

## Autoimmune Cytopenias

Autoimmune diseases are caused by a combination of genetic, epigenetic, and environmental factors and affect approximately 5% of the population [9] [12]. Over 80 autoimmune diseases have been identified and their incidence is increasing [11] [12].

While each individual autoimmune disease may have a prevalence between 0.01-3% and affect a relatively small number of people, multiple autoimmune or inflammatory diseases are observed within the same individuals or family members more frequently than would be expected if the genetic factors and biological pathways involved with different diseases were completely independent, suggesting that some diseases share common processes [4] [11]. The cost of DNA sequencing has decreased substantially and has allowed for more genetic studies [54]. However, understanding how genetic variations affect gene function and ultimately lead to the development of autoimmune diseases has been challenging [12].

Immune thrombocytopenia (ITP) and autoimmune hemolytic anemia (AIHA) are two autoimmune cytopenias that are characterized by the antibody-mediated destruction of platelets and red blood cells, respectively [10]. As will be discussed later, both diseases are rare, heterogeneous, involve similar pathways, and can occur simultaneously or sequentially in some individuals. There is significant overlap in the therapies used for ITP and AIHA, particularly corticosteroids, splenectomy, and rituximab. Finding genetic factors that are associated with increased susceptibility or that can predict the progression of symptoms or responsiveness to different treatments has been difficult because of the rarity and complexity of the diseases.

### *Genetic Factors*

It is well understood that variations in genes within the human major histocompatibility complex (MHC) are significantly involved with increased susceptibility to autoimmune diseases [11] [12]. Genome-wide association studies (GWAS) have discovered approximately 100-200 common genetic variations outside the MHC locus that are associated with one or sometimes more autoimmune diseases [11] [12]. Interpreting the results of GWAS is difficult for several reasons. Many of these genetic variations have relatively low odds ratios ( $OR \leq 1.5-2$ ), so each individual variation does not contribute significantly to susceptibility [12]. GWAS explain only a small percentage of heritability and do not take gene-gene interactions into account [53]. While identifying interactions between genes can be extremely complicated, ignoring their existence may result in incorrect estimates of heritability [53]. Another issue is that some genetic variations that increase susceptibility to one disease can actually be protective against a different disease [4] [11].

GWAS have focused primarily on searching for common single nucleotide polymorphisms (SNPs) [54]. Common genetic variants are defined as having a minor allele frequency of greater than or equal to 0.5% [54]. Genetic variants with minor allele frequencies above 5% can usually be readily identified, while variants with frequencies between 0.5-5% are somewhat more challenging [54]. There can be value in searching for common variants that occur less frequently; for example, the discovery of low-frequency nonsense mutations in the *PCSK9* gene facilitated the development of novel treatments for LDL cholesterol [54].

Rare variants (minor allele frequency  $< 0.5\%$ ) have started to receive more attention as well [54]. It has been hypothesized that the human genome contains significantly more rare than common genetic variants and that rare variants could make “substantial” contributions to the heritability of multi-factorial diseases [47]. Finding and analyzing rare variants is also challenging. The effects of rare missense mutations on gene function are not immediately obvious, especially when the mutations occur in non-coding regions, and accurate interpretations may require additional biological knowledge with respect to regulatory sequences for the gene [54].

Other genetic variations include copy number variations, insertions, deletions, translocations, and tandem duplications [2]. Studies have indicated that “more base pairs are altered as a result of structural variation –

including copy number variation – than as a result of point mutations” in the human genome [2]. Copy number variations are associated with a number of autoimmune diseases [26] and it is possible that other structural variations also contribute to susceptibility. However, identifying structural variations is complicated since they are typically situated in repetitive DNA and vary in size [2].

The rarity of some autoimmune diseases increases the difficulty of conducting larger studies [11]. Statistical significance thresholds in genetic studies must be set very high in order to avoid false positives, but studies may have too few participants and genetic variants may have too high statistical significance to be associated with increased susceptibility to a disease [4]. Applying biological knowledge of gene function and pathways may justify lowering the threshold to broaden the search and could help discover new genetic variants that would have otherwise been disregarded due to low statistical significance [4]. Genetic variants that are associated with multiple diseases are also of interest [4] [11]. Cotsapas et al developed a cross-phenotype meta-analysis statistic to analyze the associations between 107 SNPs outside of the human MHC and multiple autoimmune diseases and discovered that 47 of the SNPs were associated with at least two diseases [11]. One of the goals of the study was to “identify groups of diseases that should be considered as a unified phenotype and analyzed together”, which can provide additional information that would be more challenging to obtain by studying only individual diseases [11].

### *Epigenetic Factors*

Concordance rates in monozygotic twins for autoimmune diseases are oftentimes low, indicating that susceptibility is influenced by other factors besides genetics [9].

Epigenetics is the study of changes in gene expression that are heritable, potentially reversible, and do not involve modifications in the DNA sequence [9] [34]. Epigenetic modifications regulate gene expression throughout the cell cycle and are applied or reversed, sometimes rapidly, in response to biological or environmental factors [9]. Epigenetic modifications include DNA methylation and histone modifications, which regulate transcription, and microRNAs (miRNAs), which provide post-transcriptional regulation and may also have roles in the regulation of transcription [6] [40]. These modifications may interact with each other to regulate gene expression; for example, DNA methylation and miRNAs have been observed to support or work against each other to regulate gene expression in rheumatoid arthritis [40].

DNA methylation involves the addition of a methyl group to the 5' carbon of the cytosine ring in CpG dinucleotides [9] [20]. CpG dinucleotides occur at significantly higher frequencies in certain regions of the genome, which are known as CpG islands [20]. The rest of the genome is mostly devoid of CpG dinucleotides [20]. Approximately 60% of human genes have promoter regions that contain CpG islands, and methylation at or within close proximity of transcription start sites can suppress transcription initiation, although elongation is not affected [20]. Most CpG islands at transcription start sites are not methylated unless the gene has been silenced [20].

Abnormal changes in DNA methylation can result in pathogenic cytosine to thymine mutations and reduced expression of tumor suppressor genes, which are associated with the development of cancer and other diseases [20]. DNA methylation also helps maintain genome stability by suppressing the transcription of transposable and repetitive elements, which constitute between half and two-thirds of the human genome [13], that reside within gene bodies without obstructing transcription of the host genes [20]. Approximately 8% of the human genome consists of endogenous retroviral elements and when methylation of regions containing them is disrupted, the expression of retroviral proteins may be activated [9].

miRNAs are short (usually around 22 base pairs [15]) RNA sequences that provide post-transcriptional regulation by binding with mRNAs for cleavage or translational repression based on sequence complementarity [6]. They may also contribute to transcriptional silencing [6]. miRNAs are produced from untranslated long primary miRNA transcripts (pri-miRNAs), which are cleaved in the nucleus at their 5' ends by the Drosha RNase III endonuclease and transported to the cytoplasm for processing at their 3' ends by the Dicer RNase

III endonuclease [6] [9]. Over 2000 miRNA genes have been discovered in humans [15].

Epigenetic modifications have been associated with the development of cancer [20] and numerous autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes mellitus, and multiple sclerosis [9] [34] [40]. Since epigenetic modifications are potentially reversible, there is hope that they could potentially be leveraged as therapeutic targets [40].

### *Tregs and Th17 Cells*

Autoreactive T and B lymphocytes develop even in the absence of autoimmunity [12]. In healthy individuals, autoreactive lymphocytes are removed through complicated positive and negative selection processes during their development [12]. The threshold for removal is different for each individual [12].

CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (Tregs) are responsible for regulating the activation of immune responses and suppressing them after infectious agents have been successfully cleared [34] [44]. Tregs also have roles in maintaining self-tolerance [34]. Tregs are primarily produced within the thymus but can also differentiate from CD4<sup>+</sup> cells in the peripheral circulation [34]. T helper 17 (Th17) cells differentiate from CD4<sup>+</sup> cells and are responsible for upregulating inflammatory responses and maintaining adaptive immune responses to infectious agents [34]. Overproduction of Th17 cells has been observed in autoimmune diseases [10] [34].

FOXP3 and ROR $\gamma$ t are two transcription factors that compete with each other to induce CD4<sup>+</sup> cells in the peripheral circulation to differentiate into Tregs or Th17 cells, respectively [34]. Epigenetic modifications in highly conserved non-coding regions of the *FOXP3* gene affect its expression [34]. The promoter region is hyperacetylated in Tregs but not in conventional T cells and contains CpG islands that are not methylated in Tregs but are weakly methylated in conventional T cells [34]. Another region is completely demethylated in Tregs and methylated in conventional T cells; methylation of this region is associated with the stability of *FOXP3* expression in Tregs [34]. Loss-of-function mutations in the *FOXP3* gene are associated with fatal autoimmune diseases in both mice and humans [44].

A variety of cytokines and transcription factors, such as IL1, IL-6, IL-23, and TGF $\beta$ , are involved with the differentiation of CD4<sup>+</sup> cells into Th17 cells [34]. The epigenetic factors that regulate the development of Th17 cells are not currently well understood but could help explain why production of Th17 cells is increased in autoimmune diseases and may even represent potential therapeutic targets [34].

### *Immune Thrombocytopenia*

Immune thrombocytopenia (ITP), also known as idiopathic thrombocytopenic purpura, is an organ-specific autoimmune disorder characterized by the autoantibody-mediated destruction of normal platelets and the impaired production of platelets [30] [33]. The destruction of platelets occurs primarily within the spleen and liver [10] [22]. The accelerated destruction and reduced production of platelets may occur simultaneously, and the extent to which each process occurs may contribute to the effectiveness of different therapies in individual patients [22] [29].

ITP is typically characterized by the presence of anti-platelet autoantibodies, although not all anti-platelet autoantibodies are detectable with currently available commercial assays and between 30-40% of ITP patients have no identifiable antibodies [10] [24]. Antibodies target multiple platelet antigens, including GPIb, GPIIb, and GPIIIa, and sometimes other cytoplasmic proteins [10] [22]. It is not known what causes the development of autoantibodies against structurally unrelated proteins in ITP, but “[t]his situation appears to be quite different from warm autoimmune hemolytic anemia, with which ITP is often compared, where antibodies are typically confined to epitopes within the Rh locus” [10].

Platelet production is regulated by thrombopoietin (TPO) and is oftentimes normal in individuals with ITP,

although it can be impaired when megakaryocytes, which produce platelets and also express platelet antigens during their development, are targeted by autoantibodies [10] [22] [30]. Autoantibodies may selectively target megakaryocytic antigens, suppressing their development and causing them to undergo premature apoptosis [10] [22]. In ITP patients, platelets still bind and remove TPO before being destroyed, so TPO levels are usually normal or slightly elevated [10] [22].

Dysregulation of T cells has been implicated in the pathogenesis of ITP [30]. Platelets express a variety of cytokines that maintain the balance of T helper 1 and 2 (Th1 and Th2) cells, and disrupting this balance can result in the activation and differentiation of autoreactive B cells [30]. Anti-platelet autoreactive T cells exhibit “decreased apoptosis, undergo clonal expansion and create a cytokine imbalance that can lead to lower levels and abnormal function of Tregs” [30]. Increased levels of Th17 cells and reduced levels of Tregs have been observed in ITP patients, and overexpression of the Th17 transcription factor ROR $\gamma$ t has been shown to induce the development of anti-platelet antibodies in mice [10] [30].

Primary ITP is diagnosed based on a persistent platelet count of less than  $100 \times 10^9/L$  and the absence of other medical conditions and secondary causes of thrombocytopenia [33]. No test to diagnose ITP exists, so the diagnosis is made by exclusion [33]. Secondary ITP is associated with other medical conditions or causes:

- Evans syndrome, which is currently defined as the simultaneous or sequential development of two or more autoimmune cytopenias, such as ITP, autoimmune hemolytic anemia, or autoimmune neutropenia [1] [3]
- Autoimmune disorders, such as systemic lupus erythematosus, rheumatoid arthritis, antiphospholipid antibody syndrome, or autoimmune lymphoproliferative syndrome [10] [30] [51].
- Immunodeficiency disorders, such as chronic lymphocytic leukemia or common variable immunodeficiency [10] [32]
- Bacterial or viral infections, such as *Helicobacter pylori*, hepatitis C, and HIV [30]
- Side effects of immunizations, such as the measles-mumps-rubella vaccine [33]
- Adverse reactions to drugs [30] [33]

ITP affects 1.9-6.4 per 100,000 children every year with higher incidence in boys, usually has an acute onset, and spontaneously resolves within 6-12 months in approximately 80% of cases [10] [24] [16]. ITP in children is usually preceded by bacterial or viral infections, which may induce molecular mimicry and cause the immune system to attack platelets until the infectious agents have been cleared [10]. Children who develop chronic ITP are usually adolescent, female, and have higher platelet counts when initially diagnosed [16] [33].

ITP affects between 1.5-4 per 100,000 adults every year and is observed somewhat more frequently in females than males [16]. The incidence of ITP increases with age and is no longer correlated with gender after around 60 years of age [16] [29]. It is estimated that between 5-11% of adults with ITP spontaneously recover [16], although the actual rate of spontaneous remission is unknown because most patients are treated with corticosteroids [28]. Up to 53% of adults with ITP go into remission within three years after being diagnosed [28]. Remission rates are not consistent across all studies for all treatments used, and “[t]he literature contains an unexplained, extreme variation of 3% to 50% in reported remission rates on glucocorticoids” [38].

Familial cases of ITP are rare and thrombocytopenia can be caused by other inherited diseases, such as Wiscott-Aldrich syndrome [16]. An estimated 2% of children have a family history of ITP [10]. However, close relatives may have other autoimmune diseases [10].

Symptoms include increased bruising, petechiae, ecchymoses (dry purpura), mucosal bleeding, oral bleeding, epistaxis, and in women, heavy menstrual bleeding [27] [29]. Low platelet counts increase the risk of bleeding events, such as gastrointestinal bleeding or intracranial bleeding [27]. Approximately 3% of children with

ITP experience severe bleeding symptoms, such as gastrointestinal bleeding [28], although intracranial hemorrhage, which affects an estimated 2.67% of adults with ITP [28], is very rare in children [33]. The risk of thrombosis is increased for patients with ITP [10] [29]. Mortality rates for patients with newly diagnosed ITP and chronic ITP are estimated at 0.8-2.1% and 5.4-6.6%, respectively [33].

ITP is a relatively benign disease for many, but not all, patients [38]. It is extremely difficult to predict the risk of severe bleeding events or the effectiveness of different treatments [7] [28]. The primary goal of treating ITP patients is to maintain platelet counts that are sufficiently high to prevent serious bleeding symptoms rather than attempting to raise platelet counts to normal ( $\geq 100 \times 10^5/L$ ) levels [33]. Treating children who have ITP does not decrease the probability that the disease will become chronic, and long-term corticosteroid therapy is associated with detrimental side effects, so careful observance and refraining from physical activity that could result in injuries are advised if no significant bleeding symptoms are present [33]. Adults with ITP have higher risks of bleeding events and other complications that increase with age [24], and the American Society of Hematology evidence-based treatment guidelines for ITP recommends that newly diagnosed adults with platelet counts below  $30 \times 10^5/L$  receive treatment [33]. Most clinicians use  $30 \times 10^5/L$  as a threshold for treatment, although it is “unclear whether offering treatment to all patients with ITP at these levels will result in decreased bleeding” [33].

First-line therapies for ITP include corticosteroids and intravenous immunoglobulin (IVIg) [33]. Platelet kinetics measurements have shown that corticosteroid therapy increases platelet counts but not survival in patients with ITP [22]. Anti-D immunoglobulin may be given to patients who do not have autoimmune hemolytic anemia, are Rh-positive, and have not undergone splenectomy [33].

Second-line therapies include splenectomy, rituximab, thrombopoietin receptor agonists, and other immunosuppressant medications [33] [45]. Splenectomy is the only potentially curative treatment for ITP and the only treatment that results in sustained remission after 1 year in a large percentage of patients [33]. Approximately 60-70% of adults with ITP who undergo splenectomy go into complete remission [28] [45]. Due to the increased risk of infections and septicemia, many patients are reluctant to undergo splenectomy [28] [33].

Rituximab, a chimeric monoclonal antibody that targets the CD20 antigen expressed on B cells [32] [52], was developed for other diseases, including non-Hodgkins lymphoma, chronic lymphocytic leukemia, and rheumatoid arthritis [50], and is used off-label as a second-line therapy for ITP [33]. Approximately 40% of ITP patients respond to rituximab initially, while approximately 20% of adults and 26% of children with chronic ITP have a response lasting for at least 5 years [25] [45]. Some ITP patients who relapse after receiving rituximab develop long-lived plasma cells in their spleens that produce anti-platelet antibodies [25]. These cells do not express CD20, are not targeted by rituximab, and have not been found in patients with ITP who were not treated with rituximab, which suggests an association between the depletion of peripheral B cells and the establishment of long-lived plasma cells in the spleen [25] [39]. Rituximab is associated with a variety of side effects, including infusion reactions and serious infections [50]. Other rare adverse effects, such as progressive multifocal encephalopathy and hepatitis B reactivation, have also been reported [50].

Thrombopoietin receptor agonists (TPOs) are designed to bind to the TPO receptor and stimulate the growth of megakaryocytes [22]. First-generation TPOs prompted safety concerns when autoantibodies developed against one of them [22]. Two second-generation TPOs, romiplostim and eltrombopag, have been FDA-approved for use in patients with chronic ITP [33]. While TPOs can be effective at increasing platelet production, they do not improve platelet survival and platelet counts sometimes decrease once treatment is discontinued [22] [33]. Furthermore, they are expensive and limited to patients who have already failed to respond to corticosteroids, IVIg, or splenectomy or are unable to undergo splenectomy [28].

Currently available therapies for ITP cannot always sustain increased platelet counts over longer periods of time or after discontinuation and many of them are nonspecific and associated with increased risks of infection or other complications [45]. Prospective therapies under investigation attempt to target more specific pathways, such as Fc receptors [45]. New TPOs and other therapies that stimulate platelet production may also be in development [45].

Treating chronic ITP can be expensive [21]. Single-center studies conducted in Germany and in France and a retrospective study conducted in the United States observed that the costs of treating adults with chronic ITP can be high, especially when hospital stays or emergency room visits are needed, and can be comparable to the costs of treating other chronic diseases, such as diabetes [8] [21] [41]. A single-course treatment of IVIg costs approximately \$9,648, while a single-course treatment of rituximab costs approximately \$15,596.64 USD; the costs of single-course or one-month treatments with romiplostim and eltrombopag have been estimated at \$5,732.40 and \$5,934.54, respectively<sup>1</sup> [39].

Patients with chronic ITP who fail to respond to multiple lines of therapy may have an increased risk of death from bleeding or infections [38]:

However, it has to be determined whether the relatively toxic treatment of ITP has additional adverse effects on survival. For example, corticosteroids as well as immunosuppressive drugs and the splenectomized state can induce susceptibility to severe infections. In our cohort, more patients died due to infection than due to bleeding, and the lethal infections were probably related to treatment in at least half of the cases.

Chronic ITP may also decrease the quality of life for patients [27] [28] [31]. It is estimated that between 90-100% of adults with chronic ITP have received corticosteroids at some point after being diagnosed [28]. ITP patients who receive corticosteroids over longer periods of time (> 1 year) have higher incidences of diabetes, obesity, gastrointestinal bleeding, osteoporosis, and myocardial infarction than patients without ITP who are also receiving corticosteroids [27]. Although frequently described side effects of corticosteroid therapy include “hypertension, hyperglycemia, cataracts, and osteoporosis”, patients who receive long-term therapy oftentimes express dissatisfaction with other side effects as well [28]:

Instead, the side effects most bothersome to patients receiving prednisone and dexamethasone are weight gain, increased appetite, changes in personality, mood or emotions, “moon face” or puffy cheeks, bloating, swelling, and sleep disturbances. Children often experience hyperactivity. Patients rank treatment-bother with corticosteroids higher than with any other ITP therapy.

Several studies have attempted to understand the impact of chronic ITP on quality of life [27] [31]. Each study has limitations, particularly with respect to sample sizes, the severity of the disease in the patients studied, and difficulties with determining whether the disease itself or treatment-related side effects has a more significant impact on quality of life, but patients with chronic ITP generally report chronic fatigue, anxiety, depression, and financial problems [27] [31]. McMillan et al concluded that patients with chronic ITP may not perceive the disease as being “mild” [31]:

[T]his study shows that chronic ITP is not a “mild” chronic disease from the perspective of the affected patient [31]. The impact of ITP on patients’ quality of life is substantial. In our study, patients felt worse than not only the general U.S. population but also patients with other important chronic conditions.

### *Autoimmune Hemolytic Anemia*

Autoimmune hemolytic anemia (AIHA) is an acquired disease in which healthy red blood cells are destroyed by the immune system [51] [52]. Unlike congenital hemolytic anemias, red blood cells are not “intrinsically defective” in AIHA [51]. AIHA is diagnosed based on the presence of hemolytic anemia and detection of

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<sup>1</sup>The drug prices described in [39] were provided by the National Institutes of Health Clinical Center in 2013.

anti-red blood cell autoantibodies using the direct antiglobulin test (DAT), also known as the Coombs test [51] [52]. In some cases, multiple tests may be necessary to identify autoantibodies [52].

Warm AIHA is the most common form and is associated with antibodies that bind more strongly at temperatures above 37°C [51]. These antibodies usually target antigens within the Rh locus in red blood cells and sometimes glycoproteins or other red blood cell proteins as well [10] [51]. IgG and C3d antibodies are frequently present in warm AIHA, and in rare cases IgM antibodies are also present [52]. The spleen and liver are the primary sites of red blood cell destruction in warm AIHA [51].

Cold AIHA occurs less frequently than warm AIHA and accounts for 15-20% of AIHA cases [51]. Cold-reactive antibodies bind more strongly at temperatures below 4°C and are associated with intravascular hemolysis [51]. Antibodies that are active in temperatures up to 30°C are associated with cold agglutinin disease [51]. IgM and C3d autoantibodies are typically present in cold AIHA [52].

Around 7-8% of AIHA patients have a “mixed” form with both warm- and cold-reactive antibodies [52]. Between 5-10% of AIHA patients do not have antibodies that can be detected even with more sensitive tests; these patients must be diagnosed based on exclusion of other causes of hemolysis and their responsiveness to treatments [52]. Atypical cases have been found with increasing frequency and pose additional challenges because symptoms tend to be more severe and diagnosis of AIHA takes longer, which may result in delays in receiving treatments [52].

Imbalances in the ratio of Th1/Th2 cells are seen in AIHA patients [17] [32]. Levels of the IL-17A cytokine produced by Th17 cells are significantly increased in patients with warm AIHA versus healthy controls, and there appears to be an inverse correlation between IL-17A and hemoglobin levels [17]. Th17 cells have been observed to stimulate B cells to switch to the production of IgG antibodies and proliferate in mouse models [17]. Increased levels of Th17 cells and decreased levels of Tregs could therefore be associated with the pathogenesis of AIHA [32].

Approximately half of all AIHA patients are diagnosed with primary AIHA [52]. Secondary AIHA is associated with other medical conditions or causes, including:

- Chronic lymphocytic leukemia or other lymphoproliferative disorders [52]. Approximately 10% of AIHA patients have chronic lymphocytic leukemia, while approximately 14% of patients with chronic lymphocytic leukemia have AIHA [51].
- Common variable immunodeficiency [32]
- Approximately 20% of AIHA patients have other autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis) [32] [51]. AIHA affects an estimated 5-10% of individuals who have systemic lupus erythematosus and may manifest before other symptoms [32].
- Approximately 10% of AIHA patients have bacterial or viral infections [51] [52]. Molecular mimicry may contribute to the development of AIHA [51]. Direct infection by the EpsteinBarr virus may lead to the development of AIHA [51].
- Adverse reactions to drugs [51]

The estimated incidence of AIHA is 0.8-3 per 100,000 people per year, while the prevalence is 17 per 100,000 people [52]. AIHA appears to affect both men and women equally and prevalence increases with age [51]. AIHA is rarer in children, affecting 0.2 per 100,000 children per year [52], and bacterial or viral infections can be associated with the initial development of symptoms [1].

AIHA is oftentimes considered “benign”, but severe hemolysis and reticulocytopenia can constitute medical emergencies [1] [5]. Approximately 20% of adults and 39% of children with AIHA have reticulocytopenia, which could indicate that red blood cell precursors are also damaged and subsequently undergo apoptosis

or are destroyed by the immune system [5] [52]. Some patients need red blood cell transfusions and should be given limited quantities of leukocyte-depleted red blood cells slowly to minimize adverse effects [52]. Transfusions are challenging because some autoantibodies react to all red blood cell types and AIHA patients are more likely to develop anti-red blood cell alloantibodies after receiving transfusions [52].

Familial cases of AIHA are rare [1]. However, a nationwide study of 265 children in France discovered that up to 8% of children with primary AIHA from certain ethnic backgrounds had close relatives with AIHA, while 14% of children with primary AIHA across all ethnic backgrounds had close relatives with other immunological diseases [1]. Furthermore, 37% of the children had Evans syndrome<sup>2</sup>, and 22% of them had close relatives with immunological diseases [1]. Approximately 63% of the children enrolled in the study later developed other immunological diseases after a median follow up time of 3 years [1].

Symptoms of AIHA include anemia, lethargy, pallor, jaundice, splenomegaly, and hepatomegaly, and severe hemolysis can result in hemoglobinuria [51]. Other symptoms may be present in secondary AIHA [51]. AIHA may have an acute onset and hospitalization and red blood cell transfusions may be necessary, or it may manifest more slowly over time [23] [52].

Spontaneous remission is rare in AIHA patients and earlier onset appears to be associated with an increased risk of relapse [5] [23]. Corticosteroid therapy does not always result in long-term remission, unlike in ITP [23]. Treating AIHA is difficult because of the lack of clinical trials and evidence-based treatment guidelines [1] [52]. Similar to ITP, treatments for AIHA do not attempt to restore hemoglobin levels to “normal”, but rather to the minimum level that ensures the safety of the patient [23].

Corticosteroids are usually used as the first-line treatment for warm AIHA and are initially administered for 3 weeks [52]. If the patient responds well to corticosteroids, the dose is gradually decreased over a period of at least 3-4 months [52]. Low doses of corticosteroids for at least six months have been associated with longer periods of remission and lower relapse rates [52]. Although corticosteroids appear to be effective in patients with warm AIHA, this has not been confirmed in clinical trials [52]. While 80-85% of patients with warm AIHA respond well to corticosteroids, 50-60% may subsequently become dependent on corticosteroid therapy [32]. Diabetes is a “major risk factor for treatment-related deaths from infections” and patients receiving corticosteroids for long periods of time should be closely monitored [23].

IVIg is not very effective in warm AIHA and is not recommended except in severe cases that do not respond to corticosteroids [32].

If no improvements are observed after the first 3 weeks of corticosteroid therapy, second-line therapies are started [23]. Splenectomy causes between 38-82% of warm AIHA patients to go into partial or complete remission, although increased susceptibility to infections that have been reported for ITP patients who underwent splenectomy may be relevant to AIHA patients as well [23] [52]. Rituximab has also been used off-label as a second-line therapy for warm AIHA and appears to be effective, with overall and complete response rates estimated at 83-87% and 54-60%, respectively [23] [32].

Corticosteroid therapy and splenectomy have not been found to be effective at treating cold AIHA [5] [51] [52]. Protecting patients with cold AIHA from cold temperatures may reduce hemolysis, and the results of several studies have indicated that between 60-80% of cold AIHA patients who were treated with rituximab had responses that lasted one year or longer [51] [52].

Erythropoietin has been used to stimulate the production of red blood cells in patients with severe AIHA and reticulocytopenia [5].

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<sup>2</sup>Evans syndrome is currently defined as the development of more than one autoimmune cytopenia either simultaneously or sequentially, but the definition that Aladjidi et al used specifically referred to patients with both AIHA and ITP [1].

## Genetic and Epigenetic Factors in ITP and AIHA

The identification of genetic and epigenetic factors that contribute to the development of ITP and AIHA, affect the severity of symptoms, and influence responses to different treatments has been challenging because of sample sizes, the complexities involved in the immune processes, and the wide variety of phenotypes [16]. Many studies are small and find associations with only borderline statistical significance [16], and results from different studies sometimes conflict with each other [35] [42]. Conflicting results could be partially attributed to the fact that some genetic variants are much rarer in certain populations than others [42].

A number of genetic variations that are associated with ITP are also associated with other autoimmune diseases [30]. While the pathways for ITP and AIHA are not identical, both diseases are associated with dysfunction of T cells and antibody-mediated destruction of hematological cells and it would probably not be unreasonable to suspect that some of the genetic variations that increase susceptibility to ITP could increase susceptibility to AIHA as well.

Certain human leukocyte antigen (HLA) alleles are strongly associated with the development of autoimmune diseases, and an estimated 60% of warm AIHA patients have the HLA-DR15 allele [51].

*PTPN22* is a non-HLA gene that is highly associated with autoimmunity [46]. The PTPN22 protein regulates T-cell activation by dephosphorylating signaling proteins involved with T-cell activation [46]. The *PTPN22* 1858C>T polymorphism is nonsynonymous, has been associated with an increased risk of ITP, along with several other autoimmune diseases, including rheumatoid arthritis, Addison disease, autoimmune thyroid disease, and systemic lupus erythematosus, and is believed to have an adverse effect on the interactions between the PTPN22 protein and tyrosine-protein kinase CSK [46]. Other *PTPN22* polymorphisms may impair the deletion of autoreactive B cells [46].

Variations within the Fc receptor locus are associated with autoimmune diseases. Both low and high copy number variations in the *FCGR3B* gene are associated with increased susceptibility to systemic lupus erythematosus and primary Sjögren's syndrome [26]. Copy number variations in *FCGR2C* are associated with ITP [26]. Polymorphisms in *FCRG2A* and *FCRG3A* have been associated with ITP and responsiveness to splenectomy, corticosteroids, and rituximab [10] [35]. The New Zealand Black mouse is genetically predisposed to spontaneously developing AIHA and displays decreased expression and function of Fc $\gamma$ RIIb, which may result in the increased activity of IgG antibodies that target red blood cells [51].

Cytotoxic T-lymphocyte protein 4 (CTLA-4) is a receptor that is expressed on the surfaces of activated T cells and has a significant role in the negative regulation of T cell responses [48]. Variations in the *CTLA4* gene are associated with an increased risk of insulin-dependent diabetes, autoimmune thyroid disease, systemic lupus erythematosus, rheumatoid arthritis, AIHA, and other autoimmune diseases [12] [36] [48]. In a study of 50 patients with AIHA (including 30 patients with both chronic lymphocytic leukemia and AIHA), 60 patients with ITP, 100 patients with chronic lymphocytic leukemia, and 100 healthy controls, Pavkovic et al observed that an A  $\rightarrow$  G polymorphism in the first exon of the *CTLA4* gene appeared significantly more frequently in AIHA patients, with highest frequency in patients with both chronic lymphocytic leukemia and AIHA [36]. They did not observe a statistically significant difference between ITP patients and healthy controls [36].

Basciano et al investigated a possible association between the most common beta 1 tubulin SNP and responsiveness to different treatments in ITP patients and found that ITP patients who were heterozygous or homozygous for the minor allele had higher failure rates to corticosteroid, rituximab, and immune-modulatory treatment, while homozygous minor allele patients also had a higher failure rate for anti-D treatments [7]. Beta 1 tubulin polymorphisms may affect platelet structure, function, and turnover, and while the SNP investigated does not appear to contribute to the pathogenesis of ITP, Basciano et al concluded that it could have potential value with respect to predicting responsiveness to different treatments [7].

A study of 206 Caucasian patients with primary ITP and 618 healthy controls found a potential association between a common SNP in the tumor necrosis factor alpha (TNFA) gene and primary ITP, although the

results were of borderline statistical significance and genetic studies conducted with other populations (Japanese and Brazilian) failed to discover any associations between the SNP and increased susceptibility to ITP [42]. However, the SNP is significantly rarer in non-Caucasian populations, including Japanese, Chinese, and African populations, and genetic differences across ethnic populations could explain why the other studies obtained conflicting results [42].

Polymorphisms in the promoter region of *TNFSF13B* gene, which encodes B-cell activating factor (BAFF) [49], have been observed more frequently in individuals with ITP than in healthy controls and could be associated with higher expression of BAFF in patients with active ITP [30].

A missense mutation in the *CNR2* gene is associated with increased susceptibility to chronic ITP in children [30].

Variations in the *Lbw2* locus may increase susceptibility to autoimmune hemolytic anemia in New Zealand Black mice [43].

The role of DNA methylation in ITP is not well understood [37]. A SNP in the promoter region of the DNA methyltransferase 3B (*DNMT3B*) gene and a variable number tandem repeat polymorphism in an intron in the IL-1 receptor type 1 gene were found at higher frequencies in a small study of Cretan children with ITP [37]. The *DNMT3B* SNP was not found to be associated with ITP in a Chinese population, although the results of another study indicated that *DNMT3A* and *DNMT3B* are expressed at significantly lower levels in patients with ITP compared to healthy controls [37].

Oxidative stress has been linked to the production of autoantibodies and the development of cancer [10] [14]. Oxidative stress can damage DNA in many ways, such as by deleting or modifying nucleotides, and inhibit binding with DNA methyltransferases, which may result in hypomethylation [14]. Recent studies have attempted to understand the effects of oxidative stress on the pathogenesis of ITP and have associated overexpression of the *VNN1* gene, which encodes Vanin-1, with an increased risk of progression to chronic ITP in children [10] [30]. Oxidative stress has also been associated with the pathogenesis of AIHA in New Zealand Black mice [18] [43].

A study conducted by Jernås et al observed that quantities of some miRNAs were significantly different between patients with chronic ITP and healthy controls [19]. In particular, miRNAs targeting CXCL13 and IL-21 were significantly increased in patients with chronic ITP [19].

### Conclusion

Although much progress has been made towards understanding the pathogenesis of many autoimmune diseases, including immune thrombocytopenia and autoimmune hemolytic anemia, we may still be a long way from being able to predict the progression of symptoms and responsiveness to treatments for individual patients. Chronic ITP has been associated with high medical costs and decreases in the quality of life that could be partially related to the treatments used, and any information that could improve the ability to provide individualized treatment plans for patients would be of significant benefit. Further investigation of genetic and epigenetic factors that are involved with disease processes, such as the production of Th17 cells, could reveal additional therapeutic targets.

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