Utilizing ML, we recently demonstrated that there are four unique SLE clusters defined by longitudinal autoantibody profiles alone (188). While these clusters are predictive of disease activity, treatment requirements, complications, and mortality, it also points to autoantibodies as being a fundamental underlying mechanism of immune dysregulation and disease pathogenesis of SLE. This approach can be adopted to study pathogenesis for other SARD and inform more personalized monitoring and treatment plans. The focus of current SLE ML models is on the identification of patients with established disease or the prediction of specific SLE manifestations, e.g., nephritis, neuropsychiatric disease. This includes a validated diagnostic algorithm called the SLE Risk Probability Index (SLERPI) where a SLERPI score of greater than 7 was highly accurate (94.2%) and sensitive for detecting early disease (93.8%) and severe manifestations including kidney (97.9%) and neuropsychiatric involvement (91.8%) (189). Future studies to develop ML models that predict the development of new-onset SLE utilizing datasets that include environmental exposures are needed.

Conclusions

Our examination of risk and protective factors for SARD development, including adherence to multiple healthy lifestyle behaviors, has helped our understanding of the pathogenesis of autoimmunity that involves immune dysregulation, epigenetics, and an altered microbiome. Multiple environmental exposures, including social and behavioral factors throughout our lifespan are likely synergistic and interactive with each other and with genetic factors, influencing the immune system in a complex interplay of epigenetic, hormonal, and microbiome influences, leading to systemic inflammation and eventual organ damage in some. While a major focus has been placed on identifying new targets for disease treatment, shifting the care paradigm to disease prevention is an attractive proposition, especially as our ability to identify high-risk individuals improves. In the few prevention trials that have been conducted, the importance of identifying patients at the highest risk and the likelihood of benefiting from preventative treatment has been highlighted, and thus far, biomarkers have played a critical role in risk stratification. Given the complexity and vast clinical heterogeneity of SARD, ML approaches will become increasingly relied upon to study SARD pathogenesis and prevention.

Author contributions

MC: Conceptualization, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. KC: Conceptualization, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. MF: Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

MC has received consulting fees from AstraZeneca, Mallinckrodt Pharmaceuticals, MitogenDx, Werfen, Celltrion, Organon, and GlaxoSmithKline. MF is the Medical Director of Mitogen Diagnostics Corp and a consultant to Werfen.

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

Publisher's note

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Maternal seafood intake, dietary contaminant exposure, and risk of juvenile idiopathic arthritis: exploring gene-environment interactions

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Objectives: Juvenile idiopathic arthritis (JIA) originates from a complex interplay between genetic and environmental factors. We investigated the association between seafood intake and dietary contaminant exposure during pregnancy and JIA risk, to identify sex differences and gene-environment interactions.

Methods: We used the Norwegian Mother, Father, and Child Cohort Study (MoBa), a population-based prospective pregnancy cohort (1999–2008). JIA patients were identified through the Norwegian Patient Registry, with remaining mother-child pairs serving as controls. We assessed maternal seafood intake and dietary contaminants typically found in seafood using a food frequency questionnaire completed during pregnancy, mainly comparing high (≥90th percentile, P90) vs low (<P90) intake. Multivariable logistic regression calculated adjusted odds ratios (aOR), including sex-stratification analyses. A polygenic risk score (PRS) for JIA was used in a subsample to assess gene-environment interactions.

Results: We identified 217 JIA patients and 71,884 controls. High vs low maternal intake of lean/semi-oily fish was associated with JIA (aOR 1.51, 95% CI 1.02-2.22), especially among boys (aOR 2.13, 95% CI 1.21-3.75). A significant gene-environment interaction was observed between total fish intake and PRS, with high fish intake associated with JIA primarily in those with low PRS (p<0.03). We

found no associations between high vs low exposure to other types of seafood or environmental contaminants and JIA.

Conclusions: We found a modestly increased risk of JIA associated with high intake of lean/semi-oily fish during pregnancy, not explained by estimated exposure to dietary contaminants. Our data suggest a more pronounced association in children with a lower genetic predisposition for JIA.

KEYWORDS

juvenile idiopathic arthritis (JIA), MoBa, fish, contaminants, heavy metals, polygenic risk score, gene-environment interaction, sex differences

1 Introduction

Juvenile idiopathic arthritis (JIA), the most common inflammatory rheumatic disease of childhood, manifests as arthritis before the age of 16 years which persists more than six weeks, and without an apparent cause. It consists of seven heterogeneous subgroups, reflecting the complex interplay between genetic predisposition and environmental influences that contribute to the diverse clinical manifestations (1). Known genetic variants are estimated to account for 13-25% of the risk for JIA, while the remaining risk is attributed to environmental factors and their interaction with genetic predisposition (1, 2). Limited high-quality data and modest sample sizes have constrained prior attempts to pinpoint environmental risk and protective factors (3). Furthermore, despite JIA being more prevalent in girls than in boys (4), few studies have investigated this sex disparity, which is important for understanding the underlying pathomechanisms of disease development.

Diet is an example of an environmental factor that remains underexplored in relation to JIA risk (3). Results from a Swedish prospective cohort study showed that fish intake more than once per week during pregnancy and the first year of life was associated with increased risk of JIA, which was mainly attributed to high heavy metal exposure (5).

Among the environmental contributors, heavy metals like mercury and cadmium, and persistent organic pollutants (POPs), have emerged as potential triggers of autoimmunity (6–9). Mercury is associated with subclinical autoimmunity in humans through the production of autoantibodies and cytokines (10–13), while in individuals with a genetic predisposition, cadmium may exacerbate autoimmunity (14) and increase the risk of rheumatoid arthritis (RA) (15, 16). Furthermore, exposure to POPs has also been linked to autoimmune diseases, with research suggesting increased risk of celiac disease, especially in girls (8), and of RA (17).

Diet serves as a major source of these contaminants (18), with seafood being a significant contributor to mercury (19) and shellfish contributing to cadmium exposure (20). Individuals consuming high amounts of seafood are also at greater risk of POPs exposure (21, 22). It has been suggested that diseases with a sex disparity should be investigated for environmental risk factors like contaminant exposure, as differences in vulnerability and susceptibility between the sexes may account for the prevalence disparities (23).

Our primary aim was to explore the association between seafood intake and dietary environmental contaminant exposure during pregnancy and JIA risk. Secondary aims included exploring sex disparities and possible interactions between seafood intake and genetic predisposition to JIA.

2 Material and methods

2.1 Study population and design

We used data from the Norwegian Mother, Father, and Child Cohort Study (MoBa), which was linked by national identification (ID) numbers to the individual records in the following population-based health registers: the Norwegian Patient Registry (NPR) and the Medical Birth Registry of Norway (MBRN).

MoBa is a population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health. Participants were recruited from all over Norway from 1999-2008. Of those invited to participate, 41% of women consented. The cohort includes approximately 114,500 children, 95,200 mothers, and 75,200 fathers. The current study is based on version 12 of the quality-assured data files released for research in 2019 (24). Genotype data was available for a subsample of 51,804 children, which is further described under "Genotyping Data, Polygenic Risk Score (PRS) for JIA".

Three questionnaires were sent to the mothers during pregnancy, the second being a semi-quantitative food frequency questionnaire (FFQ). The FFQ was distributed in gestational week 22 and covered the average intake of 255 food items and beverages during the first half of pregnancy (25). The MoBa FFQ has been validated and found to be a reliable tool to estimate intake of

nutrients and foods during pregnancy, including various types of fish and seafood (26, 27). The FFQ was introduced in March 2002 and all pregnancies recruited between 2002 and 2008 are included in our study. Figure 1 outlines the flow of subject for inclusion in our study from the MoBa cohort.

2.2 Outcome

The Norwegian Patient Registry (NPR) contains data with personal ID numbers from all Norwegian public hospitals and specialists with public funding from 2008. We defined a JIA case as having at least two International Classification of Diseases (ICD)-10 codes (≥2 M08, ≥2 M09, or 1 M08 *and* 1 M09). We recently validated this case definition and have found a positive predictive value of 93.4% (28). For cases where the child received their first ICD-10 code in 2021, we accepted a single relevant ICD-10 code (M08 or M09), recognizing that there might have been only one doctor visit before we received our latest updates from NPR in December 2021. Controls were defined as live births that were non-JIA cases. We excluded children with only one registration of ICD-10 code M08 or M09 between 2008-2020 because they might have JIA.

2.3 Exposure variables: intake of seafood and environmental contaminants

We estimated maternal seafood intake and the exposure to dietary contaminants by the FFQ that was developed and validated for pregnant women in MoBa (25).

Food frequencies reported in the FFQ were converted to food amounts (grams/day) using FoodCalc and the Norwegian food table (26). Seafood intake was allocated into five variables, of which three were strictly related to fish intake: 1) oily fish (more than 8% fat, such as herring, mackerel, salmon), 2) lean/semi-oily fish (up to 8% fat, such as cod, haddock, saithe), and 3) total fish (total amount of oily fish and lean/semi-oily fish). In addition, we included 4)

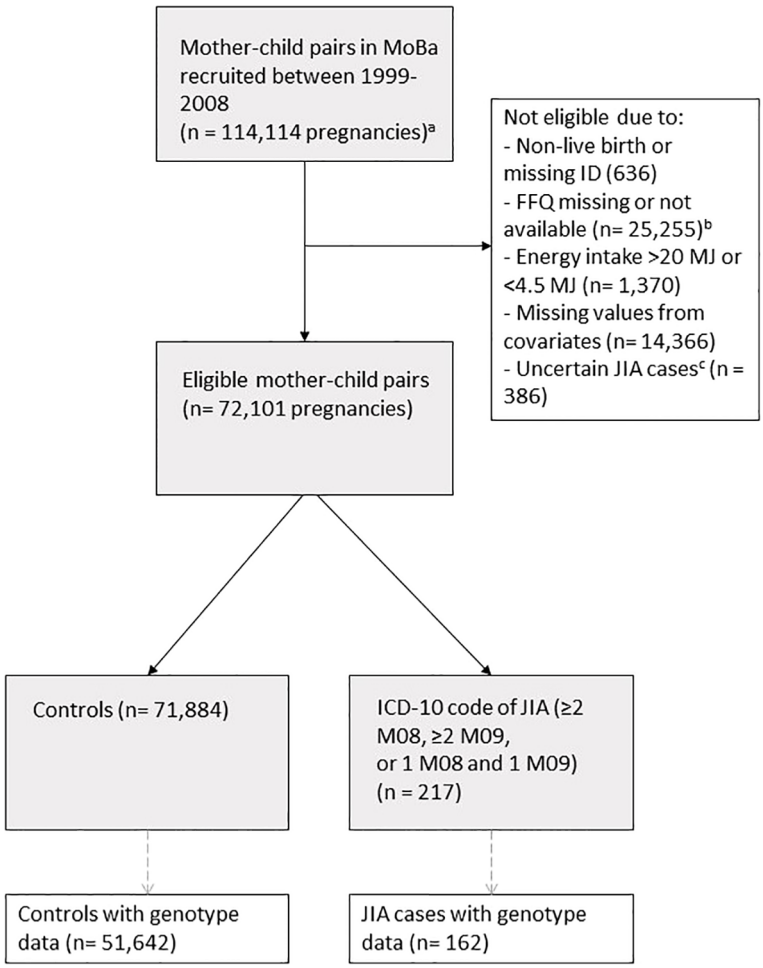

FIGURE 1
Flowchart of study population with exclusion criteria. [a] This number includes siblings. [b] The MoBa FFQ was introduced in 2002. [c] We excluded all controls with a single ICD-10 code (M08 or M09) to rule out potential JIA cases, except for those who received their first code in 2021. For these cases, we accepted a single relevant ICD-10 code, acknowledging that they might have had only one doctor visit before our latest NPR update in 2022.

shellfish intake (capturing crab, shrimp, and mussels), and 5) total seafood (total fish and shellfish, including fish liver, roe, and fish liver/roe spread). We converted these continuous variables (grams/day) into categorical variables in the following way:

We categorized the seafood variables into high intake, defined as equal to or exceeding the 90th percentile (≥P90) of the population, and low intake, defined as less than 90th percentile (<P90). In secondary analyses, the five seafood variables were also divided into quintiles with the lowest group serving as reference. Lastly, because the Norwegian Directorate of Health recommends between 300-450 grams of fish each week (29), an exposure variable was also set at ≥300 grams of fish per week, which was compared to intake <300 grams/week.

The exposure to dietary environmental contaminants was estimated by combining consumption data from the FFQ with concentrations of contaminants in Norwegian food, based on data across various Nordic studies and databases, with the mean or median values from these studies used for the estimation of dietary contaminant exposure. The food contamination data spans several years, corresponding with the period when the FFQ was completed, and is described elsewhere (30, 31). Dietary contaminant exposure was categorized into two main groups: 1) heavy metals and 2) persistent organic pollutants (POPs). Heavy metals included a) mercury, and b) cadmium, while POPs included c) dioxins and dioxin-like (dl) compounds, and d) non-dioxin-like (non-dl) polychlorinated biphenyls (using PCB-153 as a proxy). The exposure to dioxins and dl-compounds is expressed as toxic equivalents (TEQ) when assessing their combined effect (32). The dietary contaminant variables were calculated per kilogram of pre-pregnancy body weight (kg bw), which was self-reported. We analyzed high vs. low intake and across quintiles as defined above.

2.4 Covariates and confounders

Potential confounding factors included maternal education, and parity (categorical variables); maternal age, pre-pregnancy BMI, daily energy intake (continuous variables); and maternal history of inflammatory rheumatic disease (see definition below), parental smoking status, and maternal supplement use during pregnancy (e.g., fish oil, vitamin D, folate) as dichotomous variables (yes/no). Associations with lean/semi-oily and oily fish were mutually adjusted due to their correlated intake.

When analyzing dietary environmental contaminants, we included the child's birth year from the Medical Birth Registry as a possible confounder because contaminant levels in fish may have varied over the years, and because the cumulative risk of JIA increases with the child's age. Information about region of birth was also obtained from the Medical Birth Registry.

Mother's history of inflammatory rheumatic diseases was obtained via linkage to NPR and included following ICD-10 codes: M05, M06, M07, M08, M09, M30, M31, M32, M33, M34, M35, M45, M46, and L94.

2.5 Genotyping data, polygenic risk score for JIA

In MoBa, umbilical cord blood samples were collected at birth and DNA was stored at the Norwegian Institute of Public Health (33). Genotyping was carried out over several years through various research projects (34). MoBaPsychGen genotype quality control (QC) pipeline was developed to manage the complex relationships within the cohort. This pipeline includes steps for pre-imputation QC, phasing, imputation, and post-imputation QC, and it accounts for array and batch effects (35).

We focused on individuals of European ancestry, identified by visually comparing the first seven genetic principal components (PCs) to those from unrelated samples in the 1000 Genomes phase 1 project (35). Related individuals with a kinship coefficient >0.05 had one member excluded, prioritizing the retention of JIA cases, with other exclusions made randomly.

To estimate the genetic risk for JIA, we calculated PRSs using data from a genome-wide association study (GWAS) of JIA (36). The calculation was done using PRSice version 2.3.3 (37), applying different P-value thresholds as 5E-8, 1E-6, 1E-5, 1E-4, 1E-3, 1E-2, 5E-2, 1E-1, 5E-1, and 1. We then extracted the first PC of PRSs across all P-value thresholds, following a widely used method (38). The standardized PRS was then converted into a binary variable with cut-off at 0, of which the PRS <0 was regarded as "low", whereas the PRS ≥0 was regarded as "high".

2.6 Statistical analysis

Stata V.17.0 statistical software (StataCorp) and R version 4.2.3 (39) were used to conduct all statistical analyses. Characteristics of high vs low consumers of fish were reported as mean (SD) or median (IQR), as appropriate for continuous variables and by distribution differences (counts and percentages) for categorical variables. We used multiple logistic regression to examine the associations between seafood intake, dietary environmental contaminant exposure and risk of JIA. All associations are reported as odds ratios (OR) with 95% confidence intervals (CI), and as adjusted ORs (aOR) when adjusted for possible confounding factors listed above. The number of subjects with missing values on covariates was low for both cases (n=40, 18%) and controls (n=14,366, 20%), and all estimates are therefore based on complete case analyses. All analyses were further stratified by sex. In a sensitivity analysis, we included the region of birth (South-East, West, Middle and North), and thus presumably the region where the pregnancy took place, as a possible confounder because research shows a two-fold increased incidence of JIA in northern compared to southern Norway (28) and reports of geographical variations in fish intake (40).

To assess potential interactions between fish intake and genetic predisposition to JIA, we conducted multiple logistic regression analyses with an interaction term between fish intake and PRS. We

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included the same variables as in the main model except maternal history of inflammatory rheumatic disease to avoid over-adjustment. The Wald test was used to assess statistical significance of an interaction, and a p-value <0.05 was regarded as significant. We further investigated the interaction between fish intake and PRS by calculating the products of fish intake and dummy variables of each PRS group and replacing the interaction term in the multiple logistic regression with those products. This allowed us to estimate the association between fish intake and JIA in the low and high PRS groups separately. We used this model to visualize the relationship between fish intake and JIA in both groups predicting JIA risk in a simulated dataset of n = 200. As an additional test for interactions, we applied a case-only analysis by testing for associations between seafood intake and PRS in the cases only (41).

3 Results

3.1 Study sample characteristics

Our final analytical sample included 72,110 mother-child pairs; 217 children with JIA were identified (Figure 1). Of JIA cases, there were 139 (64.1%) girls and 78 (35.9%) boys. The median weekly maternal fish intake was 218 grams. Baseline characteristics categorized by high (≥P90) vs. low (<P90) total fish intake are shown in Table 1.

TABLE 1 Baseline characteristics categorized by high and low total fish intake in 72,101 MoBa participants 2002-2008.

Characteristics	High total fish intake (P≥90)*	Low total fish intake (<P90) *
Population	7,209 (10.0)	64,892 (90.0)
Maternal age at delivery, years, mean (SD)	31.0 (4.8)	30.3 (4.5)
Maternal education		
High school or less	2,722 (37.8)	21,839 (33.7)
College, up to 4 years	2,675 (37.1)	27,202 (42.0)
College, more than 4 years	1,812 (25.1)	15,851 (24.4)
Maternal pre-pregnancy BMI, mean (SD)	24.0 (4.4)	24.1 (4.3)
Maternal parity		
0	3,097 (43.0)	29,714 (46.0)
1	2,487 (34.5)	23,185 (36)
2 or more	1,624 (22.5)	11,993 (18.5)
Inflammatory rheumatic disease in mother		
Yes	209 (2.9)	1,853 (2.9)
No	7,000 (97.1)	63,039 (97.1)
Maternal daily caloric intake, kcal, median (IQR)	2462 (2053, 2939)	2207 (1866, 2620)
Maternal smoking status during pregnancy		
Yes	642 (8.9)	5,048 (7.8)
No	6,567 (91.1)	59,844 (92.2)
Paternal smoking status		
Yes	1448 (20.1)	12,624 (19.5)
No	5761 (79.9)	52,268 (80.6)
Dietary supplement use during pregnancy		
Yes	6,167 (85.6)	56,032 (86.4)
No	1,042 (14.5)	8,860 (13.7)
Region of birth		
South-East	3,365 (46.7)	35,794 (55.2)
West	1,955 (27.1)	16,132 (24.9)
Middle	1,207 (16.7)	9,296 (14.3)
North	682 (9.5)	3,670 (5.7)

*High is defined as equal to or above 90th percentile, while low is defined as below 90th percentile.
Numbers are n (%), mean (SD) or median (IQR).

3.2 Seafood intake and JIA

High vs low intake of lean/semi-oily fish during pregnancy was associated with JIA (aOR 1.51, 95% CI 1.02-2.22) (Table 2). After adjusting for region of birth, the confidence interval included 1 (aOR 1.45, 95% CI 0.99-2.18) (Supplementary Table 1). Additional results with region of birth as a covariate are presented in Supplementary Table 1-Supplementary Table 2. We found no other evidence of associations between high vs low intake of other seafood variables and JIA risk (Table 2).

After sex-stratification, we found an association with lean/semi-oily fish intake among boys (aOR 2.07, 95% CI 1.17-3.66), but not in girls (Table 2 and Supplementary Table 1). Similarly, high shellfish intake was associated with increased risk among boys (aOR 1.86, 95% CI 1.02-3.38), but not girls (Table 2). Additionally, consuming fish ≥300 vs. <300 grams/week during pregnancy, regardless of fat content, was linked to higher odds of JIA in boys (aOR 1.92, 95% CI: 1.22-3.04), but not in girls (Supplementary Table 3). When analyzing by quintiles, no other convincing evidence of associations were observed (Supplementary Table 4).

3.3 Interactions between fish intake and polygenic risk score

The following results are based on a smaller sample than our main analyses (controls n= 51,642, JIA case n= 162) due to lack of genetic data on all observations. To account for this, we ran the main analyses on the smaller dataset as a sensitivity analysis, with the results provided in Supplementary Table 5.

We found evidence of an interaction between total fish intake and PRS (aOR 0.33, 95% CI 0.12-0.90, p-value 0.03), but not with the other seafood variables (Supplementary Table 6). The association between total fish intake and JIA was only apparent in

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TABLE 2 Overall and sex-stratified associations between high vs. low seafood intake and JIA.

	All (controls n= 71,884, JIA cases n= 217)		Boys (controls n= 36,784 and JIA cases n= 78)		Girls (controls n= 35,100, JIA cases n= 139)	
	Unadjusted OR (95% CI)	aOR[a] (95% CI)	Unadjusted OR (95% CI)	aOR[a] (95% CI)	Unadjusted OR (95% CI)	aOR[a] (95% CI)
High total fish intake						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥423.5 grams/week)	1.02 (0.65-1.58)	1.02 (0.65-1.59)	1.78 (0.98-3.23)	1.80 (0.98-3.31)	0.63 (0.32-1.24)	0.63 (0.32-1.24)
High lean/semioily fish intake						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥249.5 grams/week)	**1.50 (1.03-2.20)**	**1.51 (1.02-2.22)**	**2.13 (1.21-3.75)**	**2.07 (1.17-3.66)**	1.18 (0.70-1.99)	1.21 (0.72-2.06)
High oily fish intake						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥156 grams/week)	0.81 (0.50-1.32)	0.80 (0.49-1.31)	1.45 (0.76-2.74)	1.36 (0.71-2.62)	0.49 (0.23-1.04)	0.49 (0.23-1.06)
High shellfish intake						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥65 grams/week)	1.12 (0.73-1.71)	1.14 (0.74-1.74)	**1.83 (1.01-3.33)**	**1.86 (1.02-3.38)**	0.76 (0.41-1.41)	0.78 (0.42-1.44)
High seafood intake						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥492 grams/week)	0.91 (0.58-1.49)	0.92 (0.57-1.46)	1.62 (0.87-3.00)	1.64 (0.88-3.07)	0.56 (0.27-1.13)	0.55 (0.27-1.14)

[a]Adjusted for maternal age, education, pre-pregnancy BMI, parity, daily caloric intake, history of inflammatory rheumatic disease in mother, parental smoking status during pregnancy and supplement use during pregnancy. When lean/semioily fish is the main exposure, it is also adjusted for oily fish intake, and vice-versa. Bold text indicates statistically significant results.

TABLE 3 Associations between high seafood intake and JIA risk in groups of high or low genetic risk (PRS of JIA).

Exposure	PRS group[a]	aOR[b] (95% CI)
High total fish	Low	**2.26 (1.08-4.71)**
	High	0.75 (0.38-1.49)
High lean/semioily fish	Low	**2.23 (1.06-4.66)**
	High	1.14 (0.63-2.05)
High oily fish	Low	0.65 (0.20-2.11)
	High	0.84 (0.43-1.61)
High shellfish	Low	1.65 (0.74-3.71)
	High	1.38 (0.81-2.36)
High seafood	Low	1.67 (0.74-3.77)
	High	0.82 (0.43-1.58)

[a]The standardized PRS was converted into a binary variable with cut-off at 0, of which the PRS <0 was regarded as "low", whereas the PRS ≥0 was regarded as "high".
[b]Adjusted for: maternal age, education, pre-pregnancy BMI, parity, daily caloric intake, parental smoking status during pregnancy, supplement use during pregnancy, high PRS and PCs 1-10. When lean/semioily fish is the main exposure, it is also adjusted for oily fish intake, and vice-versa. (controls n= 51,642, JIA cases n= 162).
Bold text indicates statistically significant results.

the low PRS group (aOR 2.26, 95% CI 1.08-4.71) (Table 3 and Figure 2). Furthermore, we also found an association between lean/semi-oily fish and JIA in the low PRS group (aOR 2.23, 95% CI 1.06-4.66), but not with the other seafood variables (Table 3 and Supplementary Figure 1-Supplementary Figure 2). A case-only design was used to test the interaction between fish intake and PRS, which further confirmed the findings from the case-control analyses: the high total fish intake was negatively associated with PRS in the cases, whereas none of the other seafood variables reached statistical significance (Supplementary Table 7).

3.4 Estimated environmental contaminants and JIA

We found no evidence of associations between estimated dietary intake of environmental contaminants and risk of JIA, whether analyzed by high vs. low intake (Table 4) or by quintiles (Supplementary Table 8).

After sex-stratification, we found a positive association between non-dl PCBs and JIA in boys (aOR 2.24, 95% CI 1.03-4.86), when comparing a dietary exposure corresponding to the 4th quintile to the 1st quintile (Supplementary Table 8). Among girls, being in the

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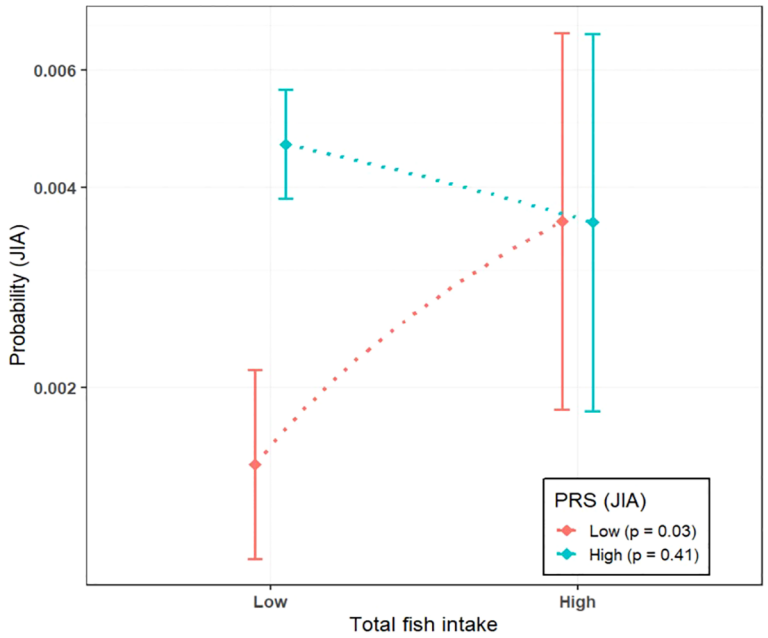

FIGURE 2
Association between total fish intake and JIA risk grouped by high (=>0) and low (<0) polygenic risk score (PRS) for JIA. P-values indicate the significance of the associations between fish intake and JIA risk within each PRS group.

TABLE 4 Overall and sex-stratified associations between high vs. low dietary contaminant exposure[a] and JIA.

	All (controls n= 71,884, JIA cases n= 217)		Boys (controls n= 36,784 and JIA cases n= 78)		Girls (controls n= 35,100, JIA cases n= 139)	
	Unadjusted OR (95% CI)	aOR[b] (95% CI)	Unadjusted OR (95% CI)	aOR[b] (95% CI)	Unadjusted OR (95% CI)	aOR[b] (95% CI)
Mercury						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥0.3 ug/kg bw/week)	0.91 (0.58-1.45)	0.92 (0.57-1.46)	1.17 (0.58-2.35)	1.22 (0.60-2.48)	0.77 (0.42-1.44)	0.76 (0.41-1.42)
Cadmium						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥2.1 ug/kg bw/week)	1.39 (0.94-2.06)	1.42 (0.94-2.14)	1.47 (0.78-2.79)	1.51 (0.77-2.99)	1.35 (0.82-2.21)	1.36 (0.80-2.29)
Dioxins and dioxin-like (dl) compounds						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥7.5 pg TEQ/kg bw/week)	0.86 (0.54-1.38)	0.83 (0.51-1.34)	1.46 (0.77-2.77)	1.45 (0.74-2.82)	0.56 (0.27-1.14)	0.52 (0.25-1.08)
Non-dioxin-like PCBs (PCB-153)						
<90th percentile	Ref	Ref	Ref	Ref	Ref	Ref
≥90th percentile (≥13.2 pg/kg bw/week)	0.91 (0.58-1.45)	0.90 (0.56-1.43)	1.03 (0.49-2.14)	1.02 (0.48-2.14)	0.85 (0.47-1.54)	0.83 (0.46-1.51)

[a]Contaminants were estimated by combining consumption data from the FFQ with concentrations of contaminants in Norwegian food.
[b]Adjusted for maternal age, education, pre-pregnancy BMI, parity, daily caloric intake, history of inflammatory rheumatic disease in mother, parental smoking status during pregnancy, supplement use during pregnancy and the child's birth year.

5th quintile of either dl-compound or non-dl PCB intake, was negatively associated with risk of JIA (aOR 0.40, 95% CI 0.20-0.79 and aOR 0.44, 95% CI 0.23-0.83; Supplementary Table 8).

4 Discussion

In this large population-based study, we found a modestly increased risk of JIA associated with high maternal intake of lean/semi-oily fish (approximately 250 grams or more per week) during pregnancy. No clear associations were found between JIA and overall maternal intake of fish, oily fish, shellfish, or seafood intake. Sex-stratified analyses suggested a stronger positive association between high maternal seafood intake and JIA risk in boys. For instance, an intake of >300 grams of fish per week as recommended by the Norwegian Directorate of Health (29), was linked to increased risk of JIA in boys but not in girls. We observed no clear associations with estimated maternal dietary contaminant exposures. The risk associated with total fish intake depended on genetic predisposition: high fish intake significantly affected JIA risk only in individuals with a low genetic predisposition to JIA.

Our results are partly in line with a Swedish study (5), which found positive associations between fish intake of more than once per week during pregnancy and JIA risk, although our effect sizes were of substantially lower magnitude. The Swedish study did not specify portion sizes, complicating direct comparisons. Furthermore, our study specifically associates lean/semi-oily fish with increased JIA risk, while the Swedish study identified the strongest association with total fish intake without distinguishing between fish varieties (5).

We found no evidence of robust associations between exposure to dietary environmental contaminants and risk of JIA. This differs from the Swedish study which attributed the heightened risk of JIA to increased heavy metal exposure, including mercury, through fish intake (5), and another study showing that prenatal exposure to environmental contaminants can alter the cord serum metabolome, potentially increasing the risk of immune-mediated diseases such as JIA (42). Despite seafood accounting for 88% of total dietary mercury exposure – with lean fish contributing to more than half of this - as well as being a considerable source of other contaminants (20, 43, 44), we found no evidence that it contributed to JIA risk in MoBa. In fact, our sex-stratified analyses show an inverse relationship between exposure to POPs and JIA in girls. Unlike the Swedish study, which measured blood concentrations, our study relies on self-reported dietary data, but includes a much larger sample size (217 vs. 41 JIA cases) (5).

JIA is more prevalent in girls than boys (4), yet our study suggests that high seafood intake is more strongly associated with JIA risk in boys. Sex-stratified analyses showed no indication of increased risk of JIA when comparing high vs low intake of seafood and contaminant exposure (except lean/semi-oily fish and cadmium) in girls, on the contrary, estimates indicated a lower risk of JIA with high intake. In contrast, for boys, all associations indicated an increased risk of JIA.

Most studies on sex disparities in pediatric illnesses do not explore underlying causes (45), making our sex-stratified analyses valuable for addressing this knowledge gap. Although estrogen levels are often suggested as a cause for the higher prevalence of autoimmunity in women, the low and stable levels during childhood suggest other mechanisms (4). The varying patterns of JIA risk between boys and girls with seafood intake may be due to lack of statistical power given the sample size (girls, n = 139, boys, n = 78), and the results should be interpreted cautiously. The inverse relationship between POP exposure and JIA risk in girls observed in our study may not be directly linked to POPs, but could reflect a spurious association with oily fish, which was estimated to have a protective association in girls. This protective association may be related to nutrients in oily fish rather than POPs. A study on diabetes type 1 observed similar findings (46). A separate MoBa study on prenatal exposure to POPs showed immunosuppressive effects (32), which could potentially explain a protective association in girls. Inherent biological differences may also influence these sex-specific trends.

Gene-environment interaction analyses suggest that genetic predisposition modifies the effect of fish intake on JIA risk, and vice versa. Specifically, fish intake had a stronger estimated association with JIA risk in individuals with low genetic predisposition, while its impact was estimated as less pronounced in those with a high genetic risk. Our previous findings show that the PRS is more strongly associated with JIA in girls than in boys, with a higher proportion of female JIA cases having a standardized PRS >0 (submitted for publication)[1]. This might explain why we observe a stronger association between fish intake and JIA risk in boys, as male JIA cases, on average, have a lower genetic risk of JIA.

Our study's strengths include its prospective design, comprehensive data collection with genetic liability, a large study population, and linkage to national registries, ensuring minimal loss to follow-up. A significant and novel strength is the incorporation of a PRS within a subset of our cohort, enabling us to study gene-environment interactions in JIA. By sex-stratification, we discerned variations in risk estimates between boys and girls. To our knowledge, this is the largest population-based prospective cohort study exploring environmental risk factors for JIA, identifying 217 cases.

While including more JIA cases than in previous studies, the sample size remains the main limitation of the study, as it reduces the power to detect small effects, especially in stratified analyses and for the subset with genotype data. We also lack data on JIA subtypes, which is important given the disease's heterogeneity; different subtypes may have distinct pathomechanisms or vulnerabilities. We did not exclude controls with other systemic autoimmune diseases, potentially diluting the observed effects. Additionally, while the recruitment into MoBa was population-based, the cohort is not fully representative of the general population (47). For instance, the homogenous ethnic background of MoBa participants (48) may limit the generalizability of our findings to more diverse populations. The

[1]Haftorn KL, Rudsari HK, Jaholkowski PP, Dåstøl VØ, Hestetun SV, Andreassen OA, et al. *Nonlinearity and sex differences in the performance of a polygenic risk score for juvenile idiopathic arthritis.* (2024).

self-reported dietary data may result in exposure misclassification as the FFQ provide rough estimates, even though it has been validated (26). We cannot study exact dietary intake for the second half of the pregnancy as the FFQ was completed in week 22, however, we assume consistent dietary patterns throughout the pregnancy. Additionally, we lack measured blood concentration of contaminants. Our contamination estimates rely on broader Nordic averages rather than location-specific data, so this approach may not adequately capture exposure differences across Norway, especially in areas of higher contamination, highlighting the need for future research to measure blood concentrations. Although we adjusted for potential confounders, residual confounding cannot be ruled out due to the observational nature of the study. Lastly, since NPR data begins in 2008, JIA cases diagnosed and in remission between 2002-2008 may be missing. Some of the older-diagnosed JIA cases are also missing, because follow up ended in 2021.

In conclusion, we observed an increased risk of JIA in children whose mothers consumed high amounts of lean/semi-oily fish during pregnancy, particularly in boys. Despite lean fish being an important source of dietary mercury exposure, the heightened JIA risk was not explained by contaminant exposure in our study. Our findings also suggest a stronger association between fish intake and JIA in those with a low genetic predisposition to JIA. Further studies are warranted to explore the underlying mechanisms of seafood and JIA, as definitive causation cannot be inferred. This includes more precise assessments of contaminant exposure via blood samples, and the need to clarify the observed sex differences and genetic interactions.

Data availability statement

Data from the Norwegian Mother, Father and Child Cohort Study and the Medical Birth Registry of Norway used in this study are managed by the national health register holders in Norway (Norwegian Institute of public health) and can be made available to researchers, provided approval from the Regional Committees for Medical and Health Research Ethics (REC), compliance with the EU General Data Protection Regulation (GDPR) and approval from the data owners. The consent given by the participants does not open for storage of data on an individual level in repositories or journals. Researchers who want access to data sets for replication should apply through helsedata.no. Access to data sets requires approval from The Regional Committee for Medical and Health Research Ethics in Norway and an agreement with MoBa.

Ethics statement

The establishment of MoBa and initial data collection was based on a license from the Norwegian Data Protection Agency and approval from The Regional Committees for Medical and Health Research Ethics. The MoBa cohort is currently regulated by the Norwegian Health Registry Act. The current study, part of the MoBaRheuma project, was approved by The Regional Committees for Medical and Health Research Ethics (REK), which includes linkages with NPR and MBRN. REK ref. nr. 2019/1222.

Author contributions

VD: Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Methodology, Visualization. KH: Conceptualization, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. HR: Formal analysis, Methodology, Validation, Writing – review & editing. PJ: Formal analysis, Methodology, Supervision, Writing – review & editing. KS: Methodology, Writing – review & editing. SH: Conceptualization, Methodology, Resources, Writing – review & editing. CW: Methodology, Writing – review & editing. LR: Methodology, Writing – review & editing. OA: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. AB: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. IC: Conceptualization, Formal analysis, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. HS: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

OA has received consulting fees from Cortechs.ai and Precision Health AS and declares future stock options with Cortechs.ai and Precision Health AS. OA has also received speaker fees from Lundbeck, Janssen, Otsuka, and Sunovion.

The remaining 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.

Generative AI statement

The authors declare that Gen AI was used in the creation of this manuscript. ChatGPT was solely used to improve the language clarity and precision, as English is not the primary language of the authors. It was not used for data analysis or any other purpose beyond improving language.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1523990/full#supplementary-material

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The toll like receptor 7 pathway and the sex bias of systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) predominately affects women with a ratio of females-to-males of about 9:1. The complement of sex chromosomes may play and important role in the mechanism of the sex bias. Previous work has shown that men with Klinefleter's syndrome (47,XXY) as well as women with 47,XXX are found in excess among SLE patients well as among Sjogren's disease, systemic sclerosis and idiopathic inflammatory myositis. in cells with more than one X chromosome, all but one is inactivated. However, X chromosome inactivation, as mediated by the long noncoding RNA X-inactive specific transcript, or XIST, is not complete with approximately 10% of genes in the non-recombining region of the X chromosome escaping X inactivation. In the TLR7 signaling pathway, both the TLR7 and TLR adaptor interacting with endolysosomal SLC15A4 (TASL) escape X inactivation. Comparing male and female immune cells, there is increased TLR7 signaling related to increased expression of these genes in cells with more than one X chromosome. Cells with more than one X chromosome also express XIST, while cells with one X chromosome do not. XIST, as a source of ligand for TLR7, has also been shown to increase TLR7 signaling. Thus, we propose that both these mechanisms operating in immune cells with more than one X chromosome may act in a mutual way to mediate an X chromosome dose effect for the sex bias of autoimmune disease.

KEYWORDS

systemic lupus erythematosus, sex bias, TLR7, TASL, XIST

Sex bias in lupus

Systemic illness among patients with the rash of lupus erythematosus was first noted by Moriz Kaposi in Vienna during the late 19th century (1). During the remainder of the 19th century and through the middle of the 20th century, the entity of systemic lupus erythematosus was established (2). The bias of this disease to affect women was also noted during this period, with assembled cohorts comprised by ~90% of women (3). This ratio of ~9:1 women to men in

cohorts of SLE has continued to be true into the 21st century with modern epidemiological methods (4). This relationship holds true in all racial and ethnic groups studied.

Sex hormones in lupus

While there are sex hormone differences between SLE patients and matched controls, be they men or women (reviewed in (5)), a fundamental biological explanation for these findings and their relationship to the gender-bias of SLE has not been forthcoming (6). Clearly, some men with SLE have primary hypogonadism. For instance, Mok, et al, found that 5 of 35 men with SLE had low serum testosterone and high luteinizing hormone (LH) while none of 33 control men did (7). The etiology of the hypogonadism in these men was not determined. Higher serum prolactin is also found in both men and women with SLE compared to controls (8, 9). However, men with SLE have the same degree of hypogonadism and low testosterone as do men with other non-female biased chronic illnesses (10), suggesting chronic illness causes hypogonadism in SLE rather than vice versa. Furthermore, at the onset of disease, prior to treatment, there are no sex hormone differences between SLE patients and a matched control population (11).

X chromosome in lupus

Seeking another explanation to the sex bias of SLE, we examined the complement of sex chromosomes, initially among men with SLE. We found that these SLE-affected men were much more likely than matched control men to have Klinefelter's syndrome, that is, 47,XXY (12). Subsequent work found that 47,XXX was found in excess among women with SLE (13). We have also found the rare mosaic, 45XO/46XX/47XXX, is associated with SLE (14), while Turner's syndrome (female 45,XO) was not found in excess among SLE patients (15). We have now extended these findings to other female-biased autoimmune diseases (16, 17), and others have replicated the findings in SLE (18, 19). Thus, this work established that the number of X chromosomes was a risk factor for SLE, and that the number of X chromosomes might underly the female predominance of the disease.

Discussing the potential mechanisms by which an X chromosome dose effect might operate requires a brief review of the biology of the sex chromosomes, which are in mammals, of course, are the X and Y. The X and Y chromosomes pair in meiosis and mitosis by virtue of short regions at the distal ends of both chromosomes known as the pseudoautosomal regions (PAR); namely PAR1 and PAR2. Each PAR contains a handful of genes, which behave identically to autosomal genes. That is, there is expression of one copy on X and one copy on Y with genetic crossover occurring within PAR1 and PAR2 of the X and Y chromosomes. Meanwhile, on the X chromosome, centromeric to the two PARs are about 2000 genes that are X-linked. Similarly, on the Y chromosome centromeric to the two PARs are about 40 genes in the non-recombining region of Y. Almost all these Y genes are expressed in male gonadal tissue and function in spermatogenesis. In contrast, X-linked genes, like other chromosomes, are not functionally organized; and, generally do not have a Y homologue (although there are exceptions).

In cells with 2 or more X chromosomes, all but one is inactivated by methylation through the action of the X inactive-specific transcript (*Xist)* gene (Figure 1), which encodes a long non-coding RNA (20). That is, since women have two X-chromosomes and men have one, the imbalance in X chromosome gene expression is equalized by each cell with 2 or more X chromosomes randomly undergoing inactivation (which is mediated by methylation of CpG) of all but one X chromosome. However, despite the fact that the inactive X chromosome makes up the cytoplasmic Barr body, X inactivation is not an all-or-none phenomenon. On the inactivated X chromosome (X_i), about 15% of the genes escape methylation partially or completely giving women (and Klinefelter men) more phenotypic variability compared to normal (i.e., 46XY) men (21).

Continued presence of *Xist* transcripts were not thought to be needed for maintenance of X inactivation (22). However, recent data demonstrate that this may not be the case in immune cells. Yu and colleague showed that deletion of Xist in CD11c-positive atypical memory B lymphocytes along with TLR7 activation induced isotype switching. In addition, *Xist* down regulation was found among B cells from women with SLE (23). Also, Anguera has found different localization patterns of the Xist non-coding RNA in B cells with upregulation of 20 X chromosome genes in female cells (24, 25). In a published preprint, conditional knock of Xist in female mice (BALB/c and C57BL/6) produced a spontaneous lupus phenotype (26). Thus, there may be differences in the physiology of this long non-coding RNA in B cells that change X chromosome inactivation in such a way that predisposes to a SLE.

X chromosome and immune genes

The idea that immune genes are enriched on the X chromosome is frequently evoked. However, we find this is not the case. All human genes and Gene Ontology (GO) categories were downloaded from NCBI's FTP server (ftp.ncbi.nlm.nih.gov/Gene/DATA/) on August 6th, 2024. Only protein-coding and RNA-producing (eg, ncRNA) transcripts with at least one GO category annotation were selected for analysis. GO categories associated with all transcripts on each human chromosome were then identified, summed, and hypergeometric tests performed to determine relative chromosomal enrichments or depletions in each GO category. False Discovery Rate (FDR) corrections for the most significant p-value (enriched or depleted) were performed to correct for multiple testing. As a positive control, we find the Y chromosome highly enriched (p-value = 0) in the GO categories "spermatogenesis" and "gonadal mesoderm development". We find that, although there are many immune-related genes on the X chromosome, it is not particularly enriched for immune-related genes more than any other chromosome. This was true for all genes related to immune function with 50 of 1,482 (3.4%) on the X chromosome. Furthermore, no individual category of immune function had

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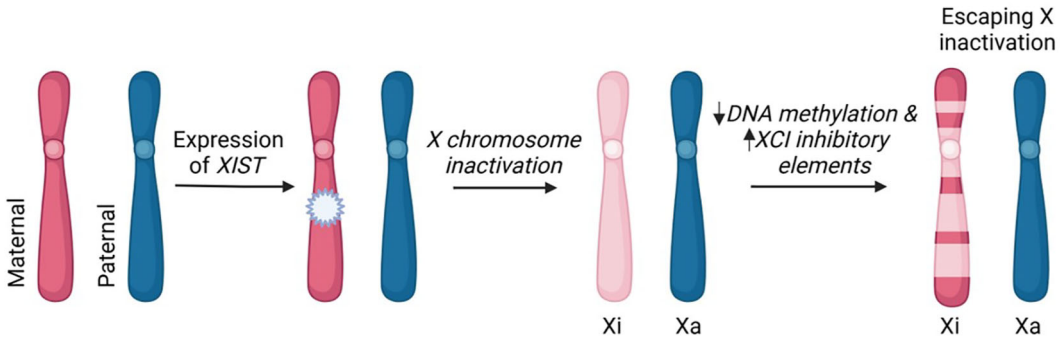

FIGURE 1
X chromosome inactivation and escaping X inactivation. The process of XCI occurs in mammalian cells that have two or more X chromosomes. In early stages of embryonic development, the maternal or paternal X chromosome is randomly silenced. This X-inactivation is initiated by long non-coding RNA, *XIST*, and subsequent DNA methylation and histone modifications. The incomplete inactivation of the X chromosome (pseudoautosomal region and variable genes throughout the X chromosome) results in approximately 15% of X-linked genes remaining transcriptionally active. These "escapee" genes contribute to differential expression of X-linked genes between men and women. Xi-inactive; Xa-active.This image was created in Biorender.com.

enrichment on the X chromosome (Table 1). In fact, we found significant immune-related transcript enrichment on other chromosomes, particularly chromosome 9 (Table 2), and we found other GO categories enriched on the X chromosome (Supplementary Table S1). Some of the categories in Supplementary Table S1 might impact immune processes (eg, miRNA-mediated gene silencing), but none are not immune-specific.

TABLE 1 Immune related gene categories for the X chromosome.

GO group name/ID	on X/total	OR	FDR p value
innate immune response/0045087	14/485	0.81	0.6494
immune response/0006955	8/310	0.72	0.6494
adaptive immune response/0002250	4/193	0.58	0.6494
AHIRMAP/0061844	2/99	0.56	0.6494
activation of innate immune response/0002218	2/32	1.82	0.6494
positive regulation of innate immune response/0045089	2/30	1.95	0.6494
positive regulation of Ig production/0002639	2/28	2.10	0.6494
immunoglobulin mediated immune response/0016064	2/24	2.48	0.6494
immunological synapse formation/0001771	1/13	2.28	0.6494
negative regulation of immune response/0050777	1/12	2.48	0.6494
negative regulation of Ig production/0002638	1/8	3.90	0.6494
positive regulation of adaptive immune response/0002821	1/8	3.90	0.6494
regulation of immunoglobulin production/0002637	1/7	4.56	0.6494
T cell mediated immunity/0002456	1/16	1.82	0.6497
regulation of innate immune response/0045088	1/21	1.37	0.6521
regulation of immune system process/0002682	1/39	0.72	0.6585
innate immune response in mucosa/0002227	1/27	1.05	0.6617
regulation of immune response/0050776	1/27	1.05	0.6617
immune response-regulating signaling pathway/0002764	1/37	0.76	0.6625
humoral immune response/0006959	2/60	0.94	0.6642
positive regulation of immune response/0050778	1/36	0.78	0.6656

AHIRMAP, antimicrobial humoral immune response mediated by antimicrobial peptide.

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TABLE 2 Gene ontology categories that are significantly found increased on a given chromosome.

chromosome	GO category/ID	#/total	OR	FDR p value	
9	0002286	TCA	17/24	57.99	0
9	0002323	NKCA	17/19	202.96	0
19	0002764	IRRSP	35/37	258.04	0
9	0006959	HIR	18/60	10.23	1.40E-08
6	0050778	PRIR	16/36	13.54	2.15E-08
19	0002682	RISP	15/39	9.21	4.00E-06
20	0045087	IIR	36/485	2.83	6.69E-05
9	0002250	AIR	25/193	3.56	0.0001
6	0002250	AIR	30/193	3.12	0.0001
4	0061844	AHIRMAP	14/99	4.15	0.006
8	0002227	IIRM	7/27	9.37	0.009
17	0045087	IIR	12/485	0.37	0.010
17	0061844	AHIRMAP	17/99	2.99	0.036
12	0061760	AIIR	6/18	8.62	0.045

TCA, T cell activation involved in immune response; NKCA, natural killer cell activation involved in immune response; IRRSP, immune response-regulating signaling pathway; HIR, humoral immune response; PRIR, positive regulation of immune response; RISP, regulation of immune system process; IIR, innate immune response; AIR, adaptive immune response; AHIRMAP, antimicrobial humoral immune response mediated by antimicrobial peptide; IIRM, innate immune response in mucosa; AIIR, antifungal innate immune response.

Candidate X genes in lupus

X chromosome genes that escape X inactivation; and, thus have expression of the gene from each of X chromosome, are candidates to mediate the X chromosome dose effect. Our attention was drawn to two genes in the toll like receptor 7 (TLR7) pathway that routinely escape X inactivation; namely, *TLR7* itself and *TASL* (TLR Adaptor Interacting With Endolysosomal SLC15A4). The TLR7 pathway is critical for the pathogenesis of SLE, both in murine models and humans. For instance, rare gain-of-function TLR7 mutations can cause monogenic pediatric SLE (27–29) and mice with TLR7 over-expression due to a translocation between the X and Y chromosome develop a lupus-like illness (30, 31). The TLR7 protein is localized to the endosome and is critical for recognition of viruses and subsequent activation of the innate immune system. TLR7 binds single-stranded RNA or metabolites thereof, which activates the pathway, leading to production of interferon as well as other cytokines (32). Furthermore, common population variants of genes encoding protein that function in the TLR7 pathway show genetic association to the SLE phenotype. These include TLR7, TASL, SLC15a4 (a binding partner of TASL (33)), and UNC93B1, a regulator of TLR7 movement into the endosome (34–37). Many functional studies also implicate the TLR7 pathway in SLE pathogenesis in both human and murine lupus models (30, 31, 38–43).

Given the critical nature of the TLR7 pathway in SLE and the association of X chromosome number with the sex bias of the disease, we elected to study the role of TASL in the TLR7 pathway. As described above, the TASL gene routinely escapes X inactivation and TASL is expressed in several immune cells, including B lymphocytes and monocytes, contains an SLE risk allele (19, 35) and binds SLC15A4 on the lysosomal surface (44). SLC15a4 regulates lysosomal pH, to which TLR7 signaling is highly sensitive (45, 46). In addition, knockout of the gene is known to abrogate TLR7 signaling (47).

Given these data, we undertook studies to examine the role of TASL in the TLR7 pathway (48). In particular, since TASL and SLC15a4 are binding partners and SLC15a4, at least in part, determines lysosomal pH, we studied lysosomal pH. First, we examined expression of the TASL protein in human primary monocytes, B cells and lymphoblastoid cells lines. In each case, TASL was expressed more highly in female cells compared to male cells (49). Additional studies from Odham et al, also found TASL was more highly expressed in female cells and this sexual dimorphism was magnified when stimulated with type I interferons (50). Using a ratiometric measurement of lysosomal pH via fluorescence in unstimulated female monocytes, we found lysosomal pH averaged 4.9 versus 5.6 in male cells (p=0.0001) (48). A similar difference in lysosomal pH was also found between male and female B cells and dendritic cells, while we did not find a female: male dichotomy for lysosomal pH in NK or T cells, neither of which express TASL (48). Thus, the sex difference in lysosomal pH is likely to be associated with increased TLR7 signaling, and may be dependent upon increased expression of TASL in female cells.

In order to determine if, in fact, TASL participates in lysosomal pH regulation and TLR7 signaling, we undertook a series of knockdown experiments using CRISPR-Cas9 and primary human monocytes (CD14+/CD16−). In female cells treated with a TLR7 agonist, TASL knockdown abrogated interferon-alpha, IL-6 and TNF production (49). Thus, TASL is critical for TLR7 pathway signaling. Furthermore, knockdown of TASL expression resulted in a rise in lysosomal pH in female monocytes to the pH we found in

male monocytes. And, intracellular transport of NOD1 antigens, a function of SLC15a4, was also abrogated by TASL knockdown (49). However, it should be noted that these results have not been independently replicated; and, thus, are not confirmed.

Several other lines of evidence support a sex-biased function of the TRL7 pathway (51–53). Our studies in primary monocytes and LCLs suggest TASL is involved in the TLR7 in a sexually dimorphic manner such that lysosomal pH is lower and TLR7 signaling greater in female versus male cells. As of late, studies on TASL have shown that the once uncharacterized protein functions as enzyme that regulates interferon regulatory factor 5 (IRF5), colocalizes with TLR7 and is interferon inducible. TASL ability to increase interferon production (our work and others) and its own protein level to be subsequently amplified by interferon stimulations suggest a positive feedforward response that would result in increased production that is often found in SLE affected subjects. Thus, increased expression of both TLR7 (54) and TASL (48, 49) may underlie not only improved outcome of women compared to men in some infections (55) but also female disposition to autoimmunity mediated via TLR7 (56).

XIST in lupus

Other investigators have taken a different tack in studying the role of the X chromosome in the sex bias of SLE (57, 58); however, the data generated also concern the TLR7 pathway. As mentioned above, XIST long non-coding RNA mediates X chromosome inactivation (Figure 1); and, thus, is expressed only in cells with more than one X chromosome. Dou and colleagues preformed a series of experiments that indicate XIST is a source of ligand for TLR7; and, of course, this is a sex specific source of ligand (57, 58).

First, these investigators noted that XIST is rich in potential TLR7 ligands. A putative TLR7 stimulatory motif, the UU dinucleotide, was found 2,140 times in XIST RNA. XIST was the sex-biased transcript with the highest degree of UU dinucleotide gene expression; and,

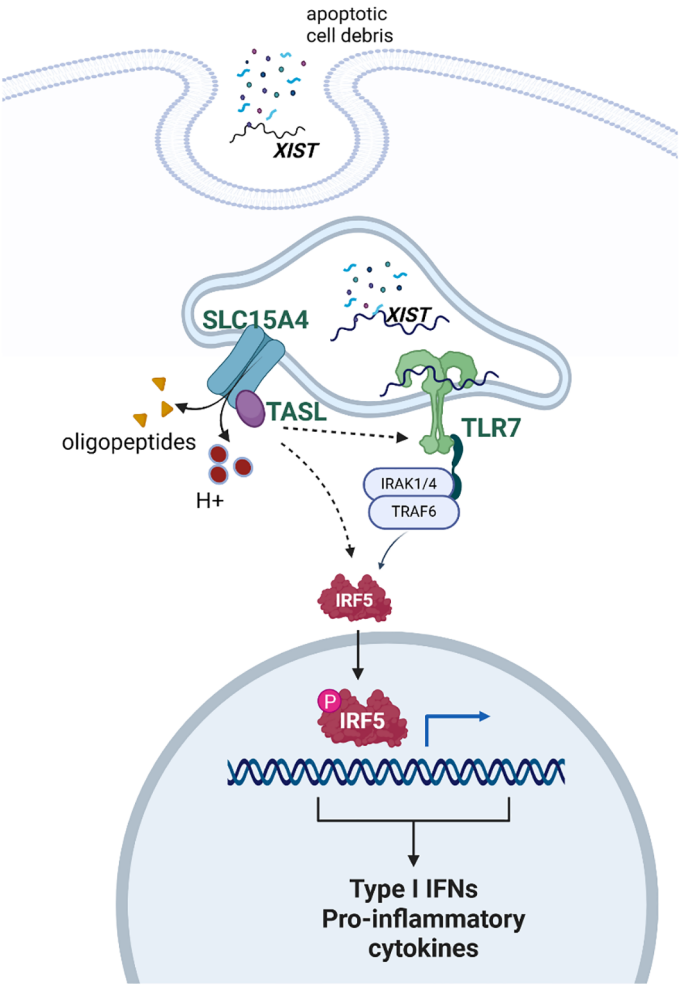

FIGURE 2
Schematic depicting the proposed interaction of XIST, TLR7, and TASL in response to self-antigen. XIST provides ligand for TLR7. Once TLR7 signaling is activated, there is a feed forward stimulation of the pathway. The genes for both TLR7 and TASL are on the X chromosome and escape X inactivation. Thus, some data suggest that TRL7 signaling is more robust in female cells, compared to male cells, on this basis. Created with Biorender.com.

further, was the only sex-biased expression source of the extended TLR7 motif 5′-GUCCUUCAA-3′ (57, 58). Overall, XIST was the strongest sex biased source of self TLR7 ligand.

Next, these investigators turned to stimulation of TLR7 by XIST nucleotides using HEK-hTLR7 cells as a reporter. The extended TLR7 motif found in XIST as well as a longer sequence of XIST (containing the A-repeat, UU dinucleotide rich region) were also found to stimulate TLR7 signaling as indicated by production of interferon-alpha. Further, not only was the response due to specific binding of XIST nucleotide and dose-dependent, the TLR7 response was inhibited by depletion of XIST as well by hydroxychloroquine (57). Additional studies found that XIST levels were higher in peripheral leukocytes among women with SLE compared to non-SLE affected matched controls, and that levels of XIST correlated with disease activity. The investigators concluded, and we certainly agree, that the XIST long non-coding RNA is the most potent source of sex biased TLR7 ligands in female cells.

XIST, TLR7, TASL in lupus and other autoimmune diseases – an hypothesis

We further conclude that these two sets of data suggest synergism for a female biased expansion of the TLR7 signaling pathway that could underlie the X chromosome dose effect found in various autoimmune diseases, including SLE (12–15), Sjögren's disease (13, 16), polymyositis/dermatomyositis (17), and systemic sclerosis (17). The idea, we think, is straight forward. XIST RNA supplies TLR7 ligand in female cells. In addition, female B lymphocytes, dendritic cells, and monocytes have enhanced TLR7 pathway signaling by virtue of the over-expression (compared to male cells) of not only TLR7 but also TASL. Enhanced TLR7 signaling activity deploys a feed forward loop in the TLR7 pathway that leads to increased expression and activity of the pathway (59). Thus, both increased ligand and enhanced activity support further enhancement of TLR7 signaling in female cells. Of course, these phenomena are universal in cells with more than one X chromosome; that is, from women or Klinefelter men. So, other factors must be in play such as other genetics or environmental exposure.

TLR7 signaling and environmental triggers in lupus

What environmental exposure might interact with this sex-biased enhancement of TLR7 signaling induced by Xist and genes in the TLR7 pathway that escape X inactivation? One candidate is Epstein Barr virus (EBV). Epidemiological evidence supports the idea that this near ubiquitous infection is necessary but not sufficient for the expression of SLE as well as multiple sclerosis, and there some evidence in Sjögren's disease (60–65). Recent studies have found that single nucleotide polymorphisms demonstrating genetic association with SLE or Sjögren's disease are more likely to be found in promoter regions bound by the EBV transcription factor EBV nuclear antigen 2 (EBNA2) (66, 67). Overall, the preponderance of evidence indicates that EBV infection is likely one of the environmental triggers for disease. Furthermore, EBV infects B lymphocytes, a cell type with expression of TASL, engaging and increasing expression of TLR7 (68). B cell hyperplasia is one of the hallmarks of systemic autoimmune disease (69). Thus, these data concerning enhanced expressed XIST, TLR7 and TASL in female cells impacting TLR7 signaling may interact with data concerning a role of EBV in promoting SLE and other autoimmune diseases (62, 63, 70). Of course, estrogen and differential expression of estrogen-regulated genes remain a potential biological trigger of the disease. The sex bias of SLE is present in prepubescent children at about 5 to 1, but of course is less pronounced than after puberty (71). These data suggest an effect of estrogen. Further, there are clear effects of estrogen on B lymphocytes and humeral immunity (72, 73) with effects on development, immune tolerance, immunoglobulin somatic hypermutation, and class switching. In addition, some estrogen effects in B cells may be mediated through cell surface (as opposed to nuclear) estrogen receptors (74).

Summary

The evidence is strong that the number of X chromosomes is important for the female bias of some, but not all, autoimmune diseases. The mechanism by which a dose effect for the X chromosome is not understood. Available evidence suggests that multiple factors may play roles that are complementary. These include expression of XIST, which provides TRL7 ligand, and escape of X inactivation by genes whose protein products are critical for TLR7 signaling (see Figure 2).

Author contributions

RHS: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. JW: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. VL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1479814/full#supplementary-material

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Cancer in connective tissue disease

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The association between cancer and autoimmunity is well-recognized, as represented by the increased incidence of cancer among patients with systemic autoimmune diseases; however, the underlying mechanisms remain only partially understood. On the one hand, malignancy may trigger a breakdown of immune tolerance in predisposed individuals, as autoimmune syndromes often emerge shortly after cancer diagnosis, suggesting that tumor antigens might initiate an autoimmune response. However, by involving persistent responses and the creation of a pro-inflammatory environment, the chronic immune activation characteristic of autoimmunity may promote oncogenesis. This scenario is further complicated by the use of immunosuppressive therapies for autoimmune conditions, which, as seen in transplant immunology, are associated with a higher risk of cancer, although data in rheumatology have not yielded definitive conclusions. Connective tissue diseases include systemic lupus erythematosus, primary Sjögren syndrome, idiopathic inflammatory myopathies, systemic sclerosis, mixed connective tissue disease, and undifferentiated forms. These conditions have been variably associated with an increased risk of cancer, both at the time of disease onset and in patients with long-standing autoimmune conditions, providing a paradigm for investigating this complex interplay. Despite recent progress, many unmet needs remain that warrant further research.

KEYWORDS

malignancy, immunology, autoimmunity, autoantibodies, connective tissue disease (CTD)

Why cancer and connective tissue disease

The relationship between malignancy and autoimmunity is well established, as supported by the increased incidence of cancer in patients with autoimmune diseases (1); however, several questions remain unanswered regarding the fundamental mechanisms of this association and their translation into clinical practice. In line with the established pathogenic model of autoimmune diseases, malignancy may trigger the breakdown of tolerance in predisposed individuals (2). This is illustrated by the occurrence of autoimmune syndromes, often with distinctive features, in close temporal proximity to cancer diagnosis (3). On the other hand, autoimmunity may serve as a fertile ground for the

development of malignancy, possibly due to persistent immune activation against autoantigens and the setting of a pro-inflammatory milieu, thus acting as a precancerous condition (4). Furthermore, autoimmune diseases are often treated using immunosuppressive therapies. While evidence from transplant immunology indicates that immunosuppression increases the risk of cancer (5), data are inconclusive when it comes to rheumatology and clinical immunology (6).

Connective tissue diseases (CTDs) are classic forms of systemic autoimmune disorders, including systemic lupus erythematosus (SLE), primary Sjögren syndrome (pSS), idiopathic inflammatory myopathies (IIM), systemic sclerosis (SSc), mixed connective tissue disease (MCTD), and undifferentiated forms (UCTD) (7–12). These diseases are characterized by unique clinical features and pathogenic mechanisms but also share a female predominance, overlapping clinical manifestations (e.g., arthralgia and arthritis, fatigue, interstitial lung disease, myositis, and Raynaud's phenomenon) (7–12), and similar immunological pathways (e.g., type I interferon activation, B-cell infiltration, activation, and proliferation) (13, 14). Within this shared framework, an increased risk of malignancy has frequently been reported across CTDs, reflecting the intricate interplay between cancer and autoimmunity (Figure 1). We speculate that some entities reflect the causal relationship of autoimmunity as a paraneoplastic phenomenon, as seen in cancer-associated myositis (CAM) or -scleroderma, where the temporal closeness between the two diagnoses is linked to peculiar environmental and pathophysiological changes (15). In other scenarios, subclinical chronic inflammation may constitute a precancerous condition contributing to the development of cancer-associated mutations and malignancy late in disease history (16, 17).

By evaluating the spectrum of CTDs, we present a critical analysis of the relationship between cancer and autoimmunity, with a focus on clinical associations, relevance of serum autoantibodies, impact of disease-specific risk factors, and role of immunosuppressive therapies. Different scenarios will be presented to support the proposed concept that certain CTDs can represent a paraneoplastic phenomenon, whereas the onset of malignancy is observed more frequently in specific longstanding CTD-related contexts. To ensure a consistent approach, similar sections will be summarized for different diseases. However, there are major differences in the available evidence, and considering that our work aims to provide a critical review of the state of the art while identifying clinical and research needs, the content of certain sections will need to be heterogeneous and vary from one condition to another. This is particularly evident in the section on immunological features, which lacks a uniform distribution in myositis and SSc compared to pSS and SLE. Table 1 summarizes the unmet needs in the management of malignancy in patients with CTDs and outlines a contextual research agenda based on the discussions presented throughout the text.

Methods and search strategy

We conducted a comprehensive critical review by searching PubMed for "idiopathic inflammatory myopathies," "systemic sclerosis," "Sjogren Disease," "systemic lupus erythematosus," and "cancer." The search focused on articles published in English from January 2010 to October 2024 and yielded 3,652 results. Papers of key relevance published outside of this period were included if they focused on relevant findings and approaches that could have influenced subsequent publications. Thus, 196 papers were included in the final review. A balanced discussion was provided by including studies that supported or challenged our perspective, ensuring a comprehensive and evidence-based analysis. Multiple reviewers (AT, AC, EG, and SC) independently evaluated the included studies; their interpretation was discussed by the full author panel to minimize bias and reach consensus, and different viewpoints were considered during the synthesis of the results. Owing to the heterogeneity of study designs, patient populations, and outcome measures, which made direct comparisons

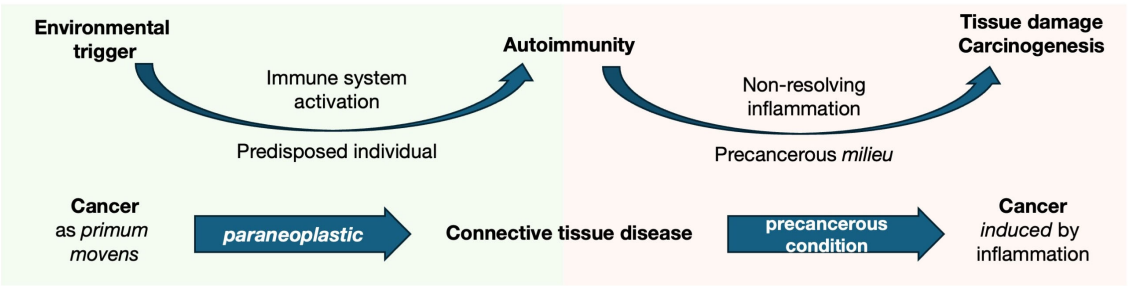

FIGURE 1
Cancer as both an environmental trigger and pathological consequence of autoimmunity in the paradigm of CTDs. The pathogenesis of autoimmune diseases involves a hypothetical environmental trigger that induces immune system response. In genetically predisposed individuals, this leads to an aberrant immune activation, which becomes dysregulated and persists over time, resulting in chronic inflammation. The chronic inflammatory milieu causes tissue damage due to ongoing inflammation but synchronously provides a precancerous condition (i.e., an environment that predisposes to the development of cancerous lesions). From this perspective, CTD are at a crossroads between cancer and autoimmunity. On the one hand, strong evidence supports the role of cancer as a trigger of autoimmune responses (as seen in cancer-associated myositis and scleroderma). However, the disease itself increases the risk of malignancies, particularly in tissues undergoing chronic inflammatory remodeling (such as the lung in SSc and lymphopoiesis in pSS).

TABLE 1 The unmet needs and research agenda in cancer management in patients with CTDs.

	IIM	SSc	pSS	SLE
Risk assessment	Can we measure the risk of cancer in new-onset IIM? Can we further stratify the risk in patients with specific phenotypes? (e.g., different autoantibodies associated with DM, ASyS) Can we better stratify patients at high risk according to disease phenotypes and autoantibodies? (e.g., anti-TIF1γ DM who do not develop cancer) Can we assess the risk of IIM in patients newly diagnosed with cancer?	Can we measure the risk of cancer in new-onset SSc? Can we measure the risk of cancer in longstanding SSc? What is the contribution of SSc to this risk? Which sites are the most involved? **In cancer-associated scleroderma**: what are the risk factors beyond anti-POLR3+ dcSSc? **Cancer-associated scleroderma**: which anti-POLR3+ patients will not develop cancer? **Late-onset cancer in SSc**: which patients should be thoroughly and repeatedly screened?	**Lymphoma**: can we measure or score the risk of developing lymphoma in pSS? Can we predict the time-to-lymphoma interval in pSS? Will any novel autoantibody provide more insights in estimating the risk of lymphoma in SSc? **Non-lymphoma**: Is pSS a risk factor for solid neoplasms? Which are the most common neoplasms? What are they associated with?	Which disease categories and phenotypes are at high vs. low risk of cancer? Can we identify any patient cluster? What is the timing of cancer onset in patients with SLE? Which autoantibodies are associated with cancer in patients with SLE, if any? Are overlap diseases (pSS, thyroiditis, autoimmune hepatitis or cholangitis) a concern in patients with SLE? Is elderly-onset SLE a risk factor for cancer?
Screening	How long and how often should patients with IIM be screened for malignancy? Should patients receive long-term screening for specific cancers in case of select internal organ involvement (e.g., ILD)? Does this apply to all IIM patients independently from the risk estimated according to the IMACS guidelines?	**Cancer-associated scleroderma**: How long and often should we screen patients? How should we screen patients? **Longstanding SSc:** When should we start screening patients? How often should we screen them? For which cancers should we screen them? Which diagnostic tests should be used and how should they be used? Which age, if any, should we start or stop searching for cancer in SSc?	**Lymphoma**: How should we screen patients? Which sites should be screened? When should we start screening patients? How often should patients be screened? **Non-lymphoma**: Should patients with pSS be offered a dedicated cancer screening because of specific risk factors?	Should patients with SLE be offered a dedicated cancer screening because of specific risk factors? In patients with overlapping CTDs, should patients follow the same screening procedures according to the overlapping entity?
Treatment	Does immunosuppressive treatment increase the risk of cancer in patients with CTDs? What is the correct management of immunosuppressive therapies in CTD patients with newly diagnosed malignancy? To what extent does disease activity enhance the risk of cancer in patients with CTDs? Which immunosuppressive treatments contribute to reduce vs. increase such risk by controlling disease activity?			

anti-POLR3, anti-RNA polymerase III autoantibodies; ASyS, antisynthetase syndrome; CTDs, connective tissue diseases; DM, dermatomyositis; IIM, idiopathic inflammatory myopathies; ILD, interstitial lung disease; IMACS, International Myositis Assessment and Clinical Studies Group; pSS, primary Sjogren Syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

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challenging, a narrative approach was adopted instead of a systematic review. To ensure a broad and speculative perspective on the topic, rigid predefined inclusion and exclusion criteria were not applied. However, studies included were original peer-reviewed research articles, systematic reviews, and meta-analyses. Case reports and small case series were considered only when they provided unique insights into novel clinical associations. Non-peer-reviewed sources and studies were excluded to maintain the robustness of the analysis.

Cancer and idiopathic inflammatory myopathies: the key role of synchronous malignancy

The heterogenous family of IIM encompasses dermatomyositis (DM), polymyositis (PM), antisynthetase syndrome (ASyS), immune-mediated necrotizing myopathy (IMNM), inclusion body myositis (IBM), juvenile inflammatory myositis, and paraneoplastic myositis or CAM (10, 18, 19). CAM is defined as a malignancy occurs within three years from the onset of myositis in adult patients (20, 21), and the risk of developing CAM varies according to the disease phenotype and the presence of selected myositis-specific autoantibodies (MSA) (22–24). Since the earliest reports dating back to 1916 (25), several studies have confirmed a strong link between cancer and IIM, particularly with DM and in the presence of autoantibodies targeting transcription intermediary factor 1γ (TIF1-γ) and the nuclear matrix protein 2 (NXP2) (26, 27).

Clinical features of paraneoplastic myositis

DM is the most common IIM clinical phenotype associated with the risk of CAM, presenting as heliotrope rash, Gottron's sign, or papules (28, 29). Patients with inclusion body myositis and ASyS do not seem to have an increased risk of malignancy (26, 30), even when presenting with signs of DM (30), whereas the risk remains unclear in subjects diagnosed with IMNM (31). In addition to the diagnosis of DM, risk factors for CAM include older age at IIM onset, male sex, smoking history, signs of cutaneous necrosis (32), dysphagia (33), rapidly progressive disease, and elevated inflammatory markers (34–37). Histological features on muscle biopsy, such as minimal lymphocytic infiltration, should also raise suspicion for CAM (38) while interstitial lung disease, arthritis, and Raynaud's phenomenon correlate with a lower risk of malignancy (34, 36, 37). Different types of malignancies have been reported with CAM, most commonly solid neoplasms, which seem to reflect the incidence observed in the general population. For instance, a large cohort from Northern Europe reported a high risk of ovarian, gastric, colorectal, and pancreatic cancers, and non-Hodgkin's lymphoma (NHL) (39). In contrast, nasopharyngeal carcinoma was confirmed as the most common neoplasm diagnosed in patients with IIM in the Taiwanese population, followed by lung, breast, and hepatic malignancies (40, 41). Moreover, slight differences in the type of incident neoplasms have been hypothesized by comparing patients with CAM according to the clinical phenotype, i.e., DM vs. PM (39). These differences warrant further investigation across different clinical subsets and ethnicities (Table 1).

Immunological features of paraneoplastic myositis

The immune pathogenesis of CAM involves several complex mechanisms, including the presence of shared antigens between tumor cells and normal tissues, molecular mimicry, and exposure to neo-self-antigens (42). These can be presented to tumor-infiltrating lymphocytes through class I (CD8+ cells) and class II (CD4+ cells) HLA complexes. This process leading to lymphocyte activation may result able to provide cancer elimination; on the other hand, activated lymphocyte may cross react with self-antigens and pathologically infiltrate normal tissues (e.g., skeletal muscle, skin), leading to inflammation and damage (42–44).

Serum autoantibodies, including both myositis-specific (MSA) and myositis-associated (MAA) autoantibodies, are of major use in the diagnosis of IIM and correlate with the development of particular manifestations among different clinical subsets (22). Most importantly, the presence of autoantibodies can further stratify patients with IIM according to cancer risk, as summarized in Table 2.

While malignancies often occur in association with DM, a 2012 meta-analysis including 312 adult patients with DM found that 80% of DM patients with cancer were anti-TIF1-γ-positive, whereas only 10% without cancer had this autoantibody (45). Overall, among patients with DM, the presence of anti-TIF1-γ autoantibodies had a positive predictive value for CAM of 58% and a negative predictive value of 93% (45). These findings were confirmed in another large cohort study, particularly raising concern for breast and ovarian neoplasms (26), and in an up-to-date meta-analysis (34). Moreover, it seems that the risk of cancer significantly increases in patients displaying high anti-TIF1-γ autoantibody titers, specifically in patients with the IgG2 isotype, compared with their respective counterparts (46, 47). TIF1-γ, also known as TRIM33, is an enzyme involved in post-translational peptide modifications, an E3-ubiquitin ligase and being involved in small ubiquitin-like modifications (SUMO). In particular, TIF1-γ has been demonstrated to participate in cell cycle regulation, DNA repair, and the regulation of TGF-β signaling (44). Alterations in the TIF1-γ gene have been described in cancer cells from patients with CAM, possibly representing the neo-self and thus triggering the anti-cancer immune response, which can culminate in autoimmunity to native TIF1-γ antigens (48). As a proof of concept, high expression of TIF1-γ has been observed in the skin and skeletal muscle, which represent the main targets of anti-TIF1-γ DM compared to other tissues (49, 50). Recently, the role of anti-TIF1-γ as a risk factor for synchronous cancer in DM patients has been redefined. Indeed, the coexisting immune response against autoantigens, such as Sp4 and CCAR1, would reduce the risk of cancer, perhaps accounting for a more robust antitumor immunological response (51–53). Further

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TABLE 2 Myositis-specific and -associated autoantibodies, associated phenotypes and current risk of cancer in IIM patients.

Autoantibody	Target molecule and function	Clinical phenotype	Clinical associations	Cancer risk
anti-TIF1γ/α	Transcription intermediary factor 1γ/α—transcriptional elongation, DNA repair	DM, JDM	DM, no ILD	High
anti-MJ/NXP2	Nuclear matrix protein-2—transcriptional regulation and activation of the tumor suppressor p53	DM, JDM	DM, calcinosis, subcutaneous edema, severe myopathy, dysphagia	High
anti-SAE	Small ubiquitin-like modifier 1 activating enzyme—post-translational modifications	DM	Severe cutaneous disease, dysphagia, systemic symptoms, mild myopathy, mild ILD (50%)	Intermediate
Anti-PUF60 (FIRs)	poly-U-binding factor protein	DM, pSS	Less ILD; in pSS frequently with Ro60, Ro52, La	Intermediate-High (200)
Anti-HMGCR	HMG-CoA reductase—rate-limiting enzyme for cholesterol synthesis	IMNM (statin-induced myopathy)	Necrotizing myopathy	Intermediate
anti-Jo-1	Histidyl-tRNA synthetase	ASyS	Classic ASyS with frequent muscle involvement	Standard
anti-PL-7	Threonyl-tRNA synthetase	ASyS	Severe ILD	Standard
anti-PL-12	Alanyl-tRNA synthetase	ASyS	May present with ILD only	Standard
anti-EJ	Glycyl-tRNA synthetase	ASyS	ASyS, ILD (with anti-Ro52)	Standard
anti-OJ	Isoleucyl-tRNA synthetase	ASyS	ASyS (severe myositis), ILD	Standard
anti-KS	Asparaginyl-tRNA synthetase	ASyS	CADM, ILD, overlap subset with *sicca*	Standard
anti-ZO	Phenylalanyl-tRNA synthetase	ASyS	Classic ASyS, rare (<1% ASyS)	Unknown
anti-YRS (Ha)	Tyrosyl-tRNA synthetase	ASyS	ASyS, rash, arthritis, rare	Unknown
anti-KJ	Translocation factor	ASyS-like	Rare	Unknown
anti-MDA5/IFIH1	Melanoma differentiation-associated gene 5—innate immune responses against viruses	DM, JDM	CADM, severe ILD, peculiar skin involvement (reverse Gottron, vasculitis, ear lesions), mechanic's hands, MIP-C	Intermediate
anti-TIF1-β	Transcription intermediary factor 1β—regulation of gene expression and chromatin structure	DM	CADM, no ILD	Unknown
anti-Ku	Heterodimer complex of 2 subunits that binds to free DNA termini—DNA repair, transcription regulation	SLE, SSc, MCTD, PM	Raynaud, arthralgia, myopathy, overlap with other connective tissue diseases	Standard
Anti-SRP	Signal recognition particle—co-translational translocation of proteins across the endoplasmic reticulum	IMNM	Necrotizing myositis, myocarditis, low ILD	Standard
anti-PM/Scl	complex of 100 KDa and 75 KDa—processing and degradation of RNAs	PM, DM, SSc, PM/SSc overlap, SLE	ASyS-like (myositis, Raynaud, arthritis, ILD, mechanic's hands)	Standard
anti-Mi-2	helicase of the nucleosome remodeling deacetylase—transcriptional regulation	DM	Classic DM (no ILD)	Standard
Anti-cN-1A	Cytosolic 5'-Nucleotidase 1A protein – nucleotide hydrolysis	IBM	Bulbar muscle weakness, wrist flexor involvement	Unknown
Anti-FHL1	Four-and-a-Half LIM domain 1—intracellular protein–protein interactions mainly with cytoskeletal proteins	DM, PM	Severe myositis, dysphagia, vasculitis	Unknown
Anti-RuvBL1/2	Ruv BL1/2 double hexame—DNA repair, chromatin remodeling, gene transcription	SSc, PM	Higher age at onset, men, diffuse SSc and myositis overlap, GI dysmotility, myocarditis	Unknown

(Continued)

TABLE 2 Continued

Autoantibody	Target molecule and function	Clinical phenotype	Clinical associations	Cancer risk
anti-SMN	Survival of motoneuron complex—transcriptional regulation and small nuclear RNP formation	MCTD, PM	MCTD with clinical features of all components of SLE, SSc and IIM; high prevalence of PAH and ILD	Unknown
anti-Nup	Nucleoporins	Not known	Myositis, ILD, Raynaud	Unknown

Cancer risk is reported as 'high' (i.e., increased compared to same-age general population), 'intermediate', or 'standard' (i.e., not different to same-age general population), according to the recent International Myositis Assessment and Clinical Studies Group (IMACS) guidelines (36). Otherwise, for rarer or novel autoantibodies, an estimate of the risk of cancer is given according to the references in the Table, linked to observational cohort studies, whereas 'Unknown' risk is reported if little (e.g., case reports, small case series) or no evidence showing cancer association is available.

ASyS, antisynthetase syndrome (myositis, ILD, polyarthritis, Raynaud's phenomenon, mechanic's hands and the presence of an antisynthetase antibody); CADM, clinically amyopathic/hypomyopathic DM; DM, dermatomyositis; GI, gastrointestinal; IBM, inclusion body myositis; ILD, interstitial lung disease; IMNM, immune-necrotizing myopathy; JDM, juvenile dermatomyositis; MCTD, mixed connective tissue disease; MIP-C, MDA5-associated autoimmunity and interstitial pneumonitis contemporaneous to the COVID-19 pandemics; PAH, pulmonary arterial hypertension; PM, polymyositis; pSS, Sjogren syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

implementation of these observations in clinical practice is required (Table 1).

NXP2, also known as MORC3, is a nuclear protein involved in the activation of the tumor suppressor protein p53 (54), a key regulator of cell cycle and senescence. Downregulation of NXP2 has been described in different malignancies, correlating with an enhanced type I IFN signature and, most importantly, with increased expression of the immune checkpoint antigen PD-L1, which is known to suppress T-cell response by binding to the cognate receptor PD-1 (55). Autoantibodies against MJ/NXP2 have been extensively associated with the risk of cancer in IIM patients (27, 56–58), even though some large studies (59) and meta-analyses (60) failed to demonstrate an association with malignancy compared to other patient subsets. The heterogeneity of the results obtained when detecting myositis autoantibodies using different methods (59, 61) suggests that one possible explanation for this discrepancy may be the varying techniques used to identify anti-NXP2 autoantibodies across different studies (58). For instance, in one of the largest studies conducted on anti-NXP2-positive DM, the presence of these autoantibodies was confirmed by immunoprecipitation in only 62% of the patients who tested positive using commercial line blots (59).

Recent studies have reported the risk of malignancy in patients with other rare serum autoantibodies. A higher incidence of cancers was observed with anti-SAE, a hallmark of erythrodermic DM (62–64), with malignancies diagnosed also many years after the onset of myositis in an American cohort (65). SAE1 is a subunit of the E1 complex constituting a SUMO activator protein that plays crucial roles in the activation of type I IFN synthesis but is also involved in tumorigenesis (66). For instance, overexpression of SAE1 has been observed in different types of cancers, correlating with a higher disease burden, metastatic disease, and worse prognosis (67–69). Concerning IMNM, it has been suggested that the risk of developing malignancies increases only in seronegative forms (70, 71), despite some reports suggesting a slightly higher rate in subjects with anti-HMGCR (71–73). Nevertheless, other autoantibodies, namely anti-Ku and anti-Mi-2, have been confirmed not to harbor any increased risk of malignancy in patients with IIM (22, 34, 74). Rare and novel MSA have been identified in short reports of small IIM cohorts, but their association with cancer is still unknown and needs to be studied more extensively in larger cohorts worldwide (Table 1). For instance, this is the case with anti-FHL1 (75), anti-RuvBL1/2 (76–78), anti-Nup (79), and anti-SMN (80, 81) autoantibodies, which have been identified in small subsets of IIM patients, as well as in SSc and MCTD.

Cancer screening in IIM: the IMACS initiative

In 2023, the International Guideline for Idiopathic Inflammatory Myopathy-Associated Cancer Screening was released by the International Myositis Assessment and Clinical Studies Group (IMACS) (36) to provide guidance on the management of patients with suspected CAM. These guidelines enable the stratification of each patient with new-onset IIM into a 'standard,' 'moderate,' or 'high' risk of malignancy, by combining the clinical features, autoantibody status, and demographic factors such as age and sex. For instance, patients should be considered at high risk if they meet at least two of the following criteria: DM phenotype, positivity for anti-TIF1-γ or anti-NXP2, age >40 years at the onset of IIM, persistent high disease activity despite therapy, dysphagia, and cutaneous necrosis. Second, the guidelines outline a 'basic' and an 'enhanced' screening panel to be performed in a tailored manner in patients with IIM, according to their previously established cancer risk.

Therefore, all patients with IIM should participate in country- or region-specific age- and sex-appropriate cancer screening programs regardless of their individual cancer risk. Additionally, basic or enhanced screening panels should be conducted at the time of diagnosis. The 'basic screening panel' should include comprehensive history taking and physical examination, routine laboratory investigations (i.e., complete blood count, liver function tests, acute phase reactants, serum protein electrophoresis, and urinalysis), and chest X-ray. Instead, the 'enhanced screening panel' includes total body CT scan, cervical screening, mammography, dosage of the prostate-specific antigen or CA-125 (while other neoplastic markers are not recommended for general screening), pelvic or transvaginal ultrasonography, and search for fecal occult blood. Additional screening with ^{18}FDG-PET/CT and upper and lower gastrointestinal endoscopy should be considered in selected patients, based on clinical evaluation.

When evaluated in retrospective cohorts, these recommendations displayed excellent sensitivity in identifying patients with malignancy but with lower specificity. Indeed, most patients with IIM were classified as high or intermediate risk of cancer, with only a minority of subjects being represented in the standard-risk group. The ability of these guidelines to detect patients developing long-term cancers seems comparable to their effectiveness in identifying malignancies occurring close to the onset of IIM (82, 83). Further multicentric, long-term cohort studies are needed to evaluate the application of the IMACS guidelines for cancer screening and their impact on follow-up strategies (Table 1). Additionally, there is a recognized need to incorporate emerging evidence on novel risk factors to improve patient stratification (Table 1), particularly concerning serum autoantibodies, as outlined in Table 2.

Cancer and Sjogren syndrome: a model of autoimmunity-induced malignancy

PSS is a chronic autoimmune disease characterized by lymphocytic infiltration of exocrine glands, leading to glandular dysfunction and development of systemic manifestations (9). In patients with pSS the overall risk of cancer is higher compared to the general population, with an estimated standardized incidence ratio (SIR) of 2.17 (95% confidence interval—CI 1.57–3.00) (84).

Clinical features of cancer in pSS

Hematological malignancies are the most frequent life-threatening complication of pSS, with one-third of cancers being B-cell lymphomas (85). Among these, NHL is the most frequently reported, with an SIR of 13.71 (95%CI 8.83–21.29) (84), reflecting a seven to 15 times higher incidence compared with the general population (86). Although autoimmunity-promoting lymphoma is frequently observed in autoimmune diseases, this association is highly expressed in patients with pSS. Mucosal-associated lymphoid tissue (MALT) lymphoma constitutes the majority of pSS-associated NHL cases (up to 65%) and mainly originates from the salivary glands. However, additional mucosal sites can be affected, including the stomach, thyroid gland, and lungs (85). In MALT-NHL, lymphomagenesis represents the last stage of the persistent polyclonal activation of marginal zone B cells. In pSS, this activation can evolve into monoclonality, typically resulting in low- or intermediate-grade lymphomas.

In recent years, efforts have been made to identify clinical features and serological biomarkers that predict the development of MALT lymphoma in patients with pSS. Data from the HarmonicSS cohort identified positive serum rheumatoid factors as the earliest and most persistent independent predictor of lymphoma. Simultaneously, B-cell manifestations (including cryoglobulinemia and glandular, cutaneous, and hematological manifestations) appear to signal a more advanced stage in the lymphomagenesis process (87). Additional biomarkers predictive of a higher risk of NHL development have also been identified, including leukopenia, low complement C4 levels, and presence of anti-La/SSB autoantibodies (88). Major salivary gland enlargement and salivary gland focus score evaluated at the time of diagnosis have also been established as independent risk factors for lymphoma in patients with pSS. In particular, a shorter time interval from pSS to lymphoma has been described with an increasing focus score (89), highlighting the importance of histological evaluation in these patients.

A higher risk of hematologic malignancies, other than lymphoma, has been reported in patients with pSS. In these patients, the detection of monoclonal gammopathy of undetermined significance (MGUS) is common, and as a result, the documented higher prevalence of multiple myeloma is not surprising. The risk of MGUS seems restricted to patients with anti-Ro/SSA and anti-La/SSB autoantibodies (90); however, studies on its evolution to multiple myeloma are limited. Thus, further epidemiological investigations are required to precisely determine the incidence and prevalence of this complication in patients with pSS.

Solid cancers were also more frequently observed in patients with pSS (SIR 1.39). In particular, an association between thyroid and other ENT cancers, nonmelanoma skin cancer, hepatocellular carcinoma, lung cancer, prostate carcinoma, kidney, and urothelial cancers has been reported (84). Among these, thyroid cancer is the most frequently recognized, with a 2.6 SIR reported in a pSS cohort of over 7,000 patients (91). These data were confirmed by Britton Zeron et al., who described thyroid cancer as the most common solid tumor in pSS after hematological neoplasms (SIR 5.05) (92). The explanation for this association remains unclear. However, considering that the risk of developing thyroid cancer is higher in patients with autoimmune thyroiditis (93), and that autoimmune thyroiditis is one of the most frequent comorbidities in pSS (94), it is reasonable to hypothesize that the co-occurring autoimmune disease affecting the thyroid might contribute to the development of this neoplastic manifestation.

Current evidence on the established and putative risk factors for malignancy in patients with pSS is summarized in Supplementary Table 1.

Immunological features of cancer in pSS

MALT lymphoma is thought to result from local antigen-driven B-cell selection within tertiary lymphoid structures (TLS), which are typically referred to as ectopic germinal centers (GCs). It is now recognized that during pSS, ectopic GCs form in the minor salivary and/or parotid glands of approximately 30%–40% of patients (95). Since these structures host crucial phenomena, such as oligoclonal B cell expansion and somatic hypermutation of Ig variable genes (96), ectopic GCs are currently considered the 'beating heart' of the autoimmune reaction (97). However, despite these functions, the association between ectopic GC formation and lymphoma development remains unclear. While some studies have indicated

that the presence of ectopic GCs in minor salivary gland biopsies is a risk factor for NHL lymphoma development (98, 99), more recent studies have not confirmed their predictive value (100). Nevertheless, the view that ectopic GCs are markers of more active and severe diseases is widely accepted (101). Peripheral biomarkers associated with ectopic GCs formation, such as CXCL13, have been identified (102) and are currently being used in clinical trials to monitor disease progression. Notably, elevated peripheral levels of CXCL13 appear to be associated with an increased risk of NHL, further strengthening the relationship between ectopic GC formation and hematologic malignancy development (103, 104).

Cancer screening in pSS

Lymphoproliferative disease surveillance remains a challenge in patients with pSS even after stratification according to patient risk. Recent studies have shown that patients without clinical suspicion of lymphoma or increased systemic disease activity are unlikely to benefit from major salivary gland imaging screening for detecting this complication (105). This issue is compounded by evidence of the poor reliability of salivary gland ultrasound protocols and scores in identifying lymphoma in patients with pSS and high clinical suspicion (106). It has been proposed that combining salivary gland ultrasound with histology could improve the detection of patients at the highest risk of lymphoma (106). However, evidence is still lacking regarding optimal screening strategies, imaging modalities, and timing. Efforts should also focus on detecting lymphoproliferative diseases at sites other than the major salivary glands, including both the nodal and extranodal sites. Furthermore, identifying the risk factors and screening protocols for non-lymphoproliferative neoplasms should also constitute a priority in the research agenda (Table 1).

Cancer and systemic sclerosis: a unique scenario for both malignancy-induced autoimmunity and autoimmunity-induced malignancy

Systemic sclerosis (SSc) is associated with an increased risk of malignancy, with cancers being diagnosed at a significantly younger age compared to the general population (17, 107–112), and is a leading cause of death among patients (113–115). Cancer strongly affects the disease course of SSc (110, 116), particularly when diagnosed close to the onset of rheumatological manifestations (117). Breast, lung, and hematologic cancers, including lymphoid and myeloid neoplasms, are most frequently diagnosed in patients with SSc (17, 118–120), but increased rates of melanoma and non-melanoma skin cancers, hepatocellular carcinoma, urothelial (119), and thyroid cancers, particularly in cases of coexistent autoimmune thyroiditis (121), have also been reported.

Risk factors for cancer in patients with SSc include demographic and clinical features, disease duration, selected complications, and the presence (or absence) of particular autoantibodies (120, 122–124). However, a clear profile of the patient with SSc 'at risk of malignancy' remains elusive due to the complex interplay between such characteristics and additional risk factors (e.g., family history, exposure to smoking, air pollutants, ionizing radiation, etc.). Compelling evidence suggests that in patients with SSc, some cancers are diagnosed close to the onset of autoimmune manifestations, akin to paraneoplastic phenomena, whereas others exhibit a characteristic delay, often correlating with an increased burden of organ damage (125). These aspects will be discussed in the following sections and summarized in Figure 2.

Clinical features of cancer in SSc

Given the short interval that is seldom observed between the onset of SSc and the diagnosis of cancer, a subset of SSc cases is thought to represent a paraneoplastic syndrome (120, 125, 126), referred to as 'cancer-associated scleroderma.' This subset may include patients in whom the antitumor immune response culminates in the onset of autoimmunity (127). From a clinical perspective, early diffuse and rapidly progressive SSc is associated with a high risk of synchronous malignancy (128, 129), particularly in the presence of certain serum autoantibodies.

A second peak of incident malignancies occurs in patients with a long history of SSc and related complications (125), such as pulmonary arterial hypertension and interstitial lung disease (ILD) (119, 124), particularly in cases of progressive fibrosis (120). Chronic inflammation has long been associated with an increased risk of malignancy (4), and what is observed in the SSc scenario could fit within this frame. For instance, this is the case for lung cancer, which arises more frequently in patients with ILD and established disease (123). However, while esophageal involvement is common in SSc, no increased risk of esophageal malignancy has been reported to date. Further research is warranted to test whether the presence of factors considered as 'protective' from cancer (i.e., limited cutaneous disease, anticentromere autoantibodies—ACA) (130, 131) is linked to smoldered cancer incidence in this patient subset.

Immunological features of cancer in SSc

Positivity for anti-RNA polymerase III (POLR3) autoantibodies has traditionally been linked to an increased risk of overall (120, 130, 132, 133) and synchronous cancers (111, 130, 133–135), mostly in patients with diffuse disease (131). Support for the association between the two conditions was elegantly provided by the evidence of alterations in the *POLR3A* locus in samples of synchronous cancers derived from patients with anti-POLR3^{+} SSc, but not in negative cases (15). However, conflicting data on the risk of malignancy with anti-POLR3 autoantibodies have been reported

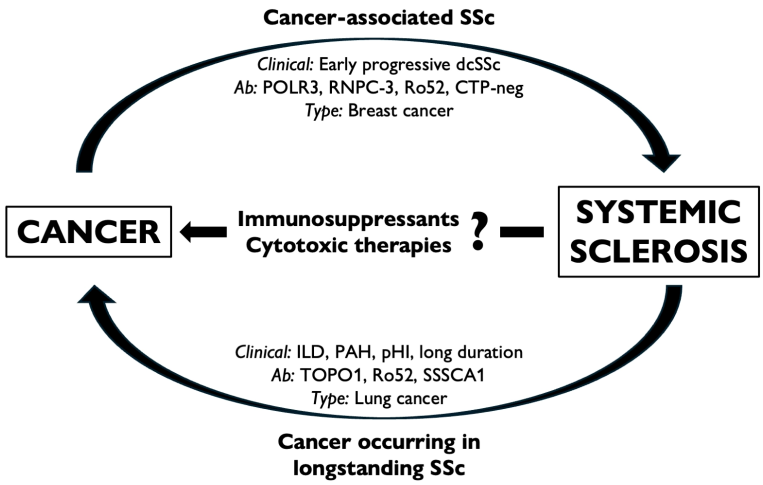

FIGURE 2
The interplay between cancer and SSc. Some forms of SSc can be regarded as cancer-associated (or paraneoplastic) scleroderma, in which the putative etiological role of malignancy is supposed to trigger the onset of autoimmunity in predisposed individuals (a). Cancer can also occur in longstanding SSc, particularly at specific sites and is associated with the selection of risk factors, phenotypes, and disease complications (b). Immunosuppressive and cytotoxic treatments are commonly adopted to treat SSc-related complications; however, the putative role of such therapies remains elusive (c). CTP-neg, 'CTP-negative' patients; dcSSc, diffuse cutaneous SSc; ILD, interstitial lung disease; PAH, pulmonary arterial hypertension; pHI, primary heart involvement.

in some cohorts (111, 136, 137). Apart from possibly reflecting genetic or epigenetic differences, such heterogeneity could also indicate the role of multiple autoantibody specificities in modulating the rate of cancers (127, 138). Indeed, similar to what was recently described in DM (51, 52), multiple serum autoantibody specificities likely confer a protective role against malignancy in patients with another autoantibody traditionally linked to an increased risk of cancer. A significant difference in the rate of neoplasms has been observed in anti-POLR3 positive patients with or without concurrent autoantibodies (130, 137). An increased risk of cancer-associated scleroderma has been also reported in patients without anticentromere (ACA), anti-Topoisomerase-I (TOPO1), and anti-POLR3 autoantibodies, the so-called CTP-negative cases (131), as well as in ANA-negative SSc cases (139). Mecoli et al. demonstrated a protective role of anti-Th/To in cancer-associated scleroderma (140). Since the Th/To complex is composed of four molecular subunits (140), it would be useful to investigate correlations between the rate of malignancies based on the presence of single vs. multiple autoantibodies directed towards the different subunits. Similar considerations could be made in patients with anti-POLR3, notably directed to RP155 and/or RP11 subunits of RNA polymerase III (141), and autoantibodies to the PM/Scl complex, which includes a 75 KDa and a 100 KDa subunit and have been associated with malignancy in Spanish patients (120, 142).

Among the rarer autoantibodies, anti-U3-RNP/fibrillarin (138) and anti-RNPC-3, usually associated with limited cutaneous disease but severe organ involvement, have been correlated with cancer-associated scleroderma, along with a worse prognosis, comparable to that observed with anti-POLR3 (143). In particular, a short SSc-cancer interval has been described for anti-RNPC-3 in an American cohort (143), although no association with malignancy was found in another European cohort (144). However, while the first study primarily focused on the characteristics of anti-RNPC-3+ patients and their association with cancer, the European study aimed to characterize the features of patients who tested positive vs. negative for that autoantibody. Moreover, different autoantibody detection methods have been used (143, 144), which could have influenced the results.

Breast cancer is the most frequent malignancy diagnosed as cancer-associated scleroderma, particularly in the presence of anti-POLR3 (136) and diffuse disease (131). Interestingly, breast cancer and SSc share select molecular pathways, including hyperactivation of the mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K), and transforming growth factor beta (TGF-β) (145). In addition, tumor-infiltrating lymphocytes are more abundant in breast cancers of patients compared than in those without autoimmune disease (145). These observations support the hypothesis of a possible interplay between the anticancer response and the onset of autoimmunity in cancer-associated scleroderma. Further research is required to understand the prognostic role and therapeutic impact of these observations from both the oncological and rheumatological perspectives.

Serum autoantibodies also played a significant role in stratifying patients according to the risk of late-onset malignancy (Table 1). Anti-topoisomerase I (TOPO1) positivity is a potential risk factor, particularly for lung cancer. However, it is unclear whether autoantibodies themselves, their association with ILD, or both are putative risk factors for malignancy (110, 146). Late-onset cancer occurs more frequently with the recently described anti-SSSCA1 antibody, an emerging predictor of SSc-related primary heart involvement, which may support the hypothesis of a correlation between long-standing SSc, organ damage, and incident malignancies (147). Anti-SSA/Ro autoantibodies, often detected in patients with

SSc and high burden of visceral involvement (148, 149), have been associated to late-onset cancers in a French SSc cohort. A large case–control study attributed this correlation specifically to positivity for the anti-Ro52 subset (130). This result was retrospectively validated by our group in an independent cohort of patients with SSc (137), suggesting a more intricate role of anti-Ro52 positivity. Indeed, cancer-associated scleroderma was more frequently reported when anti-Ro52 was found to be the sole autoantibody, whereas its positivity in combination with other specificities correlated with higher rates of overall cancer throughout the disease history of patients with SSc (137).

Table 3 summarizes the current evidence on the association between serum autoantibodies and cancer risk in patients with SSc.

Cancer screening in SSc

Patients with SSc represent an ideal population for implementing tailored cancer screening strategies because of the potential existence of different risk categories, as recently proposed for IIM (36). Recommendations for cancer screening were proposed by a panel of experts and are specifically meant for patients with new-onset SSc and anti-POLR3 autoantibodies (133). The panel pointed to the need to exclude synchronous malignancy, particularly of the breast, with regular screening suggested thereafter according to age- and sex-related risk factors (133). Despite preliminary evidence demonstrating the predictive role of seriate monitoring of tumor-associated antigen serum levels (150), a panel of experts discouraged their dosage *a priori* in patients with SSc, similar to that in the general population (133). However, the proposed recommendations are only applicable to anti-POLR3 positive patients. Thus, a tailored cancer-screening strategy for SSc remains largely speculative.

Cancer screening should be a priority, and tools to allow patient stratification into different risk clusters are needed. Such clusters may ideally benefit from different screening strategies at different time points during the disease course. As mentioned in the previous sections, the interplay of a wide range of features should be considered to assess the risk of malignancy in patients with SSc, including the disease phenotype, presence and severity of complications, serum autoantibodies, and traditional risk factors, such as tobacco exposure and family history. Finally, it would be interesting to verify whether repeated testing for serum autoantibodies could intercept changes in the autoimmune repertoire, which might help stratify the risk of incident cancer in patients with SSc during the follow-up period (Table 1).

Immunosuppressive treatments and cancer in SSc

Patients with SSc-related organ involvement are treated with immunosuppressive and/or cytotoxic therapies, raising concern for secondary cancers (151, 152) as supported by the observation of urothelial cancers occurring after exposure to cyclophosphamide (119, 120, 153). Mycophenolate mofetil (MMF) is commonly used for the treatment of SSc and is particularly effective in ILD (154, 155). Evidence mostly derived from transplant immunology has not raised

TABLE 3 Systemic sclerosis-specific and -associated autoantibodies, clinical associations and current evidence regarding cancer risk.

Autoantibody	Target antigen	Clinical associations	Cancer risk
anti-TOPO1/Scl-70	Topoisomerase I	dcSSc, ILD	Likely increased** (110, 146)
anti-CENP-A/B	Centromere proteins	lcSSc, PAH, DU, calcinosis, gastrointestinal disease	Not increased (130)
anti-POLR3	RNA polymerase III	Rapidly progressive dcSSc, SRC, GAVE	Increased*** (133, 137, 138)
anti-Th/To	RNase P Nucleolar Protein Complex	lcSSc, ILD, PAH	Not increased (140)
anti-NOR90	Nucleolar Organizer Region 90 KDa	lcSSc, mild disease	Not increased (130)
anti-PM/Scl	Nucleolar macro-molecular complex of 75 KDa and 100 KDa	arthritis, myositis, ILD	Likely increased (120)
anti-Ro52	Tripartite motif-containing protein 21	lcSSc, ILD, PAH, overlap pSS	Likely increased# (124, 130, 137)
anti-U3-RNP	Fibrillarin	higher mRSS, myositis	Likely increased## (138)
anti-RNPC-3	RNA Binding Region Containing 3 (U11/U12-RNP)	ILD, gastrointestinal dysmotility	Increased## (143)
anti-SSSCA1	autoantigen p27 (centromere-associated protein)	cardiac involvement*, pSS overlap	Increased## (147)

Due to relatively poor evidence concerning cancer risk, compared to IIM, cancer risk is reported as 'increased,' 'possibly increased,' or 'not increased,' according to relevant literature discussed in the main text. Results are mainly derived from observational cohort or case-control studies. In particular, multicentric cohort studies were available for anti-TOPO1, anti-POLR3, anti-CENP-A/B, anti-Th/To, and anti-PM/Scl autoantibodies.

dcSSc, diffuse cutaneous systemic sclerosis; DU, digital ulcers; GAVE, gastric antral vascular ectasia; ILD, interstitial lung disease; lcSSc, limited cutaneous systemic sclerosis; mRSS, modified Rodnan skin score; PAH, pulmonary arterial hypertension; pSS, Sjogren syndrome; SRC, scleroderma renal crisis.

* Defined as evidence of impaired left ventricle function and/or signs of right failure and/or clinically significant arrhythmia.

** Evidence suggests particularly for long-term incidence of lung cancer.

*** Conflicting evidence pointing towards increased risk only in the absence of multiple autoantibody positivity.

Evidence suggesting increased risk particularly in patients without multiple autoantibody positivity.

Evidence from single studies or small case series.

major concerns regarding the oncological risk of MMF (155–157), except for the possibly increased rate of non-melanoma skin cancers (158). While no study has specifically evaluated the risk of cancer in patients with SSc treated with MMF, drug safety was suggested in a large cohort of patients treated for fibrotic lung diseases (159), as well as in patients with SSc (138). We hypothesized that the antiproliferative effects of MMF (155) modulate the humoral immune response without affecting cell-mediated immunity (160), thus minimally impairing immune surveillance towards malignancy. Finally, current data are insufficient to establish any association between cancer incidence and more innovative treatments (e.g., rituximab and tocilizumab) in patients with SSc (161) (Table 1).

Cancer and systemic lupus erythematosus: still an unclear scenario

The dual role of immune activation in SLE—driving autoimmunity while potentially influencing tumor suppression or promotion—creates a paradox that is central to understanding the relationship between SLE and cancer. A recent meta-analysis revealed a pronounced increase (2.87-fold; 95%CI 2.49–3.24) in the standardized mortality ratio (SMR) for all-cause mortality among SLE patients compared to the general population (162). Despite the heterogeneity among the included studies, an elevated cancer-related mortality risk (SMR 1.7-fold) was reported in SLE patients (163). The overall cancer risk profile in SLE is shaped by a heterogeneous set of factors, including disease activity and damage, immunosuppressive treatments, genetic predisposition, and environmental exposure (164).

From an epidemiological perspective, SLE displays a unique cancer risk profile. Hematologic malignancies (NHL, Hodgkin lymphoma, leukemia, and myeloma), and lung, cervical, thyroid, gastrointestinal, hepatobiliary, and liver cancers occur more frequently in SLE, which is partly attributed to chronic immune activation and persistent inflammation. Conversely, breast, endometrial, and prostate cancers and melanoma are less common, possibly due to alterations in hormonal pathways and immune surveillance mechanisms (163, 165).

Clinical features of cancer in SLE

Specific features of SLE, such as hematological and pulmonary manifestations, may contribute to cancer risk, namely NHL and lung cancer. However, despite the well-established association between idiopathic pulmonary fibrosis and lung neoplasms, pulmonary fibrosis is rarely reported in SLE and has not shown statistically significant associations, despite evidence of increasing trends (166). A higher SLICC/ACR Damage Index has emerged as a risk factor for cancer (167, 168); however, the relationship with disease activity risk remains unclear (168) (Table 1).

Secondary and overlapping autoimmune diseases, such as Sjogren's syndrome, autoimmune liver disease, scleroderma, and autoimmune thyroiditis, may contribute to cancer risk in SLE (169) (Table 1). For instance, secondary Sjogren's syndrome increases the risk of NHL (168), although the predominance of the DLBCL subtype raises questions about Sjogren's status as the primary driver (170). Autoimmune thyroiditis is strongly linked to thyroid cancer in SLE patients, as supported by evidence of thyroid autoimmunity in most cases of thyroid cancer in this population (171).

Childhood-onset SLE (cSLE) is a disease subset that warrants particular attention regarding cancer risk. Lymphomas and solid tumors have been reported at a significant rate, with a median time of 10 years after cSLE diagnosis. Distinct clinical presentations, risk factors, and treatment challenges have been outlined in this population, underscoring the need for heightened vigilance and tailored management strategies for young patients (172).

Finally, patients with SLE may be more susceptible to oncogenic viruses such as Epstein–Barr virus (EBV) (169), human papillomavirus (HPV), and hepatitis B virus (HBV). Impaired immune surveillance could lead to higher rates of viral persistence and reactivation, contributing to the development of lymphomas (173), cervical dysplasia and cancer (174), and hepatocellular carcinoma. By weakening the antiviral defenses, immunosuppressive therapies may further increase this risk.

Current evidence on the established and putative risk factors for malignancy in patients with SLE is summarized in Supplementary Table 2.

Immunological features of cancer in SLE

Chronic inflammation plays a key role in fostering a pro-oncogenic microenvironment via DNA damage, oxidative stress, and cytokine-mediated pathways (175, 176). For instance, the increased risk of lymphoma may be driven by cytokines upregulated in SLE, such as BAFF, APRIL, IL-6, and IL-10, which promote B-cell survival, proliferation, and inflammation (177). These factors are linked to non-germinal center B-cell-like DLBCL, the predominant lymphoma subtype in SLE (169, 178).

SLE-associated autoantibodies, a hallmark of the disease, are hypothesized to promote tumor development by entering cells and causing DNA damage (179). Notably, an anti-DNA autoantibody named 3E10 has been shown to enter cell nuclei, bind to DNA, and impair key DNA repair pathways, thereby contributing to genomic instability. By increasing susceptibility to DNA damage, 3E10 provides a compelling link between SLE autoimmunity and malignancy (180).

Moreover, specific genetic variants (e.g., SNPs in CD40 and HLA alleles) have been associated with both SLE and malignancy, particularly DLBCL and lung cancer (181), although some findings suggest pleiotropy or linkage disequilibrium rather than direct biological causation (182). Emerging research has also identified epigenetic mechanisms, particularly microRNA dysregulation, implicated in both SLE pathogenesis and hematologic cancers, highlighting the potential role of shared post-transcriptional regulatory pathways in the concurrent development of autoimmunity and malignancy (183).

SLE might also confer protection against hormone-sensitive cancers, possibly because of lower exposure to estrogens and androgens. Indeed, women with SLE often experience earlier menopause (184) and are less frequently prescribed estrogen-containing medications (185), whereas men with SLE have lower androgen levels (186). Moreover, certain autoimmune mechanisms may yield protective effects, as in the case of 5C6 anti-DNA autoantibodies that selectively target tumor cells with defects in DNA repair processes (e.g., BRCA2-deficient cancer cells) (187). While the rates of hormone-susceptible breast cancers are similar among SLE patients and the general population, patients with SLE experience a significantly lower incidence of triple-negative cancers, which are mostly characterized by genetic mutations in DNA repair pathways (188).

Cancer screening in SLE

Established recommendations for cancer screening in patients with SLE are unavailable. Thus, these procedures largely rely on expert opinions, substantially overlapping with what is recommended in the general population (189). In particular, cervical screening and/or HPV vaccinations, periodic mammograms, and fecal occult blood testing are advised for all patients according to age- and sex-specific local guidelines (189). Moreover, clinical screening through regular lymph node examination and routine chemistry is recommended for hematological malignancies, while thyroid enzymes, autoantibodies, and ultrasound should be performed because of the risk of thyroid neoplasms (189). Apart from pursuing smoking cessation, lung cancer screening with annual chest CT scans is recommended only in patients with a high-risk profile (i.e., aged 50 years–75 years and with a history of smoking) (189), while hepatobiliary screening is not recommended unless in cases of positive HBV or HCV serologies (189), and urinary cytology is recommended periodically in patients who have undergone cyclophosphamide.

However, a large cohort study demonstrated that adherence to cancer screening is an issue in patients with SLE, with at least 25% of patients not being regularly screened, particularly in cases of established and longstanding disease (190). This seems particularly crucial regarding cervical cancer screening, since patients with SLE are at higher risk of abnormal test results compared with controls (191).

Immunosuppressive treatments and cancer in SLE

Immunosuppressive treatments can influence the risk of cancer in SLE (192) because their long-term use may impair immune surveillance (193). Prolonged and cumulative high-dose cyclophosphamide has been strongly linked to an elevated risk of bladder cancer (with oral cyclophosphamide) and hematological malignancies (189). Similarly, azathioprine has been associated with a risk of hematologic malignancies (164), highlighting the need for careful monitoring and optimal dosing. Moreover, the use of immunosuppressive therapies is associated with a higher risk of cervical neoplasia than antimalarials (194), underscoring the importance of regular screening in these patients.

Calcineurin inhibitors have been associated with an increased incidence of cancers in solid organ transplant recipients (195), with previous studies suggesting their role in impairing DNA repair, promoting angiogenesis, and facilitating tumor invasion (196). However, a recent large cohort study of SLE patients with consistent follow-up found no significant difference in cancer risk between those using calcineurin inhibitors and those who did not, even after adjusting for potential confounders (197). Biologics that target B-cell pathways, such as rituximab and belimumab, are generally considered safe; however, their effects on cancer remain the subject of ongoing investigation. Finally, owing to the close association between drug exposure and disease activity, many studies face challenges in distinguishing the individual contributions of these factors to cancer risk (Table 1).

Compared to immunosuppressants, hydroxychloroquine, which is universally prescribed for SLE, has been associated with a decreased cancer risk (198), particularly for breast and non-melanoma skin cancer (193), possibly because of its anti-proliferative and anti-angiogenic activity.

Limitations and concluding remarks

While this study aimed to provide insight into the dual-faceted clinical relationship between cancer and CTDs (i.e., cancer-associated CTDs vs. cancer occurring subsequently or within the context of CTDs), we acknowledge certain limitations. Although our literature review was comprehensive and sought to analyze evidence that supports and challenges our hypotheses, we did not follow a systematic review approach, which would be necessary to address more specific research questions based on the current evidence. A consistent approach was attempted across diseases, but the major differences in evidence availability led to some degree of heterogeneity, particularly in the immunological feature sections related to myositis and SSc *versus* pSS and SLE. Publication bias should also be considered, particularly regarding data on rare and emerging autoantibody specificities, along with the relatively greater abundance of studies on certain diseases, primarily IIM and SSc, compared to pSS and SLE. There are also biases in the races and ethnicities that have been studied in different diseases, which should be addressed in future investigations. The heterogeneity of analytical methods for autoantibody detection (e.g., immunoprecipitation, line blot, and ELISA) should also be considered when comparing different studies, as the sensitivity and specificity vary depending on the techniques used and the target autoantigen (199). Moreover, our objective was to highlight unmet needs and identify avenues for future research in autoimmunity and rheumatology, with potentially significant implications from the clinical, pathophysiological, and therapeutic perspectives.

Patients with CTDs exhibit distinct cancer risk profiles, which are influenced by the etiological role of malignancy in certain contexts and the precancerous environment created by chronic inflammation and autoimmune activation. Similarities in immune pathogenesis are thought to occur among patients with

paraneoplastic forms of CTDs, as seen when comparing findings from anti-TIF1-γ+ DM and anti-POLR3+ SSc, in which the complex interplay between cancer-related mutations and aberrant tumor immune editing is thought to culminate in the activation of self-reactive lymphocytes, ultimately leading to tissue damage and CTD onset. On the other hand, chronic immune activation reflecting specific pathogenic clues can be considered a potentially premalignant condition, as suggested by the evidence of an increased risk of lung cancer in patients with longstanding SSc-ILD. From this point of view, the example provided by pSS is paradigmatic, since the disease itself is responsible for the generation of autoreactive lymphocyte clones with lymphoma-prone behavior, ultimately culminating in MALT-NHL onset. Most importantly, a correlation between disease activity and lymphoma risk has been clearly demonstrated in pSS. The role of immunosuppressive therapies in cancer risk in these patients remains unclear. Therefore, further research is needed to unravel the complex interplay between CTDs and malignancy, which requires a multidisciplinary approach that integrates clinical and pathophysiological aspects (Table 1). Addressing this challenge is essential to improve cancer screening, prevention, and treatment strategies in this patient population.

Author contributions

AT: Conceptualization, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. AC: Conceptualization, Supervision, Visualization, Writing – original draft, Writing – review & editing. EG: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. SC: Writing – original draft, Writing – review & editing. MD: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. CS: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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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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The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1571700/full#supplementary-material

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Commentary: Cancer in connective tissue disease

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KEYWORDS

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A Commentary on

Cancer in connective tissue disease

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I read with great interest the comprehensive review by Tonutti et al. titled "Cancer in Connective Tissue Disease" (1), which provides a timely analysis of the bidirectional relationship between malignancy and autoimmunity in connective tissue diseases (CTDs). The authors adeptly synthesize current evidence on cancer risk stratification, autoantibody profiles, and screening challenges across systemic lupus erythematosus, systemic sclerosis, idiopathic inflammatory myopathies (IIM), and Sjögren's syndrome (SS). Their work underscores the critical need for multidisciplinary collaboration to address unmet needs in early detection and management.

I commend the authors for highlighting the paradoxical role of autoimmunity—where chronic inflammation may promote oncogenesis, yet autoimmune responses can also exert antitumor effects. This duality is exemplified by the contrasting implications of autoantibodies like anti-TIF1-γ (high cancer risk in IIM) and anti-Sp4/CCAR1 (potentially protective). However, I emphasize the urgent need for standardized autoantibody detection methods. As noted, discrepancies in anti-NXP2 results across assays (e.g., line blot vs. immunoprecipitation) complicate clinical interpretation (2). Harmonizing laboratory techniques is essential to refine risk stratification and validate guidelines like the IMACS cancer-screening algorithm (3).

I also support the call for disease-specific screening frameworks. While IMACS offers a model for IIM, similar protocols are lacking for systemic sclerosis and Sjögren's syndrome, where lymphoma risk escalates with biomarkers like ectopic germinal centers or CXCL13. Tailored strategies must integrate serological, clinical, and imaging data (e.g., salivary gland ultrasound in SS) while balancing cost-effectiveness and accessibility.

Finally, the impact of immunosuppressants on cancer risk warrants deeper exploration. Although the review notes inconclusive data on therapies like mycophenolate in systemic sclerosis, real-world studies are needed to clarify risks associated with newer biologics (e.g.,

rituximab) and the potential protective role of hydroxychloroquine. Pharmacovigilance registries could illuminate these associations.

In conclusion, Tonutti et al. have delivered an invaluable review that crystallizes the complex cancer-CTD interplay. Future efforts should prioritize validating autoantibody panels, expanding screening guidelines, and elucidating treatment-related oncogenic risks through international cohorts.

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The author declares 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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Nonlinearity and sex differences in the performance of a polygenic risk score for juvenile idiopathic arthritis

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Background: Juvenile idiopathic arthritis (JIA) is an immune-mediated pediatric disease believed to result from a complex interplay of genetic and environmental factors. Genome-wide association studies have enabled calculation of polygenic risk scores (PRS) for JIA. Understanding how the PRS associates with JIA and whether it performs similarly across sexes is essential for its utility in future studies.

Methods: We studied the relationship between a PRS developed from a previously published genome-wide association study of JIA and JIA in children from the Norwegian Mother, Father and Child Cohort Study (MoBa; total n = 57,630; JIA cases = 238). Generalized linear models (GLM) and generalized additive models (GAM) were used in logistic regression to assess the association. Furthermore, we investigated whether the relationship between PRS and JIA differed by sex by applying GAM models with interaction terms.

Results: PRS was significantly associated with JIA using both GLM ($p< 2e-16$) and GAM ($p< 2e-16$) models, and our results indicated a nonlinear relationship between PRS and JIA (effective degrees of freedom, EDF = 1.96). We found a significant interaction between sex and JIA PRS in relation to JIA ($p = 0.017$), and indications of a stronger and more logit-nonlinear relationship in females (EDF = 1.82) versus males (EDF = 1.06).

Conclusion: The relationship between PRS and JIA was slightly logit-nonlinear for females and logit-linear for males. The PRS for JIA can likely be used either as a continuous or discrete variable in analyses, but sex-stratification is recommended for future studies.

KEYWORDS

juvenile idiopathic arthritis, polygenic risk score, nonlinearity, sex differences, gene-sex interaction

1 Introduction

Juvenile idiopathic arthritis (JIA) is an immune-mediated disease characterized by joint inflammation lasting for at least six weeks and presenting before the age of 16 (1). It is a heterogenous disease with seven subtypes, and it is more prevalent in girls (2, 3). JIA imposes a significant burden on patients, their families, and society. It is believed to result from a complex interplay of genetic and environmental factors, although causal factors and underlying mechanisms remain largely unknown (4).

Familial, twin, and genome-wide association studies (GWAS) have helped to approach and dissect the genetic contribution to complex diseases, including JIA (5, 6). The monozygotic twin concordance rate of JIA has been estimated as 25-40%, and the sibling recurrence risk ratio as 11.6 (1). In the so far largest GWAS of JIA, including 3305 cases and 9196 controls, López-Isac et al. identified numerous susceptibility loci for JIA with a total SNP-based heritability of 0.61 (7).

The results from GWAS studies can be exploited by constructing polygenic risk scores (PRS), comprising aggregated effects of variants across the genome, which can be used to estimate the individual's genetic risk for the outcome of interest (8). PRS have been widely applied in studies of a range of different diseases and phenotypes and can be particularly useful in studies assessing the relationship between genetic and environmental risk factors for disease (9). Although PRSs have been suggested as potential clinical tools in the future, there are several obstacles that need to be addressed before they can be implemented into a clinical setting (9). PRSs are therefore so far mainly useful as research tools for studying genetic risk.

Recently, we developed a PRS for the children in the Norwegian Mother, Father and Child cohort study (MoBa) based on results from the aforementioned GWAS by López-Isac et al. (7, 10). When including a PRS in statistical models, either as a main effect or interaction variable, it is important to know how it relates to the outcome, in our case JIA. Understanding how the risk of JIA changes depending on the PRS can inform whether the PRS can be used as a continuous variable in the model or if it should be grouped into a discrete variable, and if so, how the discrete variable should be defined (11). Traditional logistic regression assumes a linear relationship between predictors and the log-odds of the outcome. However, some biological associations, including those between genetic risk scores and disease, may not follow a strictly linear pattern. Using nonlinear methods for modelling can therefore be useful because they are flexible enough to capture more complex relationships between the PRS and JIA. Furthermore, the PRS may be performing differently in specific subgroups, such as males and females, which can also be important to uncover when including the PRS in studies of risk and disease development (12).

Sex-specific genetic associations appear to play a role in a number of autoimmune and immune-mediated diseases, but the degree to which these differences contribute to JIA susceptibility has not been fully studied (13). A recent study on JIA patients found that the presence of antinuclear antibodies (ANA) was associated with specific genes, and this was observed more frequently in females, suggesting an interaction between certain genes and sex (14). Furthermore, a female-specific association between the *PTPN22* SNP rs2476601 and JIA has been confirmed across several different populations (15, 16), and evidence of a sex-specific association of *PSMA6/PSMC6/PSMA3* genetic variants with subtypes of JIA has also been reported (17). However, genome-wide studies of JIA, including the GWAS on which our PRS is based, were not stratified by sex (7). To address potential sex differences, it is thus important to assess whether the PRS performs similarly in males and females.

To fill these knowledge gaps, our aims of this study were 1) to investigate the relationship between the PRS for JIA and the probability of a JIA diagnosis, and 2) to explore whether the relationship between the PRS and JIA risk is different between males and females.

2 Methods

2.1 Study population and design

MoBa is a large-scale pregnancy cohort study led by the Norwegian Institute of Public Health (NIPH), which recruited participants across Norway between 1999 and 2008. 41% of the eligible women participated. The cohort comprises around 114,500 children, 95,200 mothers, and 75,200 fathers (18, 19). The present study uses version 12 of the MoBa data files, which underwent quality assurance and were made available for research in January 2019. We included MoBa children who had previously been genotyped (20).

2.2 Outcome

Information about JIA status was collected by linkage to the Norwegian Patient Registry (NPR), which includes data with personal ID numbers from all Norwegian public hospitals and specialists with public funding from 2008 (21). In Norway, the university hospitals with specialists within pediatric rheumatology have the main responsibility of diagnosing and following JIA patients. Cases were born between 1999 and 2009 and diagnosed with JIA before December 2021. We defined a JIA case as having at least two International Classification of Diseases (ICD)-10 codes (≥2 M08, ≥2 M09, or ≥1 M08 *and* ≥1 M09). In a recent validation of this case definition, we found a positive predictive value of 93.4% (10), ensuring a low number of false positive diagnoses. It is therefore reasonable to assume that our case definition largely reflects accurate diagnoses. For cases who received their first ICD-10 code in 2021, we accepted a single relevant ICD-10 code (M08 or M09), as we received our latest updates from NPR in December 2021. Controls were defined as non-JIA cases, and we removed all controls who had one ICD-10 code (M08 or M09) because they might have JIA.

2.3 Polygenic risk score for JIA

Umbilical cord blood samples were collected at birth, and the extracted DNA was frozen and stored at NIPH. The genotyping, quality control and imputation of the genetics data of the samples in MoBa have been extensively described previously (20). We calculated PRSs from the results of a previously published GWAS of JIA (7) by applying PRSice, version 2.3.3 (22). We chose *p*-value thresholds of 5E-8, 1E-6, 1E-5, 1E-4, 1E-3, 1E-2, 5E-2, 1E-1, and 1 to calculate PRSs and then extracted the first principal component (PC) for PRSs across all the thresholds, using this first PRS-PC as our final PRS for JIA (23). We then, using the whole dataset, standardized the PRS to a mean of zero and a standard deviation (SD) of 1 (24) and we used the standardized PRS for all analyses. In sensitivity analyses, the PRS was categorized into (1) quartiles, forming four equal-sized categories, (2) three categories containing the top 10%, middle 80% and bottom 10% of observations, and (3) a binary variable based on the median (Supplementary Table 1).

2.4 Statistical analysis

R version 4.2.3 was used to conduct all statistical analyses (25), and all scripts are available in our GitHub repository (https://github.com/KristineLH/PRS-JIA-sex). We used multiple logistic regression and generalized additive models (GAM) to examine the relationship between PRS and JIA. The top 10 PCs from the whole genotype dataset, together with sex, and year of birth were included as covariates in the models.

Nonlinear modeling approach

To account for potential logit-nonlinearity, we applied GAM using the *gam* function from the mgcv package (26). GAM extends traditional regression by allowing flexibility in how predictors influence the outcome, fitting smooth, data-driven curves rather than assuming a fixed logit-linear form. In our model, PRS was modeled as a smooth function using a regression spline, which adapts to the shape of the data. The effective degrees of freedom (EDF) from the GAM output served as an indicator of nonlinearity, with an EDF of 1 representing a linear relationship and values greater than 1 suggesting a nonlinear relationship (27).

Modeling sex differences

To investigate whether the relationship between the PRS and JIA differed by sex, we first included an interaction term between the PRS and sex in the multiple logistic regression model. The Wald test was used to assess statistical significance of the interaction, and a p-value < 0.05 was regarded as significant. However, interaction terms in standard regression models assume a constant, linear modification of the association by sex, which may not fully capture potential differences in the way the PRS is associated with JIA in males and females. To address this, we further investigated sex-specific patterns by fitting separate smooth splines for the PRS in males and females. Specifically, we created new variables by multiplying PRS with dummy variables for each sex and then modeled these products as smooth terms in the GAM framework. This allowed us to estimate the association between the PRS and JIA in each sex separately.

Visualization

To aid interpretation, we visualized the relationship between PRS and JIA for each model. Using the *predict* function, we calculated the probability of JIA across a range of PRS values (-4.5 to 4.5 with an increment of 0.1), while keeping other covariates (10 PCs, year of birth) at their mean values. This enabled direct comparison of PRS effects across methods (Figure 1) and sexes (Figure 2).

3 Results

3.1 Study sample characteristics

Our final analytical sample included 57,630 children of whom 238 were identified as JIA-cases (Table 1). Male participants comprised 51.0% (n = 29,139) of the controls, compared to only 39.9% (n = 91) of the JIA cases. The JIA cases had a mean PRS of 0.58 (+/- 1.10 SD), whereas the mean PRS in controls was -0.002 (+/- 1.00 SD).

3.2 Association between PRS and JIA

We assessed the association between PRS and JIA using a standard logistic regression model (GLM) and a generalized additive model (GAM), results shown in Figure 1. In both

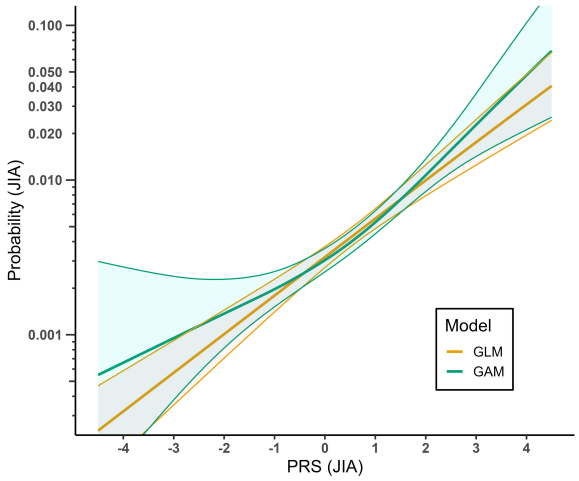

FIGURE 1
Relationship between PRS for JIA and probability of JIA modelled by a generalized linear model (GLM) compared to a generalized additive model (GAM). The lines show the fitted prediction model of JIA probability ~ PRS + sex + year of birth + top 10 principal components for each of the models. The colored areas represent the 95% confidence intervals for the corresponding models.

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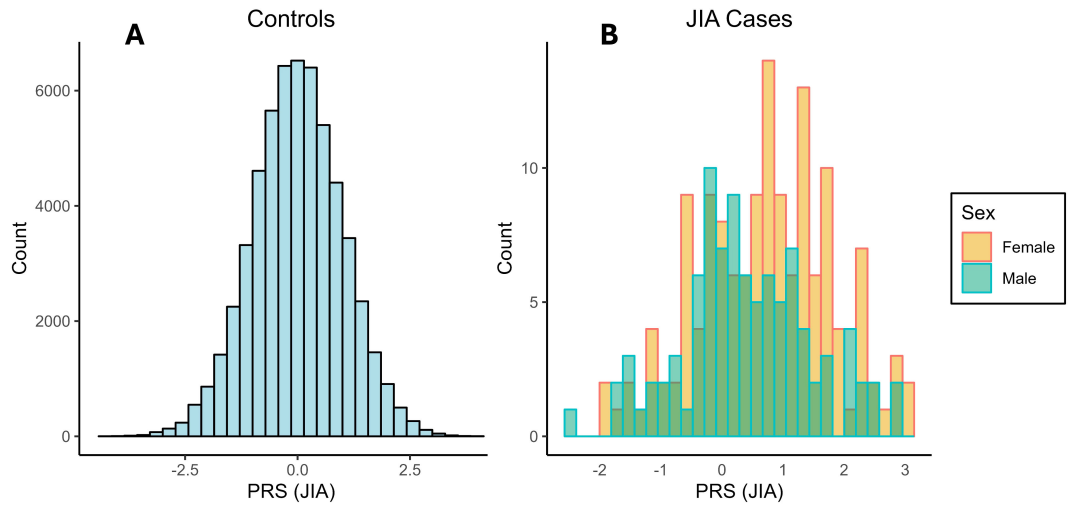

FIGURE 2
Distribution of JIA PRS in **(A)** controls, and **(B)** JIA cases grouped by sex.

models, PRS was significantly associated with JIA ($p < 2e\text{-}16$ for both models), and the results were similar for the categorized PRS variables (Supplementary Figure 1). The EDF in our GAM model was 1.939, indicating a logit-nonlinear relationship between PRS and risk of JIA.

3.3 The association between PRS and JIA differs by sex

In Figure 2, we show the distributions of PRS in controls, as well as cases stratified by sex. The PRS distributions for controls show a mean of 0.01 in males and -0.01 in females. In contrast, JIA cases demonstrate higher PRS means. Specifically, the PRS mean for male cases is 0.40, while for female cases, it is 0.70, indicating a stronger association between PRS and JIA diagnosis in females compared to males.

We further investigated the interaction between sex and PRS in association with JIA. In a simple logit-linear model, the interaction term between sex and PRS was significantly associated with JIA ($p = 0.017$). We then investigated this interaction further by conducting a semi-stratified analysis allowing for nonlinear relationships (Figure 3). This model showed that PRS was significantly associated with JIA in both females ($p < 2e\text{-}16$) and males ($p < 0.001$). Interestingly, the relationship between PRS and JIA was approximately logit-linear in males (EDF = 1.06) but showed a larger tendency of logit-nonlinearity in females (EDF = 1.82). We detected a similar pattern when defining the PRS as high- and low-risk variable divided into top 10%, bottom 10% and middle 80% of observations (Supplementary Figure 2).

4 Discussion

Our results show that the relationship between PRS and JIA is weakly logit-nonlinear. The notable difference in PRS distribution between male and female JIA cases underscores a sex-specific variation in PRS among JIA cases in the MoBa cohort. Furthermore, we show a significant interaction between sex and PRS in relation to JIA, with sex acting as a PRS effect measure modifier. Interestingly, the logit-nonlinearity of the relationship seems to be driven by the females, whereas in males the relationship seems to be logit-linear.

Understanding the relationship between a PRS and the outcome of interest is important when the PRS is to be used in further analyses, such as when investigating interactions between environmental exposures and genetic predisposition to develop JIA. Particularly, for the PRS to be used as a continuous variable

TABLE 1 Study sample characteristics.

Characteristics	JIA cases			Controls		
	All	Male	Female	All	Male	Female
Sample size (n,%)	238 (100)	95 (39.9)	143 (60.1)	57,392	29,319 (51.0)	28,073 (48.9)
Year of birth (mean, SD)	2005 (2.18)	2004 (2.19)	2005 (2.15)	2005 (2.17)	2005 (2.17)	2005 (2.16)
PRS (mean, SD)	0.579 (1.10)	0.399 (1.08)	0.699 (1.11)	-0.002 (1.00)	0.005 (1.00)	-0.010 (0.99)

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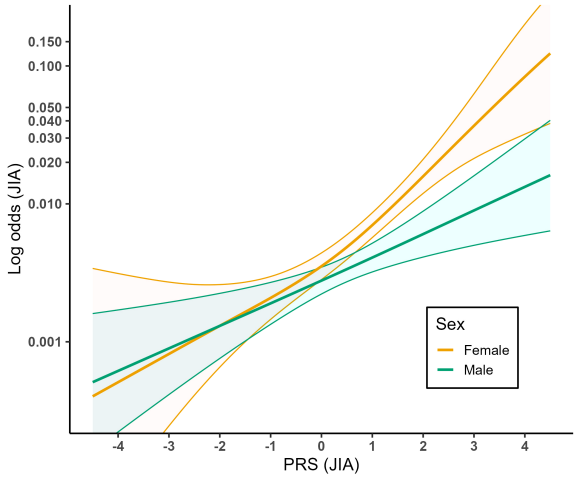

FIGURE 3
Relationship between PRS of JIA and probability of JIA in females and males. The lines show the fitted prediction model of JIA probability ~ PRS + sex + year of birth + top 10 principal components for each of the sexes. The colored areas represent the 95% confidence intervals for the corresponding models.

in analyses of JIA, the relationship between PRS and JIA should be well modelled (11). A non-linear relationship between the PRS and JIA could suggest that, for risk prediction, the PRS should be grouped into a discrete variable. Our results indicate a somewhat logit-nonlinear relationship between the PRS for JIA and risk of JIA, with a stronger effect with higher PRS compared to lower PRS. However, as shown in Figure 1, the GAM model taking logit-nonlinear associations into account is not vastly different from the simple logit-linear model. It should be noted, however, that the relatively small number of JIA cases in our dataset may have limited our power to detect subtle nonlinear interactions, particularly for males. Although grouping the PRS into a categorical variable as shown in Supplementary Figure 1 gave a similar fit, the predicted probabilities of JIA were lower than with the continuous PRS, especially for the high-risk groups. This indicates some loss of information and shrinkage towards the mean due to grouping the PRS. Thus, we suggest using PRS as a continuous variable in future studies when possible. Grouping the PRS into high- and low-risk groups of top 10%, bottom 10% and middle 80% gave the most similar fit compared to using the PRS as a continuous variable and may therefore be an alternative way of modelling the PRS. However, males and females appear to require distinct models for use of this PRS for JIA.

Sex-specific and sex-dependent effects of PRSs for other diseases, like schizophrenia and coronary artery disease have also been reported (28–30). The difference we observe in PRS performance between the sexes could reflect differences in the sex ratio among cases and controls in the GWAS our PRS is based on (12), with the girl cases outnumbering the boys and consequently having more influence on the formation of the score. However, the sex ratios were not stated in the GWAS paper, which may limit our results (7). Furthermore, different subtypes of JIA are associated with different genetic loci, and sex distribution also differs depending on the subtype (3). Some subtypes, such as oligoarticular and polyarticular JIA, which constitute around 70% of all cases, occur 2–3 times more frequently in girls, but not all JIA subtypes are more common in females (3). Thus, the PRS may be mainly reflecting genetic predisposition for the more common subtypes which are also more common in females and therefore show a stronger association with JIA in females compared to males. We did not have access to information on subtypes in our dataset and were thus not able to account for this in our analyses. Given that certain JIA subtypes differ in their genetic patterns, this represents a limitation of our study. Furthermore, gene-environment interactions involving exposures that differ by sex, such as hormones, have not been accounted for and may have influenced our results. Finally, our results may indicate that the effect of genetic predisposition on JIA development is dependent on biological processes that differ between the sexes.

When using the PRS for JIA in association and interaction analyses, researchers should be aware of the sex-specific associations and consider sex-stratification when possible. Our findings suggest that future studies on the genetic predisposition to JIA, including GWAS and the development of PRS, should incorporate sex-specific analyses to identify genetic loci that may contribute to disease development in males and females separately, as well as those shared between sexes (31, 32). Developing a set of distinct PRS scores specifically for sex-by-subtype categories could prove to be even more usefully predictive, but this would require a very large genetic dataset with detailed information on sex and JIA subtypes. We also suggest exploring potential susceptibility loci for JIA on the X-chromosome (33) as this was not included in our study nor, to our knowledge, in any GWAS of JIA thus far. As sex differences are common in autoimmune diseases in general, investigating sex-specific associations of PRS may be relevant also for other autoimmune and immune-mediated diseases (34).

In conclusion, our results show that the relationship between our PRS and JIA is slightly logit-nonlinear, but only for females.

The PRS for JIA can likely be used either as a continuous or discrete variable in analyses, but sex-stratification should be considered. Future studies should further investigate sex-differences in genetic predisposition of JIA and other autoimmune diseases.

Data availability statement

Access to MoBa data can be obtained by applying to the Norwegian Institute of Public Health (NIPH). Restrictions apply regarding the availability of these data, which were used under specific approvals for the current study and therefore not publicly available. Access can only be given after approval by the Regional Committees for Medical and Health Research Ethics (REC) in compliance with the EU General Data Protection Regulation (GDPR) and approval from the data owners. The consent given by the participants does not open for storage of data on an individual level in repositories or journals. Requests to access these datasets should be directed to helsedata.no/en.

Ethics statement

The studies involving humans were approved by The Regional Committees of Medical and Health Research Ethics South East (project number 28469) and the Norwegian Data Protection Agency. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

KH: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. HR: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing, Methodology. PJ: Writing – original draft, Writing – review & editing. VD: Writing – original draft, Writing – review & editing. SH: Writing – original draft, Writing – review & editing. OA: Data curation, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. CW: Methodology, Writing – original draft, Writing – review & editing. HS: Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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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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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

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A possible role for immunogenetic factors in myositis developing after vaccination in the pre-covid-19 era

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Introduction: Vaccinations have had a transformative impact on public health, reducing the incidence of many infectious diseases and increasing survival. However, there remains uncertainty about the potential of vaccines to trigger autoimmune diseases such as the idiopathic inflammatory myopathies (IIM). Myositis after vaccination (MAV) is a rare clinical entity, but given immunogenetic associations with other adverse events, we explored genetic risk factors, particularly human leukocyte antigen (HLA) alleles and GM/KM immunoglobulin allotypes, that may predispose individuals to develop MAV.

Methods: We examined clinical characteristics, vaccination history, autoantibodies, HLA alleles and GM/KM allotypes from 56 patients who developed MAV, 133 myositis cases with no documented vaccination within 6 months of onset (non-MAV), and 527 healthy controls from the pre-COVID-19 era. Genotyping for HLA and GM/KM allotypes was performed by standard assays. Differences in allele frequencies in race-matched groups were evaluated using chi-square tests, odds ratios (OR) and 95% confidence intervals (CI). Multivariate logistic regression adjusted for age, sex, and vaccination type. Statistical significance was defined as a Holms corrected p-value of less than 0.05.

Results: No clinical or serologic differences were found between MAV and non-MAV patients. However, the HLA-DQA1*03:03 allele was a unique risk factor for MAV in Caucasians (OR=3.87, 95% CI=1.56-9.54, p=0.002), while the known myositis risk factor, HLA-DRB1*03:01, was a protective factor for MAV (OR=0.41, 95% CI=0,18-0.94, p= 0.033). GM2, GM13, and KM1 allotypes were more frequently observed in MAV patients than healthy controls, and other HLA alleles were risk or protective factors for specific vaccines given in patients who developed MAV.

Conclusion: Immunogenetic factors may influence the likelihood of developing MAV. Further studies of larger, deeply phenotyped populations are needed to confirm these associations and could inform personalized risk assessments and targeted interventions, thereby enhancing vaccine safety.

KEYWORDS

polymyositis, dermatomyositis, vaccination, adverse events, HLA, GM/KM, juvenile dermatomyositis

1 Introduction

The idiopathic inflammatory myopathies (IIM) are a group of rare systemic autoimmune conditions characterized by muscle inflammation and weakness that arise from chronic immune activation in genetically predisposed individuals in response to certain environmental triggers (1). Major strides have been made in defining the genetic risks for IIM and other autoimmune conditions (2), but identifying the even more important environmental risk factors has been hampered by the lack of validated measures and the constantly changing mixtures of exposures that occur over a lifetime (3). Vaccines, while highly beneficial, can in rare cases, cause chronic immune activation followed by the development of a number of autoimmune diseases, including myositis (4, 5).

Certain polymorphic immune response genes have been associated with IIM. One of the strongest genetic associations for autoimmune diseases is located on chromosome 6p21.3 that includes the human leukocyte antigen (HLA) locus in addition to other immune system-modulating genes (6). Alleles of the 8.1 ancestral haplotype (8.1 AH), *HLA-DRB1*03:01* and *HLA-B*08:01*, show the strongest association with IIM in Caucasians (7, 8). Other polymorphic genes associated with autoimmune diseases, including IIM, are the immune response genes that encode immunoglobulin gamma heavy chains (GM) and immunoglobulin kappa light chains (KM) (9). These have also been identified as genetic susceptibility factors across different ages and ethnicities for various clinical and serological IIM phenotypes (10).

There is no doubt that vaccines have significantly improved global public health by boosting immune responses to many infectious agents, preventing infections, and minimizing morbidity and mortality. However, it is plausible that vaccines, often given intramuscularly, could cause initial immune activation in muscles to progress to a chronic systemic inflammatory response in those with certain immunogenetic backgrounds. While many patients develop myositis without any documented recent vaccination, the concept that vaccinations may be linked to the onset of some cases of myositis has been previously suggested in case reports (11–14). The first identified cases of myositis following vaccination (MAV) included myositis developing in a temporally related way to diphtheria-tetanus-pertussis vaccines (11, 15, 16) and smallpox vaccines (17) in adult and pediatric patients. Additional reports of vaccine constituents, including aluminum hydroxide, and not the immunization antigens themselves, have led to macrophagic myofasciitis (18).

Certain adverse events to drugs, medical implants and vaccines have previously been associated with clinical, serologic or immunogenetic features (19–21). Based on our observation that some myositis cases were temporally associated with vaccinations, we systematically compared those patients who developed myositis within 6 months of a documented vaccination to those who had no documented vaccinations within 6 months of myositis disease onset and to healthy controls (HC) from the pre-COVID-19 era to assess possible clinical, serological, and immunogenetic differences.

2 Materials and methods

2.1 Study participants

Myositis patients and HC were enrolled into investigational review board-approved clinical protocols at the National Institutes of Health (NIH) Warren Grant Magnuson Clinical Center and the United States Food and Drug Administration from 1983 to 2002. These protocols studied the natural history of myositis and twins and siblings discordant for myositis.

Per our protocol criteria, all patients met Bohan and Peter criteria for definite or probable myositis (22, 23). They were all diagnosed with IIM, including dermatomyositis (DM), juvenile dermatomyositis (JDM), polymyositis (PM), juvenile polymyositis (JPM), and inclusion body myositis (IBM) based on the accepted criteria at the time of enrollment. Patients with myositis and another connective tissue disease (CTM) were also included. IIM patients who received a documented vaccination within six months prior to first myositis symptom onset were included in the myositis after vaccination (MAV) group (n=56), while those who did not receive vaccination during this time interval (documented by history and review of medical records) were categorized as non-MAV (n=133). All patients underwent a comprehensive medical history and physical examination, which included detailed protocol

questionnaires completed by the patients and their enrolling physicians.

The clinical data included age, self-classified race, gender, and signs and symptoms. Since gene frequencies differ by race, the HLA and GM/KM data were assessed in Caucasian patients, which was the largest cohort and the only one adequate for reliable statistical analysis. The HC groups were race-matched.

2.2 HLA typing

HLA allele typing was performed using purified genomic DNA, using laboratory-designed and commercial reagents (Genovision, West Chester, PA; Dynal Biotech, Lafayette Hill, PA) and PCR-mediated sequence-specific oligonucleotide probe hybridization and sequence-specific priming technique via standard techniques (24).

Allele frequencies per patient (carriage rates) were determined by the number of allele-positive subjects divided by the total number of subjects for which complete HLA data were available at a given locus. All patients in the HLA allele analysis were self-identified as Caucasians and divided into MAV (n=48) and non-MAV (n=93) groups. For comparison, the HC data (n=527), who did not have myositis, were obtained through the NIH HLA laboratory.

2.3 GM and KM allotyping

Immunoglobulin gamma heavy chain (GM) and immunoglobulin kappa light chain (KM) allotyping was performed using standard hemagglutination inhibition methods to type for IgG1m, IgG2m, and IgG3m and for IgKM1 and IgKM3 (25). Allotype and phenotype frequencies were determined by the number of allotype-positive subjects divided by the total number of subjects for which data were available at a given locus. All patients in the GM and KM allotype analysis were Caucasian and divided into MAV (n=19) and non-MAV (n=34) cases. Race-matched HC (n=266) were used for comparison.

2.4 Autoantibody identification

Myositis-specific autoantibodies (anti-synthetases, anti-signal recognition particle (anti-SRP), anti–Mi-2 and myositis-associated autoantibodies (anti-Ku, anti-La, anti-Ro, anti–URNP, and anti–PM-Scl), were identified from frozen serum samples using previously validated methods of protein and RNA immunoprecipitation (IPP) and double immunodiffusion (10). The NXP2 and TIF1 autoantibodies were identified with IPP, followed by immunoblotting (26).

2.5 Statistical analysis

Analyses were performed using GraphPad Prism (GraphPad, Inc., La Jolla, CA). For both the HLA allele analysis and the GM/KM allotype analysis, the allele or allotype frequencies were compared by chi-square test or Fisher's exact test for counts below 5, for 2x2 contingency tables between MAV and controls, MAV and non-MAV, or non-MAV and controls. The odds ratios (OR), 95% confidence intervals (CI) were determined. The MAV group was also divided and compared to non-MAV and HC by the four most frequent vaccines: Hepatitis B, Influenza, Tetanus, and Mumps-Measles-Rubella (MMR).

A p-value was considered significant if below 0.05 using the Holm procedure to adjust for multiple comparisons (27). The U-test, or Mann-Whitney test, was used to compare non-parametric variables, such as the months from vaccine to first symptom, calculations between children and adults, and between the different vaccines.

Chi-square tests were performed to examine differences in the frequency distributions between the MAV and non-MAV groups. An analysis in which the distribution of clinical subgroups significantly differed between the MAV and non-MAV groups led to performing a sensitivity analysis, in which a random sample of patients were selected in similar clinical subgroups. This was also performed with the MAV group within 6 months and 3 months from vaccination. If the genetic results differed from the primary analysis, the difference in clinical subgroup distribution was interpreted to have affected the result, however, if the genetic results remained the same, the difference in clinical subgroup distribution was interpreted as not affecting the genetic results.

3 Results

3.1 Clinical findings

There were 56 patients, including 28 females, in the MAV group, 48 of whom were Caucasian, three African American, and five of mixed race, and 133 patients, including 92 females, in the non-MAV group, of which 98 were Caucasian, 12 African American, six Asian or Hispanic, and 17 of mixed race. Of these, 48 MAV patients and 95 non-MAV patients were Caucasian and HLA-typed, while 19 MAV patients and 34 non-MAV patients were Caucasian and also underwent GM/KM typing. The clinical and autoantibody subgroup, race, gender, and signs and symptom distributions were similar in the MAV and non-MAV groups for all patients included in the study (Table 1), as well as for the HLA-analyzed groups. The patients in which GM/KM was examined had a lower frequency of JDM in the MAV group (21.1%) and a higher frequency of JDM in the non-MAV group (73.5%) ($p = 0.0004$). The median age of disease onset for the MAV group was 5.4 years in children and 43.8 years in adults, which was similar to the non-MAV group (6.7 and 45.4 years, respectively).

Of the 56 MAV patients, 17 received a form of the tetanus vaccine, 15 received a Hepatitis B vaccine, 15 received an influenza vaccine, and 13 received a MMR vaccine (Table 2). The median time to myositis symptoms after vaccination was 2.2 months with a range of 0–6 months and an IQR of 3.5 months, while the median time to diagnosis of myositis after vaccination was 7.0 months.

TABLE 1 Distribution of clinical and autoantibody subgroups, and signs and symptoms of myositis patients developing symptoms of myositis within 6 months of vaccination (MAV) and those without documented vaccination within 6 months of symptom onset (non-MAV).

Clinical Groups*	MAV (n=56)	Non-MAV (n=133)
	N (%)	N (%)
JDM	26 (46.4)	84 (63.2)
DM	10 (17.8)	17 (12.8)
PM	13 (23.2)	15 (11.3)
CTM	3 (5.4)	7 (5.3)
IBM	2 (3.6)	6 (4.5)
JPM	2 (3.6)	4 (3.0)
Myositis-Autoantibody Groups*+		
MSA and MAA Negative	36 (64.3)	89 (66.9)
p155 (TIF1)	14 (25.0)	40 (30.1)
Mi-2	5 (8.9)	6 (4.5)
MJ (NXP2)	3 (5.4)	23 (17.3)
SRP	3 (5.4)	6 (4.5)
Aminoacyl tRNA-Synthetases	4 (7.2)	11 (8.3)
Ro60	5 (8.9)	12 (9.0)
PM-Scl	2 (3.6)	4 (3.0)
U1RNP	1 (1.8)	6 (4.5)
Clinical Features*		
Myalgia	35 (63.6)	88 (67.2)
Distal muscle weakness	29 (51.8)	62 (47.0)
Muscle atrophy	27 (50.0)	51 (38.6)
Falling	25 (46.3)	62 (47.3)
Dysphagia	24 (44.4)	61 (46.2)
Cuticular overgrowth	22 (40.7)	41 (31.3)
Fever	20 (35.7)	42 (31.8)
Arthritis	17 (30.4)	58 (43.9)
V-sign rash	17 (31.5)	38 (29.0)
Asymmetric weakness	12 (22.2)	21 (16.2)
Raynaud's Phenomenon	12 (22.2)	18 (13.6)
Shawl-sign rash	9 (16.7)	25 (19.1)
Mechanic's hands	7 (13.0)	13 (9.9)
Palpitations	4 (7.4)	12 (9.2)
Carpal Tunnel Syndrome	4 (7.4)	7 (5.3)
Interstitial lung disease	2 (3.8)	8 (6.1)

#MAV, myositis symptoms developing within 6 months of documented vaccination; non-MAV, no documented immunization within 6 months of onset of myositis; JDM, juvenile dermatomyositis; DM, dermatomyositis; PM, polymyositis; CTM, connective tissue disease overlap with myositis; IBM, inclusion body myositis; JPM, juvenile polymyositis; MSA, myositis-specific autoantibody; MAA, myositis-associated autoantibody; p155 (TIF1), anti-transcription intermediary factor 1 autoantibodies; MJ (NXP2), anti-nuclear matrix protein autoantibodies 2; SRP, anti-signal recognition particle autoantibodies; Ro60, autoantibodies to the 60kD protein of the heterogeneous antigenic complex; PM-Scl, autoantibodies to the 75kD and 100kD proteins seen in the polymyositis/scleroderma complex; U1RNP, autoantibodies to the U1 ribonucleoprotein complex.
+ Sum is > 100%, as some patients have both MSA and MAA.
*No significant differences were detected between the MAV and Non-MAV groups.

Tetanus, influenza, and MMR had a similar period from vaccination to first myositis symptom. However, for those who received Hepatitis B vaccine, there was a significantly shorter latency period, with a median of 1 month from vaccination to first myositis symptom ($p = 0.045$). In the cases where vaccines were given in a series, there was a median of 2.2 months from the time of first vaccination to first myositis symptom, a median of 3.0 months after the second vaccine, and a median of 3.5 months after the third vaccine.

In total, 98 vaccines were administered to the 56 patients (Table 2). Sixteen patients received multiple vaccines on different days within the 6-month period and nine patients received 2 or 3 doses of Hepatitis B vaccine. Among 16 Hepatitis B patients, five developed MAV after the 1st dose, five developed MAV after the 2nddose, and six developed MAV after the 3rd dose.

3.2 HLA analysis

The frequency of DQA1*02:01 was significantly higher in the MAV group compared to non-MAV (OR = 3.80, 95% CI = 1.36-10.58, $p = 0.007$), however, it was protective for non-MAV versus HC (OR = 0.25, 95% CI = 0.11-0.55, $p = 0.0004$) (Table 3). The frequency of DRB1*03:01 was significantly lower for MAV compared to non-MAV (OR = 0.41, 95% CI = 0.18-0.94, $p = 0.033$) but it was a risk factor for the non-MAV versus HC (OR = 3.42, 95% CI = 2.14-5.48, $p < 0.0001$), but not for MAV vs. HC. DRB1*15 was a protective factor for the non-MAV group compared to HC (OR = 0.44, 95% CI = 0.22-0.88, $p = 0.017$). DQA1*05 was a risk factor for the non-MAV group (OR = 2.25, 95% CI = 1.40-3.45, $p = 0.004$). Adult and juvenile data were similar in the overall HLA analysis and showed no significant differences.

Several risk and protective alleles for the non-MAV group were also shared by the MAV group, including DRB1*10:01 (OR = 6.29, 95% CI = 1.78-22.20, $p = 0.001$) and DQA1*03:01 (OR = 3.43, 95% CI = 1.92-6.13, $p < 0.0001$) as risk factors. DRB1*02 (OR = 0.06, 95% CI = 0.01-0.25, $p < 0.0008$) was a protective factor for the non-MAV and MAV groups (Table 3). Homozygosity of HLA alleles did not show a significant impact for either risk or protective factors for the MAV or non-MAV groups.

Several HLA alleles demonstrated significant associations in the MAV versus HC groups (Table 3). The DRB1*10:01 allele was significantly associated with MAV (OR = 8.95, 95% CI = 2.05-39.00, $p = 0.012$) compared to HC. The DQA1 03:01 allele (OR = 4.23, 95% CI = 1.92-9.32, $p = 0.007$) and DQA1*03:03 (OR = 3.86, 95% CI = 1.56-9.54, $p = 0.002$) were also risk factors for MAV when compared to HC. HLA DQA1*03:03 was the only unique risk factor allele for MAV that was not also a risk for the non-MAV group when compared to HC (Table 3). However, the frequency of DRB1*02 (OR = 0.03, 95% CI = 0.01-0.46, $p < 0.0001$) was lower in MAV, indicating a lower likelihood of MAV in individuals with this allele. A sensitivity analysis of HLA alleles of MAV cases developing within three months of vaccination resulted in the same findings.

The frequencies of the linked alleles DQA1*02:01 and DRB1*07:01 were significantly higher in the MAV group receiving the Hepatitis B or influenza vaccines compared to non-

TABLE 2 Distribution of the number of patients receiving vaccines and the number of vaccines administered prior to first symptoms in 56 patients who developed myositis within 6 months after vaccination#.

Vaccine	Patients receiving a vaccine within 6 months of onset (% of all 56 patients)+	Vaccinations administered within 6 months of onset (% of all 98 vaccinations)
Any Tetanus (DPT, DTaP, or Td)	17 (30.4)	18 (18.4)
Hepatitis B	15 (26.8)	27 (27.6)
Influenza A/B	15 (26.8)	15 (15.3)
MMR or Measles	13 (23.2)	13 (13.3)
OPV or IPV	6 (10.7)	6 (6.1)
Prevnar or Pneumococcal	3 (5.4)	3 (3.1)
Hepatitis A, Hemophilus influenzae type B, Varicella, Meningococcal, Typhoid, or Yellow fever *	12 (21.4)	12 (12.2)
Rabies, Japanese Encephalitis, Influenza A virus subtype H1N1, or Lyme **	4 (7.2)	4 (4.0)

*2 patients each received one of these 6 vaccines, and 2 vaccinations were administered for each vaccine listed.
**1 patient each received one of these 4 vaccines, and 1 vaccination was administered for each vaccine listed.
DPT, diphtheria pertussis tetanus vaccine; DTaP, diphtheria tetanus acellular pertussis vaccine; Td, tetanus booster; MMR, measles mumps rubella vaccine; OPV, oral polio vaccine; IPV, inactivated polio vaccine.
+Ten patients received more than 1 vaccine at the same time and the combinations of vaccines given within 6 months of developing myositis were: Patient 1 - 1st HepB, influenza; Patient 2 - 1st HepB, MMR; Patient 3 – OPV, MMR; Patient 4 - DTP, Haemophilus influenzae type B, 3rd HepB, pneumococcal conjugate vaccine; Patient 5 – DTaP, HIB, 3rd HepB; Patient 6 – DTaP, IPV, MMR; Patient 7 – DTaP, OPV; Patient 8 – Td, MMR; Patient 9 – Varicella, MMR; and Patient 10 – Td, HepA.

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TABLE 3 Differences in HLA types in Caucasian myositis after vaccination (MAV) patients, non-MAV patients, and healthy controls*.

HLA Alleles	MAV % (n=48)	Non-MAV % (n=93)	Control % (n=527)	MAV vs. Non-MAV		MAV vs. Healthy Control		Non-MAV vs. Healthy Control	
				P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)
HLA-DRB1									
*02	0.0	2.2	26.8	0.543	1.05 (0.09-11.93)	<0.0001	0.03 (0.01-0.46)	<0.0008	0.06 (0.01-0.25)
*03:01	26.3	45.6	20.3	0.033	0.41 (0.18-0.94)	0.498	0.71 (0.33-1.51)	<0.0001	3.42 (2.14-5.48)
*07:01	26.3	12.5	23.9	0.099	0.40 (0.15-1.04)	0.888	0.88 (0.42-1.86)	0.173	0.45 (0.23-0.88)
*10:01	7.9	5.7	0.9	0.697	0.70 (0.16-3.10)	0.0125	8.95 (2.05-39.0)	0.0012	6.29 (1.78-22.20)
*15	12.5	10.8	21.4	0.976	0.84 (0.28-2.47)	0.202	1.91 (0.79-4.61)	0.0172	0.44 (0.22-0.88)
HLA-DQA1									
*02:01	23.4	7.4	24.3	0.007	3.80 (1.36-10.58)	0.920	1.04 (0.51-2.11)	0.0004	0.25 (0.11-0.55)
*03:01	19.1	22.3	7.7	0.823	1.21 (0.51-2.91)	0.007	4.23 (1.92-9.32)	<0.0001	3.43 (1.92-6.13)
*03:03	14.9	7.4	4.3	0.231	0.46 (0.15-1.40)	0.002	3.86 (1.56-9.54)	0.293	0.56 (0.23-1.35)
*05	47.9	62.1	42.6	0.105	1.78 (0.88-3.59)	0.544	0.81 (0.45-1.46)	0.004	2.2 (1.40-3.45)

*Carriage rates were determined by the number of allele-positive subjects divided by the number of subjects for whom complete HLA data were available at a given locus. Abbreviations per prior tables.

MAV (Table 4). The DQA1*03:03 allele was a risk factor for MAV patients who received influenza vaccines compared to HC (Table 4).

3.3 GM/KM analysis

The GM phenotype 1, 2, 3, 5, 13, 17, 21, 23 and allotypes GM 2 (OR = 3.17, 95% CI = 1.24-8.13, p = 0.012) and GM13 (OR = 12.5, 95% CI = 1.64-95.05, p = 0.001) were risk factors for MAV compared to HC, but were not risk factors for the non-MAV group (Table 5). KM1 (OR = 3.43, 95% CI = 1.30-9.03, p = 0.009), and KM1,3 (OR = 5.19, 95% CI = 1.47-18.29, p = 0.008) were also risk factors for MAV.

The allotypes GM 2 (OR = 3.61, 95% CI = 1.09-11.99, p = 0.0319), KM 1 (OR 5.57, 95% CI = 1.64-18.94, p = 0.004), and the phenotype KM1,3 (OR 5.19, 95% CI = 1.47-18.29, p = 0.0078) were risk factors for MAV compared to Non-MAV. Because the JDM subgroup was more frequent in the non-MAV than MAV groups, we performed a sensitivity analysis with the MAV group that received their last vaccination within three months and selecting a random sample of JDM patients to create a similar proportion of myositis clinical subgroups in the non-MAV group as in the MAV group in the three month window. In this analysis, the MAV group's GM/KM risk alleles remained unchanged comparing the MAV and non-MAV groups.

4 Discussion

Gene-environment interactions appear to play an important role in the development of autoimmune diseases (28). Immunogenetic factors are critical for immune responses to vaccines and have been proposed to modulate risk for the development of vaccine adverse reactions (21). This study suggests possible genetic associations with the development of myositis after vaccinations. HLA alleles have been associated with the development of many autoimmune diseases, including multiple sclerosis, systemic lupus erythematosus, type 1 diabetes mellitus, Sjogren disease and IIM (29–34), as well as possible risk factors for some vaccine adverse events (21).

Our study identified HLA-DQA1*03:03 as a unique risk factor for MAV versus HC, as this allele is not known to be associated with any other IIM groups. This unique risk factor for MAV suggests a different immune response pathway leading to myositis after vaccinations. Interestingly, the known myositis risk factor DRB1*03:01 was present in lower frequency in the MAV group compared to non-MAV group.

The frequency of HLA-DQA1*02:01, a known risk factor in Caucasians for anti-Mi-2 autoantibodies, was significantly higher in patients with MAV, particularly after the Hepatitis B and influenza vaccines, compared to non-MAV, but no association of MAV was seen with anti-Mi-2 autoantibodies. However, HLA-DQA1*02:01

TABLE 4 Differences in HLA types in Caucasian myositis after vaccination (MAV) patients, non-MAV patients, and healthy controls by vaccine types*.

Vaccine	HLA Alleles	MAV vs. Non-MAV		MAV vs. Control	
		P-value	OR (95% CI)	P-value	OR (95% CI)
Hepatitis B (n=7)	DRB1*07:01	0.006	14.00 (2.84-76.39)	0.018	7.92 (1.81-41.83)
	DQA1*02:01	0.002	16.57 (3.63-71.83)	0.037	5.23 (1.38-20.89)
Influenza (n=14)	DQA1*01	0.038	0.28 (0.01-0.93)	0.021	0.27 (0.10-0.79)
	DQA1*02:01	0.001	7.77 (2.05-26.21)	0.323	1.96 (0.71-6.26)
	DQA1*03:01	0.497	1.54 (0.20-2.06)	0.017	5.30 (1.73-17.83))
	DQA1*03:03	0.102	3.72 (0.92-14.26)	0.020	6.61 (1.84-25.68))
Tetanus (n=10)	DRB1*16	0.030	7.46 (1.64-36.91)	0.011	8.98 (2.38-35.76)

*Carriage rates were determined by the number of allele-positive subjects divided by the number of subjects for whom complete HLA data were available at a given locus; MAV patients in each group were compared to 93 non-MAV and 527 controls.

appeared to be a protective factor for the non-MAV group compared to the HC. Although DRB1*07 had previously been described to be associated with myositis in certain racial populations (1), we found this allele to be significantly more frequent in Caucasians with MAV after Hepatitis vaccines compared to the non-MAV group. These findings highlight the complex gene-environment interactions involved in MAV and suggest potential areas for future research and interventions.

The results of comparing both MAV and non-MAV to HC revealed significant associations between specific HLA alleles and risk of myositis, showing further alleles of interest in the immunogenetic profiles of these patients. DRB1*10:01 and DQA1*03:01 were linked to an elevated risk of MAV, indicating a genetic predisposition to myositis following immunization. The protective association with DRB1*02 suggests a reduced likelihood of developing myositis in carriers of this allele, potentially due to its

TABLE 5 Differences in GM/KM allotypes and phenotypes in Caucasian myositis after vaccination (MAV), Non-MAV, and control groups*.

GM/ KM Markers	MAV % (n=19)	Non-MAV % (n=34)	Control % (n=266)	MAV vs. Non- MAV		MAV vs. Control		Non-MAV vs. Control	
				P-values	OR (95% CI)	P-values	OR (95% CI)	P-values	OR (95% CI)
Allotypes									
GM 2	52.6	23.5	25.9	0.0319	3.61 (1.09-11.99)	0.012	3.17 (1.24-8.13)	0.7642	1.14 (0.49-2.63)
GM 13	94.7	73.5	59.0	0.0756	0.15 (0.02-1.33)	0.0012	12.5 (1.64-95.05)	0.1483	0.52 (0.23-1.15)
KM 1	62.3	23.5	33.3	0.0043	5.57 (1.64-18.94)	0.0087	3.43 (1.30-9.03)	0.3173	1.53 (0.66-3.51)
Phenotypes									
GM 1, 2, 3, 5, 13, 17, 21, 21, 23	26.3	8.8	3.4	0.1181	0.27 (0.06-1.30)	0.001	10.2 (3.01-34.50)	0.1434	0.36 (0.09-1.41)
KM 1, 3	52.6	17.6	25.9	0.0078	5.19 (1.47-18.29)	0.012	3.17 (91.23-8.13)	0.3994	1.63 (0.65-4.11)
KM 3, 3	42.1	76.5	63.9	0.0124	0.22 (0.07-0.75)	0.0984	2.43 (0.95-6.26)	0.2087	0.54 (0.23-1.25)

*Conventions and abbreviations per prior Tables.

role in modulating immune responses. Previous literature has not elucidated any association of these alleles with myositis, warranting further investigations.

Immunoglobulin genes are important risk and protective factors for many autoimmune diseases, and GM13, KM1 and KM3 allotypes have been described as risk factors for myositis (9, 10). The GM/KM analysis identified GM2 and GM13 as risk factors for MAV compared to HC, but not for the non-MAV group. Similarly, KM1 and the KM1,3 phenotype were also linked to increased MAV risk. These findings suggest that specific GM/KM allotypes may serve as additional non-HLA genetic markers for MAV risk, warranting further research into their potential for personalized risk assessment.

Among the 56 MAV patients, there was a median interval of 2.2 months from vaccination to the first myositis symptom. Previous case reports showed the interval between vaccination and the development of symptoms of myositis ranged from 24 hours to 2 months, which generally aligns with our observations (13, 35–37). It has been postulated that when patients develop myositis after repeated vaccine exposure, it is likely due to an amplified immune response triggered by the repeated doses. While our data showed a delayed onset of myositis symptoms following the influenza vaccine, this contrasts with previous case reports that reported a shorter latency period of less than a month after receiving the influenza vaccine (36, 38). As there was a significantly shorter latency period, with a median of one month from vaccination to first myositis symptom for those developing MAV after Hepatitis B vaccine ($p = 0.045$), it is possible that a different mechanism of immune activation may be at work in these cases.

Our study has several limitations. First, our cohort was relatively small and was collected before the onset of the COVID-19 pandemic, and as a result, it does not include patients who developed myositis after receiving COVID-19 vaccinations. This a notable limitation, particularly in light of numerous case reports that have been published during and after the pandemic documenting the onset of autoimmune diseases, including myositis and specifically anti-melanoma differentiation-associated protein 5 (MDA5) autoantibody-positive DM following COVID-19 vaccination (5, 39–41). Other recently approved vaccines, including those to rotavirus, human papillomavirus, and herpes zoster were also not included in our study. It is interesting that so many different vaccine antigens might be associated with myositis, which suggests a single mechanistic explanation is not likely, and also raises the question of the role of the various adjuvants used in these many vaccines. However, given the small numbers of cases and variations in adjuvants from vaccine to vaccine, from manufacturer to manufacturer, and over time, it was not possible to carefully evaluate this. Furthermore, our investigation did not include certain recently identified myositis autoantibodies, including anti-MDA5, and did not include the most recent genotyping methods. And some non-MAV cases may have received vaccinations that were not recalled or documented, potentially biasing the comparisons. Nevertheless, our study lays the groundwork for future research on MAV. We hope that future research will build on this foundation, incorporating more recent methods and including all vaccines and phenotypes of myositis to provide a more comprehensive understanding of MAV.

5 Conclusion

Our study highlights the complex relationship between vaccinations and the onset of myositis. Our findings are generally consistent with previous studies and reports of MAV, although our data showed a somewhat more delayed onset of myositis symptoms after vaccination, particularly following the influenza vaccine. The novel identification of the HLA-DQA1*03:03 allele as a unique risk factor for MAV and the protective factor of HLA-DRB1*03:01 suggests the role of a genetic predisposition in the MAV group that differs from non-MAV myositis patients. GM/KM associations and other HLA genes were noted among specific vaccines and MAV. These genetic associations could provide insights into the pathogenesis of myositis, suggesting that specific gene-environment interactions may influence the susceptibility of developing MAV. Studies in larger populations exploring greater numbers of deeply clinically, immunologically, and genetically phenotyped subjects, and including all currently available vaccines, are needed to understand possible associations among vaccines and myositis and the genetic risk and protective factors involved. A larger study population would also be instrumental in determining the possible epistatic or interactive effects of HLA, GM, and KM alleles on MAV.

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 humans were approved by National Institutes of Health Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants or their legal guardians/next of kin.

Author contributions

EA: Data curation, Writing – original draft, Writing – review & editing. AP: Data curation, Writing – original draft, Writing – review & editing. ES: Data curation, Resources, Writing – review & editing, Conceptualization. JP: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing, Data curation. LR: Data curation, Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing, Funding acquisition. FM: Conceptualization, Funding

acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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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. The authors declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Decreased cigarette smoking may partially explain the increased prevalence of antinuclear antibodies in the United States

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Introduction: Despite well-known harmful health effects of smoking, research supports an inverse association with some autoimmune diseases. High-titer antinuclear antibodies (ANA) are associated with autoimmune diseases, and ANA prevalence in the US increased between 1988 and 2012. Tobacco smoking decreased during those years while vaping of electronic cigarettes (e-cigarettes) increased after their introduction in 2007. Carbon monoxide (CO) may ameliorate autoimmunity, and e-cigarettes deliver much less CO than regular cigarettes. We explored interdependencies among ANA, smoking, and time.

Methods: We analyzed cross-sectional data on ANA and the primary nicotine metabolite, cotinine, in 13,288 participants ≥12 years old from three time periods (1988-1991, 1999-2004, 2011-2012) of the US National Health and Nutrition Examination Survey. Smoking exposure (none, passive, active) was inferred from serum cotinine. We used logistic regression to analyze ANA prevalence, adjusted for sex, age, and race/ethnicity.

Results: Over the study periods, ANA prevalence was highest (13.3-19.2%) for nonsmokers but non-trending; lower (11.1-15.5%) for "passive" smokers but steadily increasing; and even lower for active smokers but increasing from 7.4% in 1999–2004 to 13.3% in 2011-2012. The increases in ANA among passive and active smokers were mainly in adolescents (ages 12–19 years). Smokers had reduced odds of ANA in 1999-2004, with an odds ratio (OR) of 0.65 and a 95% confidence interval (CI) of 0.45-0.93, but this association was weaker in 1988-1991 (OR=0.80; 95% CI:0.52-1.22) and 2011-2012 (OR=0.82; 95% CI:0.56-1.21).

Discussion: Although smoking causes harmful health effects, ANA data are consistent with smoking playing a role in decreasing autoimmunity. Recent vaping among adolescents may partially explain their large increase in ANA prevalence. The inverse ANA association with smoking strengthened between 1988-1991 and 1999-2004 but then weakened by 2011-2012. The initial

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strengthening was potentially because nonsmokers were exposed to progressively less CO (and/or other components of secondhand smoke), due to tightened smoking restrictions, while the potential nicotine-associated protection against ANA may have weakened after e-cigarettes became a source. Smoking should not be recommended given its negative health impacts. However, further studies could elucidate new mechanisms, perhaps involving components of tobacco smoke or vaping, possibly enabling development of novel preventative or treatment measures.

KEYWORDS

antinuclear antibodies (ANA), autoimmune diseases, carbon monoxide (CO), cotinine, e-cigarettes, National Health and Nutrition Examination Survey (NHANES), tobacco smoking, vaping

1 Introduction

High-titer antinuclear antibodies (ANA) are biomarkers associated with many autoimmune diseases (1–6), some of which have increased in incidence over recent decades for unknown reasons. Previously, based on data from the US National Health and Nutrition Examination Survey (NHANES), we reported an increasing ANA time trend (7) and investigated possible ANA associations with 253 xenobiotics (8). Our initial goal was to explore whether temporal changes in the levels of any xenobiotics associated with ANA could help explain the increase in ANA prevalence over time. However, many xenobiotics were evaluated at only one point in time or had mostly undetectable levels. We ultimately focused on serum cotinine, which was measured in nearly all participants.

Smoking tobacco is a major cause of preventable deaths, illnesses, and health care costs worldwide (9, 10), but despite overwhelming evidence of harmful effects of smoking in general, smoking has appeared to be inversely associated with ANA (7). Cotinine has often been used as a biomarker for tobacco smoke exposure (11–13), and as the primary metabolite of nicotine, cotinine has long been regarded as the most reliable indicator of active and passive exposure to tobacco smoke (11, 14). However, cotinine can also signal other nicotine exposures such as nicotine gum, chewing tobacco, snuff, and snus. Recently, an increasingly popular nicotine-delivering alternative to regular cigarettes, electronic cigarettes (e-cigarettes), has expanded the opportunities for smokeless exposure to nicotine (15).

In this article, we explore whether the decrease in cigarette smoking over the past few decades (16, 17) could plausibly account for some of the increase in ANA. We assess associations seen in the large NHANES database, some of which were observed previously (7), and postulate a potentially protective (or immunosuppressive) effect of carbon monoxide (CO) that might help explain the apparent inverse correlation between cigarette smoking and ANA.

The effects of smoking and CO on autoimmune diseases depend on individual variability, exposure levels, and the disease in question. Perricone et al. (18) discuss numerous studies of the relationship between smoking and autoimmune diseases. While smoking is a risk factor for many autoimmune diseases, smoking appears to have a protective effect for others, including ulcerative colitis, celiac disease, Behcet's disease, type 1 diabetes, and autoimmune hypothyroidism. Epidemiologic studies have suggested that smoking may protect against ulcerative colitis (19–21), Behcet's disease (21), autoimmune hypothyroidism (22–24), and Sjogren's syndrome (19, 20), and that CO may protect against discoid lupus erythematosus (25). Rodent studies have suggested that CO may have therapeutic effects for various autoimmune diseases, including multiple sclerosis (26, 27), collagen-induced arthritis (28), systemic lupus erythematosus (29), type 1 diabetes (30), uveitis (31), and autoimmune hepatitis (32).

Starting early this century, many smokers began using e-cigarettes, either in addition to or instead of regular cigarettes (15, 33–36). Among 116 adult e-cigarette users in one study (37), 68% self-reported as current smokers, 24% as former smokers, and 8% as never smokers. The use of e-cigarettes doubled between 2010 and 2013 among US adults, with over 20 million having tried them (34), and the use among high school students increased from 1.5% in 2011 to 16% in 2015 (15). E-cigarettes supply nicotine and thus cotinine (15, 38) but produce much less CO than regular cigarettes (39, 40). We hypothesize that a decrease in CO and/or other possibly "protective" smoking byproducts, either due to quitting all forms of smoking or switching fully or partially from regular cigarettes to e-cigarettes (or other nicotine delivery systems, such as chewing tobacco, snuff, or snus), may have contributed to the recent increase in ANA in the US.

2 Subjects and methods

2.1 Study participants

Data on ANA were available for 13,519 participants from five NHANES cycles: 1988-1991, 1999-2000, 2001-2002, 2003-2004, and 2011-2012. The NHANES sampled representative members

of the noninstitutionalized civilian US population and provided sampling weights to adjust for selection probabilities and nonresponse (41), which enables inference that generalizes to most of the US population. All participants signed informed consent documents and completed questionnaires, and most were physically examined and provided blood and urine specimens. Available data included demographic characteristics, health covariates, measured factors, and constructed variables. The NHANES protocol was approved by the Human Subjects Institutional Review Board of the US Centers for Disease Control and Prevention (CDC).

2.2 ANA assessment

All serum samples were evaluated for ANA in the laboratory of Dr. Edward K.L. Chan between 2016 and 2017 by indirect immunofluorescence at a 1:80 dilution using the NOVA Lite HEp-2 ANA slide with DAPI kit (Inova Diagnostics, San Diego, California, USA), with a highly specific fluorescein isothiocyanate-conjugated secondary antibody (goat anti-human IgG). Immunofluorescence staining intensities were graded 0–4 compared to standard references (42). Grades 1–4 were considered positive for ANA and grade 0 was considered negative. For more assay details see Dinse et al. (7).

2.3 Data on ANA, cotinine, and smoking

For cost and other practical reasons, ANA were only assayed in a subset of participants ≥12 years old in each of the five cycles. The ANA subsamples from 1999-2000, 2001-2002, and 2003–2004 were each roughly one-third the size of those from 1988–1991 and 2011-2012. Thus, as in our earlier studies (7, 8), we combined the three middle cycles to create three time periods with similar sample sizes: 1988-1991 (N=4,727), 1999-2004 (N=4,527), and 2011-2012 (N=4,265). As before, we focused on these three periods rather than the five cycles.

All analyses were restricted to the 13,519 participants with ANA data. The CDC adjusted the sampling weights to account for analyzing this ANA subsample. Data were available on cotinine, and thus smoking exposure as defined by cotinine concentration, for 13,288 participants; on self-reported smoking history for 12,278 participants; and on both smoking exposure and smoking history for 12,063 participants. Supplementary Table S1 shows the numbers of participants in each time period (and overall) with data on ANA, smoking exposure, and smoking history. Throughout this article, "cotinine" refers to serum cotinine and not urinary cotinine.

2.4 Model variables

The ANA outcome variable was a binary indicator of ANA positivity/negativity. Cotinine concentration (ng/mL) was a quantitative variable and was used to classify smoking exposure as none (≤0.05), passive (>0.05 to 10), or active (>10), as recommended by the CDC and the US Environmental Protection Agency (EPA) (13), though a sensitivity analysis applied a more recent recommendation of >3 ng/mL for defining active smoking exposure. Combining the first two exposure categories produced an indicator of smoking status: nonsmoker (none or passive exposure) versus smoker (active exposure). Smoking history was based on questionnaire data, with individuals self-reporting as never, former, or current smokers.

Except where otherwise noted, our primary analyses adjusted for sex, age, race/ethnicity, and the survey design variables (i.e., strata, clusters, and weights proportional to the inverse probability of sampling), each of which was available for all participants. Age was measured in years and categorized by decade (12-19, 20-29,…, 70-79, ≥80), though sensitivity analyses explored the use of fewer age categories, a quantitative age variable, or a restricted cubic spline in age. Self-reported race/ethnicity was categorized as non-Hispanic White, non-Hispanic Black, Mexican American, or Other. Secondary analyses adjusted for body mass index (BMI), alcohol intake, poverty income ratio (PIR), and education, as defined previously (42). Secondary analyses also investigated CO content in cigarettes, pack-years of smoking, lifetime years of smoking, and years since former smokers quit smoking, though these data were very limited.

2.5 Statistical analysis

When analyzing ANA prevalence, we used logistic regression models to allow the probability of ANA positivity to depend on explanatory variables. All models adjusted for the survey design variables. The basic model for estimating overall ANA prevalence and its 95% confidence interval (CI) did not include adjustment covariates, but we did include a categorical covariate for period when estimating ANA prevalence in each of the three time periods. When assessing ANA time trends, we adjusted for sex, age, and race/ethnicity and calculated an ANA prevalence odds ratio (OR) and its 95% CI for each period relative to the first period. The statistical significance of an ANA time trend was evaluated by replacing the categorical period covariate with a quantitative time covariate and then inspecting its p-value, where time was defined as the number of years between the midpoints of the participant's period and the first period.

When analyzing the cotinine data, we calculated the geometric mean cotinine concentration for each time period. We also derived a trend line by using linear regression to model individual log-transformed cotinine concentration as a function of the number of years between the midpoints of the participant's period and the first period. Any concentration below the limit of detection (LOD) was replaced by an imputed value of LOD/$\sqrt{2}$ (43, 44). The cotinine LOD was initially 0.05 ng/mL but was lowered to 0.015 ng/mL during the second period due to an improvement in the assay; the corresponding imputed values were 0.035 and 0.011 ng/mL. We also evaluated mean cotinine concentrations over time (and estimated trend lines) within subgroups of self-reported never,

former, and current smokers, and we used kernel density plots to assess the full cotinine concentration distribution for each smoking-history subgroup and time period.

When analyzing smoking time trends, we used logistic regression to estimate the prevalence of smokers in each time period. Overall prevalence estimates were adjusted for the survey design variables but not for any covariates. Also, after further adjusting for sex, age, and race/ethnicity, we estimated a prevalence OR (and a 95% CI) for each period relative to the first period.

When investigating the relationship between ANA and smoking, we performed logistic regression analyses similar to those described above for ANA prevalence. First, we stratified by smoking and analyzed ANA prevalence and time trends separately in each stratum. Second, we stratified by both age and smoking to see whether the ANA association with smoking depended on age. Third, we added a smoking covariate (instead of stratifying) and assessed whether that smoking covariate affected the ANA association with time or whether removing the period covariate altered the ANA association with smoking. Fourth, we also added a smoking-by-period interaction to evaluate whether smoking modified the ANA time trend. Fifth, we stratified by period and compared ANA prevalence for smokers versus nonsmokers to gauge how the ANA association with smoking changed over time.

Finally, we conducted sensitivity analyses to assess whether our results changed when using an alternative age covariate (fewer categories, quantitative, or restricted cubic spline) or when only considering adults (ages ≥20 years). We also explored the use of a more recent recommendation of >3 ng/mL for the cotinine cutpoint when defining active smoking exposure. In addition, we investigated several other covariates (BMI, alcohol intake, PIR, and education) and the limited data on cigarette CO content, pack-years, years of smoking, and years since quitting.

All analyses were performed with SAS software (version 9.4, SAS Institute, Cary, NC) and accounted for the survey design variables by using special survey procedures. Domain statements were used to properly handle the sampling weights in subgroup analyses. Variance estimates for the 95% CIs were obtained using the Taylor series method. Reported p-values were 2-sided. All plots were constructed in SAS except the kernel density plot, which was created in R (version 4.4.0, R Foundation, Vienna, Austria).

3 Results

3.1 ANA time trend

The prevalence of ANA rose over the 25-year span for which NHANES data on ANA were available, with most of the increase occurring between the second and third time periods. Accounting only for time period and the survey design variables, the weighted estimates of ANA prevalence were 11.0% (95% CI: 9.7-12.5%) in Period 1 (1988–1991), 11.4% (95% CI: 10.2-12.8%) in Period 2 (1999-2004), and 16.1% (95% CI: 14.5-17.9%) in Period 3 (2011-2012). These overall estimates, along with sample sizes and numbers of ANA-positive participants, are shown in the last row of Table 1. Relative to Period 1 and after further adjustment for sex, age, and race/ethnicity, the ANA prevalence OR was 1.02 (95% CI: 0.84-1.24) for Period 2 and 1.49 (95% CI: 1.23-1.82) for Period 3 (Table 2), and there was strong statistical evidence of a positive trend in ANA prevalence over time (p=0.0001). We reported these results earlier (7), with slight discrepancies due to minor differences in analysis, but repeat them here for context.

3.2 Cotinine time trend

Supplementary Figure S1 shows the geometric mean cotinine concentration and its 95% CI for each period, along with the best-fitting trend line. There was strong statistical evidence (p<0.0001) of a steady decrease over time. When stratified by self-reported smoking history, the mean cotinine levels ranged from 0.04 to 0.27 ng/mL for never smokers, 0.08 to 0.59 ng/mL for former smokers, and 104.2 to 158.5 ng/mL for current smokers (top half of Supplementary Table S2). The best-fitting trend line had a negative

TABLE 1 Sample sizes, ANA-positive counts, and ANA prevalence estimates by time period and smoking exposure.

Smoking	Period 1: 1988-1991		Period 2: 1999-2004		Period 3: 2011-2012		All Periods Combined	
Exposure [a]	N+/N	Prev (95% CI) [b]	N+/N	Prev (95% CI) [b]	N+/N	Prev (95% CI) [b]	N+/N	Prev (95% CI) [b]
None	93/429	19.2 (13.6-26.3)	264/1,884	13.3 (11.3-15.7)	401/2,379	17.4 (14.7-20.4)	758/4,692	16.0 (14.2-18.0)
Passive	343/2,739	11.1 (9.6-12.8)	190/1,581	12.7 (10.5-15.4)	141/1,001	15.5 (13.2-18.1)	674/5,321	12.5 (11.3-13.7)
Active	168/1,357	8.6 (6.4-11.4)	89/1,034	7.4 (5.6- 9.7)	127/884	13.3 (11.0-15.9)	384/3,275	9.5 (8.2-10.9)
Total	643/4,727	11.0 (9.7-12.5)	545/4,527	11.4 (10.2-12.8)	669/4,265	16.1 (14.5-17.9)	1,857/13,519	13.0 (12.1-13.9)

ANA, antinuclear antibodies; CI, confidence interval; LOD, limit of detection; N, total number of participants (sample size); N+, number of ANA-positive participants; Prev, ANA prevalence (as a percent).

[a]Smoking exposure categories were based on serum cotinine concentrations (None, ≤0.05 ng/mL; Passive, >0.05 to 10 ng/mL; and Active, >10 ng/mL). Due to a technical improvement in the cotinine assay, the cotinine LOD decreased from 0.05 to 0.015 ng/mL during Period 2. The number of participants with a missing cotinine value also decreased over time from 202 in Period 1 to 28 in Period 2, and then to 1 in Period 3.

[b]ANA prevalence was estimated under two logistic regression models for ANA positivity (yes/no), adjusted for the survey design variables (sampling weights, strata, and clusters). One model included only an intercept, which produced an overall estimate for all time periods combined. The other model included a categorical covariate for time period, which produced a separate estimate for each period. Both models were applied initially to all participants with data on ANA regardless of data on smoking exposure (Total) and then within subgroups with data on both ANA and smoking exposure (None, Passive, and Active). The subgroup counts sum to less than the total sample size because some participants were missing data on smoking exposure (i.e., cotinine).

TABLE 2 Covariate-adjusted assessments of ANA time trends by smoking exposure.

Smoking	ANA Prevalence Odds Ratio for Time Period (95% CI) [b]			Time Trend
Exposure [a]	Period 1: 1988-1991	Period 2: 1999-2004	Period 3: 2011-2012	p-value [b]
None	1.00 (reference)	0.70 (0.45-1.09)	0.99 (0.62-1.57)	0.2139
Passive	1.00 (reference)	1.28 (0.96-1.69)	1.72 (1.33-2.22)	0.0001
Active	1.00 (reference)	0.81 (0.53-1.23)	1.45 (1.01-2.08)	0.0661
Total	1.00 (reference)	1.02 (0.84-1.24)	1.49 (1.23-1.82)	0.0001

ANA, antinuclear antibodies; CI, confidence interval.
[a]Smoking exposure categories were based on serum cotinine concentrations (None, ≤0.05 ng/mL; Passive, >0.05 to 10 ng/mL; and Active, >10 ng/mL).
[b]ANA time trend assessments were based on two logistic regression models for ANA positivity (yes/no). Each model adjusted for the survey design variables (sampling weights, strata, and clusters) and categorical covariates for sex, age, and race/ethnicity. One model added a categorical covariate for time period, which allowed estimates of the ANA prevalence odds ratio for each period relative to the first period. The other model instead added a continuous covariate for time, as measured by the number of years between period midpoints relative to the first period, and produced a p-value from a t-test to assess a linear ANA time trend. Both models were applied initially to all participants with data on ANA regardless of data on smoking exposure (Total) and then within subgroups with data on both ANA and smoking exposure (None, Passive, and Active).

slope in all three subgroups but was steeper for never and former smokers than for current smokers (Figure 1). Also, the decrease over time was statistically significant for both never and former smokers ($p<0.0001$), but not for current smokers ($p=0.08$). Thus, on average, current smokers had cotinine levels that were high and fairly constant over time, while former and never smokers had levels that were low and decreasing, likely due to steady reductions in secondhand smoke exposure. Similar results were obtained when excluding participants under age 20 years (bottom half of Supplementary Table S2) to account for smoking history data being available for different age ranges across time periods (≥17 years in Period 1, ≥12 years in Period 2, and ≥20 years in Period 3).

Rather than focusing on means, Figure 2 displays kernel density estimates of the entire distribution of cotinine concentrations by time period and smoking history. These plots clearly show the differences in cotinine levels for never and former smokers (low) versus current smokers (high), as well as the consistency over time for current smokers. The cotinine distributions for never and

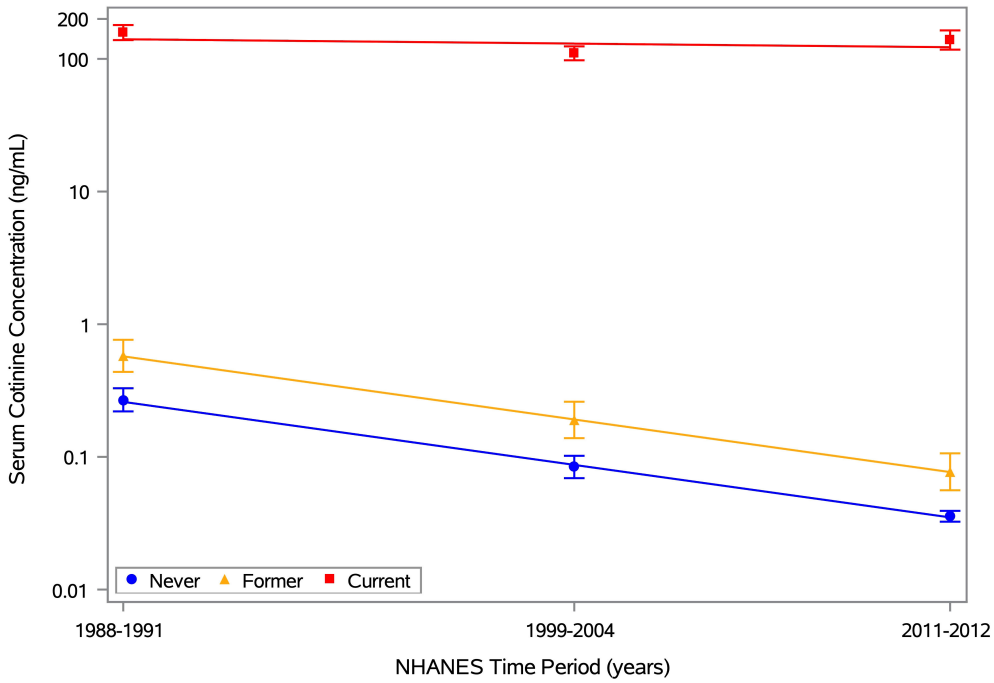

FIGURE 1
Mean serum cotinine concentration by time period and smoking history. Estimates of the geometric mean serum cotinine concentration and its 95% CI are plotted for each of 3 time periods (1988-1991, 1999-2004, and 2011-2012), along with the best-fitting trend line. Separate estimates are shown for self-reported never, former, and current smokers, based on the 12,063 NHANES participants aged ≥12 years with data on ANA, serum cotinine, and smoking history. The means for never, former, and current smokers are depicted by blue circles, yellow triangles, and red squares, respectively, with the same colors used for the 95% CI error bars and trend lines. Any concentration below the limit of detection (LOD) was replaced by an imputed value equal to LOD/ $\sqrt{2}$. The horizontal axis is linear in time, defined as the number of years between the midpoints of the participant's period and the first period, and the vertical axis is logarithmic in serum cotinine concentration (ng/mL).

former smokers were less consistent, with a notable shift toward lower values as time progressed. Much of this shift was likely due to many never and former smokers having cotinine levels below the LOD, which decreased from 0.05 to 0.015 ng/mL in the second time period. Nondetectable levels were replaced by imputed values of 0.035 and 0.011 ng/mL, respectively, which match well with the peaks of the period-specific cotinine distributions for never smokers. The cotinine distributions were more spread out for former smokers than for never smokers, perhaps due to a larger proportion of former smokers interacting with a current smoker.

3.3 Smoking time trend

Cigarette smoking in the US has decreased for a half-century (16, 17). We confirmed this downward trend in the NHANES data by examining the proportions of active smokers (defined by cotinine levels) and current smokers (based on self-reports), both of which clearly decreased over time. Unadjusted period-specific estimates of smoking prevalence for both classifications demonstrated similar decreases among all participants and among adults only (Supplementary Table S3), as did covariate-adjusted estimates of the smoking prevalence ORs for time period (Supplementary Table S4).

3.4 ANA time trends by smoking exposure

Estimates of ANA prevalence exhibited different temporal patterns in the three smoking exposure subgroups. For individuals with no exposure, these estimates were highest but did not show a clear trend; for passive exposure, they were intermediate and increased steadily across all periods from 11.1% (95% CI: 9.6-12.8%) to 12.7% (95% CI: 10.5-15.4%) to 15.5% (95% CI: 13.2-18.1%); and for active exposure, they were lowest and initially flat but then rose markedly from 7.4% (95% CI: 5.6-9.7%) in Period 2 to 13.3% (95% CI: 11.0-15.9%) in Period 3 (Table 1). Covariate-adjusted estimates of the ANA prevalence OR for Period 3 relative to Period 1 were 0.99 (95% CI: 0.62-1.57) for no exposure, 1.72 (95% CI: 1.33-2.22) for passive exposure, and 1.45 (95% CI: 1.01-2.08) for active exposure (Table 2). When assessing a linear trend in ANA prevalence across all three periods, the p-values for the three exposure subgroups were 0.2139, 0.0001, and 0.0661, respectively (Table 2). We reported similar estimates previously (7), but with smoking exposure categories defined by slightly different cutpoints for cotinine concentration.

To investigate whether age modified the association between smoking and temporal patterns of ANA, in addition to stratifying by smoking exposure, we further stratified by three age groups (12-19, 20-49, and ≥50 years) instead of including a categorical covariate for

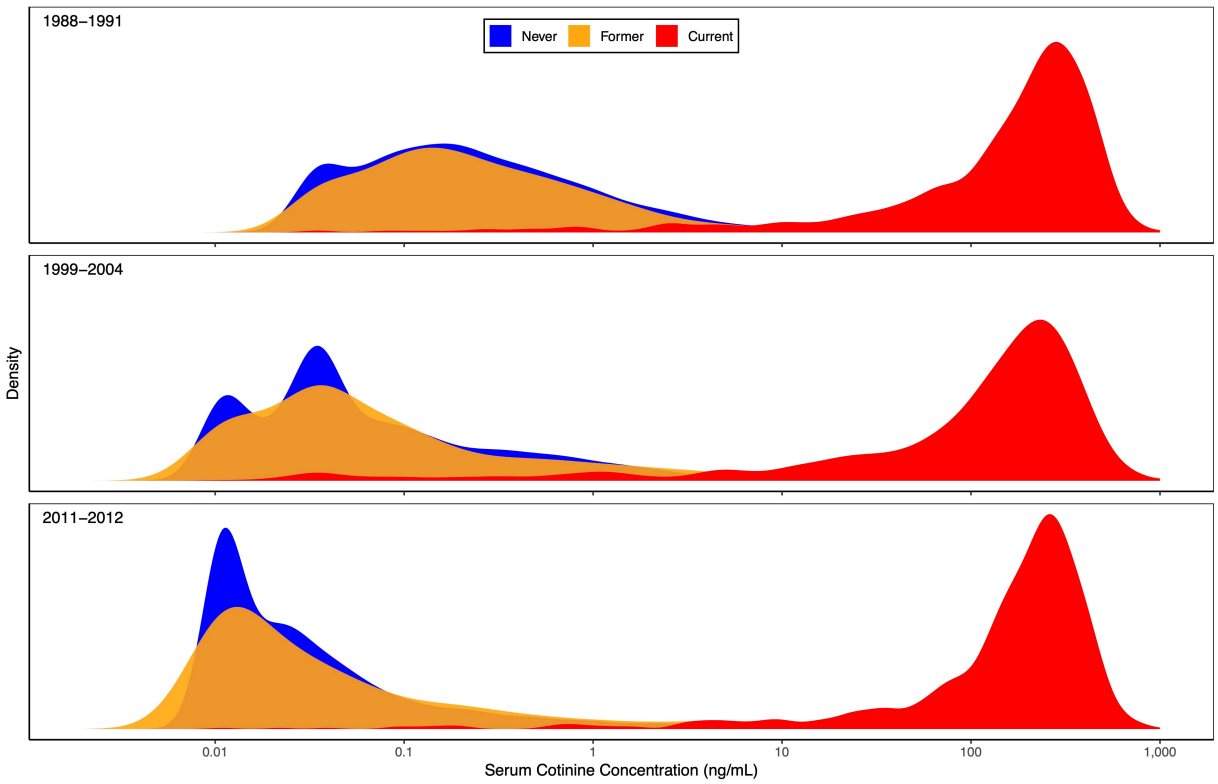

FIGURE 2
Serum cotinine concentration distribution by time period and smoking history. Kernel density estimates of the entire serum cotinine concentration distribution are plotted for each of 3 time periods (1988-1991, 1999-2004, and 2011-2012). Separate estimates are shown for self-reported never (blue), former (yellow), and current (red) smokers, based on the 12,063 NHANES participants aged ≥12 years with data on ANA, serum cotinine, and smoking history. Any concentration below the limit of detection (LOD) was replaced by an imputed value equal to LOD/ $\sqrt{2}$. The horizontal axis is logarithmic in serum cotinine concentration (ng/mL).

age. This approach essentially allowed for interactions between age and the covariates (sex, race/ethnicity, and time period). Despite the larger number of subgroups leading to smaller counts within each, there was statistical evidence that the observed increase in ANA prevalence over time was associated mainly with 12–19 year-olds who were passive (p=0.005) or active (p=0.003) smokers (Table 3). Among adolescents and relative to Period 1, the ORs and 95% CIs for passive smokers were 1.63 (0.83-3.23) in Period 2 and 2.64 (1.37-5.08) in Period 3, and for active smokers they were 3.01 (0.53-17.3) in Period 2 and 9.92 (2.20-44.7) in Period 3. The wide CIs are indicative of the small counts, but the ORs are large, especially for adolescents who were active smokers (which would have included vapers), for whom the odds of being ANA positive were roughly 10 times greater in Period 3 compared with Period 1. The differences across age categories, based on assessing an interaction between age group and time period, were statistically significant (p=0.009).

3.5 ANA associations with smoking by time period

In an alternative covariate-adjusted analysis, we focused on smoking status and assessed the odds of ANA positivity for smokers relative to nonsmokers (Table 4). Overall, smokers were less likely to have ANA than nonsmokers (OR=0.73; 95% CI: 0.58-0.92; p=0.007). When stratified by time period, the ANA prevalence ORs for smoking status varied in magnitude and statistical significance but not in direction. The odds of having ANA were significantly lower for smokers than nonsmokers in Period 2 (OR=0.65; 95% CI: 0.45-0.93; p=0.020), but that inverse association was weaker and not statistically significant in Period 1 (OR=0.80; 95% CI: 0.52-1.22; p=0.297) and Period 3 (OR=0.82; 95% CI: 0.56-1.21; p=0.310). This nonmonotonic temporal pattern is illustrated in Figure 3, where ANA prevalence estimates are smaller for smokers than nonsmokers in all three time periods, but the difference is much greater in Period 2 than in Periods 1 and 3.

3.6 Additional analyses

We performed several sensitivity analyses by adding covariates to a base model that was adjusted for sex, age, race/ethnicity, and time period (Supplementary Table S5). Rather than stratifying by smoking exposure, including it as a categorical covariate led to the same basic pattern of ANA prevalence not changing much between Periods 1 and 2, followed by a marked increase in Period 3. When we also added a smoking-by-period interaction, the main effects of both smoking and period were statistically significant, but the interaction was not. On the other hand, excluding time period significantly worsened the model fit (p<0.0001), suggesting that calendar time was important and that smoking on its own could not fully explain the observed ANA differences.

We also performed secondary analyses that accounted for BMI, which had been shown previously to modify ANA time trends (7). Adding a 3-level categorical covariate for BMI (underweight/healthy, <25; overweight, 25 to <30; or obese, ≥30) to the base

TABLE 3 Covariate-adjusted assessments of ANA time trends by smoking exposure and age group.

Smoking	ANA Prevalence Odds Ratio for Time Period (95% CI) [b]			Time Trend
Exposure [a]	Period 1: 1988-1991	Period 2: 1999-2004	Period 3: 2011-2012	p-value [b]
Age Group 1: 12–19 years old				
None	1.00 (reference)	3.64 (0.99-13.4)	2.84 (0.75-10.8)	0.5002
Passive	1.00 (reference)	1.63 (0.83-3.23)	2.64 (1.37-5.08)	0.0047
Active	1.00 (reference)	3.01 (0.53-17.3)	9.92 (2.20-44.7)	0.0032
Age Group 2: 20–49 years old				
None	1.00 (reference)	0.39 (0.21-0.72)	0.58 (0.32-1.06)	0.9447
Passive	1.00 (reference)	1.09 (0.70-1.70)	1.53 (0.91-2.60)	0.1435
Active	1.00 (reference)	0.73 (0.43-1.24)	1.28 (0.82-2.00)	0.4474
Age Group 3: ≥50 years old				
None	1.00 (reference)	0.87 (0.47-1.60)	1.29 (0.70-2.36)	0.1303
Passive	1.00 (reference)	1.37 (0.91-2.06)	1.41 (0.96-2.06)	0.0452
Active	1.00 (reference)	0.85 (0.48-1.47)	1.43 (0.78-2.63)	0.2353

ANA, antinuclear antibodies; CI, confidence interval.
[a]Smoking exposure categories were based on serum cotinine concentrations (None, ≤0.05 ng/mL; Passive, >0.05 to 10 ng/mL; and Active, >10 ng/mL).
[b]ANA time trend assessments were based on two logistic regression models for ANA positivity (yes/no). Both models stratified by smoking exposure and age group, and both adjusted for the survey design variables (sampling weights, strata, and clusters) and categorical covariates for sex and race/ethnicity. One model added a categorical covariate for time period, which allowed estimates of the ANA prevalence odds ratio for each period relative to the first period. The other model instead added a continuous covariate for time, as measured by the number of years between period midpoints relative to the first period, and produced a p-value from a t-test to assess a linear ANA time trend.

TABLE 4 Covariate-adjusted assessments of ANA associations with smoking status by time period.

Time Period	ANA Prevalence Odds Ratio for Smoking Status (95% CI) [a]		p-value [a]
	Nonsmoker	Smoker	
Period 1: 1988-1991	1.00 (reference)	0.80 (0.52-1.22)	0.297
Period 2: 1999-2004	1.00 (reference)	0.65 (0.45-0.93)	0.020
Period 3: 2011-2012	1.00 (reference)	0.82 (0.56-1.21)	0.310
All Periods Combined	1.00 (reference)	0.73 (0.58-0.92)	0.007

ANA, antinuclear antibodies; CI, confidence interval.
[a]Assessments of the association between ANA and smoking status were based on a logistic regression model for ANA positivity (yes/no) that adjusted for the survey design variables (sampling weights, strata, and clusters) and categorical covariates for sex, age, and race/ethnicity. The model also included a categorical covariate for smoking status, as defined by serum cotinine concentrations (Nonsmoker, ≤10 ng/mL; Smoker, >10 ng/mL), which allowed estimates of the ANA prevalence odds ratio for smokers relative to nonsmokers. The model was applied separately for each time period and also for all periods combined. The p-value for assessing statistical significance was based on a t-test.

model did not change the ANA time trends, nor did also adding a BMI interaction with any factor in the base model or in an expanded model that also included a covariate for smoking exposure (Supplementary Table S5). Similarly, the original ANA time trends within smoking exposure subgroups (as shown in Table 2) did not change much when augmenting the base model with covariates for a BMI main effect and a BMI-by-age interaction (Supplementary Table S6).

Replacing the 8-category age covariate with a 3-category age covariate, a quantitative age covariate, or a restricted cubic spline in age did not alter the basic pattern of ANA prevalence being relatively flat between Periods 1 and 2, and then increasing substantially in Period 3 (Supplementary Table S5). Similarly, that basic ANA pattern also remained consistent when other covariates were added to the base model, such as an individual main effect for alcohol intake, PIR, or education; those same main effects plus a

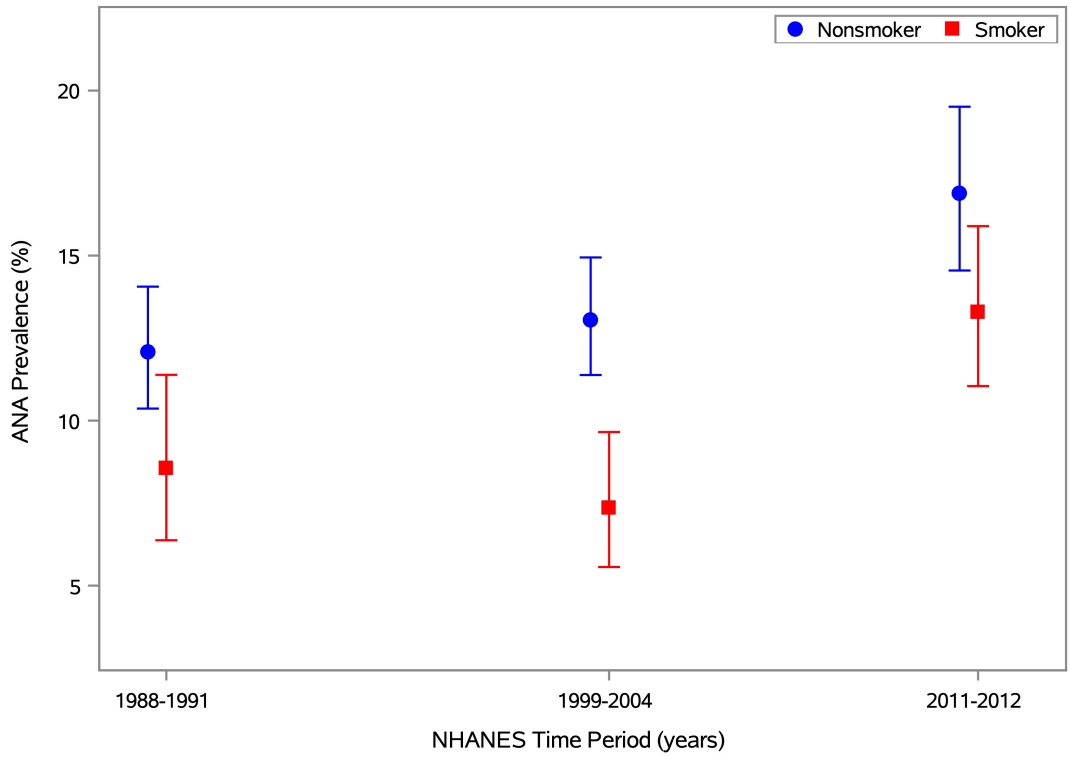

FIGURE 3
ANA prevalence by time period and smoking status. Estimates of ANA prevalence and its 95% CI are plotted for smokers and nonsmokers in Period 1 (1988-1991), Period 2 (1999-2004), and Period 3 (2011-2012), based on the 13,288 NHANES participants aged ≥12 years with data on both ANA and smoking status (i.e., serum cotinine). The prevalence estimates for nonsmokers and smokers are shown by blue circles and red squares, respectively, with the same colors used for the 95% CI error bars. Separately for each smoking status, the prevalence estimates and 95% CIs were derived from a logistic regression model for ANA positivity, adjusted for the survey-design variables and a categorical covariate for time period. The horizontal axis is linear in time, defined as the number of years between the midpoints of the participant's period and the first period, and the vertical axis is linear in ANA prevalence (as a percentage).

main effect for BMI; and those same main effects plus both a main effect for BMI and an interaction between BMI and each of those other covariates.

Data were available on the CO content in the brand of cigarettes used by each of 1,157 current smokers from Periods 2 and 3 aged ≥20 years. After adjusting for sex, age, race/ethnicity, and time period, there was mild evidence that ANA prevalence decreased as CO content per cigarette increased (OR=0.92; 95% CI: 0.85-1.00; p=0.042). We also multiplied CO content by average number of cigarettes smoked per day to estimate total CO, but the covariate-adjusted analysis showed no evidence of an association with ANA (p=0.65). In additional covariate-adjusted analyses of participants of all ages from all time periods, we found no evidence of an ANA association with pack-years among 4,795 ever (former or current) smokers (p=0.91), years of smoking among 5,047 ever smokers (p=0.18), or years since quitting among 2,738 former smokers (p=0.56). We also allowed for various pack-year threshold values, but no ANA associations with pack-years were significant.

4 Discussion

In summary, we assessed representative US data regarding ANA, time, smoking, CO and their interdependencies. Table 5 lists several relevant concepts and results, including the following information. High-titer ANA are associated with autoimmune diseases (1–6). In the US, ANA increased from 1988 to 2012, primarily in the second half of that interval (7); both active and passive exposure to smoke from regular cigarettes decreased during those years (16, 17); and e-cigarette use rapidly increased after being introduced in 2007 (15, 33–36), especially among adolescents (ages 12–19 years). CO may protect against ANA and certain autoimmune diseases (18–20, 25–32). E-cigarettes deliver much less CO than regular cigarettes (39, 40).

Our general observation is that something related to smoking cigarettes appears to have been inversely associated with ANA and any potentially protective effect waned in the later time period, possibly because people were smoking less and vaping more, or because something else about smoking changed. In most cases, our use of the word "protective" refers to a statistical association and not a proven biological protection. We hypothesize that reduced CO from decreased exposure to cigarette smoke may account for some of the overall increase in ANA. This reduction in CO could have come from current smokers cutting back on their cigarette consumption (including some degree of switching to vaping), from former smokers who quit (and possibly switched to vaping), and from never or passive smokers being exposed to less secondhand smoke (due to regulations and social pressure). We also hypothesize that the rapid increase in e-cigarette use after 2007, especially among teenagers, may partially explain why the increase in ANA prevalence was larger during the latter half of the study years and why the increasing ANA time trend was the most pronounced in teenagers (7). Our two-part hypothesis is consistent with what is already known about ANA, smoking, and CO, as well as with the results from our analyses of the NHANES data. Specifically, we assessed how the ANA time trend depended on smoking exposure levels, including within age subgroups, and how the ANA association with smoking depended on calendar time. Both are described below.

TABLE 5 Concepts and results related to the hypothesis that decreased cigarette smoking may partially explain the increased prevalence of antinuclear antibodies in the United States.

1. High-titer antinuclear antibodies (ANA) are associated with some autoimmune diseases, and ANA prevalence estimates increased over time: a little between Period 1 (1988-1991) and Period 2 (1999-2004) and a lot between Period 2 and Period 3 (2011-2012).
2. Viewing ANA time trends by smoking exposure, there was no clear trend over time in ANA prevalence estimates for individuals with no exposure (negligible serum cotinine), a steady increase for individuals with passive exposure (low serum cotinine), and a flat-then-increasing trend for individuals with active exposure (high serum cotinine). The ANA time trends among passive and active smokers were associated mainly with 12–19 year-olds.
3. Viewing ANA associations with smoking by time period, the estimated odds of having ANA were less among active smokers (high serum cotinine) than nonsmokers (negligible or low serum cotinine) in all time periods, but only the difference in Period 2 was statistically significant.
4. Serum cotinine steadily decreased over time, primarily in self-reported never and former smokers, but not in self-reported current smokers.
5. Smoking of regular cigarettes and secondhand exposure to their smoke steadily decreased over time.
6. Vaping of electronic cigarettes (e-cigarettes) began after Period 2 (in 2007) and rapidly increased over time.
7. Both regular cigarettes and e-cigarettes deliver nicotine and hence produce cotinine, but e-cigarettes produce much less carbon monoxide (CO) than regular cigarettes.
8. Some studies suggest that low levels of CO may be protective against ANA and certain autoimmune diseases.
9. In summary, less smoking of regular cigarettes may have led to less low-level CO exposure and more ANA. The hypothesized explanation involving potential CO protection against ANA is consistent with the observed patterns of ANA prevalence estimates, the long-term decreases in secondhand smoke exposure, and the recent increases in vaping, especially among adolescents (12–19 years old).

The ANA time trends across the three smoking-exposure subgroups (as defined by serum cotinine level) are consistent with our hypothesis. Individuals with no smoking exposure had negligible cotinine levels and presumably were not affected by changes in vaping or secondhand smoke. Thus, we infer that their exposure to CO from cigarette smoke was minimal and, consistent with our hypothesis, their ANA prevalence showed no clear time trend. Individuals with passive exposure to smoke had detectable but relatively low cotinine levels, which means they would have been affected by changes in secondhand smoke but probably were not regular vapers. Hence, these individuals might have experienced a small but steady increase in ANA prevalence across all time periods, which we speculate could be due to the steady decrease in their low-level CO "protection" from decreasing secondhand smoke (and possibly also from reduced exposure via air pollution (https://www.epa.gov/air-trends/carbon-monoxide-trends)). Active smokers had high cotinine levels, which could result from either regular cigarettes or e-cigarettes, and would have been affected by

changes in vaping but not secondhand smoke. Thus, these individuals presumably would not have had any change in potential CO protection or ANA prevalence between Periods 1 and 2, since vaping did not begin until 2007, but would have had a decrease in potential CO protection and, consistent with our hypothesis, a corresponding increase in ANA prevalence between Periods 2 and 3, as some of them took up vaping. Therefore, our hypothesis regarding potential smoking-associated CO protection from ANA is consistent with the possibility that the observed ANA patterns could be at least partially explained by the continued decrease in secondhand smoke exposure and the recent increase in vaping. In fact, when viewed by age group, the largest increase in ANA prevalence was between Periods 2 and 3 in teenagers who were active smokers, the timeframe and age group most associated with vaping.

The ANA associations with smoking seen across the three time periods are also consistent with our hypothesis and may relate to events that affected nonsmokers in the early years and smokers in the later years. Active smokers had significantly lower odds of having ANA than nonsmokers in Period 2, as would be expected if CO is protective, but this evident reduction was weaker (and not significant) in Periods 1 and 3. Between the first two periods, secondhand smoke exposure decreased (which would only affect nonsmokers) but vaping had not yet been introduced (which could only affect smokers who later started switching to e-cigarettes). All smokers had active smoking exposure, but nonsmokers were a mix of individuals with no exposure and passive exposure. The proportion of nonsmokers with passive exposure decreased over time, as presumably did their potential CO protection from secondhand smoke, and thus their ANA prevalence would have increased. However, neither potential CO protection nor ANA prevalence would have changed among smokers. Hence, the odds of having ANA for smokers versus nonsmokers would be smaller in Period 2 than in Period 1 (as we observed). Between Periods 2 and 3, secondhand smoke exposure again decreased (which would only affect nonsmokers) while vaping increased rapidly (which would mainly affect cotinine-identified active smokers). As described above, the level of potential CO protection from secondhand smoke among nonsmokers would have decreased, increasing their ANA prevalence. Concurrently, potential CO protection among active smokers (some of whom were vapers) would also have decreased due to increased vaping, and thus their ANA prevalence would have increased. The increase in ANA due to increased vaping among smokers could have more than offset the increase in ANA due to decreased secondhand smoke among nonsmokers, resulting in the ANA prevalences for smokers and nonsmokers to appear more similar in Period 3 than in Period 2 (as we observed).

Although we hypothesize that decreased CO and increased vaping may help explain both the changes in ANA time trends across smoking exposure levels and the changes in ANA associations with smoking across time periods, other factors may also have played a role. For example, cigarette smoke is composed of many chemicals with a wide array of effects on the body and we have an incomplete understanding of their immune impacts that could include both stimulatory and inhibitory elements that may vary from product to product (18, 45). Also, certain components of e-cigarettes, such as flavoring agents (46), may potentially increase the risk of developing ANA in users, and vaping may introduce additional chemical contaminants contributing to bystander health effects from secondhand exposure (47). In addition to vaping, there are other nicotine-delivering alternatives to regular cigarettes, including nicotine gum, chewing tobacco, snuff, and snus (48), that can have immune system effects (49). Another consideration is that some ANA subtypes may be more relevant than others. In a previous study (50), we found that time period and smoking exposure were more strongly associated with anti-dense fine speckled 70 autoantibodies than with total ANA. Miller (51) discussed a wide range of other potentially relevant factors such as elements of the environment, various lifestyles, and even climate change that could impact recent increases in autoimmunity and autoimmune diseases.

Our study had several strengths. The NHANES cohort with data on ANA was very large and spanned 25 years (1988–2012), with all ANA assays performed in the same laboratory, using the same evaluators, methods, and equipment. All statistical analyses were weighted to enable analytic results that generalize to the civilian noninstitutionalized US population ≥12 years old. Many of our analyses of ANA, cotinine, and smoking accounted for sex, age, and race/ethnicity as potential correlates or modifiers, and some analyses also adjusted for BMI, alcohol intake, PIR, or education.

On the other hand, our descriptive findings are subject to certain limitations. There may be concerns about the age of serum samples used for ANA assessment, some of which were nearly three decades old when assayed. However, there were no gross differences in appearance or behavior to suggest degradation, and antibodies are stable over time in frozen storage (52). Some NHANES data were obtained from questionnaires, such as smoking history, but self-reported nicotine product use has been shown to be valid (53). As vaping has increased, high cotinine levels have become less reliable for identifying persons who only smoke regular cigarettes (and thus are exposed to more CO). We considered using self-reported smoking history instead, but that information was often missing and it was not clear whether persons who replaced some or all of their regular cigarettes with e-cigarettes would classify themselves as former or current smokers. Also, we used 10 ng/mL of cotinine to distinguish passive and active smokers, as recommended by the CDC and EPA (13), but some researchers have suggested using a lower cutpoint, such as 3 ng/mL (54). However, our sensitivity analysis found that using the lower cotinine cutpoint had little effect on the results. No participant was followed longitudinally; thus, both cotinine and ANA were assessed cross-sectionally at only one point in time per participant, so measured cotinine levels may poorly reflect the levels when ANA developed. Reported associations, even if confirmed, may not correspond to causal effects. In fact, there could be reverse-causal effects if immune system or other changes associated with ANA influence smoking behavior or the metabolism of nicotine, cotinine, or other byproducts of smoking.

Perhaps the most serious deficiency in our data is the lack of direct information about e-cigarette use. At the time of our analyses, there were limited NHANES data on vaping in the 2013-2014, 2015-2016, and 2017–2018 cycles, but none in cycles with data on ANA. However, despite this absence of direct data, we might assume that most self-reported current smokers in Period 3 with a high cotinine level probably smoked regular cigarettes, whereas most self-reported former smokers with a high cotinine level had probably switched to e-cigarettes. The first group included 89/639 (13.9%) with ANA, while the second group included 18/85 (21.2%) with ANA, a difference that is consistent with our hypothesis of a potentially protective effect of CO derived from smoking regular cigarettes (and also consistent with an effect of something in e-cigarettes on ANA). Also, direct information on individual CO levels would have been helpful, though we found some evidence that lower ANA prevalence was associated with cigarette brands having higher CO content, which provides additional indirect support for our hypothesis.

In conclusion, cigarette smoking decreased over the past several decades and ANA prevalence increased, which we corroborated with analyses of NHANES data. However, the degree to which these two time trends might be causally related is unclear. Cotinine was used to infer exposure to cigarette smoke, and average levels steadily declined between 1988 and 2012 in the NHANES cohorts, with a downward-sloping straight line providing a good fit to log-transformed cotinine concentrations. The prevalence of ANA rose between 1988 and 2012, but this upward trend was not linear, showing a relatively small increase from 1988–1991 to 1999-2004, followed by a much larger increase from 1999–2004 to 2011-2012. The latter time interval coincides with the introduction of vaping, with many smokers replacing at least some of their regular cigarettes with e-cigarettes. That change might not have affected cotinine levels but should have reduced CO levels. We suggest that such a drop in CO levels potentially could be causally associated with the concurrent increase in ANA, as there is evidence that low levels of CO are protective against ANA and certain autoimmune diseases. However, while CO may be one factor in this process, one should keep in mind that there are many additional byproducts of smoking that possibly could play a role. Nonetheless, decreased smoking exposure (active and passive) across all study years could have contributed to a general increase in ANA, which could have been greatly supplemented in the later years by the rapid increase in vaping. Thus, smokers who reduced their use of regular cigarettes in favor of vaping may have lost some of the hypothesized protective effect afforded by CO, which could have increased their risk of developing autoimmunity.

We searched the literature for additional mechanisms and contributing factors that might help explain why decreased smoking could lead to increased ANA and found conflicting data on the complex mixtures that make up tobacco smoke and e-cigarette vapor. One parallel mechanism to CO is nicotine itself. Reduced cigarette smoking, if not replaced by other nicotine sources (48), would decrease the nicotine anti-inflammatory processes, which could then increase inflammation and ANA. For example, despite smoking being an established risk factor for rheumatoid arthritis (RA), several investigators have discussed a possible therapeutic effect of nicotine on RA (55–57). In the end, we concluded that exact mechanisms for why less smoking is associated with more ANA are unclear and further research is needed to identify the causes of the recent dramatic increases in ANA in the US. Hopefully, future studies will collect data on vaping history and CO biomarkers, which could provide direct evidence to assess our hypothesis.

In closing, given the many negative effects of smoking on increasing deaths, illnesses, and health care costs worldwide, we are certainly not recommending that smoking should be considered as an approach to prevent autoimmunity or autoimmune diseases. Rather, we believe that further studies in this area are needed as they may elucidate new mechanisms, perhaps involving certain components of tobacco smoke or e-cigarette vapor, that could allow for the development of novel preventative or treatment measures in the future.

Data availability statement

All data are publicly available. The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by CDC Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from the CDC NHANES study. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

GD: Conceptualization, Writing – review & editing, Formal analysis, Methodology, Resources, Writing – original draft, Software, Visualization. CW: Conceptualization, Methodology, Writing – review & editing. CP: Conceptualization, Methodology, Writing – review & editing. CC: Methodology, Writing – review & editing, Formal analysis, Data curation, Software. JP: Formal analysis, Methodology, Writing – review & editing, Software, Visualization. EC: Writing – review & editing, Conceptualization, Data curation, Resources. FM: Conceptualization, Writing – review & editing, Funding acquisition, Project administration.

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Conflict of interest

Authors GD, CC, and JP were employed by DLH, LLC.

The remaining 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.

The reviewer GK declared a past co-authorship with the author EC to the handling editor.

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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.2025.1537043/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Mean Serum Cotinine Concentration by Time Period. Estimates of the geometric mean serum cotinine concentration and its 95% CI are plotted for each of 3 time periods (1988-1991, 1999-2004, and 2011-2012), along with the best-fitting trend line. These estimates are based on the 13,288 NHANES participants aged ≥12 years with data on both ANA and serum cotinine. Any concentration below the limit of detection (LOD) was replaced by an imputed value equal to LOD/ $\sqrt{2}$. The horizontal axis is linear in time, defined as the number of years between the midpoints of the participant's period and the first period, and the vertical axis is logarithmic in serum cotinine concentration (ng/mL).

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