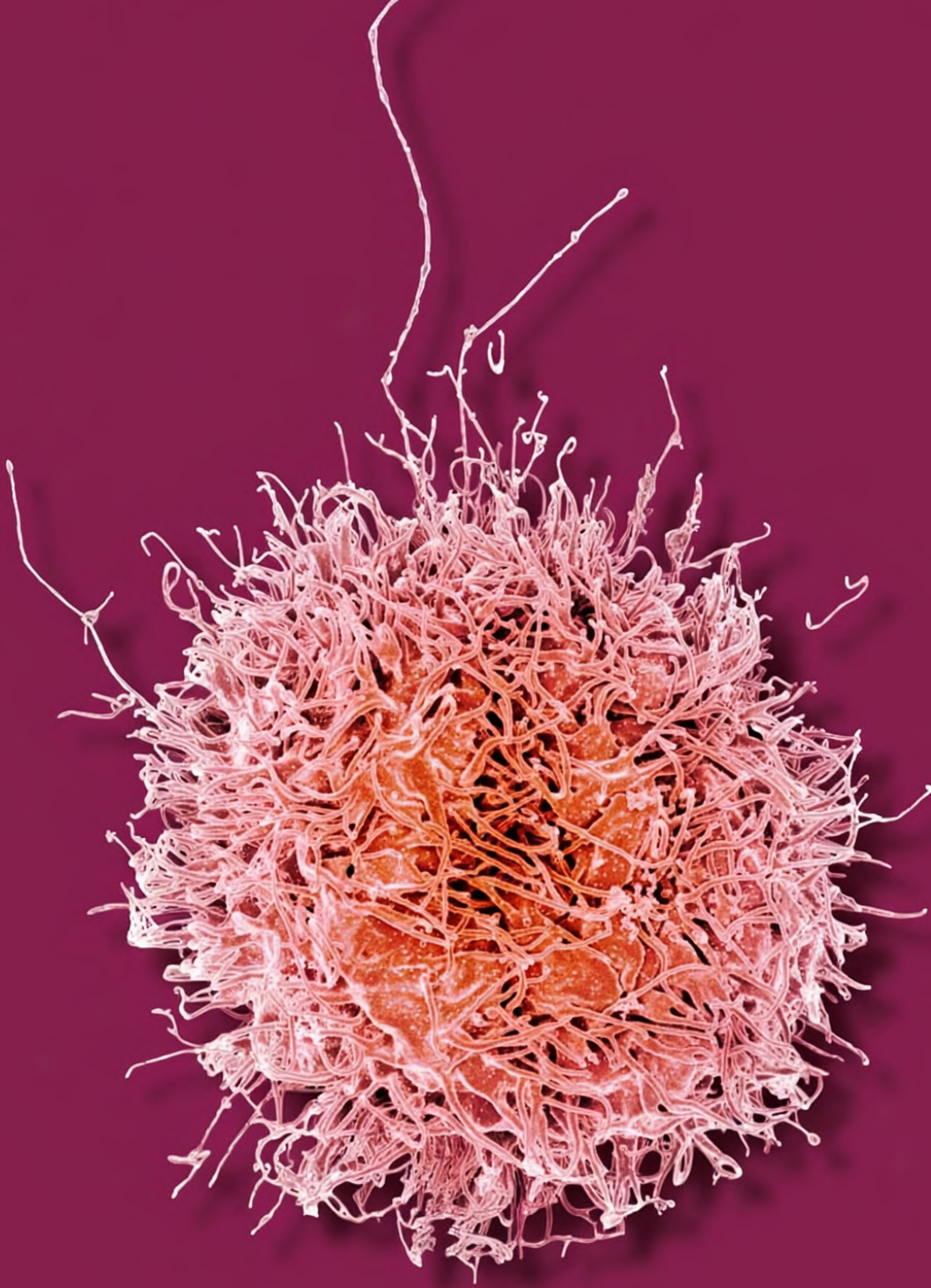


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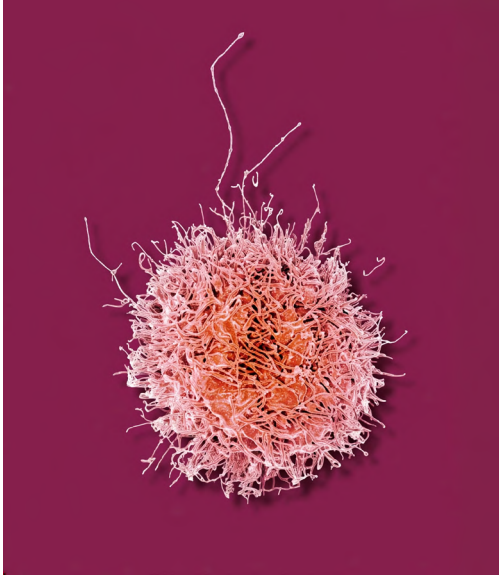
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Cytotoxic T Lymphocytes

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When Immune Tolerance Fails: From Mechanisms to Clinical Phenotypes

Dear Colleagues,

Immune tolerance preserves the balance between an effective immune response and the protection of healthy tissues. Its failure can lead to autoreactive responses and a wide range of autoimmune and immune-mediated diseases. Despite their diverse clinical presentations, these disorders often share common features, including impaired regulatory mechanisms, altered immune checkpoints, environmental influences, and autoantibody production.

In the second issue of the *Turkish Journal of Immunology* in 2026, one review and five original articles examine different aspects of immune dysregulation, ranging from basic mechanisms to potential clinical biomarkers.

The review, *Immune Dysregulation: Mechanisms of Tolerance Failure, Clinical Spectrum, and Emerging Therapeutic Strategies*, provides an overview of the mechanisms responsible for defective immune tolerance and discusses new therapeutic approaches aimed at restoring immune homeostasis.

The experimental study, *Maternal Gut Dysbiosis Exacerbates Offspring Susceptibility to Collagen-Induced Arthritis in Mice*, draws attention to the influence of the maternal gut microbiota on immune development and autoimmune susceptibility in offspring. It also raises important questions about how early-life and intergenerational factors may contribute to the later development of immune-mediated disease.

In the article *Association Between Vitamin D Status, Regulatory T Cells, and Disease Severity in Pediatric Systemic Lupus Erythematosus*, the relationship between vitamin D levels, regulatory T cells, and clinical severity is evaluated in children with systemic lupus erythematosus. The study brings together metabolic, immunological, and clinical parameters in the assessment of systemic autoimmunity.

The article *Immune-Biochemical Stratification Identifies the Pure Primary Biliary Cholangitis Phenotype in AMA-Positive Individuals* examines whether combined immunological and biochemical assessment can improve the characterization of individuals positive for anti-mitochondrial antibodies (AMA). This integrated evaluation may contribute to more accurate patient classification and follow-up.

The final two studies address different aspects of psoriasis. *Association of CTLA-4 Gene Polymorphisms and Serum Soluble CTLA-4 Levels with Psoriasis Vulgaris* investigates the relationship between psoriasis and both genetic and soluble components of the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathway. *Anti-ADAMTSL5 and Anti-K17 Autoantibodies in Psoriasis: Diagnostic Performance and Associations with*

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Systemic Inflammation focuses on autoreactivity against ADAMTSL5 and keratin 17. Together, these studies offer complementary perspectives on the roles of immune regulation and autoantigen recognition in psoriasis.

The articles presented in this issue remind us that immune dysregulation rarely results from a single defect. It more often reflects the interaction of genetic susceptibility, regulatory immune cells, checkpoint pathways, microbiota, metabolic factors, and tissue-specific responses. A better understanding of these relationships will support the development of more informative biomarkers and more precisely targeted treatments.

We thank the authors, reviewers, and editorial team for their contributions, and we hope our readers will find this issue both informative and stimulating.

With warm regards,

Prof. Günnur Deniz 

On behalf of the Editorial Board

Turkish Journal of Immunology

Immune Dysregulation: Mechanisms of Tolerance Failure, Clinical Spectrum, and Emerging Therapeutic Strategies

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Abstract

The immune system sustains health through a carefully calibrated equilibrium between activation and restraint, a balance that, when disrupted, gives rise to a clinically heterogeneous spectrum of disorders collectively termed immune dysregulation. Spanning autoimmune diseases, primary and secondary immunodeficiencies, hypersensitivity reactions, and chronic inflammatory states, these conditions collectively affect hundreds of millions of people globally and impose an enormous burden on healthcare systems as well as individual quality of life. Central to the pathophysiology of nearly all immunological disorders is the failure of immune tolerance, including both the thymic mechanisms that eliminate autoreactive lymphocytes during development and the peripheral checkpoints, including regulatory T cells (Tregs), co-inhibitory molecules, and cytokine circuits, that restrain self-reactive clones escaping central deletion. Beyond tolerance failure, immune dysregulation emerges through a convergence of genetic mutations affecting key regulatory genes (e.g., *FOXP3*, *AIRE*, *RAG*, and *IL10*), epigenetic reprogramming driven by environmental exposures, microbial dysbiosis, and perturbations in cytokine networks. The clinical consequences range from organ-specific destruction in type 1 diabetes mellitus and multiple sclerosis (MS) to life-threatening systemic syndromes including cytokine storm and multi-organ failure. Current therapeutic strategies, including immunomodulatory drugs, biological agents, Janus kinase (JAK) inhibitors, hematopoietic stem cell transplantation (HSCT), and emerging chimeric antigen receptor T (CAR-T)-cell approaches, have transformed outcomes but fall short of providing a cure for most conditions. This review synthesizes the current mechanistic understanding, maps the clinical landscape of immune dysregulation, critically evaluates the evidence for existing and novel therapies, and identifies key gaps where future research must focus to achieve disease-modifying and curative outcomes.

Keywords: Immune dysregulation, autoimmune disease, immune tolerance, immunodeficiency, hypersensitivity, cytokine storm

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Introduction

Immunology occupies a unique position among the biomedical sciences: it is simultaneously the study of protection and the study of destruction. The very mechanisms that defend the host against an almost infinite variety of microbial threats -pathogen recognition, lymphocyte activation, antibody production, inflammatory cascades- can, under circumstances that are still incompletely understood, turn against the self and cause disease (1,2). The immune system consists of interconnected networks of molecules, proteins, cells, tissues, and organs that work in concert to maintain host integrity against both external pathogens and internal threats (3). This duality is not a design failure; it is an inherent consequence of a system built for exquisite discrimination, where errors at any level exact a biological cost. Immunological disorders, broadly defined as conditions arising from aberrant immune function, encompass autoimmune diseases, primary and secondary immunodeficiencies, hypersensitivity reactions, and chronic inflammatory states (4,5). Their collective global prevalence, which has risen markedly over recent decades, places them among the leading causes of chronic morbidity (6,7). Yet, the mechanistic heterogeneity across and within these categories has historically made unified frameworks difficult to construct.

What unites this clinical diversity at a biological level is the concept of immune homeostasis, the dynamic equilibrium between immune activation and restraint maintained by an intricate architecture of cellular, molecular, and epigenetic regulatory mechanisms (8). When homeostasis breaks down, the pathological consequences reflect the specific layer at which regulation fails: defective thymic selection allows autoreactive lymphocytes to mature; regulatory T-cell (Treg) dysfunction removes the brake on self-reactive responses; cytokine imbalances amplify inflammatory signals beyond the capacity for resolution; and genetic mutations or environmental insults disrupt signaling pathways that normally coordinate immune activity (9–11). The downstream effects are clinically heterogeneous, ranging from the focal destruction of pancreatic β -cells in type 1 diabetes mellitus to the multi-system inflammation of systemic lupus erythematosus (SLE), and from the immunological silence of severe combined immunodeficiency (SCID) to the hyperinflammatory crescendo of cytokine storm syndrome (12–14). Central to virtually all of these conditions is a failure, partial or complete,

transient or permanent, of immune tolerance, the state in which the immune system withholds a destructive response against specific antigens (15,16).

Immune tolerance is established and maintained through two complementary layers. Central tolerance, operating in the thymus for T cells and the bone marrow for B cells, eliminates lymphocytes bearing high-affinity receptors for self-antigens through a process of clonal deletion governed in part by the autoimmune regulator (*AIRE*) gene (17). Peripheral tolerance acts as a secondary checkpoint, encompassing anergy induction, Treg-mediated suppression, and co-inhibitory receptor signaling via cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) (15,18). Regulatory T cells, defined by expression of the master transcription factor *FOXP3*, are indispensable for active immune suppression. Loss-of-function mutations

Highlights

- Central and peripheral tolerance failure, driven by genetic, epigenetic, and environmental factors, constitutes the common mechanistic thread across the spectrum of immunological disorders.
- Loss of *FOXP3*⁺ regulatory T-cell function, whether genetic (immune dysregulation, polyendocrinopathy, enteropathy, X-linked [IPEX] syndrome) or acquired, is sufficient to precipitate severe, multisystem autoimmunity, underscoring the indispensable role of active immune suppression.
- Cytokine imbalance, characterized by excess tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-17 (IL-17), and interferon- γ (IFN- γ), together with deficient interleukin-10 (IL-10) and transforming growth factor- β (TGF- β), drives chronic inflammation and can escalate to cytokine storm with multi-organ failure.
- Janus kinase (JAK) inhibitors, biologic agents targeting specific cytokines or co-stimulatory pathways, and clustered regularly interspaced short palindromic repeats (CRISPR)-based gene correction represent the most promising near-term therapeutic advances.
- The role of gut microbial dysbiosis in shaping immune homeostasis remains an underexplored but rapidly evolving area with significant translational potential.

in *FOXP3* cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, a devastating neonatal multiorgan autoimmune disease that illustrates the non-redundant requirement for active immune restraint (19,20). Immune dysregulation arises when tolerance mechanisms are overwhelmed, bypassed, or genetically disrupted, resulting in inappropriate immune activation, deficient host defense, or both.

The classification of immunological disorders reflects the predominant mode of immune failure. Hypersensitivity reactions, classified by Gell and Coombs into four types according to their effector mechanisms, represent exaggerated or misdirected responses to antigens that pose no genuine threat (21). Type I (immunoglobulin E [IgE]-mediated) reactions, including asthma, allergic rhinitis, and anaphylaxis, have increased substantially in prevalence over the past half-century, with food allergies disproportionately affecting children (21,22). Auto-immune diseases, affecting an estimated 4–5% of the global population across more than 80 distinct clinical entities, arise from failure of self-tolerance, resulting in immune attack on host tissues (23,24). They display a consistent female predominance, reflecting incompletely understood contributions of sex hormones and X-chromosome-linked immune regulatory genes (25). Immunodeficiency states, whether primary (inborn errors of immunity [IEI]) or secondary (human immunodeficiency virus infection/acquired immunodeficiency syndrome [HIV/AIDS], malnutrition, or iatrogenic), result from inadequate immune responses that permit opportunistic pathogens to establish life-threatening infections (13,26,27). Chronic inflammatory diseases, in which unresolved immune activation damages tissues without a specific self-antigen target, complete this spectrum (28).

Understanding immune dysregulation has accelerated dramatically over the past two decades. The characterization of rare monogenic immunodeficiencies has illuminated critical regulatory nodes (9,29); genome-wide association studies have identified hundreds of susceptibility loci for complex autoimmune diseases (30); and advances in single-cell transcriptomics, epigenomics, and spatial proteomics have revealed cellular heterogeneity at unprecedented resolution. Concurrently, the therapeutic landscape has been transformed by targeted biological agents, small-molecule Janus kinase (JAK) inhibitors, and early-phase immune resetting strategies including chimeric antigen receptor T (CAR-T)-cell

therapy (9,31). Yet translating mechanistic depth into clinical benefit remains an ongoing challenge: biologics targeting tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-17 (IL-17) manage rather than cure immune-mediated diseases; hematopoietic stem cell transplantation (HSCT) offers immune reconstitution with substantial procedural risk; and the prospect of durable remission without continuous immunosuppression, while increasingly plausible, has not yet been achieved at scale (32,33).

This review integrates current mechanistic understanding with the clinical spectrum of immune dysregulation and critically evaluates the evidence base for existing and emerging therapeutic strategies. It identifies the knowledge gaps that constrain progress, particularly the incomplete understanding of tolerance induction in adult tissues, the therapeutic exploitation of the gut microbiome, and the challenge of achieving durable immune reset without global immunosuppression and frames the questions that will define the next decade of immunological investigation.

Immune Tolerance: The Foundation of Self-Discrimination

Central Tolerance

The thymus and bone marrow serve as the primary censors of the immune repertoire, subjecting developing lymphocytes to a dual-selection process that is remarkable in its stringency and, when it fails, in its consequences. In the thymus, as illustrated in Figure 1, developing T cells first undergo positive selection. Only those cells capable of recognizing peptide-major histocompatibility complex (MHC) complexes with sufficient affinity to receive survival signals are permitted to mature, ensuring a self-MHC-restricted repertoire (34). The subsequent step, negative selection, addresses the inherent self-reactivity enriched by positive selection: T cells bearing T-cell receptors (TCRs) with excessively high affinity for self-antigen-MHC complexes are eliminated by apoptosis (clonal deletion), a process mediated in part by medullary thymic epithelial cells and dendritic cells (3).

Central to thymic negative selection is the *AIRE* gene, whose product drives the ectopic expression of thousands of tissue-restricted antigens within medullary thymic epithelial cells. Mutations in *AIRE* cause autoimmune polyendocrinopathy-candidiasis-ectodermal

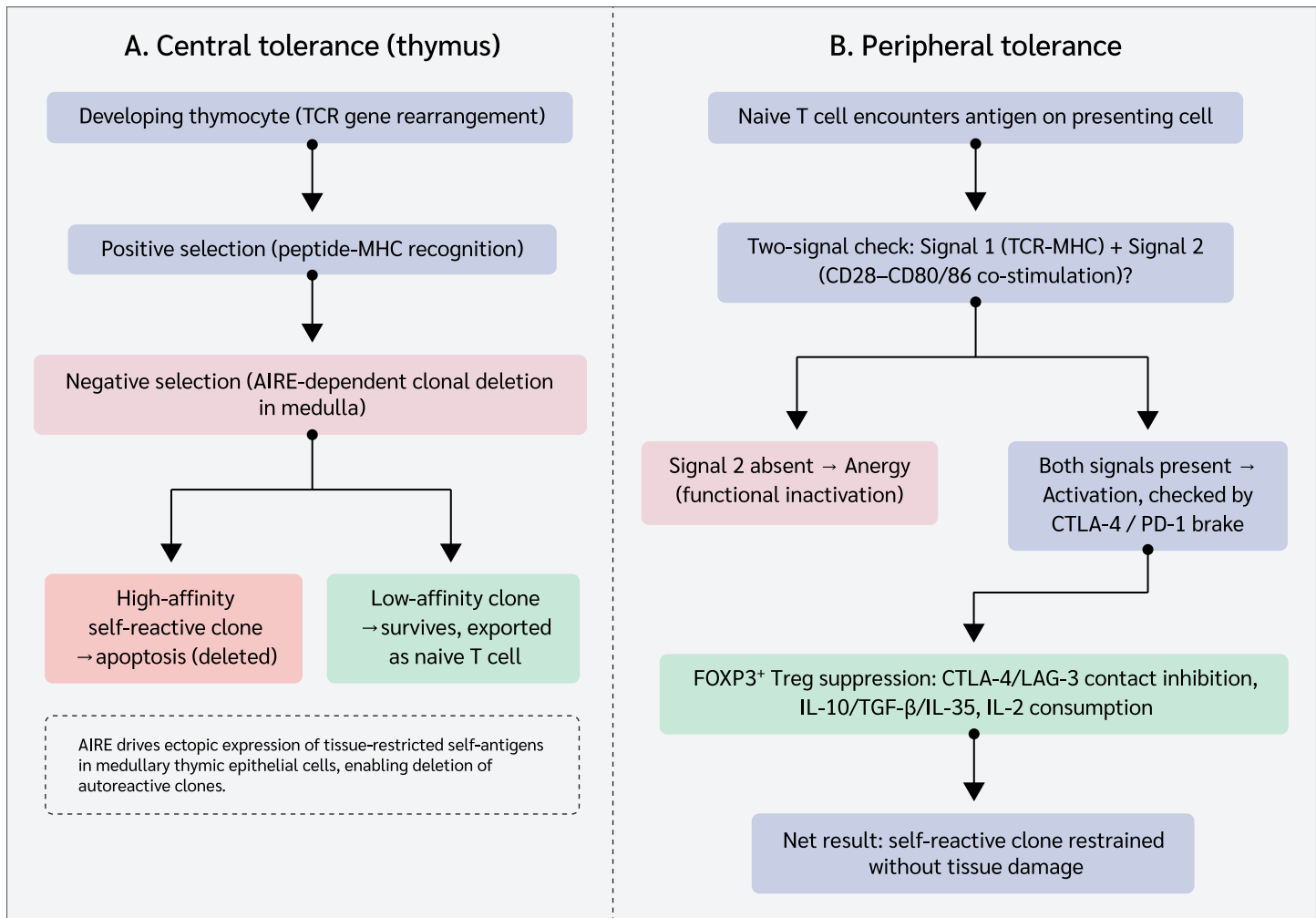


Figure 1. Mechanisms of immune tolerance.

Left panel: Central tolerance in the thymus and bone marrow—positive selection (MHC recognition), negative selection (AIRE-dependent clonal deletion of high-affinity autoreactive clones), and survival of low-affinity mature naïve lymphocytes. **Right panel:** Peripheral tolerance mechanisms including anergy, $FOXP3^+$ Treg-mediated suppression (IL-10, TGF- β , CTLA-4), and the two-signal model of lymphocyte activation. Failure at either level contributes to autoimmunity and immune dysregulation. Panel A shows the sequential thymic selection process culminating in AIRE-dependent clonal deletion; Panel B shows the two-signal checkpoint and Treg-mediated suppressive mechanisms that constrain autoreactive clones that survive central selection.

dystrophy (APECED), illustrating with near-experimental precision the indispensability of this mechanism for self-tolerance. Analogous central processes operate in the bone marrow for B lymphocytes: autoreactive B cells undergo receptor editing or clonal deletion, and those that survive carry receptors of sufficiently low self-antigen affinity that peripheral regulatory mechanisms can normally contain them (15,34).

Peripheral Tolerance

No central selection process operates with perfect efficiency, and autoreactive lymphocytes circulate in healthy individuals without causing disease, a testament to the robustness of peripheral tolerance. The

principal peripheral mechanisms are anergy (functional inactivation through the absence of co-stimulation), clonal deletion, and active suppression by $FOXP3^+$ Tregs (15,18). The two-signal model of lymphocyte activation is fundamental here: Signal 1 (MHC-TCR binding) in the absence of Signal 2 (cluster of differentiation 80 [CD80]/CD86-CD28 co-stimulation) induces anergy rather than activation. Negative regulators, most notably CTLA-4 on Tregs, which competes with CD28 for CD80/CD86 with far higher affinity, function as physiological brakes on T-cell activation (15).

These $FOXP3^+$ Tregs deploy multiple suppressive mechanisms: contact inhibition via CTLA-4 and lymphocyte

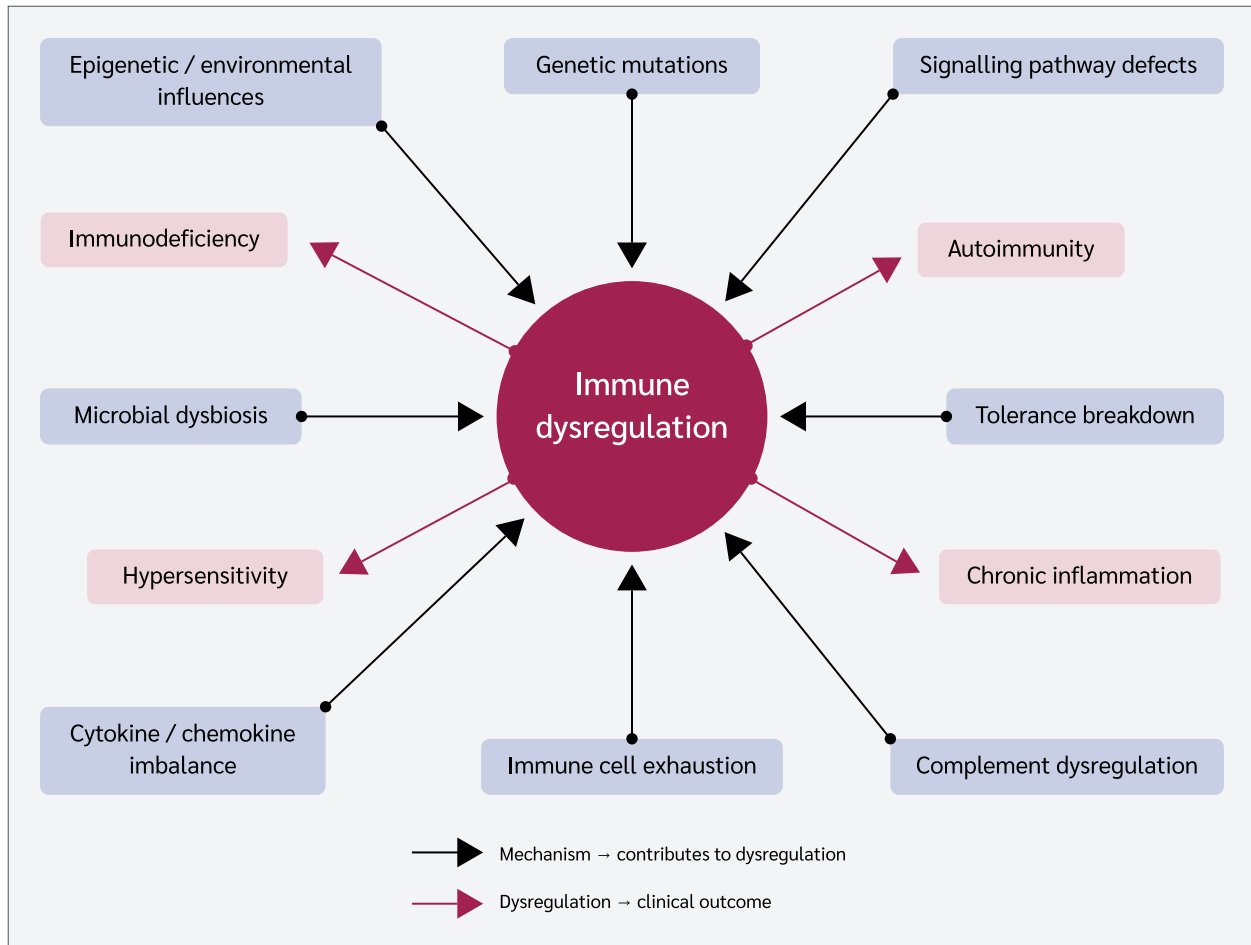


Figure 2. Integrated mechanisms of immune dysregulation. Eight interconnected pathological processes-genetic mutations, epigenetic/ environmental influences, microbial dysbiosis, cytokine/chemokine imbalance, immune cell exhaustion, complement dysregulation, tolerance breakdown, and signaling pathway defects-all converge on a central state of immune dysregulation. Inward-pointing arrows indicate the eight contributing mechanisms; outward-pointing arrows indicate the four clinical outcomes (autoimmunity, immunodeficiency, hypersensitivity, chronic inflammation) that result from dysregulation, so that cause and consequence are visually distinguished.

SCFA: Short-chain fatty acids, EBV: Epstein-Barr virus.

activation gene 3 (LAG-3), immunosuppressive cytokines (IL-10, TGF- β , and IL-35), and competitive consumption of IL-2 (18,20). The centrality of *FOXP3* to immune homeostasis is illustrated by IPEX syndrome, caused by loss-of-function mutations in *FOXP3*, which produces severe neonatal diabetes, inflammatory enteropathy, eczema, and multi-organ autoimmunity, often fatal in infancy without HSCT (19,20). Additionally, IL-10-producing type 1 regulatory T (Tr1) cells and TGF- β -producing T helper 3 (Th3) cells represent distinct regulatory lineages that complement canonical *FOXP3*⁺ Treg function (18). Immune homeostasis dysregulation arising from these failures can lead to a range of pathologies from debilitating autoimmune diseases to severe immunodeficiencies (8).

Mechanisms of Immune Dysregulation

Genetic Architecture of Immune Dysregulation

Single-gene disorders have delineated the non-redundant functions of immune regulatory molecules with remarkable clarity. Severe combined immunodeficiency (SCID), caused by mutations in genes encoding components of the TCR signaling pathway or the common γ -chain of cytokine receptors, ablates T-cell development and leaves affected infants profoundly susceptible to life-threatening infections (35,36). Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome arises from mutations in the *FOXP3* gene, as described in Figure 2. Type I hypersensitivity

susceptibility has been associated with gain-of-function mutations in caspase recruitment domain family member 11 (*CARD11*) and loss-of-function mutations in mucosa-associated lymphoid tissue lymphoma translocation protein 1 (*MALT1*), both converging on aberrant nuclear factor kappa B (NF- κ B) signaling in lymphocytes (37,38). Mutations in recombination-activating genes (*RAG*) have been associated with autoimmunity (9), while damaging mutations in interleukin-10 (*IL10*), interleukin-10 receptor subunit alpha (*IL10RA*), and interleukin-10 receptor subunit beta (*IL10RB*) cause severe, early-onset inflammatory bowel disease (IBD) (39–41). Genome-wide association studies of SLE have identified hundreds of susceptibility loci, many in genes encoding complement components and interferon-pathway regulators (5,30). While mutations in these genes or pathways predispose to immune dysregulation, environmental triggers are essential for progression to clinical disease (9,11).

Epigenetic and Environmental Contributions

Epigenetic modifications, including DNA methylation, histone acetylation, microRNA dysregulation, and chromatin remodeling, alter gene expression in immune cells without changing the underlying DNA sequence, and accumulating evidence implicates them in immune dysregulation (6,42). In SLE, hypomethylation of interferon-stimulated genes in T cells has been documented, and *in vitro* demethylation has been shown to induce autoantibody production, a causal link between epigenetic state and autoimmune potential (42). Environmental exposures serve as epigenetic triggers in genetically predisposed individuals: ultraviolet B radiation activates keratinocyte apoptosis and nucleosomal antigen release in SLE, as shown in Figure 2; Epstein-Barr virus (EBV) molecular mimicry between EBV nuclear antigen 1 (EBNA-1) and the Ro/La autoantigens represents one of the strongest epidemiological links between infection and systemic autoimmunity; and chemicals such as hydralazine and procainamide induce drug-related lupus through demethylation of immune regulatory genes (5,30). Dietary patterns exert immunomodulatory effects both directly and indirectly through the gut microbiota and its metabolites, with high-fat, low-fiber diets reducing circulating short-chain fatty acids (SCFAs) that normally drive Treg differentiation (10).

Microbial Dysbiosis and Mucosal Immunity

The gut harbors the largest concentration of immune cells in the body, and the continuous dialogue between

commensal microbiota and intestinal immune cells shapes systemic immune tone throughout life (43). Commensal microbes drive intestinal Treg differentiation, mucosal tolerance, and appropriate activation thresholds; dysbiosis, defined as qualitative and quantitative shifts in microbial community composition, has been associated with IBD, rheumatoid arthritis (RA), multiple sclerosis (MS), and SLE (43). Mechanistically, altered microbial metabolites, such as SCFAs, can disrupt epithelial integrity and immune signaling pathways. Persistent infectious agents, most notably *Mycobacterium tuberculosis* and HIV, additionally cause T-cell exhaustion characterized by progressive loss of cytokine secretion and sustained upregulation of PD-1 and CTLA-4, which compromises both pathogen clearance and immune regulation (27,44). Molecular mimicry between microbial antigens and host proteins represents another pathway to tolerance breakdown, generating cross-reactive immune responses that inadvertently target self-tissues following infection (5).

Cytokine and Chemokine Imbalance

Cytokines are not merely biomarkers of immune activity; they are the causal agents of the tissue damage and systemic symptoms that define immune-mediated diseases (9,21). The pathogenic cytokine landscape in autoimmunity is dominated by TNF- α , IL-1 β , IL-6, IL-17, and IFN- γ , which collectively promote dendritic cell maturation, Th17 polarization, macrophage activation, and osteoclast activation, as shown in Figure 3. Dysregulation of type I interferons and pro-inflammatory cytokines, particularly IL-6 and TNF- α , plays a critical role in SLE pathogenesis (30,45). Dysregulation of IL-1 production causes autoinflammatory diseases including deficiency of IL-1 receptor antagonist and cryopyrin-associated periodic syndromes (CAPS) (21,29). Disruption of key signaling pathways such as NF- κ B and JAK-signal transducer and activator of transcription (STAT) leads to abnormal immune activation or suppression (9). In extreme cases, dysregulated cytokine release culminates in a cytokine storm, a hyperinflammatory state characterized by systemic inflammation, multiorgan dysfunction, and high mortality (12,46).

Immune Cell Dysfunction and the Exhaustion Paradigm

Effective immunity depends not merely on the magnitude of the immune response but on the functional integrity of its cellular constituents. T-cell exhaustion, arising from chronic antigen stimulation, is defined

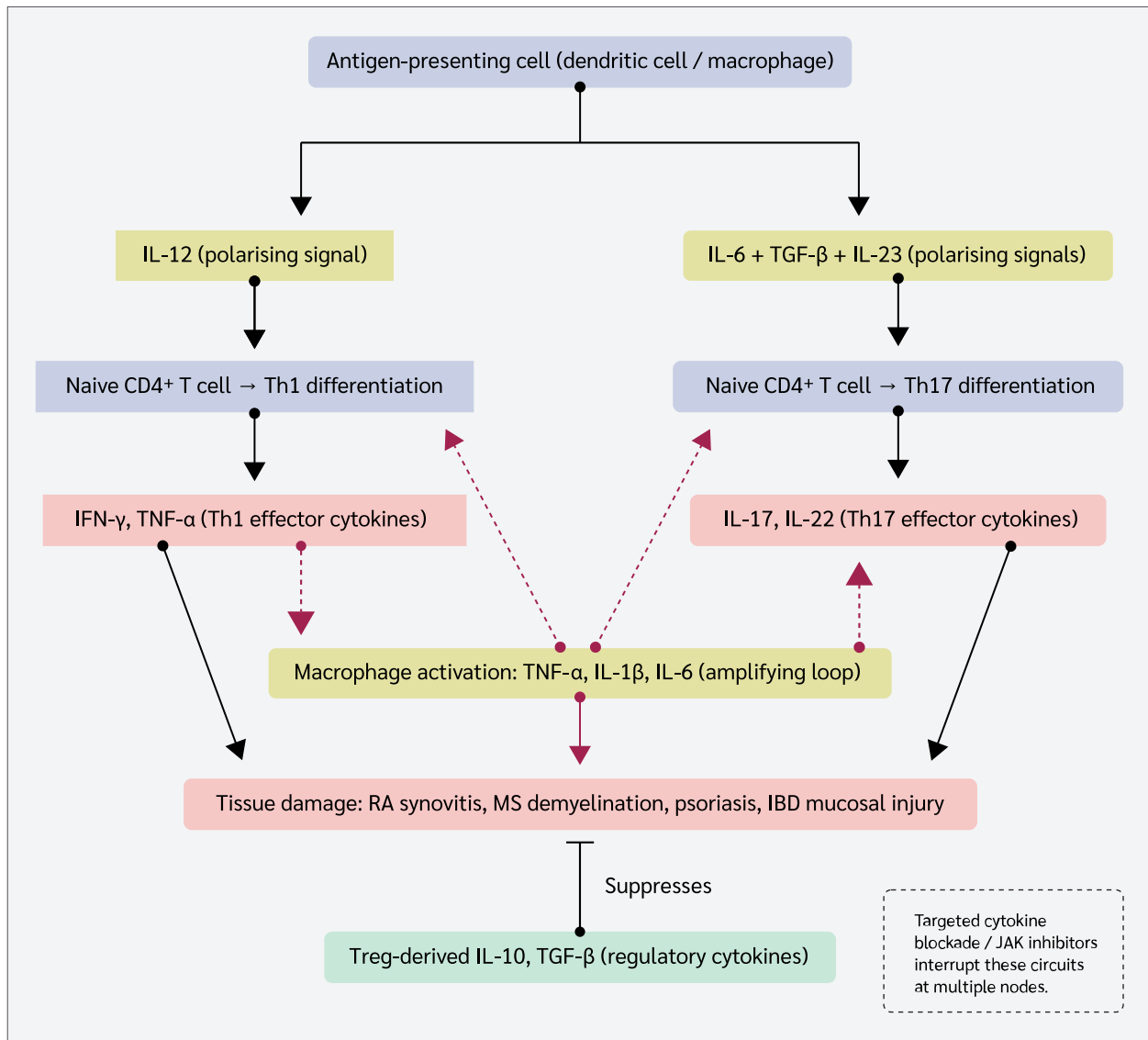


Figure 3. Cytokine networks in immune dysregulation. Pro-inflammatory Th1 (IFN- γ , TNF- α) and Th17 (IL-17, IL-22) cytokines, amplified by macrophage-derived TNF- α , IL-1 β , and IL-6, drive tissue damage in autoimmune diseases. Treg-derived IL-10 and TGF- β oppose these pathways; deficiency of regulatory cytokines or excess of inflammatory mediators sustains chronic disease. Targeted cytokine blockade and JAK inhibitors represent the primary pharmacological strategy for interrupting these circuits. Arrows trace the antigen-presenting cell-driven differentiation of naive CD4⁺ T cells into Th1 (IL-12-driven) and Th17 (IL-6/TGF- β /IL-23-driven) subsets, the amplifying macrophage loop these effector cytokines sustain, and the opposing Treg-derived IL-10/TGF- β axis; the direction of each arrow reflects established upstream-to-downstream signaling relationships rather than a simple co-occurrence.

RA: Rheumatoid arthritis, **SLE:** Systemic lupus erythematosus, **MS:** Multiple sclerosis, **IBD:** Inflammatory bowel disease.

by graded loss of effector functions, reduced proliferative capacity, and increased expression of inhibitory receptors including T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), PD-1, LAG-3, and CTLA-4 (44). B-cell dysregulation contributes to autoimmunity through loss of tolerance

checkpoints that allow autoreactive germinal center reactions, producing high-affinity autoantibodies that mediate pathology through fragment crystallizable (Fc) receptor activation and complement fixation (23). In SLE, dysfunction of B cells, T cells, and dendritic cells, together with impaired clearance of apoptotic cells, collectively amplifies inflammation (30,45). Dysfunction of B-cell and T-cell subsets, particularly Tregs

and Th17 cells, as well as dendritic cells, contributes to the inflammatory milieu characteristic of SLE and other systemic autoimmune diseases (5).

Complement System Dysregulation

The complement system, activated through the classical, lectin, and alternative pathways, serves as a crucial effector arm of innate immunity facilitating pathogen clearance, opsonization, and inflammation (2,21). Deficiencies in early complement components (C1q, C2, and C4) are paradoxically associated with SLE through impaired immune complex clearance, while uncontrolled activation, as in atypical hemolytic uremic syndrome caused by factor H mutations, drives endothelial injury through deposition of the membrane attack complex (2). Regulatory proteins including factor H and decay-accelerating factor (DAF) are essential for preventing excessive complement activation on host tissues; mutations or functional impairments in these regulators tip the balance toward pathological inflammation. These mechanisms of immune dysregulation rarely operate in isolation; their convergence in genetically susceptible individuals exposed to environmental triggers exemplifies the multifactorial complexity of immunological disorders, underscoring the need for systems-level approaches to research and management (9,11).

Classification and Clinical Spectrum of Immunological Disorders

Hypersensitivity Reactions: Exaggerated Immune Activation

The Gell and Coombs classification (summarized in Table 1) retains conceptual utility as an organizing framework for the diverse mechanisms by which inappropriate immune responses against innocuous antigens cause pathology (21). Type I hypersensitivity reactions, illustrated in Figure 4, are mediated by allergen-specific IgE cross-linking of FcεRI receptors on mast cells and basophils and are the most clinically prevalent. Their global increase has been attributed to the hygiene hypothesis, positing that reduced exposure to commensal organisms deprives the immune system of regulatory signals that normally constrain Th2 responses (22,48). Type II reactions, mediated by cytotoxic IgG or IgM antibodies directed against cell-surface antigens, operate through complement activation and antibody-dependent cellular cytotoxicity (ADCC), exemplified by autoimmune hemolytic anemia, Goodpasture syndrome, myasthenia gravis, and Graves disease (21). Type III (immune complex-mediated) reactions are illustrated by SLE, serum sickness, and reactive arthritis, and are characterized by complement activation and neutrophil recruitment at sites of complex deposition (21). Type IV delayed-type

Table 1. Classification of major immunological disorder categories.

Category	Key examples	Primary mechanism	Immune response	G-C type*	Ref.
Autoimmune disease	SLE, RA, T1DM, MS, Hashimoto thyroiditis	Loss of self-tolerance; autoreactive T/B cells; autoantibody production	Excessive (self-directed)	II, III, IV	5,23
Primary immunodeficiency	SCID, CVID, IPEX, CGD, XLA	Genetic mutation → absent or dysfunctional immune cells or pathways	Deficient	N/A	13,26
Secondary immunodeficiency	HIV/AIDS, chemotherapy-induced, malnutrition	Extrinsic depletion of immune competence (infection, drugs, nutrition)	Deficient (acquired)	N/A	4,27
Hypersensitivity reaction	Anaphylaxis, hemolytic anemia, serum sickness, contact dermatitis	Exaggerated or inappropriate response to non-threatening antigen	Excessive (antigen-directed)	I, II, III, IV	21
Chronic inflammatory	IBD, psoriasis, chronic granulomatous disease	Unresolved inflammation; defective anti-inflammatory regulatory circuits	Dysregulated activation	IV	28,47
Autoinflammatory disease	FMF, CAPS, DIRA, NLRP3 disorders	Innate immune dysregulation; no adaptive self-antigen response	Innate hyperactivation	N/A	29

*Gell-Coombs classification applies primarily to hypersensitivity reactions.
SLE: Systemic lupus erythematosus, **RA:** Rheumatoid arthritis, **T1DM:** Type 1 diabetes mellitus, **MS:** Multiple sclerosis, **SCID:** Severe combined immunodeficiency, **CVID:** Common variable immunodeficiency, **IPEX:** Immune dysregulation, polyendocrinopathy, enteropathy, X-linked, **XLA:** X-linked agammaglobulinemia, **CGD:** Chronic granulomatous disease, **IBD:** Inflammatory bowel disease, **FMF:** Familial Mediterranean fever, **CAPS:** Cryopyrin-associated periodic syndromes, **DIRA:** Deficiency of IL-1 receptor antagonist, **G-C:** Gell-Coombs, **Ref.:** Reference numbers as listed.

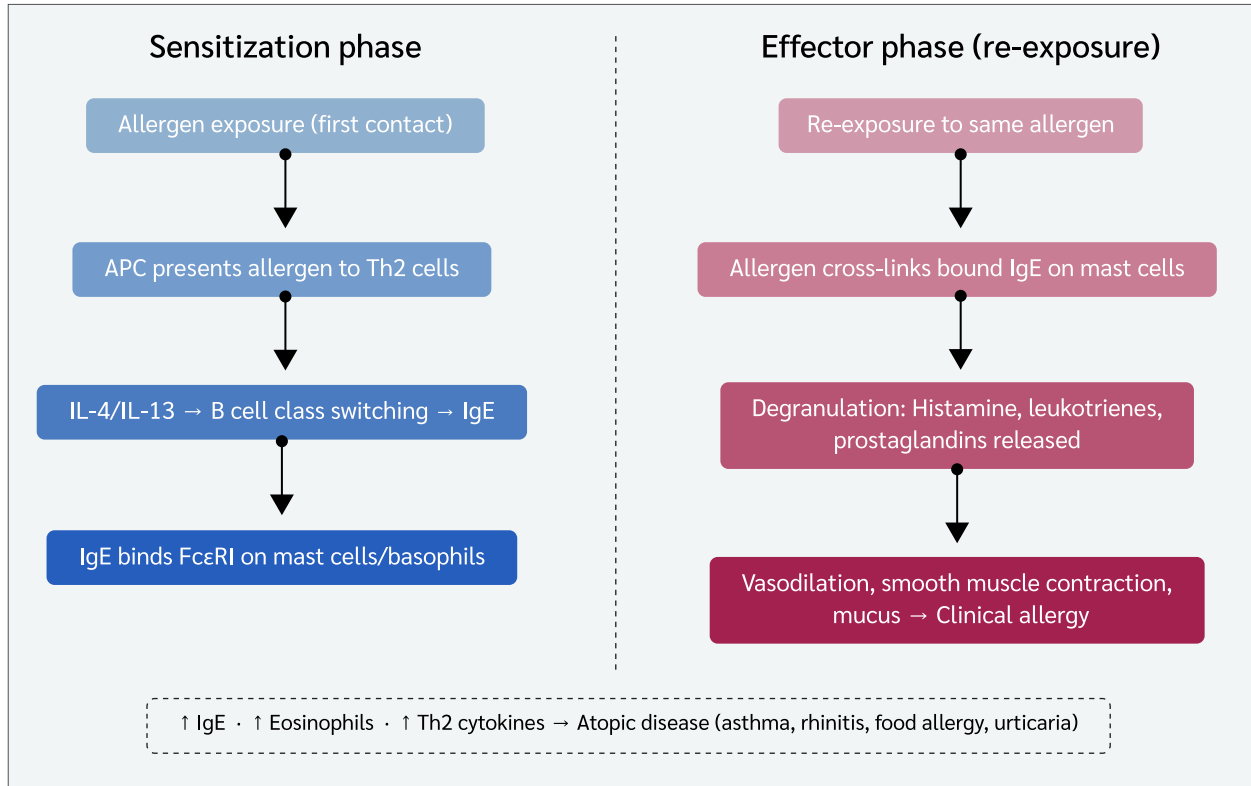


Figure 4. Type I (IgE-mediated) hypersensitivity: sensitization and effector phases. Upon initial allergen exposure, Th2-polarized responses drive IgE class switching and IgE binding to high-affinity FcεRI receptors on mast cells and basophils (sensitization). Re-exposure cross-links surface-bound IgE, triggering immediate degranulation and release of histamine, leukotrienes, and prostaglandins, followed by a late-phase response driven by Th2 cytokines and eosinophil recruitment.

APC: Antigen-presenting cell, **FcεRI:** High-affinity IgE receptor.

hypersensitivity, driven by antigen-experienced T cells, manifests 48–72 hours after antigen challenge, underpinning contact dermatitis, tuberculin reactions, and graft rejection (3,21).

This framework has been refined over the past decade. The original hygiene hypothesis, framed around reduced childhood infection, has been largely superseded by the biodiversity hypothesis, which attributes the rise in Type I hypersensitivity less to fewer infections per se and more to reduced early-life exposure to the broader range of environmental and commensal microorganisms that historically calibrated regulatory immune development; this distinction matters clinically, since it reframes prevention away from deliberately permitting infection and toward restoring microbial diversity, while also helping explain why some epidemiological data linking infection frequency directly to allergic disease risk have been inconsistent across cohorts.

Autoimmune Diseases: Tolerance Failure at Scale

More than 80 distinct autoimmune entities are recognized, collectively affecting approximately 4–5% of the global population with a consistent female predominance (4,24,25). All share a breakdown in the distinction between self and non-self, though their underlying mechanisms, effector pathways, and target organs are highly heterogeneous (5,23). Autoantibodies function as pharmacological agents at their targets: in Graves disease, thyroid-stimulating hormone (TSH)-receptor antibodies act as agonists driving thyroid overactivity; in myasthenia gravis, acetylcholine receptor antibodies act as antagonists blocking neuromuscular transmission (3). In SLE, nuclear antigen-antibody immune complexes deposit in glomeruli, skin, and synovium, activating complement and recruiting neutrophils that release neutrophil extracellular traps (NETs). These structures expose additional nuclear antigens, creating a self-amplifying autoimmune cycle (5,14). Helper CD4⁺

T cells, particularly Th17 cells producing IL-17, are key pathogenic effectors in RA, MS, psoriasis, and IBD, while FOXP3⁺ Treg cells that would normally restrain these responses appear to adopt a dysfunctional pro-inflammatory phenotype, a phenomenon designated Treg plasticity, whose molecular basis remains incompletely understood (4,9).

This literature is not fully settled, however. Human fate-mapping and lineage-tracing studies have produced conflicting estimates of how frequently bona fide FOXP3-stable Tregs convert to an IL-17-producing, pro-inflammatory phenotype *in vivo*, as opposed to a minority of unstable, low-FOXP3 cells that are selectively expanded under inflammatory conditions. This distinction has materially different therapeutic implications: the former would argue for stabilizing existing Tregs, while the latter would argue for excluding unstable clones from adoptive Treg therapy protocols. Reconciling these positions, rather than treating Treg plasticity as an established fact, remains an open methodological priority for the field.

Immunodeficiency: The Consequences of Immune Absence

Primary immunodeficiencies, now formally designated inborn errors of immunity (IEIs), encompass over 450 recognized disorders affecting innate immunity, adaptive immunity, immune regulation, or combinations thereof (13,26). Their clinical presentations reflect the specific immunological compartment affected: antibody deficiencies (such as X-linked agammaglobulinemia [XLA] and common variable immunodeficiency [CVID]) manifest with recurrent sinopulmonary infections; T-cell and combined immunodeficiencies confer susceptibility to viral, fungal, and opportunistic pathogens; and complement deficiencies predispose to *Neisseria* infections and autoimmune diseases through impaired immune complex clearance (2,26). Secondary immunodeficiencies, which are more prevalent globally, arise from HIV infection, iatrogenic immunosuppression, and malnutrition, each creating predictable patterns of immune deficiency (4,27). Infection with HIV remains paradigmatic: selective CD4⁺ T-cell depletion disrupts coordinated adaptive immune responses, permits opportunistic infections, and results in chronic immune activation that accelerates non-infectious morbidities (27).

Clinical Manifestations of Immune Dysregulation

The clinical manifestations of immune dysregulation are shaped by three intersecting variables: the nature of the immune defect (excessive, deficient, or misdirected), the organ systems targeted, and the phase of disease. Organ-specific manifestations serve as important diagnostic indicators of the underlying immunological disorder, as mapped out in Table 2 (2,24,49). Dermatological manifestations frequently serve as the diagnostic entry point, particularly in SLE and psoriasis, where the Th17-mediated keratinocyte hyperproliferation at sites of mechanical trauma (the Koebner phenomenon) provides visible evidence of systemic immune dysregulation (14,50). Neurological involvement has emerged as an especially dynamic field, with the discovery of specific neural autoantibodies transforming the classification and management of autoimmune encephalitis, a condition previously misdiagnosed as psychiatric disorders (14,55). Severe complications, cytokine storm syndrome, hemophagocytic lymphohistiocytosis, and acute respiratory distress syndrome, represent the extreme consequence of immune regulatory failure and are associated with high short-term mortality (12,57,60). In cytokine storm, positive feedback loops between immune cells result in persistent cytokine secretion and overwhelming systemic inflammation, driving vascular leakage, coagulopathy, and multi-organ failure (12,46,61).

Prevention, Treatment, and Emerging Therapeutic Strategies

Therapeutic strategy in immune dysregulation is shaped by two competing imperatives: the need to suppress pathological immune responses and the imperative to preserve host defense against infection and malignancy (31). Most current treatments resolve this tension imperfectly. The clinical success of TNF- α blockade in RA, IBD, and psoriasis provides direct pharmacological proof that cytokine excess drives tissue pathology. Janus kinase inhibitors provide a valuable oral therapeutic option with broader cytokine inhibition but carry class-specific risks, including thromboembolism, which remain under active regulatory scrutiny, as listed in Table 3 (9). Hematopoietic stem cell transplantation remains the only reliably curative approach for many monogenic immunodeficiencies and severe autoimmune phenotypes, but its risk-benefit

Table 2. Organ-system manifestations of immune dysregulation: a clinically oriented summary.

Organ system	Manifestations	Representative conditions	Key mechanism	Key references
Dermatological	Malar rash, photosensitivity, urticaria, psoriatic plaques, eczema, vasculitis purpura, alopecia areata	SLE, psoriasis, atopic dermatitis, urticarial vasculitis	Th17/IL-17 axis; IgE-mast cell activation; autoantibody-complement deposition; Th2 skewing	14,50,51
Respiratory	Recurrent sinusitis, bronchiectasis, airway hyper-responsiveness, interstitial lung disease, pulmonary fibrosis	CVID, allergic asthma, RA-ILD, Goodpasture syndrome	Impaired phagocyte/antibody defense; IgE/eosinophil-mediated bronchoconstriction; immune complex deposition	31,51,52
Gastrointestinal	Chronic diarrhea, mucosal ulceration, protein-losing enteropathy, autoimmune hepatitis, cholangitis	IBD, CVID enteropathy, autoimmune hepatitis, PSC	Gut dysbiosis; TNF- α /IL-23 axis; autoantibody-mediated hepatocellular injury; Th17 effectors	39,43,47
Musculoskeletal	Synovitis, joint erosion, cartilage destruction, myositis, tendinitis, osteopenia	Rheumatoid arthritis, dermatomyositis, spondyloarthropathy	Anti-CCP/RF autoantibodies; TNF- α /IL-6 cytokine excess; Th17 effectors; osteoclast activation	31,53
Neurological	Demyelination, peripheral neuropathy, autoimmune encephalitis, cognitive dysfunction, neuropsychiatric features	Multiple sclerosis, NMO-SD, NMDAR encephalitis, neuropsychiatric SLE	Autoreactive CD8 ⁺ T cells; AQP4/NMDAR autoantibodies; neuroinflammation; blood-brain barrier disruption	14,54,55
Cardiovascular	Accelerated atherosclerosis, myocarditis, pericarditis, Libman-Sacks endocarditis, vasculitis, thrombosis	SLE, RA, Kawasaki disease, giant cell arteritis, antiphospholipid syndrome	Endothelial activation by inflammatory cytokines; immune complex deposition; plaque instability	31,56
Renal	Proteinuria, hematuria, nephrotic/nephritic syndrome, hypertension, renal failure	Lupus nephritis, IgA nephropathy, ANCA-associated vasculitis, membranous nephropathy	Complement/IC deposition in glomeruli; pANCA/cANCA-mediated vessel injury; complement activation	2,14
Hematological	Hemolytic anemia, thrombocytopenia, neutropenia, pancytopenia, coagulopathy, DIC	AIHA, ITP, HLH, antiphospholipid syndrome	Autoantibody-mediated cell destruction; cytokine-driven ineffective hematopoiesis; macrophage hyperactivation	57–59

SLE: Systemic lupus erythematosus, **CVID:** Common variable immunodeficiency, **IBD:** Inflammatory bowel disease, **RA-ILD:** Rheumatoid arthritis-associated interstitial lung disease, **PSC:** Primary sclerosing cholangitis, **NMO-SD:** Neuromyelitis optica spectrum disorder, **ANCA:** Anti-neutrophil cytoplasmic antibody, **AIHA:** Autoimmune hemolytic anemia, **ITP:** Immune thrombocytopenic purpura, **HLH:** Hemophagocytic lymphohistiocytosis, **DIC:** Disseminated intravascular coagulation, **IC:** Immune complex, **AQP4:** Aquaporin-4, **NMDAR:** N-methyl-D-aspartate receptor, **Th17:** T helper 17 cell, **Th2:** T helper 2 cell, **TNF- α :** Tumor necrosis factor- α [typically TNF- α : Tumor necrosis factor- α], **IL-17:** Interleukin-17, **IL-23:** Interleukin-23, **IgE:** Immunoglobulin E, **IgA:** Immunoglobulin A, **RF:** Rheumatoid factor, **pANCA:** perinuclear Anti-neutrophil Cytoplasmic Antibody, **cANCA:** cytoplasmic Anti-neutrophil Cytoplasmic Antibody.

profile constrains its use to conditions sufficiently severe to justify procedural mortality; low survival and increased rates of graft failure have been reported in some subsets (9,32). Abatacept and belatacept have shown promising results in CTLA-4 deficiency as first-line therapy to control immune dysregulation, though increased infection susceptibility during treatment remains a concern for long-term use (32,33). Gene therapy has achieved regulatory approval for adenosine deaminase (ADA)-SCID and is in advanced trials for recombination-activating gene 1/recombination-activating gene 2 severe combined immunodeficiency (RAG1/RAG2 SCID), chronic

granulomatous disease, and Wiskott-Aldrich syndrome; its autologous nature eliminates graft-versus-host disease (9,13). Early-phase trials of anti-CD19 CAR-T-cell therapy in refractory SLE and myositis have generated extraordinary excitement with preliminary durable drug-free remissions, but replication in larger cohorts is needed (9). Immunomodulatory drugs, including ruxolitinib for inflammatory and proliferative disease, novel peptide drugs, and anti-T-cell antibodies, expand the pharmacological toolkit, while prevention strategies, including vaccination, dietary microbiome intervention, and neonatal SCID screening (via T-cell receptor excision

Table 3. Therapeutic landscape for immune dysregulation: mechanisms, applications, and limitations.

Therapeutic Class	Mechanism of action	Key agents	Primary indications	Limitations	Key Ref.
Conventional immunosuppressants	Broad suppression of lymphocyte proliferation and inflammation	Corticosteroids, methotrexate, azathioprine, cyclophosphamide	RA, SLE, IBD, vasculitis, organ transplantation	Non-specific; infection risk; metabolic toxicity; teratogenicity	31
TNF- α inhibitors	Neutralize soluble and membrane-bound TNF- α	Adalimumab, infliximab, etanercept, certolizumab pegol	RA, IBD (CD/UC), psoriasis, SpA	Reactivation of latent TB; increased infection risk; primary non-response ~30%	31
IL-6 pathway inhibitors	Block IL-6 receptor or cytokine signaling	Tocilizumab, sarilumab, siltuximab	RA, cytokine storm, giant cell arteritis, Castleman disease	Risk of GI perforation; hepatotoxicity; masking of fever obscuring infection	12
IL-17/IL-23 inhibitors	Block Th17 axis cytokines or upstream polarizing signal	Secukinumab, ixekizumab, ustekinumab, guselkumab	Psoriasis, PsA, axSpA	Increased Candida infection; IBD exacerbation with IL-17 agents	50
JAK inhibitors	Inhibit JAK1/JAK2/JAK3, disrupting cytokine receptor signaling via JAK-STAT pathway	Ruxolitinib, tofacitinib, baricitinib, upadacitinib	RA, IBD, alopecia areata, atopic dermatitis, myelofibrosis, GvHD	Thrombosis risk (higher doses); herpes zoster reactivation; MACE risk; ongoing regulatory scrutiny	9
Anti-CD20 (B-cell depletion)	Eliminate CD20 ⁺ B cells, reducing autoantibody production and B-cell antigen presentation	Rituximab, obinutuzumab, ocrelizumab	RA, SLE, MS (ocrelizumab), ANCA vasculitis, AIHA	Hypogammaglobulinemia; PML risk (rare); prolonged B-cell depletion	31,54
CTLA-4-Ig fusion proteins	Block CD80/86-CD28 co-stimulation, impairing T-cell activation	Abatacept, belatacept	RA, CTLA4 haploinsufficiency, LRBA deficiency, renal transplant (belatacept)	Increased infection susceptibility; IV administration burden; not curative	32,33
HSCT	Immune reconstitution via donor hematopoietic stem cells	Allogeneic, autologous, gene-corrected autologous HSCT	SCID, IPEX, HLH, severe refractory autoimmune disease	GvHD; graft failure; transplant-related mortality; limited donor availability; low survival in some subsets	32,42
Gene therapy / editing	Correct causative mutation in autologous stem cells via lentiviral vector or CRISPR-Cas9	ADA-SCID (approved), RAG-SCID, CGD, WAS (in trials)	Monogenic IEI without suitable HSCT donor or after failed HSCT	Insertional mutagenesis (viral vectors); off-target edits (CRISPR); limited to monogenic disorders; access/cost	9,42
CAR-T-cell therapy	Redirect cytotoxic T cells to deplete autoreactive B cells or other specific cellular targets	Anti-CD19 CAR-T (investigational in autoimmunity)	Refractory SLE, myositis, systemic sclerosis (early-phase clinical trials)	Cytokine release syndrome; neurotoxicity; manufacturing complexity; uncertain durability; high cost	9

RA: Rheumatoid arthritis, **SLE:** Systemic lupus erythematosus, **IBD:** Inflammatory bowel disease, **CD:** Crohn disease, **UC:** Ulcerative colitis, **SpA:** Spondyloarthritis, **PsA:** Psoriatic arthritis, **axSpA:** Axial spondyloarthritis, **GvHD:** Graft-versus-host disease, **HSCT:** Hematopoietic stem cell transplantation, **IEI:** Inborn errors of immunity, **SCID:** Severe combined immunodeficiency, **MACE:** Major adverse cardiovascular events, **PML:** Progressive multifocal leukoencephalopathy, **WAS:** Wiskott-Aldrich syndrome, **CAR-T:** Chimeric antigen receptor T cell, **AIHA:** Autoimmune hemolytic anemia, **ANCA:** Anti-neutrophil cytoplasmic antibody, **LRBA:** Lipopolysaccharide-responsive beige-like anchor protein.

circle [TREC] assays) broaden the public health approach to immune dysregulation (9,26).

It is worth being explicit about the limits of this evidence base at the present time: the published experience consists of small, uncontrolled, single-arm case series from a handful of centers, typically including fewer than 20 patients per report, with follow-up rarely exceeding two to three years; no randomized controlled trial has yet

been reported, the durability of remission beyond this window is unknown, and the risk of long-term B-cell aplasia or secondary malignancy with CAR-T-cell therapy in a nonmalignant, potentially younger population has not been systematically characterized. These findings should accordingly be considered as hypothesis-generating rather than practice-changing evidence at this stage.

This regulatory scrutiny was substantially sharpened by the ORAL Surveillance trial, a large, randomized safety study in RA patients aged 50 years or older with cardiovascular risk factors, which found higher rates of major adverse cardiovascular events, malignancy, and venous thromboembolism with tofacitinib compared with TNF- α inhibitors, prompting boxed-warning label changes for the entire JAK inhibitor class. Whether this risk generalizes to younger, lower-cardiovascular-risk patients, and to other diseases treated with JAK inhibitors beyond RA, remains actively debated, and current guidance favors reserving JAK inhibitors for patients in whom TNF- α inhibitors have failed or are contraindicated, rather than using them as a first-line biologic-sparing option.

Future Directions and Knowledge Gaps

Despite remarkable advances in understanding and treating immune dysregulation, the field faces several fundamental challenges. Single-cell multi-omics technologies, combining transcriptomics, epigenomics, proteomics, and spatial profiling, are beginning to reveal the heterogeneity of immune states across disease stages and between individuals, potentially enabling prediction of disease trajectories before clinical manifestation. The prospect of durable immune reset, rather than ongoing suppression, is supported by CAR-T-cell therapy data. Regulatory T-cell therapeutics, including expanded polyclonal Treg infusions and antigen-specific engineered Tregs, are currently in clinical trials as biologically elegant approaches to restoring immune regulation. The microbiome-immune interface holds substantial promise: fecal microbiota transplantation is well established in IBD, and its application in systemic autoimmunity is under active investigation. Artificial intelligence (AI) and machine learning applied to multi-modal immunological datasets are producing predictive models for disease flares, treatment response, and at-risk-to-disease transition whose clinical implementation will require careful attention to interpretability and regulatory frameworks. With advances in technologies such as spatial transcriptomics and AI, further breakthroughs in immunology are anticipated. Molecular therapies, including monoclonal antibodies, B-cell depleting agents, and CAR-T-cell treatments, represent a step toward more precisely targeted therapies with potentially fewer off-target side effects; however, as detailed below, this potential remains to be confirmed in controlled trials, and a cure for most

autoimmune diseases remains an aspiration rather than a demonstrated outcome.

Controversies and Evidence Gaps

Several topics covered in this review remain genuinely unsettled rather than representing incremental refinements of an agreed understanding and are highlighted here explicitly rather than being folded silently into the narrative above. First, whether Treg dysfunction in autoimmune disease principally reflects numerical deficiency, functional impairment of otherwise normal-numbered Tregs, or outright lineage instability (Treg plasticity) remains contested, and different answers imply different therapeutic strategies (Treg expansion, Treg stabilization, or exclusion of unstable clones from adoptive therapy). Second, the JAK inhibitor cardiovascular and malignancy signal identified in ORAL Surveillance was generated in an older, cardiovascular-risk-enriched RA population, and whether it applies with similar magnitude across the younger, more diverse populations now being treated with JAK inhibitors for atopic dermatitis, alopecia areata, and inflammatory bowel disease is an open, clinically consequential question. Third, the CAR-T-cell literature in autoimmunity, discussed above, currently rests entirely on small, uncontrolled case series; the field lacks any randomized comparison against existing biologic therapy, and claims of a durable immune reset should therefore be regarded as preliminary until such data become available. Finally, the therapeutic microbiome literature is asymmetric: fecal microbiota transplantation has strong evidence in *Clostridioides difficile* infection and some support in ulcerative colitis, but its extension to systemic autoimmune disease rests largely on small pilot studies and preclinical models and should not be presented with the same evidentiary weight as the gut-specific indications. We identify these four areas as priorities for future systematic review and, where feasible, randomized controlled trials, rather than as settled components of current practice.

Conclusion

Immune dysregulation is not a single disease but a mechanistic category encompassing the full spectrum of conditions that arise when the immune system loses its capacity for discriminating, proportionate, and

self-limited responses. The shared language of tolerance failure, cytokine dysregulation, cellular exhaustion, and genetic predisposition connects conditions as clinically diverse as IPEX syndrome, SLE, allergic asthma, and HIV-associated immunodeficiency into a coherent framework that illuminates both pathogenesis and therapeutic logic. The past decade has brought transformative advances: biological agents that precisely interrupt individual cytokine pathways, JAK inhibitors that broadly dampen cytokine signaling, and the first glimpses of curative approaches through gene therapy and immune resetting strategies. To date, no curative therapy is available for most autoimmune diseases. However, recent research indicates that molecular therapies, monoclonal antibodies, B-cell depleting agents, and, more speculatively, CAR-T-cell treatments may benefit patients by addressing some limitations of traditional approaches.

Nevertheless, as discussed above, the evidence for several of these newer strategies remains preliminary, and a cure for most autoimmune and immunodeficiency conditions has not yet been demonstrated at scale. The path toward closing persistent gaps depends on mechanistic clarity regarding how genetic susceptibility, environmental exposures, and microbial ecology converge on threshold-dependent transitions from immune health to immune disease, together with therapeutic innovation in antigen-specific tolerance induction, Treg-based therapies, and clustered regularly interspaced short palindromic repeats (CRISPR)-enabled gene correction. Achieving durable, curative outcomes will require the full integration of cellular, molecular, and clinical immunology with the technological and analytical tools that are already reshaping what is possible to know and to do about the human immune system.

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Maternal Gut Dysbiosis Exacerbates Offspring Susceptibility to Collagen-Induced Arthritis in Mice

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Abstract

Objective: The gut microbiota plays a critical role in immune system development and the maintenance of immune tolerance. Dysbiosis, particularly during the maternal period, has been implicated in autoimmune diseases. This study investigated whether maternal antibiotic-induced dysbiosis exacerbates collagen-induced arthritis (CIA) in offspring and whether fecal microbiota transplantation (FMT) can reverse this effect.

Materials and Methods: To establish a dysbiosis model, pregnant BALB/c mice were administered a combination of amoxicillin (40 mg/kg) and vancomycin (30 mg/kg) from the last week of pregnancy until the male pups used in the experiment (n=5 per group) reached 8 weeks of age (total treatment duration, 9 weeks). In the FMT group, oral FMT was administered after completion of antibiotic therapy. The bacterial profiles of the mice gut microbiota were analyzed by sequencing, and CIA was subsequently induced in all groups.

Results: Maternal dysbiosis significantly reduced bacterial populations in the gut microbiota, whereas FMT restored these populations. Additionally, dysbiosis was associated with increased arthritis severity in the CIA model, whereas FMT appeared to reduce it. These results demonstrate that the maternal gut microbiota plays a critical role in the pathogenesis of autoimmune arthritis and that FMT may represent a potential therapeutic approach.

Conclusion: Maternal dysbiosis in the gut microbiota and its treatment with FMT affected body weight in mice, particularly causing low birth weight. Dysbiosis increased disease severity in the CIA model, whereas FMT treatment reduced it.

Keywords: Gut microbiota, dysbiosis, fecal microbiota transplantation, collagen-induced arthritis, rheumatoid arthritis, autoimmunity

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Introduction

The critical role of the gut microbiota in organismal homeostasis and immune system development is increasingly being understood through numerous studies (1,2). This microbial diversity plays a central role in the development of immune tolerance, in addition to performing essential functions such as nutrient metabolism, colonization resistance against pathogens, and immunomodulation (3,4). Dysbiosis, which refers to a disruption in the functional balance of the gut microbiota, is associated with various inflammatory and autoimmune diseases (5).

Factors in early life are particularly important in the development of dysbiosis. Colonization of the microbiota begins in the prenatal period, and maternal factors such as the mother's diet, medications (particularly antibiotics), and delivery mode shape the newborn's microbial composition (6,7). Studies have shown that dysbiosis caused by maternal antibiotic use disrupts intestinal barrier integrity, thereby triggering systemic inflammation and having long-term effects on immunological maturation (8,9). This is particularly important in the pathogenesis of autoimmune diseases, which are characterized by impaired immune tolerance.

Rheumatoid arthritis (RA), a chronic, systemic autoimmune disease, can develop as a result of dysbiosis (10,11). In addition to genetic predisposition, environmental triggers play a role in the pathogenesis of RA, and recent studies have indicated the potential role of the gut microbiota in this process (12,13). For example, microbiome studies in RA patients have shown that the prevalence of some bacterial genera is increased, whereas that of others is decreased; similarly, experimental arthritis models have demonstrated that changes in the microbiome are associated with disease severity (14,15).

One strategy for treating dysbiosis is fecal microbiota transplantation (FMT). In this approach, the gut microbiota from a healthy donor is transferred to a recipient to restore microbial balance. It has been proven to be effective in various gastrointestinal diseases, including *Clostridium difficile* infection (16,17). However, the immunomodulatory effects of FMT on autoimmune diseases, particularly RA, and the underlying mechanisms remain incompletely understood.

The effects of the microbiome on the immune system, the effects of maternal dysbiosis on the development of

autoimmune diseases such as RA, and the treatment of these effects with FMT have been discussed in only a few studies (18). Therefore, this study aimed to investigate experimentally the effects of maternal gut dysbiosis induced by antibiotic exposure, as well as subsequent FMT, on the development and severity of collagen-induced arthritis (CIA) in offspring. We further sought to reveal the long-term programming effects of the maternal gut microbiota on the immune system and the therapeutic potential of FMT.

Materials and Methods

Animals and Experimental Protocol

In this study, we used 12 pregnant female BALB/c mice aged 2–3 months. The pregnant mice were divided into three groups (n=4 per group). Five male offspring from each maternal group were randomly selected for experimental analyses after weaning. This experimental study was conducted after receiving approval from the Bursa Uludağ University Animal Experiments Local Ethics Committee (Approval No: 2023-06/05, Date: April 11, 2023).

Induction of Maternal Dysbiosis

Pregnant mice were divided into three groups designated as the control, dysbiosis, and FMT groups (Figure 1A; five male offspring per group). The control group received sterile drinking water throughout the experiment, and the male offsprings born to these mothers received sterile drinking water after weaning. This group did not receive antibiotics.

To induce dysbiosis in the dysbiosis and fecal microbiota transplantation (FMT) groups, an antibiotic mixture was administered daily. This mixture consisted of amoxicillin (Bostonchem Co., Boston, MA, USA, 40 mg/kg) and vancomycin (Bostonchem Co., Boston, MA, USA, 30 mg/kg), added to the drinking water. The treatment lasted for a total of 5 weeks, starting in the last week of pregnancy and continuing until the offspring were 4 weeks old and weaned. Antibiotics were transferred to the newborn pups through maternal milk. By the third week, the pups consumed a combination of maternal milk and water, which also contained the antibiotics. After weaning, the same antibiotic mixture was administered to the male offspring for an additional 4 weeks, resulting in a total of 9 weeks (1 prenatal week and 8 postnatal weeks) of antibiotic-induced dysbiosis.

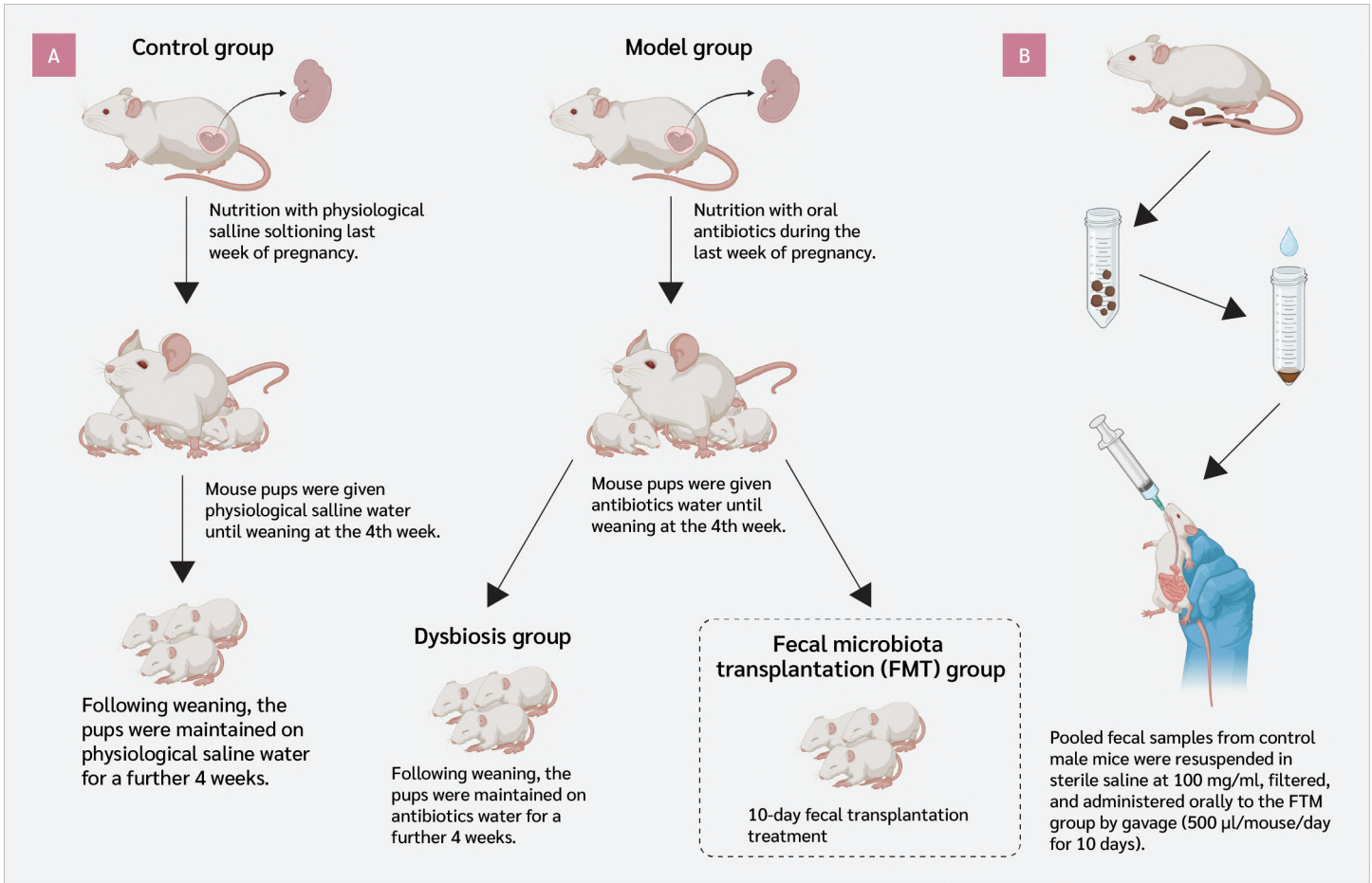


Figure 1. Experimental design and schematic representation of the fecal microbiota transplantation (FMT) protocol. **(A)** Pregnant mice were divided into a control group and a model group. The model group is shown first because both the dysbiosis and FMT groups received initial antibiotic treatment. No intervention was performed in the control group from the last week of pregnancy until the end of the experiment. Mice in the model group received oral antibiotics starting in the last week of pregnancy. Antibiotic treatment continued until 4 weeks of age, which is the age of weaning. Until this age, the pups consumed both their mother's milk and antibiotic-containing drinking water. At 4 weeks of age, the pups were separated from their mothers, and antibiotic administration continued for an additional 4 weeks. When the pups reached 8 weeks of age, antibiotic treatment was discontinued, and the model group was divided into a dysbiosis group and an FMT group. **(B)** Feces from the control group mice were collected and suspended, then administered orally to the FMT group mice by gavage for 10 days.

Fecal Microbiota Transplantation (FMT)

After inducing dysbiosis in the FMT group (i.e., after completion of the 1-week prenatal and 8-week postnatal antibiotic regimen), FMT was administered to the pups. Considering the antibiotic half-life, FMT was initiated 1 day after the last antibiotic dose. Healthy donor mice from the control group were used for FMT treatment (n=5). Fecal samples obtained from the control group mice were suspended and administered orally to the FMT group via gavage once daily for 10 days. Fecal samples were collected fresh daily. Fecal microbiota transplantation was not administered to the dysbiosis group (Figure 1B). All animals were housed in individually ventilated

cage (IVC) (Tecniplast S.p.A., Buguggiate, Italy) systems with sterile feed and bedding. The experimental procedure used to induce microbial changes is summarized in Figure 1.

After microbial changes were completed, fecal samples were collected from all offspring groups to assess whether dysbiosis had developed and to evaluate the effect of FMT. Fecal pellets were collected into sterile, DNase- and RNase-free tubes free of DNA and RNA contamination. Samples from each group were pooled to create a single sample per group. Shotgun metagenomic sequencing was performed by Sapiens Genetic Tanı Inc.

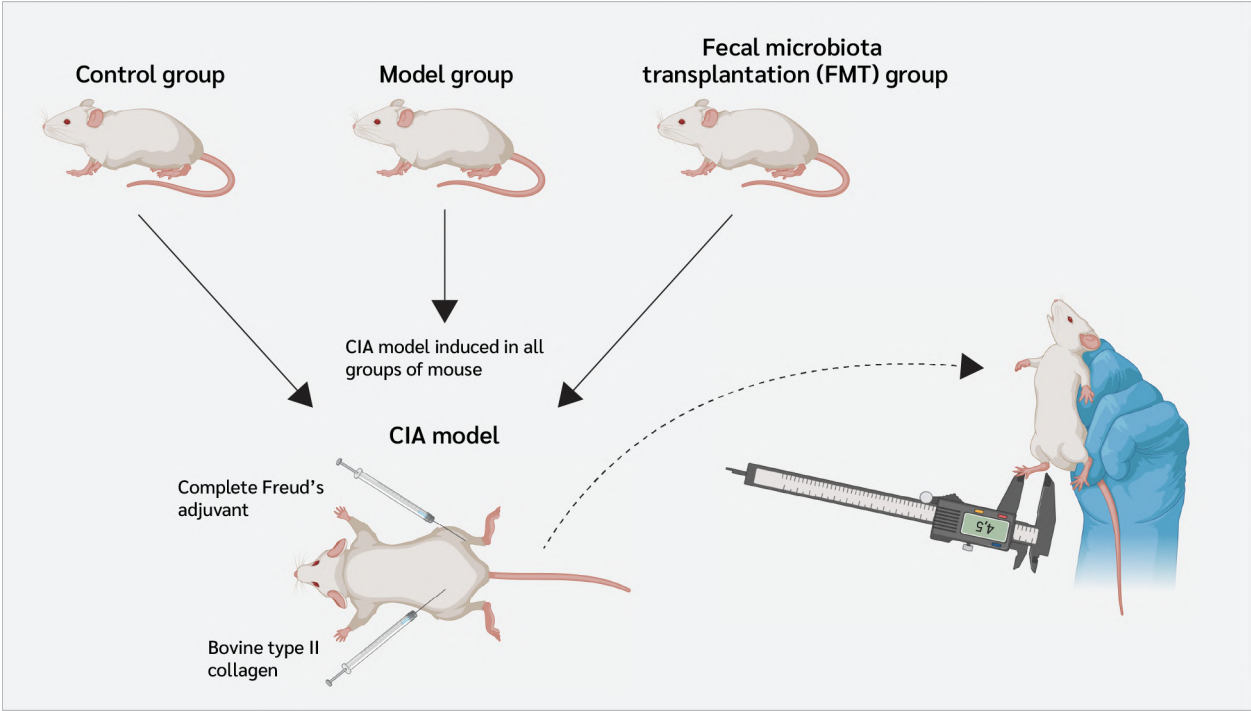


Figure 2. Induction of CIA model in mice with altered microbiota (control group, dysbiosis group, and FMT group) and measurement of joint thickness. For arthritis induction, 10 μ L of collagen emulsion containing 100 μ g bovine type II collagen was injected intra-articularly into the right hind leg (between the femur and tibia). Simultaneously, 100 μ L of CFA was injected subcutaneously. This immunization protocol was repeated 14 days later.

(İstanbul, Türkiye). In summary, library preparation was performed using the Illumina DNA Prep Kit (Illumina, San Diego, CA, USA,) according to the manufacturer's instructions, including DNA shearing, indexing, amplification, and purification. Library fragment distribution was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to obtain paired-end 150-bp reads.

Induction of Collagen-Induced Arthritis (CIA)

Following the alterations in microbial composition, CIA was initiated in male offspring across all groups, in accordance with a previously established method for BALB/c mice (19). To minimize potential variability associated with fluctuations in sex hormones and the estrus cycle in mice, only male offspring were included in the study. In summary, 10 μ L of collagen emulsion (containing 100 μ g bovine type II collagen; Sigma-Aldrich, St. Louis, MO, USA) was injected intra-articularly into the right hind leg (between the femur and tibia). At the same time, 100 μ L of complete Freund's adjuvant (CFA;

Table 1. Clinical scoring criteria for collagen induced arthritis.

Symptom	Score
Normal	0
Slight redness and swelling in the ankle and some claws	1
Moderate redness and swelling in the ankle and some claws	2
Severe redness and swelling in the ankle and all toes	3
Multiple joint involvement and severely inflamed limb	4

containing heat-killed *Mycobacterium tuberculosis*; Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously. This immunization protocol was repeated 14 days later. Arthritis development was monitored for 21 days. The left hind leg of each animal that did not receive an injection served as a control (Figure 2).

Clinical arthritis was assessed by measuring joint thickness with a digital caliper (Mitutoyo, Kawasaki, Japan) (Figure 2) and by scoring the severity of joint inflammation according to the clinical scoring system (Table 1). Body weight was measured every 3–4 days for 10 weeks.

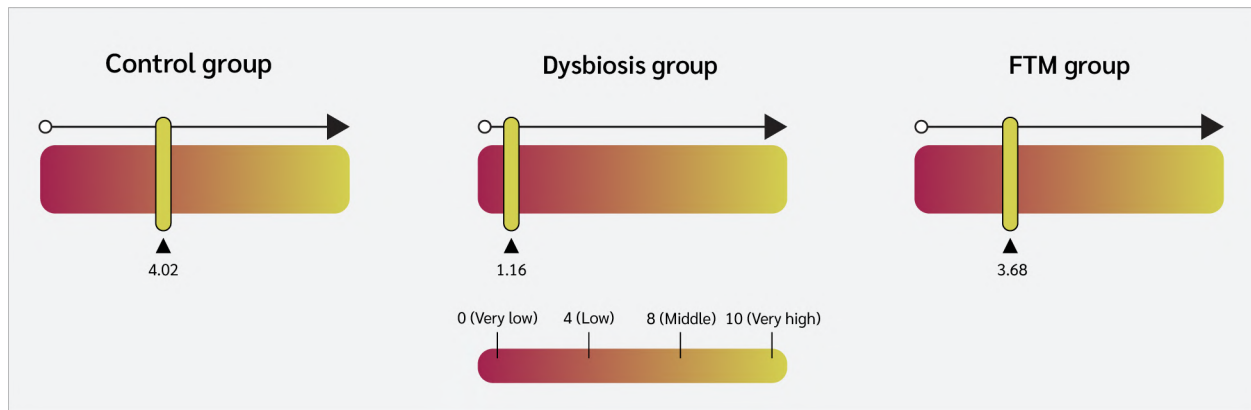


Figure 3. Gut microbiota alpha diversity (Shannon index). Fecal samples were collected from five animals in each group and pooled for analysis.

Histopathological Analysis

At the end of the experiment (day 21), the mice were euthanized by cervical dislocation. Joint tissue samples were collected from the arthritic (right) and control (left) legs. The legs were disarticulated at the coxofemoral joint, skinned, and fixed in 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA). Following fixation, bone decalcification was performed using 14% EDTA (pH 7.2; Sigma-Aldrich, St. Louis, MO, USA), and the solution was changed every three days. After decalcification, the joints were cut horizontally at the genu articulation region and embedded in paraffin following a standard tissue processing procedure (increasing alcohol concentrations, xylene, paraffin).

Sections 4 μ m thick were cut from the paraffin blocks and stained with hematoxylin and eosin (Merck Millipore, Burlington, MA, USA) for examination under a light microscope (Olympus CX41 Phase Contrast & Darkfield Microscope, Tokyo, Japan). Histological scoring was performed using a standardized semi-quantitative scale (0–4), evaluating synovial hyperplasia, pannus formation, inflammatory cell infiltration of the synovial membrane, neovascularization, cartilage hypocellularity, and degenerative/necrotic changes accompanied by fissure formation. All sections were evaluated independently by two blinded investigators to ensure consistency and minimize observer bias.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Continuous variables, including joint thickness values and body weight, were analyzed using one-way ANOVA followed

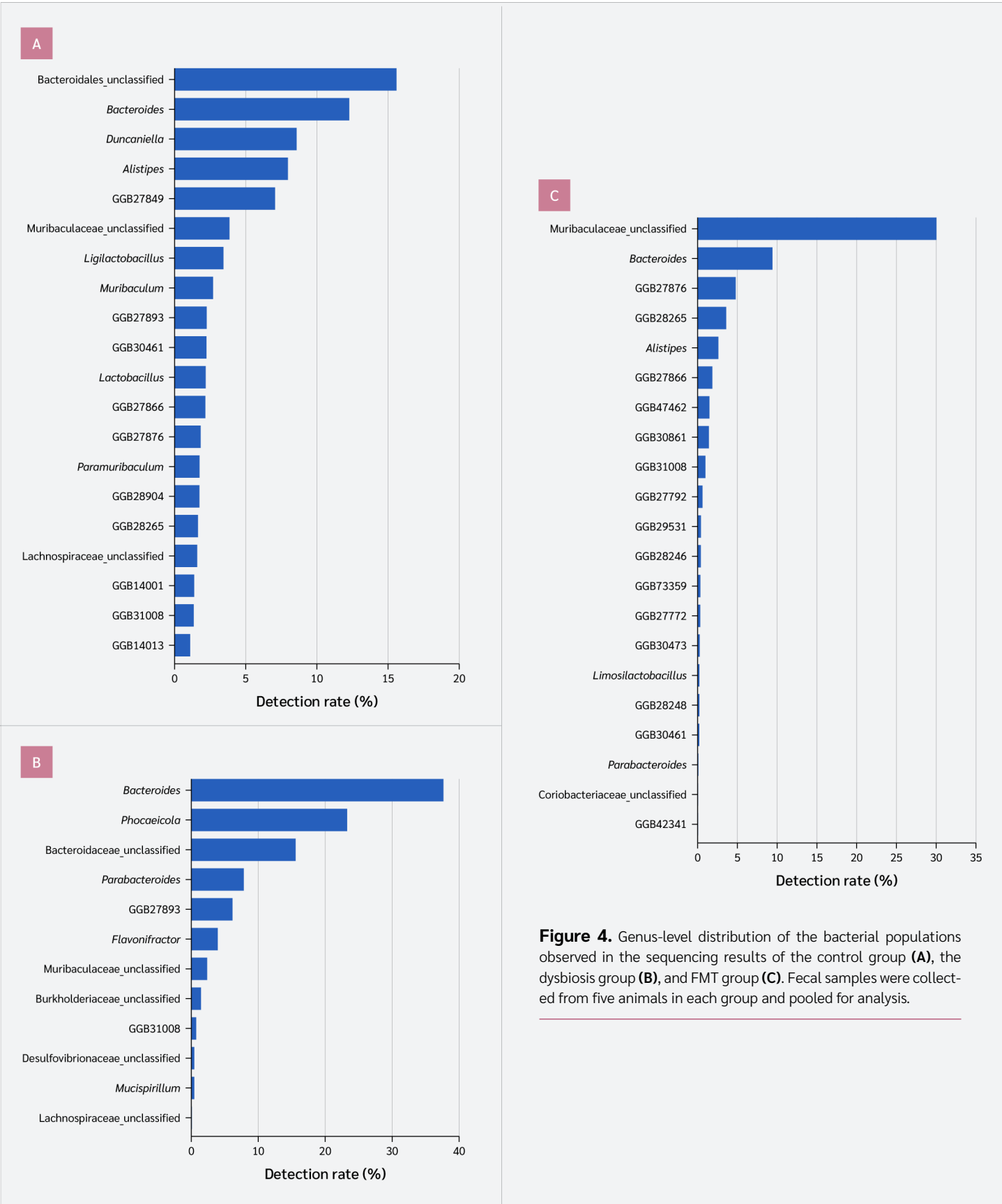
by Tukey's post hoc test. Ordinal data, including clinical arthritis scores and histopathological scores, were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. All data were presented as mean $\pm$ standard error of the mean. Statistical comparisons were performed using the independent samples t-test. A p -value <0.05 was considered statistically significant.

Results

Shotgun metagenomic analysis of fecal samples collected prior to arthritis induction revealed significant changes in the gut microbiota. The Shannon index, an indicator of alpha diversity, was 4.02 in the control group and 1.16 in the dysbiosis group. These findings indicate that alpha diversity in the dysbiosis group was significantly reduced compared with that of the control group. The Shannon index of 3.68 in the FMT group indicated that FMT substantially restored this loss of diversity (Figure 3).

Genus-level bacterial distribution analysis showed that the microbial population was significantly reduced in the dysbiosis group. While 124 different bacterial genera were identified in the control group, this number dropped to 12 genera in the dysbiosis group. In the FMT group, bacterial diversity increased, with 109 genera identified, indicating restoration of the microbial diversity toward that observed in the control group (Figure 4).

Maternal-initiated dysbiosis had a significant effect on growth. Animals in the dysbiosis and FMT groups had



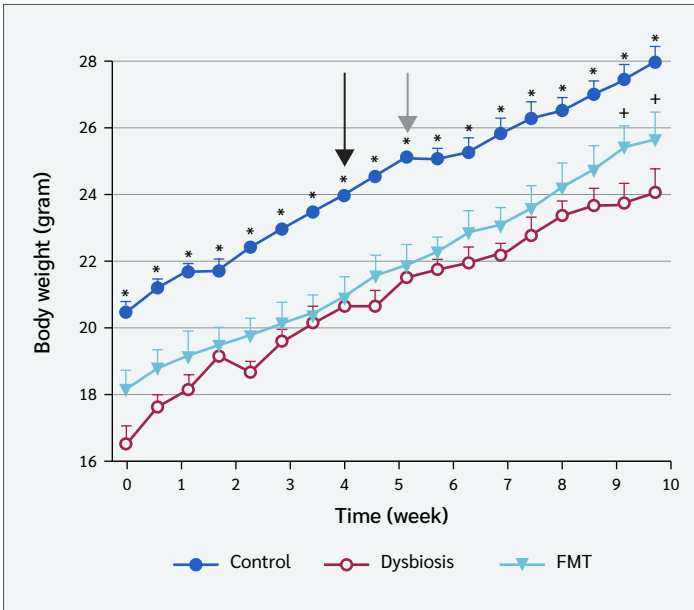


Figure 5. Changes in body weight during the experimental period. Body weight (g) was measured over a 10-week period in the control, dysbiosis, and FMT groups. Antibiotic administration was performed during weeks 0–4, after which treatment was discontinued and FMT was initiated at week 5. At baseline (week 0), the control group displayed significantly higher body weights than the antibiotic-treated groups ($p=0.0001$). At week 4, both antibiotic-treated groups remained significantly lower than the control group ($p=0.0058$). Following FMT initiation at week 5, the FMT group progressively diverged from the dysbiosis group, reaching statistical significance at weeks 9 and 10 ($p=0.038$ and $p=0.0375$, respectively). Data are presented as mean $\pm$ standard error ($n=5$ per group). Statistical comparisons were performed using an independent samples t-test. Asterisks (*) denote significant differences between the control group and the antibiotic-treated groups (dysbiosis and FMT), whereas plus signs (+) denote significant body weight recovery in the FMT group compared with the Dysbiosis group. The black arrow indicates the end of antibiotic treatment, and the gray arrow indicates the first injection for induction of arthritis.

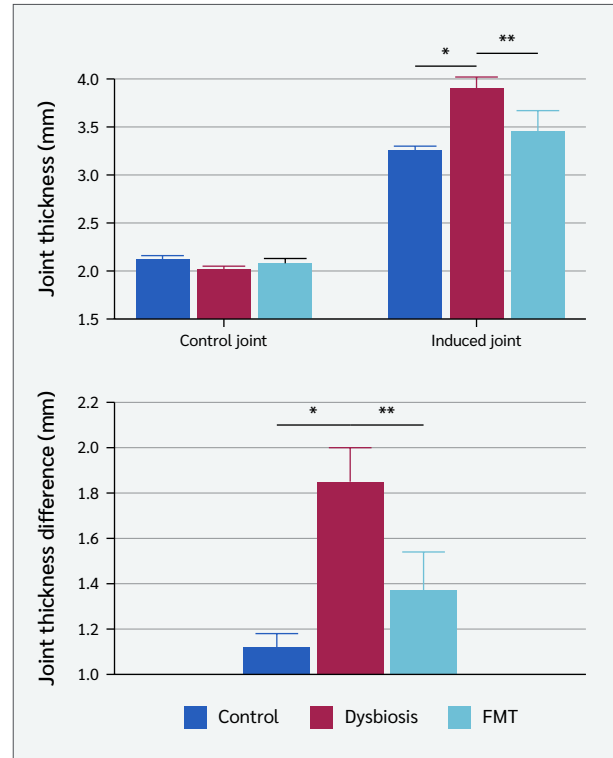


Figure 6. Effect of fecal microbiota transplantation on joint thickness. Data are presented as mean $\pm$ standard error (SE; $n=5$ per group). **(A)** Joint thickness (mm) across experimental groups. Joint thickness was significantly increased in the dysbiosis group compared with the control group (*, $p=0.0028$). Treatment with FMT significantly reduced joint thickness relative to the dysbiosis group (**, $p=0.046$). **(B)** Joint thickness difference (arthritic joint thickness - contralateral control joint thickness). A significant increase was observed in the dysbiosis group compared with the control group (*, $p=0.0095$). Treatment with FMT significantly reduced the difference in joint thickness compared with the dysbiosis group (**, $p=0.023$).

Table 2. Clinical arthritis scores in the experimental groups.

	Control group	Dysbiosis group	FMT group
Clinical arthritis score	3.25 $\pm$ 0.25	3.00 $\pm$ 0.32	3.20 $\pm$ 0.20

Note: Values are presented as mean $\pm$ standard error.

FMT: Fecal microbiota transplantation.

significantly lower body weights than those in the control group from the time of weaning (week 0). This difference persisted throughout the 4-week postnatal antibiotic administration period. After FMT treatment began, the FMT group showed an improving trend in body weight gain compared with the dysbiosis group. Following

arthritis induction, body weight gain slowed in all groups as expected (Figure 5).

In the CIA model, joint thickness was significantly higher in the dysbiosis group than in the control group ($p=0.0028$). Joint thickness was significantly lower in the FMT group than in the dysbiosis group ($p=0.046$) (Figure 6).

When the difference in thickness between the arthritic and control joints was calculated, it became evident that dysbiosis increased the severity of arthritis, while FMT partially prevented this increase (Figure 6). Clinical arthritis scores showed no statistically significant differences between the groups (Table 2). Images of the joints in the experimental CIA model are shown in Figure 7.



Figure 7. Representative images of joint thickness in the control (A), dysbiosis (B), and FMT (C) groups. In all images, the right legs have arthritis, whereas the left legs served as controls.

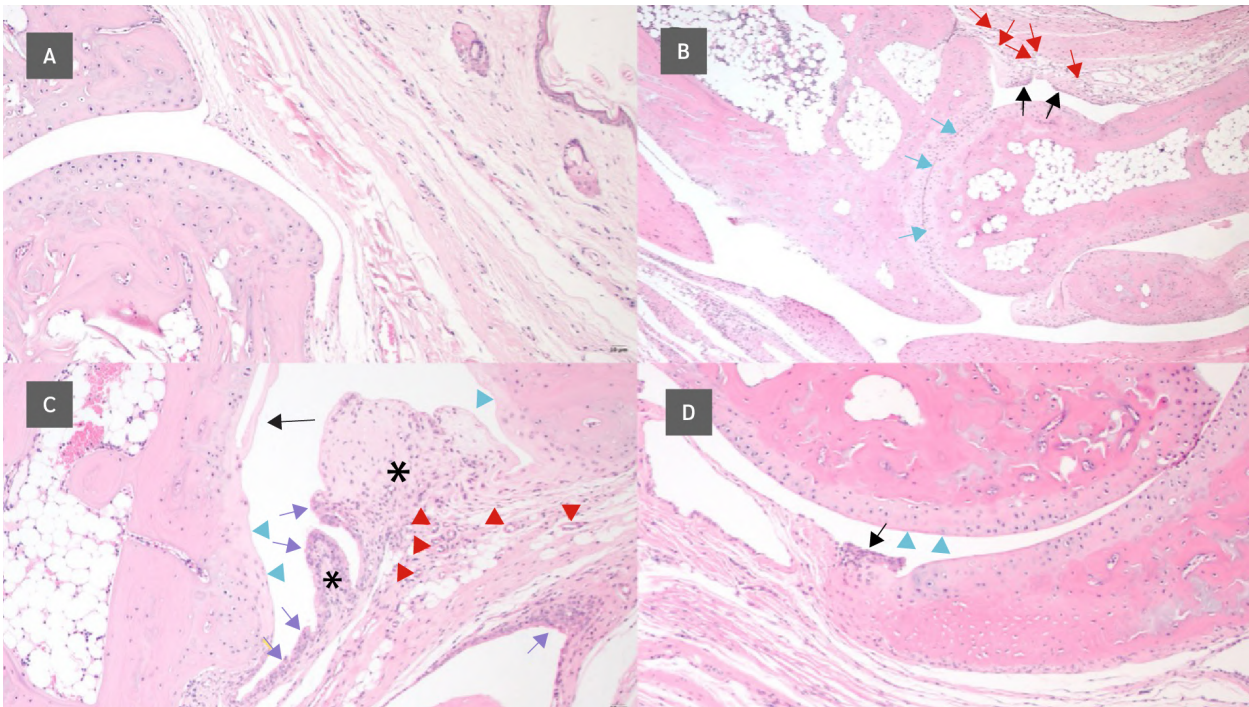


Figure 8. Histopathological evaluation of joint tissue samples. Representative histopathological images of joint tissues from the experimental groups: (A) control joint, (B) arthritic joint from the control group, (C) arthritic joint from the dysbiosis group, (D) arthritic joint from the FMT group. Blue arrows indicate hypocellularity along with degenerative-necrotic changes in chondrocytes, yellow arrows indicate necrotic changes in cartilage tissue, black arrows indicate synovial hyperplasia, red arrows indicate neovascularization, and asterisks indicate mononuclear cell infiltration. Original magnification: A, C, and D, $\times 10$; B, $\times 4$.

Histopathological examination of joint tissues revealed that dysbiosis exacerbated arthritis-related tissue damage. In the dysbiosis group, more severe synovial hyperplasia, pannus formation, cartilage erosion, and near-complete loss of the joint space were observed compared with the control group. In the FMT group, the severity of these pathological changes was reduced

compared with the dysbiosis group (Figure 8). Histopathological scoring confirmed higher damage scores for parameters such as synovial hyperplasia and pannus formation in the dysbiosis group, while demonstrating that FMT reduced this damage (Table 3).

Table 3. Histopathological scores in the experimental groups.

Histopathological parameter	Control group	Dysbiosis group	FMT group
Synovial hyperplasia	2.50 ± 0.24	3.50 ± 0.58	3.25 ± 0.48
Development of pannus	2.25 ± 0.24	3.25 ± 0.58	3.25 ± 0.48
Infiltrations in the synovial membrane	2.25 ± 0.37	3.00 ± 0.03	2.75 ± 0.29
Neovascularization	3.00 ± 0.37	2.75 ± 0.33	2.00 ± 0.29
Hypocellularity in cartilage tissue	3.00 ± 0.20	3.00 ± 0.33	2.75 ± 0.41
Degenerative necrotic changes and fissure development in cartilage tissue	3.50 ± 0.20	3.50 ± 0.33	3.00 ± 0.25

Note: Values are presented as mean ± standard error.

FMT: Fecal microbiota transplantation.

Discussion

This study examined the impact of antibiotic-induced dysbiosis during the maternal period on the severity of CIA in offspring and the possible therapeutic effects of FMT.

In this study, we demonstrated that maternal dysbiosis reduced gut microbial diversity, led to reduced body weight gain in offspring, and increased arthritis pathology. Importantly, FMT partially restored microbial diversity and attenuated arthritis severity. The most important finding of our study is that maternal dysbiosis exacerbates the pathogenesis of arthritis.

Our findings suggest that maternal dysbiosis was associated with reduced body weight gain in male offspring, which may indicate a potential impact on early developmental parameters. However, further studies are needed to draw more definitive conclusions regarding developmental outcomes. This result is consistent with previous research showing an association between antibiotic use and an increased prevalence of RA (20).

Similarly, an increase in arthritis severity has also been reported in experimental models where antibiotics were administered postpartum (21,22). However, our study makes a significant contribution to the literature by showing that the risk occurs not only in the postnatal period but also in the maternal period. This finding highlights the critical role of the gut microbiota in the early development of the immune system.

Our microbiota analyses showed that the combination of amoxicillin and vancomycin caused significant dysbiosis

and that FMT was able to substantially restore microbial diversity. We also found that the combination of amoxicillin and vancomycin led to a significant decrease in the Firmicutes/Bacteroidetes ratio within the bacterial population. This finding can be explained by the well-documented activity of vancomycin against Gram-positive bacteria (23).

Our analysis further demonstrated that the number of bacterial genera increased after FMT. The increase in the number of bacterial genera supports the ability of FMT to restore microbial diversity, and this finding is consistent with previous studies (24,25). For example, a clinical study conducted in 2018 (24) demonstrated that FMT increases gut microbial diversity in humans and is a reliable method. Similarly, another study (25) reported an increase in the microbial population and Shannon index following FMT, consistent with our findings. Numerous animal models have also demonstrated that FMT can reshape the gut microbiome, supporting our results (26,27). According to our results, it can be considered that dysbiosis may disrupt the integrity of the intestinal epithelial barrier, increasing the passage of microbial products (such as lipopolysaccharide [LPS]) into the systemic circulation, thereby triggering the production of pro-inflammatory cytokines (tumor necrosis factor- α [TNF- α], interleukin-6 [IL-6], and interleukin-17 [IL-17]) and exacerbating joint inflammation.

Additionally, the decrease in Lachnospiraceae observed in the dysbiosis group is significant because this family is a major producer of short-chain fatty acids (e.g., butyrate) with anti-inflammatory effects. The decrease in these metabolites may have contributed to impaired immune tolerance and enhanced autoimmune responses. The

protective effect of FMT is likely related to its ability to restore these beneficial bacteria, thereby promoting the production of immunomodulatory metabolites.

There are some limitations to our study. First, the number of male offspring obtained after birth was relatively low. Second, to explain the mechanism more clearly, measurements of intestinal permeability, analysis of serum cytokine levels, and examination of immune cell subpopulations in the lamina propria or spleen (such as the regulatory T cell [Treg]/T helper 17 [Th17] balance) would be required. Additionally, metabolomic analyses would further improve our understanding of the functional capacity of the microbiota alterations.

Another limitation of our study relates to the metagenomic sequencing methodology. Due to budget constraints, fecal samples from five animals in each experimental group were pooled together, creating a single composite sample for sequencing analysis for each group. While this approach provided valuable insights into the microbial community structure at the group

level and successfully demonstrated the profound effects of antibiotic-induced dysbiosis and FMT-mediated restoration, it prevented us from assessing inter-individual differences in gut microbiota composition. Consequently, we were unable to evaluate the heterogeneity of microbial responses within treatment groups or perform statistical analyses on alpha- and beta-diversity metrics at the individual level. Future studies with larger sample sizes and individual sequencing will be valuable for characterizing the full spectrum of microbial diversity and elucidating individual differences in response to dysbiosis and FMT treatment.

In conclusion, this study demonstrated that maternal-derived gut dysbiosis can influence the severity of CIA development in mice. It has been shown that FMT treatment holds therapeutic potential to reduce this severity. Our findings highlight the importance of the microbiome in the development and severity of autoimmune diseases and suggest that microbiota-focused treatment approaches may represent a promising preventive or therapeutic approach.

Ethical Approval: This experimental study was approved by the Bursa Uludağ University Animal Experiments Local Ethics Committee on April 11, 2023 with decision number 2023-06/05.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – H.B.O., M.Y., D.Y.E.; Design – H.B.O., M.Y., D.Y.E.; Supervision – H.B.O., M.Y., D.Y.E.; Funding – H.B.O.; Materials – H.B.O., D.Y.E., E.Y.; Data Collection and/or Processing – F.D., G.A., E.Y., M.A., A.A.; Analysis and/or Interpretation – F.D., G.A., E.Y., M.A., A.A., M.Y.; Literature Review – F.D., H.B.O., M.Y., D.Y.E., G.A.; Writing – F.D., H.B.O., M.Y., D.Y.E., A.A.; Critical Reviews – H.B.O., M.Y., D.Y.E.

Conflict of Interest: The authors declared that they have no conflict of interest.

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Association Between Vitamin D Status, Regulatory T Cells, and Disease Severity in Pediatric Systemic Lupus Erythematosus

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Abstract

Objective: Systemic lupus erythematosus (SLE) is characterized by loss of immune tolerance that is often associated with regulatory T (Treg) cell dysfunction. Vitamin D is known to enhance Treg differentiation and expansion. This study aimed to investigate the associations between serum vitamin D and zinc levels, Th17 and Treg cell percentages, and disease activity in pediatric patients with SLE.

Materials and Methods: A cross-sectional study was conducted among children aged 7.5–18.9 years who were diagnosed with SLE at Dr. Saiful Anwar Hospital, Malang, Indonesia. Serum 25-hydroxyvitamin D [25(OH)D] levels were measured using an enzyme-linked immunosorbent assay (ELISA). Treg cell percentages were determined via flow cytometry. Disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score.

Results: The study included 32 pediatric patients with SLE. Among the study participants, 25.0% had sufficient vitamin D levels, whereas 75.0% had suboptimal vitamin D status, including 28.1% with vitamin D insufficiency and 46.9% with vitamin D deficiency. Moderate-to-severe disease activity (SLEDAI ≥ 7) was observed in 84.4% of the participants. A significant positive correlation was observed between serum 25(OH)D levels and Treg cell percentages ($r=0.402$, $p=0.0225$), indicating that higher vitamin D levels were associated with increased Treg cell percentages.

Conclusion: Pediatric patients with SLE show a high prevalence of vitamin D deficiency and insufficiency. Adequate vitamin D status is associated with increased Treg cell percentages, suggesting a potential role for vitamin D in enhancing immune regulation and managing disease severity in pediatric SLE.

Keywords: Autoimmunity, SLE, SLEDAI, T cell, vitamin D

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Introduction

Contrary to its name, vitamin D is not a vitamin, but a steroid hormone (1). Vitamins act as antioxidants or cofactors in enzymatic reactions and are primarily obtained through the diet. In contrast, vitamin D is produced by activating sterol fractions derived from plant and animal sources, namely phytosterols and cholesterol, and through exposure to sunlight (2). Some food sources, such as salmon, sardines, tuna, cod liver oil, egg yolks, and shiitake mushrooms, contain small amounts of vitamin D (100–200 IU) (3). By contrast, sun exposure can produce approximately 10,000 to 20,000 IU of vitamin D if 30% of the total body surface area is exposed to sunlight for 15 to 30 minutes per day (4). Steroid hormones regulate gene expression and activate or deactivate protein production according to the body's needs. Vitamin D also plays an important role as an immunomodulator (5).

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease that requires long-term treatment. When it occurs in children under 18 years of age, it is referred to as childhood-onset SLE (6). The incidence of SLE in children ranges from 0.3–0.9 per 100,000 children per year, with a prevalence of 3.3–8.8 per 100,000 children (7). Compared with adult-onset SLE, childhood-onset SLE is generally associated with more severe clinical symptoms, with a higher prevalence of complications such as lupus nephritis, hematological anomalies, and photosensitivity (8). The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) is commonly used to assess disease activity in patients with SLE. The SLEDAI score is calculated based on clinical and laboratory parameters (9).

SLE is characterized by the production of antibodies that attack the body itself, including antinuclear antibodies (ANA), antiphospholipid antibodies, anti-Smith antibodies, and anti-ribonucleoprotein antibodies. These autoantibodies promote the overproduction of pro-inflammatory cytokines and lead to dysregulation of the innate and adaptive immune systems (10). In addition, SLE is associated with impaired T-cell function and imbalance between T helper 17 (Th17) cells and regulatory T (Treg) cells (11). An increase in the ratio of Th17 cells to Treg cells has been reported to play a role in the severity and disease activity of several autoimmune diseases, including SLE (12).

Several studies have shown that vitamin D levels are negatively correlated with ANA, anti-dsDNA, and SLEDAI

scores, but positively correlated with bone mineral density (13). Vitamin D [$1,25(\text{OH})_2\text{D}_3$] suppresses cytokine responses by Th1 and Th17 cells, induces Treg cells, promotes IL-4 production, and enhances natural killer cell function (14). Within the innate immune system, vitamin D enhances cellular chemotaxis and phagocytosis while activating the transcription of antimicrobial peptides, such as β -defensin and cathelicidin. Specifically, dendritic cells are an important target of the immunomodulatory effects of vitamin D (15). Dendritic cells play a critical role in maintaining peripheral tolerance by preventing autoreactive T cells, which can cause autoimmune damage (16). Furthermore, *in vitro* studies have demonstrated that vitamin D inhibits IL-17 synthesis, suppresses Th17 cell differentiation, and increases the number of $\text{CD4}^+\text{CD25}^+$ Treg cells, which subsequently produce IL-10 and promote a shift from Th1 cells toward type 1 Treg cells (17).

Besides vitamin D, studies have shown that trace elements such as zinc may help maintain the optimal function of the immune system, and that zinc is involved in many aspects of cellular metabolism (18). The main source of zinc intake is meat and meat products, but zinc is also present in mollusks, milk, soybeans, and spinach (19). Zinc depletion may improve clinical manifestations in patients with SLE and reduce anti-dsDNA antibody levels (20). Zinc modulates the pro-inflammatory response by targeting nuclear factor kappa B (NF- κ B), controls oxidative stress, and regulates inflammatory cytokines. Zinc deficiency reduces both innate and adaptive immunity. The effects include impaired host defense through reduced neutrophil and natural killer cell function, impaired phagocytosis and intracellular killing, altered cytokine production, and diminished T- and B-cell-mediated immune responses (21).

Based on these findings, further research is needed to investigate the relationship between serum vitamin D and zinc levels and the percentages of Th17 cells and Treg cells in children with SLE. Accordingly, this study investigated the associations among serum vitamin D and zinc status, Th17 and Treg cell percentages, and disease activity in pediatric patients with SLE.

Materials and Methods

Study Design and Participants

Pediatric patients who met the inclusion criteria were enrolled in this study. The inclusion criteria were patients aged 7.5 to 18.9 years with a diagnosis of SLE established

according to the Systemic Lupus International Collaborating Clinics (SLICC) criteria at the Dr. Saiful Anwar Hospital, Malang, Indonesia, who had either recently commenced or were currently receiving standard treatment (corticosteroids or immunosuppressants), and had no other organ disorders (22). The exclusion criteria included patients with poor nutritional status, congenital diseases, malignancies, and other immunodeficiency or autoimmune diseases.

Patients who agreed to participate in this study included both those who had recently been diagnosed and those who had previously received treatment for SLE. However, this study did not include a control group of healthy children for comparison. Research participants were enrolled using consecutive sampling.

Written informed consent was obtained from all parents or legal guardians, and informed assent was obtained from all patients prior to study initiation. All research protocols complied with institutional guidelines and were approved by the Health Research Ethics Commission, Faculty of Medicine, Universitas Brawijaya (No. 208/EC/KEPK/07/2021). This study was registered on ClinicalTrials.gov (NCT07069348).

Laboratory Measurements and Biochemical Analysis

Venous blood samples (12 mL in total) were collected from each participant at baseline at the Central Laboratory of RSUD Dr. Saiful Anwar. The blood was collected using a closed vacutainer system and distributed into three designated tubes for the following analyses:

Serum 25-Hydroxyvitamin D [25(OH)D] Analysis

A 3-mL venous blood sample was collected in a yellow-cap Vacutainer Serum Separator Tube (SST) (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 3000 rpm for 5 minutes at 4°C to obtain serum. Serum 25(OH)D concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., London, Ontario, Canada) according to the manufacturer's instructions. The assay had an analytical sensitivity (lower limit of detection) of 5.5 ng/mL, with a calibration range of 10 to 160 ng/mL to define its functional linearity. Based on the serum concentrations, vitamin D status was categorized as deficient (<20 ng/mL), insufficient (20–<30 ng/mL), or sufficient (≥ 30 ng/mL).

Serum Zinc Measurement

An additional 3-mL venous blood sample was collected in a second yellow-cap Vacutainer SST and centrifuged to obtain serum for zinc measurement. Serum zinc concentrations were determined using atomic absorption spectrophotometry (AA-6200; Shimadzu Corporation, Kyoto, Japan). These measurements were carried out at the Analysis and Measurement Unit Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.

PBMC Cell Isolation Protocol

A 6-mL venous blood sample was collected in a purple-cap EDTA Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ, USA) and layered onto Ficoll-Hypaque (density, 1.077 g/mL; Thermo Fisher Scientific, Grand Island, NY, USA) for density-gradient centrifugation (23). Samples were centrifuged at 1600 rpm for 30 minutes at room temperature, resulting in the formation of distinct cellular layers. The peripheral blood mononuclear cell (PBMC) layer was carefully aspirated using a micropipette and transferred to a new 15-mL centrifuge tube. Cells were washed with 1 mL of phosphate-buffered saline (PBS) and centrifuged again at 1200 rpm for 10 minutes at room temperature. Following centrifugation, the PBMC pellet was resuspended in PBS. Cell counts and viability were subsequently assessed using the trypan blue exclusion assay with a hemocytometer, confirming a viability greater than 95% in all samples prior to flow cytometric analysis.

Flow Cytometric Analysis of Th17 and Treg Cells

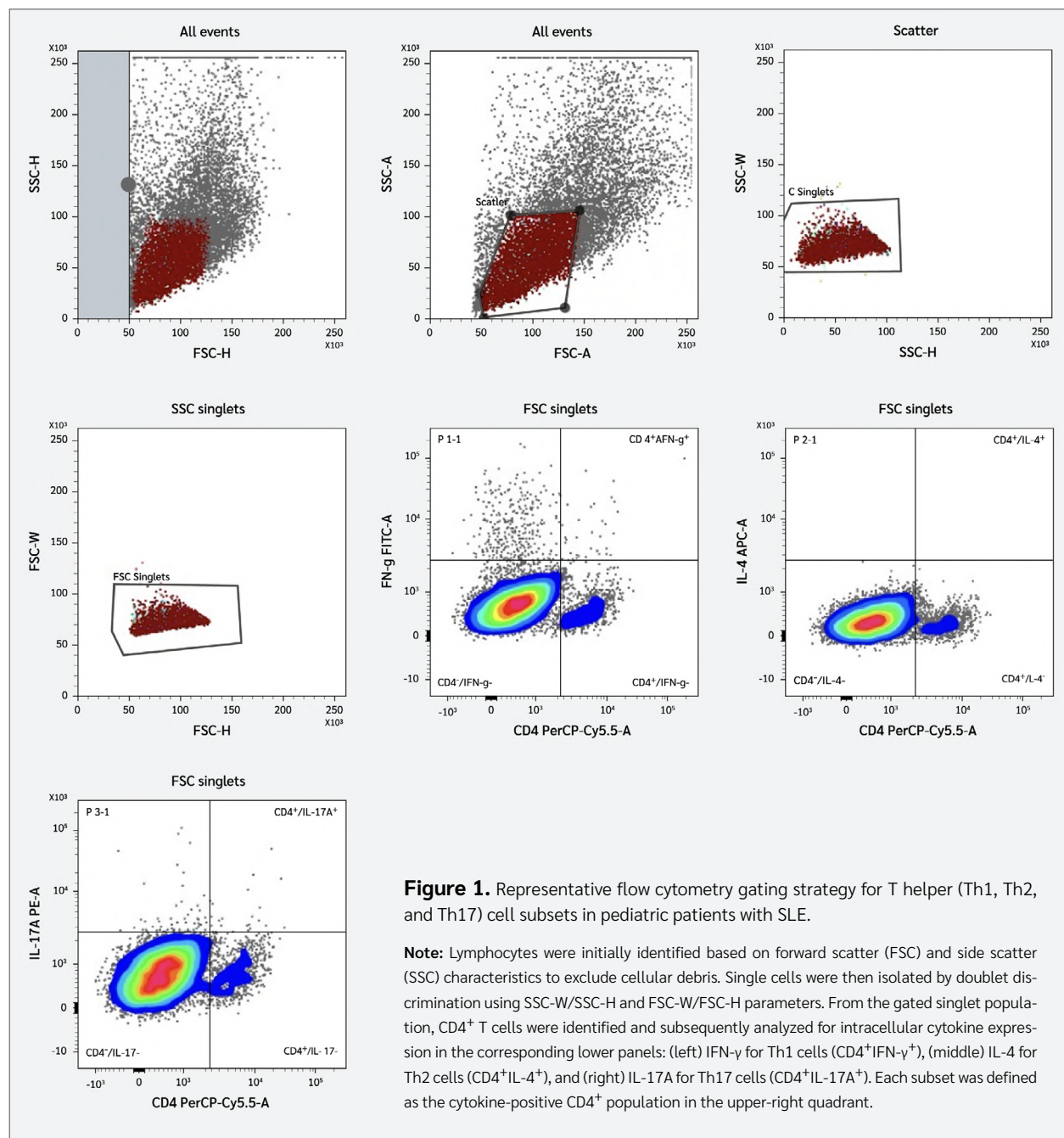
A 96-well flat-bottom tissue culture plate was coated with anti-CD3 (sc-1179; Santa Cruz Biotechnology, Dallas, TX, USA). PBMCs were suspended in complete RPMI 1640 medium (ATCC modification; Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine. The cell suspension was adjusted to a concentration of 1×10^6 cells/mL, and 200 μ L (approximately 2×10^5 cells) was added to each anti-CD3-coated well. The plate was incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂.

To facilitate intracellular cytokine detection for Th17 cells, BD GolgiStop™ (containing monensin) (BD Biosciences, San Jose, CA, USA) was added during the final 6 hours of stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, St. Louis, MO,

USA) before cell harvest (24). Following stimulation, the cells were collected and centrifuged at 2500 rpm for 5 minutes at 4°C. The supernatant was collected and stored for secreted cytokine analysis, while the cell pellet was immediately processed for immunophenotyping.

For Th17 enumeration, the pellet was stained using a Human Th17 Phenotyping Kit (560751; BD Pharmingen, BD Biosciences, San Jose, CA, USA), targeting the CD4⁺

IL-17A⁺ population. The detailed hierarchical gating strategy for Th17 cells is shown in Figure 1. For Treg identification, the cells were stained for surface markers CD4 and CD25, followed by intracellular staining for FoxP3 using the Human FoxP3 Buffer Set (560133; BD Pharmingen, BD Biosciences, San Jose, CA, USA), as shown in Figure 2. For both cell populations, fixation and permeabilization were performed using BD Cytofix/Perm/Wash[™] buffers to allow antibody



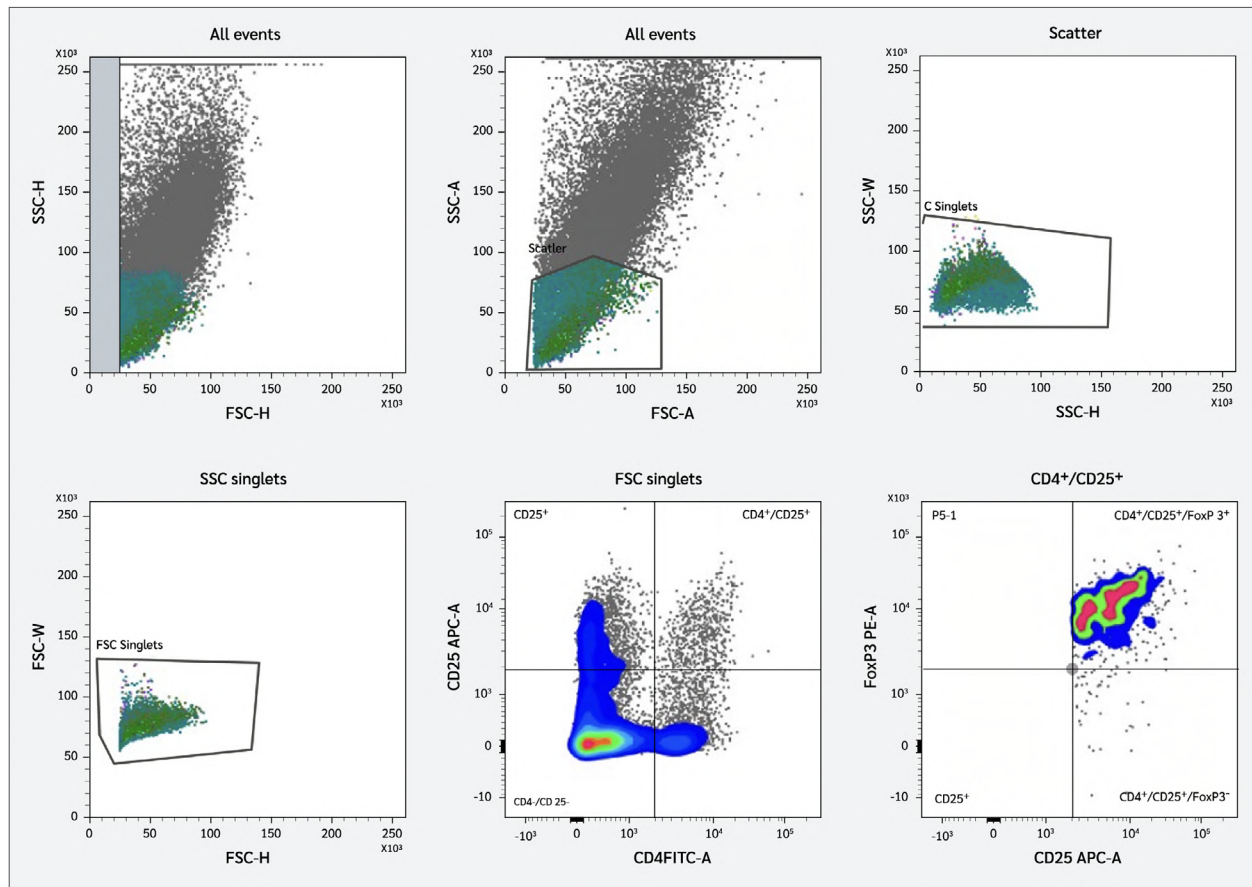


Figure 2. Representative flow cytometry gating strategy for regulatory T (Treg) cells in pediatric patients with SLE.

Note: Lymphocytes were initially identified based on forward scatter (FSC) and side scatter (SSC) characteristics to exclude cellular debris. Single cells were then isolated by doublet discrimination using SSC-W/SSC-H and FSC-W/FSC-H parameters. From the gated singlet population, $CD4^+CD25^+$ T cells were identified (left panels). Regulatory T cells were subsequently identified and quantified based on intracellular FoxP3 expression within the $CD4^+CD25^+$ population and are shown as $CD4^+CD25^+FoxP3^+$ cells in the upper-right quadrant (right panels).

access to the intracellular IL-17A and nuclear FoxP3 proteins. Data acquisition was performed on a FACSCalibur flow cytometer and analyzed using CellQuest Pro software, version 5.2.1 (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD). Data distribution was assessed for normality using the Shapiro-Wilk test. Group comparisons were performed using Student's t test for normally distributed variables. Correlations between variables were analyzed using Spearman's rank correlation test, and correlation coefficients (r) were reported accordingly. A two-tailed $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism software version 10.0.3 (GraphPad Software, San Diego, CA, USA).

Results

Pediatric SLE Patient Characteristics

A total of 32 children aged 7.5–18.9 years, with a mean age of 13.39 years, were included in this study. Other patient characteristics are presented in Table 1, while nutritional status is presented in Table 2. Based on the patients' medical histories, it was noted that none of the recruited subjects received vitamin D supplementation during the study period.

Nutritional status is a complex concept that encompasses a variety of factors in children, including body weight, height, age, and body mass index (BMI). These factors are interrelated and can be used to assess a child's overall health and well-being and to categorize nutritional status according to the World Health

Table 1. Baseline characteristics of the study participants.

Characteristics	Mean	SD
Age (years)	13.39	3.28
Weight (kg)	40.41	14.23
Height (cm)	142.7	15.09
IBW (%)	103.81	22.83
BMI (kg/m ²)	19.65	5.73
Baseline SLEDAI score	13.88	5.456
Baseline PedsQL score	79.50	10.34
Vitamin D (ng/mL)	20.29	10.23
Zn (ppm)	4.32	1.85
Rate of Treg cells (%)	9.17	7.35
Rate of Th17 cells (%)	12.54	13.26
Th17/Treg ratio	1.73	1.72

BMI: Body mass index, **IBW:** Ideal body weight, **PedsQL:** Pediatric Quality of Life Inventory, **SD:** Standard deviation, **SLEDAI:** Systemic Lupus Erythematosus Disease Activity Index, **Th17:** T-helper 17, **Treg:** Regulatory T cells, **Zn:** Zinc.

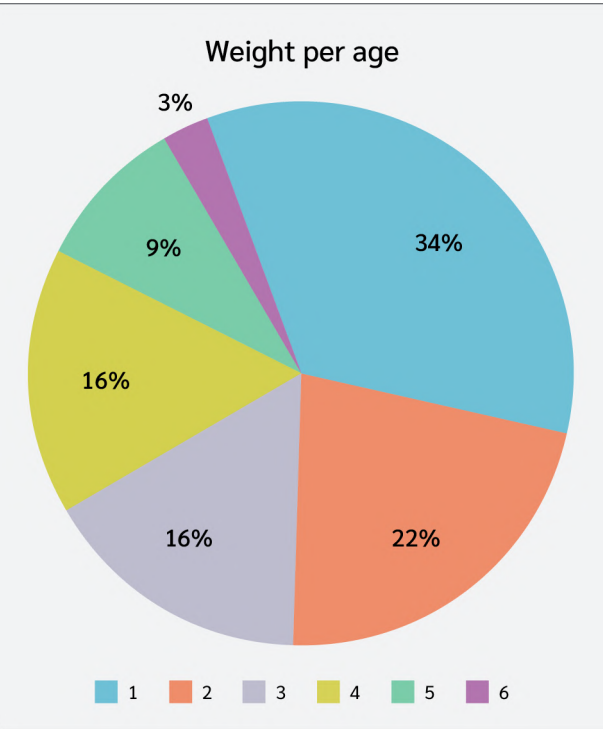


Figure 3. Weight-for-age distribution of the study participants.

Note: Participants were categorized into six percentile groups based on standardized growth charts: (1) <P5; (2) P5–P10; (3) P10–P25; (4) P25–P50; (5) P50–P75; and (6) P75–P90.

Table 2. Nutritional status of the study participants.

Characteristic	Mean	SD
Weight-for-age	2.53	1.50
Height-for-age	1.67	0.48
BMI-for-age (WHO)	2.34	1.23
BMI-for-age (CDC)	3.13	1.77
WHO nutritional status	1.25	0.57
CDC nutritional status	1.25	0.57

BMI: Body mass index, **CDC:** Centers for Disease Control and Prevention, **SD:** Standard deviation, **WHO:** World Health Organization.

Note: Values for nutritional status variables represent the coded categories used for analysis, Weight-for-age: (1) <P5; (2) P5–P10; (3) P10–P25; (4) P25–P50; (5) P50–P75; and (6) P75–P90. Height-for-age: (1) Normal; (2) Short. BMI-for-age (WHO): (1) –2 SD to –1 SD (31%); (2) –1 SD to the mean (28%); (3) the mean to +1 SD (22%); (4) +1 SD to +2 SD (13%); and (5) >+2 SD (6%). BMI-for-age (CDC): (1) P5–P10 (22%); (2) P10–P25 (19%); (3) P25–P50 (22%); (4) P50–P75 (19%); (5) P85–P90 (6%); (6) P90–P95 (6%); and (7) >P95 (6%). WHO and CDC nutritional status: (1) Normal; (2) Obese; (3) Overweight.

Table 3. Seasonal sunlight conditions during the study period.

Month	Seasonal sunlight conditions
March	Low to moderate sunlight
April–May	Moderate to high sunlight
June–July	High sunlight levels with intermittent cloudy periods
August	Peak sunlight with predominantly clear skies
September	Beginning of the rainy season with decreasing sunlight

Organization (WHO) and Centers for Disease Control and Prevention (CDC) criteria. Samples were collected from March to September 2025. As a tropical country, Indonesia receives high-intensity sunlight throughout most of the year, particularly between 10:00 AM and 2:00 PM. Detailed information regarding sun exposure is presented in Table 3.

Anthropometric Profile and Growth Distribution

The initial assessment focused on physical development. As shown in Figure 3, the distribution of weight-for-age categories revealed a significant trend toward malnutrition, with the largest proportion of subjects (34%) falling below the 5th percentile (<P5). This was followed by children in the P5–P10 range (22%), while 16% of participants were distributed across each of the P10–P50

categories. In contrast, higher percentiles were less common, with only 12% of the subjects in the P50–P90 range.

Complementing these findings, Figure 4 presents the distribution of height-for-age. While the majority (66%) exhibited normal height, 34% were classified as having short height. Together, these data indicate a high prevalence of growth impairment and underweight status among the study participants.

Nutritional Status Assessment Based on WHO and CDC Criteria

To gain a more detailed understanding of nutritional status, BMI-for-age was further analyzed using both the WHO and CDC growth references. Figure 5 shows the WHO distribution, where the largest group (31%) fell within the -2 to -1 standard deviation (SD) range, indicating that most subjects were toward the lower end of the normal reference range. This pattern remained consistent when applying the CDC growth reference (Figure 6), where the majority of participants

also clustered in the lower- to mid-percentile ranges (P5–P75).

Figure 7 summarizes the overall nutritional status. While 81% of the participants were classified as having normal nutritional status, 19% were categorized as either overweight (13%) or obese (6%). These findings suggest that although underweight-related growth patterns were common, a subset of participants also exhibited excess body weight.

Correlation of SLEDAI Score with Vitamin D and Immunological Parameters

Beyond physical growth, this study evaluated the relationship between clinical disease activity and biochemical markers. Figure 8 illustrates the relationship between the SLEDAI score and the measured biomarkers, showing a strong inverse correlation with serum vitamin D levels. Unlike zinc levels and the percentages of Treg and Th17 cells, which showed dispersed distributions,

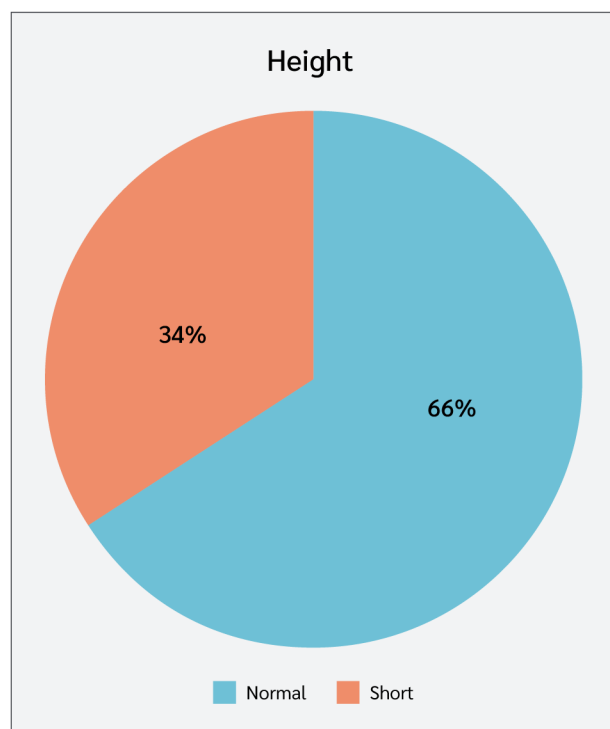


Figure 4. Height distribution of the study participants.

Note: The pie chart illustrates the proportions of participants with normal height (66%) and short stature (34%). Participants were classified according to height-for-age percentiles using standardized growth charts, with short stature defined as height below the 3rd percentile for age and sex.

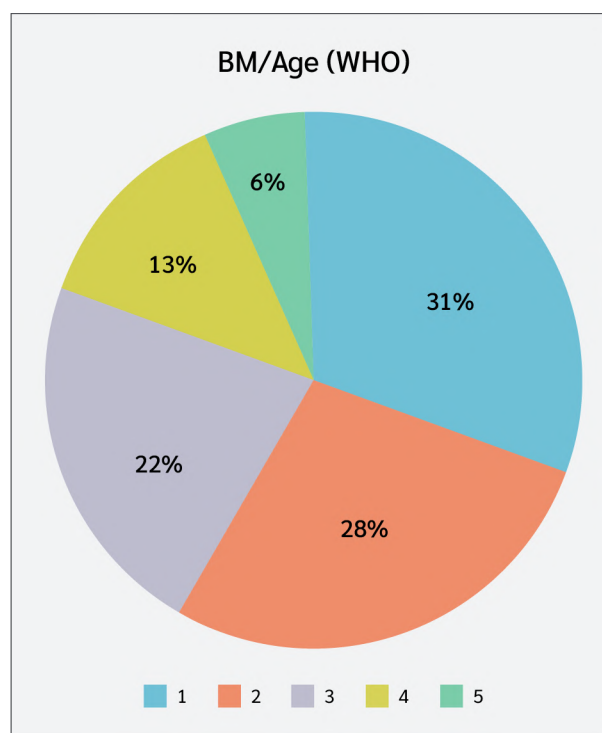


Figure 5. Distribution of BMI-for-age z-score categories according to the WHO growth standards for children and adolescents aged 5–19 years.

Note: The pie chart illustrates the proportions of participants in the following BMI-for-age z-score categories: (1) -2 SD to -1 SD (31%); (2) -1 SD to <0 SD (28%); (3) 0 SD to $<+1$ SD (22%); (4) $+1$ SD to $+2$ SD (13%); and (5) $>+2$ SD (6%).

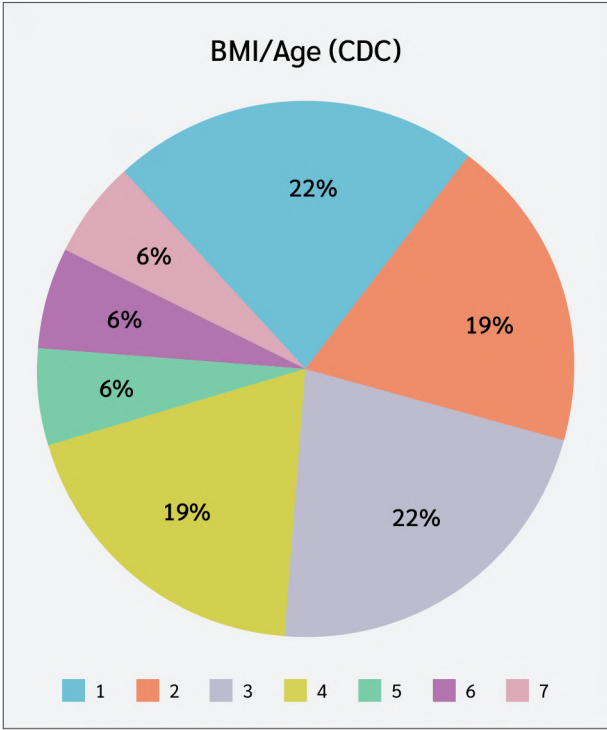


Figure 6. Distribution of BMI-for-age percentile categories according to the CDC growth charts.

Note: The pie chart illustrates the proportions of participants aged 2–19 years in the following BMI-for-age percentile categories: (1) P5–P10 (22%); (2) P10–P25 (19%); (3) P25–P50 (22%); (4) P50–P75 (19%); (5) P85–P90 (6%); (6) P90–P95 (6%); and (7) >P95 (6%).

Table 4. Correlation between SLEDAI score and vitamin D, zinc, Treg cells, and Th17 cells.

Variable	R	p-value	95% CI
Vitamin D	-0.957	<0.0001***	-0.9794 t– -0.9111
Zinc	-0.1976	0.2782	-0.5191 – 0.1728
Treg cells	-0.345	0.0531	-0.62599 – 0.015
Th17 cells	0.02069	0.9105	0.330 – 0.366

CI: Confidence interval, **SLEDAI:** Systemic Lupus Erythematosus Disease Activity Index, **Th17:** T-helper 17, **Treg:** Regulatory T cells.

vitamin D levels consistently decreased as disease activity increased. Based on serum 25(OH)D levels, the patients were categorized into three groups: 8 (25.0%) with sufficient levels, 9 (28.1%) with insufficient levels, and 15 (46.9%) with deficient levels.

This observation was confirmed by the statistical analysis presented in Table 4. Only vitamin D exhibited a

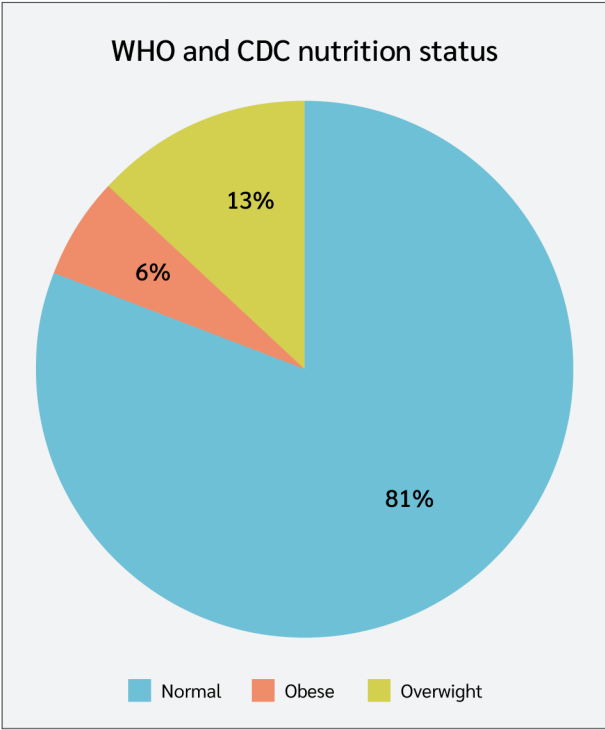


Figure 7. Nutritional status classification based on combined WHO and CDC criteria.

Note: The pie chart illustrates the proportions of participants classified as having normal nutritional status (81%), overweight (13%), or obesity (6%).

Table 5. Correlation between vitamin D and zinc, Treg cells, and Th17 cells.

Variable	R	p-value	95% CI
Zinc	0.2176	0.2316	-0.1525 – 0.5341
Treg cells	0.4021	0.0225*	0.05133 – 0.6646
Th17 cells	0.06892	0.7078	0.287 – 0.408

CI: Confidence interval, **Th17:** T-helper 17, **Treg:** Regulatory T cells.

significant relationship with the SLEDAI score ($r=-0.957$; $p<0.0001$), with a 95% confidence interval (CI) ranging from -0.9794 – -0.9111 . In contrast, no significant correlations were observed between SLEDAI score and zinc levels ($r=-0.1976$; $p=0.2782$), Treg cell percentages ($r=-0.345$; $p=0.0531$), or Th17 cell percentages ($r=0.02069$; $p=0.9105$).

Figure 9 shows the relationship between vitamin D levels and immune cell populations. A positive correlation was observed between vitamin D levels and Treg cell

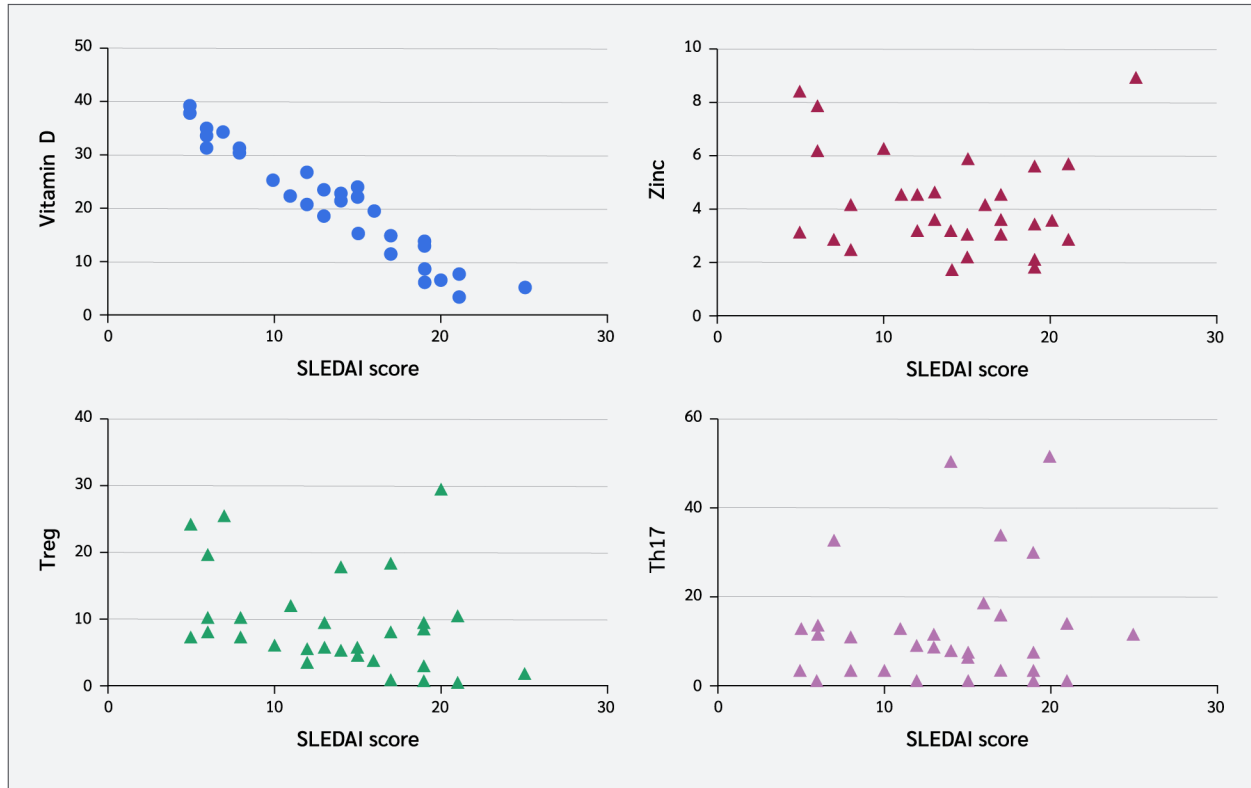


Figure 8. Correlation between SLEDAI score and serum vitamin D and zinc levels, and Treg and Th17 cell percentages.

Note: The scatter plots demonstrate a significant inverse correlation between the SLEDAI score and serum vitamin D levels. No significant correlations were observed between the SLEDAI score and serum zinc levels, Treg cell percentages, or Th17 cell percentages.

percentages, indicating that higher vitamin D levels were associated with increased Treg cell percentages.

Table 5 confirmed this association. Vitamin D showed a significant positive correlation with Treg cell percentages ($r=0.4021$; $p=0.0225$; 95% CI, 0.05133–0.6646), whereas no significant correlations were found with zinc levels ($r=0.2176$; $p=0.2316$) or Th17 cell percentages ($r=0.06892$; $p=0.7078$). These findings collectively suggest a selective association between vitamin D status and the regulatory immune compartment in patients with SLE.

Consistently, the analysis of the Th17/Treg ratio presented in Table 6 further supported this observation. The Th17/Treg ratio showed a moderate positive correlation with SLEDAI score ($r=0.483$; $p=0.005$; 95% CI, 0.161–0.712), indicating that a higher ratio was associated with increased disease activity. In contrast, a moderate negative correlation was observed between the Th17/Treg ratio and vitamin D levels ($r=-0.441$; $p=0.012$; 95% CI, -0.684 to -0.109), suggesting that higher vitamin D levels were associated with a lower Th17/Treg ratio.

Table 6. Correlation between the Th17/Treg ratio, SLEDAI score, and vitamin D levels.

Variable	r (Spearman)	p -value	95% CI
SLEDAI Score	0.483	0.0051	0.161 – 0.712
Serum vitamin D	-0.441	0.0116	-0.684 – -0.109

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

Discussion

Our findings reveal a significant inverse correlation between serum 25(OH)D concentrations and SLEDAI scores, reinforcing the hypothesis that vitamin D deficiency is associated with increased SLE disease activity (25). In our study, the striking prevalence of suboptimal vitamin D levels, whereby 75% of our pediatric exhibited insufficiency or deficiency, mirrors the high prevalence reported in larger adult cohorts, such as the 170-patient study by Schoindre et al. (26). In the present study, sufficient vitamin D levels (≥ 30 ng/mL) were identified in

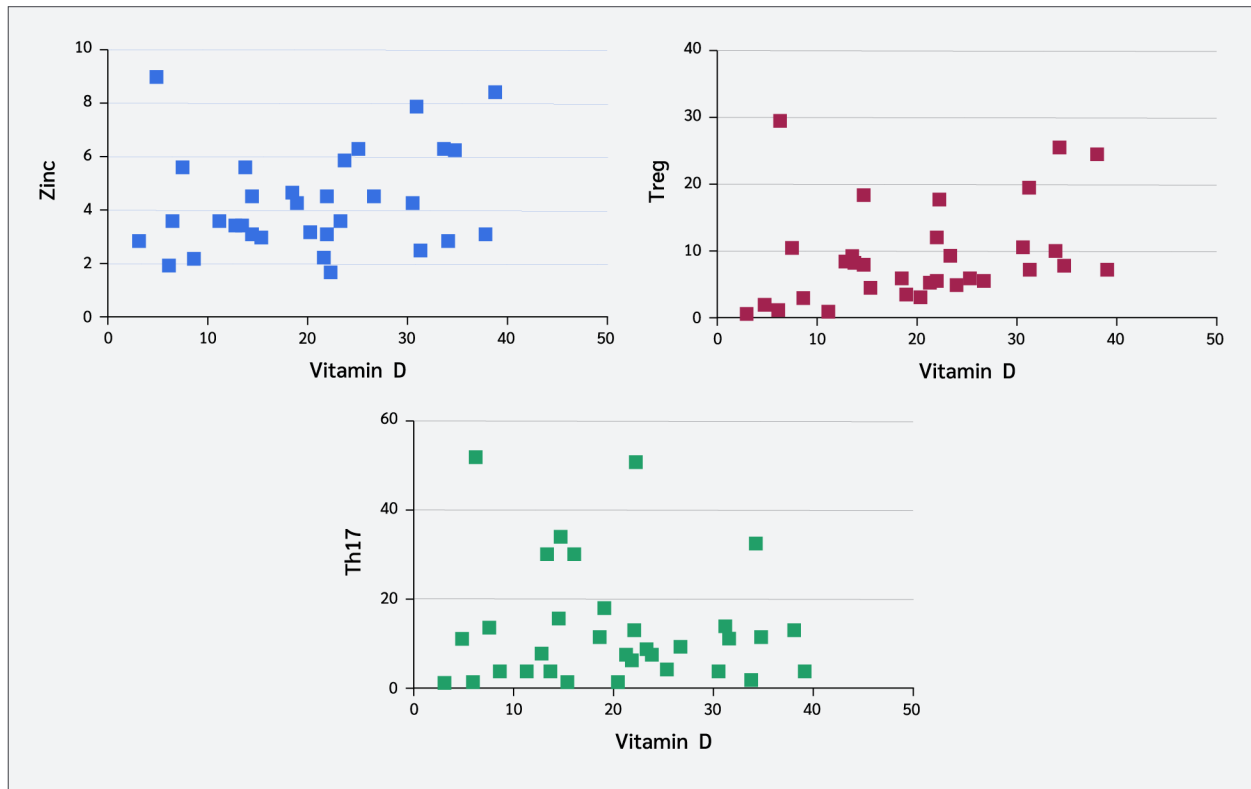


Figure 9. Correlation between serum vitamin D levels and serum zinc levels, and Treg and Th17 cell percentages.

Note: The scatter plots demonstrate a significant positive correlation between serum vitamin D levels and Treg cell percentages. No significant correlations were observed between serum vitamin D levels and serum zinc levels or Th17 cell percentages.

only 8 patients (25.0%), whereas vitamin D insufficiency ($10 \leq 25(\text{OH})\text{D} < 30$ ng/mL) and deficiency (< 10 ng/mL) were found in 9 (28.1%) and 15 patients (46.9%), respectively. The high proportion of patients with suboptimal vitamin D levels further supports the notion that vitamin D depletion is not merely an incidental nutritional deficit but a persistent, disease-related feature of SLE pathophysiology. These findings are reinforced by pediatric-specific data from a cross-sectional study of 45 children with SLE, which demonstrated significantly lower serum 25(OH)D levels than those of 109 healthy controls (14). Similar findings across pediatric and adult populations underscore the high prevalence of vitamin D insufficiency in SLE and suggest that it may reflect both chronic inflammation and disease-related factors.

This observation suggests that reduced vitamin D levels in pediatric SLE cannot be explained solely by environmental or age-related factors. Similar findings have also been reported in Asian populations. A study from Taiwan reported a mean serum vitamin D₃ level of 19.7 ± 7.9 ng/mL (27). Collectively, these consistent findings across

diverse populations support an association between vitamin D insufficiency and SLE disease activity.

Multiple risk factors predispose patients with SLE to vitamin D deficiency or insufficiency, reflecting the complex interplay between disease characteristics, organ involvement, and therapeutic interventions. One of the principal contributors to vitamin D insufficiency in SLE patients is photosensitivity, a hallmark clinical feature of the disease. Exposure to ultraviolet B (UV-B) radiation can trigger photosensitive reactions, leading patients to avoid sunlight deliberately. In addition to provoking cutaneous manifestations, UV-B exposure has been shown to exacerbate dermatological disorders and precipitate systemic disease flares in SLE (28). Consequently, strict photoprotection practices, although clinically necessary, significantly limit cutaneous vitamin D synthesis and contribute to chronically low serum vitamin D levels.

Beyond photosensitivity, disease-related complications also play an important role in disrupting vitamin D metabolism. Renal involvement, particularly lupus

nephritis, is especially important because the kidneys are responsible for the 1α -hydroxylation of vitamin D into its biologically active form. Impaired renal function may therefore reduce the conversion of circulating 25(OH)D to active vitamin D, rendering patients with lupus nephritis especially susceptible to deficiency (29). This mechanism highlights how organ damage intrinsic to SLE can directly influence vitamin D homeostasis.

Pharmacological therapy further compounds this risk. Corticosteroids, which remain a cornerstone of SLE management, adversely affect vitamin D metabolism by reducing intestinal calcium absorption and accelerating the catabolism of both 25(OH)D and $1,25(\text{OH})_2\text{D}_3$ (30). As a result, patients receiving long-term corticosteroid therapy often exhibit persistently low vitamin D levels despite standard supplementation. Indeed, these patients frequently require higher doses of vitamin D to achieve and maintain adequate serum concentrations (31). Collectively, these findings indicate that vitamin D deficiency in SLE is multifactorial, reflecting the combined effects of disease activity, organ involvement, and treatment-related factors.

Systemic lupus erythematosus (SLE) is characterized by profound immune dysregulation, including the presence of pathogenic autoantibodies, impaired Treg cell function, and expansion of pro-inflammatory Th17 cells. In this context, adequate vitamin D status may help maintain immune homeostasis. Vitamin D participates in antimicrobial defense, macrophage differentiation, and modulation of adaptive immunity, particularly by promoting CD4^+ T-cell differentiation toward anti-inflammatory phenotypes such as Th2 and Treg cells. These immunomodulatory properties are essential for preserving immune tolerance and limiting excessive inflammatory responses.

Conversely, vitamin D deficiency may disrupt these regulatory pathways and contribute to immune imbalance. Vitamin D insufficiency has been associated with altered dendritic cell maturation and dysregulated cytokine production, including reduced regulation of key inflammatory cytokines such as IL-6, IL-12, and $\text{TNF-}\alpha$, which are central to SLE pathogenesis (32). Through these mechanisms, low vitamin D levels may facilitate persistent immune activation and autoantibody production.

Clinical interventional studies provide further support for the immunoregulatory role of vitamin D in SLE. A prospective study in patients with hypovitaminosis D receiving 100,000 IU of cholecalciferol weekly for six months

demonstrated a significant increase in mean serum 25(OH)D levels, from 18.7 ± 6.7 ng/mL at baseline to 41.5 ± 10.1 ng/mL after treatment ($p < 0.001$) (33). Importantly, vitamin D supplementation was associated with favorable immunological changes, including increased proportions of Treg cells, enhanced FoxP3 and $\text{TGF}\beta$ expression, expansion of naïve CD4^+ T cells, and reductions in memory B cells, Th1 and Th17 effector cells, IL-6 expression, and anti-DNA antibodies (34). Collectively, these findings suggest that vitamin D supplementation may help restore immune homeostasis in patients with SLE.

Evidence regarding the clinical benefits of vitamin D supplementation in SLE continues to evolve, with several studies suggesting a favorable impact on disease activity. One study demonstrated that vitamin D₃ supplementation at a dose of 8000 IU/day for eight weeks in deficient patients and for four weeks in insufficient patients resulted in significant improvement in SLEDAI-2K scores ($p = 0.028$). In the same cohort, anti-double stranded DNA (anti-dsDNA) antibody titers also decreased significantly ($p = 0.045$), suggesting potential immunological benefits alongside clinical improvement (35).

A systematic review further supports these observations. Three out of four included studies reported improvements in SLEDAI scores together with favorable changes in inflammatory markers, fatigue, and endothelial function following vitamin D supplementation. However, one study did not demonstrate a significant improvement in disease activity (36). Moreover, other investigations have failed to identify significant differences between vitamin D supplementation and placebo groups (37). Collectively, these mixed findings indicate that the clinical benefit of vitamin D supplementation may depend on baseline vitamin D status, supplementation dose, treatment duration, and disease heterogeneity, underscoring the need for further well-designed randomized controlled trials.

Conclusion

In conclusion, vitamin D deficiency is common in pediatric SLE and is significantly associated with higher disease activity. Although optimizing vitamin D status may contribute to improved immune homeostasis and disease control, further well-designed prospective studies and randomized controlled trials are needed to clarify the therapeutic role of vitamin D supplementation in pediatric SLE.

Ethical Approval: The study was approved by the Health Research Ethics Commission, Faculty of Medicine, Universitas Brawijaya (Approval No. 208/EC/KEPK/07/2021). It was also registered at ClinicalTrials.gov (Identifier: NCT07069348).

Informed Consent: Written informed consent was obtained from all parents or legal guardians, and informed assent was obtained from all participating children before study enrollment.

Peer-review: Externally peer-reviewed

Author Contributions: Concept – W.B., D.W.; Design – W.B., D.W.; Supervision – W.B.; Funding – W.B., D.W.; Materials – W.B., D.W.; Data Collection and/or Processing – C.M., T.R., R.P.F.; Analysis and/or Interpretation – C.M., T.R.; Literature Review – R.P.F., D.W., C.M., T.R.; Writing – R.P.F., D.W.; Critical Review – W.B.

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Immune-Biochemical Stratification Identifies the Pure Primary Biliary Cholangitis Phenotype in AMA-Positive Individuals

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Abstract

Objective: The presence of antimitochondrial antibodies (AMAs) is strongly associated with primary biliary cholangitis (PBC), but serological reactivity alone may not consistently characterize a distinct clinical presentation. We aimed to determine whether a combination of mitochondrial-specific autoimmunity (M2 seropositivity) and cholestatic burden, quantified by alkaline phosphatase (ALP), is associated with a physiologically coherent pure PBC phenotype within an AMA-positive cohort.

Materials and Methods: This retrospective cross-sectional study included 300 AMA-positive adult patients assessed at a tertiary referral institution. Clinical phenotypes were categorized as pure PBC, PBC-autoimmune hepatitis (AIH) overlap, or non-PBC based on accepted diagnostic criteria.

Results: The study included 300 AMA-positive individuals, of which 80 were diagnosed with pure PBC, 37 with PBC-AIH overlap, and 183 were categorized as non-PBC. Seropositivity for M2 and ALP levels were independently associated with the pure PBC phenotype. M2 positivity was strongly associated with pure PBC (adjusted odds ratio [aOR] 14.60, 95% confidence interval [CI] 1.56–136.37, $p=0.019$), whereas each 10 U/L increment in ALP was associated with 18% higher odds of pure PBC (aOR 1.18, 95% CI 1.05–1.32, $p=0.005$). The multivariable model showed good internal discrimination (area under the receiver operating characteristic curve [AUC] 0.86, 95% CI 0.76–0.93), achieving 69.0% sensitivity and 89.2% specificity at the optimal probability threshold. In sensitivity analyses involving overlap syndromes, ALP remained independently correlated with disease phenotype, whereas the influence of M2 seropositivity diminished.

Conclusion: Antimitochondrial antibody positivity alone does not define a consistent clinical phenotype. The integration of M2 seropositivity with ALP levels may provide a more informative immune-biochemical framework for phenotypic stratification among AMA-positive individuals.

Keywords: Primary biliary cholangitis, antimitochondrial antibody, M2 seropositivity, alkaline phosphatase, cholestasis, autoimmunity

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Introduction

Primary biliary cholangitis (PBC) is a chronic immune-mediated cholestatic liver disease marked by progressive destruction of small intrahepatic bile ducts, potentially leading to fibrosis and cirrhosis if left untreated (1,3). Historically considered an organ-specific autoimmune disorder, contemporary evidence indicates a multidimensional immunopathogenic framework in which adaptive autoimmunity, innate immune activation, cholangiocyte-intrinsic stress responses, and bile acid-mediated epithelial injury collectively contribute to chronic biliary inflammation (1–3). Genome-wide association studies have identified susceptibility loci within human leukocyte antigen (HLA) class II and immune regulatory pathways, reinforcing the concept of systemic immunological dysregulation as an underlying determinant of disease risk (1,2). The significant female predominance and frequent association with other autoimmune diseases further suggest a common immunogenetic and immunoregulatory basis (1).

The serological marker of PBC is the development of antimitochondrial antibodies (AMAs), specifically those directed against the E2 subunit of the pyruvate dehydrogenase complex (AMA-M2), which are present in around 90–95% of individuals with confirmed disease (4,5). Mitochondrial-directed autoimmunity constitutes a highly distinctive humoral signature of autoimmune liver disease, indicating a selective breach of tolerance to highly conserved intracellular antigens (5,6). Cholangiocyte apoptosis, abnormal antigen presentation, and bile acid-induced epithelial stress are mechanistically suggested to reveal immunogenic mitochondrial epitopes, thus sustaining autoreactive B- and T-cell responses (3,6).

Although AMA and M2 antibodies are highly specific for established PBC, serological reactivity alone does not reliably identify disease manifestation or progression. Community-based studies have shown that AMA positivity occurs in approximately 0.2–0.4% of the general population, although anti-M2 positivity is significantly rarer (7). Large observational and longitudinal studies have shown that only a small proportion of seropositive individuals without early cholestasis develop clinically evident PBC during follow-up (8–10). These findings demonstrate a clear distinction between autoimmune reactivity and clinically significant cholangiopathy, implying that additional immunological or biochemical signals are required to characterize clinically relevant phenotypes.

In addition to standard AMA responses, disease-specific antinuclear antibodies, such as anti-gp210 and anti-sp100, have been associated with distinct clinical subgroups and, in rare cases, more severe disease outcomes (5,11). From a biological perspective, portal infiltration by autoreactive CD4⁺ and CD8⁺ T cells, T helper 1 (Th1)– and T helper 17 (Th17)–driven immunological polarization, and dysregulated cytokine signaling all contribute to biliary epithelial damage (1,6). Recent studies have increasingly suggested that innate immune activation and cholangiocyte-specific inflammatory signaling pathways are critical to the breakdown of immune tolerance (6). Collectively, these findings support a multidimensional immunologic hypothesis in which serological markers constitute only one aspect of disease biology.

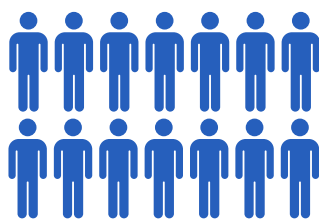
However, despite progress in understanding disease mechanisms, a significant translational gap persists. In AMA-positive populations, the relationship between mitochondrial-specific autoimmunity and measurable biochemical damage in identifying clinically significant disease phenotypes remains unclear. In clinical practice, AMA-positive individuals without persistent cholestasis frequently constitute a diagnostic gray area, while overlap syndromes further obscure phenotypic distinctions (8,12). A systematic framework that integrates immunological specificity with the biochemical manifestation of biliary damage may improve phenotypic resolution.

The objective of this study was to investigate whether the combination of mitochondrial-specific autoimmunity (M2 seropositivity) and cholestatic burden, measured by alkaline phosphatase (ALP) levels, can differentiate the pure PBC phenotype within an AMA-positive population. We hypothesized that an integrated immune-biochemical framework would be associated with improved phenotypic stratification compared with AMA status alone and would better reflect an immunologic gradient among AMA-positive individuals.

Materials and Methods

This retrospective cross-sectional study included consecutive AMA-positive adult patients identified through the Medical Microbiology Laboratory of Ankara Bilkent City Hospital between February 2019 and July 2023. Antimitochondrial antibody and antinuclear antibody (ANA) indirect immunofluorescence (IIF) intensity grades, ANA

STUDY POPULATION



AMA-positive
individuals (n=300)
Tertiary referral center

CLINICAL PHENOTYPES

- Pure PBC (n=80)
- PBC-AIH overlap (n=37)
- Non-PBC (n=183)

FUTURE PERSPECTIVE



These findings should be interpreted strictly as associations. Longitudinal studies and external multicenter validation are required before this framework can be applied in routine clinical practice.

IMMUNE-BIOCHEMICAL FRAMEWORK

AMA POSITIVITY



Indicator
mitochondrial-directed
autoimmunity
Heterogeneous serologic state



M2 SEROPOSITIVITY (AMA-M2 / anti-PDC-E2)



Reflects mitochondrial-specific
humoral autoimmunity



ALP ELEVATION (Cholestatic burden)



Reflects cholestatic bile duct
injury and biochemical damage



Integration of M2 seropositivity and
ALP elevation is more closely
associated with the pure PBC
phenotype

PHENOTYPIC STRATIFICATION

PURE PBC PHENOTYPE

M2
positive ALP
elevated



More closely associated
with the pure PBC phenotype

OVERLAP PHENOTYPE (PBC-AIH overlap)

M2
positive ALP
elevated



Clinically heterogeneous group with mixed
cholestatic and inflammatory features

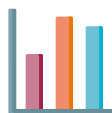
NON-PBC PHENOTYPE

M2
negative ALP
normal or
mildly elevated



Lacks the combined immune-biochemical
profile suggestive of pure PBC

KEY FINDINGS



Multivariable analysis

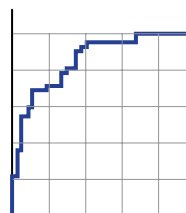
M2 seropositivity and ALP levels were
independently associated with
the pure PBC phenotype.



M2 positivity
aOR 14.60
(95% CI 1.56–136.37), $p=0.019$



ALP (per 10 U/L increase)
aOR 1.18
(95% CI 1.05–1.32), $p=0.005$



Model performance

AUC 0.86 (95% CI 0.76–0.93)
Sensitivity: 69.0%
Specificity: 89.2%
(Optimal probability threshold)

In sensitivity analyses including overlap cases, ALP remained independently associated with phenotype, whereas the association of M2 seropositivity diminished.

CONCLUSION



AMA positivity alone does not define a consistent clinical phenotype. Integrating M2 seropositivity with ALP levels may provide a more informative immune-biochemical framework for phenotypic stratification among AMA-positive individuals.



LIMITATIONS

- Retrospective, cross-sectional, single-center design (selection bias)
- Absence of external validation (exploratory model)
- Disease heterogeneity and temporal variability
- Lack of longitudinal follow-up

staining patterns, AMA-M2 immunoblot results, and clinical diagnoses were retrospectively reviewed.

All consecutive adult patients (≥ 18 years) with confirmed AMA positivity were eligible for inclusion. Antimitochondrial antibody positivity was established based on the laboratory-specific cutoff levels determined by the IIF assay. Patients were required to have available data on M2 serostatus and baseline biochemical parameters, including ALP, to be included in the final analysis.

Patients with active viral hepatitis (positive hepatitis B virus [HBV] DNA, hepatitis C virus [HCV] RNA, or acute hepatitis E), decompensated cirrhosis (Child-Pugh class B/C, active ascites, variceal bleeding, or hepatic encephalopathy), active malignancy, active cholestasis (including biliary obstruction on ultrasonography, magnetic resonance cholangiopancreatography [MRCP], or computed tomography [CT]; choledocholithiasis; biliary malignancy; or primary sclerosing cholangitis) or insufficient clinical, serologic, or biochemical data for multivariable analysis were excluded. These criteria were applied to ensure that identified immune-biochemical relationships accurately represented intrinsic autoimmune characteristics while minimizing confounding from secondary cholestatic or inflammatory liver injury.

Clinical phenotypes were determined according to established international diagnostic criteria and multidisciplinary specialist evaluation. Patients were categorized as pure PBC, PBC-autoimmune hepatitis (AIH) overlap, or non-PBC within the AMA-positive cohort based on clinical, serologic, and biochemical findings at the time of assessment.

Pure PBC was defined as the presence of AMA and persistent cholestatic liver biochemistry, particularly elevated ALP, without significant hepatitic inflammatory features. The diagnosis was established according to internationally accepted diagnostic criteria and confirmed by the attending hepatologists.

The PBC-AIH overlap syndrome was defined by the coexistence of diagnostic features of both PBC and AIH. These features included elevated transaminases, increased immunoglobulin G (IgG) levels, and consistent histopathological findings when available. The diagnosis was established using accepted clinical criteria, including the Paris-type criteria, and multidisciplinary specialist consensus. Patients who did not meet the criteria for

either pure PBC or PBC-AIH overlap were categorized as non-PBC. All diagnoses were confirmed by experienced hepatologists and gastroenterologists during the clinical evaluation.

Hospital records were reviewed to obtain data on alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP, gamma-glutamyl transferase (GGT), international normalized ratio (INR), albumin, immunoglobulin M (IgM), and IgG. Laboratory parameters were assessed either simultaneously with ANA testing or within one week thereafter. Furthermore, the records included clinical details such as age, sex, and diagnosis.

An ANA IIF test was performed using HEp-20-10/liver tissues (Euroimmun AG, Lübeck, Germany). The screening technique began with a preliminary dilution of 1:100 as recommended by the manufacturer. At the screening dilution of 1:100, fluorescence intensity was graded semiquantitatively as 0 (negative), 1+, 2+, 3+, or 4+, with 4+ representing the strongest fluorescence intensity using a EUROSTAR III Plus fluorescent microscope (Euroimmun AG, Lübeck, Germany) at $\times 40$ magnification. The presence of antibodies against extractable nuclear antigens (ENA) was assessed using the EUROLINE ANA Profile 3 plus DFS70 (IgG) test kit manufactured by manufactured by Euroimmun AG, Lübeck, Germany.

The study was conducted in compliance with the Declaration of Helsinki and was authorized by the Ankara Bilkent City Hospital Non-Interventional Clinical Research Ethics Committee on July 12, 2023, with decision number E2-23-4480.

Statistical Analysis

R version 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA) were used for statistical analyses. The Shapiro-Wilk test and visual examination of histograms were used to determine whether continuous variables were normally distributed. Most continuous variables were presented as medians (interquartile range [IQR]) because they were not normally distributed. Categorical variables were expressed as frequencies and percentages.

The Kruskal-Wallis test for continuous variables and the chi-square test or Fisher's exact test for categorical variables, as applicable, were used to compare the three clinical groups (pure PBC, overlap, and non-PBC).

A multivariable logistic regression model was used to identify independent predictors of the pure PBC phenotype based on characteristics that were deemed clinically significant or showed a potential association in univariate analysis. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to present the results. Multicollinearity among predictor variables was assessed using the variance inflation factor (VIF) analysis before multivariable logistic regression modeling. Variables with VIF values below 5 were considered to have no significant multicollinearity.

Receiver operating characteristic (ROC) curve analysis based on the predicted probabilities from the adjusted multivariable logistic regression model was performed to evaluate discriminative performance. The area under the curve (AUC) was calculated to quantify model performance. The Youden index was used to determine the optimal probability threshold, which was then applied to calculate the model's sensitivity and specificity. A two-sided $p < 0.05$ was considered statistically significant.

Results

From February 2019 to July 2023, 35,195 autoimmune liver antibody panel tests (ANA, anti-smooth muscle antibody [ASMA], and liver-kidney microsomal [LKM] antibody) were performed at our institution. Of these tests, 1009 were positive for antimitochondrial antibodies (2.9%). Additionally, 40,297 M2 immunoblot tests were performed, of which 1349 were positive (3.3%). The study included a total of 300 patients who tested positive for AMA and underwent M2 immunoblot testing. The mean age of the included patients was 57.0 (48.0–66.0) years.

Primary biliary cholangitis was diagnosed in 80 patients, and PBC-AIH overlap in 37 (12.3%). Among the remaining 183 patients, 46 (25.1%) had rheumatologic diseases (ankylosing spondylitis, Behçet disease, mixed connective tissue disease, myositis, psoriatic arthritis, Sjögren syndrome, Raynaud phenomenon, scleroderma, systemic lupus erythematosus, and adult-onset Still disease); 45 (24.6%) had neurologic diseases (Alzheimer disease, Bell's palsy, epilepsy, tension-type headache, migraine, multiple sclerosis, hemiplegia, myopathy, neuropathic bladder, polyneuropathy, cerebrovascular accident, vertigo, and optic neuritis); 30 (16.4%) had endocrine diseases (diabetes mellitus, hypothyroidism, hypoparathyroidism, multinodular goiter, pituitary adenoma, and

chronic thyroiditis); 18 (9.8%) had gastroenterological diseases (cryptogenic cirrhosis, pancreatitis, hepatic fibrosis, Crohn disease, nonalcoholic fatty liver disease, pancreatic cyst, pangastritis, and hepatic hemangioma); 12 (6.6%) had renal diseases (chronic renal failure and hydronephrosis); 12 (6.6%) had pulmonary diseases (asthma, chronic obstructive pulmonary disease, pneumonia, interstitial lung disease, and pulmonary arterial hypertension); 4 (2.2%) had cardiovascular diseases (hypertension, heart failure, and pericarditis); and 16 (8.7%) had other conditions (joint pain, leukocytoclastic vasculitis, immune thrombocytopenic purpura, anemia, dyspepsia, xerosis cutis, allergy, and urticaria).

The study population comprised 300 AMA-positive individuals categorized into three distinct phenotypes: pure PBC, PBC-AIH overlap, and non-PBC AMA-positive patients. A distinct serologic gradient developed among phenotypes: pure PBC ($n=80$, 26.7%), PBC-AIH overlap ($n=37$, 12.3%), and non-PBC ($n=183$, 61.0%). Intensity grades of AMA IIF differed significantly among the clinical phenotypes ($p=0.0002$), with higher grades observed in the cholestatic phenotypes.

M2 positivity was more frequent in pure PBC (92.5%) and PBC-AIH overlap (75.7%) than in non-PBC patients (59.0%) ($p < 0.001$), underscoring its phenotypic specificity among AMA-positive populations. Although overall ANA positivity rates (pure PBC, 43/80 [53.8%]; overlap, 22/37 [59.5%]; non-PBC, 84/183 [45.9%]) did not differ significantly ($p=0.224$), semiquantitative ANA IIF intensity grades differed modestly among the clinical phenotypes ($p=0.022$).

Immunoglobulin profiling showed modest but statistically significant increases in IgM levels in pure PBC patients (1.90 [1.48–2.81] g/L) compared to non-PBC patients (1.30 [1.00–2.10] g/L; overlap: 1.44 [1.04–2.67] g/L) ($p=0.047$), while IgG levels remained comparable ($p=0.276$), indicating a predominance of humoral cholestatic immune activation over generalized hypergammaglobulinemia. Liver biochemistry revealed a strong cholestatic burden in both pure primary biliary cholangitis and overlap groups. Alkaline phosphatase and GGT levels were markedly elevated in these phenotypes (ALP: pure PBC 149.0 [102.5–252.8], overlap 160.0 [126.0–261.0], non-PBC 86.0 [67.8–112.3] U/L; GGT: pure PBC 68.5 [30.8–200.8], overlap 87.0 [50.0–149.0], non-PBC 27.0 [18.0–61.0] U/L; both $p < 0.001$), establishing a clear biochemical distinction between cholestatic

Table 1. Baseline characteristics of antimitochondrial antibody-positive individuals according to clinical phenotype.

Variable	Pure PBC	PBC-AIH Overlap	Non-PBC	p-value
Age (years)	56.5 (51.0–66.0)	58.0 (52.0–66.0)	56.0 (46.0–66.0)	0.635
Sex (female), n (%)	71 (88.8%)	34 (91.9%)	156 (85.2%)	0.473
AMA IIF intensity grade (0–4+)	2.0 (1.0–3.0)	2.0 (2.0–3.0)	2.0 (1.0–2.0)	0.0002
AMA-M2 immunoblot intensity grade (0–3+)	3.0 (3.0–3.0)	3.0 (2.0–3.0)	2.0 (0.0–3.0)	<0.001
ANA IIF intensity grade (0–4+)	1.0 (0.0–3.0)	2.0 (0.0–3.0)	0.0 (0.0–2.0)	0.022
IgM (g/L)	1.90 (1.48–2.81)	1.44 (1.04–2.67)	1.30 (1.00–2.10)	0.047
IgG (g/L)	13.9 (11.6–17.9)	14.2 (11.9–15.6)	12.9 (11.0–14.8)	0.276
AST (U/L)	33.5 (20.8–49.0)	42.0 (29.0–79.0)	22.0 (17.0–29.8)	<0.001
ALT (U/L)	40.0 (21.0–52.3)	45.0 (31.0–85.0)	25.0 (19.0–34.0)	<0.001
ALP (U/L)	149.0 (102.5–252.8)	160.0 (126.0–261.0)	86.0 (67.8–112.3)	<0.001
GGT (U/L)	68.5 (30.8–200.8)	87.0 (50.0–149.0)	27.0 (18.0–61.0)	<0.001
Total bilirubin (mg/dL)	0.50 (0.40–0.74)	0.65 (0.50–0.80)	0.50 (0.40–0.66)	0.029
Albumin (g/L)	43.4 (39.9–45.1)	43.8 (40.2–45.1)	44.0 (41.0–45.7)	0.357
INR	1.00 (0.98–1.06)	1.00 (0.91–1.01)	1.00 (0.95–1.00)	0.147
M2 positivity, n (%)	74 (92.5%)	28 (75.7%)	108 (59.0%)	<0.001
ANA positivity, n (%)	43 (53.8%)	22 (59.5%)	84 (45.9%)	0.224

Data are presented as median (interquartile range [IQR]) for continuous variables and number (percentage) for categorical variables. AMA and ANA IIF intensity grades were assessed semiquantitatively from 0 (negative) to 4+ (strongest fluorescence intensity), whereas AMA-M2 immunoblot intensity was graded from 0 (negative) to 3+ (strongest band intensity).

ALP: Alkaline phosphatase, **ALT:** Alanine aminotransferase, **AMA:** Antimitochondrial antibody, **ANA:** Antinuclear antibody, **AST:** Aspartate aminotransferase, **GGT:** Gamma-glutamyl transferase, **Ig:** Immunoglobulin, **INR:** International normalized ratio, **PBC:** Primary biliary cholangitis, **AIH:** Autoimmune hepatitis.

Table 2. Distribution of antinuclear antibody (ANA) patterns according to clinical phenotype.

ANA pattern	Pure PBC n (%)	PBC-AIH Overlap n (%)	Non-PBC n (%)
Centromere	12 (15.0)	2 (5.4)	10 (5.5)
Speckled	7 (8.8)	5 (13.5)	21 (11.5)
Mixed	4 (5.0)	5 (13.5)	22 (12.0)
Negative	37 (46.2)	15 (40.5)	99 (54.1)
Nuclear dots	9 (11.2)	4 (10.8)	9 (4.9)
Nuclear envelope	11 (13.8)	6 (16.2)	22 (12.0)

Note: Overall χ^2 test, $p=0.098$

ANA: Antinuclear antibody, **PBC:** Primary biliary cholangitis, **AIH:** Autoimmune hepatitis.

and non-cholestatic AMA-positive patients. Transaminases (AST and ALT) were significantly higher in overlap

patients (AST: pure PBC 33.5 [20.8–49.0], overlap 42.0 [29.0–79.0], non-PBC 22.0 [17.0–29.8] U/L; ALT: pure PBC 40.0 [21.0–52.3], overlap 45.0 [31.0–85.0], non-PBC 25.0 [19.0–34.0] U/L; $p<0.001$), consistent with characteristics of hepatic inflammation. Total bilirubin levels were considerably elevated in overlap individuals (0.65 [0.50–0.80] mg/dL vs. pure PBC 0.50 [0.40–0.74] and non-PBC 0.50 [0.40–0.66] mg/dL; $p=0.029$), although synthetic function measures (albumin and INR) were maintained and equivalent among groups, indicating a preponderance of early to mid-stage disease in this cohort (Table 1).

Across the entire cohort, the distribution of ANA status and patterns did not differ significantly among the clinical phenotypes (overall $p=0.098$). ANA negativity was the most frequent category in all three groups, while centromere, speckled, mixed, nuclear dots, and nuclear envelope patterns were distributed similarly across the phenotypes. Overall, semiquantitative ANA IIF intensity

Table 3. Multivariable logistic regression analyses.

Table 3A. Multivariable logistic regression model for pure primary biliary cholangitis (PBC) versus non-PBC.

Variable	aOR	95% CI	p-value
M2 positivity	14.60	1.56–136.37	0.019
ALP (per 10 U/L)	1.18	1.05–1.32	0.005
IgM (g/L)	0.88	0.48–1.60	0.678
AMA IIF intensity grade (0–4+)	1.05	0.53–2.09	0.894
Age	0.99	0.95–1.04	0.801
Sex (female)	0.46	0.05–4.16	0.491

grades differed modestly among the phenotypes, whereas the distribution of qualitative ANA patterns did not differ significantly among the groups (Table 2). Collectively, these findings indicate that semiquantitative ANA IIF intensity grades differed modestly among the clinical phenotypes, whereas the distribution of qualitative ANA patterns did not differ significantly among the pure PBC, PBC-AIH overlap, and non-PBC groups (Table 2). Variance inflation factor analysis showed no evidence of problematic multicollinearity among the variables included in the multivariable logistic regression model (all VIF values <5; [Supplementary Table S1](#)).

In the primary multivariable analysis excluding overlap cases, both M2 seropositivity and cholestatic burden were independently associated with the pure PBC phenotype in AMA-positive individuals. M2 positivity was strongly associated with pure PBC (adjusted OR [aOR] 14.60, 95% CI 1.56–136.37, $p=0.019$). The confidence interval for M2 seropositivity was wide. Alkaline phosphatase levels, quantified in 10 U/L increments, demonstrated an independent association, wherein each 10 U/L increase in ALP was associated with 18% higher odds of pure PBC (aOR 1.18, 95% CI 1.05–1.32, $p=0.005$). In contrast, IgM levels, AMA IIF intensity grade, age, and sex were not independently associated with pure PBC after adjustment (Table 3).

A sensitivity analysis was conducted to evaluate the robustness of these findings by defining the outcome as the PBC spectrum (pure PBC + PBC-AIH overlap) compared to non-PBC AMA-positive patients. In this comprehensive clinical definition, ALP was independently correlated with disease phenotype (aOR 1.16 per 10 U/L, 95% CI 1.06–1.27, $p=0.002$). Nonetheless, the effect

Table 3B. Sensitivity analysis: multivariable logistic regression model for the PBC spectrum (pure PBC + PBC-AIH overlap) versus non-PBC.

Variable	aOR	95% CI	p-value
M2 positivity	2.81	0.82–9.63	0.101
ALP (per 10 U/L)	1.16	1.06–1.27	0.002
IgM (g/L)	0.98	0.90–1.06	0.573
AMA IIF intensity grade (0–4+)	1.26	0.69–2.31	0.454
Age	1.01	0.97–1.05	0.802
Sex (female)	0.81	0.11–6.07	0.840

aOR: adjusted odds ratio, ALP: Alkaline phosphatase, AMA: Antimitochondrial antibody, CI: Confidence interval, IgM: Immunoglobulin M, PBC: Primary biliary cholangitis, AIH: Autoimmune hepatitis.

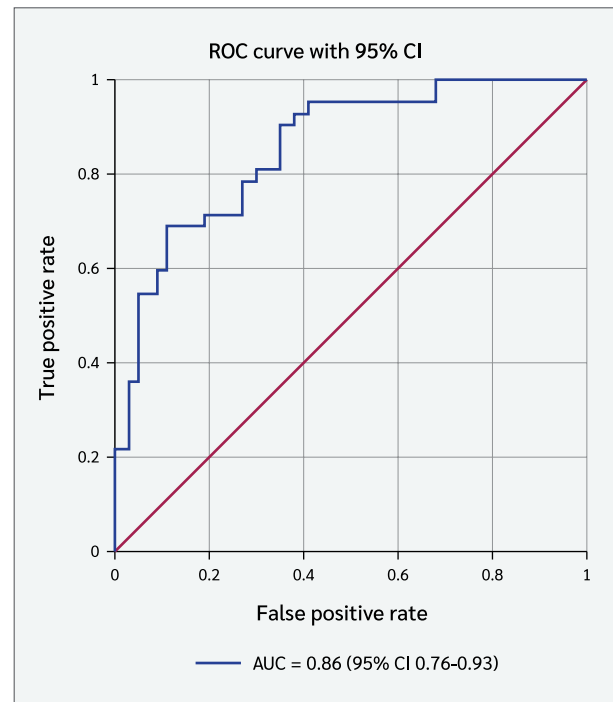


Figure 1. Receiver operating characteristic (ROC) curve of the multivariable model discriminating pure primary biliary cholangitis (PBC) from non-PBC within an AMA-positive cohort.

size of M2 positivity diminished and became statistically nonsignificant (aOR 2.81, 95% CI 0.82–9.63, $p=0.101$), suggesting that the association of M2 seropositivity may be more specific to the pure PBC phenotype than to the broader PBC spectrum. These findings collectively suggest that among AMA-positive individuals, cholestatic biochemical burden is a consistent and broad correlate

of disease phenotype, while M2 seropositivity is a more precise indicator of pure PBC (Table 3).

The discriminative performance of the primary multivariable model (pure PBC versus non-PBC, excluding overlap) was evaluated by receiver operating characteristic analysis. The model achieved an AUC of 0.86 (95% CI 0.76–0.93), with a sensitivity of 69.0% and a specificity of 89.2% at the optimal probability threshold (Figure 1).

Discussion

Our findings suggest that AMA positivity represents an immunologically heterogeneous state rather than a distinct clinical phenotype. Large real-world studies have shown that many AMA-positive individuals do not fulfill criteria for established PBC at baseline, indicating that serologic autoreactivity and overt cholangiopathy may dissociate in routine practice (8,10). In our cohort, AMA positivity alone did not characterize a uniform clinical phenotype. Instead, M2 seropositivity and ALP-defined cholestatic burden were associated with the pure PBC phenotype, with good internal discrimination. This integrated immune-biochemical relationship is visually summarized in our conceptual framework (Figure 2).

This finding should be interpreted cautiously. Longitudinal data show that many individuals with incidental AMA positivity, particularly those without baseline cholestasis, have a relatively low medium-term risk of progression to overt PBC (10,13). Therefore, the “pure PBC” phenotype observed cross-sectionally is not a fixed clinical entity but reflects the clinical status at evaluation. Conversely, cohorts enriched for AMA-M2 positivity may show higher rates of subsequent PBC features, suggesting that risk is heterogeneous and depends on baseline biochemical injury and ascertainment context (3).

Our hospital-based AMA and M2 prevalence rates should also be interpreted as referral-enriched estimates. Population-based studies report AMA positivity in approximately 0.2–0.4% of the general population, significantly lower than rates observed in hepatology referral cohorts (8,13). In a tertiary-care setting, pretest probability and case-mix distribution enrich the cohort for cholestatic and overlap phenotypes (14). Therefore, these estimates should not be directly generalized to unselected populations.

The association between M2 seropositivity and pure PBC is biologically plausible but should not be overinterpreted. Antimitochondrial antibodies, particularly the M2 subtype, target mitochondrial antigens, such as the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), and are closely linked to the humoral immune profile of PBC (15–18). Biliary epithelial stress and innate immune activation may enhance antigen presentation and sustain biliary-focused inflammation (6,19). However, our findings demonstrate an association rather than a causal immunopathogenic relationship. Furthermore, while our multivariable model confirmed M2 seropositivity as a strong predictor of the pure PBC phenotype, the wide confidence interval suggests that the exact magnitude of this association remains uncertain. This is likely attributable to the limited number of M2-negative cases within our specific pure PBC cohort, highlighting the need for larger, multicenter studies to more precisely define the effect size.

Although diagnostic-accuracy studies support their performance in established PBC (4,20,21), PBC-specific autoantibodies may also be detected in individuals without persistent cholestasis or in alternative conditions (22,23). This explains why M2 seropositivity alone was insufficient to characterize the broader PBC spectrum. In contrast, ALP remained independently associated with phenotype across models, functioning as a stable marker of cholestatic injury. This aligns with guidelines and outcome studies linking persistent ALP elevation to poorer transplant-free survival (3,24–27). Additionally, qualitative ANA pattern distribution did not differ significantly among clinical phenotypes ($p=0.098$), indicating that ANA patterns alone are insufficient to discriminate these groups in our cohort.

Overlap syndromes represent an important source of clinical heterogeneity. Criteria-based definitions, including Paris-type frameworks, are useful but may not capture the full spectrum of inflammatory cholangiopathy (28). Contemporary outcome data suggest that some clinically treated AIH-PBC variant patients respond to combination therapy without satisfying strict criteria (12). In our sensitivity analysis, the association of M2 was attenuated when overlap cases were included, whereas ALP remained significant. These findings suggest that M2 seropositivity is more closely associated with classical pure PBC, while PBC-AIH overlap syndromes represent a broader, heterogeneous inflammatory state.

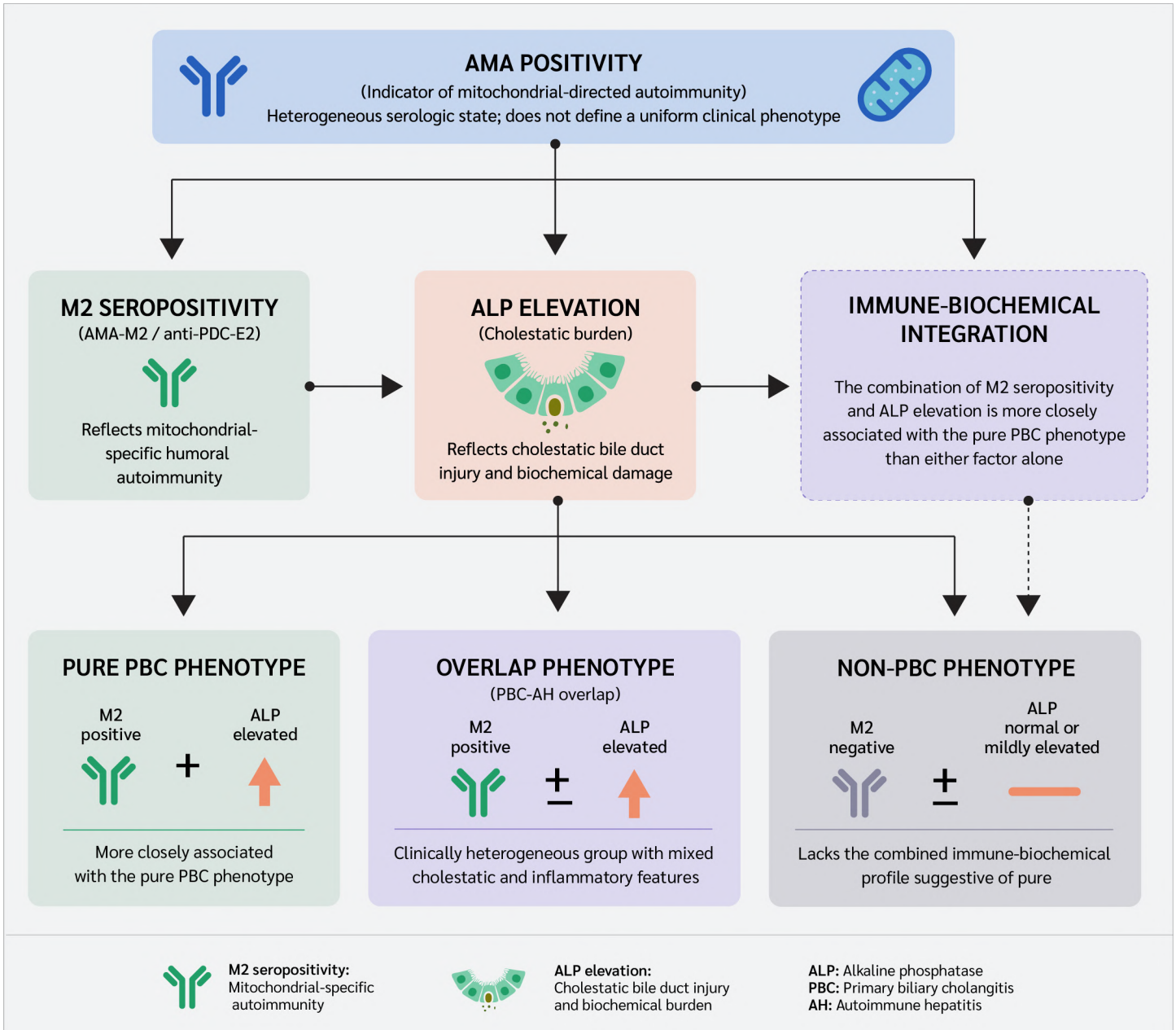


Figure 2. Conceptual framework of immune-biochemical stratification in AMA-positive individuals.

From a clinical perspective, the model should be viewed as exploratory. Although the model showed good internal discrimination, its generalizability is limited without an external validation cohort. The reported AUC and probability thresholds are internally derived and may be influenced by local assay platforms or patient case-mix. Independent, multicenter validation is required before routine clinical implementation.

Individuals who are AMA-positive display profound clinical heterogeneity, ranging from asymptomatic

seropositivity to evolving cholestasis or overlap syndromes. Accordingly, the observed immune-biochemical profile should be regarded as a cross-sectional representation of disease status, which may be influenced by temporal changes in liver biochemistry, immune activity, treatment exposure, and other clinical factors.

This study has several limitations. First, our single-center, retrospective design in a tertiary setting introduces selection bias, likely enriching the cohort with complex or overlapping phenotypes that do not fully reflect

community-based AMA-positive populations. Second, lacking an external validation cohort, our prediction model remains exploratory. Its diagnostic metrics and probability thresholds require independent, multicenter validation before routine clinical application. Third, small sample sizes in certain subgroups, particularly ANA patterns, may have reduced statistical power. Consequently, the prevalence estimates, observed effect sizes, and model performance derived from our cohort should not be broadly generalized. For instance, the wide confidence interval observed for M2 seropositivity suggests limited precision and should be interpreted cautiously.

Finally, because of the cross-sectional design, our findings should be interpreted as associations rather than evidence of causality or disease progression. Prospective longitudinal studies are needed to determine whether AMA-positive individuals maintain their phenotype over

time or transition between non-PBC, PBC-AIH overlap, and pure PBC states.

Conclusion

In conclusion, AMA positivity alone was insufficient to define a uniform clinical phenotype in this cohort. Among AMA-positive individuals, the pure PBC phenotype was more closely associated with the combined presence of M2 seropositivity and elevated ALP levels, integrating mitochondrial-specific autoimmunity with objective biochemical evidence of cholestatic injury. Compared with AMA status alone, this immune-biochemical framework may improve phenotypic stratification. However, prospective longitudinal studies and external validation are required before this model can be adopted in routine clinical practice.

Ethical Approval: The study was approved by the Ankara Bilkent City Hospital Non-Interventional Clinical Research Ethics Committee on July 12, 2023, with decision number E2-23-4480.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed

Author Contributions: Concept – E.S.; Design – E.S.; Supervision – E.S.; Materials – E.S., S.G.; Data Collection and/or Processing – E.S., S.G.; Analysis and/or Interpretation – E.S., S.G.; Literature Review – E.S., S.G.; Writer – E.S.; Critical Review – E.S.

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Association of *CTLA-4* Gene Polymorphisms and Serum Soluble CTLA-4 Levels with Psoriasis Vulgaris

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Abstract

Objective: Psoriasis vulgaris (Pv) is a chronic inflammatory condition with a genetic and immunological basis. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a key immunoregulatory molecule expressed on T cells that transmits inhibitory signals important for maintaining immune homeostasis. Single nucleotide polymorphisms (SNPs) in the *CTLA-4* gene, as well as changes in the soluble form of CTLA-4 (sCTLA-4), may influence CTLA-4 function or expression and contribute to psoriasis pathogenesis. This study aimed to examine the association of two *CTLA-4* SNPs, rs231775 (+49A/G) and rs3087243 (CT60), as well as serum sCTLA-4 levels with Pv.

Materials and Methods: The study enrolled patients with Pv and healthy controls. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and serum sCTLA-4 levels were measured using enzyme-linked immunosorbent assay (ELISA).

Results: The study included 45 patients and 45 controls. No significant association was detected between individual SNPs and Pv. However, the G/G haplotype was significantly more frequent in the Pv group, suggesting an increased disease risk. Serum sCTLA-4 levels were significantly higher in patients with Pv and positively correlated with disease severity.

Conclusion: These results suggest that haplotype interactions play a more important role than single SNP effects in psoriasis susceptibility, and that elevated sCTLA-4 may contribute to psoriasis immunopathogenesis.

Keywords: CTLA-4, sCTLA-4, SNPs, rs231775, rs3087243, psoriasis vulgaris

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Introduction

Psoriasis is a long-term, immunologically driven inflammatory cutaneous disorder characterized by recurrent flare-ups, scaling, and erythema (1). The estimated worldwide prevalence of psoriasis is around 3%–4%, with psoriasis vulgaris (Pv) representing the most common clinical subtype (2). According to the Global Psoriasis Atlas (GPA), the overall prevalence of psoriasis in Egypt is 0.4% (3). Recognizing its burden, psoriasis was classified as a serious non-communicable disease by the World Health Organization (WHO), with emphasis on concerns related to misdiagnosis, inadequate treatment, and social stigma (4).

The immunopathogenesis of psoriasis is driven by dysregulated immune responses, particularly involving helper T-cell subsets (Th1, Th17, and Th22) and regulatory T (Treg) cells, leading to persistent T-cell activation and chronic inflammation (5). Pro-inflammatory cytokines secreted by antigen-presenting cells (APCs) in response to environmental or immunological triggers activate naïve T cells and polarize their differentiation into effector T-cell subsets. These activated T cells subsequently secrete cytokines including tumor necrosis factor- α (TNF- α), interleukin (IL)-17, and IL-22, leading to hyperproliferation, epidermal hyperplasia, neovascularization, and the formation of psoriatic plaques (6).

Effective T-cell activation depends not only on antigen recognition through the major histocompatibility complex (MHC)/T-cell receptor (TCR) interaction but also on additional costimulatory signals, particularly those mediated by the B7 (CD80/86)/CD28 pathway (7). Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), a member of the immunoglobulin superfamily encoded by the *CTLA-4* gene located on the long arm of chromosome 2, is a co-inhibitory checkpoint receptor that negatively modulates T-cell activation by binding CD80 and CD86 with higher affinity than CD28, thereby attenuating T-cell-mediated immune responses (8).

Additionally, CTLA-4 negatively regulates T-cell activation by inhibiting IL-2 secretion and IL-2 receptor expression, thereby suppressing T-cell proliferation and cytokine production. It also promotes T-cell tolerance by preventing the nuclear accumulation of key transcription factors, including activator protein-1 (AP-1), nuclear factor kappa B (NF- κ B), and nuclear factor of activated T cells (NFAT), and disrupts stable APC interactions by

counteracting the TCR-induced "stop signal" required for full T-cell activation (9).

Cytotoxic T-lymphocyte-associated antigen 4 is constitutively highly expressed on Tregs and is essential for maintaining immune homeostasis, as demonstrated by the fatal autoimmunity observed in *CTLA-4* knockout mice (10). In the context of psoriasis, studies have found that impaired CTLA-4 function, together with reduced Treg numbers in skin lesions, contributes to the unchecked activation of Th1 and Th17 cells, leading to excessive cytokine production, keratinocyte hyperproliferation, and chronic inflammation (11,12).

Several *CTLA-4* single-nucleotide polymorphisms (SNPs) have been well-characterized and linked to various autoimmune disorders such as diabetes mellitus, rheumatoid arthritis, and Graves' disease (13–15). Among the most commonly studied variants are rs231775 (+49A/G), located in exon 1, and rs3087243 (CT60 G/A), located in the 3' untranslated region of the *CTLA-4* gene (16).

Additionally, a soluble isoform of CTLA-4 (sCTLA-4), produced by alternative messenger RNA (mRNA) splicing, is present in human serum and has been found at elevated levels in several autoimmune diseases, suggesting a role in the pathogenesis of these conditions (17). Nonetheless, the interplay between *CTLA-4* SNPs, sCTLA-4 levels, and Pv is still poorly understood, particularly in the Egyptian population, indicating a need for further study.

Materials and Methods

Study Design and Participants

The present case-control study was conducted from August 2023 to February 2025 at the Departments of Medical Microbiology and Immunology and Dermatology, Faculty of Medicine, Ain Shams University.

The study population included patients with clinically diagnosed Pv who had not undergone systemic therapy or phototherapy within the preceding three months, while patients with other types of psoriasis, autoimmune or chronic inflammatory diseases, malignancies, or those receiving immunosuppressive or systemic therapy were excluded. Disease severity was assessed by two physicians through a joint assessment using the Psoriasis Area and Severity Index (PASI). The control group comprised age- and sex-matched apparently healthy

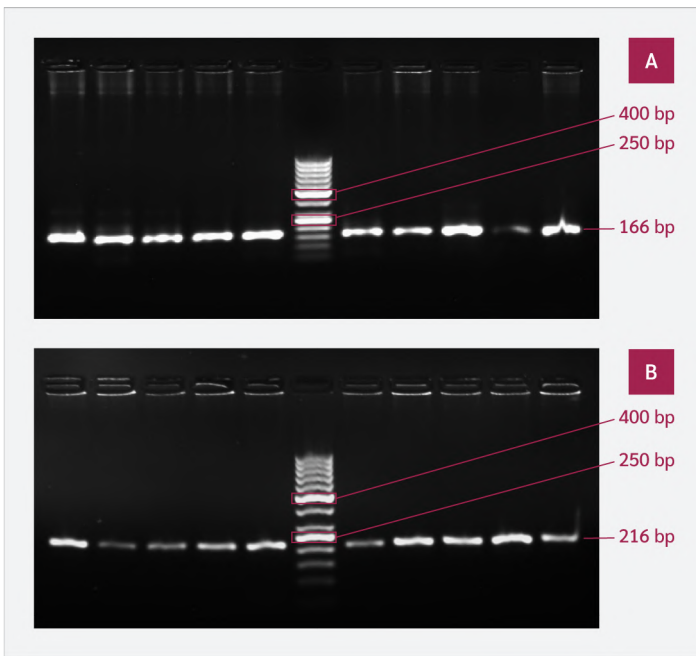


Figure 1. Agarose gel electrophoresis of PCR products.

(A) rs231775 PCR products. All lanes show a 166-bp amplicon. (B) rs3087243 PCR products. All lanes show a 216-bp amplicon. The middle lane contains a 50-bp DNA ladder.

individuals with no personal or family history of psoriasis, autoimmune diseases, or cancer.

DNA Extraction and Genotyping

From each subject, 5 mL of venous blood was collected and divided between an ethylenediaminetetraacetic acid (EDTA) tube for DNA extraction and a plain tube for serum separation. Samples were preserved at -80°C with proper labeling until analysis.

Genomic DNA was extracted using the GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was utilized for genotyping. The primer sequences were adopted from the methodology of Dursun et al. (18). The thermal cycling protocol consisted of an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C , annealing at 55°C , and extension at 72°C , each lasting 30 seconds, followed by a final extension step at 72°C for 7 minutes. The PCR products were subjected to 2% agarose gel electrophoresis to verify the expected amplicon sizes (Figure 1).

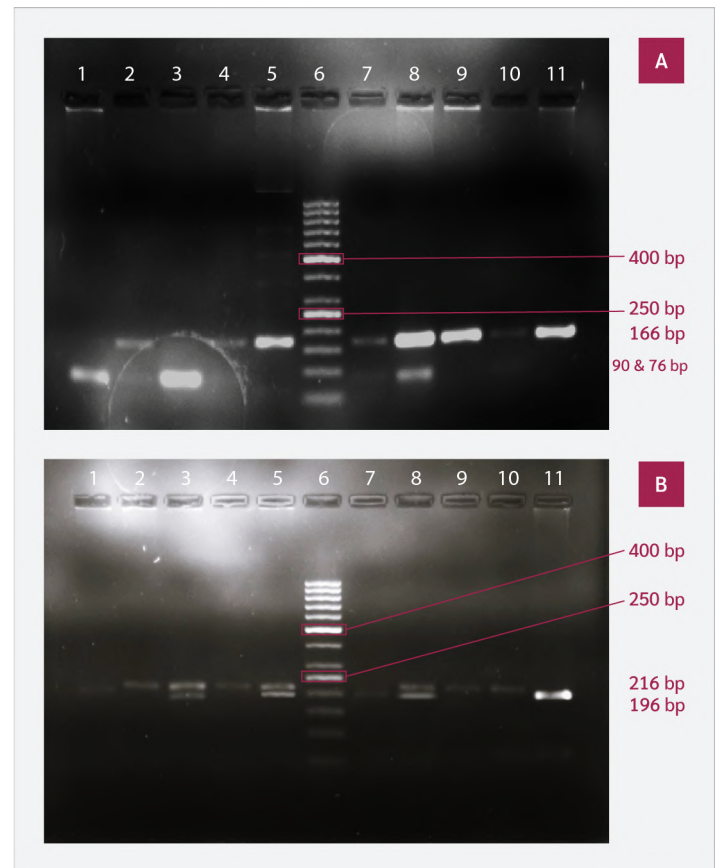


Figure 2. Agarose gel electrophoresis of restriction digestion products.

(A) rs231775 digestion products. Lanes 4, 5, 9, and 11 show 166-bp bands (AA genotype). Lanes 2, 7, and 8 show 166-, 90-, and 76-bp bands (AG genotype). Lanes 1 and 3 show 90- and 76-bp bands (GG genotype). Lane 6 contains a 50-bp DNA ladder.

(B) rs3087243 digestion products. Lanes 2, 4, 9, and 10 show 216-bp bands (GG genotype). Lanes 3, 5, and 8 show 216-, 196-, and 20-bp bands (GA genotype). Lanes 1, 7, and 11 show 196- and 20-bp bands (AA genotype). Lane 6 contains a 50-bp DNA ladder.

Note: The 20-bp fragments are too small to be clearly visualized on the agarose gel.

The PCR-amplified products of rs231775 and rs3087243 were digested with the restriction enzymes BbvI and NcoI (Enzymomics, Daejeon, South Korea), respectively, according to the manufacturer's protocol. The primer sequences, amplicon sizes, corresponding restriction enzymes, and the possible genotypes determined by restriction digestion are summarized in Table 1. The digested products were resolved by electrophoresis on a 2.5% agarose gel, and the resulting bands were visualized using the Azure 600 Gel Imaging System (Azure Biosystems, Dublin, CA, USA) (Figure 2). Samples showing borderline or unclear results were re-genotyped. Additionally, to ensure reproducibility, 20 randomly selected samples were subjected to repeat genotyping with complete concordance between repeated results.

Table 1. Primer sequences, PCR reaction preparation, amplicon sizes, corresponding restriction enzymes, and the possible genotypes of the studied SNPs.

CTLA-4 SNP	Primer sequence	PCR Reaction Preparation (μL)	Amplicon (bp)	RE (Condition)	Digestion products (bp)
rs231775	F: 5'-TTGCTCTACTTCTGAAGACCTGAA-3' R: 5'-GAAGACGTTTCCACTCACTCTGAAA-3'	PCR master mix: 12.5 F: 1 R: 1 DNA template: 5 Nuclease-free water: 5.5	166	BbvI (4 U, 37°C for 1 h)	AA: 166 AG: 166, 90, 76 GG: 90, 76
rs3087243	F: 5'-CACCCTATTTGGGATATACC-3' R: 5'-CGGAAGGACTTTATATCTGGA-3'	PCR master mix: 12.5 Forward primer: 1 Reverse primer: 1 DNA template: 5 Nuclease-free water: 5.5	216	NcoI (10 U, 37°C for 1 h)	GG: 216 AG: 20, 196, 216 AA: 20, 196

CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4, **SNP:** Single nucleotide polymorphism, **PCR:** Polymerase chain reaction, **RE:** Restriction enzyme, **bp:** Base pair, **F:** Forward primer, **R:** Reverse primer.

Table 2. Distribution of genotypes and alleles for the studied SNPs among the Pv and control groups.

CTLA-4 SNP rs231775		Pv group n (%)	Control group n (%)	χ^2	<i>p</i>	<i>q</i> -value	OR (95% CI)	HWE (<i>p</i>), Pv group	HWE (<i>p</i>), control group
Genotypes (n=45)	AA	25 (55.6)	32 (71.1)	3.112	MC 0.185	0.333	Reference	0.103	0.421
	AG	14 (31.1)	11 (24.4)				1.36 (0.75–2.45)		
	GG	6 (13.3)	2 (4.4)				2.29 (0.84–6.22)		
Alleles (n=90)	A	64 (71.1)	75 (83.3)	3.822	0.051	0.153	Reference		
	G	26 (28.9)	15 (16.7)				1.56 (0.998–2.43)		
CTLA-4 SNP rs3087243		Psoriasis group n (%)	Control group n (%)	χ^2	<i>p</i>		OR (95% CI)	HWE (<i>p</i>) cases	HWE (<i>p</i>) controls
Genotypes (n=45)	GG	10 (22.2)	9 (20.0)	0.111	0.946	0.946	Reference	0.175	0.180
	GA	27 (60.0)	27 (60.0)				0.94 (0.49–1.80)		
	AA	8 (17.8)	9 (20.0)				0.87 (0.38–1.98)		
Alleles (n=90)	G	47 (52.2)	45 (50.0)	0.089	0.766	0.946	Reference		
	A	43 (47.8)	45 (50.0)				0.95 (0.66–1.36)		

Pv: Psoriasis vulgaris, **OR:** Odds ratio, **CI:** Confidence interval, ***q*-value:** Benjamini-Hochberg (BH) adjusted *p* value, **HWE:** Hardy-Weinberg equilibrium.

Table 3. Association between the studied SNP genotypes and PASI and sCTLA-4 levels within the Pv group.

	CTLA-4 SNP rs231775			Test	<i>p</i> (<i>q</i> -value)	CTLA-4 SNP rs3087243			Test	<i>p</i> (<i>q</i> -value)
	AA n=25	AG n=14	GG n=6			GG n=10	GA n=27	AA n=8		
PASI										
Median (IQR)	5.10 (3.80–6.40)	6.40 (4.70–6.50)	10.10 (5.90–11.20)	H=2.266	0.322 (0.594)	5.10 (4.55–7.77)	5.50 (3.60–6.50)	6.30 (5.33–11.35)	H=1.611	0.447 (0.626)
sCTLA-4 (ng/mL)										
Median (IQR)	8.60 (8.20–10.00)	8.25 (7.93–9.00)	9.20 (7.25–10.03)	H=0.326	0.849 (0.849)	7.75 (7.00–8.90)	8.60 (8.20–9.20)	9.30 (8.55–12.33)	H=4.091	0.129 (0.301)

Pv: Psoriasis vulgaris, **CTLA-4:** Cytotoxic T-lymphocyte-associated antigen 4, **PASI:** Psoriasis Area and Severity Index, **sCTLA-4:** Soluble cytotoxic T-lymphocyte-associated antigen 4, **IQR:** Interquartile range, **H:** Kruskal-Wallis test, ***q*-value:** Benjamini-Hochberg (BH) adjusted *p* value.

Table 4. Haplotype frequencies among the Pv and control groups.

Haplotype (rs231775/rs3087243)	Pv group n (%)	Control group n (%)	<i>p</i>	<i>q</i> -value	OR (95% CI)
A/G	27.10 (30.1)	37.50 (41.7)	0.106	0.239	0.60 (0.33–1.12)
A/A	36.90 (41.0)	37.50 (41.7)	0.927	0.946	0.97 (0.54–1.76)
G/G	19.90 (22.1)	7.50 (8.3)	0.010*	0.045*	3.12 (1.27–7.66)
G/A	6.10 (6.8)	7.50 (8.3)	0.694	0.946	0.80 (0.26–2.43)

Pv: Psoriasis vulgaris, **OR:** Odds ratio, **CI:** Confidence interval. *: Statistically significant after Benjamini-Hochberg adjustment.

Measurement of Serum sCTLA-4 Levels

Serum sCTLA-4 levels were measured using a human sCTLA-4 enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, Shanghai, China) following the manufacturer's instructions. This kit employs a quantitative sandwich ELISA technique for the detection of circulating sCTLA-4 levels in serum samples. Standards were assayed in duplicate, 50 µL of standards and 40 µL of samples were added to the corresponding wells. Then, 10 µL of anti-sCTLA-4 antibody was added to sample wells only, followed by the addition of 50 µL of streptavidin-horseradish peroxidase (HRP) to both the sample and standard wells but not the blank well. The plate was covered with a seal and incubated for 60 minutes at 37°C. The plate was washed according to the manufacturer's instructions, and then 50 µL of each substrate solution (A and B) was added to all wells, followed by incubation for 10 minutes in the dark at 37°C. A stop solution was then added to all wells, resulting in a color change from blue to yellow. The optical density (OD) of each well was measured using a microplate reader set to 450 nm. The concentrations were determined from a standard curve and expressed in ng/mL.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 25.0 (IBM Corp., Armonk, NY, USA). Non-parametric quantitative variables were summarized as medians and interquartile ranges (IQRs), while qualitative variables were presented as frequencies and percentages.

Non-parametric continuous variables were analyzed using the Mann-Whitney U test for two-group comparisons and the Kruskal-Wallis test for multiple-group comparisons. The relationships among categorical variables were evaluated using the chi-square test or the Monte Carlo test when appropriate.

Spearman's rank correlation coefficient was utilized to determine the association between quantitative variables. HaploView software version 4.2 (Broad Institute, Cambridge, MA, USA) was used to estimate haplotype frequencies. To account for multiple comparisons, the Benjamini-Hochberg false discovery rate (FDR) correction was applied separately to case-control analyses and within-case clinical association analyses. Both raw *p*-values and adjusted *p*-values (*q*-values) were reported. Statistical significance was defined using an adjusted *p*<0.05.

Results

The present case-control study enrolled 45 patients with Pv and 45 age- and sex-matched healthy controls. The median age of the patients with Pv was 40 years, with an IQR of 26.0–50.0, while the median age of the controls was 36 years, with an IQR of 24.0–46.0. The Pv group included 26 males and 19 females, whereas the control group included 21 males and 24 females.

Regarding genotype distribution, both study groups were in Hardy-Weinberg equilibrium (HWE) for both SNPs. Furthermore, no significant differences in genotype or allele frequencies were detected between the two groups for either SNP (Table 2).

Table 3 illustrates the associations between the studied SNPs genotypes and the examined parameters within the Pv group, with no significant differences observed.

Four haplotypes defined by rs231775 and rs3087243 SNPs were identified, with the G/G haplotype occurring significantly more frequently in the Pv group (Table 4).

Regarding the sCTLA-4 immunoassay, serum sCTLA-4 levels differed significantly between the Pv group and

Table 5. Comparison of serum sCTLA-4 levels between the Pv and control groups.

Parameter	Pv group n=45	Control group n=45	Test	p	q-value
sCTLA-4 (ng/mL), median (IQR)	8.60 (7.90–10.00)	5.80 (4.90–7.00)	U=175.0*	<0.001*	0.009*

Pv: Psoriasis vulgaris, **sCTLA-4:** Soluble cytotoxic T-lymphocyte-associated antigen 4, **IQR:** Interquartile range, **U:** Mann-Whitney U test.

controls (Table 5). Notably, serum sCTLA-4 levels correlated positively with PASI scores but not with age (Table 6). Moreover, there was no significant difference in sCTLA-4 levels by sex within the Pv group, with a median (IQR) of 8.60 (8.05–9.80) ng/mL in males and 8.40 (7.75–9.60) ng/mL in females ($U=218.0$, $p=0.504$, $q=0.588$).

The receiver operating characteristic (ROC) analysis revealed excellent discriminatory performance of serum sCTLA-4 levels in differentiating the Pv group from controls, with an area under the curve (AUC) of 0.914. The optimal cutoff value was ≥ 7.35 ng/mL, yielding a sensitivity of 86.67%, specificity of 80%, and an overall accuracy of 83.3% (Figure 3).

Discussion

Due to ethnic and regional variations in SNPs within the *CTLA-4* gene, it was essential to investigate the relevance of *CTLA-4* polymorphisms to Pv susceptibility in the Egyptian population. This study examined the relationship between two *CTLA-4* SNPs (rs231775 and rs3087243) and serum sCTLA-4 levels and Pv susceptibility in Egyptian adults.

Our study revealed a male-to-female ratio of 1.37:1 in the Pv group. This reflects a slight male predominance, consistent with previous studies from Egypt (19), Saudi Arabia (20), and the Maghreb region (21), which reported ratios of 1.2:1, 1.4:1, and 1.16:1, respectively. However, this difference may be coincidental and could be attributed to a higher number of male patients attending the clinic or a higher willingness among male patients to participate in the study.

For rs231775, neither genotype nor allele frequencies differed significantly within the Pv group. Although the G allele showed a higher frequency among patients with Pv, this difference did not reach statistical significance ($p=0.051$), and this borderline value was not maintained after correction for multiple testing (adjusted

Table 6. Association of serum sCTLA-4 levels with age and PASI score within the Pv group.

Variable	sCTLA-4		
	Spearman's ρ	p	q-value
Age	-0.264	0.080	0.280
PASI score	0.611*	<0.001*	0.004*

sCTLA-4: Soluble cytotoxic T-lymphocyte-associated antigen 4, **PASI:** Psoriasis Area and Severity Index, **ρ :** Spearman's rank correlation coefficient. *: indicates significance.

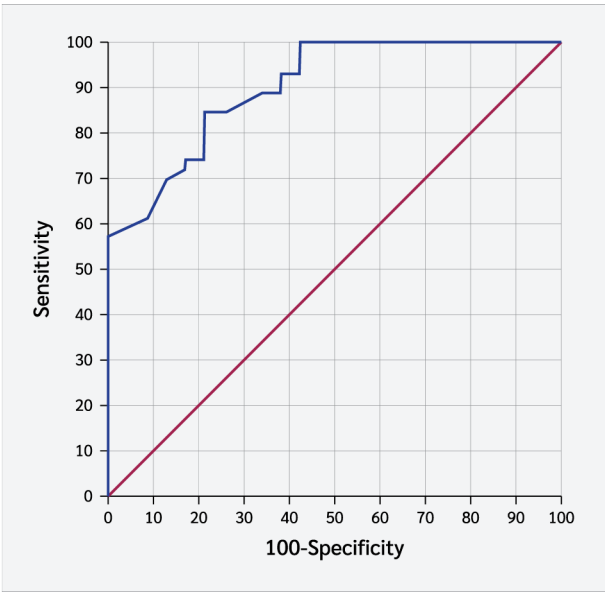


Figure 3. Receiver operating characteristic (ROC) curve of serum sCTLA-4 for differentiating patients with Pv from controls. Area under the curve (AUC)=0.914 (95% confidence interval [CI], 0.859–0.968; $p<0.0001$).

p -value=0.153). Our findings align with studies in Korean, Japanese, and Caucasian populations reporting no association with Pv (22–25).

In disagreement with our findings, Dursun et al. (18) reported a strong association between this SNP and Pv, identifying the G allele as a potential risk factor.

Similar to the findings of Dursun et al. (18) and Łuszczek et al. (25), we found no correlation between rs231775 SNP genotypes and disease severity. Additionally, our results demonstrated no association between this SNP and sCTLA-4 levels in the serum of patients with Pv, aligning with the findings of Berry et al. (17) and Huang et al. (26). In contrast, a study by Gallardo et al. (27) identified a potential association between this SNP and sCTLA-4 expression, with GG genotype carriers exhibiting reduced serum sCTLA-4 levels. Notably, rs231775 is located within an exon, rather than an intron, promoter region, or the 3' untranslated region (3'-UTR), which are more directly involved in transcription and mRNA splicing (28,29). This makes it less likely that the rs231775 SNP directly influences sCTLA-4 levels. One possible explanation for the conflicting findings is that rs231775 may be in strong linkage disequilibrium with other nearby regulatory SNPs that do affect sCTLA-4 expression.

Similarly, no significant difference was observed in the frequency of rs3087243 genotypes between the Pv and control groups, consistent with the findings of Łuszczek et al. (25) but contrasting with Dursun et al. (18), who reported significantly higher frequencies of the GG genotype and G allele in cases. However, consistent with Łuszczek et al. (25) and Dursun et al. (18), we found no relationship between rs3087243 genotypes and disease severity.

Several factors may explain the conflicting genotypic association findings, including the relatively small sample size of our study. Additionally, autoimmune diseases are multifactorial, influenced by interactions between genetic, immunological, and environmental factors, and variation in ethnic background across populations may also affect the impact of specific variants on different diseases (30–32).

The rs3087243 SNP is hypothesized to influence splicing efficiency and the production of sCTLA-4 mRNA isoforms. According to Ueda et al. (33), the G allele was reported to result in decreased sCTLA-4 mRNA expression and consequently decreased serum sCTLA-4 levels. However, we observed no correlation between sCTLA-4 levels and different rs3087243 genotypes, consistent with reports by Berry et al. (17), López et al. (34) and Dahal et al. (35), which similarly found that this SNP was not associated with sCTLA-4 levels in autoimmune diseases.

This discrepancy may be attributed to differences in the methodologies used. In 2003, Ueda et al. (33) measured

sCTLA-4 mRNA levels rather than the circulating serum protein levels. Assays based on ELISA may not differentiate between the sCTLA-4 proteins expressed from sCTLA-4 mRNA and other CTLA-4 immunoreactive forms, including those generated by proteolytic processing of membrane-bound CTLA-4 (mCTLA-4) (36). Additionally, elevated serum sCTLA-4 may reflect reduced protein turnover rather than increased gene expression, and lower mRNA levels could be the result of feedback regulatory mechanisms (17).

Haplotype analysis identified four haplotypes defined by rs231775 and rs3087243. The G/G haplotype was significantly more common in the Pv group, suggesting a potential synergistic effect. This contradicts Łuszczek et al. (25), who found no significant differences in haplotype distribution among the studied population. Comparisons with Dursun et al. (18) are challenging, as their study included an additional variant in their three-SNP analysis.

Assessment of serum sCTLA-4 revealed significantly higher levels in patients with Pv than in controls. Although ROC analysis demonstrated a strong discriminatory performance between the Pv and control groups, psoriasis vulgaris remains a clinical diagnosis, and the current findings suggest only a preliminary role for sCTLA-4 as a supportive biomarker. Furthermore, its potential clinical utility may be limited by the observation that elevated sCTLA-4 levels have also been reported in other autoimmune diseases (37). To our knowledge, this is the first study to investigate sCTLA-4 as a potential biomarker in psoriasis. Furthermore, sCTLA-4 levels correlated positively with disease severity, but showed no association with age or sex among the participants.

In agreement with our findings, Łuszczek et al. (24) also reported a significantly higher mean concentration in serum of patients with Pv, particularly in patients with higher PASI scores. Contrary to our findings, Liu et al. (38) described a lack of correlation between sCTLA-4 levels and psoriasis severity. However, in their study, severity was assessed using categorical groups (mild, moderate, and severe) rather than PASI scores as a continuous variable, as applied in our analysis.

These findings may suggest a role for sCTLA-4 in the immunopathogenesis of Pv. Previous studies have proposed that sCTLA-4 modulates immune responses by acting as a decoy receptor that competes with CD28

for B7 binding, thereby disrupting activation through the B7/CD28 co-stimulatory pathway (39–41). Experimental evidence further suggests that the biological effects of sCTLA-4 are context-dependent and may be dual in nature. While sCTLA-4 may inhibit the activation of resting T cells by limiting CD28-mediated co-stimulation, in activated immune environments, it may also compete with mCTLA-4 for B7 ligands, thereby preventing mCTLA-4 engagement and the delivery of its intracellular inhibitory signals. Consequently, the immunoregulatory functions mediated by mCTLA-4, including suppression of IL-2 production and IL-2 receptor expression, inhibition of the expression of transcription factors, and attenuation of APC activation through reduced IL-12 production, may be diminished, favoring persistent T-cell activation and inflammation (24,42). Moreover, disruption of co-stimulatory pathways by CTLA-4 Ig analogs has been shown to promote Th17 polarization, which is a vital driver of psoriasis pathogenesis (43).

Our study has several limitations, including the small sample size and the single-center design, which may have limited the statistical power to detect weaker associations and the generalizability of the results. Additionally, sequencing-based validation and gene expression analysis were not performed due to financial constraints.

Moreover, complete blinding of laboratory personnel to the clinical status of enrolled participants was not feasible.

Future studies should include larger, multicenter cohorts. We recommend investigating additional SNPs and conducting functional assays to clarify the biological effects of CTLA-4 polymorphisms. Moreover, examining the relationship between sCTLA-4 mRNA expression and serum protein levels may provide insight into potential regulatory mechanisms. Longitudinal studies are also recommended to evaluate changes in sCTLA-4 levels over time and their association with disease activity.

Conclusion

This study provides new insights into the association of CTLA-4 SNPs rs231775 and rs3087243 with psoriasis vulgaris. Although individual SNPs were not linked to disease risk, the G/G haplotype showed a significant association, suggesting a possible synergistic effect. Moreover, elevated sCTLA-4 may contribute to immune dysregulation, promoting disease severity by interfering with mCTLA-4 signaling and facilitating a shift toward Th17-driven inflammation in psoriasis vulgaris.

Ethical Approval: The study protocol was approved by the Ethical Committee of the Faculty of Medicine, Ain Shams University on June 14, 2023 with decision number FMASU MS 334/2023.

Informed Consent: Written informed consent was obtained from all participants before enrollment in the study.

Peer-review: Externally peer-reviewed

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Anti-ADAMTSL5 and Anti-K17 Autoantibodies in Psoriasis: Diagnostic Performance and Associations with Systemic Inflammation

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Abstract

Objective: Psoriasis is a chronic inflammatory skin disease that can mimic several common dermatoses, highlighting the need for objective diagnostic biomarkers. This study aimed to evaluate the diagnostic utility of serum anti-ADAMTSL5 and anti-K17 autoantibodies in psoriasis and to assess their associations with systemic inflammation.

Materials and Methods: In this case-control study, complete blood count (CBC) parameters were assessed in patients with psoriasis and healthy controls using an automated hematology analyzer, and serum anti-ADAMTSL5 and anti-K17 antibody levels were measured by enzyme-linked immunosorbent assay (ELISA). Diagnostic accuracy and associations between variables were assessed using receiver operating characteristic (ROC) curve and univariable linear regression analyses.

Results: The study enrolled 120 participants, including 80 patients with psoriasis and 40 healthy controls. Total leukocyte and absolute neutrophil counts were significantly higher in patients with psoriasis than in healthy controls ($p < 0.001$). Serum levels of anti-ADAMTSL5 (2.18 ± 0.64 ng/mL vs. 0.06 ± 0.04 ng/mL) and anti-K17 (13.74 ± 2.78 ng/L vs. 6.28 ± 0.83 ng/L) were significantly higher in patients with psoriasis ($p = 0.000$). Univariable regression analyses showed that both autoantibodies were significantly associated with the systemic neutrophilic burden. Moreover, both biomarkers demonstrated high diagnostic performance on ROC analysis, with an area under the curve (AUC) of 1.000 for anti-ADAMTSL5 and 0.998 for anti-K17.

Conclusion: Serum anti-ADAMTSL5 and anti-K17 antibodies demonstrated promising diagnostic potential for distinguishing patients with psoriasis from healthy controls in the present study. Given their strong associations with neutrophilic leukocytosis, these findings suggest a potential association between cutaneous autoimmunity and systemic inflammation.

Keywords: Psoriasis, biomarkers, autoantibodies, anti-ADAMTSL5, anti-K17

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Introduction

Psoriasis is a chronic immune-mediated inflammatory skin disease that can be challenging to distinguish from clinically similar dermatoses (1). Early-stage psoriasis may be confused with atopic dermatitis, nummular eczema, and pityriasis rosea. This diagnostic uncertainty can lead to a delay in diagnosis, which is important because timely treatment may help reduce the risk of systemic comorbidities, including psoriatic arthritis and metabolic syndrome. Thus, serological biomarkers are needed to help distinguish psoriasis from its clinical mimics (2).

One major autoantigen implicated in psoriasis is ADAMTSL5, a melanocyte-derived protein recognized by autoreactive CD8⁺ T cells (3). ADAMTSL5 is a large glycoprotein expressed by melanocytes in the epidermis. In genetically susceptible individuals, particularly those carrying HLA-C*06:02, ADAMTSL5-derived peptides can be presented to CD8⁺ T cells, triggering an autoimmune response that contributes to localized inflammation and tissue damage at the epidermal-melanocyte interface (4).

In healthy epidermis, keratin 17 (K17) expression is very low; however, inflammatory cytokines in psoriatic lesions markedly induce its expression in keratinocytes (5). Increased K17 expression and keratinocyte damage may enhance the exposure and presentation of K17-derived peptides to the adaptive immune system. In genetically susceptible individuals, loss of self-tolerance to these peptides can promote the activation of autoreactive T cells and subsequently B-cell activation and production of anti-K17 autoantibodies (6). Moreover, molecular mimicry between K17-derived peptides and streptococcal M proteins may contribute to cross-reactive immune responses, providing a possible link between streptococcal infection and psoriasis exacerbation (7). ADAMTSL5- and K17-associated immune responses have both been implicated in inflammatory pathways relevant to psoriasis, particularly the interleukin-23/interleukin-17 (IL-23/IL-17) axis, which promotes keratinocyte hyperproliferation and sustains chronic inflammation. ADAMTSL5 represents a melanocyte-associated autoantigen capable of activating autoreactive T-cell responses, whereas increased K17 expression in stressed keratinocytes may contribute to continued antigenic stimulation and inflammatory amplification (8).

Based on these previously described mechanisms, we propose that ADAMTSL5- and K17-associated immune responses may represent interconnected components of a feed-forward inflammatory framework rather than a formally established direct interaction between the two autoantigens. This conceptual framework is summarized in Figure 1.

The objective of this study was to assess systemic hematological changes by comparing complete blood count (CBC) profiles, with particular emphasis on total white blood cell (WBC), neutrophil, lymphocyte, and monocyte counts. We also investigated whether circulating anti-ADAMTSL5 and anti-K17 autoantibody levels were associated with systemic inflammatory parameters, particularly the circulating neutrophil count. Therefore, the current study aimed to evaluate the individual diagnostic performance of serum anti-ADAMTSL5 and anti-K17 autoantibodies and to investigate their statistical associations with systemic inflammatory parameters. The concurrent involvement in psoriasis was interpreted within the proposed inflammatory framework and was not evaluated as a combined diagnostic model.

Materials and Methods

Samples were collected from patients with psoriasis under the supervision of a specialist dermatologist at Ibn Sina Teaching Hospital in Mosul, Iraq, between December 1, 2025, and March 1, 2026. Scientific analyses were conducted in the specialized laboratories of the Department of Biology, College of Science, University of Mosul.

Adult participants were included in the final analysis, comprising patients with psoriasis and healthy controls. Psoriasis was clinically diagnosed and confirmed by a consultant dermatologist. Pediatric participants identified during the initial recruitment period were excluded from the final study population and statistical analyses. Eligible participants were adults aged 18–65 years with a clinically confirmed diagnosis of psoriasis. Patients with autoimmune diseases, acute or chronic infections, liver or kidney disorders, malignancies, and hormonal disorders were excluded. Participants receiving immunosuppressive medication, biological therapy, or systemic corticosteroids were also excluded, as were pregnant or breastfeeding women.

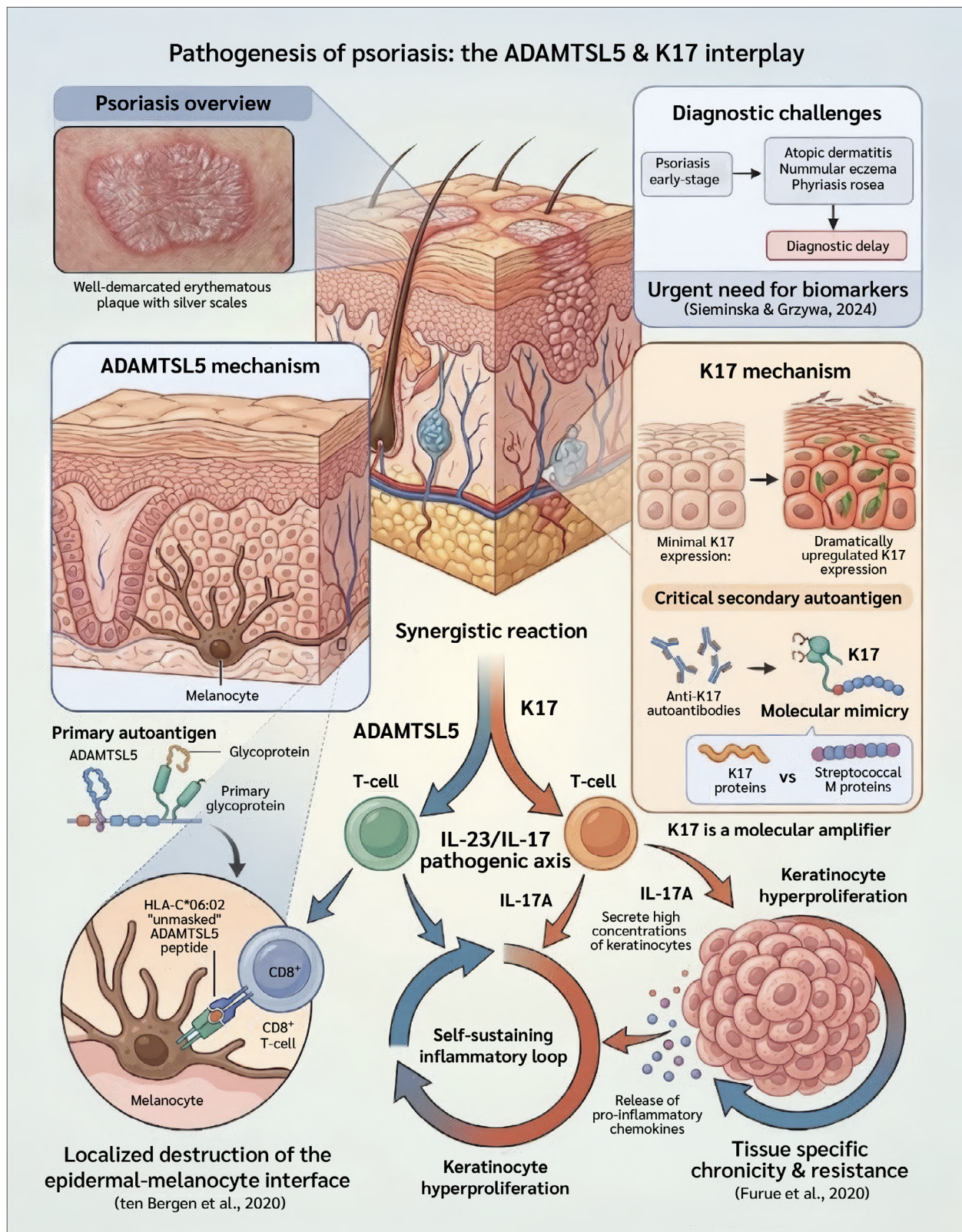


Figure 1. Proposed theoretical model of the ADAMTSL5- and K17-associated inflammatory axis in chronic plaque psoriasis. The diagram was created using BioRender (BioRender, Toronto, ON, Canada), based on previously published literature.

Venous blood (5 mL) was drawn from each participant into two tubes: 2 mL into an ethylenediaminetetraacetic acid (EDTA) tube for complete blood count (CBC) analysis and 3 mL in a gel tube for serum separation by centrifugation at 1500 rpm for 5 min. The separated serum was then collected and stored for subsequent analysis.

Hematological Analysis

Complete blood count (CBC) and five-part white blood cell differential (5-DIFF) analyses were performed using an automated hematology analyzer (Mindray BC-5000, Shenzhen, China). Whole blood samples collected in tubes containing dipotassium EDTA were analyzed (9).

Measurement of Anti-ADAMTSL5 and Anti-K17 Autoantibodies

Serum anti-ADAMTSL5 and anti-K17 autoantibody levels were quantitatively determined using commercial indirect enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions: anti-ADAMTSL5 (SunLong Biotech Co., Ltd., catalog no. SL4833Hu, Hangzhou, China) and anti-K17 (SunLong Biotech Co., Ltd., catalog no. SL0202Hu, Hangzhou, China). The assays were performed using microtiter plates precoated with the corresponding recombinant

human antigens. Standard solutions were prepared by serial dilution to obtain concentrations of 18, 12, 6, 3, and 1.5 ng/mL, and standards were analyzed in duplicate. Serum samples were diluted 1:5 by mixing 10 µL of serum with 40 µL of sample diluent. Then, 50 µL of each prepared standard or diluted serum sample was added to the designated antigen-coated wells, with one well serving as a blank control.

The plates were sealed and incubated at 37°C for 30 min, after which the wells were aspirated and washed five times with diluted wash buffer. Following washing, 50 µL of horseradish peroxidase (HRP)-conjugated detection reagent was added to each well, except the blank, and the plates were incubated at 37°C for an additional 30 min. The washing procedure was then repeated five times to remove unbound conjugate.

For color development, 50 µL of chromogen solution A followed by 50 µL of chromogen solution B was added to each well. The plates were gently mixed and incubated at 37°C for 15 min in the dark. The enzymatic reaction was terminated by adding 50 µL of stop solution, resulting in a color change from blue to yellow. Optical density was measured at 450 nm using a BioTek microplate

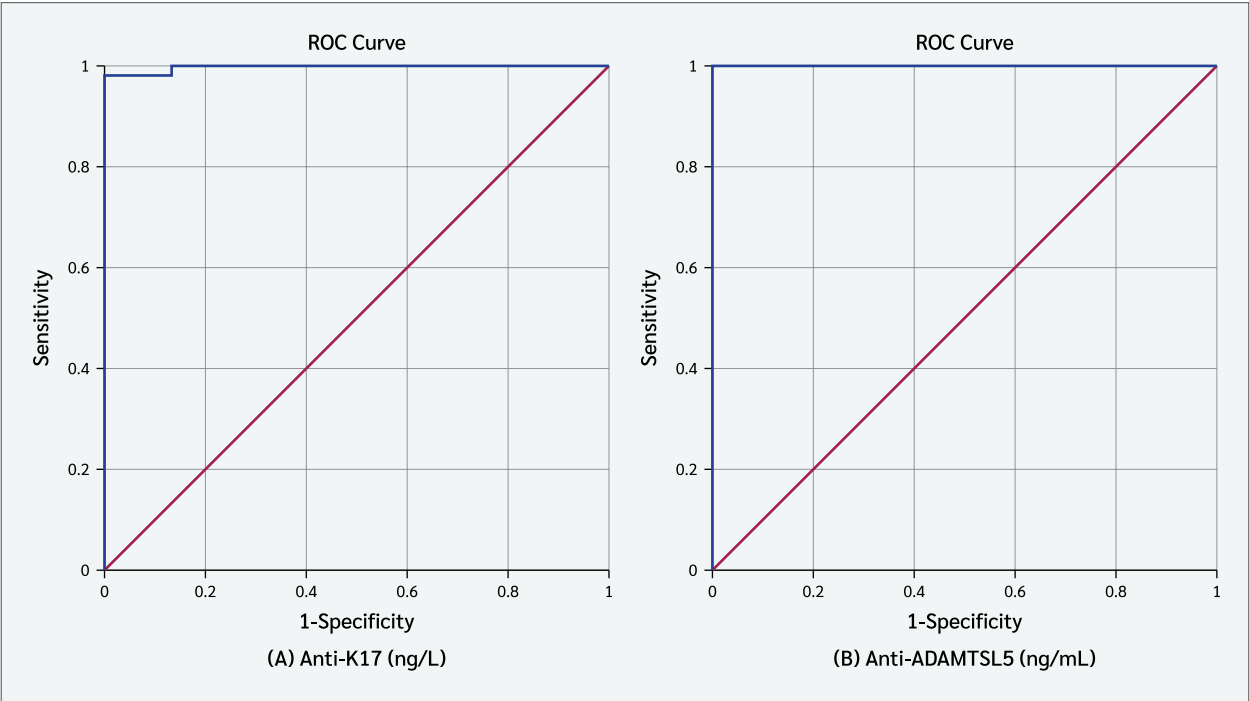


Figure 2. Receiver operating characteristic (ROC) curve analysis for diagnostic biomarkers. (A) ROC curve for anti-K17 (ng/L) showing an area under the curve (AUC) of 0.998. (B) ROC curve for anti-ADAMTSL5 (ng/mL) showing an AUC of 1.000. The diagonal line represents the line of identity (AUC=0.5).

reader within 15 min of adding the stop solution, with the blank well used for baseline correction. Autoantibody concentrations were calculated from the corresponding standard curves generated using the known standard concentrations (10).

Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA). The normality of the data distribution was evaluated using the Shapiro-Wilk test. All continuous variables, including leukocyte and differential counts, met the assumption of normality ($p>0.05$). Therefore, parametric statistical tests were applied, and data are expressed as the mean $\pm$ standard deviation (SD) and range. Independent-samples t tests and one-way analysis of variance (ANOVA) were used for group comparisons after assessing homogeneity of variance. Receiver operating characteristic (ROC) curve analysis, including the area under the curve (AUC) and 95% confidence interval (CI), was used to evaluate the diagnostic performance of anti-ADAMTSL5 and anti-K17 antibodies. Univariable linear regression models were used to evaluate the individual statistical associations of each autoantibody level with neutrophil counts. A two-tailed $p<0.05$ was considered statistically significant (11).

Results

The final analysis included 120 adult participants: 80 patients with psoriasis (35 males and 45 females) and 40 healthy controls (21 males and 19 females).

The hematological profile demonstrated significant systemic variations between patients with psoriasis and healthy controls. Total WBC counts were significantly higher in the psoriasis group than in the control group. Analysis of the five-part differential showed significant increases in absolute neutrophil, lymphocyte, and monocyte counts. Conversely, basophil (BAS) and eosinophil (EOS) counts did not differ significantly between the two groups (Table 1).

Serum anti-ADAMTSL5 concentrations were significantly higher in patients with psoriasis than in healthy controls. As shown in Table 2, patients with psoriasis had markedly higher autoantibody levels (2.18 ± 0.64 ng/mL) than healthy controls (0.06 ± 0.04 ng/mL; $p<0.001$). This finding indicates a significant difference in serum anti-ADAMTSL5 levels between the two groups.

Table 1. Comparison of peripheral white blood cell (WBC) differential parameters between patients with psoriasis and healthy controls (n=120).

Variable	Mean $\pm$ SD (cells/ μ L)	t	p-value
WBC			
Psoriasis patients	8662.41 $\pm$ 2005.24	5.59	<0.001*
Control group	5712.00 $\pm$ 691.30		
Neutrophils			
Psoriasis patients	5252.34 $\pm$ 1798.27	4.31	<0.001*
Control group	3209.53 $\pm$ 657.56		
Lymphocytes			
Psoriasis patients	2649.85 $\pm$ 927.37	3.03	0.003*
Control group	1906.34 $\pm$ 368.51		
Monocytes			
Psoriasis patients	520.61 $\pm$ 271.49	2.66	0.010*
Control group	328.70 $\pm$ 123.75		
Eosinophils			
Psoriasis patients	193.00 $\pm$ 221.23	0.70	0.483
Control group	151.39 $\pm$ 112.36		
Basophils			
Psoriasis patients	184 $\pm$ 40	0.92	0.357
Control group	190 $\pm$ 10		

Psoriasis patients, n=80; Healthy controls, n=40.

WBC: White blood cells, **SD:** Standard deviation.

* Statistically significant at $p\leq 0.05$.

Table 2. Comparison of serum anti-ADAMTSL5 levels between patients with psoriasis and healthy controls.

Variable (ng/mL)	Mean $\pm$ SD	Min.	Max.	p-value
Anti-ADAMTSL5				
Psoriasis patients	2.18 $\pm$ 0.64	0.56	4.23	<0.001*
Control group	0.06 $\pm$ 0.04	0.02	0.16	

Psoriasis patients, n=80; Healthy controls, n=40.

*Statistically significant at $p\leq 0.05$.

Serum anti-K17 concentrations were also significantly higher in patients with psoriasis than in healthy controls. As detailed in Table 3, patients with psoriasis had significantly higher autoantibody levels (13.74 ± 2.78 ng/L) than healthy controls (6.28 ± 0.83 ng/L; $p<0.001$).

This finding indicates a significant difference in serum anti-K17 levels between the two groups.

A univariable linear regression analysis was performed to evaluate the association between serum anti-ADAMTSL5 levels and neutrophil counts. As shown in Table 4, anti-ADAMTSL5 levels were positively associated with neutrophil counts ($B=720.89$, $p=0.042$). The

Table 3. Comparison of serum anti-K17 levels between patients with psoriasis and healthy controls.

Variable (ng/L)	Mean $\pm$ SD	Min.	Max.	p-value
Anti-K17 (ng/L)				
Psoriasis patients	13.74 $\pm$ 2.78	6.95	25.13	<0.001*
Control group	6.28 $\pm$ 0.83	4.97	7.92	

Psoriasis patients, n=80; Healthy controls, n=40.

*Statistically significant at $p \leq 0.05$.

model explained approximately 10% of the observed variation in neutrophil counts ($R^2=0.10$), as seen in Table 4.

A univariable linear regression analysis was performed to evaluate the association between anti-K17 levels and neutrophil counts. As shown in Table 5, anti-K17 levels were positively associated with neutrophil counts ($B=217.94$, $p=0.007$). The model explained approximately 11% of the observed variation in neutrophil counts ($R^2=0.11$), as seen in Table 5.

The diagnostic performance of anti-ADAMTSL5 and anti-K17 was evaluated using ROC curve analysis. As shown in Table 6, both biomarkers demonstrated excellent discrimination between patients with psoriasis and healthy controls. Anti-ADAMTSL5 achieved an AUC of 1.000, whereas anti-K17 showed an AUC of 0.998, indicating excellent diagnostic performance in this study population.

Table 4. Univariable linear regression analysis of the association between serum anti-ADAMTSL5 levels and neutrophil counts.

Independent variable	Dependent variable	Estimate (β)	SE (β)	Std. (β)	R^2	t	p-value
Constant	NEU (cells/ μ L)	3674.59	790.54	---	0.10	4.64	<0.001
Anti-ADAMTSL5 (ng/mL)		720.89	346.62	0.25		2.08	0.042

* $p \leq 0.05$.

NEU: Neutrophils, **SE:** Standard error, **Std.:** Standardized, **β :** Regression coefficient, **R^2 :** Coefficient of determination, **t:** t statistic.

Table 5. Univariable linear regression analysis of the association between serum anti-K17 levels and neutrophil counts.

Independent variable	Dependent variable	Estimate (β)	SE (β)	Std. (β)	R^2	t	p-value
Constant	NEU (cells/ μ L)	2257.53	1101.56	—	0.11	2.04	0.045
Anti-K17 (ng/L)		217.94	78.59	0.33		2.77	0.007

* $p \leq 0.05$.

NEU: Neutrophils, **SE:** Standard error, **Std.:** Standardized, **β :** Regression coefficient, **R^2 :** Coefficient of determination, **t:** t statistic.

Table 6. ROC curve analysis and optimal cutoff values for diagnostic biomarkers.

Variable	AUC	p-value	Sensitivity (%)	Specificity (%)	Asymptotic 95% CI	Cutoff value
Anti-K17 (ng/L)	0.998	0.001	98.8	100.0	0.992–1.000	8.79
Anti-ADAMTSL5 (ng/mL)	1.000	0.001	100.0	100.0	1.000–1.000	0.60

AUC: Area under the curve, **CI:** Confidence interval, **ROC:** Receiver operating characteristic.

Note: AUC values were interpreted as follows: 0.90–1.00, excellent; 0.80–0.89, good; 0.70–0.79, fair; 0.60–0.69, poor; and 0.50–0.59, fail.

Discussion

In our study, we demonstrated significantly elevated total WBC counts in patients with psoriasis compared with healthy controls, with the increase primarily attributable to higher neutrophil counts. This finding is consistent with the chronic inflammatory nature of psoriasis and the established involvement of neutrophils in Munro microabscess formation and amplification of T helper 17 (Th17)/T helper 22 (Th22)-mediated responses (12). Lymphocyte and monocyte counts were also significantly higher in patients with psoriasis, supporting the involvement of both adaptive and innate immune responses. In contrast, eosinophil and basophil counts were not significantly different between the groups, consistent with the predominance of Th17-mediated rather than Th2-mediated inflammation in psoriasis (13).

We found that serum anti-ADAMTSL5 levels were markedly elevated in patients with psoriasis compared with healthy controls. ADAMTSL5 is a melanocyte-derived autoantigen presented in the context of HLA-C*06:02 and recognized by autoreactive CD8⁺ T cells, providing a link between melanocyte-specific autoimmunity and psoriatic inflammation (14). The increased anti-ADAMTSL5 levels observed in our study are consistent with its proposed role as a psoriasis-associated autoantigen and may reflect persistent immune activation within the psoriatic microenvironment (15,16). The relationship between this autoimmune response and the IL-23/IL-17 inflammatory axis may also contribute to leukocyte accumulation and keratinocyte hyperproliferation (17).

Similarly, serum anti-K17 concentrations were significantly higher in patients with psoriasis than in healthy controls. K17 is an inducible intermediate filament protein that functions as a stress-associated signal and is upregulated following epidermal injury and exposure to inflammatory cytokines, including IL-17 and interferon-gamma (IFN- γ) (18). Autoimmune recognition of K17 may therefore reflect loss of immune tolerance within the chronic inflammatory environment. Previous studies have linked K17 to T-cell activation, neutrophil recruitment, and maintenance of the inflammatory response (19,20). These findings further support the potential utility of anti-K17 as a serological marker of psoriasis-associated immune dysregulation.

Univariable linear regression analysis demonstrated a statistically significant positive association between

serum anti-ADAMTSL5 levels and neutrophil counts. However, this association should not be interpreted as evidence of a direct causal effect of anti-ADAMTSL5 on neutrophil expansion. Rather, the parallel elevation of anti-ADAMTSL5 and neutrophils may reflect concurrent activation of autoimmune and innate inflammatory pathways within the systemic inflammatory environment of psoriasis. ADAMTSL5-specific autoimmunity and neutrophilic inflammation may therefore represent interconnected manifestations of the underlying inflammatory process, with neutrophil recruitment additionally mediated by chemokines such as C-X-C motif chemokine ligand 1 (CXCL1) and C-X-C motif chemokine ligand 8 (CXCL8) (21).

In a separate univariable linear regression model, serum anti-K17 levels were also positively associated with neutrophil counts. K17 is upregulated in psoriatic epidermis and participates in inflammatory pathways involving IL-17/IL-23 signaling and neutrophil-recruiting chemokines such as C-C motif chemokine ligand 2 (CCL2), CXCL1, and CXCL8 (22). Nevertheless, the relatively low R^2 indicates that anti-K17 accounts for only a limited proportion of the variation in neutrophil counts, emphasizing the multifactorial nature of neutrophilic inflammation in psoriasis (23,24).

The diagnostic performance of both biomarkers was evaluated using ROC analysis and was high in this study population. Anti-ADAMTSL5 showed an AUC of 1.000, while anti-K17 showed an AUC of 0.998, indicating excellent discrimination between patients with psoriasis and healthy controls (25,26). The corresponding cutoff values further support their potential utility as serological biomarkers. However, these findings should be interpreted within the context of the present study population and require validation in larger independent cohorts including patients with other inflammatory and clinically similar dermatological conditions before their broader diagnostic application can be established (27,28).

Conclusion

Our study demonstrated significantly elevated serum anti-ADAMTSL5 and anti-K17 levels in patients with psoriasis compared with healthy controls. The diagnostic performance of both biomarkers was high based on ROC analysis. These findings support the potential of

anti-ADAMTSL5 and anti-K17 as adjunctive serological biomarkers for psoriasis, particularly in clinical settings where psoriasis may overlap morphologically with inflammatory dermatoses such as atopic dermatitis, nummular eczema, and pityriasis rosea. However, given the relatively limited sample size and case-control design, validation in larger independent cohorts that directly include these clinical mimickers is required before their differential diagnostic utility can be established.

The significant association between autoantibody levels and systemic hematological manifestations, particularly

neutrophilic leukocytosis, suggests that these biomarkers may reflect not only local but also systemic autoimmune inflammation.

Overall, anti-ADAMTSL5 and anti-K17 showed strong individual diagnostic performance and were positively associated with systemic inflammatory features. Their potential complementary diagnostic value warrants further evaluation using combined multivariable models and independent validation cohorts.

Ethical Approval: This study was approved by the Scientific and Ethical Committee of the Department of Life Sciences, College of Sciences, University of Mosul on November 9, 2025 (Approval no. 50619). Ethical approval for the collection of patient information was also obtained under the supervision of the Ministry of Health in accordance with the Declaration of Helsinki.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

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