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Sex bias in autoimmunity: From animal models to clinical research and applications

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Sex bias in autoimmunity: From animal models to clinical research and applications

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Table of contents

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Editorial: Sex bias in autoimmunity: From animal models to clinical research and applications

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Editorial on the Research Topic
Sex bias in autoimmunity: From animal models to clinical research and applications

Considerable progress in generating and reporting scientific data disaggregated by sex has been achieved in the last decades since the Food and Drug Administration (FDA) recommendation in 1998 to provide the age, sex, and ethnicity of all participants in clinical trials. The increasing focus on recognizing sex differences in immune responses is particularly relevant for autoimmune diseases, such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS) and rheumatoid arthritis (RA) which typically affect women more than men. We are beginning to understand key differences in the immune system that may help to explain sexual dimorphisms in autoimmune disease risk and manifestations, as well as prognosis, complications, and response to treatment. There is also an increased interest in investigating how changes in human physiology (e.g., during pregnancy, lactation, menopause) and other sex and gender differences, pertaining to socio-economic and educational factors, as well as access to care and health-related behaviors can affect the immune system, advocating the need for sex and gender inclusive research. Unique gender-diverse human cohorts and animal models are aiding our understanding of how sex and gender can influence the immune system and various metabolic pathways relevant to autoimmunity (Figure 1).

There is convincing literature evidence that sex hormones differentially regulate the phenotype of various immune cells *in vitro* with implications for the pathogenesis of SLE, a disease with a profound female bias (Kim et al.). Both estrogen and progesterone have been shown to promote type-I interferon and Toll-like receptor pathway immune activation, whilst testosterone enhances T-helper 1 responses. Sex determinants also impact the clinical presentation of autoimmune rheumatic diseases,

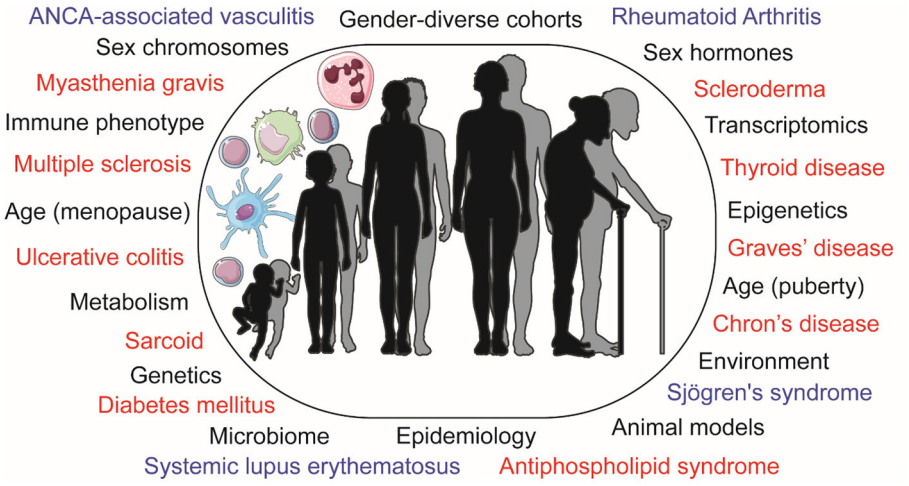

FIGURE 1
Schematic representation of the differences observed in immunology and autoimmune disease risk by sex and age, displaying pathophysiological influences and research methods used to explore these in black. Autoimmune diseases that present with a sex bias are displayed in blue (for those covered in this article) or red. ANCA, anti-neutrophil cytoplasmic antibodies.

as males with SLE present more commonly with skin involvement and renal damage (Kim et al.). The impact of endogenous and exogenous sex hormones in RA remains controversial, with the majority of studies investigating only female patients or providing conflicting evidence regarding their impact on RA risk or outcomes, despite the widely recognized anti-inflammatory properties of both androgens and progesterone, and the dichotomous effects of estrogens on immune cell functions (Raine and Giles).

An interesting area of research is the possible adverse pregnancy outcomes associated with autoimmune diseases, such as SLE. Li et al. used transcriptomics to investigate differentially expressed genes (DEGs) in placental tissue from pregnant women with SLE vs. healthy controls, as well as between SLE pregnancies with male or female fetuses. The study identified a SLE disease signature and a SLE pregnancy signature, both disaggregated by the fetal sex, and associated with unique inflammatory pathways, suggesting that fetal-sex-specificity may contribute to the pathophysiology of pregnancy complications in SLE.

Despite the frequent exclusion of the X-chromosome from genome-wide association studies, genetics allows the interrogation of the impact of sex chromosomes on sexual dimorphisms relevant to autoimmunity. Hässler et al. developed a semiparametric additive hazard model accounting for skewed X-inactivation to investigate loci associated with time-to-event data in patients with autoimmune diseases treated with biologic therapies as part of the ABIRISK consortium. Two protective single nucleotide polymorphisms were identified, which can have implications in assessing the sex-biased risk for immunogenicity to biologic treatments.

Autoimmune rheumatic diseases are also characterized by sex bias related to their comorbidity risk. Bruno et al. analyzed data from over 13,000 records of patients with SS and identified that whilst a higher proportion of male patients developed cardiovascular disease (CVD), females frequently had fibromyalgia depression, hypermobility syndromes and migraine, as well as other autoimmune conditions, such as Raynaud's syndrome, SLE and systemic sclerosis.

One of the very few autoimmune rheumatic diseases with male predominance is ANCA (anti-neutrophil cytoplasmic antibody) associated vasculitis (AAV). A meta-analysis found that men with AAV have a 1.54-fold increase of 1-year mortality risk compared to females (Zhu et al.). Additionally, significant sex differences in age of onset, clinical outcome measures, and 1-year mortality rate were observed in a retrospective analysis, highlighting the need for improved sex-tailored early management strategies in AAV.

As the field of sex disaggregated research grows in its prominence, Peckham et al. call for the expansion of such research to include gender-diverse research participants. There is a growing proportion of society frequently excluded from medical and basic research, leading to epidemiological and clinical data that is not necessarily applicable to everyone. By expanding our study designs to include transgender and non-binary people, the authors emphasize the urgent need for long-term outcome data related to gender-affirming hormonal treatments and highlight multiple ways by which future research findings could be improved.

With respect to sex-tailored disease prevention and health care, inflammation and metabolism typically drive autoimmunity and CVD risk bias toward women and men,

respectively; however, women with SLE have an increased CVD risk compared to female healthy controls (50-fold). Robinson et al. review this paradox by exploring studies in gender-diverse cohorts and highlighting an estrogen-driven atheroprotective lipoprotein profile in post-pubertal women that is absent in pre-puberty and can be induced by gender-affirming sex hormones in transgender women. Strikingly, this atheroprotective lipid profile is lost in SLE, suggesting a compromise in an autoimmune setting. This highlights potential therapeutic targets to reduce CVD risk in SLE.

Animal models of health and disease have long complemented and enhanced data from human studies. The bidirectional relationship between sex hormones and gut-microbiota—both of which are known to influence autoimmunity development is explored by Rosser et al. in a review which highlights the impact of sex hormones and the potential to modulate the gut-microbiome to influence the course of autoimmune diseases. The observations encourage to consider the therapeutic potential of the complex interplay between the myriad of microbial species that inhabit our bodies and the immune and endocrine systems.

Our understanding of sexual dimorphisms in immune responses and metabolism relevant to autoimmunity has evolved with the accelerated use of complex multi-omic techniques and analysis methods, as well as access to gender-diverse cohorts and sex/gender-specific animal models. However, many questions remain unanswered and future efforts need to account for sex and gender in human and animal research.

Author contributions

GR, HP, and CC drafted the manuscript. All authors reviewed and approved the editorial.

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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Sexual Dimorphisms of Protein-Coding Gene Profiles in Placentas From Women With Systemic Lupus Erythematosus

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Background: Systemic lupus erythematosus (SLE) may cause pathogenic changes in the placentas during human pregnancy, such as decreased placental weight, intraplacental hematoma, ischemic hypoxic change, placental infarction, and decidual vasculopathy, which contribute to high maternal and fetal mortality and morbidity. Sex-specific adaptations of the fetus are associated with SLE pregnancies. The present study aimed to determine the transcriptomic profiles of female and male placentas from women with SLE.

Methods: RNA sequencing (RNA-seq) was performed to identify differentially expressed protein-coding genes (DEGs) in placentas from women with SLE vs. normal term (NT) pregnancies with female and male fetuses (n = 3-5/sex/group). Real-time-quantitative PCR was performed (n = 4 /sex/group) to validate the RNA-seq results. Bioinformatics functional analysis was performed to predict the biological functions and pathways of SLE-dysregulated protein-coding genes.

Results: Compared with NT-female (NT-F) placentas, 119 DEGs were identified in SLE-female (SLE-F) placentas. Among these 119 DEGs, five and zero are located on X- and Y-chromosomes, respectively, and four are located on the mitochondrial genome. Compared with NT-male (NT-M) placentas, 458 DEGs were identified in SLE-male (SLE-M) placentas, among which 16 are located on the X-chromosome and zero on the Y-chromosome and mitochondrial genome. Twenty-four DEGs were commonly dysregulated in SLE-F and -M placentas. Functional analysis showed that SLE-dysregulated protein-coding genes were associated with diverse biological functions and pathways, including angiogenesis, cellular response to growth factor stimulus, heparin-binding, HIF (hypoxia-inducible factor)-1 signaling pathway, and Interleukin-17 (IL-17) signaling pathway in both SLE-F and -M placentas. Biological regulations were differentially enriched between SLE-F and -M placentas. Regulation of blood circulation,

response to glucocorticoid, and rhythmic process were all enriched in SLE-F, but not SLE-M placentas. In contrast, tumor necrosis factor production, Th17 cell differentiation, and MDA (melanoma differentiation-associated gene)-5 signaling pathway were enriched in SLE-M but not SLE-F placentas.

Conclusion: This report investigated the protein-coding gene profiles of placenta tissues from SLE patients using RNA-seq. The results suggest that the SLE-dysregulated protein-coding genes in placentas may contribute to the pathophysiological progress of SLE pregnancies in a fetal sex-specific manner, leading to adverse pregnancy outcomes.

Keywords: systemic lupus erythematosus, protein-coding RNA, placenta, pregnancy, fetal sex

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that predominantly affects women of reproductive age, typically causing damages to multiple organ systems (1, 2). SLE pregnancies are associated with maternal complications (e.g., lupus flare, hypertension, preeclampsia, and eclampsia) and fetal complications (e.g., stillbirth, spontaneous abortion, fetal growth restriction, neonatal lupus, and neonatal deaths), and increase the induced abortion rate (3–5).

SLE pregnancies are associated with many placental dysfunctions, (e.g., decreased placental weight, intraplacental hematoma, chronic villitis, thickening of the trophoblast basement membrane, ischemic hypoxic change, placental infarction, decidual vasculopathy, and fetal thrombi) (3, 6). Many mechanisms underlying SLE-induced placental and fetal dysfunction have been proposed. The fetal-placental immune system could in turn, interact with the maternal immune system and mediate maternal immune response (7). For example, anti-DNA antibodies in SLE pregnancies may inhibit trophoblast attachment and migration via cross-reacting with laminin (8). Antiphospholipid antibodies may also alter the placental phospholipid membrane and cause infarctions and edema/swelling (9, 10). In addition, anti-SSA (Sjogren's-syndrome-related antigen A) and anti-SSB (Sjogren's-syndrome-related antigen B) antibodies can cause neonatal lupus and induce fetal injury after crossing the placenta (11). Thus, defects in the placental and fetal responses to autoimmune processes and inflammation are closely related to SLE pregnancy.

Sex-specific adaptations of the fetus have been reported in many complicated pregnancies such as SLE, asthma, and preeclampsia (12–14). Specifically, a lower proportion of male fetuses were born to women with SLE, which may be partially attributed to different chronic inflammation responses between male and female fetuses in early gestation (13). During asthma pregnancy, significantly reduced birth weights were observed in women with female, but not male fetuses (12), suggesting that the inflammatory activities of asthma impacted female, but not male fetuses. Recently, Zhou et al. demonstrated that preeclampsia impaired fetal endothelial function in a fetal sex-dependent manner (14). Given that the placenta is a key organ that closely regulates fetal growth and function, a sex-specific dysregulation of placental growth and function might contribute to the adverse pregnancy outcomes induced by SLE.

Different mechanisms may govern the fetal sex-specific adaptations in various complicated pregnancies. For example, Murphy et al. (12) have reported that female but not male fetuses born to mothers with asthma are associated with a significant increase of maternal circulating monocytes and decreases in placental steroid hydroxylase activity and fetal estriol. Zhou et al. (14) have also reported that fetal-sex specific expression of genes accompanied by preeclampsia-impaired fetal endothelial function. However, mechanisms controlling sex-specific adaptations of SLE pregnancies remain unknown.

In this study, we hypothesized that SLE alters gene expression of placentas, disturbing placental biological functions in a sex-specific manner. We determined the expression profiles of protein-coding genes of placentas from SLE and normal term (NT) pregnancies using RNA sequencing (RNA-seq). Real-time quantitative PCR (RT-qPCR) was conducted to verify RNA-seq results. Functional analysis was conducted to describe the underlying biological functions of differentially expressed protein-coding genes (DEGs) in female and male placentas from SLE pregnancies.

MATERIALS AND METHODS

Ethical Approval

All procedures were conducted in accordance with the Declaration of Helsinki. Two sets of placental tissues were collected from two hospitals. The first set (SLE, $n = 10$ with five female and five male fetuses) was collected in Qilu Hospital, Shandong University. The tissue collection protocol was approved and carried out in accordance with the regulation of the Institutional Review Board of Qilu Hospital, Shandong University. SLE was defined according to the American College of Rheumatology classification criteria (15). The SLE disease activity index (SLEDAI) (16) was used to assess the disease activity of SLE patients. SLEDAI scores were assessed within 1 week before delivery. The second set (NT, $n = 10$ with five female and five male fetuses) was collected in Shanghai First Maternity and Infant Hospital affiliated with Tongji University. The tissue collection protocol was approved and carried out in accordance with the regulation of the Ethical Committee

of Shanghai First Maternity and Infant Hospital affiliated with Tongji University. All individuals included in this study were Han Chinese without information on their ancestries. Smokers and patients with cancer or diabetes mellitus were excluded.

RNA Isolation and Quality Control

Placental tissues were obtained within 30 min after vaginal delivery or cesarean section delivery. Placental villi were dissected beneath the chorionic and basal plates (∼1 × 1 cm). Placental tissues were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from placental tissues using the RNeasy mini kit (Qiagen, Germany). The concentration and quality of RNA samples were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Samples with RNA integrity number (RIN) values ≥ 7.0 were used for sequencing.

RNA-Seq and Bioinformatics Analysis of Data

We performed RNA-seq analysis of total RNA samples from placentas (n = 3-5/sex/group; **Supplementary Table 1**) as described in supplementary methods. RNA-seq strand-specific libraries were constructed using the VAHTS Total RNA-seq (H/M/R) Library Prep Kit (Vazyme, China) following the manufacturer's instructions. Purified libraries were quantified and validated by Qubit 2.0 Fluorometer and Agilent 2100 bioanalyzer to confirm the insert size and calculate the mole concentration. The library construction and sequencing were performed by Sinotech Genomics Co., Ltd Shanghai, China). Overall, more than 66 million reads per sample were generated. Reads with non-canonical letters or low quality and sequences shorter than 25 nucleotides were removed. Reads were trimmed off using FASTQ software (17). Trimmed reads were mapped to the GRCH38 genome using the HISAT2 software (18). DEGs were identified and analyzed using Cuffdiff (19) and R package edgeR (20), respectively. The fold change (FC) was estimated according to the Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) values (21, 22). The P-value significance threshold was set according to the Benjamini-Hochberg false discovery rate (FDR). DEGs were selected using the following criteria: FC > |2| and FDR adjusted P-value (q-value) < 0.05 (14), were considered as significantly modulated and recognized as SLE-dysregulated genes. The RNA-seq data have been uploaded and deposited in NCBI (National Center for Biotechnology Information) Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/, accession number: GSE177029).

Functional Analysis of SLE-Dysregulated Genes

The Metascape online analysis tool (https://metascape.org/) was used to predict the biological functions and signaling pathways of the SLE-dysregulated genes (23). Biological terms were selected using the following criteria: P-value < 0.01, enrichment factor >1.5, and term count > 3. The most statistically significant (lowest p-value) biological terms within each cluster were chosen to represent the cluster in bar graphs. A subset of representative terms from the cluster was selected and converted as a network plot. Terms with a similarity score (23) > 0.3 were connected by an edge, and the thickness of the edge represents the similarity score. Cytoscape (v3.1.2) was used to visualize the network (24).

RT-PCR

Total RNA (400 ng/sample) was transcribed into complementary DNA (cDNA) using the HiScript II Q RT SuperMix for qPCR (Vazyme Biotech, cat#: R222-01). Diluted cDNA corresponding to 4 ng of original total RNA was utilized as the template in each RT-qPCR reaction. To validate the RNA-seq results, 10 candidate genes were selected for RT-qPCR analysis (25) (n = 4 /sex/group; **Supplementary Table 1**) using NuHi Robustic SYBR Green Mix. We chose these candidate genes based on the fold changes in SLE vs. NT, relevance to placental function (i.e., angiogenesis and immune responses), expression abundances, and different expression patterns in SLE-F and -M placentas according to RNA-seq data. Primers are listed in **Supplementary Table 2**. Data were normalized to *GAPDH* and then analyzed using the $2^{-\Delta\Delta CT}$ method (25, 26).

Correlation Analysis of SLE-Dysregulated Genes and SLEDAI Scores

After RT-qPCR verification, relative expression levels of SLE-dysregulated genes were used to analyze the correlation with SLEDAI scores.

Statistical Analyses

Microsoft Excel (2016) for Windows and SigmaPlot (13.0) for Windows were used for statistical analyses. Data were represented as the medians ± standard deviation (SD) or medians with range. Data were analyzed using student's t-test or Mann-Whitney Rank Sum Test when applicable. The relationship between the relative expression levels of genes and SLEDAI scores was analyzed by Pearson's correlation coefficient. P-values < 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

All SLE patients received maintenance corticosteroids (prednisone ≤ 15 mg daily) and hydroxychloroquine (≤ 400 mg daily) during pregnancy. Three patients in the SLE group were antiphospholipid positive and were on aspirin (100 mg daily) during pregnancy. One SLE patient was positive for anti-SSA antibody. None of the newborns developed neonatal lupus. Patient ages and body mass index (BMI) were similar between NT and SLE pregnancies. However, the newborn body weight for SLE-F was significantly lower than that for NT-F (P = 0.026) (**Table 1** and **Supplementary Table 1**). There was one sample from the SLE group with a growth-restricted male fetus (27). Two patients each in SLE and NT group underwent scheduled Cesarean section delivery. Demographic and clinical characteristics are shown in **Table 1** and **Supplementary Table 1**.

TABLE 1 | Clinical characteristics.

Characteristics	SLE ($n = 10$)	NT ($n = 10$)	*P*
Age (years), median (range)	29.0 (26–36)	30.5 (28–33)	0.667
BMI, median (range)	25.4 (23.0-32.3)	28.0 (21.8-32.7)	0.663
Gestation age (weeks), median (range)	38.5 (34.9-39.7)	39.1 (38.6-40.1)	0.053
Fetal weight (grams), median (range)	2950.0 (2150.0-3850.0)	3402.0 (2895.0-3730.0)	0.026
Disease duration (months), median (range)	40.0 (10–167)	-	-
SLEDAI score, median (range)	2.5 (0-6)	-	-
ANA > 1:320, yes/no (*n*)	10/0	-	-
Anti-dsDNA, yes/no (*n*)	2/8	-	-
Anti-SSA/SSB	1/9	-	-
Anti-phospholipid, yes/no (*n*)	3/7	-	-
Preeclampsia, yes/no (*n*)	0/10	-	-
Proteinuria, yes/no (*n*)	3/7	-	-
Hypocomplementemia, yes/no (*n*)	3/7	-	-

SLE, systemic lupus erythematosus; NT, normal term; BMI, body mass index; SLEDAI, systemic lupus erythematosus disease activity index; ANA, antinuclear antibody; SSA, Sjogren's-syndrome-related antigen A; SSB, Sjogren's-syndrome-related antigen B.

Distinct Transcriptional Profile in Placentas From SLE Pregnancies

Compared with NT, SLE dysregulated 119 protein-coding genes in female placentas (**Figure 1A**; **Supplementary Table 3**), among which five and zero are located on the X- and Y-chromosomes, respectively, and four are located on the mitochondrial genome. Compared with NT, SLE dysregulated 458 protein-coding genes in male placentas (**Figure 1A**; **Supplementary Table 3**), among which 16 are located on the X-chromosomes and zero are located on the Y-chromosome or mitochondrial genome (**Figure 1A**; **Supplementary Tables 3**, **4**).

Compared to NT-F, 77 and 42 genes were upregulated and downregulated in SLE-F placentas, respectively (**Figure 1B**; **Supplementary Table 3**). Compared to NT-M, 438 and 20 genes were upregulated and downregulated in SLE-M placentas, respectively (**Figure 1C**; **Supplementary Table 3**). Twenty-four genes were commonly dysregulated in SLE-F and -M placentas (**Supplementary Table 3**), among which 21were commonly upregulated in SLE-F and -M placentas. One (*MATR3*) was upregulated in SLE-F placentas but downregulated in SLE-M placentas, while two (*CP* and *LGALS3BP*) were upregulated in SLE-M placentas but downregulated in SLE-F placentas (**Supplementary Table 3**). None of these commonly dysregulated genes is on the X- chromosome, Y-chromosome or mitochondrial genome.

The correlation RT-qPCR and RNA-seq results was performed using the fold change (SLE/NT) of 10 selected genes in RT-qPCR and RNA-seq analyses. RT-qPCR data were correlated significantly with RNA-seq analysis ($r = 0.637$, $P < 0.05$) (**Figures 1D,E**). Specifically, *CTGF*, *VEGFA*, and *GH2* were upregulated in SLE-F and SLE-M placentas. *MMP1* was downregulated in SLE-F but upregulated in SLE-M placentas. *APOLD1* was upregulated in SLE-F but not in SLE-M placentas. *FN1*, *PLAC1*, and *PSG4* were upregulated in SLE-M but not in SLE-F placentas. SLE did not alter *C3* and *DUSP1* expression in female and male placentas.

Functional and Pathway Analyses of SLE-Dysregulated Genes in Placentas

SLE-dysregulated protein-coding genes were associated with diverse biological functions and pathways (284 for SLE-F placentas and 422 for SLE-M placentas) (**Supplementary Tables 5**, **6**). Sixty-two biological functions and pathways were commonly enriched in both SLE-F and SLE-M placentas (**Supplementary Tables 5**, **6**). These biological functions and pathways included angiogenesis, cellular response to growth factor stimulus, heparin-binding, kidney development, mononuclear cell differentiation, pathways in cancer, response to calcium ion, HIF (hypoxia-inducible factor)-1 signaling pathway, IL-17 signaling pathway, and PI3K-Akt signaling pathway (**Figures 2A,C**; **Table 2**, **Supplementary Tables 5**, **6**).

Differential pathway regulations were observed between SLE-F and -M placentas. Regulation of blood circulation, regulation of cellular response to stress, response to glucocorticoid, response to temperature stimulus, response to toxic substance, response to testosterone, rhythmic process, smooth muscle cell proliferation, GnRH (gonadotropin-releasing hormone) signaling pathway, and estrogen signaling pathway were enriched in SLE-F, but not SLE-M placentas (**Figures 2B,D**; **Table 2**, **Supplementary Tables 5**, **6**). In contrast, G protein-coupled chemoattractant receptor activity, tumor necrosis factor production, T cell mediated immunity, Th17 cell differentiation, IL-27-mediated signaling pathway, MDA (melanoma differentiation-associated gene)-5 signaling pathway, phospholipase D signaling pathway, transforming growth factor beta receptor signaling pathway, type I interferon signaling pathway, and Wnt signaling pathway were enriched in SLE-M, but not SLE-F placentas (**Figures 2B,D**; **Table 2**, **Supplementary Tables 5**, **6**). **Figures 2E,F** were networks that exhibited the interactions among cluster of genes enriched in biological processes and pathways mentioned above.

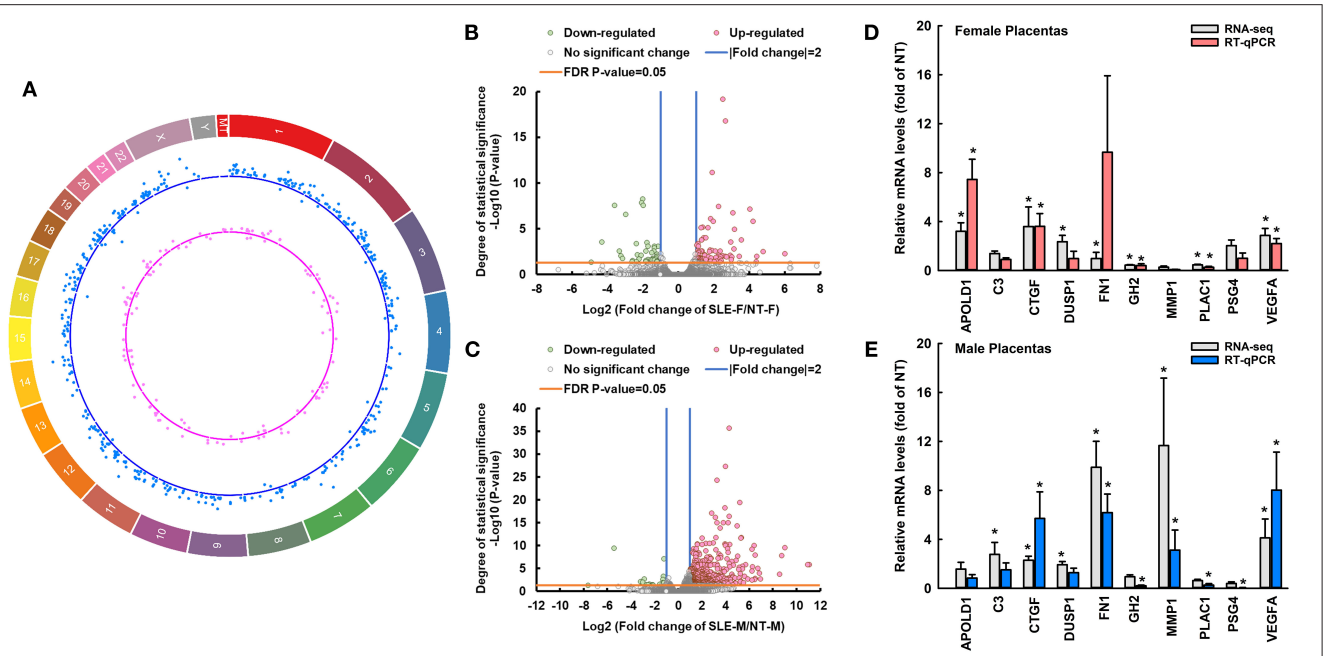

FIGURE 1 | SLE differentially dysregulates transcriptomic profiles of female and male placentas. **(A)** Circos plot illustrating the chromosome location of differentially expressed protein-coding genes between SLE-F vs. NT-F (pink dots) and SLE-M vs. NT-M (blue dots). Each dot represents one differentially expressed gene. The letters and numbers in the outer layer represent the chromosome location. For the scatter plot tracks, dots outside and inside of the centerline represent upregulated and downregulated genes, respectively. **(B,C)** Volcano plots showing differentially expressed genes between SLE-F vs. NT-F, and SLE-M vs. NT-M in RNA-seq. Gray dots: no significant difference; pink and green dots: > two-fold upregulation and downregulation, respectively (FDR-adjusted *P*-value < 0.05) in SLE vs. NT; n = 3-5/group. **(D,E)** RT-qPCR validation of SLE-dysregulated genes in female and male placentas. $^{*}P < 0.05$ vs. NT, n = 4/group. MT, mitochondrial DNA; SLE, systemic lupus erythematosus; APOLD1, apolipoprotein L domain containing 1; C3, complement C3; CTGF, connective tissue growth factor; DUSP1, dual specificity phosphatase 1; GH2, growth hormone 2; PLAC1, placenta specific 1; FN1, fibronectin 1; PSG4, pregnancy-specific beta-1-glycoprotein 4; VEGFA, vascular endothelial growth factor A; MMP1, matrix metallopeptidase 1. RNA-seq, RNA sequencing; FDR, false discovery rate. Circos plot showing the location DE-genes was generated using circa software for Windows.

Distinct transcriptional profiles in placentas from SLE pregnancies also showed that no significant correlation was found between the relative expression levels of SLE-dysregulated genes and SLEDAI scores ($P > 0.05$, **Supplementary Table 7**).

DISCUSSION

To our knowledge, this is the first report that profiles the protein-coding gene expression of human placental tissues from SLE pregnancies using RNA-seq analysis. Overall, more upregulated DEGs were identified than those downregulated. We have further demonstrated that the expression profiles are differentially dysregulated between SLE-F and -M placentas and are associated with differently regulated biological functions and pathways. These data provide clear evidence that SLE differentially regulates the expression of placental protein-coding genes in a fetal sex-dependent manner which may lead to dysregulated placental biological functions.

The mechanisms underlying the fetal sexual dimorphisms of SLE-dysregulated protein-coding gene profiles remain elusive. Expression of DEGs on X-chromosomes is likely to be a major factor that governs these fetal sexual dimorphisms since 4% of DEGs (5 from SLE-F placentas and 16 from SLE-M placentas) are located on the X-chromosome, but no DEGs are on the Y-chromosome. Given that SLE affects women more frequently than men, our current data suggest that the important contribution of X-chromosome-linked genes expression may be associated with the female sex bias in SLE (28). This is in line with a previous study that has shown that a large number of genes that may contribute to the hyperresponsiveness of the female immune system are located on the X chromosome (29). Sex hormones could be another factor that mediates the fetal sexual dimorphisms, as sexual hormones levels are different in the umbilical vein blood of female and male fetuses (30). However, no significant differential expression of androgen or estrogen receptors in SLE-F or -M placentas were detected in this study.

Differential regulation of the mitochondrial genome may also contribute to sexual dimorphisms since our data showed that 4 (*MT-ND2*, *MT-ND3*, *MT-CYB*, and *MT-ATP8*; **Supplementary Tables 3**, **4**) SLE-dysregulated genes are located on the mitochondrial genome of SLE-F, but not SLE-M placentas, which comprise 30.8% of mitochondrial protein-coding genes. The primary function of mitochondria is to generate the chemical energy to power cellular responses, which is achieved through the electron-transport chain and oxidative phosphorylation (31). The above four SLE-dysregulated mitochondrial genes participate in encoding polypeptides of the oxidative phosphorylation system (31), suggesting that mitochondrial genes might be actively

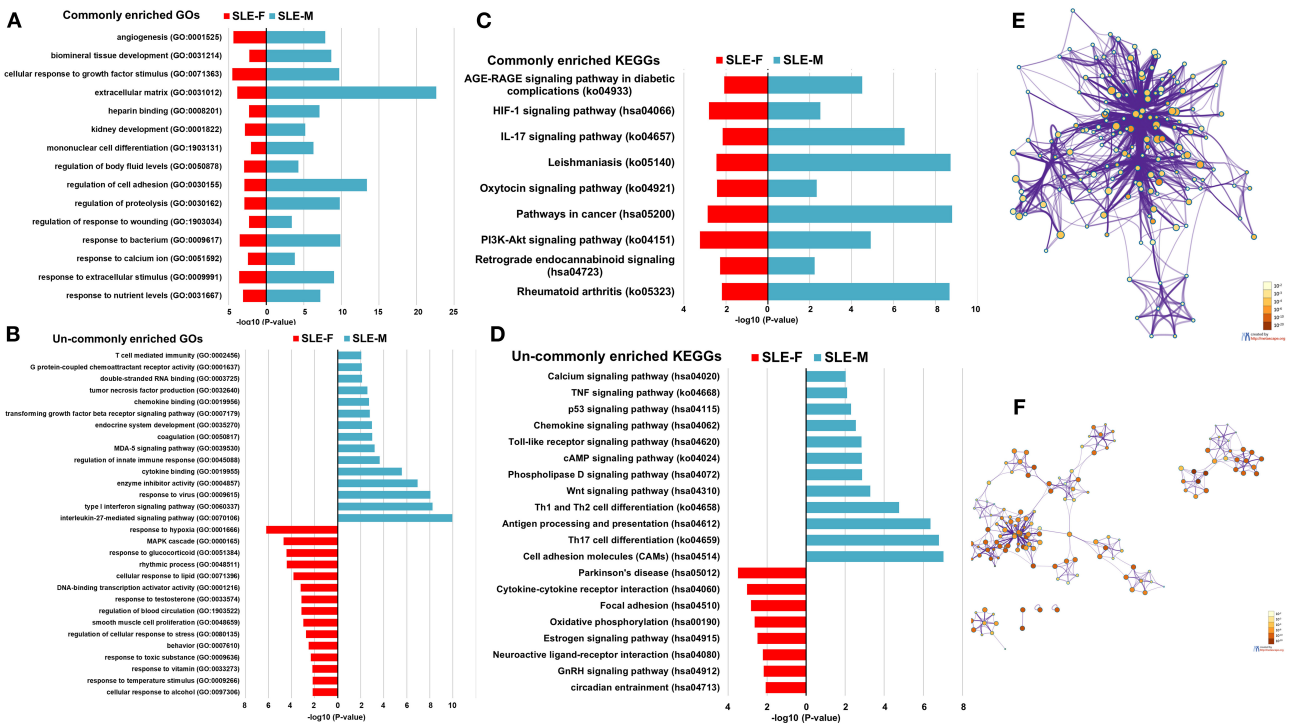

FIGURE 2 | Enrichment analysis of dysregulated protein-coding genes in SLE-F and -M placentas. **(A)** Commonly enriched GOs terms from both SLE-F and -M placentas. **(B)** Un-commonly enriched GO terms from SLE-F and -M placentas, respectively. **(C)** Commonly enriched KEGG terms from both SLE-F and -M placentas. **(D)** Un-commonly enriched KEGG terms from SLE-F and -M placentas, respectively. **(E)** Network of GO and KEGG enriched terms from SLE-F placentas colored by *P*-value, where terms containing more dysregulated genes indicate a more significant *P*-value. **(F)** Network of GO and KEGG enriched terms from SLE-M placentas colored by *P*-value, where terms containing more genes tend to have a more significant *P*-value. SLE, systemic lupus erythematosus; F, female; M, male; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

involved in the pathogenesis of SLE placentas. Mitochondrial dysfunction has been demonstrated to be associated with SLE pathogenesis (32–34). The mitochondrial dysfunction may increase not only oxidative stress but also cell apoptosis in SLE patients and defective bioenergetics (33). Oxidative stress induced by mitochondrial dysfunction is considered an essential downstream contributor for SLE pathogenesis (34). A study has shown that CD4+ T cells from an SLE mouse model have higher basal and activated mitochondrial oxidative metabolism, while inhibition of mitochondrial respiratory-chain complex 1 by treating SLE mice with metformin can prevent autoimmune activation (32). Our findings are in line with the above reports and support the notion that the mitochondrial genome of SLE may contribute to fetal sexual dimorphisms, and mitochondrial dysfunction may be present in SLE placentas.

We have also demonstrated that SLE dysregulates biological processes within placentas in a fetal sex-dependent manner. For instance, regulation of blood circulation was enriched in SLE-F but not in SLE-M placentas. In addition, published studies reported that age-standardized cardiovascular disease incidence, prevalence, and mortality rates are lower in women than men (35, 36). Similar results were reported in SLE cohorts that male lupus patients had more cardiovascular damage than their female counterparts (37). Therefore, the enrichment of regulation of blood circulation suggests a possible protective adaptation of cardiovascular diseases in SLE-F placentas.

Our findings revealed that *MDA-5* was upregulated in SLE-M, but not SLE-F placentas in association with enrichment of MDA-5 [melanoma differentiation-associated gene-5, gene name: *IFIH1* (interferon induced with helicase C domain 1)] signaling pathway only in SLE-M, but not SLE-F placentas. The relationship between MDA-5 and SLE has been proposed by several groups (38, 39). For example, the single nucleotide polymorphisms (SNP) in *MDA-5* has been implicated in the pathogenesis of SLE in Africa-Americans, American, Asian, Brazilian, European, and European-Americans (39). *MDA-5* has also been demonstrated to be associated with increased sensitivity to serum IFN-α and anti-dsDNA antibodies among SLE patients (38). Our results showed, for the first time, the aberrant expression of *MDA-5* in human SLE placentas. Therefore, it is necessary to further investigate the relationship between MDA-5 and SLE pregnancy, especially the function of MDA-5 in SLE placentas.

SLE-F and -M placentas share some common SLE-dysregulated biological processes, e.g., angiogenesis, innate immune responses, and inflammation. This is consistent with the previous observation since these biological processes are essential in placental development and function (40). These

TABLE 2 | SLE dysregulated biological functions in female and male placentas.

Biological functions	Female placentas		Male placentas	
	P-value	DEG number in SLE vs. NT	*P*-value	DEG number in SLE vs. NT
Angiogenesis	4.24×10^{-5}	11	1.44×10^{-8}	31
Cellular response to growth factor stimulus	3.08×10^{-5}	12	1.89×10^{-10}	37
Heparin binding	5.15×10^{-3}	4	8.41×10^{-8}	15
HIF-1 signaling pathway	1.56×10^{-3}	4	3.14×10^{-3}	7
IL-17 signaling pathway	6.91×10^{-3}	6	3.02×10^{-7}	11
Regulation of blood circulation	6.64×10^{-4}	3		
Response to glucocorticoid	3.69×10^{-5}	6		
Rhythmic process	3.86×10^{-5}	8		
GnRH signaling pathway	6.71×10^{-3}	4		
Estrogen signaling pathway	3.19×10^{-3}	3		
Tumor necrosis factor production			2.62×10^{-3}	9
Th17 cell differentiation			1.58×10^{-7}	12
IL-27-mediated signaling pathway			1.14×10^{-10}	6
MDA-5 signaling pathway			6.16×10^{-4}	3
Type I interferon signaling pathway			5.95×10^{-9}	10

SLE, systemic lupus erythematosus; NT, normal term; DEG, differentially expressed gene; HIF, Hypoxia-inducible factor; IL, interleukin; GnRH, Gonadotropin-releasing hormone; Th, T helper; MDA, melanoma differentiation-associated gene-5.

data demonstrate that aberrant functions of angiogenesis, innate immune responses, and inflammation may be present in SLE placentas. Specifically, our observations agree with the study that observed a significant increase in angiogenic activity in the serum samples of SLE patients, which was positively associated with SLE disease activity (41, 42). Guilherme et al. reported that serum Vascular Endothelial Growth Factor A (VEGFA) levels were significantly higher in pregnant women with active SLE nephritis than patients with inactive SLE or preeclampsia (42). VEGFA has been demonstrated to be the most potent pro-angiogenic factor and therefore is actively involved in the development of inflammation (43). The gene encoding *VEGFA* is located on chromosome 6 at 6p21.1, one of the major SLE susceptibility loci (44). Another pro-angiogenic counterpart of VEGFA, which is higher in SLE placentas in this study, is CTGF. CTGF, also known as Cellular Communication Network (CCN) Factor 2, belongs to the CCN family. *CTGF* gene is located on chromosome 6 at 6q23.2, which is also closely associated with SLE (45). CTGF is a key regulatory and signaling molecule associated with numerous biological processes, including angiogenesis, wound healing, cell proliferation, tissue regeneration, and fibrosis through interaction with many factors, such as VEGFA and TNF-α (46, 47). Based on our RT-qPCR results, *VEGFA* and *CTGF* mRNA levels were significantly higher in SLE placentas, suggesting that placental angiogenic activity is disrupted in SLE, just like in preeclampsia (48).

Although defined as an autoimmune disease, SLE is characterized by chronic and acute inflammation conditions in multiple organs with the generation of autoantibodies abnormally produced by one's immune system (49). Torricelli et al. reported that IL-17 was increased in serum from pregnant women with SLE (50). Our RNA-seq analysis also showed that the levels of *IL17D,* but not other IL17 family members (e.g., *IL17A*, *IL17B*, *IL17C*, and *IL17F)* was elevated in SLE-M placentas, while the IL-17 signaling pathway was enriched in both male and female placentas, supporting that IL-17 signaling pathway is actively involved in the dysregulation of placental immune response in SLE placentas.

Correlation analysis in this study failed to show any correlation between DEGs and SLEDAI scores. However, a small case number ($n = 8$) and narrow SLEDAI scores (ranging from 0 to 6) of SLE patients recruited in this study may have prevented us from elucidating any correlations.

In conclusion, this is the first report of protein-coding gene profiles of placentas tissues from SLE pregnancies with female and male fetuses using RNA-seq analysis. The results indicate that the differential expression of protein-coding genes in the female and male placenta may contribute to the different pathogenesis of SLE pregnancies. There are several limitations in this study, including a relatively small sample size which might not address all sex differences in SLE placental gene expression. In addition, SLEDAI scores of the recruited SLE patients were relatively low, ranging from 0 to 6. Thus, while these low SLEDAI scores may provide a valuable resource, recruiting a large cohort of SLE patients with a broader range of SLEDAI scores is needed to establish a more reliable correlation between DEGs and SLEDAI scores.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Qilu Hospital, Shandong University and the Scientific and Ethical Committee of Shanghai First Maternity and Infant Hospital affiliated with Tongji University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HHL and LTS collected tissues samples and drafted the manuscript. ST designed the tables and figures. YL participated in manuscript preparation. CF designed the figures and read the manuscript critically. KW collected tissues and read the manuscript critically. CZ analyzed the RNA-seq data and read the manuscript critically. JZ and QS conceived the concept and wrote the manuscript. YJZ designed the work and read the manuscript critically. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.798907/full#supplementary-material

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REVIEW
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Sex Differences in Lipid Metabolism: Implications for Systemic Lupus Erythematosus and Cardiovascular Disease Risk

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It is known that healthy women during childbearing years have a lower risk of cardiovascular disease (CVD) and coronary heart disease compared to age matched men. Various traditional risk factors have been shown to confer differential CVD susceptibilities by sex. Atherosclerosis is a major cause of CVD and mortality and sex differences in CVD risk could be due to reduced atherogenic low and very low-density lipoproteins (LDL and VLDL) and increased atheroprotective high density lipoproteins (HDLs) in women. In contrast, patients with systemic lupus erythematosus (SLE), a chronic inflammatory disease that predominately affects women, have an increased atherosclerotic and CVD risk. This increased CVD risk is largely associated with dyslipidaemia, the imbalance of atherogenic and atheroprotective lipoproteins, a conventional CVD risk factor. In many women with SLE, dyslipidaemia is characterised by elevated LDL and reduced HDL, eradicating the sex-specific CVD protection observed in healthy women compared to men. This review will explore this paradox, reporting what is known regarding sex differences in lipid metabolism and CVD risk in the healthy population and transgender individuals undergoing cross-sex hormone therapy, and provide evidence for how these differences may be compromised in an autoimmune inflammatory disease setting. This could lead to better understanding of mechanistic changes in lipid metabolism driving the increased CVD risk by sex and in autoimmunity and highlight potential therapeutic targets to help reduce this risk.

Keywords: sex and gender, lipoproteins, autoimmunity, atherosclerosis, SLE

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide (1, 2). The most common pathogenic process leading to CVD is atherosclerosis, the build-up of lipids and inflammation in the walls of major arteries (atherosclerotic plaque), leading to the narrowing of the interior lumen of the vessel, plaque rupture, thrombosis, and subsequent myocardial infarction or stroke due to the restricted blood flow to the heart or brain, respectively. Importantly, women of a childbearing age

have around half the CVD risk compared to age-matched men, and almost a 10-year delay to first myocardial infarction event (3–5). Whilst sex differences in CVD risk are narrowed in older age groups, the CVD-associated death rate among women never exceeds that of men (6, 7). Traditional risk factors of atherosclerosis that could be modified by sex hormones, such as lipid metabolism, are believed to explain these differential outcomes between men and women (8); however, there is a clear need to investigate these sexually dimorphic mechanisms of CVD to improve outcomes for both men and women.

Alternatively, women represent around 80% of all individuals with autoimmune disease, however, patients with autoimmunity have an increased risk of developing CVD through atherosclerosis (9, 10). With this respect, women between the ages of 35–44 with systemic lupus erythematosus (SLE), a chronic inflammatory disease with a 90% female predominance, have a 50 times increased risk of developing coronary artery disease compared to healthy individuals (11). This shows that the impact of SLE dramatically reduces the female CVD protection seen in healthy individuals. Interplay between traditional risk factors and factors associated with autoimmunity, as well as overlapping factors, such as dyslipidaemia (disrupted lipid metabolism) and inflammation, contribute to accelerated atherosclerosis in SLE patients (12–14).

This review aims to discuss differences in lipid metabolism between men and women, and why this is altered in autoimmunity leading to reduced CVD protection for women. This will aid understanding of the CVD bias by sex and could help to tailor sex specific therapeutic strategies to improve CVD outcomes for both men and women, including those with autoimmunity.

SEX DIFFERENCES IN LIPOPROTEIN METABOLISM: IMPLICATIONS FOR CARDIOVASCULAR RISK

The build-up of lipids in atherosclerotic plaques is largely due to lipoproteins, biochemical assemblies of lipids and apolipoproteins that are structured to enable hydrophobic lipids to transport freely around the blood. Lipoprotein subtypes are defined by their size, density, lipid content and specific apolipoprotein (Apo) expressed on their surface, which together determine their pathogenic contribution to atherosclerosis. Lipoproteins of lower density, including very low, low, and intermediate density lipoproteins (VLDL, LDL, IDL), predominately express ApoB on their surface and promote lipid uptake by inflammatory cells in atherosclerotic plaques following their oxidation. Alternatively, high density lipoproteins (HDLs) express ApoA1 on their surface and play a role in lipid efflux, inferring a role that is typically atheroprotective (15) (**Figure 1**). Emerging research supports that different sizes of lipoprotein sub-classes can infer differential effects on CVD risk (16), highlighting the need for more detailed analytical methods for serum lipid profiling, such as nuclear magnetic resonance (NMR) spectroscopy, to expand the standard lipid fraction routinely measured in clinical practice (LDL-cholesterol, HDL-cholesterol, total-cholesterol, and total triglycerides, TGs).

It is well established that prior to menopause, the lipoprotein profile of healthy women is more atheroprotective compared to age matched men (17). The Framingham Offspring Study is one of the largest studies to investigate sex differences in CVD risk factors, where subsequent interrogation of this data has identified an increase in smaller and more dense LDL particles in men compared to women (18, 19), a subset that has been previously associated with sex differences in CVD incidence (20–22). Since these observations, NMR spectroscopy analysis of serum from 1574 men and 1692 women (mean age of 52 years) from the Framingham Offspring Study confirmed the lower CVD risk lipid profile in women, where women had a twofold higher concentration of large HDL particles compared to men (23). Large-HDL subsets have been shown to confer higher CVD protection (16). Complimentary to previous studies, the difference in HDL particle size between men and women decreased with age in the Framingham Offspring Study cohort. Furthermore, previously established differences in conventional lipid measures, with men having higher concentrations of TGs, LDL-cholesterol and ApoB, but lower HDL-cholesterol and ApoA1, were also confirmed (23). Importantly, VLDL particles have a high content of TGs relative to other lipoproteins classes, and have also been associated with residual CVD risk independent of circulating TGs (24–26), however, this subset has been less well studied due to the focus of clinical lipid profiles on LDL and HDL-cholesterol measures.

Following menopause, women lose a large amount of their protective lipoprotein fractions which is reflected in increased CVD post-menopause. This is believed to be a result of reduced circulating oestradiol, where lower levels have been shown to infer an increased risk of developing metabolic diseases and CVD (27). A study of post-menopausal women, assessing coronary artery calcification (CAC), a measure of established atherosclerosis using electron beam computed tomography, found that small LDL and all VLDL subclasses were significantly associated with a higher extent of CAC (28). However, large HDL particles, but not small, inversely correlated with the extent of CAC, highlighting the protective role of HDL even in older women with lower oestradiol levels. In support, studies have shown that post-menopause, LDL increases in women to the levels of age-matched men, however, HDL remains higher in women compared to men at all ages despite the decrease post-menopause (29–32).

As heart disease is more common in older age groups and age is an independent risk factor for CVD, studies of CVD are more common in adults (33). However, new studies have explored lipoprotein metabolism in younger age groups, particularly surrounding puberty, where hormones have been shown to become extremely relevant for sexual dimorphisms in CVD risk factors. With this respect, Robinson et al. recently explored sex differences in detailed lipoprotein profiles using NMR metabolomics of serum from young, healthy pre- and post-pubertal individuals (34). This study showed that pre-puberty, no differences in lipoproteins exist, however, following the onset of puberty (assessed clinically using standardised Tanner stages),

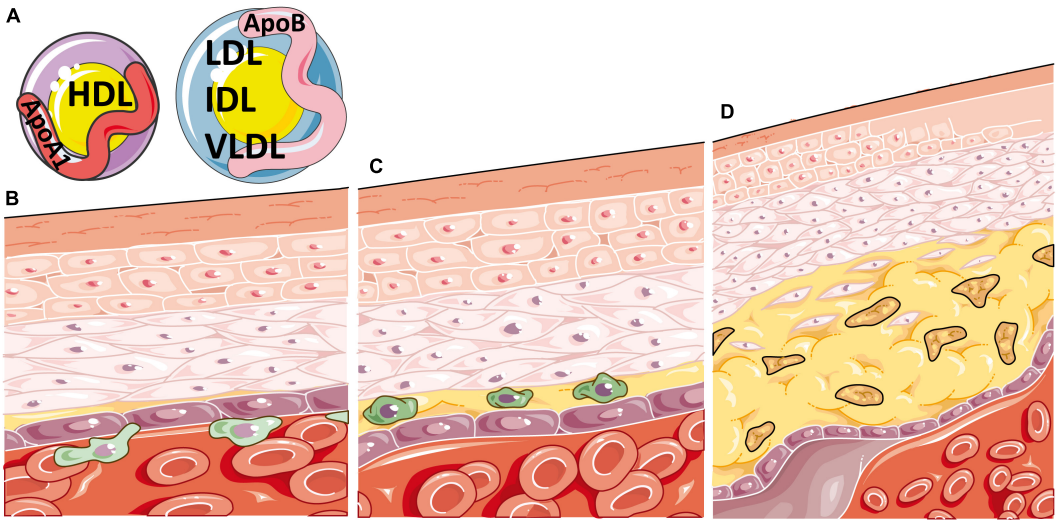

FIGURE 1 | Atherosclerosis is a disease of inflammation and lipids. **(A)** Atherosclerosis is heavily determined by the circulating balance between atheroprotective high density lipoproteins [HDL, expressing apolipoprotein (Apo)A1 on their surface] or atherogenic very low, low and intermediate density lipoproteins (VLDL, LDL and IDL, expressing ApoB on their surface). **(B)** Atherosclerosis initiates when ApoB containing lipoproteins accumulate, become oxidised, and enter the intima region of the blood vessel. This induces endothelial adhesion molecule expression and inflammatory cell recruitment, which migrate through the vessel wall, beginning the process of atherosclerotic plaque formation. **(C)** Oxidised ApoB containing atherogenic lipoproteins are taken up by macrophages in atherosclerotic plaques through scavenger receptors, increasing their cellular lipid burden and resulting in foam cell formation. **(D)** These lipid laden macrophages enlarge the plaque and produce pro-inflammatory cytokines, resulting in further immune recruitment to the plaque, damage to smooth muscle and endothelial cells, necrotic core formation from the growing mass of extracellular lipids and cell debris, narrowing of the artery and eventual thrombosis. This figure was produced using resources from Servier Medical Art, licenced under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

young men develop an atherogenic profile, consisting mostly of increased larger VLDL subsets and VLDL lipid content, whilst young women develop an increase in total, medium and larger HDL particles, HDL lipid content and levels of ApoA1. In addition, this study performed detailed serum lipoprotein profiling of a rare cohort of young transgender individuals, which validated the direct association between oestradiol and increased larger HDL and ApoA1 levels in trans-women (young individuals born phenotypically male, who were treated with puberty blockers followed by oestradiol, as gender reaffirming therapy), as well as between testosterone and increased VLDL levels in trans-men (young individuals born phenotypically female, who were treated with puberty blockers followed by testosterone, as gender reaffirming therapy). As supported by these studies of different age groups, this suggests that VLDL versus LDL could be the dominantly increased atherogenic lipoproteins in younger versus older adult men compared to age matched women. Importantly, increased circulating concentrations of LDL and VLDL in plasma have been shown to induce the development of atherosclerosis, independent of other risk factors (35). Finally, this study showed that HDL was increased by oestradiol in a dose dependent and chromosome independent manner in trans-women, suggesting that HDL may be more sensitive to changing hormones levels than atherogenic lipoproteins at this young age. Sex-specific changes in lipoproteins discussed are summarised in **Table 1**.

Together, these studies highlight that sex differences in atherosclerosis susceptibilities could be inferred from a young age by hormones and supports a role of hormones in driving lipoprotein metabolism at both ends of the age scale, as well as the importance of studying lipoproteins and CVD susceptibilities at all ages and genders (**Figure 2**).

TABLE 1 | Sex differences in lipoproteins across pubertal stages.

	Pre-puberty (girls versus boys)	Post-puberty (women versus men)	Post-menopause (women versus age matched men)
ApoB	• No difference (34)	• Increased in men (23)	
VLDL	• No difference (34)	• Increased in men (34)	
LDL	• No difference (34)	• Increased in men (23) • Increased small-LDL in men (18, 19)	• Increased in women (29–31)
ApoA1	• No difference (34)	• Increased in women (23, 34)	
HDL	• No difference (34)	• Increased in women (23, 34) • Twofold higher large-HDL in women (23)	• Lower lipid rich HDL than pre-menopause, but still increased in women (29–32)

LIPOPROTEIN METABOLISM AND DYSLIPIDAEMIA IN WOMEN WITH SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a complex and heterogeneous autoimmune disorder characterised by loss of immune cell regulation, chronic inflammation, and multiple organ damage. As well as genetic, environmental, and epigenetic contributions, hormones have

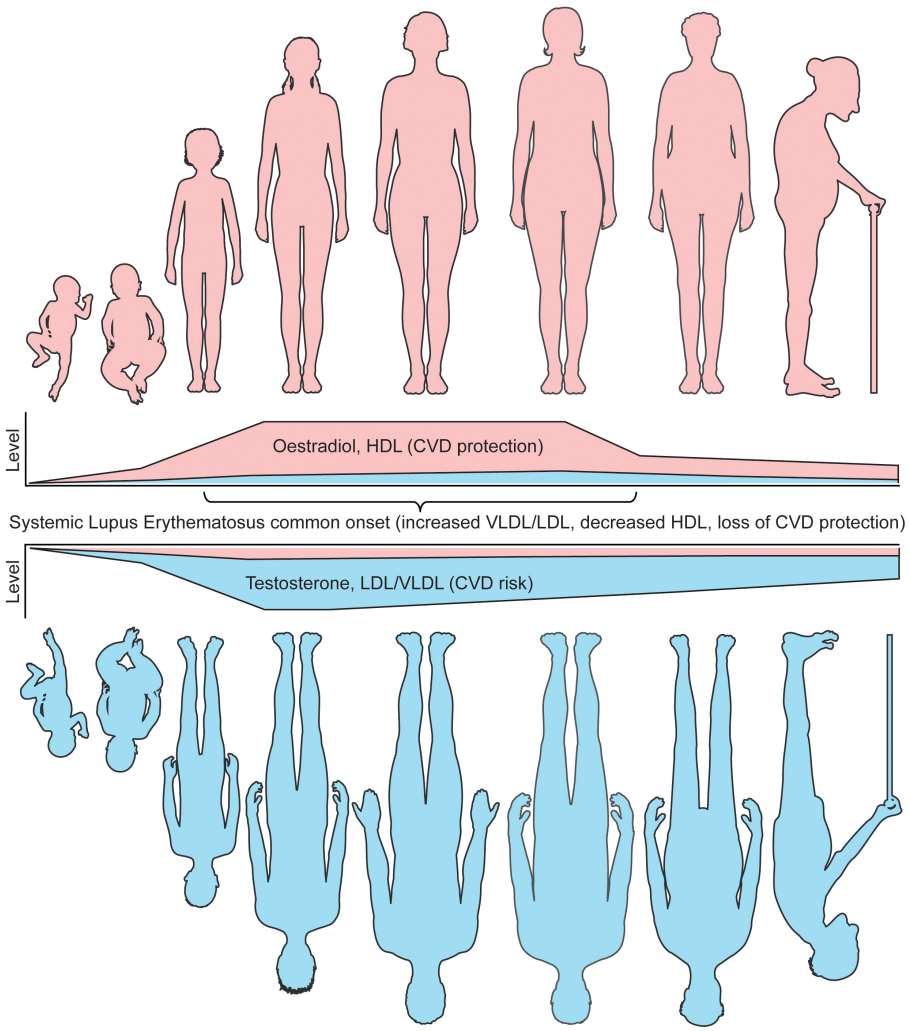

FIGURE 2 | Sex differences in lipoproteins and CVD risk coincide with age associated hormone changes. The levels of circulating sex hormones change throughout life. Pre-puberty, research has shown that girls and boys do not have differences in either atherogenic (VLDL/LDL) or atheroprotective (HDL) lipoproteins. Following the onset of puberty, oestradiol increases in young women, which in turn raises the level of circulating HDL, inferring a lower CVD risk compared to young men in healthy individuals. Post-pubertal young men, with increased testosterone and low oestradiol levels, develop a more atherogenic lipoprotein profile, inferring an increased CVD risk compared to young women in healthy individuals. Whilst age is an independent risk factor of CVD risk in both men and women, oestradiol and HDL remain high in women until menopause, where oestradiol dramatically reduces and CVD protection by HDL is less prominent. Despite this, the levels of HDL in women remain higher than men post-menopause. Older men do not experience this dramatic fall in sex hormones, however, the levels of testosterone do slowly reduce with age. A recent study has shown that sex hormone associated lipoprotein changes can be induced by cross sex hormones in young transgender individuals, supporting these CVD risk associated observations. In patients with SLE, a disease with most common onset in women of a childbearing age, where women represent around 90% of all patients, CVD protection in women is dramatically reduced due to dyslipideamia. This includes increased atherogenic lipoproteins and reduced HDL. It is speculated that this could be due to changes in levels or tissue sensitivity oestradiol, which drives inflammation and altered lipid metabolism. Understanding these fundamental differences in lipoproteins by sex will aid our mechanistic understanding of sexually dimorphic diseases and improve disease prevention and outcomes for CVD and autoimmune patients. This figure was produced using resources from Servier Medical Art, licenced under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

also been implicated in the aetiology of SLE due to the sexual dysmorphism of the disease, where the female to male ratio is 9:1 (36). Deaths attributable to disease activity in SLE have reduced dramatically over the last 50 years due to improved treatments targetting key dysregulated immune pathways, however, deaths associated with atherosclerosis and CVD are still high (37, 38). It has become apparent that the pathogenesis of atherosclerosis shares several autoimmune inflammatory pathways (39). Aside from inflammation, dyslipideamia (an imbalance between atherogenic and atheroprotective lipoproteins) is extremely common in SLE and is a conventional CVD-risk factor through atherosclerosis (40). In fact, dyslipidemia was found in over 70% of premature coronary heart disease cases and hypercholesterolaemia (elevated total and/or LDL/non-HDL-cholesterol) was present in 34–51% of SLE all patients (41). In addition, a Systemic Lupus International Collaborating Clinics'

(SLICC) cohort study reported that 36% of newly diagnosed SLE patients had hypercholesterinemia in this large international cohort, which increased to over 60% after 3 years (42, 43). Interplay between traditional CVD risk factors, including dyslipidaemia, and risk factors associated with ongoing chronic inflammation captured in disease activity scores and cumulative steroid treatment (the most used treatment for acute flares in SLE) could contribute to the accelerated development of atherosclerosis in men and women with SLE (12). Whilst healthy women of a childbearing age typically have a more atheroprotective lipoprotein profile compared to men, as the onset of SLE peaks between the ages of 15–55 and the disease has a significant female-bias (44), the atheroprotective lipid profile is replaced by dyslipideamia which is common in all patients with lupus (**Figure 2** depicts the physiological variation of lipid profile in men versus women; their associations with sex hormones and age, as well as impact of SLE-related chronic inflammation and treatment on driving a dysregulated lipid profile in all patients). Various clinical studies have found that elevated total cholesterol, TGs, circulating LDL-cholesterol and reduced HDL-cholesterol are the most common lipid abnormalities associated with SLE (40), which likely contributes to the higher lipid burden of atherosclerotic plaques, and increased CVD-risk in many patients. However, these standard clinical lipid panels, as well as many established cardio-vascular risk scores, often fail to account for the increased CVD-risk directly associated with SLE disease and treatment, and studies often do not consider how the presence of SLE modifies the impact of sex on CVD-risk (14, 45, 46). Despite this, more detailed investigations into lipoprotein subsets using NMR technology have found that women with SLE have increases in smaller LDL subfractions compared to sex matched healthy controls (HCs) (47). Further to this, a serum NMR metabolomic study by Coelewij et al., incorporating detailed lipoprotein subclass evaluation, was able to confidently differentiate between adult women with SLE and sex matched HCs by use of machine learning (48). Here, the most influential metabolites in separating SLE from HCs were medium sized HDL measures, which were reduced in SLE, as well as small HDL, VLDL, and IDL particles, which were increased in SLE compared to HCs. This suggests that different HDL sizes are important to consider when studying dyslipidaemia in SLE. In support, another study reported that smaller HDL subsets were reduced in SLE, whilst no difference in the size of VLDL or LDL were reported (49). Dyslipidaemia has also been identified by multiple studies of paediatric patients with juvenile-SLE (50, 51), where onset of SLE occurs before the age of 18 and patients typically have worse disease outcomes and an estimated 100- to 300-fold increased risk of mortality from CVD compared to age-matched healthy individuals (52, 53). Strikingly, dyslipidaemia is present in up to 63% of patients with juvenile-SLE, which is higher in patients with active disease (51). In addition, an NMR metabolomics study of these younger juvenile-SLE patients showed that small HDL subsets were the most significantly reduced lipoproteins in juvenile-SLE patients compared to HCs, validated by machine learning analysis; this reduction was also exacerbated by increased disease activity (54). Importantly, sex was adjusted for in this analysis, despite 81.5% of this cohort being female. In a more sex-specific study of young girls with juvenile-SLE, dyslipidaemia was observed in 39% of the study participants and a significant decrease in HDL-associated ApoA1 the juvenile-SLE cohort compared to HCs, supporting a more global decrease in HDL in young patients (55).

Based on available literature data, there is compelling evidence that dyslipidaemia associated with SLE could dramatically reduce the lipid protection that healthy women of a childbearing age have from CVD, even in much younger age groups. This was supported recently by Robinson et al., who investigated sex differences in lipoprotein metabolism between young, post-pubertal patients with juvenile-SLE and found that all conventional differences in lipoprotein profiles observed between age-matched healthy men and women were lost in patents with juvenile-SLE (34). A sex-specific sub-analysis showed an increase in VLDL subsets and a decrease in HDL subsets in young women with juvenile-SLE compared to HCs, supporting reduced atheroprotection in disease. This loss of protection could be due to a breakdown in conventional sex hormone signalling, and highlights that sex and age are extremely important when studying the pathogenesis of and associated dyslipidaemia in SLE, where additional factors, such as ongoing inflammation and differential sex hormones are likely to have a significant impact on the overall CVD risk. Sex differences in lipid metabolism and their impact on the CVD-risk of patients with SLE are not commonly studied due to the overwhelming female predominance of the disease; however, this needs to be a priority going forward to enable better understand of the changes in CVD risk for women with SLE of all ages.

DISCUSSION

Is it striking that the presence of SLE in women removes the sex-specific cardio-protection through dyslipidaemia and this highlights a possible role for deregulated oestradiol signalling in SLE, in addition to over-activation of proinflammatory pathways and impact of certain SLE medications on lipid metabolism, all ultimately leading to altered lipid profiles in these patients. The association between lipids and sex-hormones is not a new theory, where the combined oral contraceptive pill (oestradiol and progesterone) has been previously shown to increase circulating HDL-cholesterol and TGs, whilst the progesterone only pill has no effect (56). Oestradiol administration has also been shown to increase HDL in post-menopausal women (57, 58), supporting a direct cardioprotective role of sex hormones in lipid metabolism. The study by Robinson et al. outlined above (34), highlighted that trans-men had increased total and LDL-cholesterol and TGs as well as decreased HDL-cholesterol associated to short-term administration of exogenous testosterone as gender-reaffirming treatment (and reduced oestradiol following treatment with puberty blockers), whilst trans-women had decreased total and LDL-cholesterol associated with exposure to short-term therapeutic oestradiol doses (in the context of reduced testosterone following treatment with puberty blockers) (59, 60). Follow up studies will be critical to understand the long-term effects of these sex hormone and

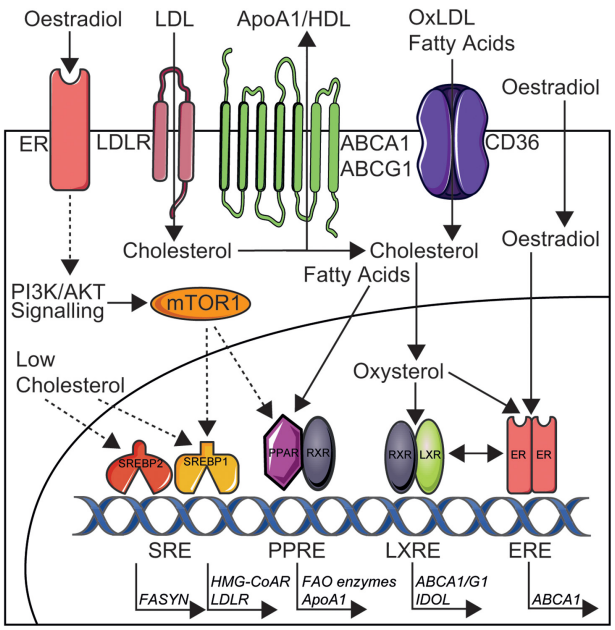

FIGURE 3 | Simplified depiction of oestradiol induced signalling pathways that may increase cardioprotection in women through altered lipid metabolism and could be targeted in SLE. Cholesterol is taken up by cells in low density lipoproteins (LDL) *via* LDL receptors (LDLR) and CD36 [oxidised (ox)LDL] or is synthesised by HMG-CoA reductase (HMG-CoAR). Cholesterol is removed from cells to ApoA1/high density lipoproteins (HDL) *via* ATP-binding cassette transporter A1 or G1 (ABCA1/G1) or converted to oxysterols intracellularly. Low intracellular cholesterol levels can induce the transcriptional activity of sterol regulatory element-binding proteins 1/2 (SREBP1/2). Fatty acids are taken up by CD36, synthesised by fatty acid synthase (FASYN), or metabolised by fatty acid oxidation (FAO). Oestradiol acts *via* membrane bound oestrogen receptors (ERs) to induce phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signalling and downstream mTOR1 activation. Activated mTOR1 increases lipogenesis *via* increased nuclear activity of SREBP1 and peroxisome proliferator-activated receptor (PPAR) transcription factors. Oestradiol can also activate transcription factor ERs in the nucleus and this increased ER nuclear activity can elevate the binding and transcriptional efficiency of liver-x-receptors (LXRs), inducing cardioprotective cholesterol metabolism through upregulated ABCA1/G1 and inducible degrader of the LDLR (IDOL). It is through these metabolic pathways that oestradiol could exert it's cardioprotective effects in healthy women and therefore could be disrupted and targetted in SLE to reduce CVD risk for patients. This figure was produced using resources from Servier Medical Art, licenced under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

lipid changes on CVD risk, as well as larger cohort studies to control for confounding factors such as BMI, hypertension and smoking. Studies in mice have also supported a protective role of oestradiol in CVD through lipid metabolism, where different stages of the menstrual cycle determine the size and lipid content of HDL produced by hepatic cells *in vivo*, relative to the levels of circulating oestradiol (61). When oestradiol levels are highest, smaller HDL particles are produced which allow more efficient cholesterol efflux from the liver *via* ATP-binding cassette transporter A1/G1 (ABCA1/G1), which in turn infers greater CVD protection. This is due to the increased nuclear activity of the oestrogen receptor, which elevates the binding and transcriptional efficiency of liver-x-receptors (ABCA1/G1), master regulators of cellular cholesterol metabolism (**Figure 3**). Where smaller HDL particles are more efficient in mice regarding cholesterol efflux, it has been alternatively shown that large- and medium-sized HDL-cholesterol is more protective of myocardial infarction and stroke in humans (16), suggesting complex differences across models. *De novo* clearance of LDL and VLDL is also increased when plasma oestradiol levels are high in mouse models (61, 62), suggesting a duel effect of oestradiol on increasing and decreasing atheroprotective and atherogenic lipoproteins, respectively. Conversely, testosterone has been shown to increase hepatic lipase activity (catalyses the hydrolysis of triglycerides), decrease the levels of HDL and reduce the size of LDL (63). Reports on the specific impact of testosterone on atherosclerosis and CVD is less researched compared to studies of oestradiol (64).

Together, these sex specific lipid changes may explain why women lose their CVD protection following menopause, however, may not explain why women of a childbearing age with SLE develop an increased CVD risk. Although a reduction in oestradiol induced signalling could be a logical explanation for the increase CVD risk in SLE, in fact, many studies have reported that oestradiol and the oestrogenic metabolite, 6 α-hydroxyestrone, are increased in women with SLE (65–68), supporting the predominance of SLE disease onset in women during their reproductive years. Alternatively, it is plausible that oestradiol may promote inflammation in SLE, which increases the impact of non-traditional CVD risk factors including chronic inflammation (36). In support, some reports show that inflammatory flares in SLE are more prominent during pregnancy (69) and that patients with SLE may have exaggerated inflammatory responses to oestradiol (70). Generalised inflammation can reduce HDL levels and increase hepatic VLDL production, whilst reducing the clearance of TG lipoproteins (71). More specifically, inflammation in SLE associated with disease flares and pro-inflammatory cytokines such as IL-6 and TNF-α can increase TG and reduce HDL levels (72, 73). This could be partly due to reduced cell-mediated cholesterol efflux in SLE (74). Larger, TG rich lipoproteins have been associated with a duel effect on inflammation and atherosclerosis, whereas smaller LDL particles can promote atherosclerosis independent of inflammation (75). In addition, VLDL particles may also have difficulty leaving the subendothelial space of blood vessels, promoting local inflammation and atherosclerotic plaque progression (76). Finally, altered liver function, the major regulator of systemic lipid metabolism, is more common in SLE due to inflammation (77–79). This liver inflammation, along with current therapies used to treat SLE, such as steroids, could contribute to the loss of atheroprotection in women through altered lipoprotein metabolism, while treatment with hydroxychloroquine which is currently recommended in all patients with SLE can counterbalance some of the negative effects SLE has on the CVD-risk profile of these patients (80).

Another master regulator of metabolism that also has an impact on lipid synthesis is the mammalian target of rapamycin (mTOR) (81). Specifically, upon stimulation through phosphoinositide 3-kinase/protein kinase B (PI3K/AKT)

signalling, mTORC1 inhibits lipolysis and induces lipogenesis *via* the activation of peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding transcription factor 1 (SREBP1) (82, 83). Separately, activated mTORC2 can induce AKT signalling and therefore mTORC1 activation indirectly. With respect to these mechanisms, liver-specific deletion of mTORC1 can induce cardioprotective effects and render mice resistant to western diet induced hypercholesterolaemia (82, 84). mTOR inhibition with rapamycin has also led to a significant reduction of atherosclerotic lesions in LDL-receptor (LDLR) deficient male mice, despite severe hypercholesterolaemia (85). Despite these beneficial effects of mTOR inhibition on cardiovascular health, mTORC1 inhibition can also cause dyslipidaemia, a common risk factor for atherosclerosis, through downregulation of hepatic LDLRs and stimulation of lipophagy, resulting in a respective increase in circulating levels of LDL-cholesterol and droplet-released lipids (86, 87), meaning that the overall contribution of mTOR to atherosclerotic risk is complex (88). To add to this complexity, sex differences in mTOR signalling have been described in mouse models, where oestradiol stimulation of the oestrogen receptor can induce PI3K/AKT signalling and downstream mTOR activation (89, 90). mTOR suppression with rapamycin can increase the lifespan of mice (91), leading to an interest in its sex-specific effects on cardiovascular health. With this respect, increased mTORC1 activity has been observed in the liver and heart tissue of young female mice compared to male mice of the same age (92), and rapamycin treatment in mice also has sex specific effects on mTORC1 and mTORC2 (93). mTOR inhibition has also been shown to improve testosterone-induced myocardial hypertrophy in hypertensive rats (94), together supporting the potential sex-specific effects of mTOR on cardiovascular health. This suggests an alternative metabolic pathway to liver-x-receptors that oestradiol may exert its cardioprotective effects in healthy women and could be disrupted in SLE (**Figure 3**). It is also reported that patients with SLE have genetic activation of mTORC1 (95), and its blockade exerts potential therapeutic efficacy in SLE through reducing pro-inflammatory T-cell and macrophage differentiation (96). mTOR activation has also been implicated in increased CVD in SLE (97). Various mTOR inhibitors, less or more selective, have been developed for use in cancers and transplant medicine, owing to their important antiproliferative and cellular effects and immunosuppressive effects, although further research is required to address the limitations of dose-related toxicity and lack of tissue selectivity (98). Therefore, the cardiovascular and inflammatory effects of mTOR appear to be model, tissue, disease, and sex specific, adding to the complexity of investigating sex differences in CVD and autoimmune susceptibilities; more human studies are warranted.

CONCLUSION AND PERSPECTIVES

Whilst CVD is more common in men, and SLE in women, sex and gender needs to be taken into account in all medical research. With this respect, CVD is the leading cause for mortality in women, representing 35% of all global deaths (99). According to a recent study by The Lancet, 275 million women were diagnosed with CVD and 8.9 million died from CVD in 2019 (100). Despite this, women are hugely under-represented in clinical trials of CVD due to the increased risk in men, which is a major global health research limitation which needs to be addressed. Not only this, but women are often under-researched, underdiagnosed and undertreated as a result of this sex bias in CVD risk. In response, The Lancet have produced The Lancet women and CVD commission, aiming to reducing the global burden of CVD on women by 2030 (101). Here, an all-female led commission outlined new recommendations to tackle inequities in diagnosis, treatment, and prevention to reduce CVD in women. With regard to lipids, a standardised case-control study of acute myocardial infarction across 52 countries, the INTERHEART study, showed that abnormal lipids were the highest population attributable risk factor for CVD with very little contributable difference between sexes (49.5% for men and 47.1% for women) (8), validating the importance of considering both sexes in CVD, particularly when studying lipid metabolism.

It is also important to note that men are underrepresented in studies and clinical trials of SLE (102). Relative to CVD, however, SLE is relatively rare in the general population and men only represent around 10% of all SLE cases. This makes equality in SLE research difficult, particularly regarding sex, where men only represent around 7% of randomised controlled trials of patients with SLE (102). Despite this, it has been shown that men with SLE tend to develop more severe renal manifestations and higher risk of end-stage renal disease, requiring increased monitoring in clinical practice (103). With this respect, men should be considered more in research and clinical trials of SLE to improve disease prognosis. As highlighted in this review, equality in research cohorts and clinical trials not only improves lives of men and women, but also helps us to understand the pathogenic mechanisms of sexually dimorphic diseases.

To conclude, whilst oestradiol conventionally promotes atheroprotective lipoprotein metabolism in healthy individuals, chronic inflammation due to altered oestradiol sensitivity in patients with SLE, as well as other SLE-related treatment factors could alter finely tuned mechanisms of lipid regulation and induce circulating lipoprotein changes toward a more atherogenic profile. It is clear that further mechanistic investigations are warranted, however, uncovering these mechanisms of fundamental sex hormone driven changes in lipid metabolism will aid disease prevention and outcomes for both patients with CVD and autoimmunity, regardless of sex or gender, highlighting the importance of considering sex hormones in medical research.

AUTHOR CONTRIBUTIONS

GR performed the literature review and wrote the first draft of the manuscript. All authors reviewed the manuscript and approved the final version.

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A Genetic Association Test Accounting for Skewed X-Inactivation With Application to Biotherapy Immunogenicity in Patients With Autoimmune Diseases

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Despite being assayed on commercialized DNA chips, the X chromosome is commonly excluded from genome-wide association studies (GWAS). One of the reasons is the complexity to analyze the data taking into account the X-chromosome inactivation (XCI) process in women and in particular the XCI process with a potentially skewed pattern. This is the case when investigating the role of X-linked genetic variants in the occurrence of anti-drug antibodies (ADAs) in patients with autoimmune diseases treated by biotherapies. In this context, we propose a novel test statistic for selecting loci of interest harbored by the X chromosome that are associated with time-to-event data taking into account skewed X-inactivation (XCI-S). The proposed statistic relies on a semi-parametric additive hazard model and is straightforward to implement. Results from the simulation study show that the test provides higher power gains than the score tests from the Cox model (under XCI process or its escape) and the Xu et al.'s XCI-S likelihood ratio test. We applied the test to the data from the real-world observational multicohort study set-up by the IMI-funded ABIRISK consortium for identifying X chromosome susceptibility loci for drug immunogenicity in patients with autoimmune diseases treated by biotherapies. The test allowed us to select two single nucleotide polymorphisms (SNPs) with high linkage disequilibrium (rs5991366 and rs5991394) located in the cytoband Xp22.2 that would have been overlooked by the Cox score tests and the Xu et al.'s XCI-S likelihood ratio test. Both SNPs showed a similar protective effect for drug immunogenicity without any occurrence of ADA positivity for the homozygous females and hemizygous males for the alternative allele. To our knowledge, this is the first study to investigate the association between X chromosome loci and the occurrence of anti-drug antibodies. We think that more X-Chromosome GWAS should

be performed and that the test is well-suited for identifying X-Chromosome SNPs, while taking into account all patterns of the skewed X-Chromosome inactivation process.

Keywords: immunogenicity, anti-drug antibodies, biotherapy, autoimmune disease, X-chromosome, skewed X-Chromosome inactivation, additive hazard model

1. INTRODUCTION

Despite the widespread recognition that genes play a role in many complex diseases, it is puzzling that one of the most important biological characteristics, the sex which is determined by the sex chromosomes, is often overlooked in genome-wide association studies (GWAS) (1–3). In practice, most of the GWAS discard this information whereas commercialized genotyping chips include thousands of Single Nucleotide Polymorphisms (SNPs) on the X chromosome. Even for autoimmune diseases that show strong sex differences in prevalence, the analyses are often restricted to the autosomes, thus neglecting X-linked information. Some potential reasons explaining this lack of interest for X chromosomes as compared to autosomes include lower coverage of chromosome X, technical issues regarding genotype calling and imputation and non-standard statistical analyses (4). In the latter case, the methodological problem is due to the fact that the statistical methods should take into account the X-chromosome inactivation (XCI) process on female X-chromosome loci (5, 6).

The main feature that makes the X chromosome different from the autosomal chromosomes is obviously the fact that, except for the pseudo-autosomal regions resulting from the divergence of evolution of sex chromosomes (X and Y), women have two copies while men have only one copy of each gene. This dosage imbalance is in part compensated by inactivation of one X chromosome in females through XCI. In each female cell, one copy of the X chromosome is inactivated. X-chromosome inactivation occurs at random (paternal or maternal), very early in embryonic life and is inherited by all daughter cells through mitosis (5). Females are mosaic, each cell having either the paternal or maternal X-chromosome inactivated. Such mosaic states can also be imposed in the case of gonosome aneuploidies. While the random inactivation process results in roughly a symmetrical (50:50) distribution in most females, skewing of X chromosome inactivation (XCI-S) is observed in some women, leading to a majority of either paternal or maternal X-chromosome inactivation. This skewing might be due either to selective pressure (negative selection) or a stochastic process (random selection in an embryonic stage where a limited number of cells give rise to the different tissues). Moreover, some genes may escape X-chromosome inactivation and remain biallelically expressed (XCE) (7).

In recent years, biopharmaceutical products (BPs) such as therapeutic monoclonal antibodies have become increasingly used in clinical practice and have led to a critical step forward in the treatment of many severe auto-immune diseases. However, for some patients these drugs activate the immune system, leading to the formation of Anti-Drug Antibodies (ADA). The mechanisms leading to drug immunogenicity can either be patient-related (genetic background, immunological status, prior exposure, prior disease) or treatment-related (drug characteristics and formulations, route, dose, frequency of administration) (8, 9). While some genomewide studies have investigated the genetic factors associated with the immunogenic potential of biotherapies (10), to our knowledge, none has investigated the X-chromosome.

To analyze the X chromosome in association studies, several test statistics have been proposed and implemented for case-control studies that consider either the XCI process or its escape (XCE). To take into account these two underlying biological processes, two genotype coding schemes are commonly used. One corresponds to the assumption of the XCI process as proposed by Clayton (11) while the other corresponds to the XCE process, as implemented in the classical PLINK software (12). For XCI, the proposed coding values are the same for homozygous females and hemizygous males while the heterozygous females fall midway between two homozygous, mimicking the fact that about 50% of cells have the minor (or alternative) allele active while the other 50% of cells have the reference allele active due to random XCI. Using this coding, Clayton derived a one degree-of-freedom score test statistic for case-control studies (11). For XCE, the coding implemented in PLINK codes female genotypes as 0, 1, or 2 copies of the minor allele and male genotypes as 0 or 1 copies of the minor allele. This genotype coding assumes that variants on both copies of the X chromosome are expressed in females. In this XCE setting, Zheng et al. (13) proposed a series of association tests that use different combinations of tests for male and female samples and rely either on genotypic counts or allelic counts in cases and controls. In practice, most of the case-control GWAS investigating X chromosome consider these two coding schemes with a logistic regression model, ignoring the XCI-S process. For time-to-event analysis, the same strategy can be considered by using the classical Cox model but with the same drawback for the XCI-S process. In a recent work, Xu et al. (1) and Han et al. (2) proposed a penalized partial likelihood approach based on the Cox model with a subject-specific random effect that takes into account the XCI-S process. However, the method is quite complex to implement and computationally burdensome for GWAS. We therefore developed a simpler genetic association test accounting for skewed X-inactivation that we used to investigate the role of X-linked genetic variants in the occurrence of ADAs in patients with autoimmune diseases treated by biotherapies.

In this paper, we first present a novel test statistic for selecting interesting loci of the X chromosome in time-to-event data investigation taking into account the XCI-S process that relies on a semi-parametric additive hazard model. It is based on a score-like test evaluated at the null hypothesis that is straightforward to implement. It avoids to compute the complex log-partial likelihood for the random effect Cox model

that requires to approximate the integration over the random effects (1, 2). Then, we apply it to the data from the real-world observational multicohort study set-up by the IMI-funded ABIRISK consortium (10) to identify susceptibility loci for drug immunogenicity.

2. MATERIALS AND METHODS

2.1. Material

The study population consists of 469 patients with genotyping information from the ABIRISK consortium real-world observational prospective multicenter cohort who suffered from multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases and who were treated by biotherapies (10). These patients were naive for the biotherapies they were given during the study, which included tumor necrosis factor (TNF) inhibitors, interferon (IFN)-beta, anti-CD20 (Cluster of Differentiation 20) and anti-interleukin 6 (IL6) receptor monoclonal antibodies.

The patients were followed up for 12 months. Clinical data were recorded in an electronic Case Report Form. DNA samples and serum samples were collected for genetic analyses and ADA testing, respectively. Serum samples for ADA testing were collected at baseline before starting BP and subsequently at each study visit thereafter. Anti-drug antibodies were detected by specific validated assays for each BP and analyzed in central ABIRISK laboratories [more information can be found in Hässler et al. (10)]. The outcome was the time between the date of the first dose of biotherapy and the first detection of the occurrence of anti-drug antibodies. Patients without ADA occurrence were censored at the date of their last clinical visit. Among the 469 subjects, 129 (27.5%) developed ADA during the 1-year follow-up.

The DNA polymorphism analysis was performed with Infinium OmniExpress-24 v1.2 BeadChip. Genotype calling was performed by Genome Studio software 2011.1 with Genotyping module v1.9 (Illumina). Genotypes were called by comparing the generated data with those in the supplied cluster file. The final report for genotype data was based on GRch38/hg38. Quality checks were applied for each sample using autosomal SNPs and removing samples with a call rate (percentage of SNPs genotyped by samples) lower than 95%, excessive observed level of heterozygosity (deviated by more than 3 standard deviations from the mean heterozygosity of the sample), ambiguous sex (genotypic sex different from phenotypic sex from the eCRF), genotyping completeness less than 99%, and non-European ethnicity admixture detected as outliers from a principal component analysis of a linkage-disequilibrium-pruned data set (with a deviation of at least 6 SDs from the mean of at least one of the first 10 principal components). For the quality control specific to the sex chromosomes, we plotted the X chromosome heterozygosity rates and the call rates for SNPs harbored on the Y chromosome. We clustered the individuals using the k-means clustering algorithm and thus eliminated four individuals. As we used the genotyping information and not the information in the measured intensities of X and Y chromosomes, we did not eliminate potential sex-chromosome aneuploidies such as Trisomy X. A total of 457 genotyped individuals were retained.

Then, we extracted 17,565 genotyped SNPs harbored on the X chromosome and conducted further quality control filtering for these SNPs. In practice, we removed samples with a call rate lower than 95% for these SNPs. The SNPs with deviation from Hardy-Weinberg equilibrium test with $p < 10^{-5}$ in females, with minor allele frequency less than 5% for both males and females were removed. Finally, a total of 456 genotyped individuals with 12,976 X-chromosome SNPs were considered for subsequent analyses.

2.2. Methods

2.2.1. Notation

Let T denote the failure time (here the time-to-ADA detection) and C the censoring time. We assume that T and C satisfy the condition of independent and non-informative censoring (14). For each subject i ($i = 1, ..., n$), $X_i = min(T_i, C_i)$ denotes the observed time of follow-up and $\delta_i = \mathbf{1}_{(X_i=T_i)}$ the indicator of failure (ADA detection) where the function $\mathbf{1}_{(.)}$ is the indicator function whose value is 1 if the argument is true and 0 otherwise. We also denote $Y_i(t) = \mathbf{1}_{(t\leq X_i)}$ the at-risk process and $N_i(t) = \mathbf{1}_{(X_i\leq t;\delta_i=1)}$ the counting process, given at time t. Let G be the genotype for a di-allelic SNP on the X chromosome and denote the two alleles as A and a with A as the rare (or alternative) allele and a as the common (or reference) allele. The genotypes are $G = ([aa], [Aa], [AA])$ for female subjects and $G = ([a], [A])$ for male subjects.

Under the unknown underlying XCI process with a potentially skewed pattern, we consider the following genotype coding variable : (i) females : $W = (0, 1/2 + U \times \mathbf{1}_{(G=[Aa])}, 1)$ for $[aa]$, $[Aa]$ and $[AA]$, respectively; (ii) males : $W = (0, 1)$ for $[a]$ and $[A]$, respectively. Here, the variable U is an unobserved (latent) subject-specific continuous random variable lying in the interval $[-1/2, 1/2]$. The values of $-1/2, 0$ and $1/2$ represent skewed XCI toward the common allele, random XCI, or skewed XCI toward the rare (or minor) allele, respectively. In the following, we use the rewritten coding : $W = Z + U \times \mathbf{1}_{(Z=1/2)}$ with $Z = 0, 1/2, 1$ for females and $Z = 0, 1$ for males. For each patient i, the data consists of $(X_i, \delta_i, Z_i, U_i)$.

2.2.2. Survival Model

In this work, we consider a semi-parametric additive hazard model with a latent variable (15, 16). The hazard function for the failure time T of individual i takes the form:

$$\lambda_i(t|Z = z, U = u) = \lambda_0(t) + \beta z + \beta u \times 1_{(z=1/2)} \qquad (1)$$

where β is the unknown regression coefficient of interest, $\lambda_0(t)$ is an unknown and unspecified baseline hazard function and U is the latent (unobserved) variable. Then, the individual-specific (conditional) survival distribution is such that:

$$S_i(t|Z = z, U = u) = exp\left[-(\Lambda_0(t) + \beta zt + \beta ut \times 1_{(z=1/2)})\right].$$

In the following, we assume that the U_i are independent and identically raised cosine distributed random variables with parameters $\mu = 0$ and $\gamma = 1/2$ (17). We recall that a continuous random variable U is said to have raised cosine distribution

with parameters $\mathbb{E}(U) = \mu$ and γ if its probability density function $f_U(x)$ is as follows: $f_U(x) = \frac{1}{2\gamma}\left[1 + \cos(\frac{x-\mu}{\gamma}\pi)\right]$ with $\mu - \gamma < x < \mu + \gamma$. In this work, U lies in the interval $[-1/2, 1/2]$ and its expectation is equal to zero. Based upon this latter assumption, when marginalized over U, the unconditional (or marginal) survival function and hazard function are given by:

$$S(t|Z=z) = \exp\left\{-\left\{\Lambda_0(t) + \beta z t + 1_{(z=1/2)} \log\left[\frac{\pi^2 \sinh(1/2\beta t)}{1/2\beta t \times (\pi^2 + 1/4\beta^2 t^2)}\right]\right\}\right\}$$

$$\lambda(t|Z=z) = \lambda_0(t) + \beta z + 1_{(z=1/2)} \times \left[-1/2\beta \coth(1/2\beta t) + \frac{1}{t} - \frac{1/2\beta^2 t}{(\pi^2 + 1/4\beta^2 t^2)}\right]$$

where sinh and coth are the hyperbolic sine function and hyperbolic cotangent function, respectively.

2.2.3. Test Statistic

In this section, a statistic accounting for skewed X-inactivation is derived for testing the null hypothesis $H_0 : \beta = 0$ (same survival distribution across genotypes) against $H_1 : \beta \neq 0$ (different survival distribution across genotypes).

Following Lin and Ying (16), under the marginal additive hazard model introduced just above, a simple semiparametric estimating function for β is constructed and a score-type function is obtained under the null hypothesis ($H_0 : \beta = 0$). Here, the intensity function for $N(t)$ is given by:

$$Y(t)d\Lambda(t|Z=z) = Y(t)\left\{d\Lambda_0(t) + \beta z + 1_{(z=1/2)} \times \left[-1/2\beta \coth(1/2\beta t) + \frac{1}{t} - \frac{1/2\beta^2 t}{(\pi^2 + 1/4\beta^2 t^2)}\right]\right\}.$$

By the Doob-Meyer decomposition (14), the counting process $N(t)$ can be uniquely broken down into the sum of a martingale and a predictable process, such that:

$$N(t) = M(t) + \int_0^t Y(t)d\Lambda(t|Z=z).$$

Under our model, we can estimate the cumulative hazard function by:

$$\hat{\Lambda}_0(t,\beta) = \int_0^t \frac{\sum_{i=1}^n \left\{dN_i(t) - \beta Z_i - 1_{(Z_i=1/2)}\left[\frac{1/2\beta \cosh(-1/2\beta t)}{\sinh(-1/2\beta t)} + \frac{1}{t} - \frac{1/2\beta^2 t}{(\pi^2+1/4\beta^2 t^2)}\right]\right\}}{\sum_{i=1}^n Y_i(t)}.$$

Then, following Lin and Ying (16) (Equation 2.7), a simple estimating function (or score-like function) for β can be written as:

$$U_\beta(\beta) = \sum_{i=1}^n \int_0^\infty (Z_i(t) - \bar{Z}(t))\Big\{dN_i(t) - Y_i(t)\beta Z_i dt - Y_i(t)1_{(Z_i=1/2)}\left[\frac{\beta/2\cosh(-\beta t/2)}{\sinh(-\beta t/2)} + \frac{1}{t} - \frac{\beta^2 t/2}{(\pi^2 + \beta^2 t^2/4)}\right]dt\Big\}$$

with $\bar{Z}(t) = \frac{\sum_{i=1}^n Y_i(t)Z_i}{\sum_{i=1}^n Y_i(t)}$. Using L'Hopital's rule, we know that the limit of $x \times \coth(x)$ as x approaches zero is equals to 1. Thus, under $H_0 : \beta = 0$:

$$U_\beta(\beta = 0) = \sum_{i=1}^n \int_0^\infty (Z_i(t) - \bar{Z}(t))dN_i(t).$$

Under the null hypothesis H_0, the random vector $n^{-1/2}U_\beta(\beta = 0)$ converges weakly to a normal with mean zero and with a variance which can be consistently estimated by $n^{-1}B(\beta = 0)$ with :

$$B(\beta = 0) = \sum \int_0^\infty (Z_i(t) - \bar{Z}(t))^2 dN_i(t).$$

Thus, the (score-like) statistic :

$$S = \frac{U_\beta^2(\beta = 0)}{B(\beta = 0)}$$

is asymptotically distributed under H_0 as a chi-square with one degree of freedom.

A stratified version of the test over k strata can be constructed by calculating $U_\beta(\beta = 0)$, and its estimated variance, separately in each stratum. Both are then summed over strata. The final stratified test is then calculated in exactly the same way presented just above.

2.3. Simulation Study

A simulation study was conducted to assess the size and power of the proposed test (herein called "Score-like") as compared to three test statistics: (i) the score test of the null hypothesis under the Cox model (herein called "Cox-XCI") using the XCI coding (*aa* (0), *aA* (0.5) and *AA* (1) for females and *a* (0) and *A* (1) for males); (ii) the score test of the null hypothesis under the Cox model (herein called "Cox-XCE") using the XCE coding (*aa* (0), *aA* (0.5) and *AA* (1) for females and *a* (0) and *A* (0.5) for males); (iii) the test proposed by Xu et al. (1) and Han et al. (2) based on a random effect XCI-S Cox model (herein called "Xu-Hao") and implemented in the R package "xlink" (18). To be in line with the Xu et al.'s XCI-S test that takes into account the sex as a potential confounding factor, we compared the results obtained by the Xu-Hao test to those obtained with the stratified versions (by the sex) of the Score-like, Cox-XCI and Cox-XCE test statistics.

Data were simulated under various scenarios assuming a locus undergoing XCI-S. The simulated variables were sex (females $K = 2$ and males $K = 1$), SNP genotype (females: $Z = 0$ for aa, $Z = 1/2$ for Aa and $Z = 0$ for AA; males: $Z = 0$ for a or $Z = 1$ for A), the individual skewness parameter and the time-to-event. In practice, genotype information for females was generated by combining the values of two Bernoulli variables ($\mathbb{B}\left(p_{[A]}\right)$) independently drawn and for males from only one Bernoulli variable with mean: 10%, 20% and 30% for both males and females. This value corresponds to a pseudo-minor allele frequency (MAF), i.e., the proportion of $[A]$ allele in the simulated population. The ratio between the female and male rate was set to 1:1. Failure times were generated from an additive hazard model with a protective effect of the minor allele such that the individual-specific hazard rate was: $\lambda(t|Z = z, K = k) = \lambda_{0(k)}(t) + \beta(z + u \times 1_{(z=1/2)})$ where $\lambda_0(t) = 5$; $\beta = 0, -1.5, -1.75, -2, -2.25, -2.5$. The baseline hazard rates were $\lambda_{0(k=1)}(t) = \lambda_0(t)$ for males and $\lambda_{0(k=2)}(t) = \lambda_0(t)\eta$ for females with $\eta = 1.2$. Three distributions for the latent variable U were investigated: (i) U was generated independently and identically from a raised cosine distribution with parameters $\mu = 0$ and $\gamma = 1/2$; (ii) U was generated independently and identically from a Beta distribution with $\mathbb{E}(U) = 1/2$ (shape parameters equal to 2); (iii) U was generated independently and identically from a truncated normal distribution ranging from -0.5 to 0.5 with mean zero and standard error of 0.18. We investigated no censoring, 20% and 40% type I censoring (administrative censoring). The total number of subjects was chosen to be 400. For each configuration of parameters, 1,000 replications were performed and the levels and powers of the tests were estimated with a 0.05 significance level.

3. RESULTS

3.1. Simulation Study

As seen from the simulation results, the estimated level of the proposed test under the null hypothesis for a threshold of 0.05 fell within the binomial range $[0.0365 - 0.0635]$ for all the studied scenarios. This is not the case for the Xu-Hao test, which gave slightly inflated type I error.

For XCI-S with a raised cosine distribution for the skewness parameter (**Table 1**), the power of the proposed test was always higher than the Xu-Hao test, with a difference varying from 1 to 6% and for each percentage of censoring. Higher power gains where observed for larger MAF. As expected, the Xu-Hao test gave higher power gains than the Cox-XCI test. The Cox-XCE test gave the worst power results. Results observed for XCI-S with a Beta distribution (**Table 2**) were quite similar. For XCI-S with a truncated Normal distribution (**Table 3**), for small and moderate MAF (10 and 20%), the power of the proposed test was always higher than the Xu-Hao test, with gains between 1 and 7%. For higher MAF (30%), the power results of the proposed test and the Xu-Hao test were close, sometimes to the slight advantage of the Xu-Hao test.

TABLE 1 | Size and power of the tests (Score-like, Cox-XCI, Cox-XCE, Xu-Hao) for XCI-S with raised cosine distribution (threshold level of 0.05).

			Cens = 0%			
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.20	49.10	63.50	75.90	85.90	91.50
Cox-XCI	5.10	40.80	55.60	70.10	81.40	88.20
Cox-XCE	4.70	34.90	48.50	61.90	73.60	82.30
Xu-Hao	7.30	44.30	59.80	72.60	83.00	89.90
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.10	65.90	80.90	91.40	96.00	98.80
Cox-XCI	5.60	61.50	78.20	89.20	94.70	98.50
Cox-XCE	5.50	53.50	70.50	81.80	92.30	96.80
Xu-Hao	7.20	65.00	79.70	92.40	95.80	98.50
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.60	75.70	91.30	95.80	99.00	99.80
Cox-XCI	4.60	72.80	89.10	94.10	98.40	99.80
Cox-XCE	5.70	63.90	83.30	90.60	97.20	99.30
Xu-Hao	7.10	75.10	90.30	95.20	98.70	99.80
			Cens = 20%			
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	6.10	42.00	52.20	64.60	76.10	84.10
Cox-XCI	5.60	32.00	43.60	55.80	67.40	76.70
Cox-XCE	6.20	27.90	37.00	48.90	59.40	69.00
Xu-Hao	8.00	36.20	47.30	59.80	70.50	80.90
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.80	55.50	73.40	81.50	91.90	97.00
Cox-XCI	5.90	49.10	67.60	76.90	89.40	96.40
Cox-XCE	6.30	43.50	57.70	70.50	83.10	91.80
Xu-Hao	7.80	53.20	71.80	80.20	91.40	96.70
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.30	67.10	81.40	92.80	97.80	99.70
Cox-XCI	4.80	61.80	77.40	90.30	97.00	99.30
Cox-XCE	4.50	54.30	69.40	85.10	93.30	97.20
Xu-Hao	6.90	64.70	79.60	91.30	97.50	99.40
			Cens = 40%			
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.70	34.90	41.70	53.40	63.00	75.00
Cox-XCI	5.10	24.60	29.70	41.80	50.20	64.00
Cox-XCE	5.80	20.60	26.20	35.80	42.40	54.30
Xu-Hao	7.20	28.10	35.00	46.30	54.90	68.00
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.20	46.10	58.00	72.80	79.00	92.20
Cox-XCI	5.00	38.80	51.40	66.30	74.40	89.10
Cox-XCE	5.90	33.10	43.60	57.40	67.30	80.50
Xu-Hao	6.40	43.30	55.40	69.70	77.80	91.30
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.00	57.60	73.90	81.50	92.60	97.40
Cox-XCI	5.30	52.50	69.10	78.60	90.80	96.20
Cox-XCE	4.80	45.70	57.60	71.00	84.30	92.40
Xu-Hao	7.40	56.70	72.20	81.50	92.50	96.90

TABLE 2 | Size and power of tests (Score-like, Cox-XCI, Cox-XCE, Xu-Hao) for XCI-S with Beta distribution (threshold level of 0.05).

Cens = 0%						
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.10	60.70	78.00	85.40	94.50	98.30
Cox-XCI	5.20	54.90	72.60	82.00	92.60	97.30
Cox-XCE	4.40	54.80	73.30	82.50	93.30	97.10
Xu-Hao	7.30	50.50	69.80	78.30	89.10	96.10
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.20	78.90	91.00	97.70	99.50	100.00
Cox-XCI	4.80	76.00	89.70	96.90	99.20	100.00
Cox-XCE	4.60	76.60	89.40	96.70	99.20	99.90
Xu-Hao	6.40	72.40	87.10	94.90	98.90	99.90
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.20	82.00	94.00	98.80	99.80	100.00
Cox-XCI	5.70	80.30	93.20	98.80	99.80	100.00
Cox-XCE	5.90	78.80	93.20	98.00	99.70	100.00
Xu-Hao	7.90	79.50	91.60	98.10	99.50	100.00
Cens = 20%						
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.00	52.80	67.00	83.00	89.00	95.10
Cox-XCI	5.30	44.20	59.90	77.40	84.80	92.50
Cox-XCE	4.40	44.20	60.10	75.50	83.40	92.60
Xu-Hao	7.60	40.70	55.70	72.80	83.30	88.90
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.10	69.90	85.50	92.60	97.90	99.80
Cox-XCI	5.10	65.50	81.40	91.40	97.60	99.70
Cox-XCE	5.90	64.90	82.80	90.70	97.20	99.30
Xu-Hao	7.10	62.80	78.00	88.90	95.70	99.10
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.50	78.20	88.50	97.30	99.60	100.00
Cox-XCI	5.20	76.60	88.00	96.70	99.50	100.00
Cox-XCE	4.70	73.70	86.50	96.10	99.20	100.00
Xu-Hao	8.20	76.10	87.40	94.20	99.20	99.70
Cens = 40%						
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.60	45.30	56.50	69.10	81.10	89.30
Cox-XCI	4.80	35.40	45.60	59.20	73.60	84.00
Cox-XCE	5.50	34.70	47.00	57.40	74.50	82.20
Xu-Hao	6.50	34.00	44.80	56.10	68.00	79.00
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.40	59.70	74.90	85.00	93.70	98.50
Cox-XCI	4.60	54.00	68.40	82.50	92.30	97.20
Cox-XCE	4.60	54.10	67.90	82.20	92.30	97.80
Xu-Hao	6.60	51.80	66.70	78.80	88.60	95.80
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.80	64.80	78.30	90.20	96.80	99.10
Cox-XCI	5.70	61.90	76.60	89.20	96.40	98.90
Cox-XCE	6.30	60.10	74.40	87.50	96.00	98.70
Xu-Hao	9.20	62.30	76.60	86.70	95.90	98.00

TABLE 3 | Size and power of tests (Score-like, Cox-XCI, Cox-XCE, Xu-Hao) for XCI-S with truncated normal distribution (threshold level of 0.05).

Cens = 0%						
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.00	47.20	61.20	75.10	85.50	93.30
Cox-XCI	5.30	40.80	53.50	69.60	80.80	89.90
Cox-XCE	5.00	34.60	46.90	62.00	73.70	84.90
Xu-Hao	7.80	43.70	57.40	71.80	83.80	91.50
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.10	65.70	81.40	90.40	96.20	98.80
Cox-XCI	5.40	61.00	77.90	87.90	95.50	98.60
Cox-XCE	5.60	54.50	70.90	81.20	91.30	96.60
Xu-Hao	7.90	65.40	79.90	90.30	96.20	98.80
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.40	77.00	88.30	94.10	99.20	100.00
Cox-XCI	5.70	74.50	87.40	93.60	99.00	99.90
Cox-XCE	6.00	63.90	82.50	90.30	97.00	99.10
Xu-Hao	8.90	78.60	89.20	94.60	99.20	99.80
cens = 20%						
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.10	41.20	52.30	65.60	76.80	84.20
Cox-XCI	5.10	31.20	42.40	56.60	69.00	77.90
Cox-XCE	5.00	26.40	36.00	49.10	60.40	70.60
Xu-Hao	8.30	34.70	47.00	61.20	72.10	79.40
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.70	56.90	69.90	84.50	92.30	97.10
Cox-XCI	4.20	50.30	66.30	81.10	90.40	96.00
Cox-XCE	4.50	44.60	58.20	74.10	84.00	92.70
Xu-Hao	6.20	55.30	69.50	83.60	91.00	97.10
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.00	68.80	81.10	92.30	97.40	98.90
Cox-XCI	4.50	65.10	78.80	90.70	96.80	98.50
Cox-XCE	5.40	55.60	72.00	84.20	93.20	96.90
Xu-Hao	7.10	69.90	82.40	93.60	97.30	98.80
Cens = 40%						
$p_A = 0.10$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	6.20	33.10	44.20	55.40	64.00	74.20
Cox-XCI	5.40	24.00	33.10	43.90	52.10	64.20
Cox-XCE	6.10	19.00	28.90	37.00	43.60	55.40
Xu-Hao	8.10	27.30	36.80	45.70	54.70	68.50
$p_A = 0.20$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	5.20	45.00	57.90	73.20	81.60	90.80
Cox-XCI	4.80	39.30	50.90	67.50	76.50	87.60
Cox-XCE	4.50	32.50	43.70	58.90	68.80	80.70
Xu-Hao	7.10	42.80	55.20	72.30	80.10	89.70
$p_A = 0.30$ β:	0	1.5	1.75	2	2.25	2.5
Score-like	4.70	56.20	70.10	84.10	91.70	96.80
Cox-XCI	4.40	51.80	66.50	80.90	89.80	96.40
Cox-XCE	4.70	43.80	57.30	73.70	83.70	92.10
Xu-Hao	8.10	58.20	71.50	84.60	91.30	96.80

3.2. Abirisk Cohort

The cohort analyzed in this work consists in 456 patients with genotyping information who successfully passed the quality-control procedures and who were suffering from auto-immune diseases and were naive for the studied biotherapies before the study. There were 309 women (68%) and 147 men (32%). Patients were aged from 18 to 87 years old and the median age was 41 years old. In this multi-cohort, 131 patients (29%, 65 males, 66 females) suffered from inflammatory bowel diseases (Crohn's disease or ulcerative colitis), 141 (31%, 42 males, 99 females) from multiple sclerosis and 184 (40%, 40 males, 144 females) from rheumatoid arthritis. Eight biotherapies were used in the study : TNF-inhibitors (Adalimumab, Etanercept, Infliximab), IFNβ (IFNβ-1a subcutaneous, IFNβ-1a intra-muscular and IFNβ-1b subcutaneous), anti-IL6R (Tocilizumab) and anti-CD20 monoclonal antibodies (Rituximab). 253 patients (55%) were taking TNF-inhibitors, 141 (31%) IFNβ, 35 (8%) anti-IL6R and 27 (6%) anti-CD20. For the 456 patients, the probability of producing ADA at 1 year was 27.5% [23.0%-31.8%]. The sex variable was not significantly related to the time to ADA detection ($p = 0.64$).

We first computed the p-values obtained with the stratified version (by the sex) of the score-like test. Then, to identify X-chromosomal loci associated with ADAs, we performed an FDR-based genome-wide analysis. Controlling the FDR at nominal level of 5% (19), we selected 24 associated signals. Results obtained using unstratified tests were similar. Among these association signals, two signals had p-values lower than 10^{-6} : rs5991366 ($p = 3.56 * 10^{-8}$ stratified test, $p = 4.58 * 10^{-8}$ unstratified test) and rs5991394 ($p = 3.74 * 10^{-7}$ stratified test, $p = 3.63 * 10^{-7}$ unstratified test). Both SNPs were located in the cytoband Xp22.2 near the gene chromobox 1 pseudogene 4 (*CBX1P4*) and the gene *REPS2* (*RALBP1* Associated Eps Domain Containing 2). For rs5991366, the frequency for the minor allele was 9.1% for females and 8.8% for males with no significant difference. For rs5991394, the frequency for the minor allele was 9.9% for females and 10.2% for males with no significant difference. This pair of SNPs are in very high linkage disequilibrium ($R^2 = 0.85$) (20).

Figure 1 displays the Kaplan-Meier survival curves for the SNP rs5991366. No event occurred among the 13 hemizygous males and 4 homozygous females for the alternative allele, whereas among the hemizygous males and homozygous females for the reference allele, 26.1% (35/134) and 27.6% (71/257) developed ADA positivity, respectively. Among the heterozygous females, 14.6% (7/48) developed ADA positivity. **Figure 2** displays the Kaplan-Meier survival curves for the SNP rs5991394. No event occurred among the 15 hemizygous males and 3 homozygous females for the alternative allele, whereas among the hemizygous males and homozygous females for the reference allele, 26.5% (35/132) and 27.5% (69/251) developed ADA positivity, respectively. Among the heterozygous females, 16.4% (9/55) developed ADA positivity.

Figures 3, **4** display the Manhattan plot of the X Chromosome genome-wide association results obtained with the score-like test (**Figure 3**) with a zoom in on the genomic region 150,000,000 bp to 20,000,000 bp (**Figure 4**). **Figures 5–7** display Manhattan plots of the X Chromosome genome-wide association results obtained with the (stratified) Cox-XCI test (**Figure 5**), the (stratified) Cox-XCE test (**Figure 6**) and the Xu-Hao test (**Figure 7**). Looking at the Manhattan diagrams in the distal Xp22.2 region, the association signals obtained from the Cox-XCI, Cox-XCE and

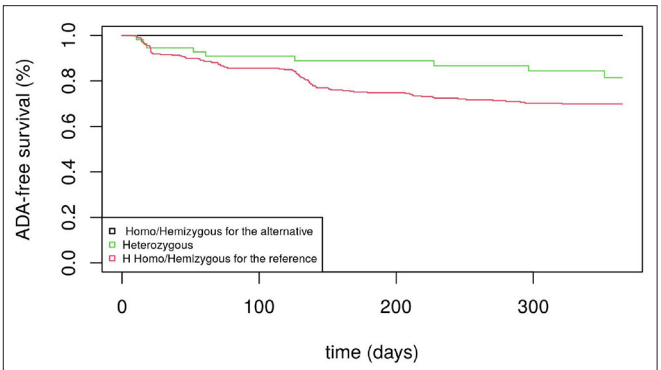

FIGURE 1 | ADA-free survival curves (men and women) for rs5991366.

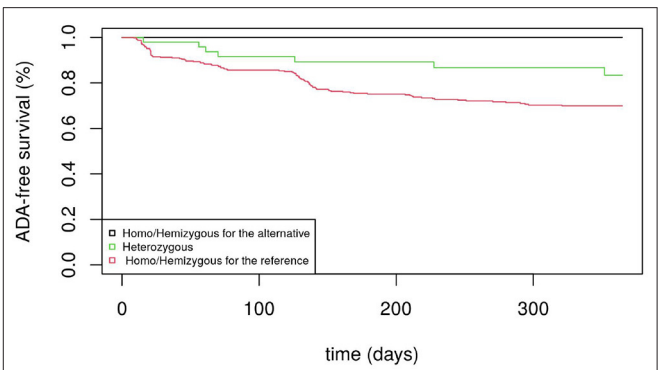

FIGURE 2 | ADA-free survival curves (men and women) for rs5991394.

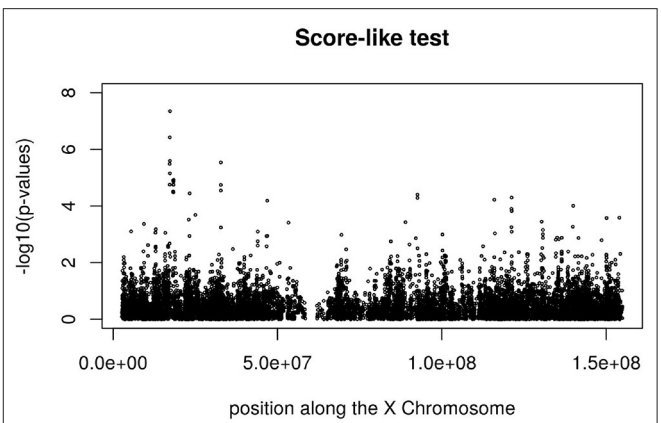

FIGURE 3 | Manhattan plot of X Chromosome genome-wide association results-Score-like test.

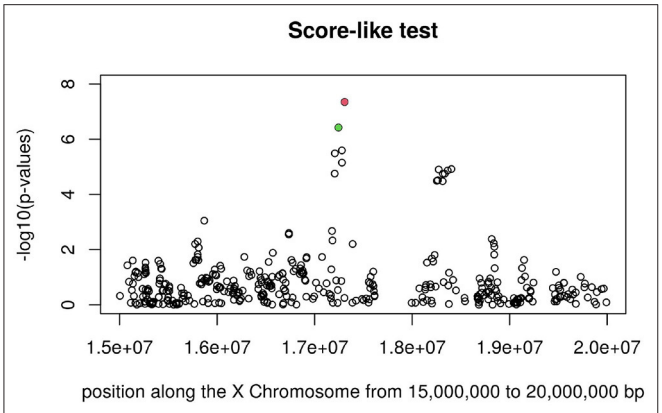

FIGURE 4 | Manhattan plot of X Chromosome genome-wide association results (Score-like test) with zoom in on area 15,000,000–20,000,000 with SNP rs5991366 (red) and SNP rs5991394 (green).

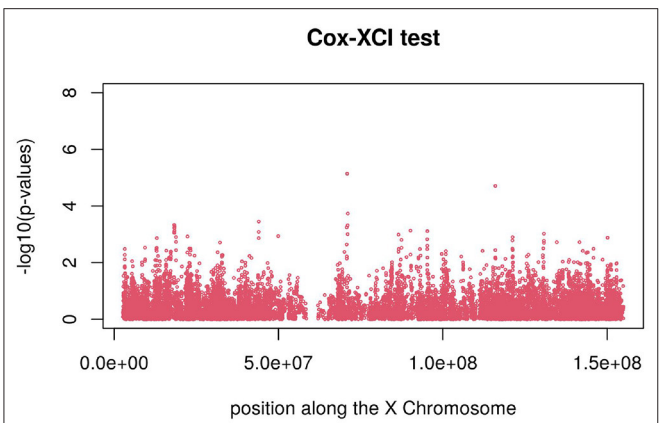

FIGURE 5 | Manhattan plot of X Chromosome genome-wide association results-Cox-XCI test.

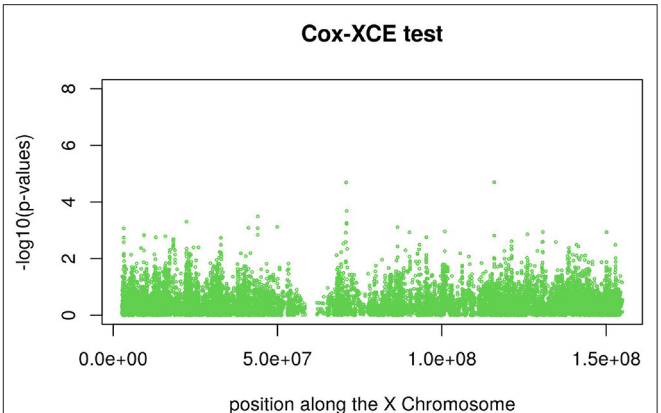

FIGURE 6 | Manhattan plot of X Chromosome genome-wide association results-Cox-XCE test.

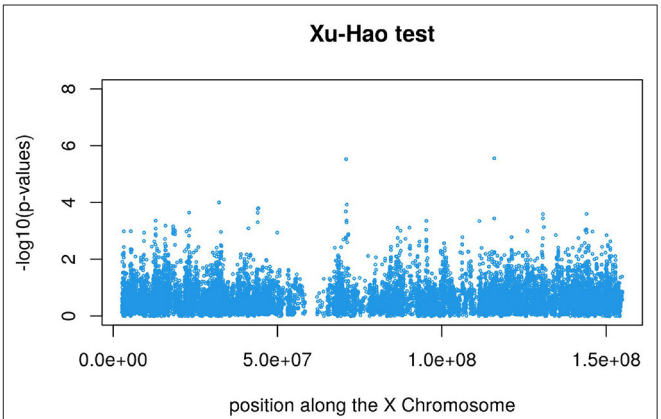

FIGURE 7 | Manhattan plot of X Chromosome genome-wide association results - Xu-Hao test.

Xu-Hao tests were weaker than those obtained from the score-like test.

4. DISCUSSION

Our aim was to investigate the association of loci located on the X chromosome with drug immunogenicity in auto-immune diseases, while taking into account different X-chromosome inactivation models: XCI (random inactivation), XCI-S (skewed inactivation) and XCE (inactivation escape). To date, few strategies have been proposed for analyzing time-to-event data, taking into account the complexity of the X-chromosome inactivation biological process. In practice, one can use statistical tests derived from the classical Cox model (with XCI or XCE coding) or a more complex and computationally burdensome test based on a random effect Cox model (1, 2).

We propose a new score-like test that takes into account for an unknown underlying XCI process with a potentially skewed pattern. We assumed a semi-parametric additive hazard model with a latent factor that provides an easy and meaningful interpretation of the skewed X-inactivation process. Results from the simulation study show that the proposed test provides higher power gains than the score tests from the Cox model (XCI and XCE coding) and to the likelihood ratio test proposed by Xu et al. (1) and Han et al. (2). With the latter test, some caution should be taken when interpreting its power results as the type I error rate is slightly inflated. For the distribution of the latent factor, we considered a raised cosine distribution that mimicks the unknown skewed X-inactivation process and leads to a closed form for the marginal survival distribution. Other choices are possible. However, as shown by the simulation study, the proposed test performs quite well even with other distributions such as the Beta and truncated Normal distributions. In the present, the additive hazard model that represents the effect of the genetic locus on the hazard rate as a linear form, serves as an alternative to the usual proportional hazards model and benefits from several useful mathematical properties. The model involves a straightforward simple testing procedure and a stratified version of the score-like test is easily obtained. However,

if the genetic effect is not confounded with sex, there is no need to stratify by sex in the analysis. Note that our main objective was not to find the best genetic model over various biological processes but to propose a novel statistic for testing the effect of a loci under an XCI-S process. The statistic could nevertheless be used for model selection, although this would require further works.

By investigating the association between the genes located on the X chromosome with anti-drug immunogenicity, the proposed test allowed us to select two SNPs with high linkage disequilibrium (rs5991366 and rs5991394) located in the cytoband Xp22.2 that would have been overlooked by the score tests from the Cox model (XCI and XCE coding) and the Xu et al.'s likelihood ratio test. Both SNPs show a similar protective effect for drug immunogenicity without any occurrence of ADA positivity for homozygous females and hemizygous males for the alternative allele. In contrast, almost 30% of the homozygous females and hemizygous males for the reference allele experienced ADA positivity. Note that for both SNPs, the frequencies of the alternative allele observed for both males and females were not significantly different from the estimates obtained in European samples (21).

The region containing the two X-linked SNPs associated with ADA occurrence is conserved between primates (99% identity) and also within mammals (70% identity with mus musculus) (22). Moreover, the SNP rs59913394 is in the proximity of a regulatory variant (rs113772781) in LD (r2=1 in Europeans), but no gene expression in immune cell types is regulated by this variant (23). In the genomic neighborhood of these two SNPs, the closest gene, *CBX1P4* (Chromobox 1 Pseudogène 4), is a pseudogene followed by the gene *REPS2* (*RALBP1* Associated Eps Domain Containing 2). The *REPS2* gene (*RALBP1* Associated Eps Domain Containing) encodes for a protein which is part of a protein complex that regulates the internalization of growth factors receptors such as *EGF* and insulin receptors and may have an inhibitory effect on growth factor cell signaling. It is downregulated in prostate cancer progression and that this downregulation is accompanied by upregulation of *NF-κB* activity (24, 25). No direct effect of *REPS2* on auto-immune disease has been recognized to date. However, the *REPS2* gene is widely expressed in several human tissues, including white blood cells and lymph nodes. Its biological targets (growth factors and *NF-κB* signaling) are also widely expressed and have a major role in inflammation and immunity. Dysregulation of the IGFs pathway has an important role in autoimmune diseases (26). In particular, IGF stimulates Treg proliferation and has a protective effect in autoimmune disease models. Further in the Xp22.2 locus, several genes have a role in immunity including *ACE2*, *TLR7*, and *TLR8*. *ACE2* (Angiotensin I converting enzyme 2) is a key actor of the renin-angiotensin system acting as a homeostatic regulator of the vascular function (blood pressure regulation). Recently it attracted much attention for its major role in SARS-CoV-2 infection through its affinity for the viral spike factor, raising the question of a possible sex bias in this disease (27). The genes *TRL7* and *TLR8* are members of the Toll-like receptor (TLR) family which plays a major role in activation of innate immunity. Studies have shown that some immunity genes such as *TLR7*, *CD40LG*, and *CXCR3* may escape XCI in several lymphoid cells (B cells, T cells, plasmacytoid cells), especially in some auto-immune diseases such as Systemic Lupus Erythematosus due to a dysregulation of the XIST long non-coding RNA involved in XCI (28–30). Here, we investigated the genes that are located in the vicinity of the SNPs of interest, but other genes that are further from the SNPs (e.g., *MNG2* multinodular goiter 2) could be relevant. Additional studies are required to strengthen our findings.

In the search for common susceptible loci, we analyzed together the time to ADA of patients treated with eight different drugs for different autoimmune diseases. This strategy, which uses information concerning various therapies and autoimmune diseases, is likely to provide significant gain in power in finding loci not associated with specific therapies or autoimmune diseases. Nevertheless, it would not be suitable for searching for loci that are specific to a particular therapy or disease.

In conclusion, we think that more X-Chromosome GWAS should be performed and that the proposed test, which is easy to implement with standard softwares, is well-suited for identifying X Chromosome SNPs, while taking into account all possibilities of the skewed X-Chromosome inactivation process.

DATA AVAILABILITY STATEMENT

The data analyzed in this study were collected in the context of the ABIRISK project by ABIRISK partners. Access to the minimal dataset underlying the findings can be obtained upon request to the ABIRISK Sustainability Scientific Committee by submission of an analysis plan. The analysis plan should explain the purpose of the use of the data and confirm the intention to use the data only for replication studies concerning anti-drug inhibitors, since this is the limitation of the ethical permission on how this data can be used. The contact person of the ABIRISK Sustainability Scientific Committee to whom the requests should be sent is MP (marc.pallardy@inserm.fr).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the General University Hospital in Prague (reference 125/12, Evropský grant 1.LF UK-CAGEKID) Institutional Committee of Heinrich Heine University, Düsseldorf, Germany (protocol reference 4451) Ethikkommission der Fakultät für Medizin der Technischen Universität München, München, Germany (reference 335/13) Ethikkommission Nordwest- und Zentralschweiz, Basel (reference 305/13) Ethikkommission der Medizinischen Universität Inns- bruck, Innsbruck (reference AN2013-0040 331/2.1) Comité Ético de Investigación Clínica de l'Hospital Universitari Vall d'Hebrón, Barcelona [reference EPA(AG)66/2013(3866)] Stockholm Regional Ethics Committee, Stockholm (reference Dnr. 2013/1034-31/3 and Dnr. 2015/749-32) Comité de Protection des Personnes Ile de France VII (reference 13-048) Medical Ethical Committee of the Academisch Medisch Centrum, Amsterdam (reference 2013-304#B20131074)

Local Ethics Committee of AOU Careggi (reference Protocol No 2012/0035982) NRES Committee London, City and East (reference 14/LO/0506) Comité de Protection des Personnes Ile de France IV (reference 2013/24) Comité d'éthique hospitalo-facultaire universitaire de Liège (reference 2015/55). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PB coordinated the project and developed the proposed statistical procedure. SH, SC-B, and PB participated in writing the original draft. SH and PB analyzed the data. SH, MA, FD, AF-H, XM, MP, and PB participated in the data collection. MP coordinated the ABIRISK project. All authors read and approved the final manuscript.

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MINI REVIEW
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Mini-Review: Gut-Microbiota and the Sex-Bias in Autoimmunity – Lessons Learnt From Animal Models

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It is well appreciated that there is a female preponderance in the development of most autoimmune diseases. Thought to be due to a complex interplay between sex chromosome complement and sex-hormones, however, the exact mechanisms underlying this sex-bias remain unknown. In recent years, there has been a focus on understanding the central pathogenic role of the bacteria that live in the gut, or the gut-microbiota, in the development of autoimmunity. In this review, we discuss evidence from animal models demonstrating that the gut-microbiota is sexually dimorphic, that there is a bidirectional relationship between the production of sex-hormones and the gut-microbiota, and that this sexual dimorphism within the gut-microbiota may influence the sex-bias observed in autoimmune disease development. Collectively, these data underline the importance of considering sex as a variable when investigating biological pathways that contribute to autoimmune disease risk.

Keywords: gut-microbiota, inflammation, autoimmunity, sex, immune system

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INTRODUCTION

One of the strongest risk factors for developing autoimmunity is female sex (1). Although the mechanistic reasons underlying the strong female sex-bias in autoimmune conditions are unclear, it is likely to be strongly influenced by sex-differences in immune system function. Generally, innate and adaptive immune responses are stronger in females than males. Classic examples include heightened interferon type 1 production by activated female plasmacytoid dendritic cells (pDCs) (2) and stronger humoral immune responses in females, with higher antibody titres at baseline and in response to vaccination (3).

Sex determinants such as sex-chromosomes and sex-hormones influence differences in immune system function. Immune system-related genes are encoded on the X and Y chromosomes (4), and sex steroid hormones such as testosterone, oestrogens and progesterone directly impact immune cells by binding to intracellular and extracellular sex hormone receptors (5). However, this is not the whole picture. Sex influences a wide variety of host responses, which could have indirect effects on the immune system. This is supported by evidence demonstrating that the sex-bias in some experimental models of autoimmunity is sensitive to environmental factors. For example, differences in disease risk or severity are less pronounced in certain housing conditions or in germ-free mice (6). These data suggest that the commensal organisms that colonize barrier surfaces–more commonly known as microbiota–may directly impact sex-bias-associated autoimmune disease risk.

The term microbiota refers to the collection of micro-organisms that share our body space, the greatest number of which are located in the gastro-intestinal tract (7). Recently, it has become evident that pathological changes to the gut-microbiota, or dysbiosis, play a central role in influencing the aberrant immune responses that contribute to autoimmune development (8). Animal models have been pivotal in demonstrating this association. For example, K/BxN mice, which develop a spontaneous erosive arthritis, and SKG mice, which develop a severe spondyloarthropathy, do not develop arthritis when housed in germ-free conditions or when treated with broad spectrum antibiotics (9, 10). Treatment with oral antibiotics suppresses disease in a wide variety of inducible models of autoimmunity, ranging from arthritis models (11) to models of multiple sclerosis (MS) and uveitis (12–15). Multiple studies have also demonstrated that changes to the gut-microbiota are associated with the progression of autoimmune models including collagen-induced arthritis (16) and systemic lupus erythematosus (SLE) (17). This review will summarize recent research suggesting that sex influences the gut-microbiota and that sex-hormones directly impact the gut-microbiota, which in turn influences the production of sex-hormones. These data highlight how two risk factors influencing autoimmunity, sex and dysbiosis, communicate and how animal research can give insights into these biological processes. We also discuss evidence from specific experimental models where sexual dimorphism in the gut-microbiota impacts autoimmune disease development. The purpose of this review is to accentuate the diverse effects sex can have on host physiology, demonstrating the importance of reporting sex-dependent effects by including both sexes in animal research.

DIFFERENCES IN GUT-MICROBIOTA OF MALE AND FEMALE MICE

There are differences in the gut-microbiota in male and female mice (a summary of sex-associated bacterial species highlighted in referenced studies is summarized in **Table 1**). In C57BL/6–the most common strain used in animal research—17 operational taxonomic units (OTU) are more abundant in male versus female mice (e.g., *Lachnospiraceae*, *Clostridium*, *Ruminoccoceae* and *Allobaculum*), and 11 OTU are more abundant in females (e.g., *Bacteroidetes* and *Barnesiella*) (18). However, in B6.129S wild-type mice *Peptococacceae* and *Streptococacceae* are more abundant in male mice, while *Turicibacter* and *Clostridiaceae* are more abundant in females (19). As these contrasting studies were carried out in different animal facilities using different analysis techniques (for example different sequencing depths) it is hard to untangle whether these differences are driven by strain background or subtle differences in housing conditions. In a study comparing C57BL/6 mice and BALB/c mice housed in the same animal facility, strain- and sex-dependent effects on the gut-microbiota remained (20). *Lactobacillus plantarum, Bacteroides distasoni, Clostridium* spp. and Turicibacter were enriched in C57BL/6 females compared to males, whilst *Bifidobacterium* was enriched in BALB/c females compared to males. Interestingly, sex-differences in the gut-microbiota correlated with the expression of several genes associated with immune system function in the intestinal tissue. The abundance of female-enriched bacteria species such as *Clostridium leptum* positively correlated with *IL-2rb, Ccr3,* and *Cd80* expression in female C57BL/6 mice, and between male-enriched bacterial species such as *Faecalibacterium prausnitzii* and *Clostridium ramosum* positively correlated with and *Apoe, IL-1β* and *Stat4* expression in male BALB/c mice (20).

Larger scale studies—one comparing sex-differences in the gut-microbiota in 8 strains from cross-collaborative mouse resource, and one independent analysis of 89 inbred mouse strains (21, 27)—demonstrate the impact of strain background, and therefore genotype, on sex-associated differences in gut-microbiota composition (21, 27). In the study comparing 89 inbred strains there were clear differences between the sexes in every strain, but the largest sex-differences were seen in C57BL/6J (females-enriched for *Coprococcus,* and males-enriched for *Bacteroides)* and C3H/HeJ mice (males-enriched for *Akkermansia, Coprobacillus, Ruminococcus, Suterella*). When the entire population analysis was interrogated together, the magnitude and direction of changes were driven by an interplay between sex and genotype (21).

Sex-dependent differences in the gut-microbiota are also impacted by diet with a high-fat diet (HFD)-fed leading to sexually divergent effects on the gut-microbiota. HFD in male mice increases the abundance of *Lactobacillus, Alistipes, Clostridium,* and *Lachnospiraceae,* whilst a HFD in female mice reduces these strains (22). In an in-depth study by Bridgewater and colleagues, sex-differences were observed in C57BL/6J mice fed standard chow, but in mice fed HFD the sex-dependent shifts were more pronounced (18). In this study, the authors did not observe opposite shifts in the bacterial species of high-fat diet fed male and female mice, but rather differences in the relative abundance of certain clades (18). More specifically, although 10 OTUs shifted in the same direction in both females and males in response to HFD (either increased or decreased in both), 31 OTUs were only affected in females and 22 OTUs were only affected in males by HFD.

Data from male and female germ-free C57BL/6J mice colonized with the same "human" microbiota (taken from one donor fed a vegetarian, high inulin diet) suggest that sex influences the ability to accommodate intestinal bacterial species (23). Despite being colonized with bacteria from the same human donor, colonized germ-free mice still displayed sex-differences in the gut-microbiota. 13 OTUs were higher in males, including *Parabacteroides distasonis* and *Blautia faecis,* whilst 33 OTUs were higher in females including *Clostridium* groups, *Escherichia fergusonii* and *Shigella sonnei* (23). Although the exact mechanisms are yet to be elucidated, these sex-difference in the ability to accommodate different bacteria is likely due to microbiota-independent sex-differences in the intestinal immune system. For example, in the study by Fransen et al. (24), sex-differences in the expression of interferon type 1 genes are present in the intestines of uncolonised mice. The authors hypothesized that lower expression of interferon type 1 genes in male mice support the colonization of bacterial groups *Alistipes, Rikenella,*

TABLE 1 | Bacterial genus/species driving post-pubescent sex differences in referenced studies.

References	Strain	Health status	Highlighted bacterial genus/species driving sex differences	Reported difference between sexes	Effect of microbiome differences on the host
Bridgewater et al. (18)	C57BL/6	Naïve	*Allobaculum*, *Bifidobacterium*, *Clostridium* XlVa	Increased in males	Males were found to be resistant to the effects of stress on activity whereas females showed decreased locomotion after stress.
			Barnesiella, *Porphyromonadaceae*	Increased in females	
Kozik et al. (19)	B6.129S	Naïve	*Peptococcaceae*, *Streptoccocaceae*	Increased in males	Males developed more severe colitis
			Turicibacter, *Clostridiaceae*	Increased in females	
Elderman et al. (20)	C57BL/6	Naïve	*Eggerthela*, *Allobaculum* (not significantly)	Increased in males	Bacteria increased in abundance in females associated with increased activation, proliferation and migration of leukocytes
			Clostridium difficile, *Clostridium leptum*, *Enterococcus*, *Turicibacter*	Increased in females	
	BALB/c	Naïve	*Eggerthela*, *Bifidobacterium*	Increased in males	Bacteria increased in abundance in males associated with proliferation of lymphocytes, T cells in particular and migration of leukocytes
			Prevotella spp., *Turicibacter* (not significantly)	Increased in females	
Org et al. (21)	C57BL/6	Naïve	*Coprococcus*, *Bacteroides*	Increased in females	N/A
	C3H/He	Naïve	*Akkermansia*, *Coprobacillus*, *Ruminococcus*, *Suterella*	Increased in males	N/A
Bolnick et al. (22)	C57BL/6	High-fed diet	*Lactobacillus*, *Alistipes*, *Clostridium*, and *Lachnospiraceae*	Increased in males	N/A
		High-fed diet	*Lactobacillus*, *Alistipes*, *Clostridium*, and *Lachnospiraceae*	Decreased in females	
Bridgewater et al. (18)	C57BL/6	High-fed diet	*Ruminococcacea*	Increased in males	N/A
		High-fed diet	*Lachnospiraceae*, *Ruminococcacea*, *Peptococcaceae*	Increased in females	
Wang et al. (23)	C57BL/6	Naïve, colonized with human microbiota	*Parabacteroides distasonis*, *Blautia faecis*	Increased in males	N/A
			Clostridium groups, *Escherichia fergusonii*, *Shigella sonnei*	Increased in females	
Fransen et al. (24)	C57BL/6	Naïve	*Ruminococcaceae* and *Rikenellaceae*	Increased in males	Male microbiota upregulates DNA repair and cell cycle genes in female recipients. Female microbiota upregulated IL-10 signaling and completement system genes, influenced by regulation of type I interferon (IFN) production in male recipients.
			Desulfovibrionaceae, *Lactobacillaceae*	Increased in females	
Zhang et al. (17)	MRL/lpr	Model of SLE	*Lachnospiraceae*	Increased in females	The increased abundance of lachnospiraceae may influence disease development
			Bifidobacterium	Decreased in females	

(Continued)

TABLE 1 | Continued

References	Strain	Health status	Highlighted bacterial genus/species driving sex differences	Reported difference between sexes	Effect of microbiome differences on the host
Yurkovetskiy et al. (6)	NOD	Model of Type 1 Diabetes	Experiment 1: *Porphyromonadceae*, *Kineospariaceae*, *Veillonellaceae*	Increased in males	Post-pubescent females develop worse disease than post-pubescent males
			Experiment 2: *Enterobacteriaceae*, *Peptococcaceae*	Increased in males	
			Experiment 3: *Lactobacillaceae*, *Cytophagaceae*	Increased in males	
			Experiment 4: *Peptostreptococcaceae*, *Bacteroidaceae*	Increased in males	
Markle et al. (25)	NOD	Model of Type 1 Diabetes	*Roseburia*, *Coprococcus*, *Bilophilia*	Increased in males	Female mice develop worse disease than males, colonization with male microbiota protects females from disease
			Lachno I.S, Parabacteroides	Increased in females	
			Rosburia, *Blautia*, *Coprococcus*	Increased in females colonized with male microbiota	
			Peptococcus	Decreased in females colonized with a male microbiota	
Gomez et al. (26)	HLA-DRB1*0402	Arthritis-resistant control mice	*Bifidobacterium pseudolongum* subsp. *Globosum*, *Parabacteroides distasonis*	Increased in males	Sex-differences are lost in arthritis-susceptible HLA-DRB1*0401 mice
			Barnesiella viscericola	Increased in females	

and *Porphyromonadaceae*, which were overrepresented in the male microbiota versus female mice (24). These bacterial species, in turn, were associated with inflammation and DNA damage when transferred to females. Thus, microbiota-independent sexual dimorphism in the immune system might lead to the selection of a sex-specific microbiota, which then drives further divergence in immune response between males and females.

BIDIRECTIONAL RELATIONSHIP BETWEEN SEX-HORMONES AND GUT-MICROBIOTA

How does sex shape the gut-microbiota? As many sex-differences in the gut-microbiota are altered by sexual maturation in mice and are modified by the surgical removal of reproductive organs *via* gonadectomy, sex-hormones probably play a dominant role. In NOD mice, which have well-documented sex-differences in the gut-microbiota, there are no sex-differences in the gut-microbiota prior to puberty (6). In male NOD mice, there is a pronounced shift in the gut-microbiota post-pubertally compared to pre-pubertal animals. In female NOD mice, puberty has limited effects on the gut-microbiota, suggesting a dominant effect of testosterone on the gut-microbiota in this model (6). To address this directly, the authors gonadectomised male mice, which shifted the gut-microbiota toward a female gut-microbiota profile compared to sham-operated male mice (6). Elegantly, to eliminate the impact of specific pathogen free (SPF) housing conditions on these observations, the authors colonized male and female germ-free NOD mice with female microbiota, finding similar differences between colonized germ-free male and female NOD mice post-pubertally (6).

Gonadectomy was also shown to impact sexual dimorphism within the gut-microbiota in other studies. However, underlining the complex interplay between diet, genotype and the gut-microbiota–the effects of gonadectomy on the gut-microbiota were different depending on strain and diet (21). Overall, gonadectomy had a greater impact on the gut-microbiota of males fed standard chow and females fed a high-fat diet (21). Testosterone treatment of male gonadectomised mice could prevent the effects of gonadectomy on the gut-microbiota of C57BL/6 and C3H/HeJ mice, but not of DBA/2J mice (21). Although this study is confounded by the fact that different strains were housed in different animal facilities, these data form the most direct evidence that sex-hormones alter the composition of the gut-microbiota.

The relationship between sex-hormones and the gut-microbiota is likely to be bidirectional. Stool levels of estradiol, progesterone and corticosterone are reduced in germ-free C57BL/6 mice compared to SPF mice (28). Male germ-free NOD mice have lower levels of testosterone (25),

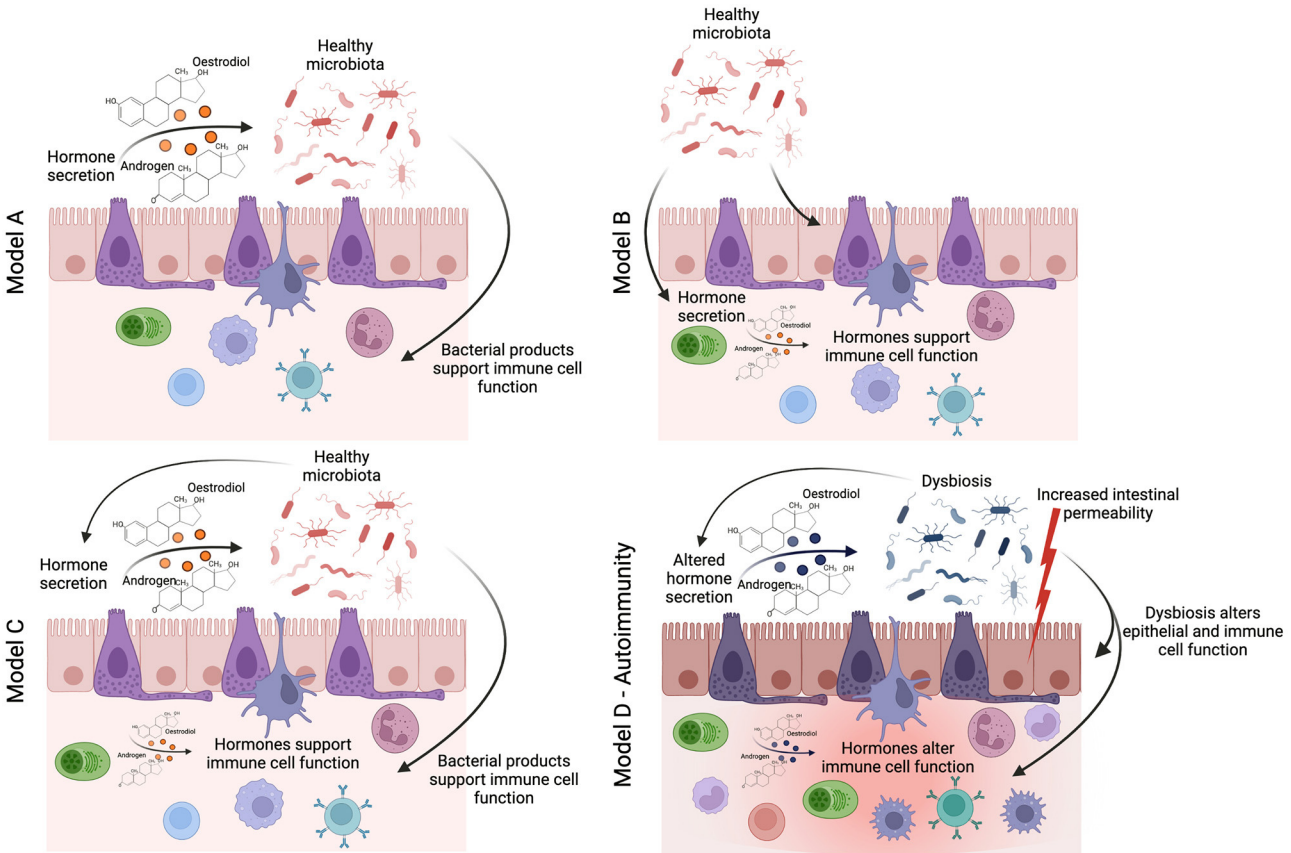

FIGURE 1 | How does sexual dimorphism in the gut-microbiota influence the immune system in health and disease? The seminal study by Yurkovetskiy et al. (6) proposes three models by which the sexual dimoprhism in the gut-microbiota may influence immune system function. In linear model A, sex-hormones regulate the identity of gut-microbes (either through immune or metabolic mechanisms), and then the gut-microbes themselves activate specific immune effector mechanisms. In linear model B, gut-microbes are regulators of sex hormone metabolism, and sex-hormones are the actual effectors on immune responses. In a two-signal model C, both microbiota and hormones could contribute in an additive fashion to influence effector mechanisms (6). We would like to add an additional model for autoimmune conditions (model D), where homeostasis is lost, leading to dysbiosis, increased intestinal permeability, potential changes in the regulation of sex-hormone production, and thus altered function of the immune cell compartment and chronic inflammation.

while female germ-free NOD mice have higher levels of testosterone compared to their colonized counterparts (25). Colonization of NOD mice with microbiota containing over-represented male-associated bacterial species modulates the levels of sex-hormones in circulation (25). Following fecal transplant in microbiota-depleted mice fed broad-spectrum antibiotics, the levels of testosterone in the donor mouse can be predicted by the gut-microbiome in the recipient mouse (29). Certain bacterial species, such as those belonging to the *Actinobacteria*, *Proteobacteria*, and *Firmicutes* phyla, can metabolize steroid hormones through the expression of enzymes such as hydroxysteroid dehydrogenase (HSD) (30). Furthermore, disrupting the microbiota through antibiotics treatment reduces the levels of steroids within the intestine (31). More directly, intestinal micro-organisms from humans regulate testosterone levels through reversible 17β reduction of androgens by HSD (32). These data suggest that the components of the gut-microbiome can directly regulate the levels of sex-hormones, and particularly testosterone, which is an important consideration for future studies studying the potential immunomodulatory impact of sex-hormones on the immune system.

EVIDENCE THAT THE GUT-MICROBIOTA INFLUENCES SEXUAL DIMORPHISM IN AUTOIMMUNE DISEASE DEVELOPMENT

Evidence from animal models of type 1 diabetes, SLE and MS–which all have a female-bias in humans–suggests that the gut-microbiota influences the sex-bias in autoimmune development. The most direct evidence comes from NOD mice, which develop a female-biased spontaneous model of type 1 diabetes. As discussed, NOD mice have a well-documented sex difference in their gut-microbiota, which influences disease development and severity. In germ-free NOD mice, the sex-bias in glucose intolerance is eradicated, suggesting a dominant role for the gut-microbiota in driving the sexual dimorphism in disease development (6). In this model, germ free mice develop diabetes

more frequently than SPF mice, suggesting a protective role for the gut-microbiota (6).

SLE has one of the most pronounced sex-biases in disease development, with a female predominance ranging from 6:1 to 15:1 depending on age/study (1). In lupus-prone MRL/lpr mice, female mice, which develop more severe proteinuria, have increased levels of *Lachnospiraceae* and less *Bifidobacterium* than male MRL/lpr mice. Interestingly, female MRL/lpr mice have more *Lachnospiraceae* than female MRL control mice, whilst these differences are not observed between their male counterparts. This suggests that the increased disease severity observed in females, which is associated with high kidney damage, may be influenced by *Lachnospiraceae* (17). Validation of this theory would involve housing male and female MRL/lpr in germ-free conditions or ablating the microbiota of MRL/lpr mice with broad-spectrum antibiotics. This would allow direct comparison of lupus severity between gut-microbiota sufficient and deficient male and female mice. To our knowledge these experiments have not been performed. The immunomodulatory potential of the gut-microbiota in MRL/lpr mice is highlighted by data showing that treatment of MRL/lpr with a mixture of 5 *Lactobacillus* strains suppresses intestinal epithelium permeability (and therefore "gut leakiness") and IgG2a production and increases IL-10 production. This leads to a reduction in lupus-associated kidney damage. In this system, treatment with *Lactobacillus* only suppresses disease in female mice and castrated male mice, but not in control males, suggesting that the impact of *Lactobacillus* on disease severity was dependent on sex-hormones (33).

In experimental autoimmune encephalitis (EAE), a mouse model of MS, treatment with high levels of the sex hormone 17-β-estradiol suppresses disease severity in C57BL/6. This is associated with alterations in the gut-microbiota, and specifically with increased abundance of *Lactobacillaceae* (34). Although the sex-bias in this model of EAE is not pronounced, the disease is known to be influenced by the microbiota, as antibiotics treatment suppresses disease development (13). This suggests an important role for the microbiota in controlling the breakdown of immunological tolerance to myelin-associated autoantigens. Models of MS that exhibit a female sex-bias, such as when EAE is induced in SJL/J mice, may offer a better system to untangle the impact of sexual dimorphism in the gut-microbiota on disease development.

Despite a pronounced sex-bias in rheumatoid arthritis development (3:1 female predominance), and the strong association between the dysbiosis and arthritis development in experimental models (9) and human patients (35), very few studies have interrogated how the gut-microbiota impacts sex-bias in experimental arthritis development. Indeed, in humanized HLA-DRB1*0401 mice, which develop a spontaneous female sex-bias disease (36), age- and sex-driven differences in the gut-microbiota of non-arthritic control mice are lost following arthritis development (26). Unpublished results from our laboratory suggest that under certain housing conditions, female K/BxN mice develop joint inflammation a week earlier than males, and that the disease incidence of collagen-induced arthritis in male DBA/1J is more consistent amongst different animal facilities than in female DBA/1J mice. Although we are unsure of the mechanisms underlying these colloquial observations, considering the strong influence of housing conditions on these subtle sex-differences in disease trajectory, it is tempting to speculate a dominate role for the gut-microbiota.

FUTURE DIRECTIONS

The studies described above provide tantalizing evidence that the interplay between sex-hormones and the gut-microbiota influences the risk of developing autoimmune conditions in females. However, there remains a distinct lack of mechanistic studies that have sought to uncover the pathways by which a sexually dimorphic gut-microbiota influences chronic inflammatory states. We, and others, have shown that the gut-microbiota influences immune system function through the production of immunogenic gut-microbiota-derived metabolites and hormones such as serotonin (37). There is initial evidence that the levels of gut-derived metabolites differ between the sexes. For example, alterations in the levels of bile acids–a major group of microbiota-dependent metabolites, associated with a high-fat diet–are different in male and female mice (38). NMR-spectroscopy analysis of 24 metabolites from the gut metabolome, including short-chain fatty acids, amino acids, and other immunogenic metabolites, shows a clear difference in the gut metabolome of healthy male and female mice (39). We believe that the impact of sex on host metabolism warrants further investigation; specifically, the effect of sex-based differences in gut-microbiota-derived metabolites on immune responses is an avenue that remains unexplored. More recently, we have demonstrated that other features of gut health, such as intestinal permeability, correlate with the onset and progression of autoimmune inflammation (40). It has been suggested that female mice have higher baseline intestinal permeability (41), and the expression of genes involved in mucus biosynthesis in the ileum–which plays an essential role in supporting a healthy gut barrier–are differentially regulated in old male and female mice (42). Moving forwards from three potential models proposed by Yurkovetskiy and colleagues of how the gut-microbiota may influences sex-hormones production or vice versa (6): we propose that future studies should consider the diverse impact of the gut-microbiota on host metabolism and barrier function, allowing the identification of mechanisms in which sexual dimorphism in the gut-microbiota influences chronic inflammatory states (**Figure 1**). This is essential to identify novel "druggable" pathways for the prevention or suppression of sex-biased inflammatory disease processes.

CONCLUSIONS

Although the impact of sex and the gut-microbiota on autoimmunity are clearly defined, the understanding of how these two risk factors may impact one another is ill-defined. In this review, we have summarized current literature from animal models that suggest that the gut-microbiota differs between the sexes in the steady state and in inflammatory

conditions. However, this relationship is complex, and is influenced by other factors including diet, housing and genotype. As translation of animal research into humans is the central tenet for the use of experimental models, these data underline the importance of collecting in-depth demographic information–such as age, ethnicity, body mass index, diet and medications–when comparing sex-differences in the human microbiota in healthy individuals and individuals with autoimmune conditions. Initial studies of the human microbiota have reported that there is sexual dimorphism in the gut-microbiota (43), and that there is a potential correlation between the diversity and richness of the gut-microbiota and urinary sex-hormones, namely estrogen, levels (44). In future studies, considering the impact of genotype on differences in the gut-microbiota between male and female mice, where possible, it would be informative to collect genotype data alongside bacterial sequencing data in large scale population studies of the human gut-microbiota. These complex large-scale studies would be critical in providing new insights into the potential directionality between sex, environment, and genetic risk when considering biological pathways contributing to autoimmune disease pathophysiology.

We have also highlighted the intimate connection between the gut-microbiota and sex-hormones, and in particular testosterone. Modulation of the gut-microbiota may represent a potential, less invasive, treatment strategy than injection of high levels of hormones, specifically in conditions where levels of sex-hormones are thought to influence disease development–such as reduction in testosterone levels in SLE patients (45, 46)–or in conditions where hormone therapy has been suggested as a potential treatment strategy–such as androgen treatment in MS (47) and in SLE (48).

Finally, we emphasize the need for mechanistic experiments that interrogate how sexual dimorphism in the gut-microbiota alters immune responses in such a way that renders them pathogenic in autoimmune conditions. Based on our previous research, we suggest that investigating how sex-differences in the gut-microbiota change immunogenic gut-derived metabolites or gut-barrier function may provide exciting new research opportunities. The reports summarized in this review show that the study of the direct and indirect pathways by which sex influences immune responses, and thus autoimmune development, is a field in its infancy. Altogether, these data highlight the need to disaggregate all aspects of medical research–including the study of the microbiota–by sex and gender, especially when considering the biological pathways that underlie the development of autoimmunity.

AUTHOR CONTRIBUTIONS

ER conceptualized the review and wrote the manuscript. NG critically reviewed the manuscript. DM reviewed the manuscript and made figures and tables. All authors contributed to the article and approved the submitted version.

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Relationship Between Gender and 1-Year Mortality in ANCA-Associated Vasculitis Patients: A Single-Center Retrospective Analysis and Meta-Analysis

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Objective: The relationship between gender and short-term prognosis of patients with anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is unclear, hence single-center retrospective analysis and meta-analysis were conducted to determine the relationship.

Methods: Initially treated patients with AAV were retrospectively enrolled. Data of clinical manifestation, laboratory indicators, Birmingham vasculitis activity score (BVAS), therapeutic treatments, and the patients' situations within 1 year were recorded. First, we compared the basic characteristics between male and female patients. Second, the risk factors associated with a 1-year mortality rate of patients with AAV were evaluated. Finally, a meta-analysis was performed to explore the effect of gender on 1-year mortality in patients with AAV.

Results: The study involved 84 patients with AAV, including 33 female and 51 male participants. In total, 14 people died (12 males and 2 females) and 70 survived in the 1st year. Statistical differences were noted in the age of onset, the course of the disease, WBC, HB, N, ESR, CRP, BUN, ALT and ALB, BVAS, and 1-year mortality rate between male and female participants. In male patients, elevated Scr, NLR, PLT, and RDW-CV were associated with poor AAV ($P < 0.05$) prognosis. The meta-analysis verified that male gender was an independent risk factor for the 1-year mortality of patients with AAV(OR = 1.54).

Conclusion: Significant sex-specific differences were found in patients with AAV. Male patients contributed to 1.54-fold of 1-year mortality risk in patients with AAV by meta-analysis. More attention should be paid to the mortality risk of male patients with AAV in the early stage.

Keywords: gender, 1-year mortality ratio, ANCA-associated vasculitis (AAV), prognosis, meta-analysis

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is a rare and severe systemic necrotizing small-vessel vasculitis (1) characterized by vascular inflammation, endothelial injury, and tissue damage (2). AAV is generally accompanied by the presence of ANCA in serum. ANCA is a serum auto-antibody for proteins present in neutrophils, which is a serological marker for small vessel vasculitis (3). The two major antigens of ANCA are myeloperoxidase (MPO) and proteinase 3 (PR3) (4). This distinctly pauci-immune form of vasculitis includes three clinic-pathological types: microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (5). The characteristic histology of MPA shows a necrotizing small-vessel vasculitis with little or absent immune deposits (pauci-immune vasculitis) and the absence of necrotizing granulomas (6, 7). GPA is mainly characterized by a typical histological triad, including granulomatous inflammation with local necrosis and necrotizing small-vessel vasculitis (8, 9). EGPA is a systemic small-vessel vasculitis associated with asthma, eosinophilia, and neuropathy. Pathologically, EGPA is considered to be a triad consisting of necrotizing vasculitis, eosinophilic infiltration, and extravascular granuloma, and the presence of ANCAs is associated with the clinical and pathological features of eosinophilic granulomatosis (10, 11). MPO–ANCA is often detected in the sera of patients with MPA and EGPA, while, PR3-ANCA is a useful marker for GPA. The clinical manifestations of AAV are roughly variable, and the gold standard for diagnosis is tissue biopsy (12). Although the etiology and pathogenesis of AAV are complex, genetic factors play a certain role which to some extent explained the geographical differences (5). The disease is more common in white and Asian populations and less common in African–American populations (12, 13).

Patients with AAV have a 1-year mortality rate of up to 80% in the natural disease course they do not receive treatment (14), and even with intensive treatment, patients still carry a 2.7-fold increased risk of death compared with the general population (15, 16). Although several studies have shown that the predictors of poor prognosis for patient survival were dialysis dependency or high creatinine level at initial diagnosis, high Birmingham vasculitis activity score (BVAS), and older age (17–19), the specific mechanism remains unknown. Recent studies had specifically focused on the association between gender and the prognosis of AAV. It was reported that male gender was an independent risk factor for all-cause mortality of patients with AAV during long-term follow-up (20); however, whether male gender was related to their short-term mortality (1-year mortality) had no unified conclusion. Among Caucasians, male patients had an increased risk of death and a higher mortality rate within 28 days of ICU admission compared with female patients (21). Male sex was also associated with an increased risk of end-stage renal disease (ESRD) (22). However, in 2007 Abe et al. found that gender was not associated with the prognosis of AAV (19). Considering there have been few studies about the relationship between gender and 1-year mortality of patients with AAV and since there were no data from mainland China, we conducted retrospective research on inpatients in our hospital and a meta-analysis to determine the impact of gender on the 1-year mortality ratio of patients with AAV. We hoped to find some correlation between gender and 1-year mortality in patients with AAV to provide a predictive index for clinicians.

MATERIALS AND METHODS

Single-Center Retrospective Analysis

Patients Enrolled

Patients with AAV admitted and first diagnosed in the Second Xiangya Hospital of Central South University between January 2014 to December 2019 were retrospectively enrolled. All patients diagnosed with AAV met the diagnostic criteria established in the 2012 Chapel Hill Consensus Conference (CHCC) (6), and those with overlap syndrome or secondary vasculitis, or severe chronic diseases such as a malignant tumor, hypertension, and diabetes were excluded. Cases lost to follow-up were also excluded. This study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University and was in accordance with the Declaration of Helsinki. This study had no adverse influence on the rights or welfare of patients. Informed consent was obtained from all patients.

Data Collection

The clinical manifestations, laboratory examination findings, BVAS, and data of treatment were collected through medical records. The laboratory examinations included blood cell count (WBC), hemoglobin (HB), platelet (PLT), neutrophils (N), lymphocytes (L), neutrophil–lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), mean platelet volume (MPV), red blood cell volume width-coefficient of variation (RDW-CV), red blood cell volume width-standard deviation (RDW-SD), erythrocyte sedimentation (ESR), C-reactive protein (CRP), complement 3 (C3), complement 4 (C4), glutamic-pyruvic transaminase (ALT), albumin (ALB), globulin (GLO), urea nitrogen (BUN), serum creatinine (Scr), uric acid (UA), hematuria, proteinuria, ANCA serotype including peripheral anti-neutrophil cytoplasmic antibody (p-ANCA), cytoplasmic anti-neutrophil cytoplasmic antibody (c-ANCA), myeloperoxidase (MPO), and protease 3 (PR3). The BVAS scores were evaluated by two experienced rheumatologists to determine the disease activity of patients with AAV after their diagnosis. In addition, the data on the treatments, such as glucocorticoid (GC), cyclophosphamide (CTX), mycophenolate mofetil (MMF), leflunomide, CD20 monoclonal antibody, and plasma exchange, were also collected. The number of patients with different conditions, such as deaths, lost visits, and survivors within 1 year, were counted via telephone follow-up.

Statistical Analysis

SPSS 26.0 software was used to compare the statistical differences and survival analysis of each index, and prism software was used to make the Kaplan–Meier (K-M) curves of patients with gender and AAV. All data between male and female patients with AAV were compared. Continuous data of normal distribution were expressed as mean standard deviation ($x \pm SD$), and

statistical differences between groups were compared using the *t*-test. Continuous data with skewed distribution were expressed with the median of the interquartile range (IQR), and statistical differences between the two groups were compared using the Mann–Whitney U test. Categorical data are expressed as ratios (%) and subjected to a chi-square test. According to the cut-off value of the ROC curve, the individual data were converted into binary classified variables, and the log-rank test and COX test were performed according to the log-likelihood ratio. In the COX model, forward stepwise regression was applied, and the default Wald's test was adopted. The standard *p*-value for variable elimination was 0.1 and the standard *p*-value for inclusion was 0.05. The results are expressed as hazard ratio and 95% confidence interval (CI). $P < 0.05$ was considered to indicate statistical significance.

Meta-Analysis

Data Sources and Searches

This meta-analysis was designed and conducted following the PRISMA statement (**Appendix 1**). Databases such as China Biomedical Library (https://www.sinomed.ac.cn), CNKI (China National Knowledge Infrastructure, https://www.cnki.net), VIP database (https://www.cqvip.com), Wan fang database (https://wanfangdata.com.cn) in China, and Cochrane Library, Embase, PubMed, and Web of Science were all applied to search for articles published up to 12 July 2021 with the following terms: ("ANCA associated vasculitis" or "ANCA vasculitis" or "ANCA related vasculitis" or "AAV" or "GPA" or "MPA" or "EGPA") AND ("Sex" or "Gender" or "Male" or "Female") AND ("Prognosis" or "death" or "survival" or "mortality" or "death rate") AND ("one year") (**Appendix 2**). In addition, a manual search of eligible studies was conducted to determine other qualified studies. This search strategy was conducted twice in total (**Figure 2**).

Inclusion Criteria and Study Selection

The inclusion criteria were original articles on AAV (including randomized controlled studies, case-control studies, cohort studies, and cross-sectional studies), which analyzed the basic characteristics and with complete data, including country, region, data source, race, follow-up time, the overall number of people, number of male and female patients, and total death toll or number of deaths per sex. The exclusion criteria were case reports, case series reviews, meta-analysis guidelines, meeting abstractions, expert opinions, etc. Studies in which the follow-up time was not 1 year were excluded. The qualification of full-text articles was determined by four reviewers (QZ, BLC, QWY, and YSW), and ambiguous articles were checked by a fifth reviewer (YG). Reviewers BLC and QWY screened the titles and abstracts of the identified references and excluded articles unrelated to the topic of interest. Reviewer FL, XX, and YG conducted a comprehensive review of related articles.

Data Extraction and Quality Assessment

Reviewers (QZ, BLC, QWY, YSW, and YG) independently extracted the data for each included study and resolved any differences through a discussion. The following variables were obtained: author, publication year, data source, research type, the total number of patients with AAV, number of male and female patients, number of deaths and survivors, number of deaths per sex, and follow-up time. All data included in the study were extracted in standard form. Case-control studies were assessed using the Newcastle-Ottawa Scale (NOS). For a total score of nine points, studies that scored 0–3 points, 4–6 points, and 7–9 points were considered low-quality, medium-quality, and high-quality studies, respectively (23).

Meta-Analysis

Review Manager 5.3 software was used for meta-analysis, and Stata14.0 software was used for Egger's test and sensitivity analysis. Dichotomous data are expressed as odds ratio (OR) and 95% CI, and continuous data were expressed as standard mean difference and 95% CI. $P < 0.05$ was considered statistically significant. The chi-square test and I^2 statistic were used to assess the heterogeneity of published studies. For studies with significant heterogeneity ($I^2 > 50\%$, $P < 0.05$), the random-effects model was used; otherwise, the fixed-effects model was employed. Funnel-plot and Egger's test were used to estimate the impact of possible publication bias (24). Subgroup analyses were further refined if heterogeneity existed.

RESULTS

Results of the Single-Center Retrospective Analysis

Clinical Characteristics of Patients With AAV

A total of 84 patients with AAV initially diagnosed were included in this study, including 33 female and 51 male patients, with an average age of 56.6 years and a disease course of 5 months. The baseline characteristics of the patients are listed in **Table 1**. MPA was the most commonly noted disease, followed by GPA and EGPA (85.7% and 9.5 vs. 4.8%). Non-specific symptoms such as fever, fatigue, and weight loss were the most common (75%), followed by the involvement of the lungs (50%), kidneys (45.2%), joints and muscles (32.1%), ear, nose, and throat (7.1%), skin (4.8%), nervous system (4.8%), mucous membrane and eye (1.2%), and cardiovascular system (1.2%). As for therapeutic agents, the majority of the patients were treated with GC combined with CTX, followed by hydroxychloroquine. A few of the patients underwent treatment with MMF, leflunomide, or CD20 monoclonal antibody.

Table 1 also displays baseline characteristics stratified by sex. The mean onset age of female patients with AAV was 52.2 years and for male patients, it was 57.9 years, and the mean disease duration before diagnosis was 7.5 months for female patients and 3.3 months for male patients with a significant difference ($P < 0.05$). The rate of organ involvement between men and women showed no statistical difference, but BVAS was higher in men than in women (12 vs. 10; $P = 0.008$). EGPA seemed more common in male patients (7.8 vs. 0%; $P = 0.042$). The laboratory indicators, including WBC, HB, N, ESR, CRP, BUN, and ALT, were all significantly higher in men, whereas ALB was lower than in women ($P < 0.05$; **Table 1**). There were no significant differences between the two groups concerning the indices of PLT, L, NLR, PLR, MPV, RDW-CV, RDW-SD, C3, C4, GLO,

TABLE 1 | Basic characteristics of patients with AAV and comparison between different genders.

	Total (*N* = 84)	Group		
		Male (*N* = 51)	Female (*N* = 33)	*P*-value
Age (Y)	56.6 ± 13.5	57.9 ± 10.5	52.2 ± 16.7	0.005*
Disease duration (M)	5.0 ± 7.1	3.3 ± 5.1	7.5 ± 8.8	0.008*
Mortality rate (*n*, %)				
3-month	4 (4.8)	4 (7.8)	0 (0)	0.105
6-month	12 (14.3)	10 (19.6)	2 (6.1)	0.056
12-month	14 (16.7)	12 (23.5)	2 (6.1)	0.024*
Classification of diseases (*n*, %)				
GPA	8 (9.5)	5 (9.8)	3 (9.1)	0.913
MPA	72 (85.7)	42 (82.4)	30 (90.9)	0.118
EGPA	4 (4.8)	4 (7.8)	0 (0)	0.042*
Types of ANCA (*n*, %)				
p-ANCA	58 (69)	31 (60.8)	27 (81.8)	0.227
c-ANCA	6 (7.1)	4 (7.8)	2 (6.1)	0.596
MPO	74 (88.1)	46 (90.2)	28 (84.8)	0.311
PR3	11 (13.3)	8 (15.7)	3 (9.1)	0.353
Organ involvement (*n*, %)				
Lung	42 (50)	28 (54.9)	14 (42.4)	0.264
Kidney	38 (45.2)	20 (39.2)	18 (54.5)	0.168
Skin	4 (4.8)	2 (3.8)	2 (6.1)	0.657
Mucosa and eye	1 (1.2)	1 (2.0)	0 (0)	0.316
Ear, nose, throat	6 (7.1)	4 (7.8)	2 (6.1)	0.754
Cardiovascular	1 (1.2)	0 (0.0)	1 (3.0)	0.170
Nervous system	4 (4.8)	2 (3.9)	2 (6.1)	0.657
Joint and muscle	27 (32.1)	16 (31.4)	11 (33.3)	0.851
Non-specific symptoms	63 (75)	35 (68.6)	28 (84.8)	0.094
Lab data (median, IQR)				
WBC (10^9/L)	9.3 [6.0–10.8]	10.2 [6.6–12.8]	8.0 [5.2–10.2]	0.044*
HB (g/l)	86 [72.3–96.5]	88 [76–98]	81.9 [69–89.5]	0.046*
PLT (10^9/L)	278.7 [181–351]	280.4 [198–351]	276.2 [177–339]	0.689
N (10^9/L)	7.4 [4.2–9.1]	8.2 [4.5–10.2]	6.2 [3.3–8.5]	0.048*
L (10^9/L)	1.3 [0.9–1.6]	1.3 [0.9–1.7]	1.2 [0.8–1.5]	0.513
NLR	7.2 [3.0–5.5]	7.5 [3.6–8.8]	6.7 [2.7–8.8]	0.165
PLR	266.7 [144.3–347.5]	254.4 [141.0–345.9]	285.6 [149.8–382.6]	0.724
MPV (fL)	10.3 [9.4–11.0]	10.4 [9.4–11.1]	10.2 [9.5–11.0]	0.706
RDW-CV (%)	14.4 [12.9–15.6]	14.3 [12.7–15.5]	14.6 [13.2–15.6]	0.463
RDW-SD (fL)	45.9 [41.7–49.0]	45.8 [41.7–49.0]	45.9 [41.3-49.2]	0.818
ESR (mm/h)	69.1 [34–99.5]	77.2 [56–101.3]	56.8 [26–93.8]	0.03*
CRP (mg/l)	46.3 [6.49–69.3]	55.4 [8.4–84.6]	27.5 [26–46]	0.019*
C3 (g/l)	0.9 [0.7–1.0]	0.8 [0.7–1.0]	0.9 [0.9–1.0]	0.781
C4 (g/l)	0.2 [0.2-0.3]	0.2 [0.2–0.3]	0.2 [0.2–0.3]	0.735
AL (u/l)	22.7 [7.7-26.6]	27.0 [10.6–27.3]	15.7 [5.2–18.8]	0.013*
ALB (g/l)	29.3 [25–33.7]	28.2 [10.6–27.2]	31.1 [26.8–30.9]	0.016*
GLB (g/l)	31.5 [25.9–36.7]	31.4 [26.8–37.4]	31.7 [25.3–36.6]	0.848
TB (umol/l)	7.1 [4.3–8.3]	7.3 [4.1–9.1]	6.8 [4.3–8.2]	0.907
Scr (umol/l)	378.7 [126.2-528.2]	402.7 [196.6–389.9]	343.1 [80.5–559.8]	0.152
BUN (mmol/l)	17.5 [9.1–23.9]	20.0 [10.7–28.9]	13.6 [6.4–18.0]	0.013*
UA (ummol/l)	396.5 [300.2–483]	418.1 [330.1–517.7]	363.3 [239.4–454.9]	0.069
Hematuria (*n*, %)	76 (90.5)	45 (88.2)	31 (93.9)	0.518
Proteinuria (*n*, %)	65 (77.4)	40 (78.4)	25 (75.8)	0.559
BVAS	12 [9.5-−14.5]	12 [10–15]	10 [9-12.75]	0.008*

(Continued)

TABLE 1 | Continued

	Total ($N = 84$)	Group		
		Male ($N = 51$)	Female ($N = 33$)	*P*-value
Treatment after diagnosis (*n*, %)				
Different doses of GC				
<0.5 mg/kg	2 (2.4)	2 (3.9)	0 (0)	0.149
0.5–1 mg/kg	23 (27.4)	16 (31.4)	7 (21.2)	0.267
>1 kg/mg	55 (65.5)	30 (58.8)	25 (75.8)	0.140
CTX	54 (64.3)	34 (66.7)	20 (60.6)	0.571
MMF	8 (9.5)	1 (2.0)	7 (21.2)	0.079
Plasma exchange	22 (26.2)	12 (23.5)	10 (30.3)	0.490
CD20 monoclonal antibody	2 (2.4)	1 (2.0)	1 (3.3)	0.756
Single drug or combination drugs				
1 drug	18 (21.4)	11 (21.6)	7 (21.2)	0.969
2 drugs	41 (48.8)	24 (47.1)	17 (51.5)	0.690
3 drugs	14 (16.7)	7 (13.7)	7 (21.2)	0.369
≥4 drugs	11 (13.1)	9 (17.6)	2 (6.1)	0.107

Y, years; M, months; %, percentage; GPA, Granulomatous vasculitis; MPA, Microscopic vasculitis; EGPA, Eosinophilic granulomatous polyangiitis; c-ANCA, Cytoplasmic anti-neutrophil cytoplasmic antibody; p-ANCA, Perinuclear anti-neutrophilic cytoplasmic antibody; PR3, Protease 3; MPO, Myeloperoxidase; IQR, Interquartile range; WBC, White blood cell; HB, Hemoglobin; PLT, Platelet; N, Neutrophils; L, Lymphocytes; NLR, Neutrophil-lymphocyte ratio; PLR, Platelet lymphocyte ratio; MPV, Mean platelet volume; RDW-CV, Red blood cell volume width-variation of red blood cells; RDW-SD, Red blood cell volume distribution width-standard deviation; ESR, Erythrocyte sedimentation; CRP, C-reactive protein; C3, Complement 3; C4, Complement 4; ALT, Glutamic-pyruvic transaminase; sALB, Albumin; GLO, Globulin; TBIL, Total bilirubin; BUN, Urea nitrogen; Scr, Serum creatinine; UA, Uric acid; BVAS, Birmingham Vasculitis Activity Score; GC, Glucocorticoid; CTX, Cyclophosphamide; MMF, Mycophenolate mofetil. $^{*}P < 0.05$.

DBIL, Scr, UA, the ratio of hematuria and proteinuria, the usage ratio of therapeutic drugs including GC, CTX, MMF, plasma exchange and CD20 monoclonal antibody, and the number of combined therapeutic drugs.

Analysis of 1-Year Survival Rate and Risk Factors

All patients underwent telephone follow-up. A total of 14 people died (12 men and 2 women) and 70 survived in the 1st year after diagnosis. During 0–3 months follow-up, there were four deaths in the male group and none in the female group. During 3–6 months follow-up, there were six deaths in the male group and two in the female group. During 6–12 months follow-up, there were two deaths in the male group and none in the female group. There was no significant difference between the 3- or 6-month mortality ratio of male and female patients with AAV, whereas the mortality ratio of male patients with AAV was significantly higher in the 1st year than female patients ($P < 0.05$; **Table 1**). The K–M survival curves indicated that the 1-year mortality ratio was higher in male patients than in female patients ($P = 0.0376$; **Figure 1**).

Univariate regression analysis was conducted to analyze the risk factors of 1-year mortality in patients with AAV. Gender, routine blood indices (WBC, PLT, N, NLR, MPV, and RDW-CV/SD), CRP, liver function (TBIL), renal function (BUN, UA, Scr), the ratio of proteinuria, the BVAS, and combined two drugs for treatment were risk factors for 1-year mortality in patients with AAV. However, only male (OR = 5.41; 95% CI 1.19-24.59), elevated Scr (OR = 4.67; 95% CI 1.10-19.92), increased PLT (OR = 7.0; 95% CI 1.42-34.57), augmented NLR (OR = 15.87; 95% CI 1.48-170.15), and raised RDW-CV (OR = 3.27; 95% CI 1.14–9.38) were independent risk factors for 1-year mortality in patients with AAV($P < 0.05$; **Table 2**).

Results of the Meta-Analysis

Study Selection

The flow chart for study selection in the meta-analysis is shown in **Figure 2**. The initial search generated 3,219 studies, of which 0 were from the China Biomedical Library, 504 were from CNKI, five were from the VIP database, 114 were from the Wan fang database in China, and 110 were from Cochrane, 1,153 were from Embase, 668 were from PubMed, and 665 were from Web of Science. After deleting 376 duplicate articles, 2,843 articles were included in the preliminary screening, among which 1,537 articles were irrelevant to this study, 87 meta-analyses or systematic literature reviews, 720 conference abstracts, 489 case reports, and 3 letters or notes were excluded. After excluding articles that did not meet the inclusion criteria, 7 articles were considered qualified, but 1 of them was also excluded for lacking adequate data after failing to contact the author. Thus, six articles from the databases were included. In addition, considering our respective study also met the inclusion criteria, a total of seven studies were applied for meta-analysis finally (**Figure 2**).

Characteristics and Quality of the Included Studies

Table 3 listed the characteristics of the included studies. All were case-control studies conducted up to 12 July 2021, of which three studies were from Asia and 4 from Europe (14, 17, 19, 25–27). The diagnostic inclusion and exclusion criteria were reported in all the studies. A total of 1,136 patients with AAV were included, and men accounted for 51.32%. The total number of deaths was

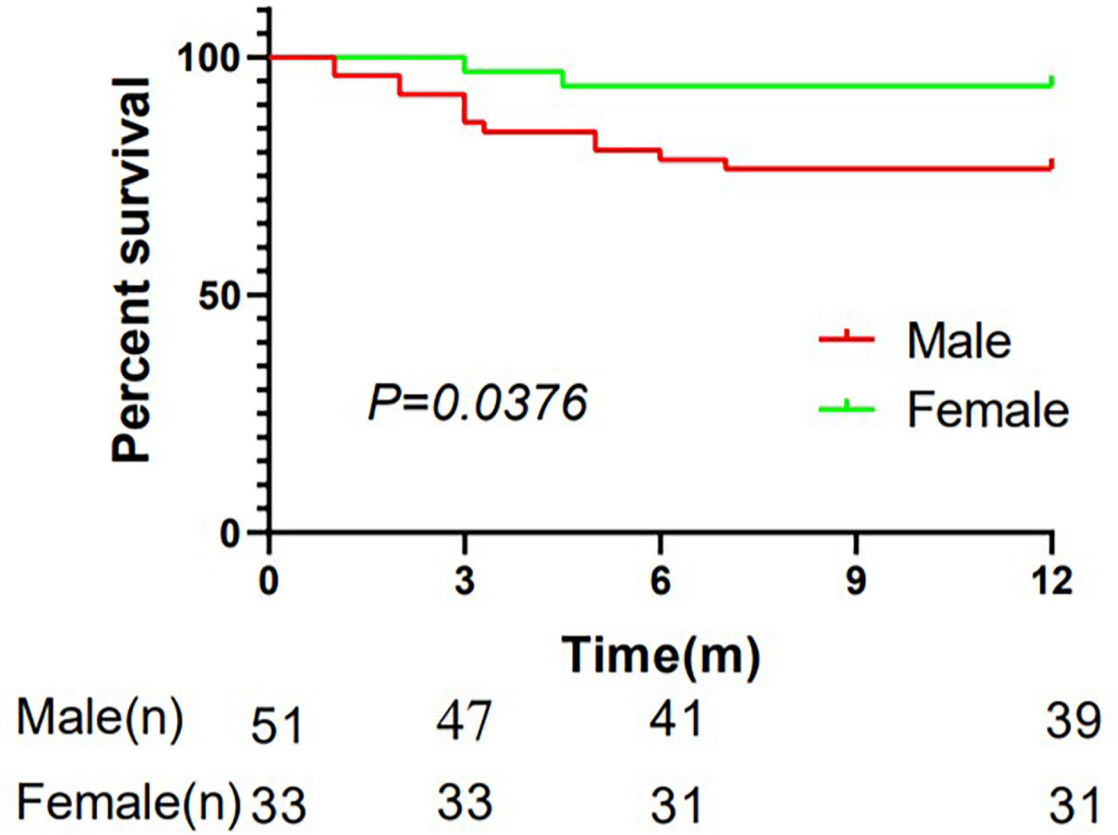

FIGURE 1 | Kaplan–Meier plots demonstrating the mortality ratio of patients with AAV in males and females in the 1-year follow-up. 84 patients were included and 70 survived in the first year after diagnosis with 39 males and 31 females. The Kaplan-Meier (K-M) survival curves indicated that the 1-year mortality ratio was higher in male patients than in female patients ($P = 0.0376$). m, months.

210, with 126 being male (60%). According to the NOS, case-control studies included in the meta-analysis were evaluated. The NOS score was six, which indicated the case-control studies were of medium quality (**Table 3**).

Meta-Analysis and Sensitivity Analysis

The forest map showed male was a risk factor for 1-year mortality of patients with AAV with OR value of 1.54 (95% CI: 1.13–2.10; $P = 0.006$) and no significant heterogeneity ($I^2 = 44\%$, $P = 0.1$) (**Figure 3A**). Therefore, the fixed-effects model was used and subgroup analyses were not further refined. Subsequently, sensitivity analysis of the above research suggested that the research findings were reliable and robust (**Figure 3D**).

Publication Bias

A funnel plot and Egger's test were performed to evaluate the publication bias of this meta-analysis. The *P*-value of Egger's test was 0.123 ($P > 0.05$), which indicated there was no publication bias (**Figure 3C**). In this study, funnel plot shapes were found to be symmetrical (**Figure 3B**).

DISCUSSION

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis is a rare disease and only a few studies are available on the relationship between gender and prognosis. To the best of our knowledge, this study was the first one to explore the relationship between gender and 1-year mortality of AAV in the population of China. This single-center retrospective study demonstrated that gender was related to the short-term prognosis of patients with AAV in southern China, with a 1-year mortality ratio significantly higher in men than in women. In addition, our meta-analysis of seven studies also showed this significant correlation. This may provide a reliable predictor for clinicians to judge the prognosis and select active treatment for patients with AAV.

Patients with AAV generally have the highest risk of acute and severe injury within 1 year of onset (28), and the all-cause mortality of male patients is higher than that of female patients in terms of long-term survival in Korea (20). In our study, the number of EGPA and GPA patients was too small compared with MPA, so we did not compare differences between the three subgroups, but we mainly aimed to compare differences between males and females (**Table 1**). Under this premise, we found there

TABLE 2 | Factors related to 1-year mortality ratio in patients with AAV according to univariate regression and multivariate regression.

	Univariate regression		Multivariate regression	
	OR	***P*-value**	**OR [95%CI]**	***P*-value**
Basic features				
Gender(M/F)	5.01	0.025*	5.41 [1.19-24.59]	0.029*
Age	4.78	0.096		
Course of disease	2.76	0.096		
Classification of diseases				
GPA	0.20	0.652		
MPA	0.00	0.978		
EGPA	0.16	0.689		
Types of ANCA				
p-ANCA	0.13	0.719		
c-ANCA	0.01	0.92		
MPO	0.36	0.548		
PR3	0.00	0.978		
Organ involvement				
Lung	1.27	0.684		
Kidney	2.33	0.124		
Skin	2.56	0.442		
Mucosa and eye	0.00	1.000		
Ear, nose, throat	0.35	0.401		
Cardiovascular	0.67	1.00		
Nervous system	0.00	0.999		
Joint and muscle	1.67	0.367		
Non-specific symptoms	1.49	0.53		
Laboratory indicators				
WBC	14.63	0.000*	2.35 [0.46–11.88]	0.303
HB	1.19	0.276		
PLT	6.32	0.012*	7.00 [1.42–34.57]	0.017*
N	14.64	0.000*	2.35 [0.46–11.88]	0.303
L	0.58	0.448		
NLR	7.03	0.008*	15.87 [1.48–170.15]	0.022*
PLR	0.00	0.984		
MPV	10.72	0.001*	2.28 [0.38-13.55]	0.367
RDW-CV	4.26	0.039*	3.27 [1.14–9.38]	0.028*
RDW-SD	16.86	0.000*	2.73 [0.29–25.92]	0.383
ESR	1.72	0.19		
CRP	10.72	0.001*	2.28 [0.38–13.55]	0.367
C3	1.62	0.204		
C4	0.33	0.567		
ALT	2.93	0.087		
ALB	0.08	0.773		
GLB	3.46	0.063		
TBIL	6.07	0.014*	2.86 [0.77–10.67]	0.117
Scr	5.61	0.018*	4.67 [1.10–19.92]	0.037*
BUN	28.61	0.000*	5.17 [0.27–99.91]	0.277
UA	5.85	0.016*	1.85 [0.48–7.09]	0.372
Albuminuria	3.87	0.049*	0.35 [0.12–1.05]	0.062
Hematuria	0.12	0.727		
BVAS	5.26	0.022*	1.57 [0.50–4.97]	0.444

(Continued)

TABLE 2 | Continued

	Univariate regression		Multivariate regression	
	OR	***P*-value**	**OR [95%CI]**	***P*-value**
Treatment after diagnosis				
Different doses of GC				
<0.5mg/kg	0.396	0.529		
0.5–1mg/kg	0.417	0.518		
>1kg/mg	0.166	0.684		
CTX	2.012	0.156		
MMF	0.192	0.661		
Plasma exchange	0.386	0.535		
CD20 monoclonal antibody	0.404	0.525		
Single drug or combination drugs				
1 drug	0.98	0.323		
2 drugs	4.441	0.035*	3.38 [0.73–15.67]	0.12
3 drugs	1.232	0.267		
≥4 drugs	0.672	0.412		

*M/F, Males/Females; OR, Odds ratio; CI, Confidence interval; GPA, Granulomatous vasculitis; MPA, Microscopic vasculitis; EGPA, Eosinophilic granulomatous polyangiitis; c-ANCA, Cytoplasmic anti-neutrophil cytoplasmic antibody; p-ANCA, Perinuclear anti-neutrophilic cytoplasmic antibody; PR3, Protease 3; MPO, Myeloperoxidase; WBC, White blood cell; HB, Hemoglobin; PLT, Platelet; N, Neutrophils; L, Lymphocytes; NLR, Neutrophil-lymphocyte ratio; PLR, Platelet lymphocyte ratio; MPV, Mean platelet volume; RDW-CV, Red blood cell volume width-variation of red blood cells; RDW-SD, Red blood cell volume distribution width-standard deviation; ESR, Erythrocyte sedimentation; CRP, C-reactive protein; C3, Complement 3; C4: Complement 4; ALT, Glutamic-pyruvic transaminase; ALB, Albumin; GLO, Globulin; TBIL, Total bilirubin; BUN, Urea nitrogen; Scr, Serum creatinine; UA, Uric acid; BVAS, Birmingham Vasculitis Activity Score; GC, Glucocorticoid; CTX, Cyclophosphamide; MMF, Mycophenolate mofetil; *$P < 0.05$.*

were statistical differences in the age of onset, the course of the disease, the proportion of EGPA, BVAS, WBC, HB, N, ESR, CRP, ALT, ALB, BUN, and 1-year mortality ratio between male and female patients with AAV.

A multicenter study in 2019 reported that male patients with AAV more often suffered from respiratory and kidney damage at an early stage, which made them seek medical help for treatment earlier than female patients (29), and this was consistent with our conclusion that male patients had a shorter course of the disease. Epidemiological data on EGPA are scarce, accounting for only 10–20% of AAV cases (30). Retrospective studies in Peru showed a significant reduction in female vs. male EGPA (1: 4) (31), which was consistent with the population distribution in our study.

A higher WBC count on initial treatment was noted in male patients with AAV with higher BVAS in our study. Cornec D et al. reported that among patients with AAV treated with rituximab, male patients had lower B cell count and lower BVAS than females (32), and naive B-lymphopenia may be a biomarker of disease activity in AAV (33). These indicated that WBC count may be associated with the disease activity of male patients with AAV. There also was a statistical difference in HB between male patients with AAV and females in our study (88 vs. 81.9 g/L). Anemia was reported to be a common complication in patients with AAV, and HB<75 g/L seemed significantly correlated with the prognosis in patients with AAV (34). Although both male patients and females suffered moderate anemia (HB>75 g/L), whether it contributed to the poor prognosis of male patients or just represented the severity of the disease requires more data to determine.

A specific index for detecting liver function is ALT. It has been reported that in a healthy adolescent population, females have a negative relationship with ALT while males have a positive relationship, but no study has suggested the same relationship in adults (35). Besides, no research has confirmed that ALT is related to the prognosis of patients with AAV. Therefore, although there was a significant statistical difference in ALT between the two groups in this study, considering that the level of ALT was normal in both male and female groups, further research is needed to confirm whether this difference is of clinical significance. Low ALB was negatively correlated with CRP and ESR and was a good indicator for disease monitoring in AAV (36). ALB was lower in men than in women in our study, while ESR, CRP, and BVAS were higher in men than in women, which highlighted the possible correlation between low ALB, elevated ESR, CRP, and high disease activity in patients with AAV. A similar study also showed low ALB was associated with the disease severity and prognosis of myeloperoxidase-ANCA-associated glomerulonephritis (MPO-ANCA-GN) (37). But no studies have confirmed an association of lower ALB with 1-year mortality in patients with AAV, and further research is needed.

We found that the 1-year mortality rate of men was higher than that of women, with a significant statistical difference ($P < 0.05$). Among the 14 dead people, 12 were men and 2 women. Similar results were discovered by Caroline et al. in Sweden that among seven patients who died from vasculitis, six were men and only one was female (14), and the reason for death was whether the curative effect of drugs was not good, or the disease progressed rapidly. We thought the main reason for death in this study was the disease progression and deterioration, for

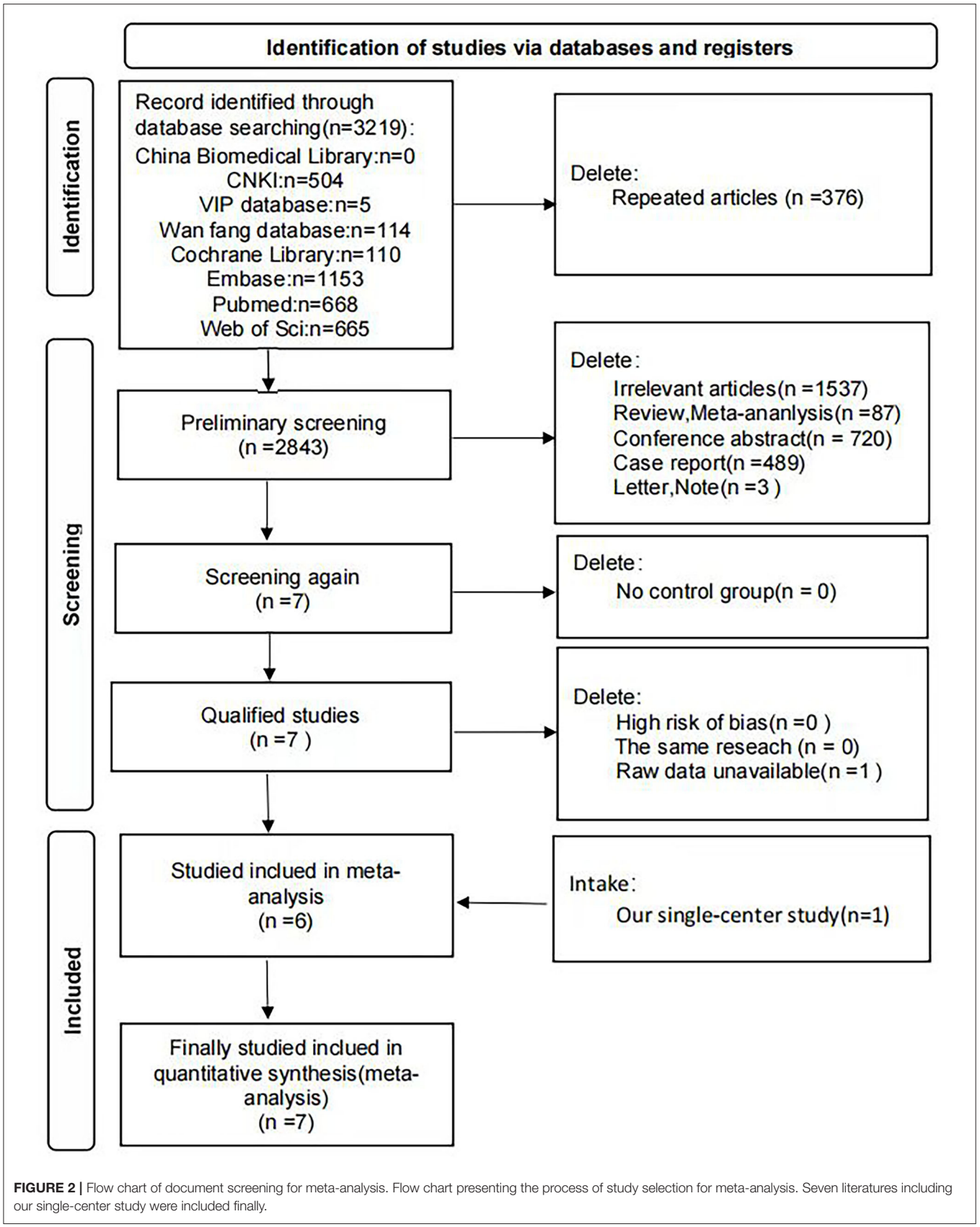

FIGURE 2 | Flow chart of document screening for meta-analysis. Flow chart presenting the process of study selection for meta-analysis. Seven literatures including our single-center study were included finally.

TABLE 3 | Main characteristics of included studies in the meta-analysis.

References	Nation	Region	Data source	Design	No. of study (*n*)	Age (Y) (median, IQR)	M/F (*n*, %)	Death of M/F (*n*)	Quality score method	Quality assessment	Quality scale
Slot et al. (17)	Holland	Europe	Single center	Case-control	85	56 [39–73]	55/30 (64.7, 35.3)	18/2	NOS	6	Moderate
Takala et al. (25)	Finland	Europe	Single center	Case-control	492	NS	243/249 (49.4, 50.6)	45/39	NOS	6	Moderate
Ono et al. (26)	Japan	Asia	Multi-center	Case-control	79	NS	36/43 (45.6, 54.4)	4/7	NOS	6	Moderate
Abe et al. (19)	Japan	Asia	Single center	Case-control	52	73.2 [62.4–84]	24/28 (46.2, 53.8)	9/9	NOS	6	Moderate
Heijl et al. (14)	Sweden	Europe	Single center	Case-control	195	69 [55–77]	97/98 (49.7, 50.3)	23/11	NOS	6	Moderate
Titeca-Beauport et al. (27)	France	Europe	Multi-center	Case-control	149	72.7 [68.5–76.8]	77/72 (51.7, 68.3)	15/14	NOS	6	Moderate
Zhu et al. 2021*	China	Asia	Single center	Case-control	84	56.6 [43.1–70.1]	51/33 (60.7, 39.3)	12/2	NOS	6	Moderate

*n, number; Y, years; IQR, Interquartile range; M/F, Male/Female; NS, Not search; NOS, the New Castle-Ottawa Scale. * Data of our single-center retrospective analysis.*

we explicitly excluded other comorbidities which may affect the mortality rate of the AAV patient at the beginning.

Given the above differences between male and female patients in our study, we wondered whether gender was an independent risk factor for poor short-term prognosis in patients with AAV, so further data was analyzed. It is surprising to find that male gender was indeed a risk factor for 1-year mortality in patients with AAV in the regression analysis. The K–M curve also showed that the 1-year mortality ratio was significantly higher in male patients than in female patients with prolongation ($P = 0.0376$).

Except for male patients, elevated Scr, increased NLR, augmented PLT, and raised RDW-CV were also found to be poor prognostic factors in patients with AAV in our study, and this might provide a simpler and more convenient means for clinicians to evaluate the prognosis of patients during follow-up. In this study, elevated Scr was identified as a poor prognostic factor for patients with AAV, which was consistent with other research (29, 38). Neutrophils trigger autoimmune diseases (39). ANCA can stimulate neutrophils to release neutrophil extracellular traps containing autoantigens, and lead patients with AAV to have autoimmune responses to these components (40). The decrease of lymphocytes is related to the low recurrence rate of the disease (41). Recently studies indicated that NLR, the ratio of neutrophils to lymphocytes, was positively correlated with the poor diagnosis of AAV. We also found that NLR was an independent risk factor for the 1-year mortality of patients with AAV. The predictive role of NLR had been speculated to be explained by a negative correlation between NLR and C3 serum levels (42), whereas a decrease in C3 level was associated with a poor renal prognosis and patient outcome (43, 44). In addition, NLR also played a positive role in renal damage, and a higher baseline NLR led to a worse renal prognosis (45). RDW is a routine measurement of the heterogeneity of circulating red blood cell size and is clinically used to distinguish different types of anemia, especially iron-deficiency anemia and chronic anemia (46). Kim et al. found that RDW$\geq$15.4% at diagnosis might increase the risk of severe GPA and predict refractory disease type (46), and this might be related to the presence of a large number of pro-inflammatory factors in patients with AAV during disease activity, while pro-inflammatory factors were associated with the development of anemia in various diseases (47). Here for the first time, we demonstrated that elevated RDW was an independent risk factor for patients with AAV, but the specific mechanism needed further investigation. We also found that an elevated PLT was an independent risk factor for patients with AAV; previous research showed that PLT count was significantly higher in patients with AAV with an active disease state than in those with a remission disease state (48), but relevant data were limited.

Among all the risk factors found in this study, the controversial relationship between gender and 1-year mortality in patients with AAV was our interest and focus as well. Hence, a systemic review and meta-analysis were conducted to confirm whether the male gender was related to the short-term prognosis of patients with AAV all over the world. We found that a total of seven studies from Asia and Europe previously covered the relationship between gender and its prognosis in patients with

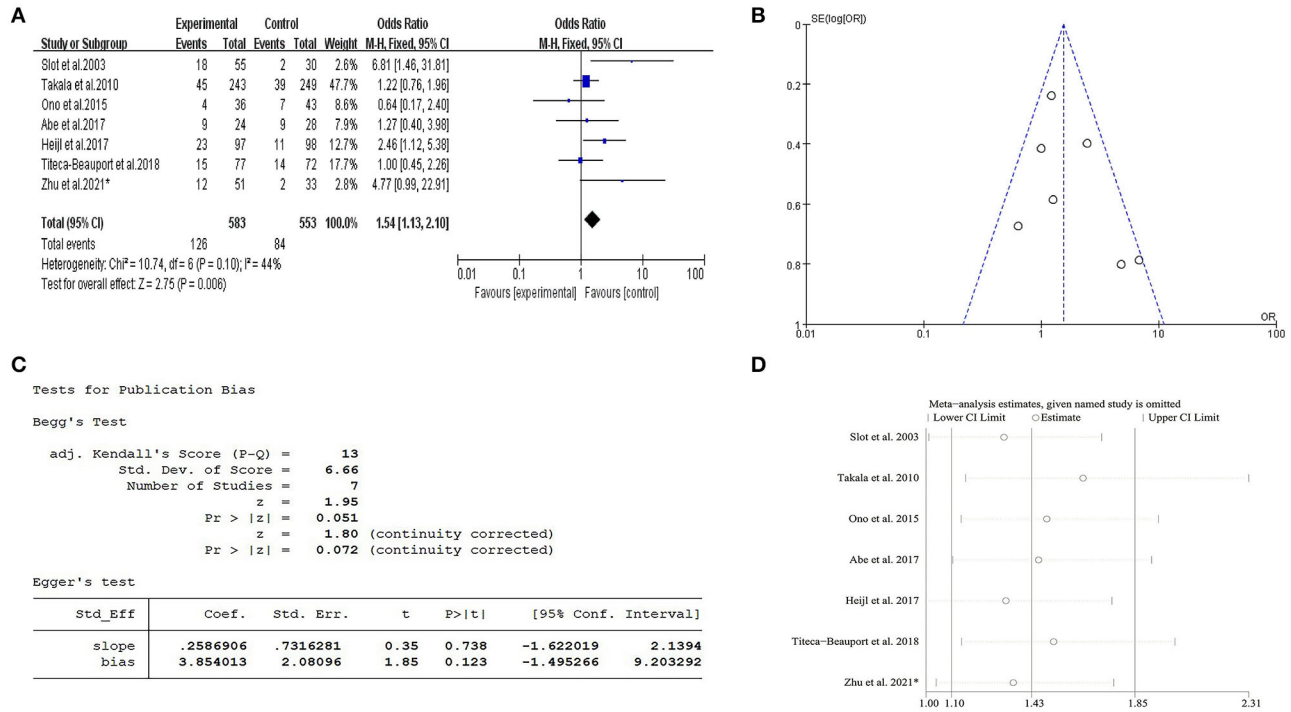

FIGURE 3 | Meta-analysis about gender vs. 1-year mortality in AAV. **(A)** The forest map showed male was a risk factor for 1-year mortality of patients with AAV with OR value of 1.54 (95% CI: 1.13-−2.10; $P = 0.006$) and no significant heterogeneity ($I^2 = 44\%$, $P = 0.1$). **(B)** Funnel plot indicated there was no publication bias. **(C)** Egger test also indicated no publication bias ($P > 0.05$). **(D)** Sensitivity analysis indicated that there was no significant change in the overall effect magnitude after removal of either study, and the results were robust and reliable. * Data of our single-center retrospective analysis.

AAV, including our single-center study. The final forest map results of our meta-analysis indicated that the male gender was a risk factor for 1-year mortality in patients with AAV and the risk of death was 1.54 times higher in male patients, which was the same as our findings in retrospective analysis. The sensitivity analysis also proved that the result of the meta-analysis was reliable. Together, we believed this provided a higher level of evidence-based evidence for the effect of male gender on the short-term prognosis of patients with AAV and a tool for early prognosis prediction for clinicians.

There were some limitations to this study. First, patients were included from a single center and were inpatients in a large general hospital whose disease state was generally severe. If conditions permit, patients with different disease activities and patients who come from the community should be investigated, together with a larger population to accurately determine the influence of gender on mortality in patients with AAV in China. Second, considering that AAV is a rare case, and we cannot obtain more data to investigate the certain relationship between gender and prognosis of MPA, GPA, and EGPA, respectively, we hope to confirm their correlations in the near future. Finally, the increased RDW-CV and PLT were both found to be prognostic risk factors for patients with AAV in our study, but given the total number of enrolled patients and information from other studies, further investigations should be performed to clarify their relationship with the short-term prognosis of patients with AAV.

CONCLUSION

Significant sex-specific differences were found in patients with AAV in Southern China. Male, elevated Scr, NLR, PLT, and RDW-CV were poor short-term prognostic factors for patients with AAV in the retrospective study. Among them, we clarified that male sex was a risk factor for 1-year mortality in patients with AAV by further meta-analysis. Clinicians should pay more attention to the mortality risk of male patients with AAV in the early stage, and intensive and careful management should be taken.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Material preparation, data collection, and analysis were performed by QZ, BC, QY, YW, and YG. The first draft of the manuscript was written by QZ and YG. The manuscript was

critically revised by FL, XX, and YG. All authors commented on previous versions of the manuscript, read and approved the final manuscript, and contributed to the concept and design of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.945011/full#supplementary-material

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Gender-Diverse Inclusion in Immunological Research: Benefits to Science and Health

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The differences between male and female immune systems are an under-researched field, ripe for discovery. This is evidenced by the stark sex biases seen in autoimmunity and infectious disease. Both the sex hormones (oestrogen and testosterone), as well as the sex chromosomes have been demonstrated to impact immune responses, in multiple ways. Historical shortcomings in reporting basic and clinical scientific findings in a sex-disaggregated manner have led not only to limited discovery of disease aetiology, but to potential inaccuracies in the estimation of the effects of diseases or interventions on females and gender-diverse groups. Here we propose not only that research subjects should include both *cis*-gender men and *cis*-gender women, but also transgender and gender-diverse people alongside them. The known interaction between the hormonal milieu and the sex chromosomes is inseparable in *cis*-gender human research, without the confounders of puberty and age. By inclusion of those pursuing hormonal affirmation of their gender identity- the individual and interactive investigation of hormones and chromosomes is permitted. Not only does this allow for a fine-tuned dissection of these individual effects, but it allows for discovery that is both pertinent and relevant to a far wider portion of the population. There is an unmet need for detailed treatment follow-up of the transgender community- little is known of the potential benefits and risks of hormonal supplementation on the immune system, nor indeed on many other health and disease outcomes. Our research team has pioneered the inclusion of gender-diverse persons in our basic research in adolescent autoimmune rheumatic diseases. We review here the many avenues that remain unexplored, and suggest ways in which other groups and teams can broaden their horizons and invest in a future for medicine that is both fruitful and inclusive.

Keywords: sex, gender, autoimmunity, sex hormones, sex chromosome, transgender

INTRODUCTION

The pertinent sex bias in the human immune system is a phenomenon that may never have come to light, were it not for significant policy changes that enforced the inclusion of female participants alongside males in medical research (1). Historically, clinical trials were conducted predominantly on male subjects only, or failed to discriminate between outcomes experienced by males vs. females (2). Justified by pragmatic reasons, predominantly healthy young males were recruited to avoid potential toxicity risks associated with pregnancy and breastfeeding, while excluding more mature patients of both sexes to decrease the risk of concomitant comorbidity. Little differed in basic scientific research, where male-only mouse models mitigated the outcome variability potentially resulting from the menstrual cycle or pregnancy, and most *in vitro* human work failed to report the sex of the cell lines used (3). This approach is not only inaccurate in answering research questions relevant to humans, irrespective of sex and gender, but is also potentially harmful in underestimating the effects of interventions on females and other gender-diverse groups. Although medical understanding and subsequent research study design have advanced significantly in recent years, this chronic failure to recognise the importance of sex as a key biological variable has by no means been fully overcome. Anecdotally, in attempting to collect data on global COVID-19 morbidity and mortality between the sexes, it was notable how few countries or local authorities were reliably disaggregating their outcome statistics according to patients' sex, even at later stages of the pandemic (4). Sophisticated national platforms detailed deaths according to geographical regions, age groups and occupational categories, but frequently neglected to mention sex. Our meta-analysis (5), alongside several other studies (6–8), showed a significant male bias in severe outcomes and deaths from SARS-CoV-2; a pattern mirrored in the vast majority of infectious diseases (9–11) and variously suggested to relate to sex hormone levels (12–14). The enhanced ability of the female immune system to clear invading pathogens is further supported by its ability to mount generally stronger responses to most vaccinations (15–17). For example, in adults given the seasonal Trivalent Inactivated Influenza Vaccine, female responses to a half-dose were comparable to those of males given a full-dose (18). The inverse of this is of course the female predisposition to developing autoimmune disorders associated with a hyper-active immune system, such as systemic lupus erythematosus (SLE), where the male:female ratio is estimated at 4–13:1, according to different studies (19–28).

Both hormonal and chromosomal factors are suggested to contribute to immunological sex differences. Oestradiol is broadly thought of as immunostimulatory, with testosterone having a more regulatory effect (29), though both have demonstrated either capability, as reviewed elsewhere (30–33). Meanwhile the X chromosome encodes the most immune-related genes of any chromosome (34) such as TLR7 [toll like receptor, responsible for sensing viral and endogenous nucleic acids to trigger release of type 1 interferons, and implicated in extrafollicular B cell class switch recombination (35)], CD40-L [co-stimulatory T cell molecule, essential for B cell class switching (36)], FoxP3 [controls regulatory T cells (37)] and CXCR3 [chemokine receptor, recruits effector T cells to sites of inflammation (38)]. This is highlighted by the abundance of X-linked immune disorders such as immunodysregulation polyendocrinopathy enteropathy X-linked (or IPEX) syndrome, X-linked agammaglobulinemia and Wiskott Aldrich Syndrome, which are associated with cellular and humoral immune deficiencies and increased risk of infections from childhood (39). Several immune genes on the X chromosome may escape the X-inactivation of one chromosome in 46,XX individuals, and thus be bi-allelically expressed, potentially resulting in altered immune regulation (40–43). Whilst studies have sought to investigate the contributions of hormonal and/or chromosomal influences on the immune response, it is recognised that it is a complex nexus and mutual interaction of the two that ultimately leads to such notable sex biases in infection and autoimmunity. With this in mind, this review seeks to highlight the importance of including subjects of both sexes, as well as transgender people in immunological research, to enable evaluation of sex-biased clinical outcomes and provide benefit to our understanding of the biology of the immune system with relevance for both science and health.

GENDER IDENTITIES AND PHYSICAL PHENOTYPES

For the majority of the population, the terms sex and gender describe the binary categories of "cisgender male" and "cisgender female"; with experienced gender matching the sex registered at birth, which is itself based upon simple observation of the genitalia of the new-born baby. Frequently assimilated within the category of "other," however, are a multitude of gender identities and physical phenotypes. By "transgender" we refer broadly to those whose experienced gender identity does *not* match that in which they were registered at birth. Thus, trans-males, are registered female at birth, typically carry a 46,XX chromosomal background, and may pursue virilisation *via* testosterone treatment and/or oestradiol blockade. Trans-females, are registered male at birth, typically of 46,XY chromosomal background, and may pursue gender-affirming oestradiol treatment and/or testosterone blockade (44). Specific treatment pathways and medications recommended by the Endocrine Society (45) are summarised in **Figure 1**. A third main category are those who are non-binary/gender fluid (not identifying exclusively and/or permanently as either gender); some of whom may seek hormonal blockade *via* treatments such as the gonadotropin releasing hormone analogs (GnRHa), or specific hormonal blockades. There is also the category of differences/disorders of sex development (previously known as 'intersex'), where people may have physical characteristics of both sexes (gonadal structures, genitalia) and this umbrella term also includes those with karyotype variations of sex development such as Klinefelter syndrome [47,XXY] and Turner syndrome [45,X] (46). Lastly but by no means exhaustively are those classified as

"agender"- not identifying with any gender at all. Many other gender-related groupings exist, beyond the scope of this review, but we have included here the main categories pertinent to immunological research.

To refer again to international COVID statistics, even fewer countries reported outcomes in those who were not cisgender. In some countries, the catch-all 'other' category was reported alongside cisgender males and females; but this was representative of so many diverse groups that granular analyses of differential gender-related outcomes could not be possible. Such is the case for the vast majority of outcome reporting in health and disease, suggesting that better characterisation of populations pertaining to self-reported gender is warranted. In the United Kingdom alone, referrals to the NHS young people's Gender Identity Development Service (GIDS) have increased by over 2000% in the last 10 years (47); this represents a growing proportion of society who are frequently not even adequately recognised in statistics, let alone included in basic science or relevant clinical research. Here we examine potential ways in which inclusion of a broader spectrum of gender groups can improve our scientific understanding of the pathogenesis of both infectious diseases and autoimmune disorders, as well as providing potentially pertinent clinical information for under-represented groups and the physicians involved in their care.

The multitude of gender-related social factors that may contribute to increased vulnerability to different medical conditions are beyond the scope of this paper and reviewed elsewhere (48). However, the physiological impact of a person's sex chromosomal makeup combined with their hormonal milieu (be this endogenous or medically supplemented) is what we propose to be an important focus of future research. In *cis*-gender people, the contributions of sex chromosomes and hormones are inextricably linked. We know both to be of significance, but researchers currently are able to separate these factors to examine how they interact and separately contribute only in animal models and *in vitro* research. By inclusion of trans or gender-diverse persons pursuing hormonal affirmation of their gender, we are able to investigate the effects of hormonal manipulation on the immune system in healthy individuals of a wide age range (usually older than 16 in the United Kingdom).

SEX BIAS IN THE EPIDEMIOLOGY AND OUTCOMES OF AUTOIMMUNE RHEUMATIC DISEASES

The majority of autoimmune rheumatic disorders (ARDs) affect *cis*-females in greater number than *cis*-males, as is the case with SLE, Sjögren's syndrome (SS) (49), scleroderma (SSc) (50) and rheumatoid arthritis (RA) (51). SLE predominantly affects females of child-bearing age, with incidence pre-puberty significantly lower (52) and pregnancy associated with increased flares in patients with recently active disease (53, 54). Taken together, these epidemiological observations strongly suggest a role for the sex hormones in disease pathogenesis. However, juvenile rheumatic diseases, defined as having onset before the age of 16–18 years depending on phenotype, such as juvenile idiopathic arthritis (JIA), juvenile lupus (JSLE), juvenile Sjögren's syndrome (JSS) and juvenile dermatomyositis (JDM) *also* exhibit sex bias, but this is less prominent than in their corresponding adult-onset phenotypes (55). JIA, for example, has no significant sex bias overall as an umbrella term, but different disease sub-types are characterised by different age at onset and sex-predominance: e.g., Enthesitis Related Arthritis (ERA) affects predominantly boys and has onset around puberty, while subtypes oligo- and poly-arthritis are more common in pre-pubertal and post-pubertal girls, respectively (56). As pre-pubertal *cis*-boys and *cis*-girls have similar serum sex hormone levels, a potential role for the sex chromosomes in the disease pathogenesis is thus also supported.

Several studies have investigated the effect of hormonal medications in SLE, where one might expect to see exacerbation of disease upon use of the oral contraceptive (OC), or hormone replacement therapy with oestradiol (HRT) given to alleviate menopausal symptoms. Commonly cited is the *Nurse Health Study*, which followed thousands of ciswomen, and reported an elevated relative risk for the development of SLE of 1.9 for women who had ever used hormonal OC (57) and of 2.1 in post-menopausal women who had ever used (HRT) (58). Although hormonal treatments have been purported to cause flares in SLE in older studies (59), recent literature has demonstrated little to no impact of OC usage on mild to moderate SLE, with the potential for unplanned pregnancies deemed a more significant risk for patients than OC use (60, 61). Several studies have demonstrated reduced androgen levels in SLE patients (62, 63), and this has been suggested to play a role in disease development or severity. Therein, the use of various forms of androgen as therapeutic agents has been tested in several incidences – with some trials showing mild efficacy (64–68) while others showed no difference from placebo (69). Thus, the current literature on *in vivo* manipulation of hormones does not provide a conclusive picture. Several case studies (70–77) detail the development of autoimmunity in trans-females upon commencement of gender-affirming oestradiol treatment, or the improvement of symptoms when taking gender-affirming testosterone (78). However, one cannot infer causality from these instances, nor can individual case studies be extrapolated to the wider population. Inclusion of trans people in bigger cohort studies on autoimmunity development is thus strongly supported – whether the increased relative risk seen in post-menopausal *cis*-females on HRT would be the same or similar in trans-women with an XY chromosomal background is yet unknown.

Although the majority of autoimmune diseases are characterised by female bias, there is evidence that type I diabetes mellitus and Crohn's disease are characterised by a male predominance, irrespective of age at onset (79, 80). Additionally, some conditions have differential disease phenotypes according to sex, which has implications in disease recognition and epidemiological data collection. This is the case with spondyloarthritis (81), which had been considered a male-predominant disease for many decades before evidence about a different clinical presentation and delays in diagnosing females with spondyloarthritis emerged (82). Further, certain treatments may be more efficacious in one sex compared to the other [recently reviewed extensively by Klein and Morgan (83)], e.g., TNF inhibitors tend to work better for males with RA

than for females (84) and female patients may be more likely to stop such drugs following the side effects they experience from them (85). Moreover, there is evidence that spontaneous puberty can completely reverse the sex bias in disorders of immune regulation such as asthma and atopy, characterised by male preponderance prepuberty, followed by a significantly increased female prevalence during reproductive years (86).

IMPACT OF AGE, PUBERTY AND MENOPAUSE ON AUTOIMMUNITY

Throughout the various life stages from infancy to old age, the immune system is also subject to great change (87, 88), and these changes are known to differ between cisgender males and females (89). The ageing immune system is a growing area of research, but less is known specifically about the immune changes that may occur during/after puberty and menopause. The coincidence of the average age of onset of several juvenile rheumatic diseases (90) with the average age of puberty onset (91) suggests that it is not merely the maturation process itself that alters one's immune system, but that the rise in sex hormone levels seen in puberty is also involved. Our systematic review of the bidirectional relationship between puberty and autoimmune rheumatic disorders demonstrated how poorly these relationships are documented in the literature, but highlighted the differences in disease outcome in those with onset pre- vs. post-puberty (92) and symptomatic differences have been noted between different age groups of SLE patients (93), with adolescent onset JSLE noted for its greater severity (94, 95). In the case of menopause, RA (96) and SSc (97) both have their peak incidence in the over 50 age bracket. SLE has classically been considered to have its peak incidence within the childbearing years in females, but a 10-year incidence study of United Kingdom patients found the peak onset to be between 50 and 54 years in females and 70–74 in males (98), and this was supported by two other shorter studies (21, 99). However, these studies were of predominantly white populations, and in studies including black (100), Arab (101) and American Indian (102) patients, younger ages of peak onset between 30.4 and 39.2 have been observed. It is unclear exactly why this might be, but this highlights the complexity of sex-based influences on the immune system, which may interact with both age- and ethnicity-related factors to give rise to autoimmunity. With the inclusion of transgender subjects of different ages and pubertal/menopausal stages among basic and clinical research, these factors could be separated out, and the impact of sex be examined without the confounders of immunosenescence and ethnically inherited risk factors.

DIFFERENTIAL EFFECT OF SEX DETERMINANTS ON IMMUNE ACTIVATION PATHWAYS

The investigation of the impact of sex-determinants on certain immune activation pathways, such as specific cell populations or pro-inflammatory pathways, where both sex chromosomal and hormonal elements have been separately suggested to be of influence is an area with great scope for new discovery. Work from our lab, published in 2019 (103), pioneered the inclusion of gender-diverse cohorts to address questions relevant to SLE, using a cohort of healthy trans- (n = 13 male, 7 female) and cisgender (n = 48 male, 51 female) young volunteers, alongside individuals with Turner Syndrome (n = 9), who are missing an X chromosome (45,X). Young transgender healthy controls were recruited from the University College London Hospital GIDS and treatment pathways are shown in **Figure 1**. Production of the antiviral cytokine family known as type 1 interferons (IFN)- predominantly by plasmacytoid dendritic cells (pDC)- is known to contribute significantly to the pathogenesis of both SLE and JSLE. We demonstrated that pDC from healthy *cis*-females produced more T1 IFN in response to TLR-7 signalling than pDC from *cis*-males, even before puberty. Using our inclusive volunteer cohort, we were additionally able to show that this related to X chromosome dosage *and* serum testosterone concentration, in a manner that was dependent upon the number of X chromosomes present. Overall, we showed that both factors were associated not just individually, but also interactively with the T1 IFN response.

More recently, we used a similar cohort (n = 17 *cis*-male; 22 *cis*-female; 10 trans-male and 10 trans-female) to examine the effects of sex and hormones on regulatory and responder CD4 + T cells (Tregs and Tresps, respectively) (104). Sex differences in Tregs are well-reported (105–109), and we firstly confirmed the observation that healthy *cis*-males have higher levels of Tregs compared to Tresps than *cis*-females both pre- and post-puberty. We then demonstrated that the ability of *cis*-male Tregs to suppress the division of Tresps was significantly enhanced compared to that of *cis*-female Tregs, supporting the concept of a pro-inflammatory phenotype in females that could contribute to autoimmunity. Then, using RNA sequencing (RNAseq), we found a significant number of differentially expressed genes (DEGs) in sorted Tregs from *cis*-males compared to females. Using our transgender healthy controls, we observed significant differences in related immune pathways following hormone treatment, demonstrating the potential for both oestradiol and testosterone to impact Tregs at a transcriptional level, even at the early stages of their treatment.

The COVID-19 pandemic has prompted several interesting studies on sex differences in viral responses, and how these translate into clinical outcomes. Takahashi et al. (8) demonstrated a more robust T cell response in females with the disease, compared to males- with poor T cell responses associated with a worse disease trajectory in males. Meanwhile males had higher levels of innate inflammatory cytokines, but higher levels of these in females were associated with more severe outcomes. Supporting these findings, Liu et al. (110) compared transcriptional differences in healthy males and females, demonstrating that males had higher expression of proinflammatory cytokines and chemokines, which they hypothesise may contribute to the 'cytokine storm' that is detrimental in COVID-19 pathogenesis. Females in this study were found to have higher expression of IFN genes, supporting

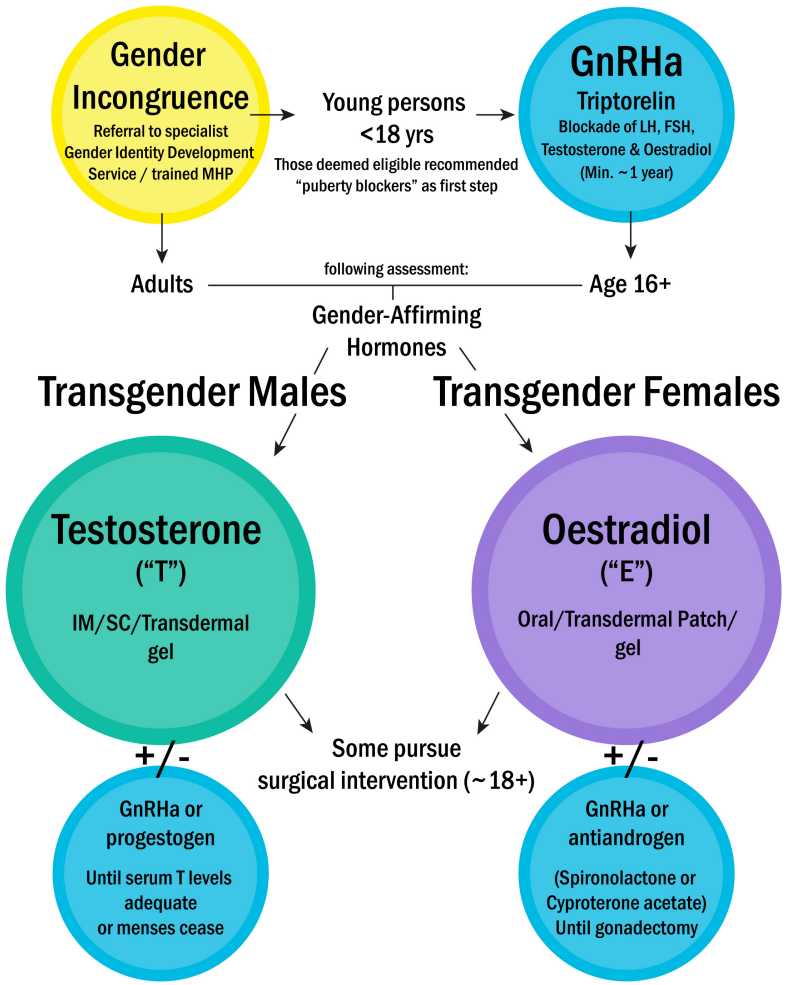

FIGURE 1 | Treatment pathway for gender incongruence, as recommended by the Endocrine Society (42). Treatment is prescribed on a case-by-case basis, based on individual country guidelines. This flowchart outlines the most commonly pursued routes. NB- Parenteral oestradiol not currently used in Europe. *MHP, Mental health professional; GnRHa, Gonadotropin releasing hormone analogs; LH, Luteinising hormone; FSH, Follicle stimulating hormone; IM, Intramuscular; SC, Subcutaneous.*

what is already known about the sex bias in IFN production in health and in autoimmunity. These data demonstrate a clear link between sexual dimorphism in the immunological systems that serve to protect us, that may also lead to damage in the context of an autoimmune disease. Inclusion of trans and gender-diverse cohorts in infection response studies, is thus equally warranted alongside those in autoimmunity.

There remain myriad of cell types and mechanisms that have been identified as potentially influenced by sex hormones or chromosomes, thus meriting *in vivo* interrogation. In addition to the further work necessitated on pDCs, the T1 IFN pathway, and Tregs/Tresps, obvious suggestions for future research directions (based on preliminary evidence of sex hormonal/chromosomal effect in animal or non-diverse cohorts) are B cells and antibody/autoantibody production (111–120), B regulatory (Breg) cells (121), CD4 T cells (116, 122–124), and specific T helper subsets (89, 125–131), CD8 cytotoxic T cells (122, 132–135), dendritic cells (136–140), Natural Killer (NK) cells (116, 141–145), neutrophils (146–149), monocytes (150) and macrophages (149, 151, 152). **Table 1** summarises a selection of notable effects of sex determinants on immune processes and cell types known to be relevant to autoimmune rheumatic disease- this is by no means an exhaustive review of the literature, and many extensive reviews are available (89, 182, 183). As a field in its relative infancy, there remain so many avenues ripe for gender-disaggregated interrogation and scintillating project proposals.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

There is an unmet need for better understanding of the long-term outcomes of sex hormone manipulation on the health of trans and gender-diverse people. This includes the effects of gender-affirming treatment on responses to natural and vaccine immunisations, on bone and muscle health, as well

TABLE 1 | Summary of notable immune system elements known to be regulated by sex determinants and their relevance to autoimmune rheumatic disease.

	***Cis*-female**	***Cis*-male**	**Relevance to autoimmune rheumatic diseases (ARD)**
Immune cells			
B cells	Oestrogens shown to: alter the threshold for B cell apoptosis/activation (112); increase capacity for class-switch recombination (114, 115, 117, 113, 119).	Androgens act *via* GPR174 to divert B cells from germinal centre formation and subsequent class-switching (120). Testosterone regulates *BAFF* – important in survival of autoreactive B cells (118).	Production of autoantibodies central to pathogenesis of many ARDs.
Immunoglobulins	Higher plasma Ig levels in females (111, 116).	–	
CD8 T cells	Lower cell frequency but higher cytotoxic capacity in females (135).	Higher cell frequencies in males (122, 123, 132).	Multiple roles across ARDs (153, 154).
CD4 T cells	Higher cell frequencies in females (116, 122, 123, 132).	–	Subset imbalance (155) and functional abnormalities in SLE (156). Pathogenic role in JIA uveitis (157).
Treg subset	Androgens enhance female CD4 + T cell FoxP3 expression *in vitro* (158).	Male Tregs had greater suppressive ability (104).	Impaired immune regulation in SLE and RA (125).
Th17 subset	Oestrogens both stimulatory (126, 127) and suppressive (128) of proliferation and IL-17 production. Activation *via* ERβ enhances Th17 response, *via* ERα suppresses (130).	Frequency of IL-17A and Th17 cells increased in males with AS compared to females with AS (129).	Role in SLE disease manifestations (159) and IL-17 in RA (160). Initiation of SS (161). Th17 axis implicated in AS pathology (162).
Th1 subset	Oestrogen and progesterone decrease Th1:Th2 and Th17:Th2 cytokine production ratios (131). Male V female Th1 or Th2 predominance varies, reviewed in (89).		SS initiation (Th1) and progression (Th2) Psianou et al. (161) Th1:Th2 imbalance in RA (163).
Th2 subset			
Macrophages and Monocytes	Macrophage phagocytic activity higher in females (146).	Testosterone increases monocyte counts in men (149).	Inflammatory damage to cartilage and bone in RA etc. (164). Defects in phagocytosis and clearance of cellular debris in SLE (165).
Dendritic Cells (DC)	E2 enhanced ability of DCs to activate CD4 + Th cells *in vitro* (136, 138).	Higher levels in hypogonadic males inversely correlated to testosterone levels (140).	Presentation of self-antigen.
Plasmacytoid Dendritic Cells (pDC)	More activated in females and produce more IFN-α (103, 166).	–	IFN production prominent role in SLE pathogenesis (167).
Neutrophils	Phagocytic activity higher in females (146). Oestrogens and progesterone can affect lifespan (147) and numbers increased during luteal phase of menstruation and in pregnancy (148).	Testosterone increases counts in men (149).	Release of proinflammatory cytokines and NET formation externalises autoantigens (168).
Natural Killer Cells (NK)	Higher cell number in females (154). Progesterone contributes to accumulation during pregnancy (144).	Increased CNS NK inflammation in males vs. females in ALS mouse model- NK depletion benefitted females but not males (145).	Cytotoxicity in inflammation and role in immunoregulation/immune homeostasis (169).
Cytokines and **Immune Mediators**			
Type 1 Interferons	IFN-α production higher in female cells post TLR stimulation (103, 170).	Testosterone correlates with IFN-α independently from X chromosome (103).	Prominent role in SLE pathogenesis (167).
Type 2 Interferons	E2 treatment in mice increased DC production of IFN-γ (138).	IFN-γ higher in stimulated lymphocyte supernatant from males (170).	Inflammatory role in SLE, SS, SSc and dermatomyositis (171).
IL-10	Higher production in stimulated lymphocyte supernatant from females (170).	Higher production in males and correlates with testosterone (172).	Breg and IL-10 role in SLE, RA and SSC (173).
Microbiota	Bi-directional relationship between hormones and microbiota, with immune impact (174, 175).		Known impact of microbiota on rheumatic disorders (176).
Transcriptional Differences			
Macrophages (MF)	Higher expression of MF IFN-stimulated genes in female mice, with sig. bias in antiviral response genes (177).	–	IFN role in SLE, SS, SSc, RA and dermatomyositis (178).
CD8 Cytotoxic cells	Greater toxicity post-stimulation in female cells: antiviral and inflammatory gene exp increased, many with oestrogen response elements in their promoters (134).	–	Multiple roles across ARDs (155, 156).
AIRE (autoimmune regulator) expression	Oestrogens inhibit (179).	Androgens enhance (180).	Necessary for self-tolerance induction in the thymus (181).

BAFF, B cell Activating Factor; Ig, Immunoglobulins; ERα, Oestrogen Receptor Alpha; ERβ, Oestrogen Receptor Beta; AS, Ankylosing Spondylitis; CNS, Central Nervous System; ALS, Amyotrophic Lateral Sclerosis; IFN, Interferon; TLR, Toll-like Receptor.

as their impact on mental health and quality of life, before moving into investigating infective and autoimmunity risk in these populations. Without accurate gender classifications in population studies, these relevant outcomes cannot be studied. There are many specific questions which need answering in relation to the impact of sex determinants on immune system functions, in particular around exposure to and timings of exposure to sex hormones. We do not know if the length of exposure to/blockade of a particular sex hormone is different from the physiological sex hormone fluctuations, especially those related to menstruation, pregnancy, or early stages of puberty/menopause. There is no research into the impact of age at which a person is first exposed to (or begins blocking) sex hormones on their risk of infections, autoimmunity, or other adverse health outcomes. Our group identified a significant impact of sex hormones in driving a pro-atherogenic lipid profile in healthy *cis*- and *trans*-male adolescents post-puberty (184). Therefore, investigating the impact of sex-affirming hormone therapy on the cardio-vascular risk of trans people has a clear clinical rationale. Further research is needed to investigate the effects of lifetime exposure to higher exogenous oestrogen or androgen therapies, especially in the context of potential reversibility and dose-dependent long-term effects. In some countries, young people are able to commence puberty blockade and gender-affirming sex hormones prior to the commencement of their natural puberty. Meanwhile in the United Kingdom, only those aged 16 + and thus likely already post-pubertal can legally be consented to start on gender-affirming hormone treatments. Others still, may not access treatment until much later into adulthood. It is important to establish whether outcomes (immunological or otherwise) would be similar or different in these groups, when their hormonal transitions have commenced at such widespread life stages. Furthermore, it is possible that different routes of hormone administration (oral, patch, gel, IM, SC.) and dosages of these may impact the systems of the human body differently. Innovative clinical trial study design, including volunteers of all gender categories, across various age ranges is required to be able to examine the relative importance of sex hormone exposure at different stages of life, against both sex chromosomal backgrounds, on various interventions or health and disease outcomes. In addition, the inclusion of subjects with altered sex chromosomal complement (such as Klinefelter and Turner syndromes) could provide suitable controls for these studies aiming to tease out the distinct effects of various sex chromosome determinants.

First steps would be establishing national and international registries with associated biological sample repositories capturing patients of various gender categories, sex chromosomal

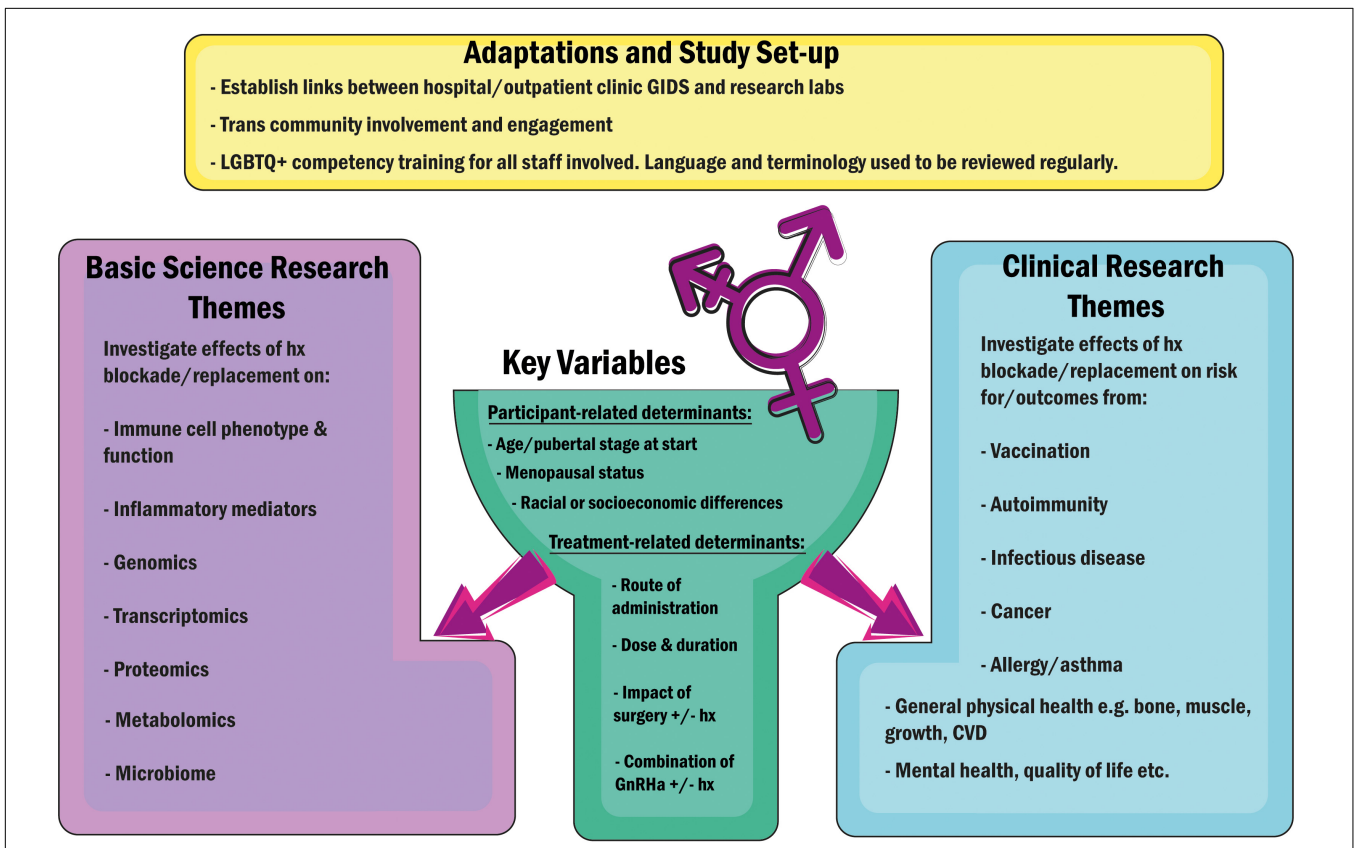

FIGURE 2 | Suggested adaptations to facilitate future research encompassing trans and gender-diverse individuals, and key research pathways proposed. *Hx, Hormones; GnRHa, Gonadotropin Releasing Hormone Agonists ("Blockers"); CVD, Cardiovascular Disease.*

backgrounds and demographic diversity to enable long-term follow-up. A number of social barriers exist, well-documented in the United States, that prevent the trans population from accessing healthcare and thus participating in research (185). Thus, it is important for such registries to be set up with advice and input from transgender charities and organisations such as *WPATH* (World Professional Association for Transgender Health) on how to overcome these barriers. This should include ensuring that all health professionals and researchers involved are trained in LGBTQ + cultural competency (186), so that all elements of study design- from language used on questionnaires, to subtlety when approaching people for recruitment- are optimised to help participants feel secure and respected. Further, recruitment must extend beyond private healthcare patients, encompassing public healthcare clinics as well as community support groups, in order to capture the true breadth of the trans population. Hospital and clinic record databases must be updated in order to capture gender definitions and associated medications more accurately, and reference ranges for clinical and laboratory tests need to be reviewed and established for gender-diverse people, as it is highly likely that they may differ from those appropriate for *cis* persons (187). If these changes were made across the world, they would not only facilitate far more impactful retrospective review of outcomes, but would vastly improve the lives and healthcare of transgender persons, who have tolerated systems that weren't designed to accommodate them for far too long. In **Figure 2**, we propose several streams of research, both clinical and immunological, as starting points for future projects. Researchers and clinicians should join forces to give people of all gender identities a voice and create opportunities for their involvement in clinical data collection and research. As more countries develop their gender identity services, and adapt to the changes outlined above, we look forward to seeing the results from further large studies such as 2021 *Michelson Prize* recipient Dr. Camila Consiglio's multi-parameter analysis of the effect of testosterone treatment on the immune systems of trans-men at the *Karolinska Institutet*, Sweden (188), and that of Professor Guy T'Sjoen's *ENIGI* consortium across Ghent, Oslo, Florence, and Amsterdam (189, 190), where long-term follow-up of participants pursuing hormonal gender affirmation will provide us with a wealth of information, pertinent to everyone – not just those it is convenient to study.

CONCLUDING REMARKS

We advocate that research should celebrate gender diversity and be as inclusive as possible to ensure that it is relevant to human society as a whole. We can only hope that in coming years, more labs and clinical teams will join us in the interrogation of sex determinants as biological variables. As personalised medicine becomes an increasingly viable and beneficial approach to healthcare, it is research like this that will be equipped to inform and steer innovation in the appropriate direction.

DISCLAIMER

Gender-related terminology is continually evolving, and terms vary in their usage between individuals and between groups across the world. Language and definitions used throughout this article have been adapted from the Gender Identity Research and Education Society (GIRES) website at time of writing (191) – we have made every effort to be inclusive, but acknowledge that these may not capture the preferences and experiences of all.

AUTHOR CONTRIBUTIONS

CC, GB, and HP contributed to conception of the review. HP wrote the first draft of the manuscript and designed the figures. CC, GB, KW, and ER wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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What is the impact of sex hormones on the pathogenesis of rheumatoid arthritis?

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Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease and has a female predominance of around 3:1. The relationship between sex hormones and RA has been of great interest to researchers ever since Philip Hench's observations in the 1930's regarding spontaneous disease amelioration in pregnancy. Extensive basic scientific work has demonstrated the immunomodulatory actions of sex hormones but this therapeutic potential has not to date resulted in successful clinical trials in RA. Epidemiological data regarding both endogenous and exogenous hormonal factors are inconsistent, but declining estrogen and/or progesterone levels in the menopause and post-partum appear to increase the risk and severity of RA. This review assimilates basic scientific, epidemiological and clinical trial data to provide an overview of the current understanding of the relationship between sex hormones and RA, focusing on estrogen, progesterone and androgens.

KEYWORDS

rheumatoid arthritis, estrogen, progesterone, androgens, pathogenesis, pregnancy

Introduction

Rheumatoid arthritis (RA) is a chronic multisystem inflammatory disease which causes a destructive symmetrical polyarthritis. It is characterized by production of the autoantibodies rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). Common to many autoimmune disorders, there is a female predominance, with a female:male distribution of around 3:1 (1), although this gender disparity is less marked than in other inflammatory rheumatic conditions such as systemic lupus erythematosus. Other observations implicating the importance of sex hormones in the pathogenesis of RA include its peak incidence at menopause (2), reduced disease activity in pregnancy and flare in the postpartum period (3). Such phenomena would suggest that declining female sex hormones, principally oestrogren and progesterone, increase the risk of RA in menopause and post-partum, while increased levels in pregnancy are protective. However, despite extensive study, the relationship between sex hormones and RA pathogenesis remains complex.

This review will synthesize lines of evidence regarding the impact of sex hormones on the pathogenesis of RA, focusing on estrogen, progesterone and androgens. We present an overview of the data from basic laboratory research through to a comprehensive evaluation of epidemiological studies and clinical trials.

Strategy

We searched PubMed for original articles, systematic reviews and meta-analyses in English, pubished between 1 January 1990 and 31 December 2021. Reference lists in retrieved articles were also reviewed to identify further articles of relevance. Search terms used comprised combinations of the following: "Rheumatoid arthritis", "sex hormones/steroids", "(o)estrogen", "progesterone", "androgens", "risk factors", "disease activity", "menarche", "pregnancy", "menopause", "contraceptive", "breastfeeding" and "hormone replacement therapy".

Rheumatoid arthritis pathogenesis

A detailed discussion of the pathogenesis of rheumatoid arthritis it outside the scope of this article, and the reader is directed to other recent excellent reviews (4–7). In brief, the etiology of RA is recognized to depend on complex interactions between genes and environment, with a resultant breakdown of immune tolerance leading to inflammation in synovial joints. The strongest genetic component is found in the human leukocyte antigen (HLA) class II molecule HLA-DRB1, with over 80 % of patients carrying the shared epitope of the HLA-DRB1*04 cluster (8), which is also associated with severity of disease (9). RA pathogenesis is thought to be initiated years before the development of symptoms and likely involves the induction by environmental factors, the most established of which is smoking (10), of post-translational modifications. These processes lead to the activation by antigen-presenting cells of an adaptive immune response, with production of the hallmark autoantibodies RF (targeting immunoglobulin G) and ACPA (6). Interactions of immune cells, including T and B cells, plasma cells and monocytes, and pro-inflammatory cytokines, most importantly tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6, lead to an infux and local activation of inflammatory cells in the synovium. Macrophage-like synoviocytes proliferate and produce TNF-α, IL-6 and IL-1, among other pro-inflammatory mediators. Infiltrating immune cells include CD4+ T cells and mature B cells producing RF and ACPAs. The synovial membrane expands and is filled with new blood vessels. Within the synovium, abundant osteoclasts are the primary mediators of bone erosion, while fibroblast-like synoviocytes secrete matrix metalloproteinases into the synovial fluid leading to cartilage degradation.

Sex hormone signaling

The sex hormones estrogen, progesterone and androgens all bind to nuclear receptors which belong to the type 1 family of nuclear hormone transcription factors (to which the glucocorticoid receptor also belongs) (11–13). In brief, binding of these hormones to their receptors in the cytosol leads to dissociation of heat shock proteins, homodimerization, translocation into the nucleus and subsequent binding to hormone response elements within regulatory regions of target genes, resulting in modification of gene transcription. Classically, oestrogens bind the cytosolic receptors estrogen receptor (ER)- α and/or ERβ to mediate genomic effects; in addition, oestrogens may also bind to membrane receptors such as G protein-coupled estrogen receptor 1 (GPER1) to trigger downstream signaling cascades that non-directly influence gene expression. The progesterone receptor (PR) and androgen receptor (AR), which are closely related, mediate classical genomic effects in a similar fashion to oestrogens, with hormone-receptor complexes binding to progresterone- and androgen-response elements respectively.

Sex hormones and genetic risk of rheumatoid arthritis

Estrogen and progesterone

Genome-wide and targeted gene analysis studies have revealed single nucleotide polymorphisms (SNPs) in sex steroid-associated genes that influence the risk and course of RA. These include SNPs in *ESR2* which encodes estrogen receptor-β (ERβ) conferring a reduced risk of erosive arthritis (14) and an improved chance of responding to anti-tumor necrosis factor (TNF) therapy (15); other SNPs associated with a reduced risk of erosive disease include those in cytochrome p450 enzymes CYP1B1 and CYP2C9, which convert oestrogens to anti-inflammatory hydroxy-oestrogens (14). We are not aware of any studies evaluating genes involved in progesterone signaling and the risk or severity of RA.

Androgens

SNPs in the cytochrome B5-encoding gene *CYB5A* have been associated with a reduced susceptibility to RA in women (16). Such polymorphisms lead to an increased production by the cytochrome B5 enzyme of androgens from precursor hormones, suggesting that higher androgen levels protect against the development of RA.

Sex hormone signaling in the immune system

There is a significant body of evidence of the immunomodulatory effects mediated by sex hormones in autoimmune disease [extensively reviewed in Hughes (17), Wilder and Elenkov (18), Hughes and Choubey (19), Kanik and Wilder (20), Cutolo and Straub (21) and Moulton (22)].

Estrogen

Oestrogens have complex interactions with the immune system which may be pro- or anti-inflammatory depending on the cell type and concentrations involved (21, 22). With relevance to B cell function, at physiological concentrations the estrogen-ERα complex binds to the promoter of the *AICDA* gene which in turn stimulates expression of the enzyme activation-induced cytidine deaminase (AID). This enzyme is the master regulator of somatic hypermutation and class switch recombination. Therefore, oestrogens support immunoglobulin class switching in B cells and would logically be expected to have a deleterious effect on autoimmune diseases characterized by autoantibody production (23).

T cell ontogeny comprises initial development as haematopoetic precusors in the bone-marrow which then migrate to the thymus. Here, a process of positive and negative selection occurs, such that T cells which react strongly with antigen presented by major histocompatibility complex survive, but those that react with self-antigen are eliminated (except a proportion of CD4+ cells that survive as regulatory T cells (T_{reg}), see below). The process of negative selection is regulated by the transcription factor AIRE, which serves to promote self-tolerance. This protein has emerged as a key factor in sexual dimorphism in autoimmunity, with females expressing lower levels of AIRE post-puberty compared to males (24). Male castration reduces expression of AIRE, while ERα deficient mice showed no difference in expression between sexes. Moreover, estrogen treatment was found to decrease AIRE expression in human thymic tissue *via* methylation of CpG sites in the *AIRE* promoter (24).

In contrast, oestrogens have been found to have a range of direct anti-inflammatory actions on T cells. Silibinin, a natural agonist of ERβ was shown to reduce the *in-vitro* expression of pro-inflammatory interleukin (IL)-17 and TNFα in T cells from healthy donors and patients with active RA (25). This effect was mediated *via* down modulation of the expression of the epigenetic modifier microRNA-155. Silibinin has also been shown to stimulate apoptosis of human RA synoviocytes *in vitro* (26) and reduce the production of inflammatory cytokines in rats with collagen-induced arthritis (26).

Oestrogens generally exert inhibitory effects on pro-inflammatory T_H1 cells, while they may inhibit pro-inflammatory T_H17 cells *via* ERα or have the opposite effect *via* ERβ. At high concentrations such as seen in pregnancy, oestrogens induce the secretion of IL-10 and suppress TNFα production in T cells, supporting an anti-inflammatory cytokine milieu (27).

Forkhead box (FOXP3) expressing T_{reg} cells are highly suppressive regulators of the immune response through secretion of anti-inflammatory cytokines such as IL-10 as well as cell-cell contact mechanisms. The *FOXP3* locus possesses sex steroid response elements enabling direct binding of hormones and subsequent modulation of FOXP3 activation. Endogenous complexes of estradiol and ERβ were shown to bind directly and activate the FOXP3 promoter in T_{reg} cells from both human cervical cancer specimens and healthy controls (28).

It has been shown that estrogen treatment before immunization with collagen can retard disease development in collagen-induced arthritis (CIA), a mouse model of RA (29). In CIA, exogenous estrogen administration ameliorated postpartum flare (30), while joint inflammation improved with treatment of non-pregnant arthritis-prone mice with estradiol or progesterone at pregnancy-like levels (31), or by treatment with high-potency estrogen alone (32). Estradiol has been linked to expansion of anti-inflammatory T_{reg} cells in pregnant mice with autoimmune encephalitis (33) and pregnancy-induced amelioration of joint inflammation has been achieved by treatment of non-pregnant SKG mice (a model of human RA) with either estradiol or progesterone at pregnancy-like levels (31).

Progesterone

In contrast to the dichotomous effects of oestrogens, progesterone exhibits broad anti-inflammatory actions (17, 21), including: inhibition of AID (in opposition to the effect of oestrogens); inhibition of the T_H1 and T_H17 response; and inhibition of NK cells, neutrophils and macrophages.

Progesterone promotes the T_H2 response by enhancing IL-4 and IL-10 production in human T cells (27). During pregnancy, lymphocytes express progesterone receptors and release a protein named progesterone-induced blocking factor, which has strong anti-natural killer (NK) cell activity, and also secrete IL-10 (34). Progesterone has been shown to induce FOXP3+ T_{regs} from naive murine CD4+ T cells *via* suppression of mammalian target of rapamycin (mTOR) signaling (35); furthermore, progesterone promotes differentiation of human fetal cord blood T cells into T_{regs} and suppresses their differentiation to T_H17 cells (36). In multiple sclerosis, T_{regs} express high levels of estrogen and progesterone receptors, and each hormone enhances the suppressive function of T_{regs} *in vitro* (37).

Recently, progesterone was shown to suppress activation (as measured by CD69 and CD25 expression) of *ex vivo* CD4+ T cells from healthy human females in a dose-dependent fashion (using doses similar to those found in the placenta) (38); furthermore, RNA sequencing analysis showed significant transcriptomic changes involving downregulation of immune-related genes and pathways important in RA, such as signal transducer and activator of transcription (STAT)-1 and STAT3.

Androgens

Androgens such as testosterone have a range of anti-inflammatory effects *in vivo*, reducing secretion of inflammatory cytokines such as TNF, IL-1 and IL-6 by monocytes and inhibiting B cell lymphopoiesis and antibody production (39). In inflammatory rheumatic diseases serum androgen levels are often reduced (40) owing to the stimulation by inflammatory cytokines such as TNF, IL-1 and IL-6 of the aromatase enzyme in immune cells and fibroblasts. Androgens are also known to bind and upregulate tyrosine-protein phosphatase non-receptor type 1 (PTPN1), which has a broad range of functions in cell growth and immune function. PTPN1 inhibits janus kinase (JAK)-2 and tyrosine kinase 2, part of the JAK-STAT pathway which is integral to T_H1 cell-mediated immune responses and the production of IL-12 and IFNγ. This signaling pathway is of especial interest in RA given the established therapeutic options targeting the JAK-STAT pathway. It is not yet known whether response to these treatments differs between men and women with RA.

Conversely to oestrogens, androgens promote AIRE transcription by recruiting androgen receptors to *AIRE* promoter regions, leading to higher expression in mice and human thymus in males compared to females (41). Male sex and androgen treatment were protective in a mouse model of multiple sclerosis, but to date this effect has not been investigated in models of rheumatic diseases including RA (41).

Similarly to oestrogens, androgens can bind to the FOXP3 androgen response element, leading to acetylation of histone H4 and activation of FOXP3; there is a strong androgen-dependent increase of FOXP3 expression in T cells from women in the ovulatory phase of the menstrual cycle but not from men (42).

Sex hormones and pathogenesis of RA: Epidemiology

There is extensive epidemiological data implicating the importance of sex hormones in the pathophysiology of RA, but results are conflicting. Large modern cohort studies and meta-analyses have often contradicted the findings of early smaller retrospective studies. We summarize the results of large modern epidemiological studies (case-control, cohort studies and meta-analyses) concerning sex hormones and RA in Tables 1–3 (43–64). Table 1 shows studies which found an association between endogenous hormonal factors and risk or course of RA, Table 2 lists the reports which found no such association, and Table 3 shows studies concerning exogenous hormonal factors and RA (Table 3) (nb, some studies are listed more than once where appropriate).

Endogenous sex hormones

Menarche

An abnormally early menarche was found to be associated with an increased risk of seropositive RA in a large prospective cohort study [relative risk (RR) 1.6 [95% confidence interval (CI) 1.1–2.4] for menarche < 10 years] (43). This finding was supported by a more recent cohort study, although the association was weaker (HR) 1.20 for menarche < 13 years (95% CI 0.9 - 1.5) (55). The data on menarche is conflicting, however, as a case-control study found an increased risk of anti-citrullinated protein antibody (ACPA)-positive RA with menarche at ≥15 vs. ≤ 12 years (44).

Pregnancy and post-partum

Hormonal changes in pregnancy

Pregnancy is associated with considerable increases in both estrogen (around four to six fold) and progesterone (around three to eight fold) (65). These hormones rise progressively through pregnancy, peaking at the third trimester and falling within weeks post-partum.

Pregnancy, parity and risk of RA

In an initial small case-control study, pregnancy was associated with a reduced risk of RA onset which did not reach statistical significance [odds ratio (OR) 0.3, 95% CI 0.04–2.6], while there was an increased incidence of RA during the first 3 months post-partum (OR 5.6, 95% CI 1.8–17.6) (66). Similar results were found in another early case-control study (67). The risk of RA onset has been shown to persist for the first year (OR 3.8, 95% CI 1.45–9.93) (68) and up to 24 months post-delivery (incidence rate ratio 1.7, 95% CI 1.11–2.70) (69).

In early studies, nulliparity was associated with a roughly two-fold risk of RA onset (70), but modern studies have shown conflicting findings regarding parity and risk of RA. Some studies have found that having more than one pregnancy increased the risk of RA (46), particularly with young age at first pregnancy (45, 46), while others have found a protective effect of parity (45, 47, 71). One retrospective cohort study found a dose-response relationship between the protective effect of parity and risk of RA (45), but this was not replicated in a later meta-analysis (47). The most recent systematic review found no association between gravidity and parity and the risk of developing RA (56).

Pregnancy and RA disease activity

RA was first observed to spontaneously remit in pregnancy in the 1930s (72). Historical studies reported that up to 90% of patients improved during pregnancy, but this effect is less apparent with modern treatment regimens (3, 73). As safety data regarding the use of traditional and biologic disease modifying drugs in pregnancy accumulates (74, 75), increasing numbers

TABLE 1 Epidemiological studies showing an association between endogenous sex hormones and rheumatoid arthritis pathogenesis.

Reference	Design	Population/country	N (Total/RA)	Hormonal factor	Summary of findings (95 % CI)
Karlson et al. (43)	Prospective cohort	Nurses aged 30–55 (at baseline), USA	121,700/674	Menarche, BF	↑ risk of RA with irregular menstrual cycles, RR 1.4 (1.0–2.0) Early menarche (<10 years)↑ risk of seropositive RA, RR 1.6 (1.1–2.4) ↓ risk of RA with ↑ duration BF, RR 0.5 (0.3–0.8) for >24 months
Pedersen et al. (44)	Case-control	Women aged 18–65, Denmark	1,284/515	Menarche	Menarche at age ≥15 years ↑ risk of ACPA-positive and -negative RA vs. menarche at age ≤ 12 years, OR 1.87 (1.23–2.85)
Jorgensen et al. (45)	Retrospective cohort	National registry/Denmark	4,400,000/7,017	Parity	↑ risk of RA with ↓ age at birth of first child ($p < 0.001$) Women with > 1 child at ↓ risk of RA vs. women with one: 2 children, RR 0.84 (0.78–0.90) 3 children, RR 0.83 (0.77–0.91)
Orellana et al. (46)	Case-control	Women aged 18–70 years, Sweden	4,946/2,035	Parity	Parity ↑ risk of ACPA-negative RA in ages 18–44, OR 2.1 (1.4–3.2) but not in ages 45—70 years ↑ risk of ACPA-negative RA with young age (<23 years) at first birth, OR 2.5 (1.5–4.1)
Ren et al. (47)	Meta-analysis	Women aged 15–84 years	2,497,580/11,521	Parity	Borderline ↓ risk of RA in parity vs. nulliparity, RR 0.90 (0.79–1.02)
Pikwer et al. (48)	Case-control	Women aged 44–74 years, Sweden	680/136	BF	Longer duration of BF ↓ risk of RA, OR 0.46 (0.24–0.91) if ≥13 months
Berglin et al. (49)	Case-control	Women aged 20–68 years, Sweden	350/70	BF	BF ↑ risk of RA, OR 4.8 (1.43–15.8)
Adab et al. (50)	Cohort	Women ≥50 years, China	7,349/669	BF	↓ risk of RA in BF, lower risk with increasing duration, OR 0.54 (0.29–1.01) if ≥ 36 months
Chen et al. (51)	Meta-analysis	Women aged 16–79 years	143,670/1,672	BF	↓ risk of RA in BF, OR 0.68 (0.49–0.92), dose response effect
Beydoun et al. (52)	Cohort	Women >60 years, USA	1,892/182	Menopause	↑ risk of RA with menopause <40 years vs. ≥50 years, OR 2.53 (1.41–4.53)
Bengtsson et al. (53)	Prospective cohort	Nurses Health Study, USA	237,130/1,096	Menopause	Early menopause (<44 years) ↑ risk of seronegative RA, HR 2.4 (1.5–4.0)
Merlino et al. (54)	Prospective cohort	Women aged 55–69 years	31,336/158	Menopause/ pregnancy/ misc	Age at menopause & age at last pregnancy ↓ risk of RA History of PCOS ↑ risk of RA
Salliot et al. (55)	Prospective cohort	Women aged 40–65 (at baseline), France	78,452/698	Multiple endogenous hormonal factors	Borderline ↑ risk of RA in early menarche (<13 years), HR 1.20 (0.9–1.5) Early age at 1st pregnancy (<22 years) ↑ risk of RA, HR 1.34 (1.0–1.7) Nulliparity ↓ risk of RA in non-smokers, HR 0.44 (0.2–0.8) ↑ risk of RA in early menopause (<45 years) in smokers, HR 1.54 (1.0–2.3)

RA, rheumatoid arthritis; RR, relative risk; BF, breastfeeding; ACPA, anti-citrullinated protein antibody; OR, odds ratio; HR, hazard ratio; PCOS, polycystic ovarian syndrome. ↑, increased; ↓, decreased.

TABLE 2 Epidemiological studies showing no association between endogenous sex hormones and RA pathogenesis.

Reference	Design	Population/country	N (Total/RA)	Hormonal factor	Summary of findings
Karlson et al. (43)	Prospective cohort	Nurses aged 30–55 (at baseline), USA	121,700/674	Parity	No effect of parity/age of first birth and risk of RA
Pikwer et al. (48)	Case-control	Women aged 44–74 years, Sweden	680/136	Parity	No effect of parity on risk of RA
Orellana et al. (46)	Case-control	Women aged 18–70 years, Sweden	4,946/2,035	Parity	No effect of parity/post-partum on risk of ACPA-positive RA
Chen et al. (56)	Meta-analysis	Women aged 15–79 years	2,385,179/13,374	Parity/pregnancy	No effect of parity, gravidity, pregnancy or post-partum on risk of RA
Orellana et al. (57)	Case-control	Women aged ≥18 years, Sweden	6,892/2,641	BF	No effect of BF on risk of RA
Salliot et al. (55)	Prospective cohort	Women aged 40–65 (at baseline), France	78,452/698	BF	No effect of BF on risk of RA
Beydoun et al. (52)	Cohort	Women >60 years, USA	1,892/182	Menopause	No effect of age at menarche and pregnancy history on post-menopausal RA

ACPA, anti-citrullinated protein antibody; BF, breastfeeding.

of patients maintain low disease activity or remission through pregnancy with a treat-to-target strategy (76).

Recent systematic reviews of the potential mechanisms of RA disease improvement in pregnancy have not identified any human studies that have specifically investigated the impact of rises in estrogen or progesterone on alterations of RA disease activity in pregnancy (77, 78).

Breastfeeding

In an early study, breastfeeding was associated with post-partum flare in inflammatory polyarthritis; this finding led the authors to suggest that prolactin may be responsible for this phenomenon (79). Since then, duration of breastfeeding has been most consistently associated with a decreased risk of RA (48, 50, 51). Other case-control (57) and large prospective cohort (43, 55) studies have found no significant association between breastfeeding and risk of RA, while a single study identified an increased risk (49). The data regarding the role of prolactin in human RA is limited and conflicting (80), but in a murine model of collagen-induced arthritis, treatment with bromocriptine, an inhibitor of prolactin secretion, suppressed post-partum flare (81).

Menopause

A consistent finding is the increased risk of RA in early menopause (52, 82, 83). In the large Nurses Health Study cohort, menopause at <44 years increased the risk of seronegative RA [hazard ratio (HR) 2.4, 95% CI 1.5–4.0] (53). The menopause has also been associated with the development of ACPA in first degree relatives of patients with RA (84).

Androgens

In men with RA, low serum levels of testosterone were found to be strongly predictive of seronegative disease (OR 0.31, 95% CI 0.12–0.85) but not significantly predictive of seropositive disease (85). Men with untreated hypogonadism have been found to be at increased risk of a range of autoimmune diseases, including RA (HR 1.31, 95% CI 1.22–1.44) (86), as are men with Klinefelter syndrome (RR 3.3, 95% CI 2.0–5.2)(87).

Exogenous sex hormones

Oral contraceptives

There are conflicting reports on the effect of oral contraceptives (OC) on the risk of RA.

Early reports suggested a beneficial effect, with a case-control study ($n = 115$) showing lower current use of OC in new cases of inflammatory polyarthritis (OR 0.22, 95% CI 0.06–0.85) (88). A further case control study found that OC use roughly halved the risk of RA, with stronger protection from earlier OC preparations (59). A matched case-control study found, conversely, an increased risk of ACPA-positive RA with OC use (44). Most recent reports, however, show no such effect (43, 50, 58, 89), although a meta-analysis has suggested that OC may reduce the risk of progression to severe disease in established RA (58). Two studies have suggested that extended OC use (>7 years) may protect against RA (49, 57). The differing

TABLE 3 Epidemiological studies of exogenous sex hormones and RA pathogenesis.

Reference	Design	Population/country	N (Total/RA)	Hormonal factor	Summary of findings (95 % CI)
Berglin et al. (49)	Case-control	Women aged 20–68 years, Sweden	350/70	OC	Use of OC ≥ 7 years ↓ risk of RA
Orellana et al. (57)	Case-control	Women aged ≥18 years, Sweden	6,892/2,641	OC	Ever use of OC ↓ risk of ACPA-positive RA, OR 0.84 (0.74–0.96) OC use > 7 years ↓ risk of ACPA-positive and ACPA-negative RA
Pedersen et al. (44)	Case-control	Women aged 18–65, Denmark	1,284/515	OC	OC ↑ risk of ACPA-positive RA, OR 1.65 (1.06–2.57)
Karlson et al. (43)	Prospective cohort	Nurses aged 30–55 (at baseline), USA	121,700/674	OC	No effect of OC use and risk of RA
Pikwer et al. (48)	Case-control	Women aged 44–74 years, Sweden	680/136	OC	No effect of OC on risk of RA
Adab et al. (50)	Cohort	Women ≥50 years, China	7,349/669	OC	No effect of OC on risk of RA
Chen et al.(58)	Meta-analysis	Women aged 16–84 years	221,022/4,209	OC	No effect on risk of RA but prevents progression to severe disease
Doran et al. (59)	Case-control	Women aged ≥18 years, USA	890/445	OC and HRT	↓ risk of RA with OC use, OR 0.56 (0.34–0.92), lower with first exposure in earlier years No association of HRT with risk of RA
Salliot et al. (55)	Prospective cohort	Women aged 40–65 (at baseline), France	78,452/698	OC and HRT	Nil effect of OC on risk of RA Nil effect of HRT on risk of RA in menopause ↓ risk of RA with oral progesterone use >24 months before menopause, HR 0.77 (0.6–0.9)
Salliot et al. (60)	Cohort	Early arthritis cohort, France	568	HRT	↓ risk of RA in women carrying HLA-DRB1 *01 and/or *04 alleles Protective effect of HRT on development of ACPA, OR 0.43 (0.24–0.77)
Orellana et al. (61)	Case-control	Women aged 18–75, Sweden	1,580/523	HRT	↓ risk of ACPA-positive RA in current users of HRT aged 50–59, OR 0.3 (0.1–0.8) ↓ risk of ACPA-positive RA in current users of combined HRT, OR 0.3 (0.1–0.7) but not estrogen-only HRT No association between HRT and ACPA-negative RA
Merlino et al. (54)	Prospective cohort	Women aged 55–69 years	31,336/158	HRT	↑ risk of RA with HRT, RR 1.47 (1.04–2.06)
Bengtsson et al. (53)	Prospective cohort	Nurses Health Study, USA	237,130/1,096	HRT	HRT use > 8 years ↑ risk of seropositive RA
Chen et al. (62)	Prospective cohort	Women aged ≥18 years with breast cancer, USA	190,620/4,460	Anti-oestrogens	↑ risk of RA with SERMs, OR 2.4 (1.9–3.0) ↑ risk of RA with AI, OR 1.9 (1.6–2.1)
Caprioli et al. (63)	Cohort	Women aged 57–74 with breast cancer, Italy	7,533/113	Anti-oestrogens	↑ risk of RA with AIs, HR 1.62 (1.03–2.56)
Wadstrom et al. (64)	Cohort/case-control	National registry, Sweden	95,362/15,356	Anti-oestrogens	No association between tamoxifen or AI and risk of RA

OC, oral contraceptive; HRT, hormone replacement therapy; ACPA, anti-citrullinated protein antibody; SERM, selective estrogen receptor modulator; AI, aromatase inhibitor; OR, odds ratio. ↑, increased; ↓, decreased.

findings regarding OC use and risk of RA have been suggested to be due to a lowering of their estrogen content over time (59, 90).

Hormone replacement therapy

Use of hormone replacement therapy (HRT) was associated with a significantly elevated risk of RA in a large prospective

study of postmenopausal women (RR 1.47, 95% CI 1.04–2.06) (54). Conversely, a case-control study found a reduced risk of ACPA-positive RA in women aged 50–70 years who were current users of combined (estrogen plus progestogen) HRT (OR 0.3, 95% CI 0.1–0.7) (61). This finding supported that of an earlier cohort study which found a protective effect of HRT on the development of ACPA in early arthritis (60). Other studies have found no association between HRT use and risk of RA (59).

Anti-estrogen agents

Selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AI) are used for the treatment of breast cancer. SERMs competitively inhibit estrogen binding to its receptor and may have agonistic or antagonistic properties in different target tissues, while AI reduce endogenous production of estrogen. A large national database study found an increased incidence of RA with use of either of these agents (OR 2.4 for SERMs (95% CI 1.9–3.0) and 1.9 (95% CI 1.6–2.1) for AI, each with >12 months treatment) (62). In another large population-based study, the use of aromatase inhibitors to treat breast cancer was associated with an increased risk of RA (adjusted HR 1.62, 95% CI 1.03–2.56) (63). In contrast, a recent large national registry study found no increased risk of RA with either tamoxifen (a SERM) or AI (64).

Clinical trials of sex hormones in RA

Estrogen and progesterone

Investigations into the therapeutic potential of sex hormones in RA have been disappointing. A randomized placebo-controlled trial of adjuvant estrogen therapy at physiological doses compatible with pregnancy in postmenopausal female RA patients was negative (91). Randomized controlled trials of ERα (92) and ERβ (93) agonists in RA were both negative. Large randomized control trials of HRT (either estrogen or estrogen and progesterone combined) in postmenopausal women with RA failed to demonstrate any benefit on symptom severity over placebo (94).

Interestingly, however, the data emerging from the field of multiple sclerosis, another disease that improves in pregnancy, is more encouraging regarding the potential therapeutic benefit of estrogen: a placebo-controlled phase 2 trial of estriol met its endpoint of a significant reduction in relapse rate (95). Progesterone or its synthetic derivatives have not been studied in clinical trials as a treatment option in RA.

Androgens

Well-designed trials of supplemental androgen therapy in patients with RA are lacking. Two preliminary studies of treatment with testosterone in the 1990s suggested positive results in male (n = 7) (96) and postmenopausal female (97) patients (n = 107). Meanwhile, two trials of dehydroepiandrosterone (DHEA) have been published. A small open-label trial of DHEA treatment in elderly RA patients (six post-menopausal female and five male) found no benefit on disease activity (98), while a randomized placebo-controlled trial of DHEA in pre-menopausal RA patients (n = 46) found improvements in quality of life but not disease activity scores (99).

Discussion

Despite extensive work in both basic laboratory and clinical studies, the exact impact of sex hormones on RA pathogenesis remains controversial. The epidemiological data described above is conflicting, with both high and low estrogen/progesterone states being found to be protective or risk factors, or to have no effect on development or progression of RA, in different studies. Namely, age of menarche, age of menopause, parity status, breastfeeding, use of the oral contraceptive, and HRT have all been found by different studies to have opposing effects on the risk of RA. Meta-analyses pertaining to parity and risk of RA found only a borderline, or zero, association (47, 56), as did that regarding the oral contraceptive (58). In contrast, the studies relating to breastfeeding have been more consistent, in that only one case-control study found an increased risk of RA with breastfeeding (49), while several others have found a protective effect, including four reporting a dose-response relationship (43, 48, 50, 51). These discrepancies are likely explained to an extent by heterogeneity in study design, variations in study populations and definitions of reproductive variables (e.g., early menarche), and inherent limitations in case-control studies such as recall bias and lack of consideration of confounding variables. On a biological level, many environmental and genetic factors may influence sex steroid signaling *via* their intracellular receptors, which may be another reason for the conflicting data from epidemiological studies.

Despite discrepancies in the published literature, several patterns do emerge. The most consistent findings are the increased risk of RA at early menopause and post-partum, and decreased disease activity in pregnancy. Therefore, declining estrogen and/or progesterone levels (in post-partum and menopause) are consistently linked to the onset of RA, while high levels of these hormones are protective during pregnancy (although many other factors may be relevant to reduced disease activity in pregnancy, as we and others have previously noted).

While there is an abundance of epidemiological data regarding reproductive factors and risk of RA, one possible avenue of research which, to our knowledge, has not been explored is the study of individuals with gender dysphoria

[except a single case report (100)]. For instance, it would be intriguing to study whether the risk of RA in transgender individuals is modified by gender affirming therapy, or whether disease activity of pre-existing RA is altered by such treatments. This topic is discussed in detail elsewhere in this chapter.

There is a wealth of basic scientific data demonstrating the immunomodulatory actions of oestrogens, progestogens and androgens. The therapeutic potential of these hormones for treating RA suggested by results from animal models has to date not translated into successful clinical trials. In humans it is likely that there is a much more complex interaction between sex hormones and a multitude of genetic and environmental risk factors (e.g., smoking, obesity, alcohol consumption) for RA. Progestogens and androgens both exhibit more broadly anti-inflammatory actions than those of oestrogens and it is interesting to note the recent finding that perimenopausal oral progestogen use reduced the risk of RA (55) and that combined HRT, but not estrogen alone, strongly reduced the risk of ACPA-positive disease (61). However, neither of these hormones have been evaluated in large well-designed trials of RA, perhaps due to concern regarding the potential side effects of systemic administration.

It is important to point that there are other sex-related factors which have been proposed as being of potential importance in RA pathogenesis that we have not considered in the present review, including microchimerism (101), sex chromosomes (102) and sex differences in gut microbiota (103). However, none of these other factors have to date shown such direct links with RA disease onset and/or progression.

In terms of future study, the rapidly expanding field of high throughput multiomics technologies (e.g., genomics, transcriptomics, proteomics and metabolomics), particularly at the single cell level, is starting to dissect the pathobiological basis of clinical heterogeneity in human disease, including in RA (104). With both epidemiological and *in-vitro* studies proving ultimately insufficient to unravel the relationship between sex hormones and RA pathogenesis, the application of these novel techniques are a tantalizing proposition for investigators in this area.

Conclusion

Sex hormones are immunomodulatory with pleiotropic effects on the immune system. There are conflicting reports regarding endogenous and exogenous sex hormones and RA pathogenesis, but declining estrogen levels in the menopause and post-partum are consistently associated with an increased risk and severity of RA. These findings, however, have not translated into improved therapies in RA, although progesterone and androgens warrant further evaluation as potential therapeutic agents in clinical trials.

Author contributions

CR and IG conceived of the article. CR performed the literature search, reviewed the source papers, and drafted the manuscript. IG independently performed the search, contributed to inclusion/exclusion of source literature, and edited the final manuscript. All authors contributed to the article and approved the submitted version.

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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

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Sex differences in comorbidities associated with Sjögren's disease

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Background: Little is known about the association of comorbidities with sex and age at diagnosis in Sjögren's disease. We tested the hypothesis that sex differences occur in comorbidities in patients with Sjögren's disease.

Methods: Patients with Sjögren's disease were identified from 11/1974 to 7/2018 in the Mayo Clinic electronic medical record and assessed for 22 comorbidities according to sex and age at diagnosis.

Results: Of the 13,849 patients identified with Sjögren's disease, 11,969 (86%) were women and 1,880 (14%) men, primarily white (88%) with a sex ratio of 6.4:1 women to men. The mean age at diagnosis was 57 years for women and 59.7 years for men, and 5.6% had a diagnosis of fibromyalgia at Sjögren's diagnosis. Men with Sjögren's disease were more likely than women to be a current or past smoker. The average time to diagnosis of comorbidities after diagnosis of Sjögren's disease was 2.6 years. The top comorbidities in patients with Sjögren's disease were fibromyalgia (25%), depression (21.2%) and pain (16.4%). Comorbidities that occurred more often in women were hypermobile syndromes (31:1), CREST (29:1), migraine (23:1), Ehlers-Danlos syndrome (EDS) (22:1), Raynaud's syndrome (15:1), SLE (13:1), systemic sclerosis (SSc) (13:1), and fibromyalgia (12:1). Women with Sjögren's disease were at increased risk of developing hypermobile syndromes (RR 7.27, CI 1.00–52.71, $p = 0.05$), EDS (RR 4.43, CI 1.08–18.14, $p = 0.039$), CREST (RR 4.24, CI 1.56–11.50, $p = 0.005$), migraine (RR 3.67, CI 2.39–5.62, $p < 0.001$), fibromyalgia (RR 2.26, CI 1.92–2.66,

$p < 0.001$), Raynaud's syndrome (RR 2.29, CI 1.77–2.96, $p < 0.001$), SLE (RR 2.13, CI 1.64–2.76, $p < 0.001$), and SSc (RR 2.05 CI 1.44–2.92; $p < 0.001$). In contrast, men with Sjögren's were at increased risk for developing myocardial infarction (RR 0.44, CI 0.35–0.55, $p < 0.001$), atherosclerosis/CAD (RR 0.44, CI 0.39–0.49, $p < 0.001$), cardiomyopathy (RR 0.63, CI 0.46–0.86, $p = 0.003$), stroke (RR 0.66 CI 0.51–0.85, $p = 0.001$), and congestive heart failure (RR 0.70, CI 0.57–0.85, $p < 0.001$).

Conclusions: The top comorbidities in Sjögren's disease were fibromyalgia, depression, and pain. Women with Sjögren's disease had a higher relative risk of developing fibromyalgia, depression, pain, migraine, hypermobile syndrome, EDS and other rheumatic autoimmune diseases. Men with Sjögren's disease had higher risk of developing cardiovascular diseases.

KEYWORDS

fibromyalgia, atherosclerosis, depression, pain, migraine, hypermobile syndrome, Ehlers-Danlos syndrome, smoking

Introduction

Sjögren's disease [often referred to as Sjögren's syndrome, which is a less accurate term (1)] is a chronic autoimmune disease with organ-specific and systemic features that has an estimated prevalence of 0.5–4.8%, affecting ∼1.5–4 million people in the US based on a total population of 300 million (2). A study using the Rochester Epidemiology Project estimated that the age- and sex-adjusted prevalence of Sjögren's disease was 10.3 per 10,000 in 2015, with a prevalence of 16.3 per 10,000 in women and 3.1 per 10,000 in men (3). The hallmark characteristic of Sjögren's disease is diminished secretory production from the primary exocrine glands, the lacrimal (involved in tear production) and/or the salivary glands. As a result, dry eye (keratoconjunctivitis sicca) and/or dry mouth (xerostomia) are among the most commonly reported symptoms. Aside from the exocrine targets, Sjögren's disease also affects the lungs, kidneys, thyroid, muscle, skin, peripheral and central nervous system (4, 5).

Previous studies of Sjögren's disease identified multiple comorbidities but did not analyze data according to sex. Several studies found that infections (particularly oral infections) and fibromyalgia/fatigue (15–30%) occur frequently in this population (6–8). In a study of 10,414 patients with Sjögren's disease the most common comorbidities were hypertension (38%), osteoarthritis (31%), rheumatoid arthritis (RA) (18%) and systemic lupus erythematosus (SLE) (15%) (9). Another study of 1,974 patients with primary Sjögren's disease were found to be at higher risk of developing hyperlipidaemia, cardiac arrhythmias, headaches, migraines and depression (10). Comorbidities in a subsequent study of 866 patients with primary Sjögren's disease included Raynaud's syndrome (14%), Hashimoto's thyroiditis (5%) and Graves' disease (3%) (11). Joint, muscle and widespread pain characteristic of fibromyalgia are also commonly observed in patients with Sjögren's disease (12). Additionally, dry eyes and dry mouth form part of the 'somatic symptoms' to be considered for a diagnosis of fibromyalgia according to the 2010 American College of Rheumatology diagnostic criteria (13). A meta-analysis of 14 studies found that patients with primary Sjögren's disease are at increased risk for cardiovascular morbidity, but did not analyze data according to sex (14). Thus, published studies have not determined whether sex differences exist in comorbidities or whether differences in age are present in comorbidities according to sex for patients with Sjögren's disease.

Sjögren's disease is known to occur more often in women, with some studies reporting a sex ratio as high as 16:1 women to men (15–18). More recently, the female to male ratio for Sjögren's disease has been reported to range from 6:1 in small US studies (19) to 14:1 in adults from large global studies (20, 21). Although Sjögren's disease can occur in women during child-bearing years, most cases are diagnosed soon after menopause around age 55–60 (22). Most studies examining sex differences in Sjögren's disease report differences in autoantibodies, other autoimmune diseases, fibromyalgia, lymphoma, and lung disease according to sex (15, 17, 18, 23) but have not examined whether sex and age differences occur in a large number of comorbidities. In this study we tested the hypothesis that sex differences occur in comorbidities associated with Sjögren's disease by examining 22 comorbidities (i.e., autoimmune diseases, cardiovascular diseases, chronic pain-related conditions) by sex and age at diagnosis in patients with primary and secondary Sjögren's disease from the Mayo Clinic medical record.

Methods

Ethics statement

Research carried out in this study was in compliance with the Helsinki Declaration. The study was approved by the Mayo Clinic Institutional Review Board and receipt of a waiver of the need to consent subjects was obtained.

Patients

Patients with Sjögren's disease were identified from the Mayo Clinic electronic medical record (EMR) using a Mayo Clinic artificial intelligence (AI) software program (i2b2) according to International Classification of Diseases (ICD)-9 (710.2) and/or ICD-10 (M35.00, M35.01, M35.02, M35.03, M35.04, M35.09) codes from 6 November 1974 to 12 July 2018. Records were filtered for birthdays after May 1, 2004 to ensure patients were ≥18 years of age. Systemic rheumatic autoimmune diseases that often co-occur with Sjögren's disease were included as comorbidities (i.e., systemic rheumatic disease such as SLE, RA, systemic sclerosis, inflammatory myopathies) so no formal distinction was made between primary and secondary Sjögren's disease. We examined comorbidities that were present at diagnosis and comorbidities that developed after diagnosis. Retrospective data were extracted from the EMR by the Mayo Clinic Statistics Department. Demographic data included age at diagnosis, race/ethnicity, sex, vitals and 22 comorbidities.

Statistical analysis

All statistical analyses were performed using R (version 4.0.3). Descriptive analysis was used to define the prevalence of comorbidities by sex among patients with Sjögren's disease. Fisher's exact test was performed to assess the association between sex and risk of comorbidities at diagnosis by sex (women vs. men) or age (<50 vs. ≥50) and shown as relative risk (RR) with 95% confidence intervals (CI). The risk of each type of comorbid condition after diagnosis of Sjögren's disease was estimated using the Kaplan-Meier method. The risk of each of these diagnoses between males and females was evaluated using Cox proportional hazards models. The hazard ratio (HR) and 95% CI for those estimates were provided for each condition. The purpose of the study was to examine comorbidities in relation to sex or age. Although a multivariate model could be applied to show whether an interaction exists between sex and age and comorbidities, we did not perform that analysis because we were most interested in understanding the individual relationships. A value of $p < 0.05$ was considered significant.

TABLE 1 Patient characteristics.

Demographics	*N*	%
All patients	13,849	
Women	11,969	86
Men	1,880	14
All patients with age at diagnosis	13,849	
<50 years of age at diagnosis	4,092	29
≥50 years of age at diagnosis	9,757	71
Women <50 years of age at diagnosis	3,641	30
Women ≥50 years of age at diagnosis	8,328	70
Men <50 years of age at diagnosis	451	23
Men ≥50 years of age at diagnosis	1,429	77
	Women ($n = 11,969$)	
White	10,541	88.1
African American	324	2.7
Asian	194	1.6
American Indian/alaskan native	69	0.6
Native hawaiian/pacific islander	14	0.1
Other/unknown	827	6.9
	Men ($n = 1,880$)	
White	1,661	88.3
African American	25	1.4
Asian	34	1.8
American Indian/alaskan native	8	0.4
Native hawaiian/pacific islander	0	0
Other/unknown	152	8.1
	Age (years)	***P*-value[a]**
Mean age at diagnosis	57	
Women		
Men	59.7	$p < 0.001$

[a]p-value result for Fisher's exact test.

Results

Patient characteristics

Patient demographics are shown in Table 1. From the 13,849 patients with Sjögren's disease identified in the Mayo Clinic EMR, 11,969 (86%) were women and 1,880 (14%) men. The sex ratio for Sjögren's disease in this study was 6.4:1 women to men. The majority of patients with Sjögren's disease were white (88% men and women), followed by African American (women 2.7%, men 1.3%) and Asian (women 1.6%, men 1.8%). The mean age at diagnosis of patients with Sjögren's disease was 57 years for women and 59.7 years for men ($p < 0.001$), indicating that diagnosis of Sjögren's disease occurred primarily in women post-menopause and after age 50 in men in this cohort. In contrast, only around 30% of women were diagnosed with Sjögren's disease prior to age 50, and only 23% of men prior

TABLE 2 More men than women with Sjögren's disease are smokers ($n = 581$).

Sex (n)	Status	n	%	Difference by sex	Relative risk (CI)	P-value[a]
Women ($n = 361$)	Smoker[b]	116	32%			
	Non-smoker	245	68%			
Men ($n = 220$)	Smoker	107	49%	17%	0.67 (0.54, 0.81)	0.0001
	Non-smoker	113	51%			

[a]Relative risk assessed using Fisher's exact test. [b]Current or past smoker.

to age 50 ($p < 0.0001$). Men with Sjögren's disease were also more likely than women to be a current or past smoker (Table 2).

Sex differences in autoantibodies and DHEA

From the 13,849 patients diagnosed based on ICD-9/10 codes with Sjögren's disease in our study, we found that 45.0% tested positive for antinuclear antibodies (ANA), 51.5% positive for Sjögren's syndrome (SS)-related antigen A/Ro (SSA), 37.9% positive for SS-related antigen B/La (SSB), 38% positive for rheumatoid factor (RF), and 15.8% had low dehydroepiandrosterone (DHEA) (Table 3). A large amount of data was missing so that the number of patients examined for autoantibodies (around 2000) and DHEA (around 200) levels were far fewer than the original number of patients (around 13,000), especially for males. More women with Sjögren's disease tested positive for autoantibodies against ANA ($p < 0.001$) and SSA ($p = 0.009$) than men (Table 3). Dehydroepiandrosterone (DHEA) was low more often in men ($p = 0.037$) (Table 3), although results may change with a larger number of patients for comparison. Low DHEA has been associated with worse Sjögren's disease (17). Other autoantibodies such as SSB and rheumatoid factor were detected at similar levels in men and women (Table 3).

Age differences in autoantibodies and DHEA

We found that patients over the age of 50 with Sjögren's disease tested positive more often for SSA ($p = 0.04$) and rheumatoid factor ($p < 0.001$) and had low levels of DHEA ($p < 0.001$) compared to younger patients (Table 4). Other autoantibodies such as ANA and SSB did not differ by age (Table 4).

TABLE 3 Autoantibodies and DHEA in women with Sjögren's disease compared to men.

Variable	Total ($n = 13,849$)	Women ($n = 11,969$)	Men ($n = 1,880$)	P value[a]
ANA[b]				**<0.001**
Missing (n)	7,970	6,976	994	
Neg	3,235(55.0%)	2,687 (53.8%)	548 (61.9%)	
Pos	2,644 (45.0%)	2,306 (46.2%)	338 (38.1%)	
SSA				**0.009**
Missing (n)	6,680	5,740	942	
Neg	3,475(48.5%)	2,982 (47.9%)	493 (52.4%)	
Pos	3,694 (51.5%)	3,247 (52.1%)	447 (47.5%)	
SSB				0.125
Missing (n)	7,647	6,598	1,049	
Neg	3,851(62.1%)	3,315 (61.7%)	536 (64.5%)	
Pos	2,351 (37.9%)	2,056 (38.3%)	295 (35.5%)	
DHEA				**0.037**
Missing (n)	13,653	11,782	1,871	
High	165 (84.2%)	160 (85.6%)	5 (55.6%)	
Low to normal	31 (15.8%)	27 (14.4%)	4 (44.4%)	
RF				0.267
Missing (n)	8,043	7,013	1,030	
Neg	3,601 (62.0%)	3,059 (61.7%)	542 (63.8%)	
Pos	2,205 (38.0%)	1,897 (38.3%)	308 (36.2%)	

[a]Fisher's Exact Test for Count Data (Bold: significant p-value). [b]ANA, antinuclear antibodies; DHEA, dehydroepiandrosterone; IgA, immunoglobulin A; M, men; Neg, negative; Pos, positive; RF, rheumatoid factor; SS, Sjögren's syndrome/disease; SSA/Ro, SS-related antigen A; SSB/La, SS-related antigen B.

Sex and age differences in autoantibodies and DHEA

The age differences found for the entire cohort were driven by women with Sjögren's disease who were more often positive for SSA ($p = 0.055$) and rheumatoid factor ($p < 0.001$) and had lower levels of DHEA ($p < 0.001$) after age 50 (Table 5), while there were no differences in the percentage of patients testing positive for autoantibodies or low DHEA in men by age (Table 6), but the number of male patients with DHEA values is too low to make conclusions.

Likelihood of having comorbidities at Sjögren's disease diagnosis by sex and age

When investigating the likelihood that a patient had a comorbidity at their diagnosis with Sjögren's disease, we found that women were more likely to have fibromyalgia at Sjögren's disease diagnosis ($p < 0.001$) (Table 7; Supplementary Table 1).

TABLE 4 Autoantibodies and DHEA in Sjögren's disease patients by age.

Variable	Total ($n = 13,849$)	<50 yrs ($n = 4,092$)	≥50 yrs ($n = 9,757$)	*P*-value[a]
ANA[b]				0.309
Missing (*n*)	7,970	2,257	5,713	
Neg	3,235 (55.0%)	991 (54.0%)	2,244 (55.5%)	
Pos	2,644 (45.0%)	844 (46.0%)	1,800 (44.5%)	
SSA				**0.045**
Missing (*n*)	6,680	1,693	4,987	
Neg	3,475(48.5%)	1,203 (50.2%)	2,272 (47.6%)	
Pos	3,694 (51.5%)	1,196 (49.8%)	2,498 (52.4%)	
SSB				0.845
Missing (*n*)	7,647	2,066	5,581	
Neg	3,851 (62.1%)	1,254(61.9%)	2,597 (62.2%)	
Pos	2,351 (37.9%)	772 (38.1%)	1,579 (37.8%)	
DHEA				**<0.001**
Missing (*n*)	13,653	3,983	9,670	
High	165 (84.2%)	103 (94.5%)	62 (71.3%)	
Low to normal	31 (15.8%)	6 (5.5%)	25 (28.7%)	
RF				**<0.001**
Missing (*n*)	8,043	2,286	5,757	
Neg	3,601 (62.0%)	1,186 (65.7%)	2,415 (60.4%)	
Pos	2,205 (38.0%)	620 (34.3%)	1,585 (39.6%)	

[a]Fisher's Exact Test for Count Data (Bold: significant *p*-value). [b]ANA, antinuclear antibodies; DHEA, dehydroepiandrosterone; IgA, immunoglobulin A; Neg, negative; Pos, positive; RF, rheumatoid factor; SS, Sjögren's syndrome/disease; SSA/Ro, SS-related antigen A; SSB/La, SS-related antigen B.

TABLE 5 Autoantibodies and DHEA in female Sjögren's disease patients by age.

Variable	W[a] <50 ($n = 3,641$)	W ≥50 yrs ($n = 8,328$)	Total ($n = 11,969$)	*P*-value[b]
ANA				0.627
Missing (*n*)	2,028	4,948	6,976	
Neg	860 (53.3%)	1,827 (54.1%)	2,687 (53.8%)	
Pos	753 (46.7%)	1,553 (45.9%)	2,306 (46.2%)	
SSA				**0.055**
Missing (*n*)	1,486	4,254	5,740	
Neg	1,068 (49.6%)	1,914 (47.0%)	2,982 (47.9%)	
Pos	1,087 (50.4%)	2,160(53.0%)	3,247 (52.1%)	
SSB				0.722
Missing (*n*)	1,833	4,765	6,598	
Neg	1,122 (62.1%)	2,193 (61.5%)	3,315 (61.7%)	
Pos	686 (37.9%)	1,370 (38.5%)	2,056 (38.3%)	
DHEA				**<0.001**
Missing (*n*)	3,535	8,247	11,782.0	
High	101 (95.3%)	59 (72.8%)	160 (85.6%)	
Low to normal	5 (4.7%)	22 (27.2%)	27 (14.4%)	
RF				**<0.001**
Missing (*n*)	2,033	4,980	7,013	
Neg	1,062 (66.0%)	1,997 (59.6%)	3,059 (61.7%)	
Pos	546 (34.0%)	1,351 (40.4%)	1,897 (38.3%)	

[a]ANA, antinuclear antibodies; DHEA, dehydroepiandrosterone; IgA, immunoglobulin A; Neg, negative; Pos, positive; RF, rheumatoid factor; SS, Sjögren's syndrome/disease; SSA/Ro, SS-related antigen A; SSB/La, SS-related antigen B; W, women. [b]Fisher's Exact Test for Count Data (Bold: significant *p*-value).

The records had a lot of missing information related to comorbidities at date of diagnosis, but fibromyalgia was found to occur in 5.7% of patients diagnosed with Sjögren's disease at the time of their diagnosis, occurring more often in women with a sex ratio of 7:1 women to men (Table 7; Supplementary Table 1). When looking at the likelihood of having a comorbidity by age (using age 50 as a cut off) we found that patients in this study that were diagnosed over 50 years of age were more likely to have fibromyalgia ($p = 0.001$), pain ($p = 0.003$) and Ehlers-Danlos syndrome (EDS) ($p = 0.007$) (Table 8).

Average time between Sjögren's disease and comorbidity diagnosis

The mean time in years between diagnosis of Sjögren's disease and a diagnosis of 1 of 22 comorbidities including rheumatic autoimmune diseases (rheumatoid arthritis, lupus), cardiovascular diseases (myocardial infarct, congestive heart failure) or pain conditions (fibromyalgia, migraine) in women or men are found in Supplementary Table 2. The overall average time (mean) to diagnosis of a comorbidity after diagnosis of Sjögren's disease was around 2.6 years for men and women together or 2.7 years for women only, and around 2.3 years for men only.

Comorbidities according to sex

The top comorbidities and sex ratios (women: men) in all patients with Sjögren's disease are listed in Table 9 and include fibromyalgia (24.9%, 12:1), depression (21.2%, 8:1), pain (16.4%, 8:1), atherosclerosis/ coronary artery disease (CAD) (14.5%, 3:1), rheumatoid arthritis (RA) (13.2%, 9:1), Raynaud's syndrome (10%, 15:1), and SLE (8.7%, 13:1). The comorbidities with the highest sex ratio that occurred more often in women in this study were hypermobile syndromes (31:1), CREST (29:1), migraine (23:1), EDS (22:1), Raynaud's syndrome (15:1), SLE (13:1), systemic sclerosis (SSc) (13:1), and fibromyalgia (12:1) (Table 9). All 22 of the comorbidities found in patients with Sjögren's disease in this study occurred more frequently in women than men, except for diseases that typically occur more often in men like lymphoma, myocardial

TABLE 6 Autoantibodies in male Sjögren's disease patients by age.

Variable	M[a] <50 ($n = 451$)	M ≥50 yrs ($n = 1,429$)	Total ($n = 1,880$)	*P*-value[b]
ANA				0.340
Missing (*n*)	229	765	994	
Neg	131 (59.0%)	417 (62.8%)	548 (61.9%)	
Pos	91 (41.0%)	247 (37.2%)	338 (38.1%)	
SSA				0.298
Missing (*n*)	207	733	940	
Neg	135 (55.3%)	358 (51.4%)	493 (52.4%)	
Pos	109 (44.7%)	338 (48.6%)	447 (47.6%)	
SSB				0.188
Missing (*n*)	233	816	1,049	
Neg	132 (60.6%)	404 (65.9%)	536 (64.5%)	
Pos	86 (39.4%)	209 (34.1%)	295 (35.5%)	
DHEA				1.000
Missing (*n*)	448	1,423	1,871	
High	2 (66.7%)	3 (50.0%)	5 (55.6%)	
Low to normal	1 (33.3%)	3 (50.0%)	4 (44.4%)	
RF				0.736
Missing (*n*)	253	777	1,030	
Neg	124 (62.6%)	418 (64.1%)	542 (63.8%)	
Pos	74(37.4%)	234 (35.9%)	308 (36.2%)	

[a]ANA, antinuclear antibodies; DHEA, dehydroepiandrosterone; IgA, immunoglobulin A; M, men; Neg, negative; Pos, positive; RF, rheumatoid factor; SS, Sjögren's syndrome/disease; SSA/Ro, SS-related antigen A; SSB/La, SS-related antigen B. [b]Fisher's Exact Test for Count Data.

TABLE 7 Likelihood of having a comorbidity by sex at Sjögren's disease diagnosis ($n = 13,849$).

Comorbidity	*n*	Diagnosis (%)	Sex ratio (W:M)[a]	*P*-value[b]
Fibromyalgia	W 682, M 95	5.7%	7:1	**<0.001**
Depression	W 454, M 52	3.8%	9:1	0.442
Pain	W 373, M 51	3.1%	7:1	0.736
Migraine	W 46, M 4	0.4%	12:1	0.149
Raynaud's syndrome	W 164, M 14	1.4%	12:1	0.409
Systemic sclerosis	W 45, M 2	0.4%	23:1	0.764
CREST	W 4, M 0	0.03%		1
Stroke	W 79, M 17	0.7%	5:1	0.885
PAH	W 118, M 20	1.0%	6:1	0.317
PH	W 105, M 20	0.9%	5:1	0.146
EDS	W 4, M 0	0.03%		1
Hypermobile	W 9, M 1	0.1%	9:1	0.29
RA	W 151, M 18	1.3%	8:1	0.788
SLE	W 144, M 13	1.2%	11:1	0.505
Polymyositis	W 19, M 0	0.2%		0.126
Dermatomyositis	W 11, M 2	0.1%	6:1	0.611
Myocarditis	W 8, M 3	0.1%	3:1	0.317
Lymphoma	W 48, M 14	0.4%	3:1	0.184
Atherosclerosis	W 235, M 81	2.0%	3:1	0.832
Myocardial infarction	W 51, M 23	0.4%	2:1	0.391
CMP	W 25, M 8	0.2%	3:1	0.645
CHF	W 100, M 29	0.8%	3:1	0.331

[a]CAD, coronary artery disease; CHF, congestive heart failure; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; EDS, Ehlers-Danlos syndrome; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; W, women. [b]Fisher's Exact Test for Count Data (Bold: significant *p*-value).

infarction/CAD, congestive heart failure, cardiomyopathy and myocarditis (Table 10) (17, 24).

Previous studies reported that women with Sjögren's disease are at an increased risk of developing RA and SLE (9). In this study we found that women with Sjögren's disease were at greater risk of developing the rheumatic autoimmune diseases/syndromes SSc (HR 2.05, CI 1.44–2.92, $p < 0.001$), CREST (HR 4.24, CI 1.56–11.50, $p = 0.005$), Raynaud's syndrome (HR 2.29, CI 1.77–2.96, $p < 0.001$), SLE (HR 2.13, CI 1.64–2.76, $p < 0.001$), and RA (HR 1.31, CI 1.11–1.55, $p = 0.001$) than men (Table 11; Figure 1A). In contrast, men with Sjögren's disease were more likely to develop cardiovascular diseases like myocardial infarction (HR 0.44, CI 0.35–0.55, $p < 0.001$), atherosclerosis/CAD (HR 0.44, CI 0.39–0.49, $p < 0.001$), cardiomyopathy (HR 0.63, CI 0.46–0.86, $p = 0.003$), congestive heart failure (HR 0.63, CI 0.46–0.86, $p = 0.003$), and stroke (HR 0.63, CI 0.46–0.86, $p = 0.003$) (Table 11; Figure 1B). Several studies found that fibromyalgia is a leading comorbidity in Sjögren's disease (6, 7), which was confirmed in this study. Here we show that women with Sjögren's disease were at an increased risk of developing fibromyalgia (HR 2.26, CI 1.92–2.66, $p < 0.001$) (Table 11; Figure 1C). Additionally, we found that women with Sjögren's disease were more likely to have hypermobile syndromes (HR 7.27, CI 1.00–52.71, $p = 0.05$), EDS (HR 4.43, CI 1.08–18.14, $p = 0.039$), migraine (HR 3.67, CI 2.39–5.62, $p < 0.001$), pain (HR 1.20, CI 1.04–1.40, $p = 0.014$), and depression (HR 1.20, CI 1.05–1.36, $p < 0.007$) than men (Table 11; Figure 1C).

Risk of developing comorbidities according to sex and age

When we examined the risk of developing comorbidities by age regardless of sex, we found that 18/22 comorbidities demonstrated age differences in the risk of developing a comorbidity with the exceptions being polymyositis, dermatomyositis, myocarditis and cardiomyopathy (Table 12). Of those comorbidities with significant differences, we found a greater risk to develop the rheumatic autoimmune diseases

TABLE 8 Likelihood of having comorbidity by age at Sjögren's disease diagnosis (n =13,849).

Comorbidity	<50 years (n = 4,092)	≥50 years (n = 9,757)	Total (n = 13,849)	P-value[a]
Fibromyalgia				**0.001**
Missing (n)	2,880	7,511	10,391	
No Dx[b]	977 (80.6%)	1,704 (75.9%)	2,681 (77.5%)	
Dx	235 (19.4%)	542 (24.1%)	777 (22.5%)	
Depression				0.710
Missing (n)	3,196	7,713	10,909	
No Dx	738 (82.4%)	1,696 (83.0%)	2,434 (82.8%)	
Dx	158 (17.6%)	348 (17.0%)	506 (17.2%)	
Pain				**0.003**
Missing (n)	3,319	8,255	11,574	
No Dx	655 (84.7%)	1,196 (79.6%)	1,851 (81.4%)	
-Dx	118 (15.3%)	306 (20.4%)	424 (18.6%)	
Migraine				1.000
Missing (n)	3,788	9,441	13,229	
No Dx	279 (91.8%)	291 (92.1%)	570 (91.9%)	
Dx	25 (8.2%)	25 (7.9%)	50 (8.1%)	
Raynaud's				1.000
Missing (n)	3,620	8,846	12,466	
No Dx	411 (87.1%)	794 (87.2%)	1,205 (87.1%)	
Dx	61 (12.9%)	117 (12.8%)	178 (12.9%)	
SSc[b]				0.856
Missing (n)	3,961	9,305	13,266	
No Dx	120 (91.6%)	416 (92.0%)	536 (91.9%)	
Dx	11 (8.4%)	36 (8.0%)	47 (8.1%)	
CREST				1.000
Missing (n)	4,078	9,650	13,728	
No Dx	14 (100.0%)	103 (96.3%)	117 (96.7%)	
Dx	0 (0.0%)	4 (3.7%)	4 (3.3%)	
Stroke				1.000
Missing (n)	4,025	9,305	13,330	
No Dx	55 (82.1%)	368 (81.4%)	423 (81.5%)	
Dx	12 (17.9%)	84 (18.6%)	96 (18.5%)	
PAH				0.684
Missing (n)	3,969	8,959	12,928	
No Dx	103 (83.7%)	680 (85.2%)	783 (85.0%)	
Dx	20 (16.3%)	118 (14.8%)	138 (15.0%)	
PH				1.000
Missing (n)	3,974	8,954	12,928	
No Dx	102 (86.4%)	694 (86.4%)	796 (86.4%)	
Dx	16 (13.6%)	109 (13.6%)	125 (13.6%)	
EDS				**0.007**
Missing (n)	4,045	9,736	13,781	
No Dx	47 (100.0%)	17(81.0%)	64 (94.1%)	
Dx	0 (0.0%)	4 (19.0%)	4 (5.9%)	

(Continued)

TABLE 8 Continued

Comorbidity	<50 years ($n = 4,092$)	≥50 years ($n = 9,757$)	Total ($n = 13,849$)	P-value[a]
Hypermobile				0.712
Missing (*n*)	4,048	9,737	13,785	
No Dx	38 (86.4%)	16 (80.0%)	54 (84.4%)	
Dx	6 (13.6%)	4 (20.0%)	10 (15.6%)	
RA				0.103
Missing (*n*)	3,674	8,347	12,021	
No Dx	388 (92.8%)	1,271 (90.1%)	1,659 (90.8%)	
Dx	30 (7.2%)	139 (9.9%)	169 (9.2%)	
SLE				0.162
Missing (*n*)	3,614	9,033	12,647	
No Dx	424 (88.7%)	621 (85.8%)	1,045 (86.9%)	
Dx	54 (11.3%)	103 (14.2%)	157 (13.1%)	
Polymyositis				0.585
Missing (*n*)	4,055	9,668	13,723	
No Dx	33 (89.2%)	74 (83.1%)	107 (84.9%)	
Dx	4 (10.8%)	15 (16.9%)	19 (15.1%)	
Dermatomyositis				0.488
Missing (*n*)	4,074	9,707	13,781	
No Dx	16 (88.9%)	39 (78.0%)	55 (80.9%)	
Dx	2 (11.1%)	11 (22.0%)	13 (19.1%)	
Myocarditis				1.000
Missing (*n*)	4,082	9,736	13,818	
No Dx	7 (70.0%)	13 (61.9%)	20 (64.5%)	
Dx	3 (30.0%)	8 (38.1%)	11 (35.5%)	
Lymphoma				0.486
Missing (*n*)	4,021	9,474	13,495	
No Dx	61 (85.9%)	231 (81.6%)	292 (82.5%)	
Dx	10 (14.1%)	52 (18.4%)	62 (17.5%)	
Atherosclerosis				0.548
Missing (*n*)	3,952	7,887	11,839	
No Dx	121 (86.4%)	1,573 (84.1%)	1,694 (84.3%)	
Dx	19 (13.6%)	297 (15.9%)	316 (15.7%)	
Myocardial infarction				1.000
Missing (*n*)	4,046	9,317	13,363	
No Dx	39 (84.8%)	373 (84.8%)	412 (84.8%)	
Dx	7 (15.2%)	67 (15.2%)	74 (15.2%)	
Cardiomyopathy				0.378
Missing (*n*)	4,026	9,533	13,559	
No Dx	61 (92.4%)	196 (87.5%)	257 (88.6%)	
Dx	5 (7.6%)	28 (12.5%)	33 (11.4%)	
CHF				0.747
Missing (*n*)	4,010	8,988	12,998	
No Dx	71 (86.6%)	651 (84.7%)	722 (84.8%)	
Dx	11 (13.4%)	118 (15.3%)	129 (15.2%)	

[a]Fisher's Exact Test for Count Data (Bold: significant p-value). [b]CAD, coronary artery disease; CHF, congestive heart failure; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; Dx, diagnosis; EDS, Ehlers-Danlos syndrome; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

TABLE 9 Percentage and sex ratio of 22 comorbidities in women and men with Sjögren's disease ($n = 13,849$).

Comorbidity	*n*	%	Sex ratio (W:M)[a]
Fibromyalgia	W 3,190, M 268	24.9	12:1
Depression	W 2,605, M 335	21.2	8:1
Pain	W 2,013, M 262	16.4	8:1
Atherosclerosis/CAD	W 1,505, M 505	14.5	3:1
Rheumatoid arthritis	W 1,645, M 183	13.2	9:1
Raynaud's syndrome	W 1,295, M 88	10.0	15:1
SLE	W 1,117, M 85	8.7	13:1
PH	W 807, M 114	6.7	7:1
PAH	W 812, M 109	6.7	7:1
CHF	W 689, M 162	6.2	4:1
Migraine	W 595, M 26	4.5	23:1
SSc	W 542, M 41	4.2	13:1
Stroke	W 423, M 96	3.8	4:1
Myocardial infarction	W 357, M 129	3.5	3:1
Lymphoma	W 296, M 58	2.6	5:1
CMP	W 231, M 59	2.1	4:1
Polymyositis	W 112, M 14	0.9	8:1
CREST	W 117, M 4	0.9	29:1
EDS	W 65, M 3	0.5	22:1
Dermatomyositis	W 61, M 7	0.5	9:1
Hypermobile syndrome	W 62, M 2	0.5	31:1
Myocarditis	W 26, M 5	0.2	5:1

[a]CAD, coronary artery disease; CHF, congestive heart failure; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; Dx, diagnosis; EDS, Ehlers-Danlos syndrome; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; W, women.

SSc ($p = 0.002$), CREST ($p < 0.001$) and rheumatoid arthritis ($p < 0.001$) in Sjögren's disease patients ≥50 years of age (Table 12). Also, an increased risk for many cardiovascular diseases were present after age 50 including stroke ($p < 0.001$), PAH ($p < 0.001$), PH ($p < 0.001$), atherosclerosis/ CAD ($p < 0.001$), myocardial infarct ($p < 0.001$), and congestive heart failure ($p < 0.001$) as well as lymphoma ($p = 0.019$) (Table 12). When analyzed alone, women were at increased risk of developing all of these conditions after age 50 (Tables 12, 13). In contrast, men with Sjögren's disease were not at increased risk of developing SSc, CREST or rheumatoid arthritis after age 50 (Table 14). However, they were at increased risk of developing the same cardiovascular conditions as women (Tables 12–14).

Discussion

A female to male bias has been reported for the rheumatic autoimmune disease's dermatomyositis (2:1), rheumatoid arthritis (3:1), SLE (7:1), SSc (12:1) and Sjögren's disease (6:1–14:1) (16, 17, 19–21). In this retrospective study of 13,849 patients with primary and/or secondary Sjögren's disease we found a sex ratio of 6.4:1 women to men which is somewhat lower than estimates from other large studies, although findings vary. Cardiovascular diseases also display strong sex differences with most heart diseases occurring more often in men like myocardial infarction, atherosclerosis/CAD, myocarditis, cardiomyopathy/dilated cardiomyopathy, and pulmonary hypertension while others occur more often in women, particularly after menopause, like hypertension, PAH, and stroke (24–28). In this study we found that men with Sjögren's disease had a greater risk of developing cardiovascular diseases than women (sex ratio, Table 1; Figure 1), while women had a greater risk of developing another rheumatic autoimmune disease and/or a chronic pain condition like EDS, migraine, hypermobile syndrome or fibromyalgia (Figure 1). At diagnosis, women were more likely to have fibromyalgia, pain and EDS (Table 8). EDS/hypermobile syndromes did not occur with high frequency in the record; however, it is a relatively common condition that occurs within the same demographic population of around 90% white women with a high percentage overlap with fibromyalgia (29), and historically has been under-diagnosed. Future research is needed to determine whether hypermobile EDS is an important comorbidity in Sjögren's disease.

Sjögren's disease is a chronic inflammatory condition where T and B cells directed against self-antigens from the exocrine glands lead to autoantibody and immune complex formation, tissue damage and inflammation (17). We found in this study that more women were positive for ANA and SSA/Ro autoantibodies compared to men (Table 3). We and others have hypothesized that increased inflammation, autoantibodies and immune complex deposition in women with autoimmune diseases increases the risk of developing other rheumatic autoimmune and cardiovascular diseases, especially after menopause (age 50) [reviewed in (17)] (17, 30, 31). Patients with Sjögren's disease have also been reported to have more hypertension and type II diabetes which increase the risk for cardiovascular disease (32), but to smoke less because of symptoms of dry mouth (30). Multiple studies have demonstrated an inverse correlation of smoking and Sjögren's disease or for smoking and focal lymphocytic sialadenitis (33–40), but differences between sex have not been reported. In this study a higher percentage of men with Sjögren's disease were smokers than women (49% men vs. 32% women), although the number of patients with data on smoking is relatively low (Table 2). Men are at an increased risk of developing cardiovascular disease compared to women (24–28) and the increased inflammation associated with Sjögren's disease would likely further promote the pathogenesis of cardiovascular disease in men.

TABLE 10 Comorbidities in women and men with Sjögren's disease by sex ($n = 13,849$).

Comorbidity	Women ($n = 11,969$)	Men ($n = 1,880$)	Total ($n = 13,849$)	P-value[a]
Fibromyalgia				**<0.001**
No Dx[b]	8,779 (73.3%)	1,612 (85.7%)	10,391 (75.0%)	
Dx	3,190 (26.7%)	268 (14.3%)	3,458 (25.0%)	
Depression				**<0.001**
No Dx	9,364 (78.2%)	1,545 (82.2%)	10,909 (78.8%)	
Dx	2,605 (21.8%)	335 (17.8%)	2,940 (21.2%)	
Pain				**0.002**
No Dx	9,956 (83.2%)	1,618 (86.1%)	11,574 (83.6%)	
Dx	2,013 (16.8%)	262 (13.9%)	2,275 (16.4%)	
Migraine				**<0.001**
No Dx	11,374 (95.0%)	1,854 (98.6%)	13,228 (95.5%)	
Dx	595 (5.0%)	26 (1.4%)	621 (4.5%)	
Raynaud's syndrome				**<0.001**
No Dx	10,674 (89.2%)	1,792 (95.3%)	12,466 (90.0%)	
Dx	1,295 (10.8%)	88 (4.7%)	1,383 (10.0%)	
SSc				**<0.001**
No Dx	11,427 (95.5%)	1,839 (97.8%)	13,266 (95.8%)	
Dx	542 (4.5%)	41 (2.2%)	583 (4.2%)	
CREST				**<0.001**
No Dx	11,852 (99.0%)	1,876 (99.8%)	13,728 (99.1%)	
Dx	117 (1.0%)	4 (0.2%)	121 (0.9%)	
Stroke				**0.001**
No Dx	11,546 (96.5%)	1,784 (94.9%)	13,330 (96.3%)	
Dx	423 (3.5%)	96 (5.1%)	519 (3.7%)	
PAH				0.123
No Dx	11,157 (93.2%)	1,771 (94.2%)	12,928 (93.3%)	
Dx	812 (6.8%)	109 (5.8%)	921 (6.7%)	
PH				0.296
No Dx	11,162 (93.3%)	1,766 (93.9%)	12,928 (93.3%)	
Dx	807 (6.7%)	114 (6.1%)	921 (6.7%)	
EDS				**0.021**
No Dx	11,904 (99.5%)	1,877 (99.8%)	13,781 (99.5%)	
Dx	65 (0.5%)	3 (0.2%)	68 (0.5%)	
Hypermobile syndrome				**0.010**
No Dx	11,907 (99.5%)	1,878 (99.9%)	13,785 (99.5%)	
Dx	62 (0.5%)	2 (0.1%)	64 (0.5%)	
Rheumatoid arthritis				**<0.001**
No Dx	10,324 (86.3%)	1,697 (90.3%)	12,021 (86.8%)	
Dx	1,645 (13.7%)	183 (9.7%)	1,828 (13.2%)	
SLE				**<0.001**
No Dx	10,852 (90.7%)	1,795 (95.5%)	12,647 (91.3%)	
Dx	1,117 (9.3%)	85 (4.5%)	1,202 (8.7%)	
Polymyositis				0.513
No Dx	11,857 (99.1%)	1,866 (99.3%)	13,723 (99.1%)	
Dx	112 (0.9%)	14 (0.7%)	126 (0.9%)	

(Continued)

TABLE 10 Continued

Comorbidity	Women ($n = 11,969$)	Men ($n = 1,880$)	Total ($n = 13,849$)	P-value[a]
Dermatomyositis				0.593
No Dx	11,908 (99.5%)	1,873 (99.6%)	13,781 (99.5%)	
Dx	61 (0.5%)	7 (0.4%)	68 (0.5%)	
Myocarditis				0.603
No Dx	11,943 (99.8%)	1,875 (99.7%)	13,818 (99.8%)	
Dx	26 (0.2%)	5 (0.3%)	31 (0.2%)	
Lymphoma				0.116
No Dx	11,673 (97.5%)	1,822 (96.9%)	13,495 (97.4%)	
Dx	296 (2.5%)	58 (3.1%)	354 (2.6%)	
Atherosclerosis/CAD				**<0.001**
No Dx	10,464 (87.4%)	1,375 (73.1%)	11,839 (85.5%)	
Dx	1,505 (12.6%)	505 (26.9%)	2,010 (14.5%)	
Myocardial infarction				**<0.001**
No Dx	11,612 (97.0%)	1,751 (93.1%)	13,363 (96.5%)	
Dx	357 (3.0%)	129 (6.9%)	486 (3.5%)	
CMP				**0.001**
No Dx	11,738 (98.1%)	1,821 (96.9%)	13,559 (97.9%)	
Dx	231 (1.9%)	59 (3.1%)	290 (2.1%)	
CHF				**<0.001**
No Dx	11,280 (94.2%)	1,718 (91.4%)	12,998 (93.9%)	
Dx	689 (5.8%)	162 (8.6%)	851 (6.1%)	

[a]*P*-values result from Fisher's test for categorical data (Bold: significant *p*-value). [b]CAD, coronary artery disease; CHF, congestive heart failure; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; EDS, Ehlers-Danlos syndrome; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; W, women.

Apart from the exocrine glands, patients with Sjögren's disease have extraglandular manifestations that affect their joints, lungs, kidneys, small vessels, as well as central and peripheral nervous system (4). Severe and chronic fatigue and pain are frequently reported by patients with Sjögren's disease and are associated with sleep disturbance and mood disorders (12, 41). Not only do these symptoms of Sjögren's disease overlap with fibromyalgia, which has been reported to occur in around 15–30% of patients with Sjögren's disease (6, 42), they also overlap with hypermobile EDS and hypermobile syndrome/hypermobile syndrome disorder (43–46). In this study we found that fibromyalgia occurred in 25% of patients with Sjögren's disease and was more frequent in women than men with Sjögren's disease (12:1), similar to other reports. Similarly, we found that more women with Sjögren's disease experienced depression (8:1), pain (8:1), migraine (23:1), EDS (22:1), and hypermobile syndrome (31:1) than men with Sjögren's disease. All of these conditions are known to occur more often in women than men (47–50). Our findings confirm known sex differences and provide an assessment of their frequency in a large cohort of patients with Sjögren's disease.

Our results show that women with Sjögren's disease are at a higher risk than men of having other rheumatic autoimmune diseases, depression, pain, migraine, fibromyalgia, EDS and hypermobile syndrome. However, we recognize that our study has certain limitations. A major limitation of the study is that as a retrospective study with a large number of patients we were not able to confirm whether the diagnosis of patients with Sjögren's disease was performed by a rheumatologist with expertise in this area. Likewise, we were not able to verify cases for such a large number of patients or distinguish primary from secondary Sjögren's disease. However, less research exists on secondary Sjögren's disease and so this data adds to that knowledge. Additionally, identifying patients using ICD-9/10 codes may over-represent the number of patients diagnosed with Sjögren's disease because the codes may be used to identify patients for work up but may not lead to a diagnosis. If patients that are included in the study do not have Sjögren's disease, this could affect the data leading to inaccurate conclusions. Even though this study included a large cohort of Sjögren's disease patients (13,849), analysis of 22 comorbidities by sex and age left small numbers of men for some comparisons and small numbers of patients for some comorbidities. If a higher number of patients were examined for those cases, the results may change. However, this study is the first to our knowledge to study comorbidities in Sjögren's disease by sex and age at diagnosis.

TABLE 11 Relative risk of developing a comorbidity in women vs. men with Sjögren's disease ($n = 13,849$).

Comorbidity	Women	Men	HR (CI)[a]	*P*-value[b]
Fibromyalgia	2,140	156	**2.26 (1.92–2.66)**	<0.001
Depression	1,935	254	**1.20 (1.05–1.36)**	0.007
Pain	1,503	196	**1.20 (1.04–1.40)**	0.014
Migraine	513	22	**3.67 (2.39–5.62)**	<0.001
Raynaud's syndrome	891	62	**2.29 (1.77–2.96)**	<0.001
SSc	431	33	**2.05 (1.44–2.92)**	<0.001
CREST	110	4	**4.24 (1.56–11.50)**	0.005
Stroke	325	75	0.66 (0.51–0.85)	0.001
PAH	646	80	1.26 (1.00–1.59)	0.053
PH	648	83	1.21 (0.97–1.53)	0.096
EDS	56	2	**4.43 (1.08–18.14)**	0.039
Hypermobile syndrome	46	1	**7.27 (1.00–52.71)**	0.05
Rheumatoid arthritis	1,274	152	**1.31 (1.11–1.55)**	0.001
SLE	821	61	**2.13 (1.64–2.76)**	<0.001
Polymyositis	85	12	1.10 (0.60–2.01)	0.76
Dermatomyositis diagnosis	45	5	1.40 (0.56–3.53)	0.48
Myocarditis diagnosis	18	2	1.41 (0.33–6.08)	0.64
Lymphoma	233	43	0.83 (0.60–1.15)	0.27
Atherosclerosis/CAD	1,198	400	0.44 (0.39–0.49)	<0.001
Myocardial infarction	284	98	0.44 (0.35–0.55)	<0.001
CMP	201	49	0.63 (0.46–0.86)	0.004
CHF	557	122	0.70 (0.57–0.85)	<0.001

[a]CAD, coronary artery disease; CHF, congestive heart failure; CI, confidence intervals; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; EDS, Ehlers-Danlos syndrome; HR, hazard ratio; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; SLE, systemic lupus erythematosus; SSc, systemic sclerosis. [b]Relative risk assessed using Cox Model (Bold- increased risk in women).

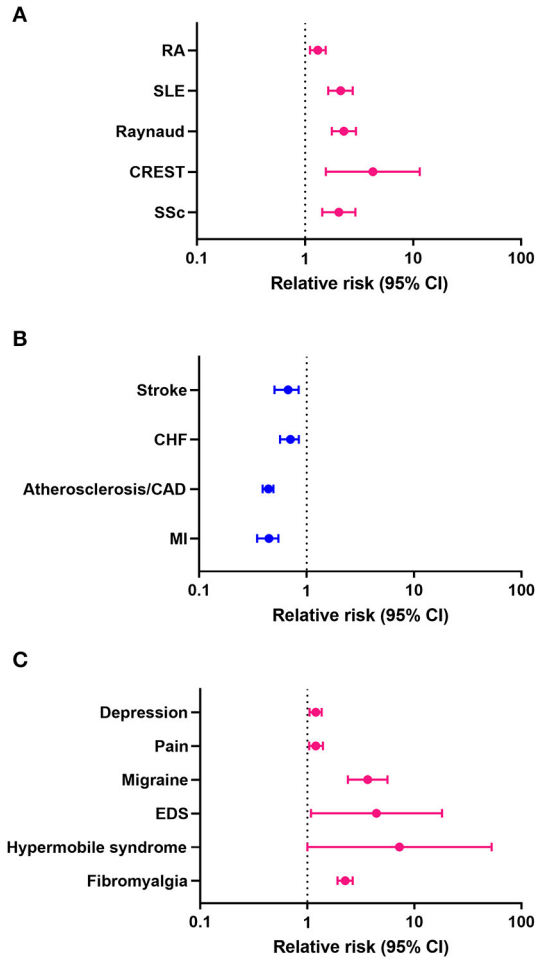

FIGURE 1
Relative risk (hazard ratio) of comorbidities in patients with Sjögren's disease ($n = 13,849$). (**A**) Rheumatic autoimmune diseases/syndromes, (**B**) cardiovascular diseases, (**C**) pain-related conditions; pink indicates increased risk in women, blue increased risk in men.

Additionally, the lower risk in men with Sjögren's disease for comorbidities may be related to the far fewer number of men in the study. However, this study is the largest to date to our knowledge examining the largest number of comorbidities and with the greatest number of men available for analysis. The study may reflect trends observed in the general population and sex differences that exist in comorbidities in the general population may influence results, such as increased cardiovascular disease in men. Future studies should examine the EMR for a similar time-period to determine whether key comorbidities such as fibromyalgia and cardiovascular disease have the same sex difference or whether these conditions occur more frequently in men or women that have Sjögren's disease. This study is the first to our knowledge to examine sex differences for these 22 comorbidities in Sjögren's disease.

Conclusions

The results of our study from patients at Mayo Clinic identified by ICD-9/10 codes showed that the top comorbidities in Sjögren's disease were fibromyalgia, depression, pain, and atherosclerosis. Women with Sjögren's disease were more likely to develop other rheumatic autoimmune diseases, fibromyalgia and experience pain, depression, migraine, EDS and hypermobile syndrome whereas men with Sjögren's disease were more likely to have cardiovascular disease and stroke. Future studies are needed to determine whether hypermobile

TABLE 12 Relative risk of developing a comorbidity in patients with Sjögren's disease that are ≥50 years of age ($n = 13,849$).

Comorbidity	<50 yr[a] (*n*)	≥50 yr (*n*)	HR (CI)	*P*-value[b]
Fibromyalgia	837	1,459	0.65 (0.60–0.71)	<0.001
Depression	678	1,511	0.86 (0.78–0.94)	<0.001
Pain	602	1,097	0.69 (0.63–0.77)	<0.001
Migraine	264	271	0.39 (0.33–0.46)	<0.001
Raynaud's syndrome	346	607	0.68 (0.59–0.77)	<0.001
SSc[c]	100	364	**1.43 (1.15–1.79)**	0.002
CREST	13	101	**3.23 (1.81–5.76)**	<0.001
Stroke	54	346	**2.59 (1.94–3.45)**	<0.001
PAH	101	625	**2.52 (2.04–3.11)**	<0.001
PH	99	632	**2.61 (2.11–3.22)**	<0.001
EDS	44	14	0.12 (0.07–0.22)	<0.001
Hypermobile syndrome	33	14	0.16 (0.09–0.31)	<0.001
Rheumatoid arthritis	336	1,090	**1.30 (1.15–1.47)**	<0.001
SLE	363	519	0.55 (0.48–0.63)	<0.001
Polymyositis	31	66	0.84 (0.55–1.30)	0.44
Dermatomyositis Diag	14	36	1.00 (0.54–1.85)	0.99
Myocarditis diagnosis	7	13	0.71 (0.28–1.78)	0.46
Lymphoma	61	215	**1.41 (1.06–1.87)**	0.019
Atherosclerosis/CAD	118	1,480	**5.35 (4.43–6.46)**	<0.001
Myocardial infarction	37	345	**3.78 (2.69–5.31)**	<0.001
CMP	59	191	1.30 (0.97–1.75)	0.077
CHF	67	611	**3.69 (2.87–4.76)**	<0.001

[a]CAD, coronary artery disease; CHF, congestive heart failure; CI, confidence interval; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; EDS, Ehlers-Danlos syndrome; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; RR, relative risk; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; yr, year. [b]Relative risk assessed using Cox Model. [c]Bold- increased risk in patients ≥50 years of age.

TABLE 13 Relative risk of developing a comorbidity in women with Sjögren's disease ≥50 years ($n = 11,969$).

Comorbidity	<50 yr[a]	≥50 yr	HR (CI)	*P*-value[b]
Fibromyalgia	788	1,352	0.67 (0.61–0.73)	<0.001
Depression	611	1,324	0.86 (0.78–0.95)	0.002
Pain	547	956	0.69 (0.62–0.76)	<0.001
Migraine	256	257	0.39 (0.33–0.46)	<0.001
Raynaud's syndrome	327	564	0.69 (0.60–0.79)	<0.001
SSc[c]	95	336	**1.44 (1.15–1.81)**	0.002
CREST	12	98	**3.49 (1.91–6.36)**	<0.001
Stroke	46	279	**2.53 (1.85–3.46)**	<0.001
PAH	95	551	**2.45 (1.97–3.05)**	<0.001
PH	91	557	**2.59 (2.07–3.24)**	<0.001
EDS	42	14	0.13 (0.07–0.24)	<0.001
Hypermobile syndrome	33	13	0.16 (0.08–0.30)	<0.001
Rheumatoid arthritis	310	964	**1.29 (1.14–1.47)**	<0.001
SLE	347	474	0.54 (0.47–0.63)	<0.001
Polymyositis	26	59	0.94 (0.59–1.50)	0.8
Dermatomyositis diag	11	34	1.25 (0.63–2.46)	0.52
Myocarditis diagnosis	7	11	0.62 (0.24–1.61)	0.33
Lymphoma	49	184	**1.56 (1.14–2.14)**	0.006
Atherosclerosis/CAD	90	1,108	**5.43 (4.38–6.74)**	<0.001
Myocardial infarction	30	254	**3.51 (2.40–5.13)**	<0.001
CMP	50	151	1.26 (0.91–1.73)	0.17
CHF	59	498	**3.54 (2.70–4.64)**	<0.001

[a]CAD, coronary artery disease; CHF, congestive heart failure; CI, confidence interval; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; EDS, Ehlers-Danlos syndrome; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; RR, relative risk, SLE, systemic lupus erythematosus; SSc, systemic sclerosis; yr, year. [b]Relative risk assessed using Cox Model. [c]Bold- increased risk in women ≥50 years of age.

EDS/hypermobile syndrome are important comorbidities in patients with Sjögren's disease.

Perspectives and significance

This study reports, for the first time, data on 22 comorbidities that occur in Sjögren's disease according to sex and age at diagnosis and after diagnosis based on ICD-9/10 codes for Sjögren's disease from the Mayo Clinic medical record. Although it is known that rheumatic diseases occur more often in women and most cardiovascular diseases occur more often in men, this relationship has not been previously reported for these comorbidities in Sjögren's disease. Importantly, this study found that women with Sjögren's disease had an increased risk of developing chronic pain syndromes like fibromyalgia, migraine, depression, pain, hypermobile syndrome and Ehlers-Danlos syndrome. In contrast, men with Sjögren's disease were at an increased risk of cardiovascular disease. Our findings reveal that rheumatic autoimmune diseases, cardiovascular diseases and pain-related conditions present clinically at a similar time as Sjögren's disease (2.6 years after diagnosis), which suggests that sex hormone effects on the immune response may be important in determining the pathogenesis of disease in a sex-specific manner. Although it is well-known that sex hormones influence immunity to promote autoimmune and cardiovascular diseases, our data suggest that this mechanism may also be important for the development of pain-related conditions like fibromyalgia and hypermobile syndrome and the development of one or more comorbidities

TABLE 14 Relative risk of developing a comorbidity in men with Sjögren's disease that are ≥50 years of age ($n = 1,880$).

Comorbidity	<50 yr[a]	≥50 yr	HR (±CI)	*P*-value[b]
Fibromyalgia	49	107	0.64 (0.46–0.90)	0.01
Depression	67	187	0.85 (0.65–1.13)	0.27
Pain	55	141	0.77 (0.56–1.05)	0.095
Migraine	8	14	0.51 (0.21–1.23)	0.13
Raynaud's syndrome	19	43	0.67 (0.39–1.16)	0.15
SSc	5	28	1.64 (0.63–4.25)	0.31
CREST	1	3	1.14 (0.11–11.49)	0.91
Stroke[c]	**8**	**67**	**2.65 (1.27–5.54)**	**0.009**
PAH	**6**	**74**	**3.76 (1.64–8.66)**	**0.002**
PH	**8**	**75**	**2.91 (1.40–6.03)**	**0.004**
EDS	2	0	0.00 (0.00–Inf)	1
Hypermobile syndrome	0	1	0.00 (0.00–Inf)	1
RA	26	126	1.52 (0.99–2.32)	0.054
SLE	16	45	0.84 (0.48–1.50)	0.56
Polymyositis	5	7	0.40 (0.13–1.26)	0.12
Dermatomyositis diag	3	2	0.18 (0.03–1.10)	0.064
Myocarditis	0	2	0.00 (0.00–Inf)	1
Lymphoma	12	31	0.76 (0.39–1.48)	0.41
Atherosclerosis/CAD	**28**	**372**	**4.56 (3.10–6.71)**	**<0.001**
Myocardial infarction	**7**	**91**	**4.14 (1.91–8.94)**	**<0.001**
CMP	9	40	1.46 (0.71–3.03)	0.3
CHF	**8**	**113**	**4.48 (2.18–9.19)**	**<0.001**

[a]CAD, coronary artery disease; CHF, congestive heart failure; CI, confidence interval; CMP, cardiomyopathy; CREST, calcinosis, Raynaud's syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia; EDS, Ehlers-Danlos syndrome; M, men; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; RR, relative risk; SLE, systemic lupus erythematosus; SSc, systemic sclerosis. [b]Relative risk assessed using Cox Model. [c]Bold- increased risk in men ≥50 years of age.

with Sjögren's disease add to the burden of disease in these patients.

Data availability statement

The datasets presented in this article are not readily available because they contain identifiable information from the Mayo Clinic electronic medical record. Requests to access the datasets should be directed to fairweather.delisa@mayo.edu.

Ethics statement

Research carried out in this study was in compliance with the Helsinki Declaration. The study was approved by the Mayo Clinic Institutional Review Board and receipt of a waiver of the need to consent subjects was obtained.

Author contributions

KB and AM-L acquired, analyzed, interpreted the data, and wrote the first draft of the manuscript. GB assisted in writing and editing the manuscript. HS and DH analyzed and interpreted data and edited the manuscript. JP, JS, GS, RP, and SW analyzed data and assisted in writing and editing the manuscript. EB, TR, PA, and PD assisted with data analysis and edited the manuscript. SL interpreted data and edited the manuscript. LS designed the study, interpreted data and assisted with writing and editing the manuscript. DF designed the study, analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

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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/fmed.2022.958670/full#supplementary-material

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Sex hormones affect the pathogenesis and clinical characteristics of systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) affects women more frequently than men, similar to the female predilection for other autoimmune diseases. Moreover, male patients with SLE exhibit different clinical features than female patients. Sex-associated differences in SLE required special considerations for disease management such as during pregnancy or hormone replacement therapy (HRT). Sex hormones, namely, estrogen and testosterone, are known to affect immune responses and autoimmunity. While estrogen and progesterone promote type I immune response, and testosterone enhances T-helper 1 response. Sex hormones also influence Toll-like receptor pathways, and estrogen receptor signaling is involved in the activation and tolerance of immune cells. Further, the clinical features of SLE vary according to hormonal changes in female patients. Alterations in sex hormones during pregnancy can alter the disease activity of SLE, which is associated with pregnancy outcomes. Additionally, HRT may change SLE status. Sex hormones affect the pathogenesis, clinical features, and management of SLE; thus, understanding the occurrence and exacerbation of disease caused by sex hormones is necessary to improve its management.

KEYWORDS

systemic lupus erythematosus, sex hormone, clinical characteristic, pathogenesis, hormone therapy

Abbreviations: BAFF, B cell-activating factor; COC, combined oral contraceptives; DC, dendritic cell; dsDNA, double-stranded DNA; E2, estradiol; ER, estrogen receptor; FSH, follicle-stimulating hormone; HC, healthy control; HRT, hormone replacement therapy; OCP, oral contraceptive; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; RCT, randomized controlled trial; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; WHI, Women's Health Initiative.

Introduction

Systemic lupus erythematosus (SLE), a chronic autoimmune inflammatory disease with heterogeneous clinical manifestations and course, affects multiple tissue and organ systems with, varying severity depending on the patient and duration of illness (1). Although many aspects of its etiology remain unclear, SLE is a complex disease known to result from the aberrant activity of the immune system due to environmental, genetic, epigenetic, and hormonal factors (2). Like other autoimmune diseases, such as rheumatoid arthritis, Sjogren's syndrome, and systemic sclerosis, SLE has a much higher prevalence in women than in men, with a female-to-male ratio ranging from 8:1 to 15:1 (3, 4). The striking difference in prevalence appears during the peak reproductive age, whereas female predominance significantly decreases during pre-puberty and post-menopause (5).

Factors associated with sex differences in SLE include sex chromosome genes, sex-dependent environmental factors, and gut microbiome composition, but considerable evidence supports that sex hormones are a major factor (6). As previously mentioned, SLE is typically more prevalent in young women of childbearing age. Indeed, its prevalence in women is only double that in men during childhood and postmenopausal periods (7). Furthermore, the disease activity of SLE can vary depending on hormonal changes such as the menstrual cycle and menopause, with a flare rate of 45–70% in pregnancy (8). In some patients with SLE, symptoms worsen each month as menstruation begins, and estrogen-containing therapies, namely, oral contraceptives (O) and postmenopausal hormone replacement therapy (HRT), are associated with an increased risk of flare (9, 10). In addition, the frequency and severity of flares decrease in most menopausal patients with SLE (11). In a lupus-prone model in NZB × NZW F1 mice, disease development was prevalent and survival time was shorter in women than in men (12).

Sex hormones include estrogen, progesterone, testosterone, dihydrotestosterone, and dehydroepiandrosterone (13). Estrogen, progesterone, and androgens are produced by the ovary in women, while testosterone precursors are produced mostly by Leydig cells in the testis in men and in the adrenal gland and thecal cells of the ovary in women (14). In women, estrogen and progesterone levels fluctuated during the menstrual cycle and life span, namely, premenopause and menopause, whereas testosterone levels remain steady during the menstrual cycle and decrease after menopause. Serum testosterone levels are higher in men than in women and decrease with age. Estrogen mainly affects reproductive function and additional processes, such as bone mass and fat distribution, while testosterone regulates physiological processes in muscle mass and strength, bone mass, fat distribution, and production of sperm and red blood cells.

In addition, sex hormones are involved in the development and function of innate and adaptive immune responses, and dysregulation of these mechanisms contributes to autoimmune abnormalities (15). Progesterone and androgens mainly have immunosuppressive and anti-inflammatory effects, thus protecting against autoimmune diseases, whereas estrogen is generally regarded as pathogenic due to its immune-stimulatory effects (16). Considering the mechanisms underlying altered immune responses and female predilection, estrogen is widely perceived as contributing to the predisposition for SLE. Recently, various clinical studies have reported sex-dependent genetic and epigenetic changes in SLE, revealing the complex role of sex hormones in addition to estrogen in the pathogenesis of SLE (17–19).

Herein, we review the current evidence regarding the role of sex hormones in the pathogenesis of SLE and describe the clinical features according to sex hormonal changes.

Methods

A systemic search of all English-language studies was performed in the Medline/PubMed, Scopus, and EMBASE databases using the following keywords: "sex hormone," "sex steroid," "systemic lupus erythematosus," "autoimmune disease," "estrogen," "progesterone," "testosterone," and "sex differences," as well as their abbreviations. Additionally, all related studies were searched manually for relevant keywords such as "oral contraceptives" and "hormone replacement therapy." Abstracts from relevant studies were reviewed, and appropriate articles were retrieved, and non-relevant papers and duplicate studies were excluded. All authors of this study conducted searches and articles were reviewed independently.

Results and discussion

Influence of sex hormones on immune response or autoimmunity (Figure 1)

Estrogen

Immune cells express two estrogen receptor (ER) subtypes, ERα and ERβ, and activation of ER-mediated or ER-independent pathways controls immune responses. ER subtypes and their mechanisms of action vary depending on the cell or environment, while hormone' concentration, density, distribution, and receptor, subtype affect immune responses. Gene expression analysis demonstrated that the expression of ERα mRNA was increased, while that of ERβ was decreased in peripheral blood mononuclear cells (PBMCs) from patients with SLE compared to healthy controls (20). However, no unique variants in ERα, ERα splice variants, and ERβ were identified in PBMCs from 19 patients with SLE compared

to 12 healthy individuals (21). Furthermore, the depletion of ERα attenuated the development of glomerulonephritis and anti-double-stranded DNA (dsDNA) antibodies, while prolonging the survival of NZB × NZW F1 mice, whereas ERβ deficiency had no effect on lupus manifestations (22, 23). Currently, the association between ER expression/action and SLE remains unclear.

B cells and autoantibody production

Estrogen stimulates B cell maturation and antibody secretion in the normal immune system. With respect to its impact on the autoimmune response, estrogen has been shown to increase the abundance of bone marrow progenitor B cells and enhance the survival of splenic B cells, which promotes the development of autoreactive B cells (24). In one study, estradiol (E2) treatment increased the production of anti-dsDNA antibodies and IgG in PBMCs from patients with active SLE, but not in patients with inactive disease or in a normal population (25). Additionally, E2 administration induced a lupus phenotype in BALB/c mice that expressed a transgene-encoded H chain of an anti-DNA antibody, rescued high-affinity DNA-reactive B cells, and led to increased Bcl-2 expression, which improved the survival of autoreactive B cells (26, 27). Meanwhile, tamoxifen treatment reduced levels of autoantibodies to IgG3, prolonged survival time, and was associated with weaker glomerular immune complex deposition in NZB × NZW F1 mice (28). Further, treatment with E2 increased levels of B cell-activating factor (BAFF) in immune cells, namely, macrophages, which promoted the survival of autoreactive B cells and autoantibody production, while ERα-knockout (KO) splenic cells showed decreased BAFF expression (29). In addition, NZB × NZW F1 mice treated with E2 exhibited more severe proteinuria and histological change in the glomerular tissue, along with increased levels of anti-C1q and anti-dsDNA antibodies (30).

T cells

Low estrogen levels enhance T-helper type 1 (TH1) cells and cell-mediated immunity, whereas high estrogen levels promote T-helper type 2 (TH2) cells and humoral immunity. E2 treatment was shown to enhance the expression of calcineurin, a T cell activation marker that acts through ERs, in the T cells of female patients with SLE, but not in those of HCs or male patients with SLE. Consistently, treatment with ERα and ERβ agonists increased the expression of calcineurin and CD154 in the T cells of patients with SLE (31, 32). E2 treatment stimulated T cells to express CD40 ligands in patients with SLE but not in normal women (33). E2 treatment induced the lupus phenotype, namely, nephritis, and production of TH2 cytokines and autoantibodies in wild-type mice, but not or minimally in ERα-KO mice (34). CD4-ERα KO mice had increased autoantibody production and CD4 + CD44 + CXCR5 + Bcl-6 + follicular helper T (TFH) cells, and E2 treatment decreased TFH cell responses, antigen-specific antibody production, and reduced IL-21 and Bcl-6 expression (35).

While different results had been reported in the populations of regulatory T (Treg) cells in SLE, incubation with E2 resulted in increased CD4, CD25, and FoxP3 mRNA expression in PBMCs from a healthy female, those from patients with SLE exhibited reduced FoxP3 mRNA expression (36). The deficiency of estrogen-related receptor γ (Esrrg), a murine lupus susceptibility gene associated with CD4 + T cell activation, has been shown to result in impaired function of Treg cells (37, 38). In addition, levels of human ESRRG, which is highly expressed in Treg cells, were reportedly lower in CD4 + T cells of patients with SLE than in those of HCs.

While increased population of CD4 + Th17 cells and IL-17A production has a pathogenic role in SLE, E2 treatment showed an inhibitory effect in Th17 cell differentiation in CNS autoimmunity (39). In addition, ERα KO mice showed TH1 and Th17 cell differentiation with IL-17 production, and estradiol inhibited Th17 cell differentiation through the downregulation of RORγt transcription (40). IL-17A production and IL-23R expression were increased in Th17 cells from female mice compared to those from male mice, both of which were decreased and Th17 cell proliferation was downregulated when ERα expression was suppressed (41).

Dendritic cells and toll-like receptor pathways

Dendritic cells (DCs), especially plasmacytoid DCs (pDCs), are activated in SLE to produce type I IFN through the Toll-like receptor (TLR)-7 or TLR-9 pathway through which endogenous DNA and RNA provoke autoimmune responses as self-antigen. Several DC subsets, namely, pDCs, express different patterns of ERs and affect ERα signaling (42). E2 treatment promoted the differentiation of DCs expressing high levels of cell surface MHC class II and CD86, whereas ER antagonists blocked DC differentiation (43). ERα deficient lupus-prone mice (strain NZM2410) had decreased frequency of pDCs and reduced endogenous expression of MHC-II and PDC-TREM, which modulates type I IFN production (44).

TLR-7-mediated IFN-α production was reportedly increased in the peripheral blood lymphocytes of healthy females compared to those of healthy males (45). E2 therapy enhanced TLR-7- and TLR-9-dependent production of IFN-α stimulated by nucleic acid-containing complexes in pDCs from postmenopausal women, while TLR-medicated IFN-α production by pDCs was restored in ERα-KO mice by E2 treatment (46). In another study, TLR-9 induced IL-6 and MCP-1 production by DCs was decreased in ERα-KO lupus-prone mice (strain NZM2410), and IL-1β and IL-23 expression were induced by a TLR-9 agonist in wild-type but not ERα-KO mice (47). Moreover, the delivery of recombinant IFN regulatory factor 5 (IRF5) protein into human pDCs increased TLR-7-mediated IFN-α secretion, while the genetic ablation of the estrogen receptor 1 gene in pDCs reduced irf5 mRNA

expression as well as IFN-α production (48). Further, estrogen treatment enhanced the expressions of TLR-8 and endosomal TLR-7 and TLR-9 in the PBMCs of patients with SLE compared to that in the PBMCs of HCs (49). Additionally, E2 exposure exacerbated proteinuria and glomerular immune complex deposition in female lupus-prone MRL^{lpr} mice through the induction of TLR-7 and -9 expression on splenic leukocytes and CD19 cells (50). Estrogen treatment was shown to enhance the expression of STAT1, which induces IFN-stimulated gene expression and upregulates TLR-8 expression (51). These data suggest that estrogen influences DC activation and IFN production through the TLR signaling pathways.

Epigenetic modulation

There were some reports finding the epigenetic changes related to the effects of estrogen or ER expression in SLE. DNA demethylation within the proximal promotor region relative to the transcription star site of the human ERα gene was associated with the overexpression of the ERα gene in SLE (52). And E2 inhibited DNA methyltransferase 1 (DNMT1) and enhances global DNA hypomethylation in SLE CD4 + T cells (53). ER agonists rescued downregulated DNMT1 and DNA hypomethylation.

E2 treatment enhances the activation of IFN-α signaling in SLE B cells *via* inhibitor of kappa B kinase ε (IKKε) by downregulating the expressions of let-7e-5p, miR-98-5p, and miR-145a-5p (54).

Estrogen treatment induced the overexpression of has-miR-10b-5p in T cells, and has-miR-10b-5p suppresses serine/arginine-rich splicing factor 1, which controls genes involved in T cell signaling and cytokine production. Has-miR-10b-5p expression was elevated in T cells from healthy women than healthy men, and elevated in T cells from patients with SLE, regardless of sex and SLE disease activity index (SLEDAI) (55).

Progesterone

Progesterone is a female reproductive steroid with immunomodulatory functions. Prolonged exposure to medroxyprogesterone acetate, synthetic progesterone used for contraception, led to lower serum IgG level, and decreased mortality in female NZB × NZW mice, although such treatment did not affect lupus phenotypes in other studies (56–58). The action of progesterone depends on its receptors, namely, progesterone receptor (PR), glucocorticoid receptor, and membrane PR. While low progesterone levels activate PRs and membrane PRs, high levels can bind not only to PRs and membrane PRs but also to glucocorticoid receptor, which is critical for reproduction. In one study, aged female PR-KO lupus-prone Nba2 mice exhibited increased IgG autoantibody production, and glomerular IgG deposition, inflammation, and damage compared to male mice (59). In addition, knockout of PR resulted in a lower splenic Treg cell population, but an increased proportion of follicular Th cells in aged female Nba2 mice.

Testosterone

Testosterone inhibits B cell differentiation in the bone marrow. Knockout of male androgen receptor, a testosterone receptor, resulted in increased levels of bone marrow B cell precursors in mice (60). In addition, male mice lacking androgen receptors had higher splenic B cell population and serum BAFF levels (61). Further, levels of plasma androgens, namely, testosterone and androstenedione, were lower in female patients with active SLE (62).

While some studies have reported the therapeutic effect of testosterone and other androgens against SLE disease activity, testosterone patches and 19-nortestosterone failed to improve disease activity or quality of life in patients with SLE (63–66). *In vitro* study assessing the effect of sex hormones on changes in Treg cells demonstrated that androgen/testosterone enhanced FoxP3 mRNA expression in CD4 + Treg cells of patients with SLE (36).

Influence of menstrual cycle on systemic lupus erythematosus

During the menstrual cycle, follicle-stimulating hormone (FSH) stimulates the ovarian follicles to produce E2. Thus, the plasma E2 concentration is increased, while that of progesterone is decreased in the follicular phase, and both E2 and progesterone plasma concentrations are high in the luteal phase (36). Increased E2 levels lead to a mid-cycle surge of luteinizing hormone, which initiates ovulation; if fertilization does not occur, progesterone levels drop. Luteolysis is inhibited during pregnancy, leading to prolonged high E2 and progesterone levels. In addition, E2 and progesterone levels are low during menopause along with the depletion of follicles.

A study comparing reproductive health histories between patients with SLE, and the general population reported no differences in hormone levels throughout the menstrual cycle (67). However, menopause occurred earlier in patients with SLE, and the use of HRT was frequent, and the family size was reduced in patients with lupus nephritis. Some data have shown that premature ovarian dysfunction is more common in patients with SLE than in HCs and is associated with the use of cyclophosphamide (68, 69). Compared to 30 HCs, abnormal and longer-length menstrual cycles were more frequent, and the median FSH level was higher, and that of progesterone was lower in 30 patients with juvenile SLE (70). A study analyzing a self-reported survey of patients with SLE revealed higher pain, fatigue, and disease activity during menses than during the hormonal surge phase, although recall bias and confusion with pre-menstrual syndrome existed (71). In examining the influence of the

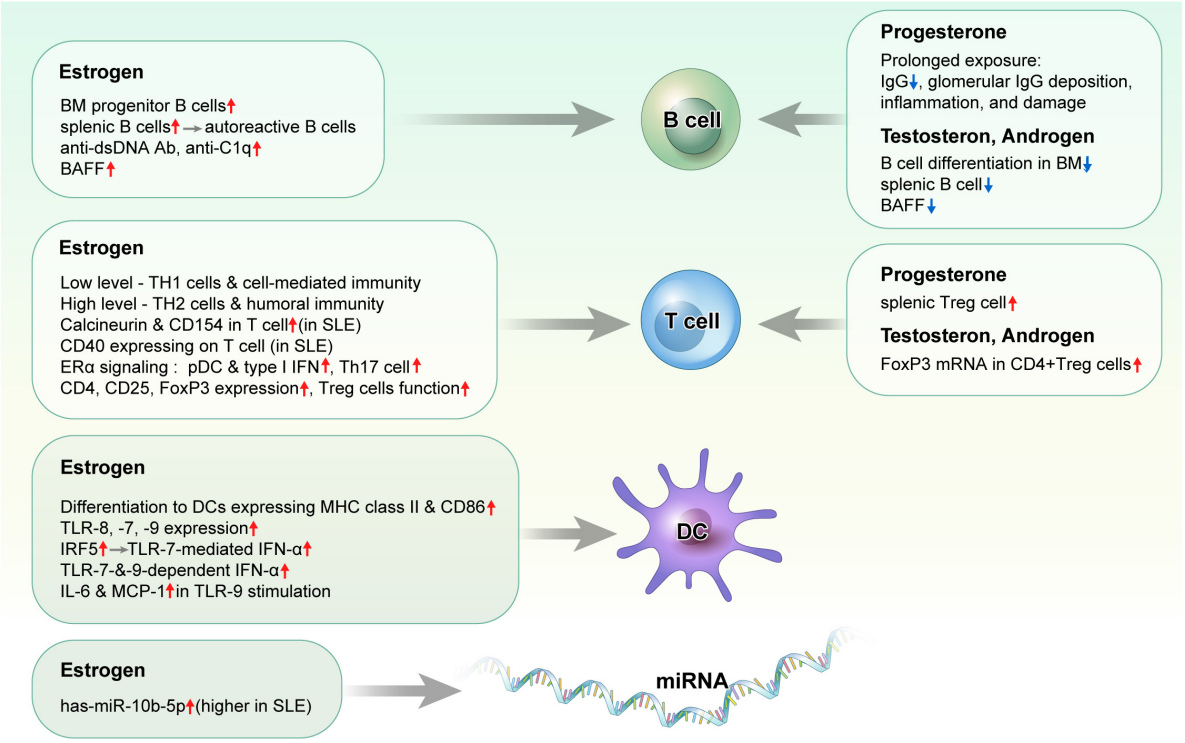

FIGURE 1
Effects of sex hormones on immune cells in systemic lupus erythematosus.

menstrual cycle or fertility on SLE, disease activity, use of medication (including glucocorticoids and cyclophosphamide), and individual differences should be considered.

Analysis of PBMCs has revealed significant changes in gene expressions, including that of TNF superfamily member 14 and signal regulatory protein-γ, during the menstrual cycle of patients with SLE compared to that of HCs (72). A study analyzing the expression of sex hormone receptor genes in PBMCs, and cell subsets reported that several immune response genes were more highly expressed during the ovulatory and mid-luteal phase (73). In addition, the level of sex hormone-binding globulin, a steroid hormone transport protein, was correlated with ERβ1 gene expression.

Characteristics of male systemic lupus erythematosus

Sex hormones and chromosomes in male systemic lupus erythematosus

Unsurprisingly, the role that sex hormones play in male and female SLE differs significantly, which has been demonstrated both in murine models and clinical studies (74). In the NZB × NZW F1 mouse model, an autoimmune disease resembling human SLE is characterized by high levels of antinuclear and anti-dsDNA antibodies, lymphadenopathy, splenomegaly, and immune complex-mediated glomerulonephritis. NZB × NZW F1 mice administered androgens exhibited reduced lupus-like phenotypes and improved survival rates, whereas autoantibodies and accelerated autoimmune disease appeared earlier in castrated NZB × NZW F1 mice administered estrogen (75, 76). A recent study with a lupus-prone mouse model reported that a male-driven immunoinhibitory milieu was related to B cell activation and differentiation, and ultimately delayed or prevented lupus-like disease, suggesting that androgens affect lupus pathogenesis and exert different therapeutic effects in males and females (77). Given the clear inhibitory effects of androgen on the immune system, a recent interest is to discover SLE therapies targeting immunomodulatory cells by elucidating mechanisms that affect the number and functionality of these cells such as regulator T/B cells, MDSCs, and M2 macrophages in genetically predisposed male mice (78).

In human studies, significantly lower androgen levels, which were inversely related to hypoandrogenism, testosterone levels, and disease activity, were detected in male patients with SLE compared to HCs (79–81). As in murine models, the clinical symptoms and serological features of men and women respond differently to synthetic androgen therapy

as a treatment for SLE. Two studies have reported that testosterone supplementation improved the clinical symptoms of male lupus patients with Klinefelter's syndrome (82, 83). Additionally, testosterone injections for cross-gender therapy resolved the skin symptoms of a transgender patient (female to male) with subcutaneous lupus erythematosus (84). In contrast, a small clinical trial reported that men treated with 19-nortestosterone exhibited decreased testosterone levels, increased serum anti-dsDNA antibody levels, and new clinical features, namely, Raynaud's phenomenon and pleuropericardial disease (64). In another clinical trial, testosterone patches did not significantly affect disease activity, quality of life, or sexual function in male patients with SLE (63). The clinical efficacy of androgen treatment in male patients with SLE remains unclear, and further studies are warranted to determine whether such treatments should be more widely provided.

In addition to sex hormones, there is growing evidence that the role of the X chromosome helps explain whether more women than men develop autoimmune diseases, namely, SLE (85–87). The first evidence supporting that the factors associated with X chromosomes cause susceptibility for SLE was a report that the prevalence of Klinefelter's syndrome (karyotype 47, XXY) is increased 14-fold in men with SLE compared to that in an unselected male population (85). With similar results, in one study, karyotype 47, XXX in females predicted an approximately 25-fold relative risk for SLE, and another report showed that 46, XX males (de la Chapelle's syndrome) were excessively present among males with SLE (86, 87). These data support that the number of X chromosomes, not phenotypes, is related to the cause of sex bias in SLE. Recently, genes in the X chromosome are also observed to be attributed to the female bias in SLE. The X chromosome encodes a greater number of genes than the Y chromosome, and X-linked genes such as KDM6a, TLR-7, CXorf21, and IRAK1 are found to be overexpressed in females' autoimmunity. Furthermore, recent data suggest that X-linked genetic factors are involved in epigenetic mechanisms to avoid X chromosome inactivation, thereby enhancing female susceptibility to autoimmune diseases (88).

Clinical features and outcomes in male systemic lupus erythematosus

Due to the perception that SLE is a disease in women of childbearing age, appropriate diagnosis and treatment are often delayed in men. Overcoming this challenge requires an awareness of the distinctive presentation of male SLE. Studies worldwide have confirmed that the peak ages of SLE incidence and prevalence are delayed for men compared to women (89). Although considerable variability is reported according to region and race, the mean age at diagnosis is 26−55 years for men, compared to 26.3−42.6 years for women (90). The peak incidence of SLE usually occurs for women in their 20s–50s, while that for men occurs in their 50s–70s (89). The prevalence curve by age tends to be similar to the distribution of incidence data, with the peak age of prevalence ranging from 45 to 69 years for women and 40 to 89 years for men (89, 91).

Serologically, anti-dsDNA and anti-Smith antibodies have been observed to occur more frequently in male SLE, whereas some studies have reported lower levels of anti-Ro/SSA and anti-La/SSB antibodies in male SLE (92–95). Although differences occur depending on the reported group, anti-U1-RNP and anti-cardiolipin antibodies and low complement appear to occur at almost the same rates in men and women (92, 96). Lupus anticoagulant positivity is more common in men, combined with smoking and alcohol use, which is related to a higher prevalence of thrombosis in male SLE than in female SLE (96–98). Additionally, renal, hematological, and neurological involvement, as well as serositis features prominently in male SLE, leading to rapid organ damage compared to female SLE (94, 99). The increase in autoantibody production and the development of lupus nephritis in male SLE is presumed to be due to the global deletion of ERα or especially in B cells (98).

Regarding skin involvement, discoid, and subacute lesions occurred more frequently in male SLE, while malar rash, photosensitivity, and Raynaud's phenomenon were much less common (74, 90, 92, 94–104). Musculoskeletal involvement and alopecia were reported less frequently in male SLE, with fewer outpatient visits and emergency department visits than in female SLE (93, 101, 105).

In relation to disease activity, SLEDAI scores and lupus severity of disease index scores did not differ significantly between the sexes in most studies (90, 94, 95, 98, 104). Conversely, renal damage is a major concern in male SLE, as several studies reported that male sex is a strong predictor of baseline damage and men have a high risk of developing chronic renal failure (91, 92, 105–109). In cohorts in the United States and Taiwan, male sex was associated with a 2-fold greater risk of end-stage renal disease (99, 110). However, in a recent study using the national data system in the United States, similar rates for both sexes were reported for end-stage renal disease and mortality (105). Increased incidence of cardiovascular events due to ischemic heart disease or stroke was also reported among male patients with SLE (90, 98, 100). In this regard, male SLE is considered to have a poorer prognosis than female SLE due to renal involvement and concomitant cardiovascular diseases. Although survival rates vary, most studies did not report statistically significant differences between men and women (90, 92, 95, 105).

The clinical characteristics and outcomes of SLE display notable sex differences, which are most influenced by sex hormones. However, considering age, race, national health system, and small cohorts of male patients with SLE, further

studies are needed to unravel the mystery of male SLE and potential therapeutic interventions for the disease.

Influence of hormone therapy on systemic lupus erythematosus

Oral contraceptives and systemic lupus erythematosus

17α-ethinyl estradiol, a synthetic analog of 17β-estradiol (E2), is a major component in OCPs and has been commonly used in hormone therapy. As the timing of pregnancy greatly contributes to success in SLE and flares cause adverse pregnancy outcomes, contraception is often considered for women with SLE, leading to the use of OCPs (111). Two major categories of OCPs, combined oral contraceptives (COCs) that contain estrogen and progesterone and progestin-only formulations, are commonly used as reversible contraceptives and are mostly safe (112). However, reflecting on the unpredictable and variable nature of SLE, OCPs have been considered unsafe and not often prescribed for women with SLE (113, 114). Several studies have reported controversial results regarding the use of OCPs in patients with SLE (**Table 1**). The role of exogenous estrogen as a flare factor was first reported in a case report in the 1960s (115). Early case reports and retrospective studies supported that the patients with established disease exposed to OCPs were at risk for developing SLE (105–120). A frequently cited and representative retrospective study reported by Jungers et al. found that flares occurred in 43% of patients with lupus nephritis when taking COCs, which was not observed with progestin-only formulations (117). In another retrospective study based on self-reported flares, 13% of patients experienced flares after initiating OCPs, particularly with musculoskeletal symptoms (120). Moreover, several cases of pulmonary hypertension and venous thromboembolism have been reported in patients with SLE after the use of OCPs (118, 121).

In addition to its association with disease activity, case reports and prospective cohort studies have reported newly diagnosed SLE after the initiation of OCPs (10, 119, 121–125). Sanchez-Guerrero et al. and Costenbader et al. reported associations between OCP use and SLE onset in 1997 and 2007, respectively, using the same population from the Nurses' Health Study cohort (124, 125). The relative risk (RR) of OCP users compared to that of never users was 1.9 (95% confidence interval [CI] 1.1−3.3) in the first study and 1.4 (95% CI 0.9−2.1) in the second study. Another highly supportive study conducted in the United Kingdom evaluates the risk of SLE incidence related to COC use (10). In this study, COC use was associated with an increased risk of SLE (RR 1.19, 95% CI 0.98−1.45), especially in women who recently started using contraceptives or at higher doses. Malignant hypertension and vascular complication have also been reported in women with incomplete SLE featuring anti-DNA and antiphospholipid antibodies (119).

However, case-control studies have reported contradictory results (126–129). The first case-control study assessing the association between OCP exposure and risk of SLE was performed in 1985 with 109 cases of SLE and 109 HCs, reporting that recent use of OCPs was independent of SLE onset (odds ratio 0.5, 95% CI 0.11−2.3) (126). A case-control study from the United States with 195 cases of SLE and 143 HCs also showed no association between SLE and either any or recent use of OCPs (127). Although studies with similar results have been reported, limitations such as selection bias have hampered the precision of these studies (128, 129). Some studies have suggested that OCP use does not equally affect all individuals and triggers SLE by inducing antinuclear antibodies in predisposed individuals with autoimmune serologies (130).

Two higher-quality randomized controlled trials (RCT) were conducted to clarify this discrepancy between conflicting results (131, 132). The first RCT was a single-blinded, non-placebo study that followed 162 patients with stable SLE randomly assigned to a COC, intrauterine device, or progestin-only pill for 12 months. In this study, disease activity, flare incidence, and time-to-first flare did not differ significantly among the groups treated with different types of contraceptive therapy (131). The second double-blind RCT, the Safety of Estrogen in Lupus Erythematosus National Assessment (SELENA) study conducted in the United States, included 183 patients with inactive or stable active SLE randomly assigned to receive placebo or COC for 12 months (132). As with the previous RCT results, the flare rates were similar between the two groups, and the discontinuation rates did not differ for any reason.

Available evidence from RCTs supports the safety of OCPs, namely, COC, in most women with SLE. Based on these results, the World Health Organization published useful information regarding contraception for women with SLE, suggesting that most OCPs can be used by women with SLE if antiphospholipid antibodies are absent or cardiovascular risk factors are unclear (113). Since both RCTs excluded patients with SLE with unstable active diseases, the results may not be applicable to all women with SLE. Considering the data to date, the effects and risks of OCPs on SLE may vary depending on the dose, duration of use, and type of hormone used. Despite being theoretically possible, the use of OCPs should be fully discussed with respect to the balance of benefits and risks for each individual patient. Most importantly, OCPs are contraindicated for women with SLE displaying positive/unknown antiphospholipid antibodies or a history of thrombosis under all circumstances (133, 134).

Hormone replacement therapy and systemic lupus erythematosus

Hormone replacement therapy (HRT) is the most effective method for relieving menopausal symptoms such as vasomotor

TABLE 1 List of studies on the risk of disease onset or flares in patients with systemic lupus erythematosus using oral contraceptives.

Study design	Study population	Oral contraceptives, dose	Main findings	References
SLE Flares				
Case report	23-year-old female	COC, 3 mg norethisterone + 50 μg ethinyl estradiol	Flare (high fever, arthritis, malar rash) in 1 week	(115)
Case report	Two cases	POC, Mestranol 100 μg POC, Mestranol 80 μg	Flare (arthritis) in 10 days Flare (skin rash) in 3 months	(116)
Retrospective study	26 Lupus nephritis	COC, 50 μg (14 patients) and 30 μg (7 patients) ethinyl estradiol POC (11 patients)	- Incidence of flare: 43% in COC groups within 3 months - No flare in POC group	(117)
Case report	16-year-old female	30 μg ethinyl estradiol + 150 μg levonorgestrel	Pulmonary hypertension in 7 months later	(118)
Retrospective study	85 SLE	COC (31 patients), 30 μg ethinyl estradiol + 150 μg levonorgestrel/75 μg gestodene POC (32 patients) Other unspecified	- Incidence of flare: 4 (13%) during the first 6 months - Incidence of flare was similar as in patients not using OCPs	(119)
Retrospective questionnaire study	55 SLE	OCP unspecified	Incidence of flare: 7 (13%) reported an exacerbation of disease activity, mostly musculoskeletal system	(120)
RCT, single blind, non-placebo, follow-up 12 months	162 SLE (≤ 40 years old, with mild or stable disease)	COC, 35 μg ethinyl estradiol + 150 μg levonorgestrel POC, 30 μg levonorgestrel IUD (TCu 380A copper device)	No difference among groups in mean activity, incidence of flares or time to first flare	(131)
RCT, double blind placebo-controlled, follow-up 12 months	183 stable or inactive SLE (91 OCP vs. 92 placebo)	Triphasic ethinyl estradiol 35μg + norethisterone at a dose of 0.5−1 mg for 12 cycles of 28 days	No differences between groups in occurrence of flares of any type (Severe lupus flare occurred in 7.7% of OCP group vs. 7.6% in the placebo group)	(132)
SLE onset				
Case report	False positive serological test for syphilis	COC, 1 mg norethisterone + 50 μg ethinyl estradiol	Developed SLE 3 weeks after the start of OCP	(122)
Case report	False positive serologic prenuptial syphilis test	1 mg ethynodiol diacetate + 50 μg ethinyl estradiol	Developed SLE 4 weeks after the start of OCP and improved with withdrawal of OCP	(123)
Case report	22-year-old female	30 μg ethinyl estradiol + 250 μg levonorgestrel	Developed pulmonary hypertension related to SLE in 9 months	(121)
Case control study	109 SLE and 109 controls	OCP unspecified	No association between OCPs and SLE	(126)
Case report	24-year-old female	30 μg ethinyl estradiol	Developed malignant hypertension who has incomplete SLE with DNA antibodies and high levels of antiphospholipid antibodies	(119)
Case control study	195 SLE and 143 controls	OCP unspecified	No association between OCPs and SLE	(127)
Prospective cohort study	99 SLE confirmed among NHS cohort 121,645 women	Use of OCPs based on self-report	- Past users vs. never users: RR 1.9 (95% CI 1.1−3.3) - No relationship with duration of OCP use	(131)
Case control study	85 SLE and 205 controls	Use of OCPs containing estrogen based on self-report	No association between OCPs and SLE	(128)
Population-based case control study	240 SLE 240 and 321 controls	OCP unspecified	No association between OCPs and SLE	(129)
Prospective cohort study	262 SLE confirmed among NHS cohort 238,308 women	Use of OCPs based on self-report	- Ever use of OCPs: RR 1.5 (95% CI 1.1–2.1) - Highest risk with short duration (< 2 years) of OCPs: (RR 1.9, 95% CI 1.3–2.8)	(125)

(Continued)

TABLE 1 Continued

Study design	Study population	Oral contraceptives, dose	Main findings	References
Population based nested case control-study	786 SLE and 7,817 controls	COC exposure First- and second-generation (ethinyl estradiol combined with the progestatives norethisterone, levonorgestrel, and norgestrel) vs. third-generation (ethinyl estradiol and either gestodene, desogestrel, or norgestimate)	- Any use of OCPs: RR 1.19 (95% IC: 0.98–1.45) - Current use of OCPs: RR 1.54 (95% IC: 1.14–5.57) - Risk was higher in current users who recently started (RR 2.52, 95% CI: 1.14–5.57), first or second-generation OC (RR 1.65, 95% CI 1.20–2.26), and increase with dose of ethinyl estradiol (RR 1.42, 1.63, and 2.92 for $\leq$ 30 μg, 31−49 μg, and $\geq$ 50 μg, respectively)	(10)

SLE, systemic lupus erythematosus; COC, combined oral contraceptives; POC, progestin-only oral contraceptives; OCP, oral contraceptives; NHS, nurses' health study; RR, relative risk; CI, confidence interval.

hot flashes, atrophic vaginitis, and urinary incontinence or frequency (135). In the 1980s and 1990s, early observational data supported that HRT reduced coronary heart disease and mortality, and initial data from the Women's Health Initiative (WHI) RCT demonstrated a decreased incidence of osteoporosis-related fractures in postmenopausal women undergoing HRT (136, 137). Thus, HRT was widely used in menopausal women for 20 years. However, in the early 2000s, data from the WHI trial suggest that HRT was associated with an increased risk of invasive breast cancer, coronary heart disease, stroke, and pulmonary embolism, leading to an abrupt decline in the use of HRT in postmenopausal women worldwide (138, 139). Although the WHI was the largest RCT on HRT, only two hormone formulations were evaluated. Subsequent studies have changed the approach to HRT by evaluating different dosages and routes of estrogen therapy, namely, transdermal HRT or an ultra-low-dose oral product, and have recently demonstrated that the benefits outweigh the risks in women within 10 years of menopause and short-term use of low-dose HRT to alleviate menopause symptoms (140, 141).

Although HRT is generally considered to relieve menopausal symptoms in the short term and protect against chronic diseases in the long term for the general female population, many inconsistencies have been reported in patients with SLE. **Table 2** lists the characteristics of studies investigating the relationship between HRT and SLE. A large prospective cohort study in 1995 reported that HRT was causally associated with an increased risk of developing SLE in postmenopausal women (142). In this study, the age-adjusted relative risks for the onset of SLE were 2.1 (95% CI 1.1−4.0) for ever users, 2.5 (CI 1.2−5.0) for current users, and 1.8 (CI, 0.8−4.1) for past users, compared with never users, and the duration of hormone use and risk of SLE were proportional. Additionally, a case-control study by Meier et al. reported that the risk of developing SLE increased as the duration of hormone use increased, and the magnitude of risk was related to estrogen dose (143). However, some studies have found no evidence of a significant increase in the incidence of SLE with HRT use (129).

Several early retrospective case-control studies failed to find an association between flares and HRT (144–146). Although these were very small studies with insufficient data, the conclusion that HRT had a minimal effect on inflammatory markers and did not change disease activity (expressed by SLEDAI) was similar in all published observational studies. Conversely, case and prospective cohort studies have provided evidence linking HRT to flares (9, 147). In one case report, a woman diagnosed with SLE maintained remission status after menopause at 38 years of age but relapsed after taking estrogen as a treatment for osteoporosis at 64 years of age (147). The largest clinical trial to date investigating the effects of HRT on disease activity in patients with SLE was part of the SELENA trial, in which mild to moderate flares were significantly increased in the HRT group (9). However, neither the occurrence of severe flares nor the mean SLEDAI scores changed significantly between the HRT and placebo groups in this study. In another prospective study conducted by Sánchez-Guerrero et al. HRT use did not change the disease activity in SLE during 2 years of treatment (148).

As with taking OCPs, the greatest concern regarding HRT is the increased risk of arterial or venous thrombosis. Most studies on HRT in patients with SLE found a link between HRT use and thrombotic events (129, 144, 145, 148–151). Although the risk of developing thrombosis increases after HRT or menopause in healthy women, the incidence of thrombosis in women with SLE increased dramatically from 0.08 to 0.11 per 1,000 person/year to 5.1 per 1,000 person/year (149, 150, 152). Several RCTs reported that HRT use alone did not increase the risk of thrombosis or coronary heart disease for patients with SLE with inactive or stable active disease, negative antiphospholipid antibodies, and no history of thrombosis (9, 153, 154). The effects of hormones on thrombosis and the reported data indicate that HRT is not safe in patients with SLE with antiphospholipid antibodies or prior vascular thrombotic

TABLE 2 List of studies on the risk of disease onset or flares in patients with systemic lupus erythematosus using hormone replacement therapy.

Study design	Study population	Hormone replacement therapy, dose	Main findings	References
SLE flares				
Case control study	60 SLE (30 HRT users and age matched 30 never users)	HRT unspecified	- No differences between the two groups in ESR, hospital admission, or medications - HRT users experienced significant improvements in general wellbeing, libido and depression.	(144)
Case control study	48 SLE (16 HRT users and age matched 32 controls)	Estrogen dose (0.3—0.625 mg) and the progestogen dose (0—10 mg of MPA)	The use of HRT does not appear to increase the rate of flares (SLEDAI change) over a 1-year follow-up	(145)
Case control study	34 SLE (11 HRT and 23 non-HRT users)	0.625 mg of CEE (Days 1–21) and MPA 5 mg daily (Days 10–21)	No difference in flares (0.12 relapses/patient-year in HRT group vs. 0.16 relapses/patient-year in the non-HRT group, $p = 0.90$) and SLEDAI change (total SLEDAI score increase during flares/patient-year in the HRT and non-HRT groups were 0.55 and 1.22, respectively, $p = 0.57$) between two groups	(146)
Case report	64-year-old female	Estrogen for osteoporosis treatment	Flare of SLE in a 64-year-old woman in remission status after taking estrogen as a treatment for osteoporosis	(147)
Randomized, double-blind, placebo-controlled non-inferiority trial	351 menopausal patients with inactive (81.5%) or stable-active (18.5%) SLE	0.625 mg of CEE daily, plus MPA 5 mg for 12 days per month	- Mild to moderate flares were significantly increased in the HRT group: 1.14 flares/person-year for HRT and 0.86 flare/person-year for placebo (RR 1.34; $P = 0.01$) - HRT did not significantly increase the risk for severe flare compared with placebo	(9)
Double-blind, randomized clinical trial	106 SLE (52 HRT users and 54 placebo)	0.625 mg of conjugated estrogen daily, plus 5 mg of medroxyprogesterone for 10 days per month	- Menopause hormonal therapy did not alter disease activity (SLEDAI score) during 2 years of treatment - Increased risk of thrombosis in hormone therapy group	(148)
SLE onset				
Prospective cohort study	45 SLE confirmed among NHS cohort 69,435 women	Use of HRT based on self-report	- Ever uses of HRT: RR 2.1 (95% IC: 1.1–4.0) - Current uses of HRT: RR 2.5 (95% IC: 1.2–5.0) - Past use of HRT: RR 1.8 (95% IC: 0.8–4.1) - HRT is associated with an increased risk for developing SLE	(142)
Case control study	41 SLE, 34 discoid lupus, and 295 age- and sex-matched controls	HRT unspecified	- Developing SLE (adjusted OR 2.8; 95% CI 0.9–9.0) or discoid lupus (adjusted OR 2.8; 95% CI 1.0–8.3) who were exposed for 2 or more years - Increased risk in estrogen only (OR 5.3; 95% CI 1.5–18.6) rather than estrogen + progesterone (OR 2.0; 95% CI 0.8–5.0), compared to non-users.	(143)
Population-based case control study	240 SLE 240 and 321 controls	HRT unspecified	No association between HRT and SLE	(129)
Prospective cohort study	262 SLE confirmed among NHS cohort 238,308 women	Use of HRT based on self-report	Ever use of HRT: RR 1.9 (95% CI 1.2–3.1)	(125)

SLE, systemic lupus erythematosus; HRT, hormone replacement therapy; ESR, erythrocyte sedimentation rate; MPA, medroxyprogesterone acetate; SLEDAI, systemic lupus erythematosus disease activity index; CEE, conjugated equine estrogens; RR, relative risk; NHS, nurses' for more details.

events. Smoking, old age, female sex, disease activity, and glucocorticoid dose are also known to increase the risk of thrombosis; therefore, HRT use should be cautioned for patients with these risk factors (155).

Furthermore, the influence of HRT on malignancy risk is a serious concern for women. In the general population, cancer risk was increased by 9% among users of HRT, which carried widely depending on the type of cancer and HRT regimen (156). In particular, the relationship between HRT and female reproductive organ cancers, such as breast, endometrial, and ovarian cancer, is of great interest. Fortunately, the risk of these cancers is rather decreased in women with SLE (157, 158). The tendency of patients with SLE to have a higher age at menarche and lower age at menopause compared to the general population leads to a decrease in lifetime estrogen exposure, reducing the incidence of female reproductive organ cancers (159). Studies on the causal relationship between cancer incidence and HRT in patients with SLE are rare, and the association between cancer and HRT in SLE has not yet been clarified in published studies (160).

In conclusion, HRT use needs to be individually tailored in consideration of various conditions. For women with SLE, transdermal or percutaneous estrogen formulations are preferred over oral preparations, and micronized progesterone or pregnane derivatives are preferred over non-pregnane when using combined estrogen and progesterone HRT. Moreover, if HRT is unavoidable in active disease, non-estrogenic drugs should be selected first (161).

Conclusion

The effects of sex hormones, estrogen, and their receptors, especially ERα, have been found to promote autoimmune responses, namely, autoantibody production, and Th17 differentiation. In addition, DC activation and a type I IFN signature are modulated through TLR-7 and TLR-9 by estrogen or its receptor. While androgens inhibit B cell activation, testosterone and other androgens have not demonstrated therapeutic effects against SLE. Although sex hormones change during the menstrual cycle, flares rarely occur according to the menstrual cycle. Defective androgens are associated with male SLE, which is characterized by more frequent skin involvement and higher risk for renal damage. Although conflicting results have been reported regarding the use of OCPs and HRT in women with SLE, their use raises the risk of flares or cardiovascular diseases in patients with antiphospholipid antibodies or a history of thrombosis; therefore, hormone therapy for patients with SLE should be decided through close consultation.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

J-WK and J-YJ: conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft preparation, review and editing, and project administration. J-WK: visualization. J-YJ: supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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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

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