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Review Article

Exploring the HLA complex in autoimmunity: From the risk haplotypes to the modulation of expression

Silvia Sartoris [a], Giovanna Del Pozzo [b,*]

[a] Dept. of Medicine, Section of Immunology University of Verona School of Medicine, Verona, Italy
[b] Institute of Genetics and Biophysics "Adriano Buzzati Traverso" National Research Council (CNR), Naples, Italy

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ABSTRACT

The genes mapping at the HLA region show high density, strong linkage disequilibrium and high polymorphism, which affect the association of HLA class I and class II genes with autoimmunity. We focused on the HLA haplotypes, genomic structures consisting of an array of specific alleles showing some degrees of genetic association with different autoimmune disorders. GWASs in many pathologies have identified variants in either the coding loci or the flanking regulatory regions, both in linkage disequilibrium in haplotypes, that are frequently associated with increased risk and may influence gene expression. We discuss the relevance of the HLA gene expression because the level of surface heterodimers determines the number of complexes presenting self-antigen and, thus, the strength of pathogenic autoreactive T cells immune response.

1. Introduction

A breakdown in immune tolerance and the activation of autoreactive T and B cells by gene-environment interactions in genetically susceptible individuals are considered the major causes of the development of autoimmune diseases. These pathologies may be systemic or organ-specific and are caused by immune cellular and antibody reactivity to self-antigens or cross-reactive antigens [1].

The most common autoimmune diseases display strong genetic components based on polygenic traits, where multiple genetic polymorphisms of immune-related loci form disease-susceptible or disease-protective genotypes. The onset of autoimmune diseases also depends on environmental triggers like pathogen infections, microbiome diet, cigarette smoke, drugs and pollution, inducing inflammatory responses and tissue damage. Among these, dysbiosis of gut microbiota could cause the alteration of the innate and adaptive local immune system, through variations in cytokines signalling and epitope spreading which would lead to autoreactive immune responses [2,3]. Some of the HLA and non-HLA susceptibility genes are common among diseases, but others differ according to ethnicity and environmental triggers. The genetic background is the major player in the physio-pathological processes, clinical outcomes, and comorbidities. A significant contribution to autoimmunity is given by gender, with females more frequently affected than males, especially for systemic and endocrine diseases, a consequence of the oestrogens and the highest reactivity of the immune system [4,5]. The age at onset varies widely depending on the disease [6] and the gene association, as in the case of myasthenia gravis, in which early-onset has been associated with HLA class I and late-onset with HLA class II genes [7].

2. HLA region and haplotypes

The primary genetic association to autoimmunity is the Major Histocompatibility Complex (MHC), the most polymorphic region of the human genome encoding the HLA molecules (Human Leukocytes Antigens), whose function is antigen presentation to antigen-specific T cells, leading to the onset of adaptive immune responses.

HLA is a region of 4 Mb on the short arm of chromosome 6 (6p21.3) divided into three subregions: the class I region, containing the classical and non-classical class I HLA genes; the class II region, which is defined

Abbreviations: Ab, Antibody; AH, Ancestral Haplotype; AIH, Autoimmune Hepatitis; APC, Antigen Presenting Cells; AS, Ankylosing Spondilytis; B-LCL, B lymphoblastoid Cell llne; CEH, Conserved Extended Haplotype; CPS, Conserved Polymorphic Sequences; DAE, Differential Allelic Expression; eQTL, expression Quantitative Trait Loci; hQTL, histone Quantitative Trait Loci; IBD, Inflammatory Bowel Disease; LD, Linkage Disequilibrium; MG, Myasthenia Gravis; MS, Multiple sclerosis; NGS, Next Generation Sequencing; PBC, Primary Biliary Cholangitis or Cirrhosis; PheWAS, Phenome-wide association studies; PCR, Polymerase Chain Reaction; PBMC, Peripheral Blood Mononuclear Cells; PSC, Primary Sclerosing Cholangitis; RA, Rheumatoid Arthritis; SE, Shared Epitope; SEnh, super enhancer; SNP, Single Nucleotide Polymorphism; TGS, Third Generation Sequencing; UC, Ulcerative Colitis.

* Corresponding author.

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Table 1
Cell lines with HLA genotype adapted by Houwaart et al. [22] and Kulski et al. [14].

Cell line	AH/CEH	MHC class II type	HLA-A	HLA-B	HLA-C	HLA-DRB3,4,5	HLA-DRB1	HLA-DQA1	HLA-DQB1
APD	60.X	DR3	A*01:01:01	B*40:01:01	C*06:02:01	DRB3*02:02:01	DRB1 *03,*11,*12,*13 or *14	DQA1*01:03:01	DQB1*06:03:01
COX	8.1	DR3	A*01:01:01	B*08:01:01	C*07:01:01	DRB3*01:01:02	DRB1 *03,*11,*12,*13 or *14	DQA1*05:01:01	DQB1*02:01:01
DBB	57.x	DR4	A*02:01:01	B*57:01:01	C*06:02:01	DRB4*01:03:01	DRB1 *04, *09 or *07	DQA1*02:01:01	DQB1*03:03:02
KAS116	51	DR1	A*24:02:01	B*51:01:01	C*12:03:01		DRB1 *01 or *10	DQA1*01:01:01	DQB1*05:01:01
MANN	44.2/ 44.3	DR4	A*29:02:01	B*44:03:01	C*16:01:01	DRB4*01:01:01	DRB1 *04, *09 or *07	DQA1*02:01:01	DQB1*02:02:01
PGF	7.1	DR2	A*03:01:01	B*07:02:01	C*07:02:01	DRB5*01:01:01	DRB1 *15 or *16,	DQA1*01:02:01	DQB1*06:02:01
QBL	18.2	DR3	A*26:01:01	B*18:01:01	C*05:01:01	DRB3*02:02:01	DRB1 *03,*11,*12,*13 or *14	DQA1*05:01:01	DQB1*02:01:01
SSTO	44.x	DR4	A*32:01:01	B*44:02:01	C*05:01:01	DRB4*01:03:01	DRB1 *04, *09 or *07	DQA1*03:01:01	DQB1*03:05:01

by the class II HLA genes; the class III region, containing many genes involved in stress response, complement cascade, inflammation and other immunology functions.

All nucleated cells express HLA class I molecules and present cytosolic antigenic peptides to $CD8^+$ T lymphocytes, while HLA class II molecules are expressed by antigen-presenting cells (APC) and present extracellular antigenic peptides to $CD4^+$ T lymphocytes.

The most distinctive features of the region are the extraordinary polymorphism of MHC class I and class II genes, the high gene density, and the linkage disequilibrium (LD) originating through a dynamic evolutionary mechanism of natural selection. These characteristics play an essential role in adaptive immune responses through the presentation of exogenous and endogenous antigens.

The MHC region exhibits recombination rates lower than the genome-wide average, resulting in long-range haplotype structures. The term "haplotype" was created by Ruggero Ceppellini [8] [9] to define the combination of alleles at the different loci present in the HLA region, linked on one single chromosome six and shown to be in LD by family studies. MHC haplotype structures are generated by multiple mechanisms, including drift, selection, and reduced crossing-over rates. The term 'allele' refers to single or multiple point mutations, insertions, and deletions identified within a single gene or in a non-coding sequence. Some HLA haplotypes were initially designated either "conserved ancestral HLA haplotypes" (AHs) or "conserved extended haplotypes" (CEHs) [10] [11] and named according to the HLA-B gene [12]. For instance, the haplotype HLA-A*01:01-C*07:01-B*08:01-DRB1*03:01-DQA1*05:01-DQB1*02:01 was named AH (or CEH) 8.1, but if two or more AH carry the same B allele, sequential numbers are added, according to the order of discovery, such as for AH 7.1 haplotype (HLA-A*03:01-C*07:02-B*07:02-DRB1*15:01-DQA1*01:02,-DQB1*06:02) and AH 7.2 haplotype (HLA-A*24:02 -C*07:02 -B*07:02 -DRB1*01:01-DQA1*01:01-DQB1*05:01). Since the two definitions identified the same haplotype structure and the assignment of the different AHs and CEHs coincided, to standardise the nomenclature, the two research groups, that created the two terms, recently published an editorial together where they introduced the common term "conserved polymorphic sequences" (CPSs) to replace AHs and CEHs [13]. Nevertheless, since the term "AH" is still the most universally used, we have chosen to use it in the present text.

An important role in determining the genomic structural variants is explained by recombination mechanisms carried out by transposable elements Alu, SVA, HERVs and LTR. These elements have caused insertions, duplications, rearrangements, deletions and gene conversion events inside the MHC locus and, as a consequence, are excellent markers for establishing the evolution of haplotypes and gene duplication [14].

Inside AHs, "blocks" of conserved DNA stretches of 5–150 kb have been identified with the ability to recombine with blocks from other AHs, generating new haplotypes. These blocks include HLA-DRB3/4/5 and DQB1 or HLA-DRB1 and HLA-DQA1 genes. The HLA-DR/DQ blocks confirmed, by their variability, that ancient recombinations or gene conversion events generated new allelic combinations affecting frequency, ancestry, and ethnicity [12]. Later, publications by Majumder et al. [15–18] demonstrated that the regulation/expression of polymorphic alleles inside the HLA-DR/DQ blocks are transcriptionally regulated by CCCTC binding protein (CTCF) and class II transactivator (CIITA) factors interacting with the regulatory XL9 motif that, through loop structures, generated long-distance chromatin interactions.

First, Dorak et al. [19] and then Kulski et al. [14] reported the AH haplotype nomenclature including classical HLA class I and class II alleles combinations and other genes involved in the antigen presentation and genetic disease predisposition.

The availability of heterozygous and homozygous cell lines at the HLA region from different populations, providing standardised reference sequences, was essential for correct HLA genotyping. The first MHC genomic sequence variations in different haplotypes were produced by the Sanger Centre MHC Haplotype Project (SCMHP) using eight homozygous cell lines [20]. These sequences are now included in the current version of GRCh38 (Genome Reference Consortium Human Build 38) human reference genome at NCBI (https://www.ncbi.nlm.nih.gov), being classified as alternative reference sequences ("alt_ref") for the MHC region. Initially, the sequences of these haplotypes were not completely resolved until, by short-range and long-range next-generation sequencing (NGS), the eight homozygous cell lines were fully and correctly sequenced. Norman et al. [21] developed a method for complete sequencing of the entire MHC region from genomic DNA to analyse a panel of 95 cell lines homozygous at the MHC region. Houwaart et al. [22] provided a comprehensive reference panel covering eight MHC class II haplotype structures completely resolved by assembly HLA sequencing data obtained with different methods. These haplotypes were classified by the copy number of the HLA-DRB1-paralogous genes HLA-DRB3, HLA-DRB4 and HLA-DRB5 and their LD with major DRB1 variants defining four haplotype structures: DR1 (DRB1*01 or *10, with no DRB3-5), DR2 (DRB1*15 or *16, with DRB5), DR3 (DRB1*03, *11, *12, *13 or *14 with DRB3), DR4 (DRB1*04, *09 or *07 with DRB4). The fully resolved MHC haplotypes included the KAS116 cell line, which lacks HLA-DRB3, -DRB4, or -DRB5 genes, thus representing one of two major MHC class II sequence structures currently not present in GRCh38 (see Table 1). The MHC reference haplotypes were defined by integrating the data obtained by recent long-read sequencing technologies with those already present in IPD-IMGT/HLA databases [23]. IPD-IMGT/HLA Database (http://www.ebi.ac.uk/ipd/mhc/align.php) is an updated repository of about 24,093 HLA and related alleles and comprising over 362,709 distinct nucleotide variants compared to the reference sequence, collected from all countries of the world [24]. HLA data sets with class I and class II alleles frequencies for worldwide populations were reported at the site http://www.allelefrequencies.net/hla.asp.

3. HLA typing in the SNP era

Genome-wide association studies (GWAS) discovered thousands of susceptibility loci related to complex traits and autoimmune diseases and confirmed their polygenic properties. The success was due to the large number of subjects analysed, concerning family studies, and the correct selection of cohorts. In a cross-sectional cohort study, data may be gathered from a population at a specific time point while, in a longitudinal study, data are collected from the same sample over an extended period, for example before and after disease onset, or during disease progression and treatment. The identification of specific disease loci is also based on the selection of patient cohorts with respect to disease-free subjects, at-risk individuals as relatives, and the general population [25].

The HLA region contains the strongest risk genes for autoimmunity. The pathologies where antibodies play a pathogenic role, such as Celiac Disease (CD), Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Type 1 Diabetes (T1D) and others, have the strongest association with class II genes, while those not determined by antibodies, such as Ankylosing Spondylitis (AS), Psoriasis, Behcet's and others are associated with class I genes. Conventional methods for HLA typing included traditional polymerase chain reaction (PCR) and Sanger sequencing which are evaluated to be labour-intensive, slow, and expensive technologies. HLA typing has become faster and cheaper by both short-read and third-generation sequencing (TGS) which improved the identification of alleles. Differently, GWAS are collections of common single-nucleotide polymorphisms (SNPs) across many genomes and large cohorts to find those statistically associated with a specific trait or disease while whole-exome sequencing (WES) and whole-genome sequencing (WGS) data allowed the identification of HLA alleles imputing functional rare variants (lower than 1% frequency) with high accuracy [23]. Phenome-wide association studies (PheWAS) is a powerful tool for examining SNPs previously mapped with GWAS and associated with a disease phenotype. PheWas has been applied to the MHC locus and allowed the identification of variants with several autoimmune diseases and cross-phenotype associations or pleiotropy. This technology aimed to identify genotype–phenotype correlations in complex human traits by comparing allele frequencies among ancestrally similar individuals but different phenotypically. However, the correct assignment of SNPs to loci and their association with various phenotypes and diseases was based not only on the accuracy of reference sequences, with high coverage of alleles and genotypes, but also on the HLA imputation algorithms that provide geneticists with appropriate tools to infer HLA alleles at the classical loci [26].

4. The AH 8.1 predisposes to several autoimmune disorders mainly in Caucasians, but it is not the only one

AHs have been used to characterise human diversity and ethnic origin or to identify and localise disease susceptibility and resistance to different immune diseases [27,28] [29]. AH frequencies vary according to the human population and disease associations. For instance, AH 8.1 and AH 7.1 show high frequencies in Caucasian populations of European origin (8-7.4% for AH 8.1 and 3.0-3.5% for AH 7.1) and lower frequencies in African Americans (1.4% for AH 8.1 and 0.9% for AH 7.1), but are almost absent in Saudi, Japanese and Chinese populations [14].

The AH 8.1 influences several aspects of the immune response by altering the balance of the cytokines produced. Indeed, in healthy carriers of the AH 8.1, a prevalent type 2 profile of cytokine production that enhances humoral responses is observed, instead of type 1 cytokines that improve cellular response. This phenotype contributes to the increased production of autoantibodies and, thus, to the incidence of autoimmune diseases [30].

Other autoimmune diseases associated with AH 8.1 are the myositis syndromes, a heterogeneous group of rare autoimmune disorders characterised by muscle weakness, inflammatory cell infiltrates, and myositis autoantibodies, with HLA-DRB1*03:01 and HLA-B*08:01 predisposing alleles [31] as well as Sjogren's syndrome, a systemic autoimmune disorder characterised by lymphocytes infiltration of salivary and lacrimal glands [32] primarily affecting women with juvenile and elderly age-onset. A recent study on Syogren's syndrome, based on the multi-omic profiling of whole blood samples from a European cohort, allowed the integrated analysis of genetic, molecular and clinical data and the identification of new biomarkers associated with the disease outcome and the development of appropriate therapies [33]. The autoimmune liver diseases comprise three primary distinct hepatic pathological conditions: autoimmune hepatitis (AIH-1 and AIH-2), primary sclerosing cholangitis (PSC), and primary biliary cholangitis (or cirrhosis) (PBC) [34]. According to autoimmune serology, the two kinds of AIH are mainly associated with HLA-DRB1*03 and -DRB1*04, while other HLA-DRB1 allele associations were found with geographical and ethnic differences. On the contrary, the DRB1*15:01 allele plays a protective role toward AIH. PBC is associated with HLA-DRB1*08 in European and Asian populations. PSC is associated with HLA-DRB1*03:01 and HLA-B*08:01 and with HLA-B*13:01 in Europeans, African, and Hispanic Americans. Most of the patients are female, and either clinical characteristics or outcome diseases are related to oestrogen function [35].

Myasthenia gravis (MG) is caused by antibodies against the nicotinic acetylcholine receptor (AChR) and includes early-onset MG associated with AH 8.1 across different populations, while late-onset MG is characterised by significant heterogeneity of HLA class II alleles associations. Indeed, a positive association with HLA-DRB1*07, HLA-DRB1*14, and HLA-DQB1*02 was found in an Italian cohort of patients, whilst HLA-DRB1*03, HLA-DRB1*11, and HLA-DQB1*03 resulted in protective alleles [36]. A study on a Spanish MG cohort confirmed the association of AH 8.1 with an early onset phenotype and identified the DQB1*05:02, DQB1*05:03 and DQB1*03:01 alleles as novel risk factors, while the protective alleles may be different according to the clinical manifestation of the disease [37].

Both T1D and CD are associated with AH 8.1. The HLA class II haplotypes, DR3 (DRB1*03:01-DQA1*05:01-DQB1*02:01) and DR4-DQ8 (DRB1*04:01/05-DQA1*03:01, DQB1*03:02) confer the highest T1D genetic risk. The risk increases in the subjects carrying DR3 or DR4-DQ8 haplotypes in homozygosis, while the DR3/DR4-DQ8 heterozygous genotype is associated with risk by over 30-fold, a substantially higher risk determined by the presence of DQ heterodimers encoded in trans, in addition to the DQ molecules encoded in cis, on the immune cell surface. The DR4 haplotypes carrying the DQB1*03:01 allele and the DQB1*06:02 allele exert a protective effect [38].

High-risk HLA haplotypes such as HLA-DR4-DQ8 develop first islet autoantibodies targeting insulin (IAA) with seroconversion peaking in the first years of life and declining over the following years. At the same time, individuals with the HLA-DR3-DQ2 haplotype are more likely to develop glutamic acid decarboxylase GADA as a first autoantibody, with the most frequent seroconversion occurring until the second year of life and remaining relatively constant. Insulinoma-associated antigen-2 (IA-2 A), and zinc transporter 8 (ZnT8A) islet autoantibodies precede the clinical onset of disease. The islet autoantibodies characterise T1D risk by defining different stages: stage 1 defined by the presence of multiple IA with normoglycemia, stage 2 by the presence of IA with asymptomatic dysglycemia, and stage 3 corresponds to the onset of clinical hyperglycemia and symptomatic disease [39].

In addition to the primary association with HLA class II genes, class I genes are also involved in T1D predisposition [40]. The reason is the autoimmune attack driven by $CD8^+$ T cells, specific to defined islet antigens, against pancreatic cells that determine islet inflammation and progressive beta-cell loss. Interferon alpha (IFNα) signalling is crucial to the early stages of T1D pathophysiology because it determines endoplasmic reticulum stress, insulitis, and HLA class I overexpression. HLA-B*39:06 and HLA-A*24:02 are the primary class I risk alleles associated with early disease onset. HLA-B*39:06 enhances the risk of T1D in

individuals carrying the DR4 or DR8-DQB1*04 genotypes, whilst HLA-A*24 was proposed to interact preferentially with HLA-DQ8 or DQ8/DQ2 and HLA-B*18 with HLA-DQ2 [41]. HLA-A*01:01 and -B*08:01 are additional independent risk alleles for Celiac disease (CD) at the HLA region, in LD with DR3-DQ2.5, as demonstrated by GWAS [42] and functional studies [43].

Many other genes have been identified outside the HLA region contributing to the genetic risk of diseases and the researchers elaborated the genetic risk score (GRS), a model based on a subset of genetic variants that reach genome-wide significance. GRS contributes to diabetes classification, between type 1 diabetes, type 2 diabetes and MODY, to the prediction of infants at risk and the progression of pathology [44].

T1D co-occurs in families with CD: approximately 4–9% of patients with T1D are affected by CD, while the patients with CD have an increased risk of developing T1D. The risk for CD associated with T1D is 95% for HLA-DQ2 (DRB1*03:01-DQA1*05:01-DQB1*02:01) carriers, 5% for HLA-DQ8 (DRB1*04-DQA1*03:01-DQB1*03:02) carriers [45]. CD and T1D share immunopathogenic mechanisms, although the autoantigens target of autoreactive T cells and autoantibodies are different: IIA, GADA, and IA-2 A in T1D [46] and tissue transglutaminase (tTG) in CD [47]. Tissue transglutaminase can modify gluten antigens in CD and islet autoantigens in T1D, increasing the binding to HLA-DQ2 molecules and activating pathogenic $CD4^+$ T cells.

Like CD and T1D, the post-translation modification (PTM) is a process also common to RA in which, following the citrullination process, the production of autoantibodies specific against citrullinated peptides and activation of the complement system was observed. RA and SLE are common autoimmune rheumatic diseases. SLE is associated with HLA-DR3/ DR2/DR8 and RA with the HLA-DR4 genotype although GWAS suggests that RA and SLE share common genetic components such as SNP profile and the overexpression of some factors of the Type I interferon (IFN) pathway [48,49]. Both diseases reached an advantage in the early diagnosis and accurate prognosis by the recent advancements in artificial intelligence (AI) thanks to the use of machine learning (ML). The analysis of clinical, laboratory, genetic data and radiological images, if available, improved the correct diagnosis and the prediction of the risk of hospitalization and complications, in addition to the response to therapy. Many other systemic autoimmune diseases will take advantage of specific algorithms able to define specific parameters that develop personalised treatment strategies, improving patient care and outcomes [50] [51].

5. AH 8.1 protects from infections but predisposes to autoimmunity

Some haplotypes carrying the genetic trait of being high responders against infections have been evolutionarily selected. However, this genetic trait predisposes individuals to develop hypersensitivity-based inflammatory diseases, like autoimmunity, as a direct complication of infection [52]. AH 8.1 contains several HLA genetic variants influencing antigen presentation, tumour necrosis factor (TNF) levels, and complement proteins. Inside this haplotype, HLA-B*08:01 and HLA-DRB1*03:01 alleles show extensive conservation, especially in Caucasians, and may develop a protective role conferring resistance to some infections. Almost all patients with cystic fibrosis (CF) suffer from lung infection by *S. aureus* and *P. aeruginosa*, followed by inflammation, causing a reduced life expectancy. The CF patients carrying AH 8.1 exhibited a statistically significantly lower frequency of bacterial colonisation and delayed onset than non-carriers [53] [54] [55].

Microbial infections are the most invoked environmental factors in the development of autoimmunity in individuals bearing susceptible HLA haplotypes. Molecular mimicry between autoantigens and microbial proteins consists of the intermolecular epitope spreading. The consequence is antibody promiscuity, which is the ability of an antibody to bind multiple different antigens or epitopes, dissimilar molecules, and structures, leading to autoimmune states. EBV (Epstein Barr Virus), CMV (Cytomegalovirus), PVB19 (Parvovirus B19), Rotavirus, Enterovirus and many other pathogens are included in the list of the aetiologic factors causing infections leading to overt autoimmunity in some patients [56]. AH 8.1 is associated in Caucasians with several autoimmune diseases involving the gastrointestinal system, such as CD and autoimmune liver diseases, resulting thus linked to alteration of gut microbiota [57] [58]. SARS-CoV-2 demonstrated that the virus interferes with several aspects of the immune system, leading to the activation of autoreactive T and B cells, and inducing the synthesis of autoantibodies related to multiple ADs such as systemic sclerosis, myositis, SLE, Sjogren's syndrome, gastrointestinal, rheumatic, and triggering latent or new onset of autoimmune thyroid diseases (AITD), such as Hashimoto Thyroiditis (HT) and Graves' disease (GD). Recent studies identified 23 peptides shared between human and SARS-Cov2 proteomes, some of which can bind to the HLA class I alleles, showing a greater potential to induce autoimmune responses through the MHC class I pathway. Specifically, Karami et al. found 4 peptides binding HLA-B*08:01, HLA-A*24:02, HLA-A*11:01 and HLA-B*27:05 [59] [56]. In acute COVID-19 patients, the presence of auto-antibodies neutralizing Type I IFNs has been implicated in almost 20% of deaths. EBV reactivation has been described to take place in SARS-Cov-2-infected individuals and EBV and SARS-Cov2 co-infections related to the onset of autoimmunity. Molecular mimicry has been found between peptide epitopes derived from several human proteins and EBV or SARS-Cov-2 protein-derived peptides [60]. Epidemiological, serological and virological evidence demonstrated the causal role of EBV in MS either through aberrant presentation of autoreactive peptides by HLA-DRB1*15:01 risk allele and alteration of immune response [51].

6. HLA expression in autoimmunity

The majority (>90%) of putative causal variants associated with autoimmune diseases are in noncoding regions of the genome affecting the gene regulation traits, such as gene expression, splicing, and chromatin phenotypes, collected by eQTLs (expression Quantitative Trait Loci) studies. Translating these noncoding variants in phenotype or clinic manifestations is difficult because linkage disequilibrium complicates their identification, as causal variants can be multiple in a single locus. In addition, during allele imputation, the high polymorphism and the extended and complex haplotype structure at the HLA region have been considered [61].

The exons encoding the peptide-binding groove have been studied to determine HLA effects on disease susceptibility/pathogenesis [62]. More recently, HLA expression levels have been implicated in autoimmune disease outcomes. The increased surface presentation of triggering self-antigens by HLA molecules may enhance the probability of activating pathogenic T cells. HLA class I genes are constitutively expressed by all nucleated cells, while class II gene expression varies across cell states within cell types, according to well-known molecular pathways. Indeed, since both HLA class I and II genes regulatory pathways including differential splicing, transcriptional and post-transcriptional regulation, epigenetic and post-translational modifications have been widely clarified [63] [64], many recent studies focused on the meaning of HLA genes differential allelic expression (DAE) in disease development and progression. DAE measures the expression of one allele of a gene relative to the other in a diploid individual and is generally caused by cis-regulatory mechanisms. HLA expression has traditionally been measured by antibody staining of cell surface molecules, microarray or quantitative PCR (qPCR) on total mRNA. Over the past decade, the high-throughput technology RNASeq allowed the evaluation of HLA expression in a genome-wide context.

Moreover, the high polymorphism and the presence of paralogue genes implicated a great difficulty in quantifying the expression. The alignment of short reads to a reference genome was needed to provide a complete representation of the HLA allelic diversity. These difficulties stimulated the development of computational pipelines and algorithms

[65], accounting for known HLA multiplicity, in the alignment step. Initially, HLA type and expression were determined through the seq2HLA algorithm utilising the RNA-Seq reads [66]. After that, the AltHapAlignR software analysed RNAseq data by alignments to the available multiple reference sequences data [67], while HLApers is a personalised pipeline provided to integrate expression quantitative trait loci (eQTL) data with HLA allele expression information from RNA-Seq. [68]. scHLAcount enables HLA allele-specific expression quantification from single-cell RNA-Seq data based on a personalised reference using UMI Unique Molecular Identifiers [69], while the scHLApers pipeline uses individual reference genomes to quantify single-cell HLA expression. By integrating four diverse datasets from multiple tissues and cell states, Kelly et al. [49] and Kang et al. [70] showed that HLA-DQ is the most variable expressed gene under cis-regulation.

7. HLA expression at the haplotype level

The eQTLs may influence gene expression in the HLA region through a single SNP or several haplotype-specific SNPs. Variants may be located in a non-coding region and affect gene expression regulation through the involvement in the splicing or chromatin phenotype or directly acting on the transcription and enhancer function. Colocalisation methods integrating GWAS and eQTL studies allowed the identification of causal variants that lead to a particular quantitative phenotypic trait or disease [71] [65].

Differences in the expression may involve the entire haplotype or a specific allele. Haplotype-specific differences in gene expression were found by analysis of homozygous B-LCLs carrying three haplotypes causing autoimmunity: COX (HLA-A1-B8-Cw7-DR3) associated with T1D, SLE and MG, PGF (A3-B7-Cw7-DR15) associated with protection from T1D and susceptibility to MS and SLE, and QBL (A26-B18-Cw5-DR3-DQ2) associated with T1D and GD (Table 1). Differences in the transcription and alternative splicing were initially considered to explain divergent expression profiles among the haplotypes [72].

More recently, by analysing the expression pattern obtained by RNASeq of HLA-homozygous B-LCLs and mapping of known eQTL, Lam et al. [73] confirmed that alleles are differentially expressed in distinct haplotypes. They demonstrated that, by comparing the two Chinese haplotypes A2-B46-DR9 and A33-B58-DR3 to the European haplotype A1-B8-DR3, eQTL SNP alleles are segregated by haplotype, exerting, therefore, haplotype-specific gene regulatory effects. Pelikan et al. [74] compared chromatin conformation and gene expression of the two risk haplotypes HLA-DR3 and HLA-DR15, associated with multiple sclerosis. They described the function of the histone quantitative trait loci hQTLs, which are variants with the potential to determine an allelic expression imbalance, altering the enhancer function through post-translational modifications of histones.

We already mentioned the presence of transposable elements in the MHC locus that determines the genomic haplotype structure but also influences gene expression. Kulski et al. analysed the regulatory properties of eight SVAs and demonstrated that were associated with differential co-expression of 71 different genes within and 75 genes outside the MHC locus, indicating that transposable elements might have potential roles in diseases [75].

Patients affected by RA, ACPA (anti-citrullinated protein antibodies)-positive, express particular HLA-DR molecules that share a 5 amino acid motif on their beta chain, charged and basic. This group of alleles, named “shared epitope (SE) alleles”, are HLADRB1*04:01, *04:04, *04:05, (HLA-DR4), HLA-DRB1*01:01(HLA-DR1), HLA-DRB1*10:01 (HLA-DR10). There is a dose effect of shared epitope positive HLA-DRB1 alleles on the risk of developing RA: subjects with two shared epitope positive HLA-DRB1 alleles are at higher risk than those with one shared epitope positive HLA-DRB1 allele, and subjects with no shared epitope positive HLA-DRB1 allele are at the lowest risk. We suggest that the risk of disease should be related to the risk alleles expression in addition to the number of epitope binding sites [76].

8. How HLA class I expression affects disease development

HLA class I mRNA and surface protein quantitative differences may influence immune response and susceptibility to several autoimmune diseases. Based on the new capture RNA-Seq method, Yamamoto et al. [77] demonstrated the highest expression of the HLA-B gene with respect to HLA-C and HLA-A, the low interallelic differences in the transcript amount and in the individual disparities of the HLA class I compared to class II loci. More recently, RNASeq and eQTL data [78] confirmed a coordinated and paired expression of both alleles of the same HLA class I genes and the same hierarchy of expression (HLA-B > C > A). Aguiar et al. [79] analysed Geuvadis (www.internationalgenome.org) consortium data from B-LCLs. They found that HLA-B is the highest expressed gene, followed by a comparable density of HLA-A. At the same time, HLA-C was about 50% of the HLA-B expression, confirmed by flow cytometry measurement [80]. Lately, an RNA-Seq method, based on UMI [69], showed that HLA-B and HLA-C gene expressions were equally abundant and about two times higher than the expression of HLA-A. Finally, eQTL analysis did not reveal variants affecting the bulk expression of the three HLA class I genes together, and each locus thus seemed to have its transcription regulatory elements [71]. In summary, by different datasets and analysis methods, the cited works reached conclusions slightly different, but they all documented the individual variation and the balanced expression among both alleles in heterozygotes [78].

The HLA-C gene has been associated with either viral infection or autoimmunity. Indeed, HLA-C high expression was correlated to more efficient recognition of HIV-1 virus by cytotoxic cells and low viremia in patients [81]. This is determined by the protective effect of a rs9264942T > C polymorphism located 35 kilobases upstream of the transcription initiation of the HLA-C gene [82]. Individuals with high expression of HLA-C alleles progress more slowly to AIDS and control viremia significantly better than individuals with low HLA-C expression alleles, causing a more efficient antigen presentation to cytotoxic T cells. The high HLA-C expression is disadvantageous in inflammatory autoimmune diseases such as Crohn’s disease. Crohn’s disease and Ulcerative colitis (UC) are two pathological manifestations of inflammatory bowel disease (IBD). They are disorders of the digestive tract, both primarily associated with HLA-DRB1*03. A second HLA association is with the HLA-C*01 molecule, which may present intestinal microbiota antigens. Moreover, in Crohn’s disease, the high HLA-C expression determines a deleterious outcome of the disease, while lower HLA-C expression exerts a protective effect [83]. This phenotype is related to the variation of miR148a expression, whose target site is located in an insertion/deletion polymorphism at position 263 of the HLA-C 3′UTR. This variation in the miR-148a expression is also linked to the polymorphism of the locus encoding miR148a, and it has a significant effect on the risk of Crohn’s disease and HIV-1 viral control.

Based on GWAS data of a Japanese population, Okada et al. [84] demonstrated that the HLA-C*12:02-B*52:01-DRB1*15:02 haplotype confers a susceptible effect on UC, but at the same time, a protective influence on Crohn’s disease. This haplotype includes the eQTL at rs9264942, above described, that regulates HLA-C expression in the Japanese population [85]. The same rs9264942C allele and the related phenotype were associated with psoriasis vulgaris, an immune-mediated inflammatory disease of the skin, in which the SNP is always in LD with HLA-Cw*06 [86].

High HLA-B27 expression level, quantified by flow cytometry on PBMCs, was reported in patients with ankylosing spondylitis (AS) and correlated with susceptibility but not disease outcome [87] [88]. Yang et al. [89] proposed that microbial and self-antigens presented by HLA-B*27 to T cells induce an inflammatory process independent from the antigenic trigger.

In GD and HT autoimmune thyroid pathologies, the activation of T and B lymphocytes reactive to the thyroid leads to the clinical manifestations of hyperthyroidism in GD and hypothyroidism in HT. GD is

primarily associated with HLA-DR3, which is in LD with HLA—B8, and immunostaining on thyroid tissue samples from patients showed HLA class I upregulation [90]. Also, HT, associated with HLA-DR3 and DR4 haplotypes, showed an upregulation of HLA-class I in thyrocytes from patients [91].

HLA class I gene expression measurement has been assessed in primary autoimmune vitiligo whose risk has been associated with an SNP haplotype 20 kb downstream and in strong LD with the HLA-A*02:01 gene. This risk haplotype includes several variants in LD, arising from a transcriptional regulatory element with an open hypomethylated chromatin configuration, causing the high expression of the HLA-A*02:01 risk gene [92].

We already mentioned the association of T1D with HLA-B*39:06, HLA-A*24:02, HLA-B*18 and the consequent activation of $CD8^+$ cytotoxic T cells leading to organ damage. We highlight here that the class I molecules are upregulated [93] following the increased expression of the transcriptional activator NLRC5 that acts as a regulator of alternative splicing mechanism and contributes to neoantigens presentation by class I molecules on Langerhans beta cells [94,95].

9. How HLA class II expression affects disease development

High MHC class II expression may influence the development of autoimmunity by facilitating the reaching of the MHC-peptide-complex threshold needed to be presented to $CD4^+$ T lymphocytes for cell activation and proliferation. Autoimmune vitiligo is associated to DR53 haplotype (with DRB3*01:01 or DRB4*01:01 genes) carrying three SNPs in LD, spanning just 47 nucleotides between HLA-DRB1 and HLA-DQA1. This SNP-haplotype maps to the intergenic region within a predicted transcriptional super-enhancer element binding several factors, active in monocytes and responsible for the HLA-DQ and HLA-DR increased molecule density, thereby facilitating enhancement of presentation of vitiligo autoantigens [96]. One of these SNPs, rs9271597A, associated with both early- and late-onset vitiligo, was found in LD with rs145954018del. Both variants, representing a high-risk haplotype associated with either severe vitiligo risk or early disease onset, are located within lymphoid-specific transcriptional enhancers and are associated with increased expression of HLA-DQ protein on peripheral monocytes and dendritic cells.

Johansson et al. [69] compared the expression of class II genes among several common haplotypes from PBMC samples of healthy individuals. They revealed that the haplotype DRB1*03:01-DQA1*05:01-DQB1*02:01 (named H1), associated with CD, was more expressed than haplotype DRB1*04:01-DQA1*03:01-DQB1*03:02 (called H6), associated with CD and T1D. Moreover, they demonstrated that low-expression DQA1 alleles were paired with DQB1 alleles with a low expression as well, and it was the same for high-expression DQA1 and DQB1 alleles. Conversely, a similar correlation was not found between DRB1 and DQ alleles. The distribution at the expression level based on reading numbers for the HLA-DQA1 alleles ranged between low expression for DQA1*01/05 alleles, intermediate expression for DQA1*02/04/06 alleles, and high expression for DQA1*03 allele. The DAE for HLA-DQB1 ranged from low expression for DQB1*02/03/04 to high expression for DQB1*05/06. Analogous results were obtained by Yamamoto et al. [77], who, through the capture RNA-Seq method, investigated the correlation between HLA-DQ haplotypes and RNA expression and confirmed very high allelic differences at DQA1 and DQB1 loci.

However, the data obtained by RNA-Seq do not agree with the results from our group obtained by qPCR. We demonstrated that, in heterozygous APC, either PBMCs or B-LCLs derived from patients affected by acute CD, the risk allele DQA1*05 is 2-3-fold more expressed than the allele DQA1*01 not associated with disease and located on the other chromosome, as well as the DQB1*02 risk allele is more abundant than DQB1*05, not associated with the pathology [97]. These CD patients carry the AH 8.1 haplotype in which the DQA1*05 and DQB1*02 disease predisposing alleles and HLA-DRB1*03 are in LD and compose the *cis* haplotype DR3-DQ2.5 [98] classified at high risk for CD in the Caucasian population. DQA1*05 and DQB1*02 risk alleles also keep high expression when are in the *trans* configuration in the genotype DR5-DR7 [99]. Although we used allele-specific primers that might have different amplification efficiency, our results of DAE for risk alleles were confirmed by measuring the effective surface amount of DQα1*05 and DQβ1*02 single chains, using specific monoclonal antibodies. We assessed that APCs carrying the genotypes homozygous DR3-DQ2.5 or heterozygous DR3-DR1/DQ2.5-DQ2.5 and DR5-DR7/DQ7-DQ2.2, express high levels of DQα1*05 and DQβ1*02 protein chains [97]. The effect was a comparable DQ2.5 heterodimer density on APCs, either homozygous or heterozygous, generating a similar number of HLA–-gliadin complexes. Consequently, the activation strength of pathogenic $CD4^+$ T cells specific for gliadin was equal and independent from the APC genotype (carrying one or two copies of risk alleles). The DQ2.5 DAE was also demonstrated in macrophages from CD patients and involved the HLA-DRB1 gene. Indeed, we showed that HLA-DRB1*03, in LD with DQ2.5 risk alleles, was more expressed than the DRB1 alleles on the other chromosome [100].

The expression analysis was extended to heterozygous B-LCLs and PBMCs from patients affected by T1D carrying HLA-DQ2.5 and HLA-DQ8 genotypes. We demonstrated, by qPCR, a differential expression of DQA1*05 and DQB1*02 in APCs from patients affected by CD and T1D comorbidity and DAE involving DQA1*03 and DQB1*03 alleles when in heterozygosis with alleles non-T1D associated [101]. High expression of DQA1*03:01 was previously demonstrated by others [102] [103]. Still, it remains to be clarified if the DAE is a consequence of autoimmunity or it is related to the APC genotype.

Several genetic studies showed that the predisposing role of HLA-DRB1*15, *03, and *01 to MS was markedly higher in people who are homozygous in comparison with heterozygotes individuals, revealing a dose effect not only for risk alleles but also for the protective allele HLA-DRB1*01 [104]. Our study concerning the HLA-DRB1 expression in PBMCs from MS patients of Southern Italy demonstrated that both MS-associated DRB1*15:01 and DRB1*03:01 alleles showed much higher expression compared to non-associated alleles in heterozygous PBMCs [105]. Exploring two expression datasets, Alcina et al. [106] detected eQTLs associated with the upregulation of HLA class II genes in several autoimmune diseases and identified the polymorphism rs3135388 correlated to high expression of DRB1*15:01.

A relevant result from our group, in both CD and MS, was that the quantitative difference between the mRNA transcribed by risk alleles associated with disease and the non-associated ones is lower in healthy subjects than in patients. If this finding is confirmed in a more significant number of healthy donors, DAE increment between patients and healthy subjects might be used as a diagnostic issue. Indeed, we propose that, in addition to the regulatory mechanisms inside the haplotype, additional factors outside the HLA locus affect HLA expression during autoimmune disease [107].

As mentioned above, Lam et al. [73] demonstrated that differential expression of several DQ and DR alleles in the three haplotypes A33-B58-DR3, A2-B46-DR9, and A1-B8-DR3 was controlled by functional variants in LD at a distance >350 KB. The comparison of their results with ours, relative to the mRNA quantization of class II alleles in CD patients, is complex since different methods were used.

A recent work [108] analysed full-length cDNA sequencing data from Oxford Nanopore Technologies' MinION and characterised HLA-DQB1 mRNA isoforms, deleted of exon 4 or 5 or both at the allele level. The skipping of exon five was determined by a G > A substitution at the splice acceptor upstream exon 5. The skipping of exon 4, observed for HLA-DQB1*03 or 06 transcripts but never HLADQB1*02 or 05, was determined by cis-acting elements in intron 3. In both cases, transcripts synthesise soluble DQ protein lacking the transmembrane domain, affecting surface expression and antigen presentation.

The two alleles HLA-DRB1*03:01 and DRB1*15 are strongly

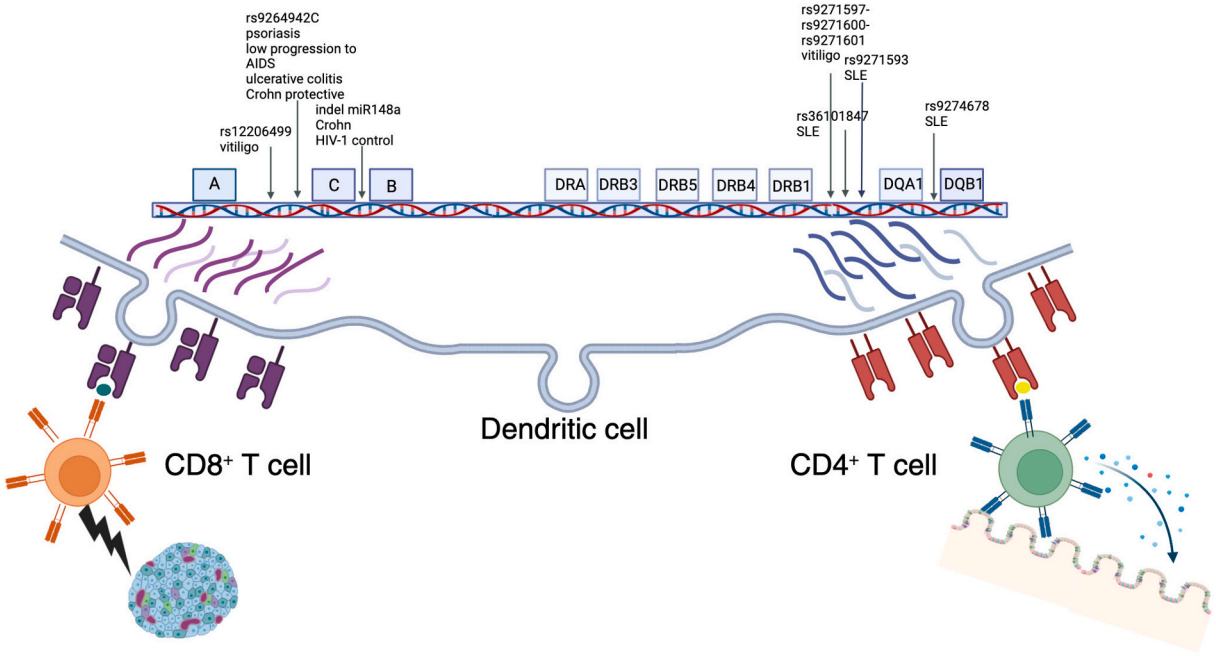

Fig. 1. Graphical representation of the position of SNPs and indels that, by enhancing the expression of HLA class I and class II genes, affect the number of complexes presenting self-antigens and influence the strength of CD4 and CD8 T cells pathogenic immune responses. The figure was realised using the graphical program Biorender.

associated with SLE susceptibility. The first is correlated with SLE in the European populations and is rare in the East Asian populations, while both populations share DRB1*15.

Raj et al. [109] identified three SNPs strongly associated with SLE, the most notable of which was rs9271593, mapping to the XL9 regulatory component within the intergenic region separating DRB1 and DQA1. Other SNPs are located upstream of the DQB1 promoter and near DRB1 exon 2. The XL9 region shows high acetylation levels associated with more accessible chromatin, in which binding sites for many transcription factors, especially CTCF and ZNF143, have been identified. These regulatory factors assemble HLA-DR and DQ proximal promoters into a transcriptional complex that facilitates coordinated and tissue-specific transcription in lymphoid, myeloid, and thymic epithelial cell lineages. The authors confirmed by RNASeq, eQTL and flow cytometry analysis that the presence of variants increases HLA-DR and -DQ expression in myeloid cells, and the increment was determined by IRF4 transcriptional factor binding to the XL9 domain. More recently, two super-enhancers (SE) defined XL9-SE and DR/DQ-SE were identified between DRB1 and DQA1 in B cells [110]. Specifically, the first does not directly regulate HLA expression but supports the function of the second by histone modification, in which deletion reduces DR and DQ gene expression. Both SEnhs regulate the three-dimensional chromatin structure through the control of CIITA and CTCF binding site interactions, already investigated by the same authors [15–18]. It should be exciting to investigate if the SNPs identified in SLE [109] or hQTL [74] disrupt the function of these SEnhs. The DAE associated with eQTL should explain the DQA1*05 and DQB1*02 expression increase that our group found in CD [97], a hypothesis to be demonstrated.

The rs9271593 variant has been found in LD with DRB1*15:01, 02, 03 in SLE Japanese patients. Kawasaki et al. [111] reported that substituting G with T in the rs2105898 variant disrupts G's binding site for the transcription factor ZNF143 in the XL9 motif, confirming the functional significance of variants inside XL9.

hQTL analysis revealed differences between the HLA-DR3 and HLA-DR15 haplotypes associated with MS [74]. Specifically, the hQTLs observed in the HLA-DR15 subjects correspond to H3K27ac sites, enhancers associated with high activation of transcription and high HLA-DQA1 and HLA-DQB1 gene expression. Analogous enhancers located in the HLA-DR3 haplotype, near the HLA-DRB1 promoter, contribute to the increased expression of this gene. Differently, hQTLs defined by the presence of H3K4me1 are weak enhancers with poor effect on the expression of flanking alleles.

The sleep disorder type 1 narcolepsy consists of the autoimmune destruction of hypocretin/orexin neurons in the hypothalamus; it is triggered by infection with the HIN1 influenza virus, and it is associated with HLA-DQA1*01:02-DQB1*06:02 alleles encoding the DQ heterodimer DQ06:02. By exploring genome-wide expression differences in PBMCs, Weiner et al. [112] demonstrated that patients homozygous for HLA-DQB1*06:02 showed a 1.65-fold higher mRNA expression than heterozygous, with a difference also evident at the protein level on the B cells surface. They suggested that partial promoter methylation and silencing of one DQB1*06:02 allele in the APCs of homozygous patients explain the expression not exactly twice that of heterozygous patients, as expected.

10. Conclusion

Polymorphic amino acid epitopes and different expression levels may drive the associations between certain HLA types and diseases. High surface expression of HLA class I or class II molecules increases self-antigens presentation. It may amplify the probability of autoreactive T-cell activation, thus leading to organ damage (Fig. 1). We highlight the relevance of haplotype structures in the association with autoimmune diseases and the peculiarity of some haplotypes to carry the susceptibility to multiple autoimmunities. In the latter case, the occurrence of one autoimmune disease rather than another might depend on the polymorphisms located at loci other than HLA and the different environmental triggers encountered. Moreover, the gene blocks inside the same haplotype may carry different SNP arrays. Thus, each haplotype contains a unique array of information encompassing the distinct allelic gene products and their quantitative expression level. In addition, the references cited demonstrated that not only the SNPs in the promoter sequences or close regulatory motives affect the allele-specific expression but also the mRNA isoforms, epigenetic factors and long-range

Table 2
Autoimmune diseases associated with differential expression of HLA risk genes/alleles and references concerned.

Autoimmune disease	HLA risk genes/ alleles	Population	Major Triggers	Reference
AS	HLA-B*27		Fungi,*Klebsiella Pneumoniae*	84,85
CD	HLA-DQ2-DR3 (DRB1*03:01, DQA1*05:01, DQB1*0201)	Caucasian, USA	Rotavirus	97–100
Crohn's disease	HLA-C	Japanese, Caucasian, USA	Smoking, Fungi, Gut microbioma	83,84
GD	HLA class I	Caucasian	*Yersinia enterocolitica, Helicobacter pylori* and Borrelia burgdorferi, gut microbioma	90
HT	HLA class I	Japanese, Caucasian	Enterovirus, PVB19 gut microbiota, dietary iodine	91
MS	HLA-DRB1*03:01 and DRB1*15	Caucasian, USA	EBV,HBV, PVB19	104–106
Narcolepsy	DQB1*06:02	Caucasian, USA	Diet, Pesticides, H1N1 influenza A	112
Psoriasis	HLA-Cw*06	Caucasian	Drugs, smoking, drinking, diet, infection and mental stress.	86
SLE	HLA-DRB1*03:01 and DRB1*15	Caucasian, Asian	Gut microbiota, smoking, UV, viral and bacterial infection	109
T1D	HLA-DQ2-DR3 (DRB1*03:01, DQA1*05:01, DQB1*0201) B*18:01, B*39:06, B*39:06	Caucasian	CMV,Rotavirus, Enterovirus, SarsCov-2	101–103, 93–94
Vitiligo	HLA-A*02:01, HLA-DRB1, HLA-DQA1	Caucasian, USA	Chemicals exposure	92,96

Abbreviations. AS: Ankylosing Spondylitis; CD: Celiac Disease; HT: Hashimoto's Thyroiditis; GD: Graves Disease; MG Myasthenia Gravis; MS: Multiple Sclerosis; SLE: Systemic Lupus Erithematosus; T1D: Type 1 Diabetes.

chromatin structures.

In conclusion, we proposed that:

1) differential expression of HLA risk alleles is a phenomenon common to different autoimmune diseases (Table 2);
2) autoimmune disorders carrying the same HLA haplotype show the same DAE;
3) the mechanism explaining the different expression of predisposing alleles might be haplotype-specific;
4) differential expression of risk alleles is a phenomenon of pathological conditions and not of healthy subjects.

CRediT authorship contribution statement

Silvia Sartoris: Writing – review & editing, Writing – original draft. **Giovanna Del Pozzo:** Writing – review & editing, Writing – original draft.

Data availability

No data was used for the research described in the article.

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