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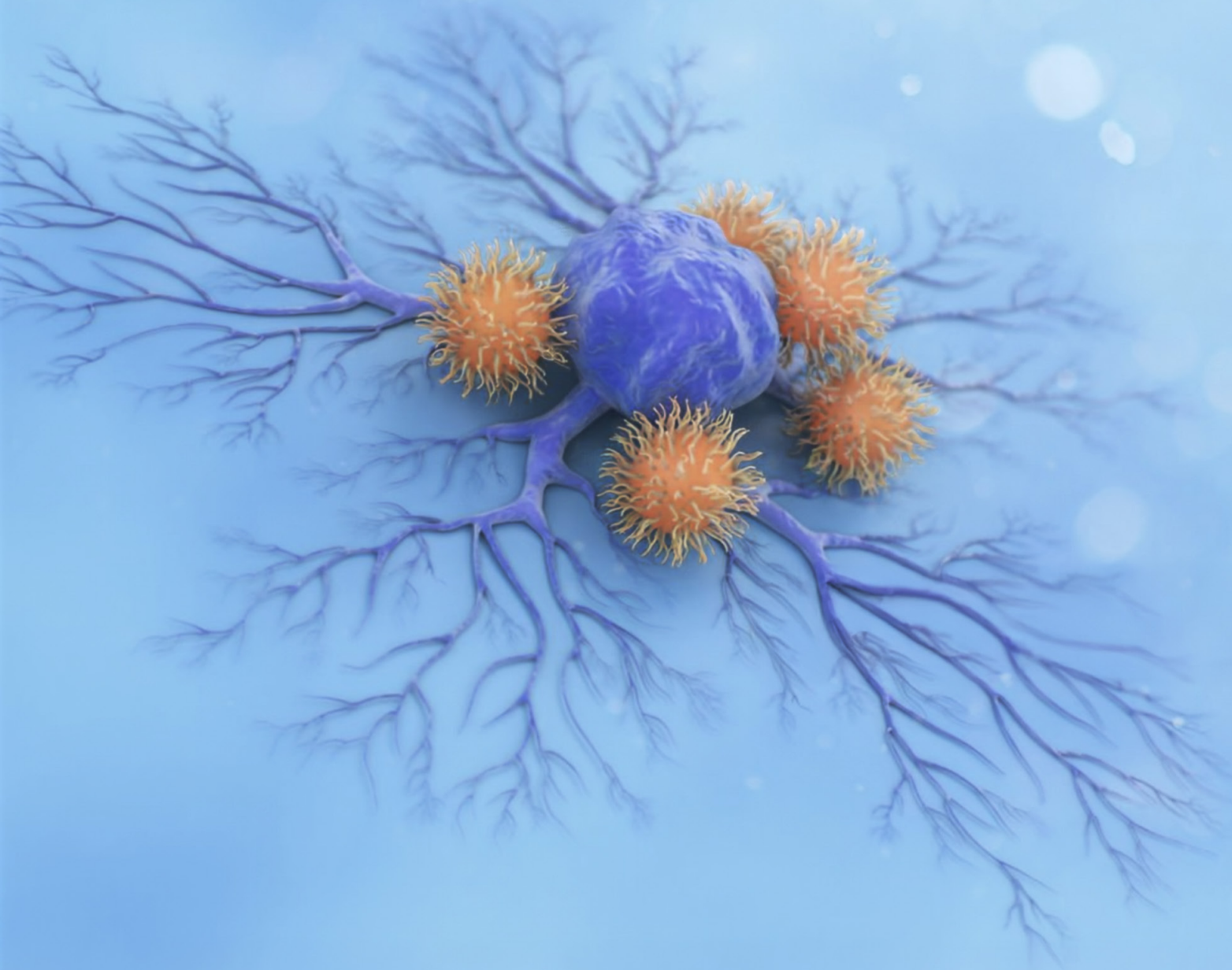

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CONTENT

Interdisciplinary Perspectives in Immunology: Highlights from the August Issue

Dear Colleagues,

On behalf of the editorial board, I am pleased to share with you the August issue of the *Turkish Journal of Immunology*.

This issue includes a comprehensive review article titled *"Non-infectious gastrointestinal tract complications in patients with common variable immunodeficiency disorders: clinical perspectives, immunologic insights and therapeutic approaches."* This work provides valuable clinical perspectives, immunological explanations, and therapeutic considerations on one of the most complex complications encountered in primary immunodeficiency.

In addition to this review, the issue features several original research articles reflecting the breadth of immunological science across oncology, autoimmunity, infectious disease, vaccine research, and translational medicine. A study from Egypt investigates **cluster of differentiation 73 (CD73) expression on T cells in de-novo acute myeloid leukemia**, offering new insights into immune checkpoint pathways and their association with short-term survival outcomes. From Türkiye, a retrospective analysis explores **the diagnostic and prognostic significance of immunoblot results in patients with systemic autoimmune rheumatic diseases**, underlining the value of **antinuclear antibody (ANA) and extractable nuclear antigen (ENA)** testing in clinical practice.

A clinical trial from Cuba reports on the **efficacy of the thymus polypeptide fraction Biomodulina T in children with thymic hypoplasia and recurrent infections**, demonstrating remarkable improvements in thymic size, reduction in infection frequency, and enhancement of immune parameters. Furthermore, an experimental study from Türkiye evaluates the **immunogenic potential of Foot-and-Mouth Disease Virus (FMDV) Antigen O-146S adjuvanted with different emulsion formulations in mice**, providing important data on adjuvant strategies for vaccine development.

Together, these contributions illustrate the dynamic and interdisciplinary nature of immunology, addressing both fundamental mechanisms and pressing clinical challenges. We extend our sincere gratitude to the authors and reviewers for their dedication, and we hope this issue will be both inspiring and informative for our readers.

With warm regards,
Prof. Günnur Deniz
On behalf of the Editorial Board
Turkish Journal of Immunology

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REVIEW ARTICLE

Turkish Journal of Immunology, 13(2):69-79

Non-Infectious Gastrointestinal Tract Complications in Patients with Common Variable Immunodeficiency Disorders: Clinical Perspectives, Immunologic Insights and Therapeutic Approaches

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Abstract

Inborn errors of immunity (IEI) are a heterogeneous group of disorders characterized by immunodeficiency and immune dysregulation. Common variable immunodeficiency (CVID) is the most frequently encountered symptomatic IEI. Patients with IEI are at risk for both infectious and non-infectious complications. Recurrent sinopulmonary infections, especially those caused by encapsulated bacteria, are often the first clinical sign. However, many patients also develop non-infectious complications, including autoimmunity, splenomegaly, chronic lung disease, enteropathy, granuloma formation, and malignancies, which significantly contribute to morbidity and mortality. Immunoglobulin replacement therapy is the primary treatment for CVID, effectively reducing infections and infection-related mortality; however, it has limited efficacy on non-infectious complications. Among these, gastrointestinal (GI) complications are particularly common and significantly impair quality of life. Gastrointestinal involvement in CVID is reported in 9% to 34% of cases. Non-infectious GI complications may involve any part of the GI tract, are highly variable in severity, and are associated with increased mortality. Common symptoms include diarrhea, bloating, and abdominal pain. Gastrointestinal involvement can resemble other diseases such as inflammatory bowel disease, celiac disease, or collagenous colitis. The pathogenesis of CVID enteropathy is complex, involving immune dysregulation, microbiota alterations, genetics, malignancy, and infections. Th1-driven inflammation plays a central role, contributing to chronic gastrointestinal symptoms. While no standardized definition exists, GI disease in CVID is generally identified by excluding infectious causes and integrating clinical and histopathological findings. Reduced or absent plasma cells in the lamina propria, nodular lymphoid hyperplasia, and intraepi-

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thelial lymphocytosis are frequent histologic findings. Currently, no established treatment guidelines exist for non-infectious complications. Expert consensus favors a personalized, stepwise, multidisciplinary approach, with steroids as first-line therapy and biologic agents considered in steroid-dependent cases. In this review, we aimed to examine the various aspects of GI tract manifestations in adult patients with CVID, with a focus on non-infectious complications.

Keywords: Common variable immune deficiency, CVID, enteropathy in CVID, Gastrointestinal tract inflammation in CVID

Introduction

Inborn errors of immunity (IEI), formerly known as primary immunodeficiency (PID), represent a diverse group of disorders, marked by varying levels of immunodeficiency and immune dysregulation (1). Although the prevalence varies by specific disease, the overall estimated prevalence is approximately 1/1200, likely lower than the true rate due to underdiagnosis (2,3). Among IEIs, predominantly antibody deficiencies (PADs), especially selective IgA deficiency and common variable immunodeficiency disease (CVID), are the most common (2-5).

Inborn errors of immunity are associated with increased risk of a wide range of comorbidities and complications, broadly classified as either infectious or non-infectious (6). Depending on the underlying disease and the affected component of the immune system, any type of infectious complication, including bacterial, viral, fungal, or opportunistic infections, may occur; these infections tend to recur and can be severe (7). Noninfectious complications comprise autoimmunity, splenomegaly, chronic lung diseases such as asthma or bronchiectasis, enteropathy, granulocytic and lymphocytic organ infiltrations, granulomas, and malignancy (8). These complications result in long-term sequelae, including structural damage due to infections such as bronchiectasis and progressive organ dysfunctions driven by immune dysregulation (9,10). These complications and their consequences are closely related to both mortality and a significant burden of the disease on patients, their families, as well as health care systems (2,4).

Mortality rates in IEIs vary based on the specific underlying diseases and the associated complications (2). The age-adjusted mortality rate was estimated to be 0.43/1,000,000 people in an epidemiological study from the United States, identifying antibody deficiency as the most common cause of death. The leading contributing factors were infections (34.6%), followed by respiratory and cardiovascular complications (17.4% and 11.4%, respectively) (11). Another epidemiological study from Europe indicated that annual and premature mortality rates in patients with CVID were 1.7–3 times higher than in the general population. Chronic respiratory disease and malignancy were the leading causes of death in this cohort (5). Notably, respiratory complications followed by gastrointestinal complications are the most common causes of morbidity in CVID patients (12).

Given this impact, it is crucial to thoroughly understand the complications from all perspectives, including clini-

Highlights

- Common variable immunodeficiency (CVID) is an inborn error of immunity with both infectious and non-infectious complications that significantly impact morbidity and mortality.
- Gastrointestinal involvement is common in CVID and presents with a wide spectrum of non-infectious pathologies, often resembling inflammatory bowel disease but with unique histopathological features.
- Common variable immunodeficiency enteropathy is marked by T helper 1 (Th1)-driven inflammation, impaired T cell responsiveness, and decreased natural killer (NK) cell levels, which together may contribute to chronic gastrointestinal symptoms.
- There is currently no consensus on the definition or management of CVID enteropathy, and no established treatment guidelines exist.
- Personalized, multidisciplinary, and stepwise treatment strategies based on symptom severity, histopathology, comorbidities, and patient preferences are recommended.

cal features, underlying mechanisms, and treatment. In this review, we examine the various aspects of gastrointestinal (GI) tract manifestations in adult patients with CVID, with a focus on non-infectious complications.

Common Variable Immunodeficiency

Common variable immunodeficiency is the most commonly occurring symptomatic IEI, with a prevalence ranging from 1/10,000 to 1/50,000 (13,14). It is a complex disorder characterized by heterogeneous clinical features and impaired antibody production due to a B-cell defect. Although the underlying pathomechanism is not well understood, in addition to the monogenic defects identified in approximately 30% of patients, T cell abnormalities related to non-infectious complications may accompany B cell dysfunction (15). In the latest update to the classification of IEIs, PADs were categorized into four main groups: 1) Severe reduction in all immunoglobulin (Ig) isotypes with profoundly decreased or absent B cells; 2) Severe reduction in at least two Ig isotypes with normal or low B cells; 3) Severe reduction in IgG and IgA with normal/elevated IgM and normal counts of B cells; 4) Isotype, light chain, or functional deficiencies with generally normal counts of B cells (16). Common variable immunodeficiency is further categorized into two main groups: 1) Common variable immunodeficiency with no gene defect specified and 2) Specific disorders caused by monogenic defects such as activated phosphoinositide-3 kinase delta syndrome (APDS), PTEN, CD19, CD81, CD20, CD21, TACI, BAFFR, TWEAK, TRNT1, ICAROS or NFKB1 and NFKB2 deficiencies (16).

Clinical Features

The most common initial clinical manifestation of CVID is recurrent sinopulmonary infections mainly caused by encapsulated bacteria. However, a substantial proportion of patients also suffer from diverse immune-dysregulation-related diseases, such as autoimmune or inflammatory disorders, allergies, granulomatous or lymphocytic inflammation of various organs, benign lymphoproliferation, and malignancy, with multiple organ systems potentially affected (1,2). On the other hand, in some patients, various non-infectious manifestations such as enteropathy, granulomatous diseases, malignancy, or autoimmune diseases may precede infectious symptoms as the initial clinical presentation (9).

Diagnosis

Common variable immunodeficiency encompasses a heterogeneous group of predominantly antibody deficiency syndromes and is generally considered a diagnosis of exclusion. Unfortunately, no clinical features or laboratory tests are pathognomonic for CVID. Therefore, clinical diagnosis and diagnostic criteria become essential for identifying cases. Several expert groups proposed distinct diagnostic criteria for CVID (8,17,18). In the late of 90s, CVID was defined by experts as the presence of hypogammaglobulinemia (a marked decrease in IgG, at least two standard deviations below the normal range for age), a marked decrease in IgM or IgA, impaired vaccine response or absence of isohemagglutinins, and the exclusion of defined causes of hypogammaglobulinemia in patients whose immunodeficiency began after 2 years of age (17). However, some authors did not agree with the strict necessity of a poor vaccine response for the diagnosis. In 2019, the European Society for Immunodeficiencies (ESID) reported clinical diagnostic criteria for a probable diagnosis of CVID (=clinical diagnosis) (Table 1). In this expert consensus, poor vaccine response is included among the supportive laboratory findings (18).

Previously, genetic analysis was recommended for patients with complications such as autoimmunity or malignancy to investigate underlying genetic defects in CVID, and was not usually performed in patients with only infections (8). With advances in genetic testing and a deeper understanding of underlying mechanisms, it has been demonstrated that some monogenic defects may respond to specific treatments, including enzyme replacement, targeted monoclonal antibodies, or stem cell transplantation (18). Therefore, genetic testing should be performed in all patients.

Diagnostic delay is a significant challenge in the management of CVID patients. A multicenter study involving 2212 patients from various parts of Europe reported a delay in diagnosis of five to seven years (6). From Türkiye, a case series of 44 pediatric patients reported a mean diagnostic delay of 4.6 years (19). On the other hand, another study from Türkiye reported an average delay in diagnosis of 14.9 years, highlighting the significance of this issue in adults (20). Diagnostic delay is crucial, as it may lead to a delay in specific treatment and the development of complications.

Treatment

In CVID, the primary cornerstone of the treatment is Ig

replacement therapy (IgRT), which is highly effective in preventing infectious diseases and, consequently, reducing infection-related mortality. However, IgRT has a limited impact on the non-infectious complications (21). Furthermore, the evidence of the effectiveness of immunosuppressive or immunomodulatory medications in treating inflammatory and autoimmune complications is inadequate. A better understanding of the underlying molecular mechanisms of the complications can pave the way for specific, safe, and effective treatment modalities. Therefore, comprehensive studies about complications are essential.

Gastrointestinal Tract Involvement in CVID

Gastrointestinal tract complications leading to malabsorption are a significant clinical feature in CVID. The gastrointestinal tract is the largest organ-system tract, playing a crucial role in the body's immune system. Therefore, GI involvement is not unusual. The frequency of GI tract complications ranges between 9% to 34% (6,22-24). Gastrointestinal tract involvement can be categorized into two main groups: infectious and non-infectious complications. Since the aim of our review is to highlight and describe non-infectious complications, infectious complications will not be discussed in this context.

Non-infectious Complications

In CVID, non-infectious pathologies are encountered across a wide spectrum of varying severity, affecting any part of the GI tract (25). Gastrointestinal tract complications and malabsorption are related to more than two-fold increased mortality in CVID patients (22).

Chronic or intermittent diarrhea is the most common GI tract-associated symptom (25). Bloating and pain are also frequently encountered (26). The clinical manifestations may mimic inflammatory bowel diseases (IBD), including both ulcerative colitis and Crohn's disease, Whipple's disease, collagenous colitis, and celiac disease (27-31). IBD-like manifestations, commonly after CVID diagnosis, can be seen in up to 10% of patients (30, 32). However, some patients presented with GI manifestations can be initially misdiagnosed with IBD since it can be challenging to differentiate the inflammatory features of IBD and CVID (27,31,33). Furthermore, duodenal histopathology can resemble celiac disease in terms of increased intraepithelial lymphocytosis with or without villous atrophy. But gluten withdrawal does not work in CVID (25). Inflammation in CVID enteropathy is thought to be multifactorial, and various factors, including immunodeficiency, immune dysregulation, autoimmunity, GI microbiota, genetics, cancer, and infections, may contribute to this inflammation (34). Interestingly, some patients may exhibit gastrointestinal inflammation in the absence of symptoms, whereas others may present with symptoms despite lacking histological evidence of inflammation (26,27).

The definition of GI tract disease in CVID, also known as CVID enteropathy, has not yet reached a consensus. Some authors combine clinical manifestations with histopathological findings, while others also include GI infections (29,35-41). The features range from mild to moderate, characterized by chronic diarrhea or increased intraepithelial lymphocytosis, and from no malnutrition or weight loss to severe cases with weight loss, malnu-

Table 1. Clinical diagnostic criteria for common variable immunodeficiency (CVID)*.

Criteria
At least one of the following: - Enhanced vulnerability to infections - Autoimmune diseases - Granulomatous manifestations - Undetermined polyclonal lymphoproliferation - Familial history of antibody deficiency
Marked decrease in level of IgG and IgA and/or IgM (≥2 SD below the age-adjusted mean, confirmed in ≥2 measurements)
At least one of the following: - **Impaired vaccine response and/or absence of isohemagglutinin** - Decreased switched memory B cells (<70% of age-adjusted normal value)
Exclusion of other causes of hypogammaglobinemia (e.g., medications, malignancies, protein loss or infections)
Older than 4 years at the time of diagnosis (symptoms may begin before)
Absence of significant T cell deficiency which is defined by ≥2 of the following: • $CD4^+$ T-cell count: - Age 2–6 yrs: <300/μL - Age 6–12 yrs: <250/μL - Age >12 yrs: <200/μL • % naïve $CD4^+$ T cells: - Age 2–6 yrs: <25% - Age 6–16 yrs: <20% - Age >16 yrs: <10% • Absent T-cell proliferation

ANA: Common variable immunodeficiency, **Ig:** Immunoglobulin, **SD:** Standard deviation, **$CD4^+$:** Cluster of differentiation 4 positive T cell.
*Adopted from Seidel et al. (18)

trition, extensive IgG loss, and increased intraepithelial lymphocytosis or histopathology, such as GVHD (25). Since the management is different, excluding infectious diseases and including both clinical and histopathological features in the definition seems logical.

The discrepancy in the definition of CVID enteropathy can lead to problems in both research studies and treatment. Therefore, precise and detailed characterization of disease phenotypes in clinical reports may lead to a better understanding and help in standardization in future studies.

In CVID patients with GI involvement, the inflammation is very heterogeneous and can be observed in any part of the GI tract, leading to various diseases (Table 2). In the majority of patients with CVID, plasma cells are decreased or absent in the lamina propria regardless of the location of the inflammation in the bowel (33,41). Another common manifestation is nodular lymphoid hyperplasia (NLH), which is defined as the formation of nodules at least 1 mm in diameter with a germinal center. NLH can be frequently detected in both the small and large intestine of CVID patients, whereas it is rarely observed in immunocompetent adults and usually occurs secondary to infections (29). The etiopathology of NLH in CVID is not clearly known. Although it is usually associated with the presence of abdominal symptoms, it is sometimes detected in asymptomatic patients coincidentally (33, 41, 42). Therefore, rather than representing a primary pathologic manifestation, it may be related to immune dysregulation.

Table 2. Non-infectious gastrointestinal tract diseases in common variable immunodeficiency (CVID).

Part of GI tract	Disease	Features
Esophagus	Reflux esophagitis	-
	IBD-like esophagitis	Ulcerations
	Non-specific esophagitis	Intraepithelial lymphocytosis with or without apoptosis
	Barrett esophagus	Without dysplasia (a patient)
Stomach	Chronic gastritis	Can be erythematous, atrophic, follicular or ulcerative
	Gastric cancer	Associated with atrophic gastritis and intestinal metaplasia
Small intestine	Celiac-like disease	Increased IEL with or without villous atrophy
	Non-specific duodenitis/ileitis	• Neutrophilic inflammation • Nodular lymphoid hyperplasia
	Granulomas	Noncaseating
	Autoimmune enteropathy	• Moderate-to-severe villous atrophy • Dense lymphoplasmacytic infiltration • Neutrophil cryptitis with or without crypt abscess
Large intestine	IBD-like colitis	• Intraepithelial or sub-epithelial lymphocytosis • Prominent apoptosis • Crypt distortion • Granulomas
	Lymphocytic/collagenous-like colitis	Increased IELs without germinal center formation
	GVHD-like colitis	Nonspecific inflammation, apoptosis
	Microscopic colitis	Macroscopically normal colon but microscopically inflamed
	Non-specific colitis	Nodular lymphoid hyperplasia
	Granulomas	Noncaseating
All part	-	Rare or absent plasma cells in the lamina propria

ANA: Common variable immunodeficiency, **GI:** Gastrointestinal, **IBD:** Inflammatory bowel disease, **IEL:** Intraepithelial lymphocytes, **GVHD:** Graft-versus-host disease.

Esophageal involvement is rare in patients with CVID, and its exact prevalence remains unclear. The most frequently reported manifestation is Candida esophagitis. Non-infectious esophageal complications include reflux esophagitis, intraepithelial lymphocytosis, esophageal ulcerations, and Barrett's esophagus (33,43).

The frequency of chronic gastritis ranges from 3% to 27% in various cohorts (25,26,35,41). Helicobacter pylori, EBV, or CMV infections were infrequently associated with chronic gastritis in CVID patients. This remarkable information suggests that the impact of continuous gastric mucosal inflammation is most likely due to immune dysregulation rather than infections in CVID (25). Gastric cancer, closely related to atrophic gastritis and intestinal metaplasia, represents another pathological condition of the stomach and has a significant impact on mortality (9,44).

The most common histopathological finding in the duodenum is increased intraepithelial lymphocytosis with or without villous atrophy, which is often less prominent than in celiac disease (33). The major distinguishing feature is the absence of plasma cell infiltration in CVID (33). Furthermore, neutrophil infiltration and follicular lymphoid hyperplasia can be observed in CVID, whereas they are rare in celiac disease (33,41,45). Patients with CVID are also insensitive to a gluten-free diet (41). On the other hand, to explore the relation between celiac disease and celiac-like findings in CVID, some authors studied the celiac-associated HLA profiles, including HLA-DQ2 and HLA-DQ8, in CVID patients. In a study, these HLA profiles were detected in 20% of the patients, whereas in another study, no association was found, suggesting that celiac-like and true celiac disease are distinct entities (26,46).

In patients with CVID, colonic histopathological findings are diverse and can mimic several gastrointestinal disorders (40). These include patterns resembling inflammatory bowel disease (IBD), as well as lymphocytic or collagenous colitis, and GVHD-like colitis (40). Notably, inflammation may not be apparent macroscopically and can only be detected through microscopic examination, sometimes presenting as non-specific patterns. Classic IBD can be distinguished from IBD-like disease in CVID by the presence of plasma cells in the lamina propria (40).

Autoimmune neutropenia (AIN) is a rare but recognized hematologic complication in CVID and is frequently accompanied by other cytopenia such as immune thrombocytopenia or autoimmune hemolytic anemia (47). Patients with AIN appear to carry an elevated risk of enteric inflammation, including chronic enteropathy, crypt distortion, villous blunting, lymphoid aggregates, and opportunistic gut infections such as cytomegalovirus colitis, especially in the context of neutropenia-related mucosal vulnerability (47). According to the United States Immunodeficiency Network (USIDNET) registry study, patients with AIN exhibited a 2.1 fold and 3.4 fold increased risk of developing enteropathy and autoimmune enteropathy, respectively. In this study, the term "enteropathy" encompassed a broad spectrum of gastrointestinal inflammatory conditions, including gastroenteropathy, enterocolitis, lymphocytic colitis, chronic and atrophic gastritis, gastroenteritis, enteritis, and duodenitis, closely resembling the histopathological features of broader CVID-associated enteropathy and IBD-like lesions (48).

Although some cases of eosinophilic inflammation in the gut of patients with CVID have been reported, the role of eosinophilic infiltration as part of the inflammatory process in GI involvement in CVID remains poorly defined (29,41,45).

Immunologic Features

Although the underlying immunopathogenesis in CVID enteropathy is not clearly defined, it is believed that a combination of defective humoral immunity, impaired regulatory T cell function, altered microbiota, and abnormal cytokine responses plays a significant role. Studies have shown that CVID patients with symptomatic GI tract inflammation have distinctive immunologic abnormalities (34,38). In symptomatic patients with CVID and GI inflammation, the level of natural killer (NK) cells in peripheral blood was lower than that of CVID patients without GI-related symptoms. Additionally, it has been demonstrated that a skewing towards a pro-inflammatory Th1 response, accompanied by elevated levels of cytokines such as interferon-γ and IL-12, contributes to chronic mucosal inflammation (34). Th1 cytokine overproduction is closely linked to GI symptoms, particularly with malabsorption (34). This Th1 response was distinct from the Th1 profile seen in Crohn's disease, where IL-23 and IL-17 responses were also involved (34). Also, while T cell hyperresponsiveness to intestinal flora antigens in the gastrointestinal tract is a hallmark of Crohn’s dis-

ease, T cell hyporesponsiveness is characteristic of CVID (34). These findings support the notion that they are different entities.

A successful personalized, targeted therapeutic approach should be guided by the underlying immunopathogenesis. Although a prominent Th1 response is observed in both diseases, the cytokine profiles differ. Anti-IL-23 therapies (targeting the p19 subunit) may not be effective in inflammatory settings where IL-12 plays a dominant role (34). Accordingly, further in-depth and comprehensive studies are warranted to fully clarify these mechanisms.

Treatment of Non-infectious Complications

Currently, no established guidelines exist for managing the non-infectious complications of CVID. Furthermore, patients were often unresponsive to standard therapeutic agents used in inflammatory conditions of the bowel. Usually, IgRT is insufficient for either preventing or treating non-infectious GI manifestations (40). According to a meta-analysis, 70% of patients required an additional agent for the treatment of GI symptoms, and only a few cases were responsive to an increased dose of Ig without additional medications (49). Steroids, immunosuppressive agents, and biological agents have been used, and inconsistent results regarding the effectiveness of these treatments have been published to date (50,51).

Personalized treatment with a multidisciplinary and stepwise approach, based on the severity of symptoms, histopathologic findings, comorbidities, and patient preferences, is proposed in an expert opinion approach (Figure 1). Moderate to severe disease is defined as the presence of severe diarrhea leading to hypoalbuminemia, weight loss, extensive and severe ulcers in endoscopic evaluation, and increased biomarkers of disease activity such as CRP and fecal calprotectin (50). Accordingly, the absence of these clinical features in symptomatic patients can be considered indicative of mild disease (50). Authors of this manuscript recommend oral prednisone for 4–8 weeks as first-line therapy for moderately or severely affected patients. If the disease becomes steroid-dependent or multiple relapses occur, vedolizumab or infliximab is recommended (50). If there is no response to these agents, alternative biologic or immunomodulatory therapies are suggested as second- and third-line options, respectively. In mildly affected patients, 5-aminosalicylic acid (for 6–8 weeks) is recommended as the first-line therapy (50). If patients are unresponsive, treatment should be stepped up to oral budesonide (for 8–12 weeks); if this also fails, patients should be managed as moderately or severely affected. The use of biologics before thiopurines is recommended, as inconsistent results have been reported with thiopurines, whereas anti-TNF and non-anti-TNF biologic agents have shown more consistent success (50). In line with these suggestions, a case series showed that biologics had the highest success rate in achieving complete remission (52). Azathioprine was used in three patients, and complete remission was achieved in one of them (52). Although this approach appears logical based on the results, given the high economic costs of the biologics, thiopurines may be considered as an alternative to biologics in selected patients.

Other Potential Treatment Modalities

For patients with CVID-associated enteropathy who do not respond adequately to the mentioned therapies, alternative and emerging treatment strategies may offer additional clinical benefit. Pediatric and adult CVID patients with refractory diarrhea were treated with oral Ig treatment (53). Furthermore, in a limited number of IEI patients with AIN-related enteropathy, cyclosporine, tacrolimus, cyclophosphamide, mycophenolate mofetil, abatacept, and hematopoietic stem cell transplantation were used, indicating that these modalities can be promising options in CVID patients with refractory autoimmune enteropathy (54).

Mesenchymal stem cell infusion has emerged as a potential therapeutic option in autoimmune enteropathy. A recent case in an adult with autoimmune enteropathy demonstrated short-term remission following this treatment (55). However, there are no further comprehensive studies that confirm the effectiveness of these treatment options.

Conclusion

Non-infectious complications affecting any organs or tissues and associated with increased morbidity and mortality in CVID patients require heightened clinical attention, careful evaluation, and prompt management through a multidisciplinary, tailored therapeutic approach. Unfortunately, many aspects of the GI tract

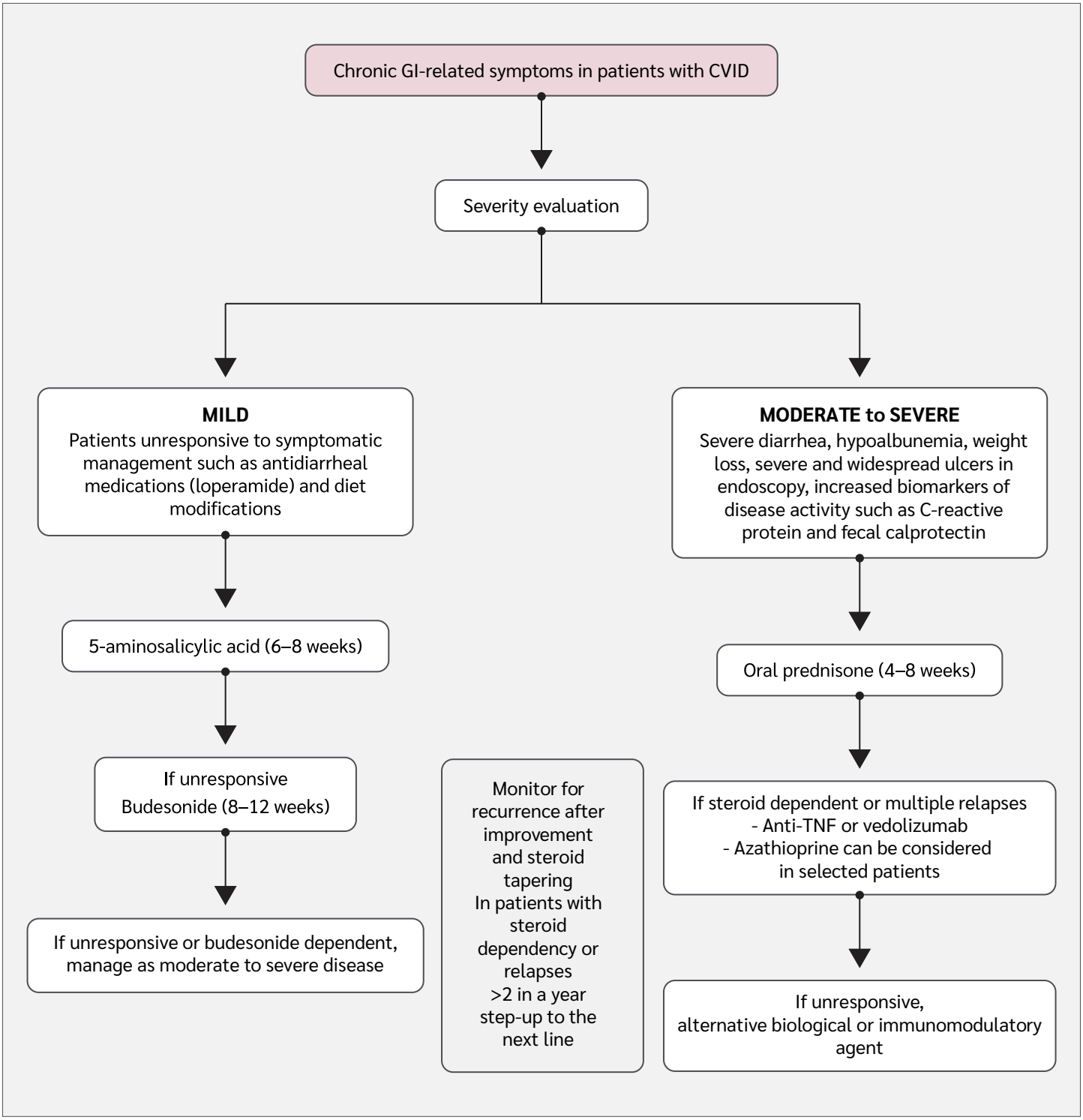

Figure 4. Non-infectious gastrointestinal tract diseases in common variable immunodeficiency (CVID).

CVID: Common variable immunodeficiency, **GI:** Gastrointestinal, **IBD:** Inflammatory bowel disease, **IEL:** Intraepithelial lymphocytes, **GVHD:** Graft-versus-host disease.

involvement in CVID mimicking other GI diseases, including celiac disease or IBD, and leading to a two-fold increased mortality rate, are not well known. This review article highlights the urgent need for studies that will help elucidate the underlying pathophysiology, thereby clarifying and facilitating the management of affected patients.

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Informed Consent: N.A.

Peer-review: Externally peer-reviewed

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ORIGINAL RESEARCH

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Relationship Between CD73 Expression on T Cells and Response to Treatment in De-novo Adult Acute Myeloid Leukemia Patients in Egypt

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Abstract

Objective: Acute myeloid leukemia (AML) is a heterogeneous blood malignancy with wide genomic aberrations, treatment approaches, and treatment outcomes. Successful treatment for AML remains challenging. CD73 is a recently recognized immune checkpoint mediator that is highly expressed in tumor microenvironment. It catalyzes the hydrolysis of adenosine monophosphate (AMP) to adenosine, which subsequently inhibits anti-tumor immune responses. This study aimed to investigate the relationship between CD73 and the response to induction therapy in adult Egyptian patients with AML.

Materials and Methods: A total of 44 blood samples were collected from 25 patients. Each survivor of induction therapy provided two samples: one on the day of initial diagnosis and another on the day of assessment of response to induction therapy (ARIT). Patients who did not survive induction therapy provided only one sample on the day of diagnosis. CD73 was measured on T cells using flow cytometric analysis and correlated with the response to treatment.

Results: Among the study cohort, median CD73 expression was 10.6% on T cells and 22.1% on $CD8^+$ T cells. There was no statistically significant difference across different risk categories, nor was there an association with treatment response. However, CD73 expression showed a positive correlation with 28-day survival.

Conclusion: CD73 expression did not show a significant relationship with any patient characteristics or disease parameters and did not affect treatment response. Nevertheless, it was positively correlated with 28-day survival.

Keywords: De-novo acute myeloid leukemia, treatment outcome, CD73

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Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults, with an estimated 20,380 new cases and 11,310 deaths reported in the USA in 2023 alone (1). According to the National Cancer Institute, only 31.7% survive beyond five years post-therapy (2). Treatment of patients with AML consists of three phases: induction, consolidation, and maintenance. The induction phase, as outlined by the National Comprehensive Cancer Network (NCCN), typically involves the "3+7" protocol, which consists of cytarabine (200 mg/m²/day) administered as a continuous intravenous infusion for seven days, combined with doxorubicin (25 mg/m²/day) given intravenously for three consecutive days (3). Following induction therapy, a follow-up bone marrow biopsy is performed to confirm whether remission has been achieved before proceeding to consolidation therapy (4). Recently, improvements have been made to the induction chemotherapy regimen, resulting in higher complete remission (CR) rates and better relapse-free and overall survival. However, focusing research efforts to achieve better long-term outcomes is still required (5).

Immune checkpoint molecules are highly expressed in the tumor microenvironment (TME), where they can modulate the response of T cells to tumor antigens and influence the patient's clinical outcome (6). CD73 is one of the recently recognized immune checkpoint receptors that is expressed on various immune and non-immune cells in the TME, including T cells, B cells, natural killer cells, dendritic cells, myeloid-derived suppressor cells, and tumor-associated macrophages. It exhibits 5′-nucleotidase activity and can hydrolyze adenosine monophosphate (AMP) to adenosine, which has immunosuppressive effects. Consequently, CD73 plays an essential role in balancing inflammation and immune suppression. Additionally, it can modulate tumorigenesis, proliferation, migration, and immune escape; therefore, it can be examined as part of tumor immunotherapy (7).

Recently, immune checkpoint therapy has demonstrated remarkable clinical outcomes for many solid tumors. It has been tested in the treatment of blood malignancies such as Hodgkin's lymphoma and has shown promising improvements in treatment outcomes (8). Currently, antibody therapy targeting CD73 is being tested in many clinical trials, either alone or in combination with other small molecular antagonists (ClinicalTrials.gov Identifiers: NCT04797468, NCT04148937, NCT03616886) (9).

The crucial role of CD73 in the therapeutic landscape of AML needs further validation. The current study aimed to investigate the relationship between CD73 expression on T cells and the treatment outcome of induction therapy in adult Egyptian patients with AML.

Materials and Methods

This prospective observational study was conducted on 25 newly diagnosed AML patients who were admitted to the Clinical Hematology and Oncology Unit, Department of Internal Medicine, University Hospital, Cairo, Egypt, and the Nasser Institute Hospital for Research and Treatment, Cairo, Egypt. The study period spanned from October 2021 to October 2023.

Patient Selection

Inclusion criteria:

- Newly diagnosed (de-novo) AML patients
- Age between 18 and 65 years
- Patients deemed fit for intensive chemotherapy of curative intent (i.e., free from cardiac, renal, or liver impairment)

Exclusion criteria:

- Age <18 or >65 years
- Relapsed AML patients
- Patients with AML who had chemotherapy initiated before enrollment in the study
- Secondary AML with a preceding hematologic disorder
- Diagnosis of acute promyelocytic leukemia (APL)
- Any acute leukemia other than AML
- History of solid malignancies
- Patients who were considered ineligible to receive intensified treatment

Clinical Evaluation and Sample Collection

A comprehensive history was taken from the patients, including demographic data, clinical data, routine laboratory tests, such as complete blood count (CBC) and C-reactive protein (CRP), and an assessment of comorbidities were done.

For flow cytometric analysis, 3 mL of peripheral blood was withdrawn from each participant under complete aseptic conditions in ethylene-diamine-tetra-acetic acid

(EDTA) tube. Blood samples were collected from each patient twice, at the time of diagnosis and on the day of assessment of response to induction therapy (ARIT). The tubes were transferred immediately to Ain Shams University Hospital laboratory for flow cytometric analysis.

Ethical Approval

The study protocol was approved by the Ethical Committee for Scientific Research, Faculty of Medicine, Ain Shams University (FWA 000017585 / MD 379 / 2019). Written informed consent was obtained from all patients or their legal guardians after providing detailed information about the study objectives and procedures.

Flow Cytometric Analysis

CD45, CD3, CD4, CD8, and CD73 monoclonal antibodies (Beckman Coulter, USA) were used to detect $CD73^{+}CD4^{+}$ T cells and $CD73^{+}CD8^{+}$ T cells. Overall, 50 µL of EDTA-treated blood was added to each of the sample tubes, and then 5 µL of each of the following anti-human monoclonal antibodies was added: anti-CD45-PC7, anti-CD3-PC5.5, anti-CD4-FITC, anti-CD8-APC, and anti-CD73-PE. After incubation for 15 minutes at room temperature, protected from light, 1–2 mL of ammonium chloride-based erythrocyte lysing solution was added to each tube. Tubes were vortexed and then analyzed using the NAVIOS flow cytometer and the NAVIOS CXP software (Beckman Coulter, USA). The gating strategy is illustrated in Figure 1. A total of 3894 cells were analyzed in 182.3 seconds.

Data Management and Analysis

Statistical analysis was performed using SPSS 23.0 statistical software (IBM Corp., Armonk, NY, USA). Non-parametric quantitative data were presented using the interquartile range (IQR) and median. The chi-square, Mann-Whitney, Kruskal-Wallis test, and Wilcoxon signed-rank test were used to compare the differences between the two groups. The correlation of paired data was analyzed using Spearman's correlation coefficient. A *p*-value of <0.05 was considered statistically significant.

Table 1. Different treatment outcomes to induction therapy on the day of ARIT as well as 28-day survival among cases.

Treatment outcome	n (%)
Response to induction (n=19)	
Resistance	4 (21.1)
Complete remission	15 (78.9)
Death	7 (28)
Survival	18 (72)

ARIT: Assessment of response to induction therapy.

Results

Demographic data

The study cohort had a median age of 41 years (range: 21–60) and consisted of 14 males (56%) and 11 females (44%). Eighty percent of the patients had no comorbidities, while 20% (n=5) had comorbid diabetes mellitus and hypertension.

Clinical information

According to the French-American-British (FAB) classifica-

Table 2. CD73 expression level on T cells in de-novo AML cases at diagnosis (n=25) and on Day of (ARIT) (n=19).

Cell type	Time point	Mean	SD	Median	IQR	Min	Max
$CD4^{+}$ T cells	Day1	1.774	1.274	1.130	0.410	0.952	5.400
	Day14	1.731	1.377	1.110	0.490	0.800	5.710
$CD8^{+}$ T cells	Day1	1.728	1.252	1.130	0.371	0.777	4.500
	Day14	1.934	1.633	1.060	2.467	0.715	6.390
Total T cells	Day 1	1.589	1.150	1.110	0.580	0.650	4.400
	Day 14	1.714	1.538	1.190	0.905	0.000	6.090

SD: Standard deviation; **IQR:** Interquartile range; **AML:** Acute myeloid leukemia; **ARIT:** Assessment of response to induction therapy.

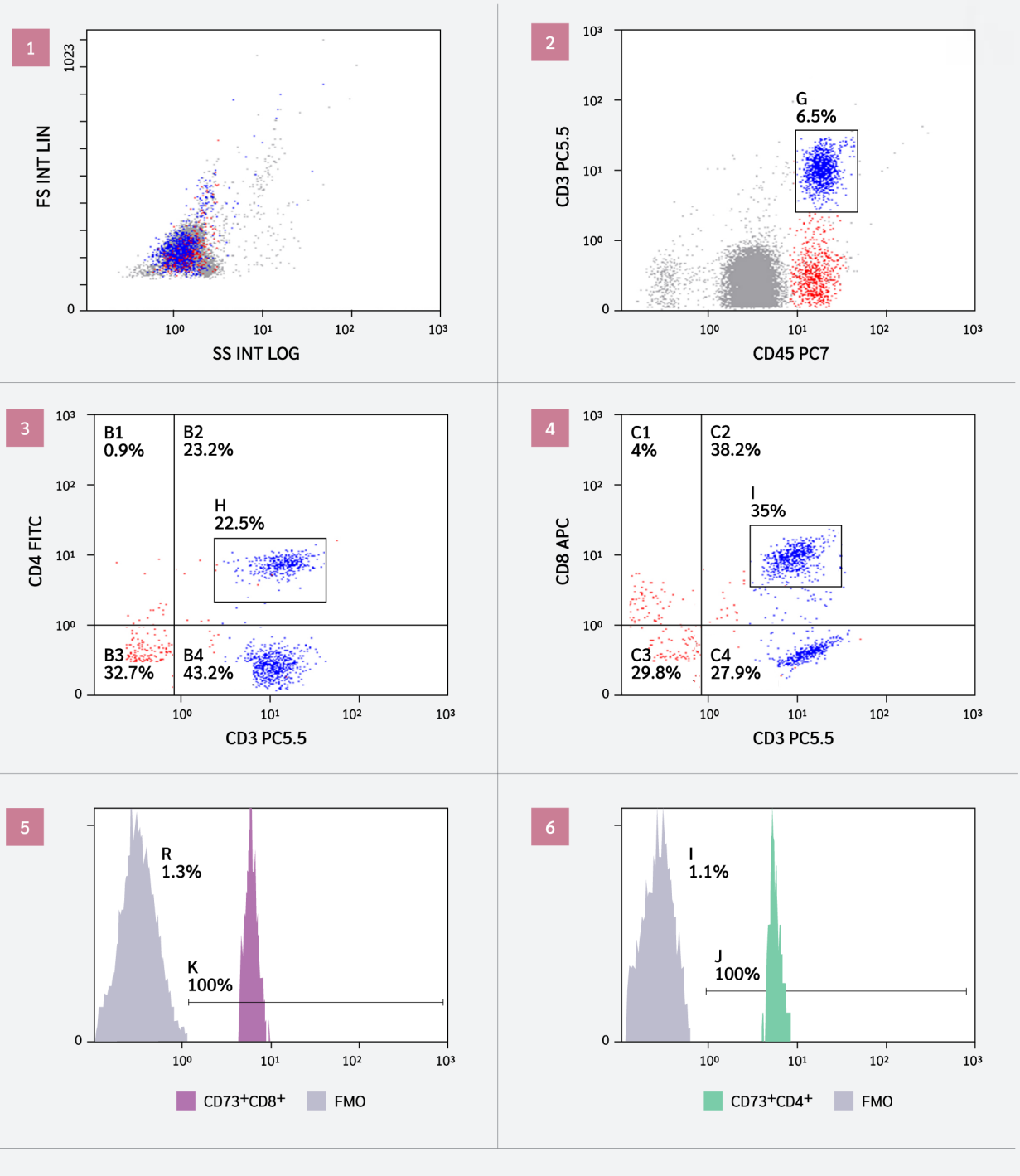

Figure 1. Gating strategy used in the study.

1. Forward scatter (FSC) vs. side scatter (SSC) plot, representing the distribution of cells based on size and internal complexity.
2. Lymphocyte population gated using anti-CD3 PC5.5 and anti-CD45 PC7.
3. Lymphocytes stained with anti-CD3 PC5.5 and anti-CD4 FITC.
4. Lymphocytes stained with anti-CD3 PC5.5 and anti-CD8 APC.
5. Identification of $CD73^+CD3^+CD8^+$ T cells using anti-CD3 PC5.5, anti-CD8 APC, and anti-CD73 PE (Gate K represents $CD73^+CD3^+CD8^+$ cells; 100%).
6. Identification of $CD73^+CD3^+CD4^+$ T cells using anti-CD3 PC5.5, anti-CD4 FITC, and anti-CD73 PE (Gate J represents $CD73^+CD3^+CD4^+$ cells; 100%).
7. Identification of $CD73^+CD3^+CD45^+$ total T cells using anti-CD3 PC5.5, anti-CD45 PC7, and anti-CD73 PE (Gate L represents $CD73^+CD3^+CD45^+$ cells; 100%).

Table 3. Comparison between CD73 expression level on Day 1 (n=19) and Day of ARIT (n=19).

Cell type	Median (IQR)		Wilcoxon signed-rank test	*p*-value*
	Day 1	Day of ARIT		
$CD4^+$ T cells (percentage)	11.2 (75.6)	3.9 (5.7)	-1.84	0.06
$CD8^+$ T cells (percentage)	26.2 (74.5)	14.3 (42.0)	-1.61	0.10
Total T cells (percentage)	20.3 (89.1)	9.7 (21.3)	-0.16	0.93

IQR: Interquartile range; **ARIT:** Assessment of response to induction therapy.

**p*-value >0.05: non-significant; *p*-value <0.05: significant; *p*-value <0.01: highly significant.

Table 4. Association between the level of CD73 expression on total T cells on Day 1 and the response to treatment and 28-day survival.

	Median CD73 expression on Total T cells on Day 1	*p*-value*
Response to induction (n=19)		
Resistance (n=4)	14.25%	0.616
Complete remission (n=15)	20.30%	
28-day survival (n=25)		
Died (n=7)	0.59%	0.042*
Alive (n=18)	21.85%	

*The statistically significant difference at $p \leq 0.05$.

tion, the distribution of AML subtypes was as follows: M0 (n=4), M1 (n=6), M2 (n=7), M4 (n=5), and M5 (n=3). Cytogenetic studies were performed according to the European Leukemia Net (ELN) recommendations for the diagnosis and management of AML (5). Based on ELN, 5 (20%) patients were classified as being at favorable risk, 13 (52%) at intermediate risk, and 7 (28%) at adverse risk. Almost all patients (96%) had no extramedullary infiltration, while only one patient had an extramedullary infiltration in the lung, presenting hemorrhagic pleural effusion.

All the patients received induction chemotherapy. However, 6 (24%) patients died before completing the induction phase: five due to septic shock and one from neutropenic enterocolitis. The patients were categorized into 15 (78.9%) responders and 4 (21.1%) with refractory disease. Patients were followed for 28 days starting from the initiation of induction therapy. During this period, one additional patient died of septicemia. By the end of the follow-up period, 18 patients (72%) remained alive (Table 1).

Immunophenotyping

Table 2 shows that the median CD73 expression on total T cells was 13.4% (range: 0–100%). $CD8^+$ T cells exhibited a higher median expression level of CD73 at 22.1%, compared to $CD4^+$ T cells, which had a median expression of only 5.6%.

Table 3 presents a comparison of CD73 expression levels on total T cells, $CD4^+$ T cells, and $CD8^+$ T cells among the 19 patients who survived induction therapy. Expression was measured on the day of initial diagnosis and again on the day of ARIT. It was evident that the median level of CD73 expression decreased across all T cell subsets following treatment. However, this decrease was not statistically significant, with *p*-values of 0.93 for total T cells, 0.06 for $CD4^+$ T cells, and 0.10 for $CD8^+$ T cells.

Among patients who survived the induction chemotherapy regimen (n=19), 15 responded to chemotherapy, achieving remission, while four were refractory. A comparison of responders versus non-responders in terms of CD73 expression revealed that the responder group had a higher median CD73 expression (20.3%) than non-responders (14.25%) (Table 4). However, the difference did not reach statistical significance (p=0.616).

A comparison between survivors (n=18) and deceased patients (n=7) based on their CD73 expression on the day of initial diagnosis revealed a significant difference. Survivors exhibited a markedly higher median CD73 level (21.85%) compared to deceased patients (0.59%), with a statistically significant *p*-value of 0.042 (Figure 2).

Figure 3 shows a comparison between CD73 expression level on total T cells on Day 1 and Day 14 after induction start in 28-day survivors and non-survivors.

The optimal cut-off value of CD73 expression on total T cells at diagnosis for predicting complete remission was >5.650 (Figure 4). The area under the ROC curve (AUC) for CD73 was 0.583. The sensitivity and specificity were 86% and 50%, respectively.

Table 5. Association between the incidence of high CD73 expression (>median rate of expression) on total T cells on Day 1 and the response to treatment and 28-day survival.

CD73 Levels		Response to induction n (%)		*p*-value	28-day survival n (%)		*p*-value
		Resistant	Complete remission		Dead	Live	
Day 1 Total T cells	Low expression	2 (50)	6 (40)	1.000*	6 (85.7)	7 (38.9)	0.073†
	High expression	2 (50)	9 (60)		1 (14.3)	11 (61.1)	

*p-value calculated using Fisher's exact test.

Table 6. Association between the level of CD73 MFI on total T cells on Day 1 and the response to treatment and 28-day survival.

		Median CD73 MFI on Day 1	*p*-value
Response to induction (n= 19)	Resistance (n=4)	0.75 MFI	0.881
	Complete Remission (n=15)	0.77 MFI	
Survival status on day 28 (n=25)	Died (n=7)	0.78 MFI	0.716*
	Alive (n=18)	0.76 MFI	

MFI: MFI: Mean fluorescent intensity. *Statistically significant difference at $p<0.05$.

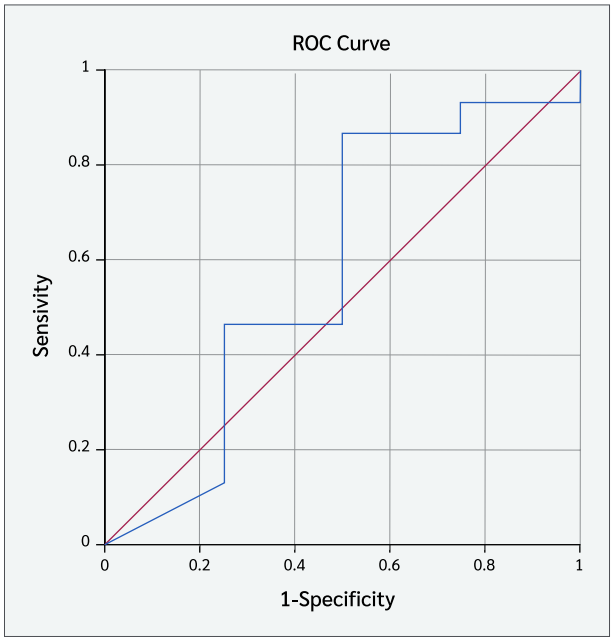

Figure 2. Receiver operating characteristic (ROC) curve for CD73 expression in predicting complete remission in newly diagnosed AML patients.

The area under the curve (AUC) was 0.583 (Standard Error: 0.187; $p=0.617$; 95% CI, 0.216–0.950), indicating a poor discriminative ability.

When comparing the incidence rate of high CD73 expression on total T cells (defined as >median expression levels) between the responder and non-responder groups, the responder group exhibited a higher rate (approximately 60%). Similarly, the incidence of high CD73 expression on total T cells was also higher in the survivor group compared to the deceased group, at 61.1% and 14.3%, respectively.

Median expression level of CD73 mean fluorescence intensity on total T cells on the day of initial diagnosis washigher in the responder group than in non-responders ($p=0.881$; Table 6).

Discussion

Although CD73 is expressed on many cells in the TME, the effector T cell is the primary cell responsible for the direct recognition and killing of tumor cells. Several studies assessed the functions of T cells in AML by high-dimensional immunophenotyping, gene expression, and functional studies (10,11) to characterize T cell function at the time of initial diagnosis and af-

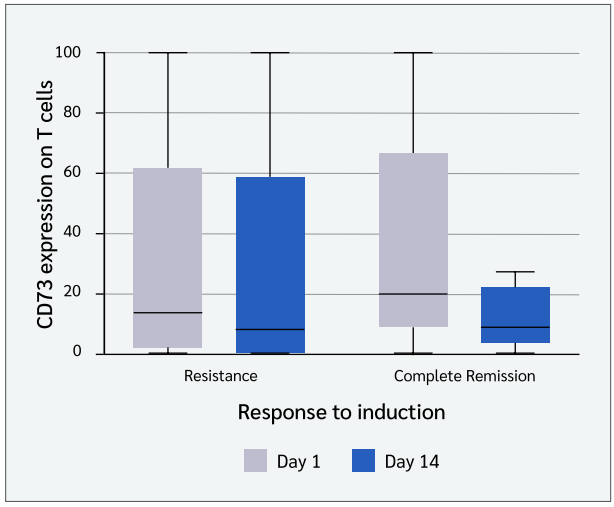

Figure 3. CD73 levels on Day 1 and Day 14 in responders vs. non-responders.

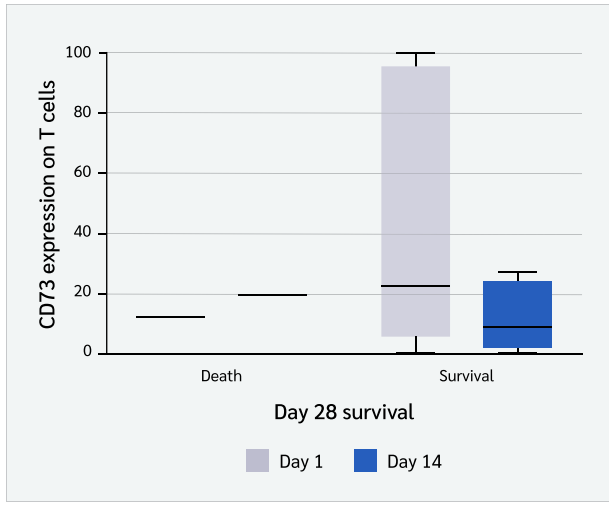

Figure 4. CD73 expression on total T cells on Day 1 and Day 14 after induction start in 28-day survivors and non-survivors.

ter induction chemotherapy. They found that multiple aspects of T cell function were deranged at diagnosis, with exhaustion and senescence being the dominant processes that reverted upon attaining complete remission.

The primary aim of this prospective study was to investigate the relation between CD73 expression on T cells and the treatment response to induction therapy in adult Egyptian patients with AML.

Regarding CD73 expression, our findings were consistent with a study conducted on 27 newly diagnosed AML patients in the USA, which reported a CD73 expression level of 22.26% on $CD8^+$ T cells (12). However, a study from Germany reported a higher CD73 expression level on $CD8^+$ T cells (41.35%) (13).

In studies assessing CD73 expression in solid tumors, methods other than flow cytometry have been predominantly used, such as immunohistochemistry and immunofluorescence. Notably, the criteria for defining high CD73 expression varied across IHC-based studies. For instance, some studies defined high CD73 expression as the percentage of positive-staining tumor cells exceeding the median expression level, while others considered staining intensity greater than 10% of positive-staining tumor cells as indicative of high CD73 expression (14). To align with these approaches, our research used the median expression level of CD73 on total Tcellsbefore the start of induction therapy (10.6%) as the cut-off value for determining high CD73 expression. This cut-off was specifically chosen to match the approach taken in previous studies, all of which examined CD73 expression in tumors before treatment. Patients whose CD73 expression met or exceeded this cut-off were classified as having high CD73 expression.

A study conducted in China on classical Hodgkin lymphoma patients reported convergent results, finding that CD73 was highly expressed in 40% of cases, as determined by immunohistochemical staining of CD73 in lymphoma samples (15). However, a higher incidence rate of high CD73 expression (61.7%) was reported by Ren et al. (16), who conducted immunohistochemical staining of CD73 in human head and neck squamous cell carcinoma (HNSCC) tissues from 162 patients with HNSCC in China. In contrast, other studies on bladder cancer and renal cell carcinoma reported lower incidence rates of high CD73 expression, at 26.4% and 15.9%, respectively, using immunohistochemical staining of CD73 in primary urothelial carcinoma tissue samples and nephrectomy tissue samples respectively (17, 18). These discrepancies in CD73 expression levels can be attributed to differences in tumor types, assessment methods, and the criteria used for defining high CD73 expression across the studies.

Upon assessing the relationship between CD73 expression and various demographic data, our results were in concordance with those of a meta-analysis that included 14 studies with a total of 2951 patients representing

12 different types of cancers. In their study, they noted no correlation between high CD73 expression and age or sex (14).

Concerning the comparison of CD73 expression in different AML immunophenotypes, our result was contrary to the study conducted by Zhang et al. (19), which reported a positive association between CD73 expression and FAB. The difference between this study and theirs may be attributed to the different types of samples analyzed, as they assessed CD73 expression on blast cells rather than on peripheral blood mononuclear cells, as was done in our study. Additionally, the larger sample size utilized in their study may also contributed to the observed differences.

Upon comparing the level of CD73 expression on total T cells, $CD4^+$ cells, and $CD8^+$ cells at diagnosis and during the assessment of response to treatment, our results were in concordance with those of a study conducted in Brazil on 33 patients with breast cancer (20). They observed a significant reduction of CD73 activity between the samples at the time of diagnosis and 6 months after treatment. However, contradictory results were reported by another experimental study on mice (21), which found that CD73 expression increased after radiotherapy compared to the baseline. These discrepancies in results may be attributed to the nature of the research, which was experimental versus prospective human study, the type of malignancy studied, as well as the different methodologies used for measuring CD73 expression, which might explain, in part, the different outcomes.

The present study demonstrated a statistically significant positive association between CD73 expression and 28-day overall survival. Similarly, positive CD73 expression was strongly correlated with longer overall survival (OS) in studies conducted on bladder cancer and breast cancer (17,22). However, Jiang et al. (14) concluded in their meta-analysis that high CD73 expression was significantly correlated with shorter OS in various cancers. Also, a more recent study conducted on 215 patients with colorectal cancer liver metastasis in France noticed that patients with high levels of serum CD73 had shorter disease-specific survival (23).

Regarding the comparison between CD73 expression and the response to treatment, our results were in concordance with those noted in a study conducted on classical Hodgkin lymphoma patients, which found that the increased expression of CD73 was associated with a better response to treatment (15). On the other hand, a study reported opposite results, finding that increased expression of CD73 was associated with a lower response to treatment in head and neck cancer patients (24).

Despite the well-known immune-suppressive role of CD73 expression on tumor and stromal cells within the tumor microenvironment, the literature focusing on CD73 expression on T cells and its relationship with prognosis is limited. The positive prognostic impact of high CD73 expression in our study is supported by a striking observation made by Kong et al. (12), who found that CD73 expression on $CD8^+$ T cells was associated with an enhanced immune response. $CD73^-CD8^+$ T cells exhibited greater functionality, while $CD73^-CD8^+$ T cells showed features of exhaustion, such as high expression of inhibitory receptors like PD-1 and TIGIT, reduced cytokine production capacity, and increased susceptibility to apoptosis. Additionally, our results may differ due to the type of tumor investigated, whereas our study focused on blood malignancies, while the aforementioned studies primarily examined solid tumors. Moreover, while those studies mainly measured CD73 expression on tumor cells, stromal cells, and Tregs, our study predominantly assessed CD73 expression on T cells. Therefore, understanding the specific distribution pattern of CD73 in each cancer type or disease status is crucial for the optimal design of clinical studies targeting CD73 in cancer treatment.

This study has several limitations, including the small number of patients enrolled, the shorter follow-up period, and the absence of a control group. As such, further studies on a larger scale of AML patients are recommended to assess the expression of CD73 on T lymphocytes. Additionally, longer follow-up studies with serial assessment of CD73 levels will be necessary to determine its utility as a predictive indicator for treatment response in AML patients.

Conclusion

Although no statistically significant association was found between CD73 expression on T cells and disease parameters or treatment response, it was positively correlated with 28-day survival, suggesting that CD73 may serve as a prognostic marker in AML. Research is required to confirm this hypothesis.

Ethical Approval: The study protocol was approved by the Ethical Committee for Scientific Research, School of Medicine, Ain Shams University (FWA 000017585 / MD 379 / 2019).

Informed Consent: Written informed consent was obtained from all patients or their legal guardians after providing detailed information about the study objectives and procedures.

Peer-review: Externally peer-reviewed

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ORIGINAL ARTICLE

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Investigation of Immunoblot Results of Antinuclear Antibody Test Positive Patients with Systemic Autoimmune Rheumatic Diseases

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Abstract

Objective: Systemic autoimmune rheumatic diseases (SARDs) are associated with antinuclear antibodies (ANAs). This study aimed to retrospectively assess the outcomes of extractable nuclear antigen (ENA) tests in patients with rheumatologic disorders who tested positive for antinuclear antibodies.

Materials and Methods: The study included 542 patients with a positive ANA result, no mixed pattern, and a positive immunoblot (IB) test. An ANA indirect immunofluorescence (IIF) test was performed on the same slide using the Hep-20-10/liver tissue kit (Euroimmun AG, Lübeck, Germany). Antibodies targeting ENAs were investigated using the EUROLINE ANA Profile 3 plus DFS70 (IgG) test kit (Euroimmun AG, Germany).

Results: The homogeneous pattern (AC-1), Sm-RNP, and ribosomal P protein exhibited significant associations with systemic lupus erythematosus (SLE) ($p<0.001$, $p=0.002$, $p<0.001$, respectively). The speckled pattern (AC 4/5), SS-A, SS-B, and Ro52 showed a strong association with Sjögren's syndrome ($p<0.001$ for each). The centromere pattern (AC-3), CENP-B, and Scl-70 were significantly correlated with systemic sclerosis ($p<0.001$ for all). The homogeneous pattern (AC-1) and Mi-2 were significantly associated with rheumatoid arthritis ($p=0.019$, $p=0.035$, respectively). The speckled pattern (AC-4/5), Jo-1, and Mi-2 exhibited significant associations with polymyositis/dermatomyositis ($p<0.001$, $p=0.006$, $p<0.001$, respectively). Systemic sclerosis was substantially correlated with mixed connective tissue disease ($p=0.021$).

Conclusion: Antinuclear antibody IIF testing should be used to evaluate autoimmune disorders. Extractable nuclear antigen testing is essential for identifying and confirming the presence of the antigen. Extractable nuclear antigen tests detect specific antigens, making them diagnostically and prognostically valuable in rheumatological diseases. Through rational laboratory use, these tests can enhance efficiency and provide more accurate clinical guidance.

Keywords: Antinuclear antibodies, systemic autoimmune rheumatic diseases, extractable antinuclear antibodies

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Introduction

Autoantibodies seen in systemic autoimmune rheumatic diseases (SARDs) generally target nuclear antigens and are known as antinuclear antibodies (ANAs) (1). Antinuclear antibody-associated rheumatic diseases encompass a diverse range of conditions characterized by the presence of positive ANAs (2). The patterns identified through the indirect immunofluorescence (IIF) technique are associated with specific diseases, and anti-cell codes (ACs) have been established based on international consensus regarding ANA patterns (3).

The IIF method using HEp-2 cells is considered the gold standard for ANA screening (4). The patterns identified by IIF are associated with specific diseases. ACs have been defined in accordance with the international consensus on ANA patterns (3). Nuclear patterns include homogeneous, speckled, dense fine speckled (DFS70), nucleolar, nuclear membrane, centromere, cytoplasmic, and mitotic staining. The association between rheumatic diseases and IIF patterns is a result of autoantibodies targeting specific cellular antigens (5). Certain autoantibodies have been isolated and designated as extractable antinuclear antibodies (ENAs).

Among ENAs, the Smith (Sm) antigen is a low-molecular-weight, non-histone, acidic ribonucleoprotein. Sjögren's syndrome-related antigen A (SS-A) is a protein involved in mRNA processing, whereas Sjögren's syndrome-related antigen B (SS-B) is a phosphoprotein that functions as a cofactor for RNA polymerase III (6). The Scl-70 antigen is identified as DNA topoisomerase I, while Jo-1 corresponds to the enzyme histidyl-tRNA synthetase (7). Analyzing ENA responses can aid in differentiating among various forms of autoimmune connective tissue disorders (8). The presence of antibodies against the Sm antigen is specific for systemic lupus erythematosus (9), while the presence of anti-SS-A or anti-SS-B antibodies indicates Sjögren's syndrome (7). Detection of ENA antibodies is not only diagnostically important but also holds prognostic significance. The presence of SS-A in the circulation of a pregnant woman may lead to neonatal lupus erythematosus (11) or congenital heart block in the baby, while the presence of anti-topoisomerase I (Topo-I) antibodies predicts a more severe progression of systemic sclerosis (SSc) (12). This study aimed to retrospectively assess the outcomes of extractable nuclear antigen tests in patients with rheumatologic disorders who tested positive for antinuclear antibodies.

Materials and Methods

This study retrospectively analyzed the simultaneous requests for IIF staining patterns, ANA tests, and IB tests for patients admitted to the Medical Microbiology Laboratory of Ankara Bilkent City Hospital from February 2019 to June 2023, along with their diagnoses.

The study included 542 patients with positive ANA results, no mixed pattern, and a positive IB test. Of these, 120 patients were diagnosed with systemic lupus erythematosus (SLE), 100 with Sjögren's syndrome (SS), 100 with systemic sclerosis (SSc), 100 with rheumatoid arthritis (RA), 100 with mixed connective tissue disease, and 20 with polymyositis/dermatomyositis. 90% of patients were recruited from the rheumatology department, with the remainder from internal medicine, physical therapy, and rehabilitation departments.

The ANA IIF test was performed on the same slide utilizing a kit (Euroimmun AG, Lübeck, Germany) containing Hep-20-10/liver tissues. The manufacturer recommended an initial dilution of 1:100 for screening purposes. Results were qualitatively evaluated using a EUROSTAR III plus fluorescence microscope at X40 magnification, ranging from 1+ (1:100) to 4+ (1:3200), based on the brightness observed at the screening dilution.

The EUROLINE ANA Profile 3 plus DFS70 (IgG) test kit (Euroimmun AG, Germany) was used to analyze antibodies targeting ENA. The examination identified 18 distinct antigens on the IB strips, including Sm/RNP, Sm, SS-A, SS-B, Scl-70, Jo-1, dsDNA, nucleosome, histone, ribosomal P-protein, AMA M2, Ro-52, PM/Scl, CENP-B, PCNA, Ku, Mi-2, and DFS70. All incubation and washing procedures were performed manually using the EUROBlotOne system (Euroimmun). Band intensities were evaluated with EUROLineScan software (Euroimmun). Certain autoantibodies were isolated and classified as ENA, which are commonly identified using enzyme-linked immunosorbent assay (ELISA) or IB techniques.

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

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY).

The data were assessed for normal distribution using the Kolmogorov-Smirnov test. Numerical variables following a normal distribution were expressed as mean ± standard deviation, while non-normally distributed data were expressed as median values. Categorical data were compared using either the Chi-square test or Fisher's exact test, as appropriate. A significance level of $p<0.05$ was considered statistically significant.

Correlation analysis and heatmap visualization were conducted using the psych and ggplot2 packages in R statistical software (version 4.5.0). Phi correlation coefficients were calculated to determine the strength and direction of associations (range: -1 to +1). Red shades indicated positive correlations (darker red denoting stronger relationships), blue indicated negative correlations, and white ($\phi = 0$) denoted no correlation.

Results

All 100 patients diagnosed with rheumatological diseases, including SLE, SS, SSc, RA, mixed connective tissue disease, and polymyositis/dermatomyositis, in a randomly selected cohort from February 2019 to June 2023 exhibited ANA positivity, a single ANA pattern, and a positive IB test. The patients' mean age was 50.5 ± 16.5 years. Women accounted for 474 cases (87.5% of the total).

Table 1. Relationship between antinuclear antibodies (ANA) patterns and immunoblot results.

	Antinuclear Antibodies Patterns (ANA)							
Line blot results (n=542 ANA positives)	Speckled (AC-4,5)	Homogenous (AC-1)	DFS (AC-2)	Nucleolar (AC-8/9/10)	Few nuclear dots (AC-7)	Multiple nuclear dots (AC-6)	Centromere (AC-3)	Envelope (AC-11/12)
SSA (n=144)	**<0.001**	**0.043**	**0.002**	0.156	0.738	0.087	**<0.001**	0.919
SSB (n=63)	**<0.001**	**<0.001**	**0.038**	0.188	0.415	0.301	**0.010**	0.301
Ro-52 (n=166)	**<0.001**	**<0.001**	**0.003**	0.331	0.648	0.058	0.094	0.231
Jo-1 (n=18)	0.135	0.202	0.288	**<0.001**	0.677	0.597	0.124	0.597
Sm/RNP (n=71)	0.135	**0.037**	**0.026**	0.753	0.073	0.269	**0.001**	0.269
Scl-70 (n=70)	0.082	**<0.001**	0.098	0.373	0.070	0.272	**0.017**	0.972
DFS (n=92)	**0.030**	0.146	**<0.001**	0.405	0.68	0.542	**0.002**	0.198
Ribosomal P protein (n=22)	0.772	**0.040**	0.238	0.366	0.644	0.558	0.088	0.558
CENP B (n=96)	**<0.001**	**<0.001**	**0.008**	0.357	0.893	0.186	**<0.001**	0.697
PCNA (n=32)	0.069	0.900	0.151	0.619	0.574	0.475	0.356	0.475
Histones (n=53)	**<0.001**	**<0.001**	0.206	0.366	0.439	0.794	0.070	0.348
Nucleosome (n=52)	**<0.001**	**<0.001**	0.062	0.082	0.464	0.353	0.075	0.353
ds-DNA (n=47)	**<0.001**	**<0.001**	0.077	0.100	0.366	0.380	**0.011**	0.698
M2 (n=80)	**0.047**	0.462	0.062	0.993	0.350	**<0.001**	0.703	**<0.001**
Sm (n=25)	0.404	0.846	0.705	0.817	**<0.001**	0.531	0.068	0.531
M-i2 (n=19)	0.211	0.525	0.374	0.954	0.669	0.587	0.114	0.587
Ku (n=19)	0.008	0.374	0.274	0.310	0.669	0.587	0.114	0.587
Pm-Scl (n=75)	0.987	0.151	0.078	0.321	0.368	0.253	0.539	0.912

ANA: Antinuclear antibody, **AC:** Anti-cell pattern code (ICAP consensus), **DFS:** Dense fine speckled, **SSA:** Anti–Sjögren's syndrome–related antigen A, **SSB:** Anti–Sjögren's syndrome–related antigen B, **Sm/RNP:** Smith/ribonucleoprotein complex, **Jo-1:** Anti–histidyl-tRNA synthetase, **Ro-52:** Anti-Ro52/TRIM21 antibody, **Scl-70:** Anti–topoisomerase I antibody, **DFS70:** Anti–dense fine speckled 70 kDa protein, **CENP B:** Anti–centromere protein B, **PCNA:** Proliferating cell nuclear antigen, **ds-DNA:** Double-stranded DNA, **AMA-M2:** Anti–mitochondrial M2 antibody, **PM-Scl:** Anti–PM-Scl complex, **Mi-2:** Anti–Mi-2 antigen antibody.

Among the ANA patterns observed, 238 were speckled (AC-4/5), 164 were homogeneous (AC-1), 61 were centromere (AC-3), 31 DFS (AC-2), 27 were nucleolar (AC-8/9/10), 8 were multiple nuclear dots (AC-6), 8 were envelope (AC-11/12), and 5 were few nuclear dots (AC-7). No cytoplasmic ANA pattern was detected.

The frequency of antigen positivity was as follows: Ro52 in 166 patients, SS-A in 144, CENP-B in 96, DFS in 92, M2 in 80, Pm-Scl in 75, Sm/RNP in 71, Scl-70 in 70, SS-B in 63, histone in 53, nucleosome in 52, dsDNA in 47, PCNA in 32, Sm in 25, ribosomal P protein in 22, Mi-2 in 19, Ku positivity in 19, and Jo-1 in 18.

Table 1 shows the correlation between ANA patterns and IB results. SS-A, SS-B, Ro52, and Ku positivity were associated with the speckled pattern (AC-4/5) ($p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, $p=0.008$, respectively). Sm/RNP, Scl-70, ribosomal p protein, histone, nucleosome, dsDNA were associated with a homogeneous pattern (AC-1) ($p=0.037$, $p<0.001$, $p=0.040$, $p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, respectively). Jo-1 was associated with the nucleolar pattern (AC-8/9/10) ($p<0.001$). DFS positivity was associated with the DFS pattern (AC-2) ($p<0.001$). CENP-B positivity was associated with the centromere pattern (AC-3) ($p<0.001$). Sm positivity was associated with the few nuclear dots pattern (AC-7) ($p<0.001$). M2 positivity was associated with multiple nuclear dots (AC-7) and envelope (AC-11/12) patterns ($p<0.001$ for both).

Table 2 illustrates the correlations between ANA patterns, IB results, and rheumatologic diseases. The homogeneous pattern (AC-1), Sm/RNP, ribosomal p protein, histone, nucleosome, and dsDNA were strongly associated with the presence of SLE ($p<0.001$, $p=0.002$, $p<0.001$, $p<0.001$, $p<0.001$, and $p=0.002$, respectively). The centromere pattern (AC-3), Scl-70, and CENP-B were significantly associated with the absence of SLE ($p=0.002$, $p<0.001$, and $p=0.005$, respectively).

The speckled pattern (AC-4/5), SS-A, SS-B, and Ro52 were significantly associated with the presence of SS ($p<0.001$ for all). In contrast, the homogeneous pattern (AC-1) and nucleosome were significantly associated with the absence of SS ($p=0.003$ and $p=0.013$, respectively).

The centromere pattern (AC-3), CENP-B, and Scl-70 were significantly associated with the presence of SSc ($p<0.001$ for all). The speckled pattern (AC-4/5), SS-A, SS-B, Ro52, Sm/RNP, DFS, nucleosome, and Sm were significantly associated with the absence of SSc ($p<0.001$, $p<0.001$, $p=0.008$, $p<0.001$, $p=0.008$, $p=0.003$, $p=0.004$, and $p=0.015$, respectively).

The homogeneous pattern (AC-1) and Mi-2 were significantly associated with the presence of RA ($p=0.019$ and $p=0.035$, respectively). Conversely, the speckled pattern (AC-4/5), SS-A, Ro52, and Scl-70 were significantly associated with the absence of RA ($p=0.008$, $p=0.002$, $p=0.021$, and $p=0.009$, respectively).

The speckled pattern (AC-4/5), Jo-1, and Mi-2 were significantly associated with polymyositis/dermatomyositis ($p<0.001$, $p=0.006$, and $p<0.001$, respectively). Sm was significantly associated with the presence of mixed connective tissue disease ($p=0.021$), whereas the homoge-

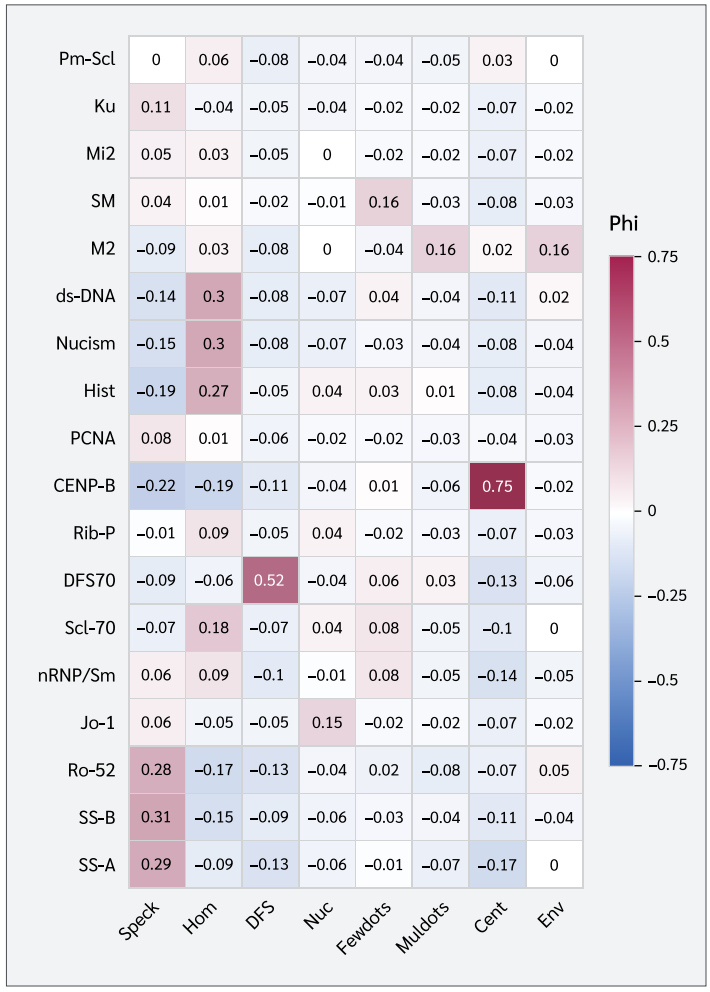

Figure 1. Heatmap of Phi correlation coefficients between ANA patterns and immunoblot autoantibodies.

Table 2. Associations of antinuclear antibodies (ANA) and immunoblot results with the diagnosis of systemic autoimmune rheumatic diseases.

	SLE (+)	SLE (-)	Sjogren (+)	Sjogren (-)	SSc (+)	SSc (-)	RA (+)	RA (-)	PM/DM (+)	PM/DM (-)	MCTD (+)	MCTD (-)
ANA Patterns (n=542)												
Speckled (AC-4/5)	50/120	188/422	**62/100***	176/442	29/100	**209/442***	32/100	**206/442***	**17/22***	221/520	48/100	190/442
Homogenous (AC-1)	**52/120***	112/422	18/100	**146/442***	31/100	133/442	**40/100***	124/442	3/22	161/520	20/100	**144/442***
DFS (AC-2)	5/120	26/422	7/100	24/442	4/100	27/442	9/100	22/442	1/22	30/520	5/1000	26/422
Nucleolar (AC-8/9/10)	5/120	22/422	4/100	23/442	6/100	21/442	6/100	21/4442	1/22	26/520	5/100	22/442
Few nuclear dots (AC-7)	1/120	4/422	0/100	5/442	0/100	5/442	0/100	5/442	0/22	5/520	4/100	**1/442***
Multiple nuclear dots (AC-6)	1/120	7/422	1/100	7/442	1/100	7/442	2/10	6/442	0/22	8/520	3/100	5/437
Centromere (AC-3)	4/120	**57/422***	6/100	55/442	**28/100***	33/442	9/100	52/442	0/22	61/520	14/100	47/442
Envelope (AC-11/12)	1/120	7/422	2/100	6/442	1/100	7/442	2/100	6/442	0/22	8/520	2/100	6/442
Lineblot Results												
SSA	38/120	106/422	**48/100***	96/442	12/100	**132/442***	14/100	**130/442***	5/22	139/520	27/100	117/442
SSB	13/120	50/422	**26/100***	37/422	4/100	**59/442***	9/100	54/442	2/22	61/520	9/100	54/442
Ro52	36/120	130/422	**57/100***	109/422	16/100	**150/442***	2/100	**145/442***	6/22	160/522	30/100	136/442
Pm-Scl	19/120	56/422	14/100	61/422	18/100	57/442	9/100	66/442	4/22	71/520	11/100	64/442
Jo-1	1/120	17/422	2/100	16/422	4/100	14/442	4/100	14/442	**3/22***	15/520	4/100	14/442
Sm/RNP	**26/120***	45/422	8/100	63/442	5/100	**66/442***	11/100	60/442	3/22	68/520	18/100	53/442
Scl-70	3/120	**67/422***	8/100	62/442	**37/100***	33/442	5/100	**65/442***	3/22	67/520	14/100	56/442
DFS	18/120	74/422	19/100	73/442	7/100	**85/442***	22/100	70/372	3/22	89/520	23/100	69/442
Ribosomal P protein	**13/120***	9/422	2/100	20/442	2/100	20/442	1/100	21/442	0/22	22/520	4/100	18/442
CENP-B	11/120	**85/422***	13/100	83/442	**36/100***	60/442	13/100	83/442	1/22	95/520	22/100	74/442
PCNA	9/120	23/422	4/100	28/442	7/100	25/442	7/100	25/442	3/22	29/520	2/100	30/442
Histones	24/120	29/422	6/100	47/442	7/100	46/442	8/100	45/442	1/22	52/520	7/100	46/442
Nucleosome	30/120	22/422	3/100	**49/442***	2/100	**50/442***	7/100	45/442	0/22	52/520	10/100	42/442
ds-DNA	19/120	28/422	4/100	43/442	6/100	41/442	6/100	41/442	0/22	47/520	12/100	35/442
M2	20/120	60/422	17/100	63/442	14/100	66/442	14/100	66/442	1/22	79/520	14/100	66/442
Sm	10/120	15/422	3/100	22/442	0/100	**25/442***	3/100	22/442	0/22	25/520	**9/100***	16/442
Mi-2	1/120	18/422	1/100	18/442	3/100	16/442	**7/100***	12/442	**4/22***	15/520	3/100	16/442
Ku	7/120	12/422	4/100	15/442	2/100	17/442	2/100	17/442	2/22	17/520	2/100	17/442

ANA: Antinuclear antibody, **AC:** Anti-cell pattern code (ICAP consensus), **DFS:** Dense fine speckled, **SLE:** Systemic lupus erythematosus, **SSc:** Systemic sclerosis, **RA:** Rheumatoid arthritis, **PM/DM:** Polymyositis/Dermatomyositis, **MCTD:** Mixed connective tissue disease, **SSA:** Anti–Sjögren's syndrome–related antigen A, **SSB:** Anti–Sjögren's syndrome–related antigen B, **Ro-52:** Anti-Ro52/TRIM21 antibody, **Sm/RNP:** Smith/ribonucleoprotein complex, **Jo-1:** Anti–histidyl-tRNA synthetase antibody, **Scl-70:** Anti–topoisomerase I antibody, **DFS70:** Anti–dense fine speckled 70 kDa protein, **CENP-B:** Anti–centromere protein B, **PCNA:** Proliferating cell nuclear antigen, **ds-DNA:** Double-stranded DNA, **M2 (AMA-M2):** Anti–mitochondrial M2 antibody, **Sm:** Smith antigen antibody, **Mi-2:** Anti-Mi-2 antigen antibody, **Ku:** Anti-Ku antigen antibody, **Pm-Scl:** Anti-PM-Scl complex antibody.

neous pattern (AC-1) and the few nuclear dots pattern (AC-7) were significantly associated with its absence (p=0.013 and p<0.001, respectively).

According to Phi correlation analysis, the speckled pattern (AC-4/5) was correlated with SS-A, SS-B, and Ro-52. The homogeneous pattern (AC-1) correlated with dsDNA, nucleosome, histone, and Scl-70. The DFS-like pattern (AC-2) showed a strong correlation with DFS70. The nucleolar pattern (AC-8/10) correlates with Jo-1. The few nuclear dots pattern (AC-7) correlated with Sm. The multiple nuclear dots pattern (AC-6) was correlated with M2. Centromere pattern (AC-3) showed a strong correlation with CENP-B. Envelope pattern (AC-11/12) correlated with M2 (Figure 1).

Discussion

Laboratories in developing countries, including Türkiye, require a dependable, cost-effective, accurate, and specific screening test. The high cost and the need for technical expertise limit its availability in many facilities.

Antinuclear antibody detection is the first step in diagnosing SARDs (13). The IIF method using HEp-2 cells is considered the gold standard (14). If ANA results are positive, it is advisable to screen for specific anti-ENA antibodies (15). In cases of strong clinical suspicion, regardless of ANA test results, physicians may request testing for specific ENA antibodies (16).

The American College of Rheumatology (ACR) and international committees recommend HEp-2 IIF as the standard method for identifying ANA (17). To date, up to 30 distinct ANA staining patterns have been described, including both nuclear and cytoplasmic staining patterns (18).The most common patterns are homogeneous (AC-1), speckled (AC-4/5), nucleolar (AC-8/9/10), and centromere (AC-3) (19). In this study, the speckled ANA pattern was the most frequently observed. Similarly, in Trabzon province, Kaklıkkaya et al. (20) reported the speckled pattern as the most common, consistent with our findings.

The homogeneous pattern is one of the most common patterns observed in the ANA test. The antigens associated with this pattern include dsDNA, histone, and nucleosomes. In a previous study, 45% of the homogenous patterns were positive in the IB test (16). Low DFS70 titers and homogeneous/speckled patterns can be difficult to distinguish; therefore, we recommend confirming them with the IB test using the DFS70 antigen. We hypothesize that the IB findings for samples with homogeneous patterns vary due to antibody diversity, particularly in patients with SLE. The antibodies detected in these patients' dsDNA, nucleosomes, and histones include SS-A, Ro-52, SS-B, Sm, U1-RNP, and ribosomal P protein (21).

In our study, the homogenous pattern (AC-1) was associated with Sm/RNP, Scl-70, ribosomal p protein, histone, nucleosome, and dsDNA. The DFS70 staining pattern is one of the most common IIF scanning patterns (22). Isolated DFS70 antibody positivity occurs in fewer than 5.7% of systemic rheumatic diseases (22), and these antibodies are often present in the serum of healthy individuals (23). In our analysis, the DFS70 pattern was the fourth most prevalent. As expected, DFS showed no correlation with any rheumatic condition in our investigation.

The centromere pattern, characterized by its structural features, is observed in localized cutaneous systemic sclerosis and Raynaud's syndrome, and is associated with CENP-A, CENP-B, CENP-C, and CENP-F antigens (24). In a previous study, only CENP-B was identified in the IB test, with a 91% positivity rate (16). The remaining negative samples may be related to other antigens. The strong agreement between the IFF centromere pattern and CENP-B positivity suggests that IFF alone may be sufficient to identify the centromere pattern. In our analysis, CENP-B positivity showed a clear correlation with SSc.

The speckled pattern is frequently observed in conditions such as mixed connective tissue disorders, SLE, and SSc (25). It is specifically associated with the SS-A, SS-B, Topo-1, Sm, U1-SnRNP, Mi2, and Ku antigens. A recent study revealed that the majority of the IB panel results showed antibodies against SS-A, Ro-52, and SS-B (16).

Depending on the HEp-2 cell line used in the IIF assay, SS-A, Jo-1, and ribosomal P-protein antibodies may be difficult to detect (17). Due to the extremely low expression levels of these antigens in HEp-2 cells or their potential denaturation during tissue fixation, false negatives may occur. In clinically suspected cases, ENA testing should be requested even if IIF results are negative.

We analyzed the correlation between IB and ANA patterns in our cohort to identify the most cost-effective fol-

low-up autoantibody test. In recent years, autoantibody testing for systemic rheumatic disorders has evolved significantly (26). The combined use of ANA patterns and extended IB panels has reduced both diagnostic time and the risk of misdiagnosis (27). Furthermore, the interpretation and reporting of ANA have been standardized to eliminate subjectivity (26). However, the testing, reporting, and interpretation of these autoantibodies remain challenging because of multiple factors, including the testing methodology and platform, the experience of immunologists and laboratory scientists, the variability of fluorescent microscopes, and the pre-test probability of SARDs (26). It is essential that physicians are aware of and approve the revised autoantibody reporting format.

As observed, ENA or anti-ENA profiling enables differentiation of various forms of SARDs. A previous study found that the presence of RNP autoantibodies is a useful marker in the diagnosis of mixed connective tissue disease (28). Similarly, ANA positivity along with dsDNA or Sm positivity serves as the diagnostic criterion for SLE (29). SS-A and SS-B antibodies are valuable immunological markers for identifying SS, subacute cutaneous SLE, and newborn lupus syndrome (30). Jo-1, histidyl-tRNA synthetase, has been established as an immunomarker for polydermatomyositis. Likewise, CENP-B and Scl-70 positivity support the identification of SSc (31). Our findings were consistent with these results.

Certain ANA patterns show strong associations with IB antigens. Fibrillarin, PM/Scl, RNA polymerase I, and other IB proteins are commonly found in the nucleolar pattern (32). González et al. (33) reported various nuclear patterns in sera positive for SS-A, SS-B, and Ro-52. Rodríguez-Orozco et al. (34) found that approximately 3.4% of IB-positive samples were ANA-negative. In our cohort, PM/Scl antibodies were detected in nucleolar patterns.

Accurate interpretation of these antibodies requires consideration of clinical features. Because many SARD manifestations are nonspecific and overlap, diagnosis can be challenging, and patients are sometimes initially diagnosed with multiple SARDs until proven otherwise (26). SLE is the prototype for SARD. Several autoantibodies can be identified in SLE, differing in their sensitivity and specificity (26). Certain autoantibodies, including SS-A, SS-B, and RNP, can be detected in multiple SARDs (35). In a prior cohort, they discovered that nearly all individuals with IB were positive for SLE, but only a few autoantibodies were positive in the remainder of the SARD. Some patients may have overlap syndromes, which could explain the occurrence of several patterns and autoantibodies (26). Nonetheless, applying the proposed algorithm may aid in narrowing down the final diagnosis, determining targeted therapy, and providing an adequate prognosis (35).

In a previous cohort, 62% of patients tested positive for both IB and dsDNA (26). One could argue that once a diagnosis is established through the detection of a specific autoantibody, extending the IB panel may be unnecessary. However, certain autoantibodies are associated with a more specific symptom. For example, SS-A and SS-B are linked to congenital heart block, while ribosomal p protein is linked to neuropsychiatric problems (35). Moreover, as many SARDs can coexist (26), IB testing is essential even when positive dsDNA antibodies are present. In a study conducted in Türkiye, SS-A (26.88%), SS-B (17.81%), and Sm/RNP (17.66%) were the most frequently detected ENA antibodies in ANA-positive samples (7). In our cohort, the most common IB findings were Ro52, SS-A, CENP-B, and DFS.

Specific ANA patterns and IB test positivity were each associated with some autoimmune rheumatic diseases. The identification of these immunologic markers underscores the predictive value of immunologic tests for disease diagnosis. Further prospective studies are necessary to expand the evidence base.

This study has some limitations. First, ANA tests were not performed on all patients, and ANA-negative patients were excluded, which prevented drawing conclusions regarding negative results. Second, data on cytoplasmic staining and accompanying IB results are limited. Future research should include larger patient cohorts, comprehensive IB results for all cellular patterns (nuclear, mitotic, and cytoplasmic), and detailed clinical associations. Employing an expanded IB panel, such as the myositis panel, may further clarify disease etiology and inform targeted therapies.

Conclusion

Antinuclear antibody IIF testing is recommended as a screening test for suspected autoimmune diseases. Extractable nuclear antigen testing should be employed to identify and confirm the presence of the appropriate

antigen. Extractable nuclear antigen tests are both diagnostically and prognostically valuable, particularly in the diagnosis of rheumatic disorders such as systemic lupus erythematosus, Sjögren's syndrome, and systemic sclerosis. The rational implementation of these tests in laboratories can improve efficiency and inform clinical decision-making.

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

Informed Consent: N.A.

Peer-review: Externally peer-reviewed

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

Conflict of Interest: The author declares no conflict of interest.

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ORIGINAL ARTICLE

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Immunogenic Potential of Foot and Mouth Virus Antigen O-146S Adjuvanted with Water-in-Oil and Water-in-Oil-in-Water Emulsion in Mice

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Abstract

Objective: Foot-and-mouth disease virus (FMDV) is a highly contagious viral pathogen that significantly impacts livestock health and productivity. This study aimed to evaluate the immunological responses to FMDV O-146S antigen adjuvanted with water-in-oil (W/O) and water-in-oil-in-water (W/O/W) emulsion in mice.

Materials and Methods: Mice were immunized intramuscularly with a vaccine formulation containing the FMDV O-146S antigen combined with oil emulsion adjuvants at a predetermined ratio and then monitored for 21 days.

Results: Compared to FMDV O-146S antigen alone, formulations containing oil emulsion adjuvants induced earlier and increased levels of anti-FMDV specific antibody responses, as demonstrated by elevated levels of anti-FMDV IgG ($p<0.0001$), IgG1 ($p<0.0002$), and IgG2a ($p<0.0018$). The increased IgG1 and IgG2a antibody isotype responses suggest activation of both Th2- and Th1-type immunity, respectively. Vaccines were also more effective at eliciting a cellular immune response, as measured by the effector T cells represented by $CD3^+CD8^+$ T cells ($p<0.0001$), IFN-γ secreting $CD8^+$ T cells ($p<0.0001$), IFN-γ secreting $CD3^+$ T cells ($p<0.0001$) and proliferation of splenocytes represented by higher stimulation index ($p<0.0001$), as well as the production of IFN-γ ($p<0.0001$), IL-4 ($p<0.0002$), and IL-1β ($p<0.0006$) cytokines.

Conclusion: Oil emulsion adjuvants containing FMDV O-146S antigen significantly boost humoral and cell-mediated immune responses to the FMDV O-146S antigen in mice.

Keywords: Foot and mouth disease, oil emulsion adjuvant, vaccine, immune response

Introduction

Foot-and-mouth disease (FMD) is a highly contagious and potentially fatal animal disease, according to the World Organization for Animal Health (WOAH), that primarily affects cloven-hoofed animals (1-3). The causative agent, foot-and-mouth disease virus (FMDV), is a member of the *Picornaviridae* family and belongs to

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the genus *Aphthovirus*. The symptoms of the disease include a sudden onset of fever and lesions on the mouth and the feet (4,5). The spread of FMD poses a significant risk to livestock, the agricultural sector, and the broader economy (6).

It is common practice to test FMDV vaccinations on susceptible pigs, cattle, and sheep for different types of vaccines. Comparatively, a mouse model can be used as a simple and inexpensive transitional model to test the immunological impact of a vaccine without the need for the extensive time, resources, and effort required by other animal models. Rodents, including water rats, moles, and brown house rats, have been shown to be susceptible to FMDV infection (7).

Water-in-oil (W/O) emulsion platforms are widely used in vaccine development due to their ability to enhance antigen presentation by modulating delivery to antigen-presenting cells (APCs) or through direct stimulation of immune cells (8). Commercially available adjuvants for FMDV vaccines include the mineral oil-based formulations such as Montanide ISA-206 and SA-201, as well as aluminum hydroxide. Oil emulsions are highly immunogenic because of their high reactogenicity (9).

This study aimed to evaluate, for the first time in mice, the immunogenic potential of novel W/O and water-in-oil-in-water (W/O/W) emulsion adjuvants developed by Coral Biotechnology, Inc. against the FMDV O-146S antigen.

Materials and Methods

Adjuvants

The emulsion adjuvants used in this study were Coralvac AT 318, Coralvac AT 318 SIS, and Coralvac 252. These adjuvants, listed in Table 1, were obtained from Coral Biotechnology, Inc. (Kartepe, Kocaeli, Türkiye).

FMDV O-146S antigen

Following approval from the Ministry of Agriculture and Forestry and the Foot-and-Mouth Disease Research Institute (Ankara, Türkiye) under permit number E-71037622-799-5127956, purified and sterilized FMDV O-146S antigen was obtained for this study. The antigen was supplied at a concentration of 110 µg/mL. Particles of the O-146S antigen, derived from FMDV strains, were used as the immunogen. The baby hamster kidney cell line (BHK21) was used for virus propagation.

Table 1. Emulsion adjuvants of water-in-oil (W/O) and water-in-oil-in-water (W/O/W) types used in the study.

Trade name	Emulsion type	Antigen/adjuvant (% v/v)
Coralvac AT 318	W/O/W	50/50
Coralvac AT 318 SIS	W/O/W	50/50
Coralvac 252	W/O	50/50

Preparation of Emulsion Adjuvant-Based FMDV O-146S Antigen-Containing Vaccine Formulations

Vaccine formulations were prepared by mixing 3 µg/mL of FMDV O-146S antigen with each adjuvant (Coralvac AT 318, Coralvac AT 318 SIS, or Coralvac 252) in a 50:50 ratio to obtain a final volume of 15 mL. The adjuvants were filtered through 0.22 µm sterile filters prior to use in the vaccine formulation.

To prepare the formulations, the antigen phase was first transferred to a beaker and stirred at 100 rpm using a mechanical mixer. While stirring, the adjuvant was slowly added to the antigen phase. For Coralvac AT 318, the stirring speed was increased to 400 rpm and continued for 30 minutes. For Coralvac 252, the speed was also increased to 400 rpm, and the mixture was stirred for 2 minutes before being transferred to a homogenizer, where it was homogenized at 15,000 rpm for 10 minutes.

The prepared vaccine formulations were stored at +4°C for 24 hours.

Physical Stability Test

This experimental setup was designed to assess the stability of formulations under varying temperature conditions. Each antigen-adjuvant formulation was placed in a 1 mL sterile Eppendorf tube and incubated at both 25°C and +4°C to compare the stability of W/O and W/O/W emulsion-based vaccines. Following incubation periods of seven days, two months, and six months under both conditions, the tubes were evaluated for phase separation and visual agglomeration to determine the physical stability of the formulations over time.

According to characterization data provided by the manufacturer, the particle size distribution of the new adjuvant formulations was determined using integrat-

ed light scattering with a Malvern Pro Red instrument. Measurements of particle diameter range, specific surface area, and surface-volume mean diameter were conducted at room temperature immediately after emulsion preparation and again after six months of storage. The volume fraction of oil in the diluted emulsions was approximately 1:1000 in all cases.

Experimental mice

All *in vivo* experiments were approved by the Ege University Animal Experiments Local Ethics Committee, with the decision numbered 2022-013 and dated January 25, 2023. Swiss albino mice, weighing 25–33 g, aged 4–6 weeks, were obtained from the Bornova Veterinary Control Research Institute. All mice were sexually mature at the time of acquisition. Prior to the experiments, the animals were acclimatized and quarantined at the Ege University Laboratory Animals Application and Research Center.

Immunological Studies

For this study, 25 Swiss albino mice (25–33 g, 4–6 weeks old) were divided into five groups (Table 2). Each mouse received a single intramuscular injection of 0.1 mL of FMDV O-146S antigen, either alone or formulated with W/O or W/O/W emulsion adjuvant. Vaccinations were administered into the hindlimb muscles on day zero. On day 21, the mice were euthanized; the injection sites were examined for local reactions, and blood and spleen samples were collected for immunological evaluation.

Detection of FMDV (O-146S-Ag)-Specific Antibody Response Using Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of specific antibodies against the FMDV O-146S antigen in serum samples were determined using an indirect enzyme-linked immunosorbent assay (ELISA), as previously described (11). Briefly, microplates were coated with FMDV O-146S antigen at a concentration of 5 µg/mL in 100 µL per well. Serum samples were diluted 1:200, and 100 µL of the diluted sera was added to each well.

Cytokine Analysis by ELISA

Serum samples from each group were analyzed for cytokine levels using commercial ELISA kits (FineTest, China), according to the manufacturer's instructions. The detection range of IL-4, IL-1β and IFN-γ was IL-4 (15.625-1000 pg/ml; catalog no. EM0119), IL-1β (12.5-800 pg/ml; catalog no. EM0109), and IFN-γ (31.25-2000 pg/ml; catalog no. EM0093), respectively.

Table 2. Vaccination groups and the number of mice in each group.

Number of groups	Number of mice	Vaccinated and control groups
1	5	Unvaccinated control (-)
2	5	Inactive FMDV (O-146S-Ag) control (+)
3	5	Inactivated FMDV (O-146S-Ag) (+) Coralvac AT 318 adjuvant
4	5	Inactivated FMDV (O-146S-Ag) (+) Coralvac AT 318 SIS adjuvant
5	5	Inactivated FMDV (O-146S-Ag) (+) Coralvac 252 adjuvant

Splenic Cell Proliferation Assay

The splenic cell proliferation assay was performed as previously described (11). For each vaccination group, spleen cells were divided into three subgroups: a non-stimulated control group, an FMDV-stimulated group (O-146S antigen), and a lipopolysaccharide (LPS)-stimulated group (12).

Mouse spleens were aseptically excised and mechanically disrupted using a sterile mesh in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with 10% bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) to obtain a homogeneous cellular suspension (13). The cell suspension was centrifuged at 450 g for 5 minutes and washed three times with RPMI-1640 medium. Cells were then resuspended in 7 mL of fresh medium and counted using a hemocytometer. A total of 5×10^7 cells/mL were seeded into 96-well plates (100 µL/well). Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2.

After 72 hours, splenocyte proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Mitochondrial dehydrogenase enzymes in proliferating cells convert MTT into a purple, water-insoluble formazan crystal, which is observed under an inverted microscope (14). The stimulation index (SI) was calculated using the following formula:

Stimulation index = (OD of stimulated cells – OD of unstimulated cells) / (OD of unstimulated cells)

Analysis of Cell-Mediated Immunity using Flow Cytometry

Using flow cytometry, the ratio of IFN-γ secreting

$CD3^+CD8^+$ cells was determined. To achieve this goal, 3 µg/mL of FMDV O-146S antigen was added to the wells. The microtiter plates were then incubated in an incubator at 37°C and 5% CO_2 for 72 hours (19). After incubation, the samples in the microplates were transferred one by one to Eppendorf tubes. The samples were centrifuged at 2000 rpm for 5 minutes, and the supernatant was removed.

After centrifugation, 50 µL of fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD3 molecular complex (BD Biosciences, USA), 50 µL of PerCP-conjugated rat anti-mouse CD8a (BD Biosciences, USA), 100 µL of fixation/permeabilization solution, and 50 µL of PE rat anti-mouse INF-γ (BD Biosciences, USA) antibodies were added to the Eppendorf tubes, respectively. Antibody working solutions were prepared separately by diluting 1 µL of anti-mouse CD3 antibody, 2.5 µL of anti-mouse CD8a antibody (in 3% fetal bovine serum [FBS]), and 2.5 µL of anti-mouse IFN-γ antibody in 1000 µL of phosphate-buffered saline (PBS) containing 3% FBS.

The samples were incubated at +4°C for 30 minutes in the dark at each stage and then centrifuged at 2000 rpm for 5 minutes between incubations. They were washed repeatedly twice with 250 µL of PBS containing 3% FBS. In the final stage, all samples were washed twice with 250 µL of washing solution, suspended in 300 µL, and analyzed in a flow cytometer device. Data analysis was conducted with BD Accuri™ C6 Software (BD Biosciences, USA).

Data Analysis

All data analyses and graphical presentations were conducted using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Statistical differences between groups were evaluated using analysis of variance (ANOVA) followed by post hoc testing. A p-value of <0.05 was considered statistically significant. Statistical significance was expressed as follows: n.s., not significant $p \geq 0.05$; $p<0.05$, significant.

Results

Stability of Prepared Oil Emulsion Adjuvant-Antigen Combinations

To evaluate the stability of the W/O and W/O/W emulsion formulations, each antigen-adjuvant combination intended for vaccination (FMDV O-146S antigen with Coralvac AT 318, Coralvac AT 318 SIS, and Coralvac 252) was incubated at two different temperatures.

At 25°C, none of the formulations showed visible agglomeration or phase separation after seven days. Following two months of incubation at this temperature, all formulations remained stable, except those containing Coralvac AT 318 and Coralvac AT 318 SIS, which exhibited signs of phase separation. After six months at 25°C, visual inspection revealed phase separation in all formulations (Figure S3).

At +4°C, all vaccine formulations were stable after seven days and two months. After six months of incubation at this temperature, phase separation was observed in all formulations, except for FMDV O-146S antigen combined with Coralvac AT 318 or Coralvac AT 318 SIS, which remained stable throughout the entire six-month storage period (Figure S4).

Particle size distribution of the emulsions was analyzed using integrated light scattering. Figures S1 and S2 display the particle size distributions immediately after preparation and after one year of storage. The resulting S-shaped curves indicate average droplet sizes ranging from 215.7 nm for Coralvac AT 318 to 249.2 nm for Coralvac 252 (Tables S1 and S2).

These findings suggest that +4°C was the ideal storage temperature for all formulations for a minimum of two months.

Determination of Specific Antibody Levels by Indirect ELISA

Twenty-one days after vaccination, serum samples were collected, and total immunoglobulin G (IgG) subclass antibody responses specific to the FMDV O-146S antigen were measured using indirect ELISA. Compared to the control group, mice that received the FMDV O-146S antigen formulated with adjuvants exhibited significantly higher absorbance values, indicating the development of an antigen-specific immune response.

As shown in Figure 1, all oil emulsion adjuvant formulations enhanced the production of IgG, IgG1, and IgG2a antibod-ies compared to the control group receiving antigen alone. Mice immunized with adjuvanted vac-cines displayed considerably higher IgG titers (****, $p<0.0001$). Specifically, IgG1 (associated with Th2-type responses) titers were increased in all adjuvant groups: FMDV O-146S

Table 3. Absorbance values (mean ± standard deviation) at 450 nm for antibody responses specific to foot-and-mouth disease virus (FMDV) O-146S antigen.

Groups	IgG	IgG1	IgG2a
Control (0.9% NaCl)	0.73 ± 0.09	0.30 ± 0.11	0.09 ± 0.01
FMDV (O-146S antigen)	1.56 ± 0.47	0.55 ± 0.10	0.30 ± 0.19
FMDV + Coralvac AT 318	3.45 ± 0.23	1.82 ± 0.25	1.56 ± 0.46
FMDV + Coralvac AT 318 SIS	3.44 ± 0.35	1.72 ± 0.27	1.56 ± 0.32
FMDV + Coralvac 252	3.39 ± 0.24	1.59 ± 0.42	1.40 ± 0.23

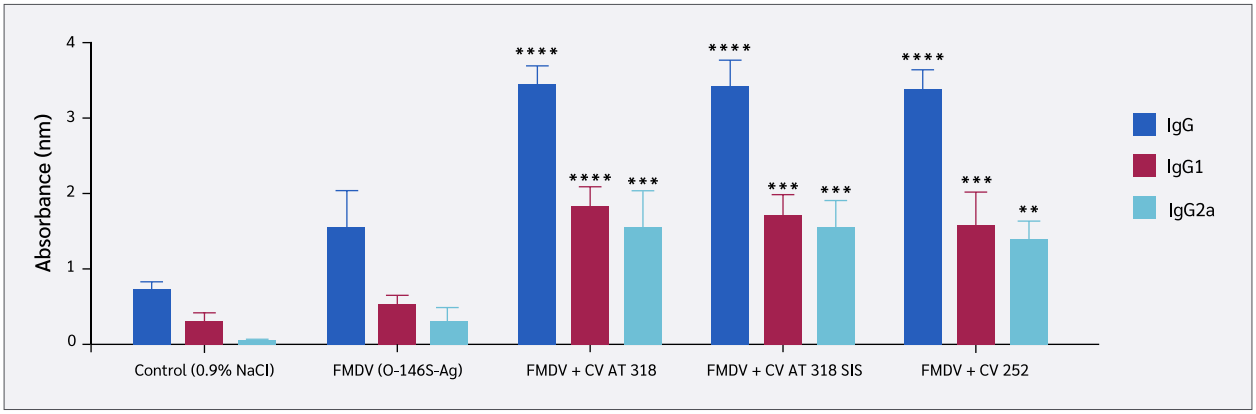

Figure 1. Absorbance values (450 nm) of foot-and-mouth disease virus (FMDV) O-146S antigen-specific antibody responses. **, $p<0.0018$; ***, $p<0.0002$; ****, $p<0.0001$.

antigen + Coralvac AT 318 (****, $p<0.0001$); FMDV O-146S antigen + Coralvac AT 318 SIS (***, $p< 0.0002$); and FMDV O-146S antigen + Coralvac 252 (***, $p<0.0005$).

Similarly, IgG2a antibody levels (indicative of Th1-type responses) were significantly higher in all adjuvanted groups compared to the control group: FMDV O-146S antigen + Coralvac AT 318 ($p<0.0002$); FMDV O-146S antigen + Coralvac AT 318 SIS ($p<0.0004$); and FMDV O-146S antigen + Coralvac 252 ($p<0.0018$) (Table 3 and Figure 1).

Determination of Cytokine Levels Following Vaccination with FMDV Formulation Groups

To evaluate the effects of each formulation on cytokine levels, serum samples from immunized mice were analyzed for Th1, Th2, and macrophage-associated cytokines. The higher levels of IFN-γ were detected in the serum from the mice immunized with FMDV O-146S antigen + CV AT 318, FMDV O-146S antigen + CV AT 318 SIS, and FMDV O-146S antigen + CV 252 compared to those from the mice immunized with the antigen alone ($p<0.0001$) (Figure 2).

Serum from mice immunized with FMDV O-146S antigen + CV AT 318 represented higher levels of IL-4 in $CD4^+$ T cells ($p<0.0002$) than serum from mice immunized with other adjuvanted groups; FMDV O-146S antigen + CV AT 318 SIS ($p<0.033$), and FMDV O-146S antigen + CV 252 ($p<0.034$) when compared with the control group (Figure 3). Serum from immunized mice showed statistically significantly increased levels of IL1-β in immune cells that produced it in the FMDV O-146S antigen + CV AT 318 ($p<0.0006$), FMDV O-146S antigen + CV AT 318 SIS ($p<0.0011$), and FMDV O-146S antigen + CV 252 ($p<0.0053$) groups compared to those from the mice vaccinated with the control group (Figure 4).

These results demonstrate that the FMDV O-146S antigen, formulated with Coralvac adjuvants, effectively stimulated a balanced immune response by prompting both

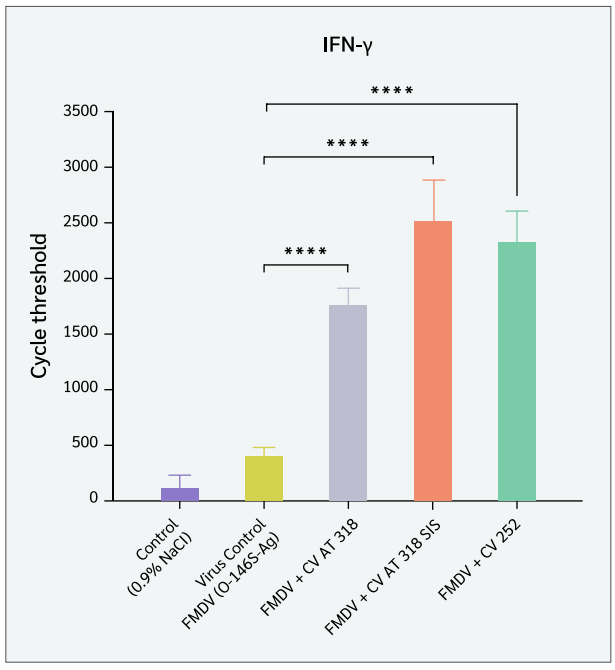

Figure 2. Effects of different FMDV vaccine formulations on interferon-γ (IFN-γ) cytokine responses. ****, $p<0.0001$.

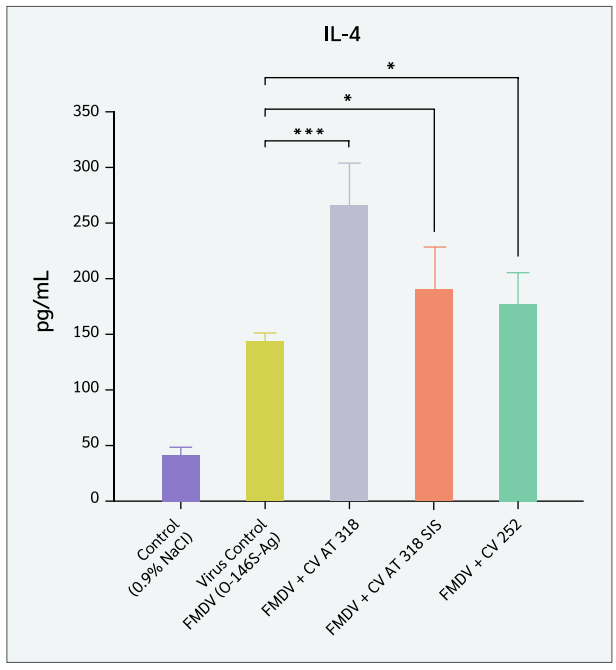

Figure 3. Effects of different FMDV vaccine formulations on interleukin-4 (IL-4) cytokine responses. *, $p<0.033$; ***, $p<0.0002$.

Th1 (IFN-γ) and Th2 (IL-4) cytokine production, as well as activating macrophage-mediated responses (IL-1β).

Evaluation of Splenocyte Proliferation in Immunized Mice

The MTT showed that the FMDV O-146S antigen + CV AT 318 ($p<0.0001$) and FMDV O-146S antigen + CV 252 ($p<0.0001$) groups exhibited significantly higher SI values and FMDV O-146S antigen + CV AT 318 SIS ($p<0.049$) compared to the group that received the FMDV O-146S antigen control alone (Table 4 and Figure 5).

Analysis of the Cellular Immune Response in Immunized Mice Using Flow Cytometry

All vaccine formulation groups exhibited an increase in $CD8^+$ T cell populations secreting IFN-γ after 72 hours of FMDV O-146S antigen stimulation. Notably, the $CD3^+CD8^+$ T cell immune response was significantly elevated in the FMDV O-146S antigen + CV AT 318, FMDV O-146S antigen + CV AT 318 SIS, and FMDV O-146S antigen + CV 252 groups compared to the control group ($p<0.0001$). The $CD3^+/CD8^+$ T cell ratio was approximately 20% across these three vaccine formulations (Figures 6a and S11).

The IFN-γ-secreting $CD8^+$ T cells were found to be significantly higher when FMDV O-146S antigen + CV AT 318

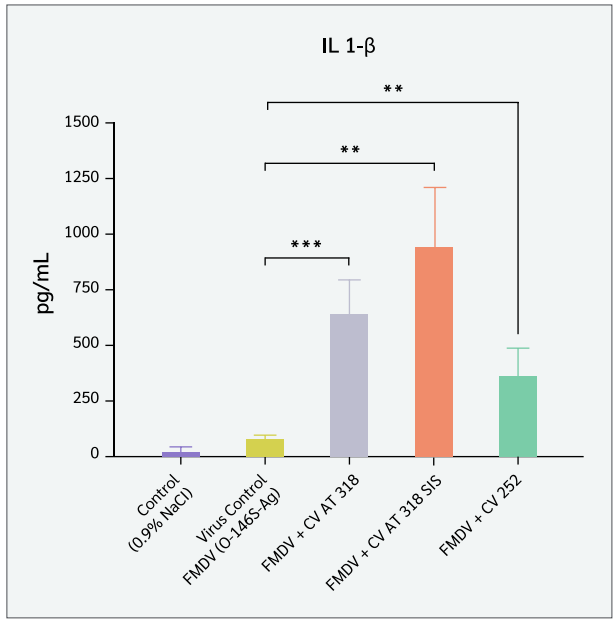

Figure 4. Effects of different FMDV vaccine formulations on interleukin-1β (IL-1β) cytokine responses. **, $p<0.053$; ***, $p<0.0006$.

(9.8%) vaccine combination was given ($p<0.0001$). In comparison to the control group, the FMDV O-146S antigen + CV AT 318 SIS group exhibited the second-highest response (7.9%) ($p<0.0001$), followed by the FMDV

O-146S antigen + CV 252 group (4.4%) (p<0.0005) (Figures 6b and S12).

When the mice were injected the FMDV O-146S antigen + CV AT 318, the IFN-γ producing CD3+ T cell ratio was 12.7%, which was statistically significantly higher than the virus control group (p<0.0001). It was 12.5% in mice that were given FMDV o 146S antigen + AT 318 SIS (p<0.0001) compared to the control group. It was demonstrated that FMDV O-146S antigen + CV 252 had the third-highest proportion of IFN-γ-secreting T cells (8.4%) among all the groups and was significantly different from the control group (p<0.0001) (Figures 6c and S13).

Discussion

The results of this study showed that the newly developed oil emulsion adjuvants significantly enhanced adaptive immune responses and exhibited strong adjuvant properties when combined with the FMDV O-146S antigen-containing vaccine formulation in mice. Adding an adjuvant to a co-administered antigen enhances the immunogenicity of the substance by stimulating a more robust and durable immune response (16). The use of a mouse model for evaluating vaccines offers several advantages, primarily in terms of reducing cost and time by allowing the assessment of the immune response before testing them in the primary host. The pathogenesis of FMDV in mice is influenced by multiple factors, including viral characteristics, the animal strain, and the method of viral penetration (17). Results from studies on target species often balance results from mouse studies. Therefore, the mouse model can reliably predict the immune response to foot-and-mouth disease viruses in cattle and pigs (17,18). Furthermore, several studies have used mice to evaluate the effectiveness of vaccines in both mice and cattle, and the results have been similar for both species (16,19,20).

Emulsion adjuvant studies have demonstrated that antigens can adsorb at the oil-water interface through both hydrophobic and electrostatic forces, resulting in significant inter-protein interactions and conformational changes in the adsorbed protein (19,20). In the current study, visual inspection of phase separation variations was used to investigate the thermal stability of FMDV vaccines across various temperatures. Inactivated FMDV is relatively stable at +4°C, but it exhibits poor stability at

Table 4. Stimulation index (SI) values (mean ± standard deviation) of splenocytes in response to lipopolysaccharide (LPS) and foot-and-mouth disease virus (FMDV) O-146S antigen.

Groups	LPS	FMDV
Control (0.9% NaCl)	0.57 ± 0.04	0.46 ± 0.05
FMDV (O-146S antigen)	0.58 ± 0.04	0.48 ± 0.06
FMDV + Coralvac AT 318	0.88 ± 0.04	0.61 ± 0.15
FMDV + Coralvac AT 318 SIS	0.62 ± 0.10	0.51 ± 0.13
FMDV + Coralvac 252	0.66 ± 0.05	0.57 ± 0.07

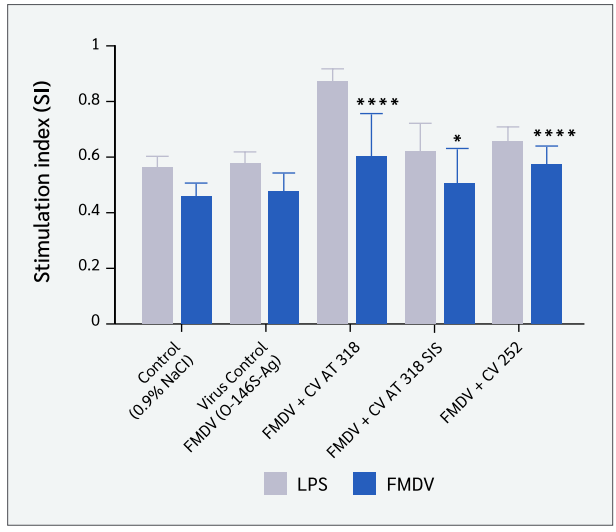

Figure 5. Splenocyte proliferation index (SI) values in response to different FMDV vaccine formulations. *, p<0.049; ****, p<0.0001.

temperatures above 37°C, consistent with findings from studies on the live virus (21). Problems with early *in vitro* characterization of the vaccine may lead to the loss of the effective antigens going undetected and, ultimately, to a failed immunization (22). As a result, there is an urgent need for an FMDV vaccination with greater stability of vaccine formulation. Figures S3 and S4 present the findings of this study, which examine the stability of FMDV O-146S antigen-containing vaccine formulations with new adjuvants over time. The results demonstrate that vaccines are more stable when stored at 25°C for seven days and at +4°C for two months.

Previous research from our group demonstrated that when the adjuvant was evaluated independently, it did not induce any adverse effects or elicit a reaction against

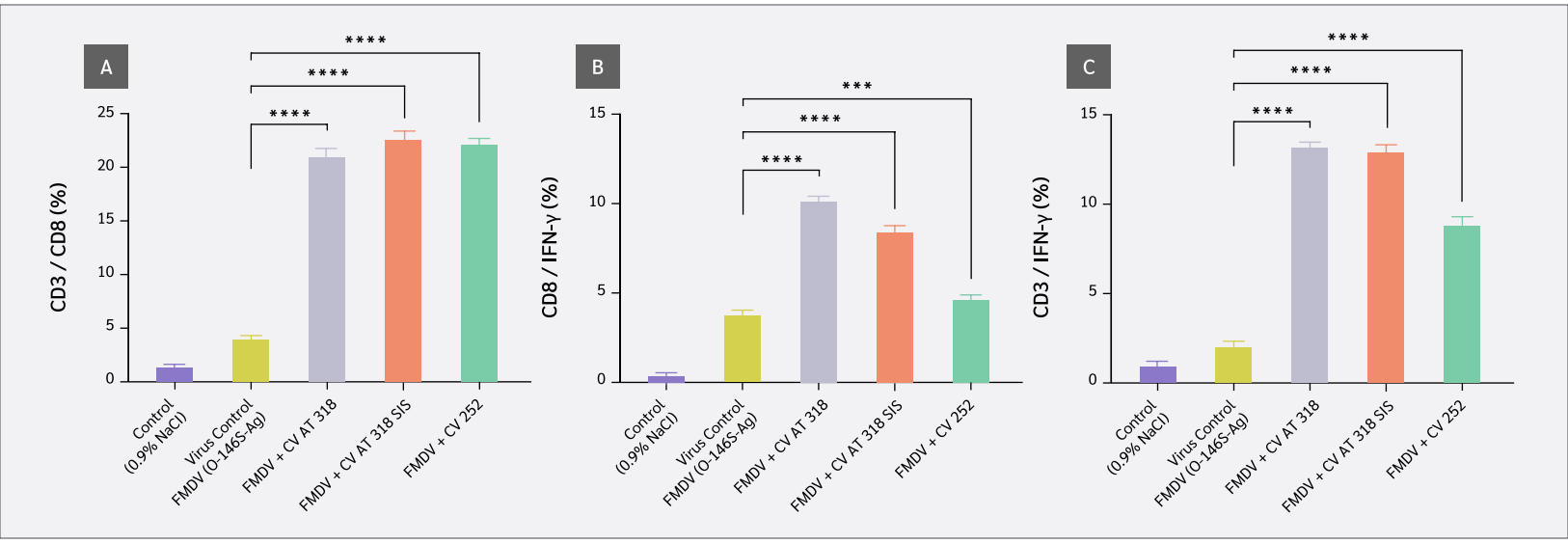

Figure 6. Effects of different FMDV vaccine formulations on splenic $CD3^+$ and $CD8^+$ T cell responses and interferon-γ (IFN-γ) production. ***, $p<0.0005$; ****, $p<0.0001$.

itself (23). The induction of neutralizing antibodies is responsible for the protective immunity to the antigen. Vaccines are designed primarily to induce specific humoral immunity. Antibody induction is typically credited as the cause of protective immunity against FMDV (24). Therefore, the efficacy of an adjuvant depends on its ability to induce faster, stronger, and more sustained responses from the immune system in the form of neutralizing antibodies (25). Specifically, total IgG antibody titers and neutralizing antibody responses can be effectively induced against FMDV using newly designed oil emulsion adjuvants. Immunization-induced IgG subclass is a predictor for the balance between Th2- and Th1-type cytokines (26).

In general, IgG2a antibodies are associated with Th1 cells, while IgG1 antibodies are induced by Th2 cells (27,28). Both IgG1 and IgG2a may have potent neutralizing abilities; however, IgG2a is more likely to contribute to virus clearance due to its important interaction with complement components and Fc receptors (29). The effectiveness of FMD vaccinations can be estimated indirectly by analyzing serological reactions following vaccination, as humoral immunity is linked to clinical protection against FMD (30). The antibody isotype response may indicate the kind of immune activity (Th1 vs. Th2) occurring *in vivo* (31). In this study, IgG isotypes against the FMDV O-146S antigen were detected using an indirect ELISA method. Following a single intramuscular injection, mice immunized with oil adjuvant formulations containing inactivated FMDV produced FMDV O-146S antigen-specific IgG1 and IgG2a, indicating the stimulation of Th1 and Th2 cells, which will have a significant effect in providing good protection against FMD virus infection through viral clearance.

A higher lymphoproliferative response was observed in mice injected with the inactive FMDV O-146S antigen + CV AT 318, FMDV O-146S antigen + CV AT 318 SIS, and FMDV O-146S antigen + CV 252 vaccine groups, as determined by studies of virus-specific cellular response. These findings suggest that the use of these adjuvants enhances the adaptive immune response against the FMDV O-146S antigen. Immunizing mice with FMDV O-146S antigen has been demonstrated to stimulate T-cell responses and increase the number of $CD8^+$ T cells in the spleen (32). In addition, oil emulsion formulations of FMDV cause FMDV O-146S antigen-specific $CD8^+$ T cells to proliferate and secrete IFN-γ. It is generally known that IFN-γ plays a role in switching the isotype of immunoglobulins, which ultimately results in a greater number of IgG2a types (33). This result is consistent with the high amounts of IgG2a that were acquired, as well as the proliferation levels that were found in the FMDV + CV AT 318, FMDV + CV AT 318 SIS, and FMDV O-146S antigen + CV 252 groups. These findings suggest that FMDV O-146S antigen adjuvanted with newly designed oil emulsion adjuvants elicits a significant cellular response.

Conclusion

The results of this study reveal that both humoral and cell-mediated immune responses against FMDV O-146S antigen can be developed in mice given a vaccine containing FMDV O-146S antigen formulated with novel W/O and W/O/W type emulsion adjuvants.

Ethical Approval: All *in vivo* experiments were approved by the Ege University Animal Experiments Local Ethics Committee, with the decision numbered 2022-013 and dated January 25, 2023.

Informed Consent: N.A.

Peer-review: Externally peer-reviewed

Author Contributions: Concept – A.N.; Design – A.N.; Supervision – A.N.; Fundings – A.N.; Materials – A.N.; Data Collection and/or Processing – A.N., K.A.M.H.A.; Analysis and/or Interpretation – A.N., K.A.M.H.A.; Literature Review – A.N., K.A.M.H.A.; Writer – A.N., K.A.M.H.A.; Critical Reviews – A.N.

Conflict of Interest: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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Efficacy of the Thymus Polypeptide Fraction Biomodulina T in Children with Thymic Hypoplasia and Recurrent Infections

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Abstract

Objective: Thymic hypoplasia (TH) in children is a known cause of recurrent infections, often indicative of immunodeficiency. This study aimed to evaluate the efficacy of Biomodulina T, a thymic polypeptide fraction, in pediatric patients with TH, with or without associated cellular immunodeficiency.

Materials and Methods: A non-controlled, phase III clinical trial was conducted among children aged 1–5 years (n=60) and registered in the Cuban Public Registry of Clinical Trials/ International Clinical Trials Registry Platform (ID code RPCEC00000247). Patients were divided into two groups: Group I (n=44), with TH without cellular immunodeficiency; and Group II (n=16), with TH and cellular immunodeficiency. Biomodulina T was administered intramuscularly (IM) in two 4-week cycles, separated by a 4-week rest period. Patients who had not achieved normal thymic size by week 16 received a third, 8-week cycle.

Results: Both groups showed significant increases in thymic size from baseline ($p<0.0001$), with no significant difference between them. The mean increase was 67% (95% CI 61–73%), and 86.5% of patients completed treatment with thymic size within the normal range. Bacterial infections decreased by 91.5%, and viral infections by 80.7%, accompanied by a reduction in antibiotic use. In patients with cellular immunodeficiency, Biomodulina T significantly increased in $CD4^+$ T lymphocytes ($p=0.018$), while no significant changes were observed in $CD8^+$ T cells, $CD19^+$ B cells, and $CD56^+$ natural killer (NK) cells. Serum immunoglobulin A (IgA) levels also increased significantly. Overall, 82.7% of patients were classified as improved, showing both normalization of the thymic size and a 50% reduction in infections.

Conclusion: Biomodulina T was clinically effective in the treatment of TH in children, regardless of the presence of cellular immunodeficiency.

Keywords: Thymic hypoplasia, cellular immunodeficiency, infections

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Introduction

The thymus is a primary lymphoid organ essential for the development and maturation of immunocompetent T cells. Its size decreases progressively throughout life, with involution typically beginning after puberty (1,2). Both genetic and environmental factors regulate thymic development. Thymic hypoplasia (TH) and aplasia refer to a small or absent thymus, particularly in small children. These conditions are most commonly associated with DiGeorge Syndrome and other congenital T-cell disorders (3,4). Additionally, viral and bacterial infections can cause thymic atrophy or impair its regenerative capacity (1,2).

Although TH is not highly prevalent, it poses a substantial burden on affected children, their families, and healthcare systems. It is associated with increased risk of morbidity and mortality, high treatment costs, and excessive use of antibiotics (5). Currently, no validated treatment exists specifically for TH. However, thymic extracts have shown promising clinical results. They influence the T cell development, maturation, and cytotoxic function, as well as enhance phagocytic and cytotoxic activity (6).

Biomodulina T (BIOCEN, Cuba) is an injectable bovine thymic extract registered in Cuba as an immunomodulatory biological drug, containing low-molecular-weight polypeptides (7). It is indicated for conditions characterized by cellular immunological dysfunction, such as recurrent infections in the elderly. Biomodulina T stimulates lymphoblastoid mitosis and influences the maturation and differentiation of T lymphocytes (7,8). Recent studies in older adults demonstrated increases in naïve $CD4^+$ and $CD8^+$ T cells, the $CD4^+/CD8^+$ ratio, as well as B and natural killer (NK) cells (9-12).

Between 1999 and 2007, Mollineda et al. (13), Christian et al. (14,15), and Rabassa et al. (16) were the first to report the use of Biomodulina T for treating TH in pediatric patients with recurrent infections. These studies showed favorable clinical outcomes, although immunological responses were not evaluated.

The objective of the present study was to evaluate the efficacy of Biomodulina T in a clinical trial involving pediatric patients with TH, with or without associated cellular immunodeficiency. In addition to clinical outcomes and changes in thymic size, the study also assessed immunological parameters, including $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets, in patients with cellular immunodeficiency.

Materials and Methods

Study Design

This study was designed as a single-arm, phase III, multicenter, non-controlled, non-randomized clinical trial involving two groups of pediatric patients receiving treatment. The trial was prospectively registered in the Cuban Public Registry of Clinical Trials (ID code RPCEC00000247), a database recognized by the World Health Organization's (WHO) International Clinical Trials Registry Platform.

The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki, the Good Clinical Practice (GCP) guidelines, and the national regulatory standards established by the Center for State Control of Medicines, Equipment, and Medical Devices (CECMED, Cuba). The study protocol was approved by the Ethics Committee of the Institute of Hematology and Immunology (Cuba) on December 13, 2016. Written informed consent was obtained from the parents or legal guardians of all participants prior to enrollment.

Patient Selection

Children aged 1 to 5 years, of any gender, who attended the immunology service at the Institute of Hematology and Immunology and at the Pediatric Hospital "Angel Arturo Aballí", were screened for inclusion. Patients were divided into two groups. Group I included children with TH and clinical manifestations of immunodeficiency, but without a confirmed laboratory diagnosis of cellular immunodeficiency. Group II included patients with TH, clinical symptoms, and a confirmed laboratory diagnosis of cellular immunodeficiency.

Thymic hypoplasia was diagnosed when the thymic area measured less than 1000 mm^2. Severity was classified as follows: severe for values less than 500 mm^2, moderate between 500 and 799 mm^2, and mild between 800 and 999 mm^2 (16). Cellular immunodeficiency was diagnosed based on age-specific reference values for $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, $CD19^+$ B cells, $CD3^-CD56^+$ NK cells, and the $CD4^+/CD8^+$ T cell ratio, following the criteria of Comans-Bitter et al. (17).

Table 1. Treatment scheme.

Weeks	Group I		Group II		
	Severe TH	Moderate TH	Severe TH	Moderate TH	Mild TH
1 to 4	1 vial IM 2×/week	1 vial IM weekly	1 vial IM 3×/week	1 vial IM 2×/week	1 vial IM weekly
5 to 8	Rest				
9 to 12	1 vial IM weekly		1 vial IM 2×/week	1 vial IM weekly	
13 to 16	Rest period				
17 to 24	1 vial IM weekly				
25 to 32	Rest period				

TH: Thyroid hormone, **IM:** Intramuscular.

Exclusion criteria included: a history of severe allergic reactions or severe generalized eczema; diagnosis of DiGeorge syndrome or other non-cellular immunodeficiencies (except IgA deficiency); steroid use within the previous 45 days; malignancy or autoimmune disease; chronic diseases such as type I diabetes mellitus (DM) or other decompensated endocrinopathy; severe congenital malformations requiring ongoing medical or surgical intervention; and hemoglobin levels below 9 g/L.

Treatment

All patients received intramuscular (IM) Biomodulina T (3 mg; BIOCEN, Cuba). Treatment consisted of an initial 4-week cycle, followed by a 4-week rest period, and then a second 4-week cycle, again followed by a 4-week rest period. Within each treatment cycle, patients received 1, 2, or 3 weekly doses depending on TH severity (Table 1). Patients whose thymic size had not normalized by week 16 received a third treatment cycle of one IM vial per week for 8 weeks, followed by an additional 8-week rest period. Patients were evaluated at the end of weeks 8, 16, 24, and 32.

Concomitant treatments included oral vitamin C and vitamin A, administered daily throughout the study period. Allergic children were also given available oral antihistamines as needed.

Outcomes and Evaluations

The primary outcome of the study was the thymic area, assessed by ultrasonography using real-time mobile ultrasound devices (Philips Affiniti 70 and MINDRAY MCI) equipped with high-resolution pediatric transducers. Scans were performed in sagittal parasternal (angled from the upper edge of the second rib to the lower edge of the fourth rib at the sternum) and axial trans-sternal planes at the level of the sternal angle. All measurements were taken during the expiratory phase. The ultrasonography was performed at baseline, and at weeks 16 and 32. Secondary outcomes included the frequency and etiology of infections (categorized as bacterial, viral, fungal, or parasitic), antibiotic consumption, and hospital admissions.

A categorical composite variable termed the General Clinical Response Criterion (GCRC) was employed to assess overall treatment efficacy. Patients were classified as follows:

- **Better:** Normalized thymic area and ≥50% reduction in infection frequency compared to baseline
- **Worse:** Unchanged or reduced thymic area and stable or increased infection frequency
- **Equal:** Any outcome not meeting the criteria for "better" or "worse"

Lymphocyte subpopulations were quantified using flow cytometry (GALLIOS cytometer, Beckman Coulter, USA) with fluorochrome-conjugated monoclonal antibodies (anti-CD3-PC5, CD4-FITC, CD8-PE, CD56-APC; Dako, Denmark; Miltenyi Biotec, Germany). Serum levels of total IgG, IgM, and IgA were measured using immunoturbidimetric assays (SPAPLUS, The Binding Site, UK).

Statistical Analysis

The statistical hypothesis was based on the results of prior studies conducted in Cuba (13-15). Using a linear

regression model, the expected slope of the thymic area was 45.9 ± 6.71 mm²/month. Sample size was calculated according to Dupont and Plummer (18) for regression-based designs, with α=0.05 and 1-β=0.9. Assuming a 60% increase in the thymic area at week 32, 55 patients were required. To account for potential dropouts, the final sample size was set at 60.

Statistical analyses were performed using SPSS version 25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Normality of data distribution was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests to determine the suitability for parametric analysis. Continuous variables were analyzed using two-way ANOVA and mixed-effects models, as appropriate. Categorical variables were compared using Fisher's exact test. A *p*-value <0.05 was considered statistically significant.

Results

The study included 60 patients: 44 in Group I and 16 in Group II (Table 2). A total of 52 patients (86.6%) completed the treatment regimen and were evaluated at week 32, thus providing valid data on the primary variable (thymic area) (Figure 1, CONSORT flow diagram). There were eight treatment interruptions (13.3%), four in each group. The leading causes of interruption were voluntary withdrawal or worsening clinical conditions.

The median age of participants was 2 years. Baseline demographic characteristics and risk factors are shown in Table 3. Overall, no significant differences were observed between the groups, except for a higher frequency of personal history of allergic diseases in Group I. No significant differences in perinatal history were found between groups.

The thymic area increased as the treatment progressed, with a significant difference from baseline at week 16, and a more pronounced increase by week 32 (p<0.0001) (Figure 2). Both groups showed similar trends without significant intergroup differences. The mean increase by the end of treatment was 67% (95% CI 61–73%) compared to baseline. At the individual level, 45 of the 52 patients (87%) achieved a thymic area within the normal range, demonstrating high efficacy of the treatment for the primary outcome.

Consistent with the increase in thymic area, the frequency of infections decreased progressively, with a significant reduction observed as early as week 8 (p<0.0001), and no significant differences between groups (Figure 3A). The mean reduction in infection frequency was 84% (95% CI 70–95%) compared to the baseline. Bacterial and viral infections predominated; fungal and parasitic infections were rare. The reduction in bacterial and viral infections was significant by week 8 (p<0.0001), with a slightly greater effect observed for bacterial infections (Figure 3B). Correspondingly, antibiotic use declined significantly from week 8 onward (p<0.0001) (Figure 3C). While 96.5% of patients used antibiotics at baseline, this figure fell to 7.69% by week 32, with no hospital admissions reported.

The composite variable CGRC, which integrates thymic area and infection frequency, showed that no patients were classified as "worse", and 82.7% were classified as

Table 2. Patients according to TH classification and assigned group.

TH Classification	Group I (without cellular immunodeficiency) n (%)	Group II (with cellular immunodeficiency) n (%)	Total n (%)
Severe	6 (13.6)	2 (12.5)	8 (13.3)
Moderate	38 (86.4)	10 (62.5)	48 (80.0)
Mild	0 (0)	4 (25.0)	4 (6.7)
Total	**44 (100)**	**16 (100)**	**60 (100)**

TH: Thyroid hormone.

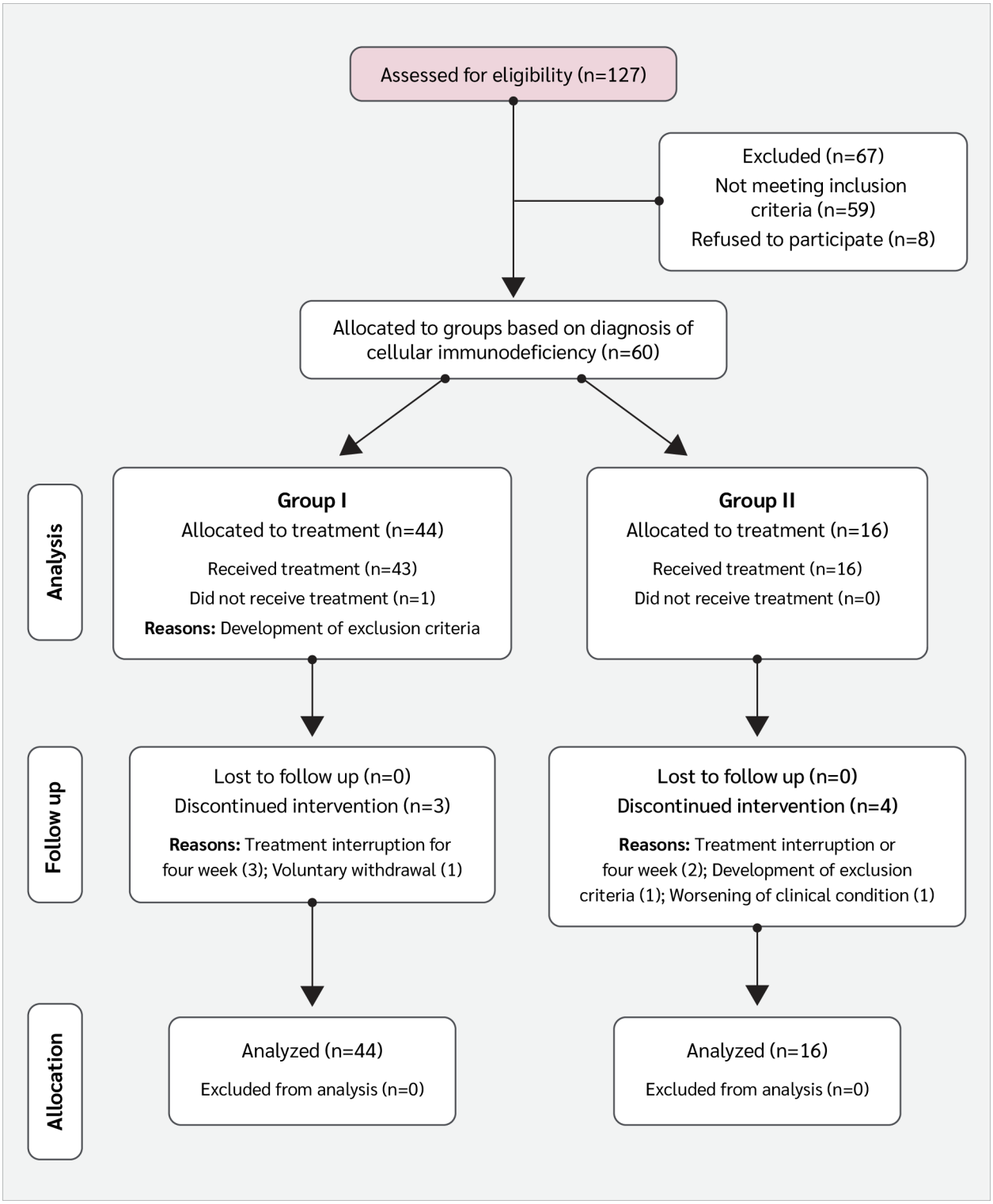

Figure 1. CONSORT flow diagram of the clinical trial.

Table 3. Demographic data and underlying disease characteristics by study group.

Characteristic	n=60	Group I (without cellular immunodeficiency) n=44	Group II (with cellular immunodeficiency) n=16	*p*-value[1]
Age, years				
Median (IQR)	2.00 (1.00–2.25)	2.00 (1.00–3.00)	1.00 (1.00–2.00)	0.52
Mean (SD)	1.90 (0.99)	1.95 (0.94)	1.75 (1.13)	
Sex, n (%)				
Female	32 (53.3)	23 (52.3)	9 (56.3)	0.78
Male	28 (46.7)	21 (47.7)	7 (43.8)	
Skin color, n (%)				
White	38 (63.3)	29 (65.9)	9 (56.3)	0.70
Mestizo	19 (31.7)	13 (29.5)	6 (37.5)	0.70
Negro	3 (5.0)	2 (4.6)	1 (6.3)	0.70
Asthma FPH, n (%)	41 (68.3)	28 (63.6)	13 (81.3)	0.19
Allergic diseases FPH, n (%)	50 (83.3)	34 (77.3)	16 (100.0)	0.050
Mean weight (SD), kg	13.33 (3.01)	13.59 (3.14)	12.63 (2.57)	0.24
BMI, mean (SD)	16.73 (2.13)	16.76 (2.29)	16.63 (1.67)	0.80

[1] Student's t test; Chi-square test of independence; Fisher's exact test.
IQR: Interquartile range, **SD:** Standard deviation, **FPH:** Family pathological history, **BMI:** Body mass index.

"better", with no significant differences between groups. According to the intention-to-treat analysis, which included patients who discontinued treatment, 71.7% demonstrated improvement based on the CGRC (Table 4).

Evaluations of T cell markers ($CD4^+$ and $CD8^+$), as well as B lymphocytes ($CD19^+$) and NK cells ($CD56^+$), were performed in Group II at baseline and week 32. A significant increase was observed in the relative proportion of $CD4^+$ T cells (p=0.018) and the sum of $CD4^+$ and $CD8^+$ T cells (p=0.016) (Figure 4). The CD4/CD8 ratio showed an increasing trend, although it did not reach statistical significance (p>0.05). No significant changes were detected in B lymphocyte and NK cell populations (data not shown).

Among the enrolled patients, 13 had decreased serum IgA concentrations. A significant increase in IgA was observed, from a mean of 0.41 (± 0.13) to 1.58 (± 1.32) (p=0.013; Wilcoxon signed rank exact test) at week 32.

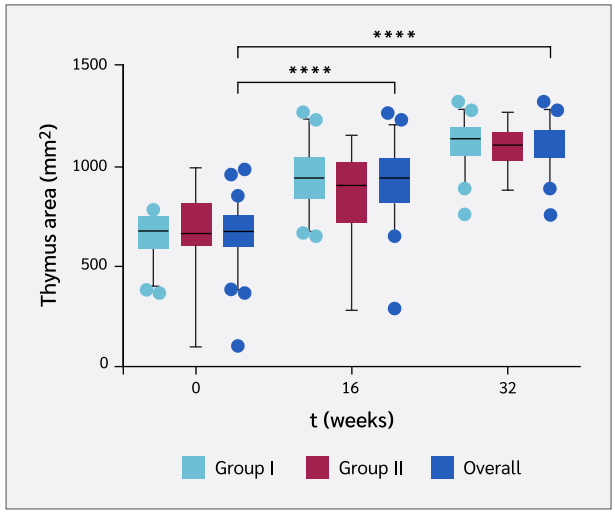

Figure 2. Increase in the thymic area following Biomodulina T treatment.

**** Indicates statistically significant difference (p<0.0001) compared to baseline (week 0) for both groups and the total sample; analysis performed using mixed-effects model.

Table 4. General results of the categorical clinical variables and comparison between treatment groups.

Characteristic	n=60	Group I (without cellular immunodeficiency) n=44	Group II (with cellular immunodeficiency) n=16	*p*-value[1]
Thymic area, n (%)				
Reaches normal value	45 (86.5)	35 (87.5)	10 (83.3)	0.65 (ns)
Equal	7 (13.5)	5 (12.5)	2 (16.7)	
Could not be determined	8	4	4	
Infections, n (%)				
Decrease ≥ 50%	48 (92.3)	36 (90.0)	12 (100)	0.56 (ns)
Decrease 0 – 50%	2 (3.85)	2 (5.00)	0 (0)	
Increase	2 (3.85)	2 (5.00)	0 (0)	
Could not be determined	8	4	4	
CGRC clinical response, n (%)				
Better	43 (82.7)	33 (82.5)	10 (83.3)	1.00 (ns)
Equal	9 (17.3)	7 (17.5)	2 (16.7)	
Worse	0	0	0	
Could not be determined	8	4	4	

[1] Fisher's exact test comparing both groups.
CGRC: Clinical global response to chelation, **ns**: Not significant.

Normal IgA values were restored in 4 patients (30.8%, p=0.012). No significant changes were observed in IgG and IgM levels.

Discussion

The present study evaluated the administration of the thymic polypeptide fraction Biomodulina T in children with TH. The results demonstrate not only clinical benefits, evidenced by a sharp reduction in infections associated with the increase and normalization of the thymic size, but also an increase in immunological markers such as the proportion of $CD4^+$ T cells in peripheral blood and the concentration of serum IgA antibodies.

The thymus is highly active during the prenatal period and grows naturally after birth, primarily during the first 4 to 6 months of life (1,2). Although it continues to increase in weight until puberty, its proportional size relative to the mediastinum decreases. After puberty, the gland undergoes gradual physiological involution. The size of the thymus, as well as its growth, has been associated with prenatal nutrition, birth weight, and breastfeeding (1,2). Children with a small thymus in the first years of life often present clinically as immunodeficient. Currently, the measurement of the thymic area is a recognized tool in the clinical practice of pediatric immunology in Cuba (19-22). These findings support further exploration of the relationship between TH and infection susceptibility in children and align with the results observed in the present investigation.

The lack of a control group in this study could be a potential source of bias. However, a control group was not included due to ethical concerns; it was not considered appropriate to withhold a treatment already in routine clinical practice in Cuba, even if it is not yet an officially

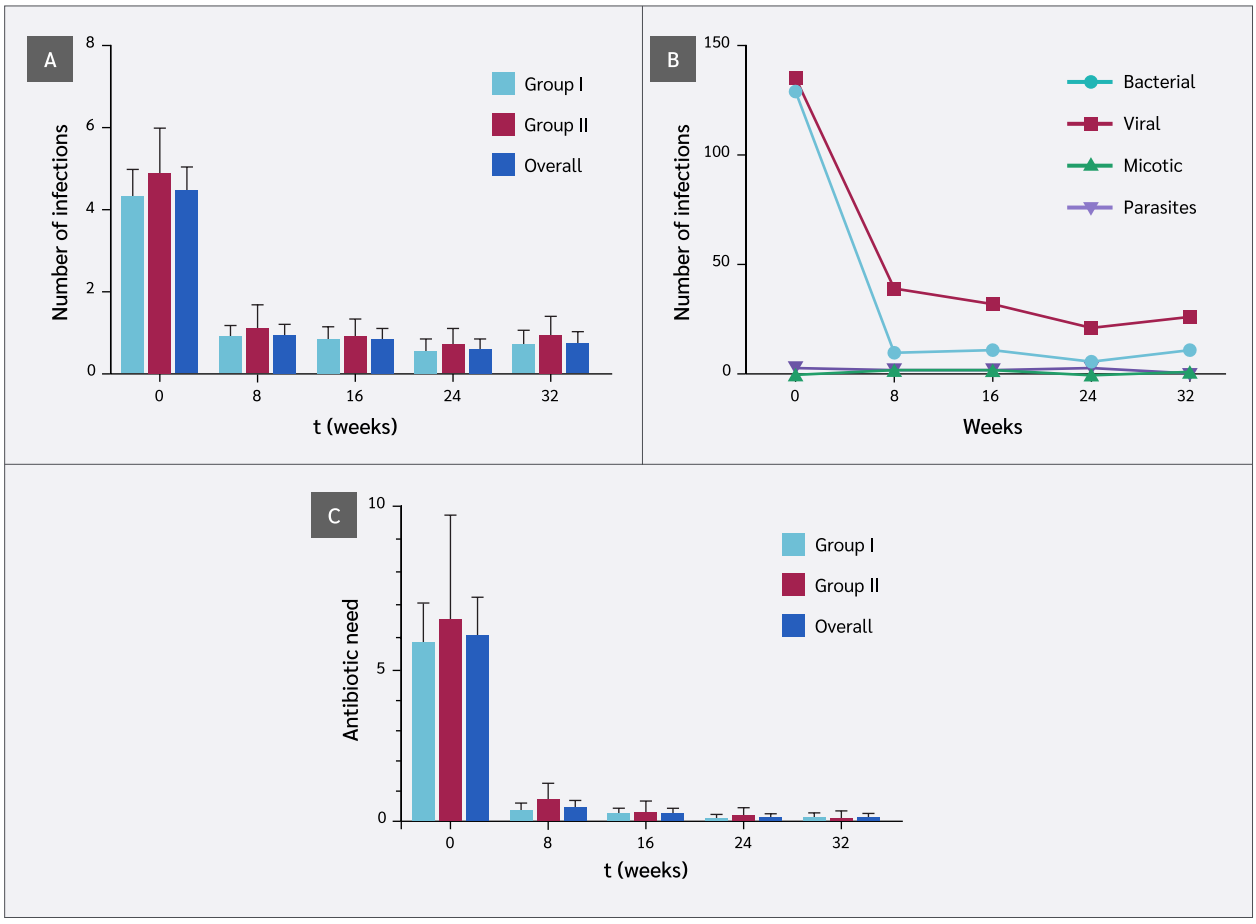

Figure 3. (A) Reduction in infection frequency by treatment group. (B) Reduction by infection etiology (bacterial vs. viral). (C) Decrease in antibiotic use.

For (A) and (C), all post-baseline time points showed a significant difference vs. baseline ($p<0.0001$, mixed-effects model). For (B), bacterial infections showed a significantly greater reduction than viral infections ($p=0.011$, ANOVA with Tukey post hoc test).

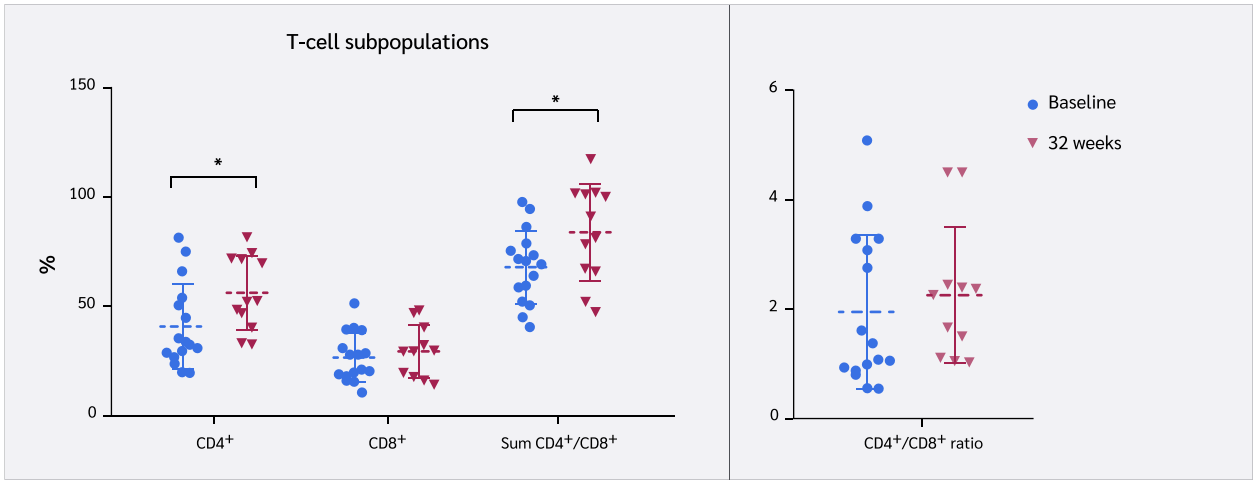

Figure 4. Effect on the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subpopulations and the sum of both at week 32 compared to baseline in Group II.

Values represent mean ± standard deviation.
*$p=0.018$ for $CD3^+CD4^+$ and $p=0.016$ for the sum, Student's t-test with Holm-Sidak correction.

approved indication, particularly given the absence of alternative curative options. Nevertheless, the demonstrated relationship between clinical results and the increase in thymic size and with T cell-dependent immune markers in a subset of patients adds robustness to the conclusions. The observed rate of thymic enlargement was consistent with the statistical hypothesis of the study (an increase above 60% within 32 weeks), which was based on data from previous controlled studies conducted in Cuba between 1999 and 2007. Given the age range of the patients (1 and 5 years) and their previous clinical histories, the outcome could hardly be explained by a spontaneous growth of the gland, not associated with the intervention.

Thymic extracts contain several polypeptides capable of promoting T lymphocyte maturation (6). Among these, pro-thymosin α and its terminal fragment thymosin α1 have received special attention (22-25). Thymosin α1, also known as timalfasin, is an approved drug in several countries, indicated for immune reconstitution and the treatment of viral and bacterial infections, particularly in older adults. In addition to its immunopotentiating activity, pro-thymosin α plays a role in intracellular processes related to cell growth (23). Another major component of thymic extracts, thymosin β4, has a powerful tissue regenerative action (26). The exact mechanisms of thymic involution and its reversal remain poorly understood. Experimental data from mouse models suggest that thymic stromal cells, rather than hematopoietic cells, drive thymic involution (27,28). Other substances, such as growth hormone, have also shown efficacy in immune system reconstitution and enhancement of thymic mass in HIV patients (28). These pharmacological activities of thymic polypeptides seem to be consistent with the findings of the present study, supporting both immune responses and thymic growth.

Previous evaluations of Biomodulina T have investigated its immunological mechanisms of action, primarily in older adults and oncology patients (9-12). However, previous clinical research in children did not address its immunological effects. In older adults, Biomodulina T has been shown to significantly increase naïve T cells (both $CD4^+$ and $CD8^+$), as well as $CD19^+$ B lymphocytes and $CD56^+$ NK cells, the latter being key components of innate antiviral immunity. In a recent clinical trial conducted in nursing homes in Cuba, Biomodulina T reduced COVID-19 lethality in a preventive setting (11). In the present study, results from children, particularly those with cellular immunodeficiency, demonstrated an increase in $CD4^+$ T cells and a non-significant trend toward increased $CD8^+$ T cells. The CD4/CD8 ratio also showed a non-significant tendency to increase within the normal range. The small sample size (only 12 patients) and variability of flow cytometry measurements limited statistical precision. Nevertheless, these results are consistent with previous findings in older adults. An increased CD4/CD8 ratio has been associated with improved responses to bacterial and viral infections (29). The results also demonstrated clinical efficacy against viral infections, although no significant effect was observed on $CD3^-CD56^+$ NK cells.

Of particular relevance is the observed increase in IgA antibodies, which are an important component of the adaptive immune response at the mucosal level. The association between decreased IgA levels and reduced thymic size may be explained by the immaturity of the immune system during the first year of life and the role of Th cells in antibody class switching (30). In addition, the high prevalence of allergic conditions in this study, particularly in the group with cellular immunodeficiency (with more than half of Group I and all of Group II exhibiting allergic symptoms), constitutes a predisposing factor for reduced IgA levels.

Few studies have reported the use of thymic extracts or synthetic thymic peptides in the treatment of aplasia or TH in children. An early study in 1989 provided preliminary evidence of clinical benefit in a small group of children with DiGeorge syndrome (31). De Mattia et al. (32) administered a bovine thymic extract to pediatric patients with recurrent infections and observed a statistically significant improvement in lymphocyte-dependent antibacterial activity, along with clinical benefits in 3-year-old children.

In Cuba, several pioneering studies have investigated children with TH, including populations similar in age to those in the present trial (13-15). In a 1996 study involving children aged 8 months to 6 years with recurrent infections, the effect of Biomodulina T (34 patients) was compared to that of Levamisole. Biomodulina T induced a mean increase in thymic area from 520.38 mm^2 to 907.35 mm^2, representing a 74.3% increase (14). In a subsequent report, eutrophic children with recurrent infections and a thymic area <1000 mm^2 were studied. Patients treated with Biomodulina T consistently showed a greater increase in thymic area, from 603.02 mm^2 to 1003.62 mm^2, a 66% increase (15). In the present study,

92.3% of patients showed clinical improvement based on reduced infections, and 82.7% showed improvement according to the CGRC variable, which also accounted for thymic size. The mean increase in thymic area was 67%, closely aligning with previously reported values. These findings confirm the clinical efficacy of Biomodulina T in the TH treatment and provide new evidence of improvements in adaptive immunity markers that support the observed clinical outcomes.

An important contribution of the current research lies in its methodological framework as a phase III clinical trial, with prospective protocol approval and compliance with GCP guidelines throughout its execution. Accordingly, the results are valid not only in terms of their contribution to scientific knowledge but also for their potential application in clinical practice in Cuba, supporting the official approval of a new medical indication (treatment of TH in children), based on evidence. Although the sample size was limited due to the low prevalence of the condition, the results provide confidence in both clinical and immunological parameters. Future observational studies are needed to expand and refine these findings, particularly in terms of safety.

Conclusion

This clinical trial confirms the efficacy of Biomodulina T in treating TH in children aged 1 to 5 years. The treatment resulted in normalization of thymic size, suppression of bacterial and viral infections, and enhancement of adaptive cellular immunity, including Th cells and IgA antibodies in immunodeficient patients. The observed clinical benefits are consistent with the immunological changes and the known pharmacological effects of the thymic polypeptides contained in the product.

Ethical Approval: The Ethics Committee of the Institute of Hematology and Immunology, Havana, Cuba approved the study protocol on December 13, 2016 with approval number 2016.12.13/01.

Informed Consent: Written informed consent was obtained from parents or legal guardians of all participants prior enrollment.

Peer-review: Externally peer-reviewed

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Correction to: The Expression of Thymic AQP7 and Perilipin 1 (PLIN1) in Rats Fed a High-Fructose Diet is Modified by Voluntary Physical Activity

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Correction Note

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